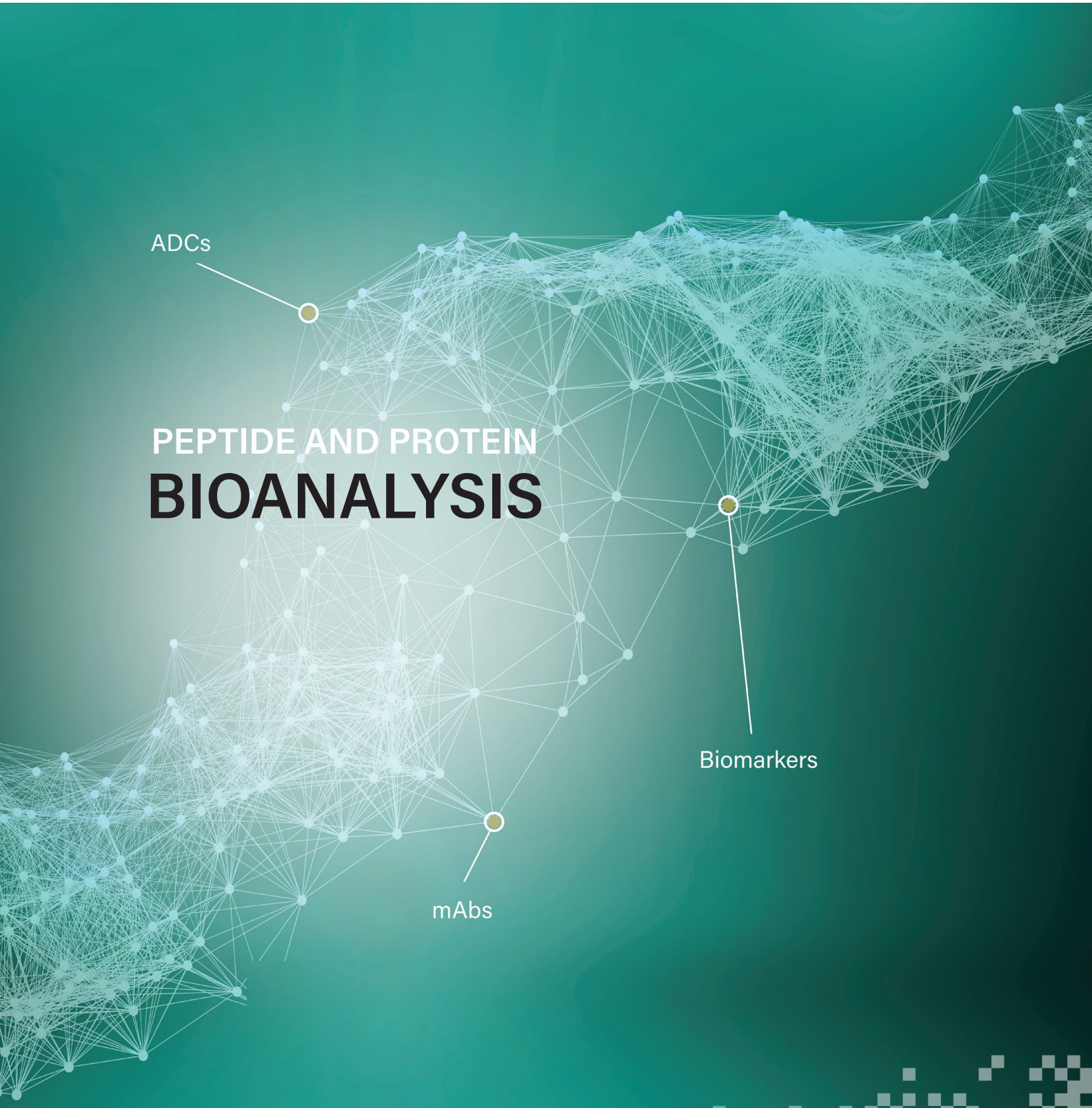


PEPTIDE AND PROTEIN BIOANALYSIS

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Yun Alelyunas, PhD

Before coming to Waters in 2012, Yun Alelyunas was a principal scientist and team leader at AstraZeneca for 20 years where she was involved in industrial chemical analysis, in vitro ADME screening, and project core team DMPK representations. Now, she applies that experience at Waters developing applications ranging from HRMS quantitation and metabolite ID to validating new informatics platforms. She enjoys working with new technologies, devising workflows, and influencing new product development. Detailed and driven, Yun finds satisfaction in working with and educating customers. Away from work, she enjoys yoga, gardening, and traveling.

Yun received her undergraduate degree in polymer chemistry from Nankai University in Tianjin, China and her PhD from the University of Oregon in physical organic chemistry.

Erin E. Chambers, Ph.D.

As a principal scientist, Erin has been working almost exclusively on peptide and protein bioanalysis for the last seven years, while managing small and large molecule bioanalysis and clinical research applications for Waters' consumables business unit. A conversation with her college dean helped put her on the path of scientific study. Ultimately, Erin graduated from Yale University with a degree in chemistry and earned her doctorate from Kings College London. Erin loves the dynamic nature of her job, helping to develop new methods and analytical systems that have a profound influence on illnesses like diabetes and Alzheimer's disease.

Catalin Doneanu, Ph.D.

A native of Romania, Catalin is a principal chemist at Waters. He develops applications and LCMS assays for peptide and protein quantitation, and methods to test drugs in bioanalysis. Catalin's biggest career influence was his 6th grade chemistry school teacher. He received his bachelors degree in chemistry at the University of Bucharest and his doctorate in analytical chemistry at Oregon State University. Away from work Catalin enjoys biking, hiking, and skating with his daughter.

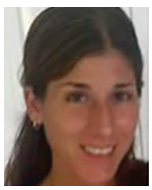
Ken Fountain, M.S.

In his role as Senior Director, Scientific Operations, Ken is responsible for the biopharmaceutical business, driving whole system solutions for protein and biologically-based therapeutics. Ken received his bachelors degree in biotechnology at Worcester Polytechnic Institute and his masters degree in chemistry from Tufts. If he could clone himself, the second Ken would be a world-class chef. He values being part of a talented group of scientists and engineers who are looking to change the way people do science.

Jay Johnson

Jay Johnson has been a research scientist at Waters since he graduated from college. That was 10 years ago. Since then, he completed graduate school at Northeastern University with a degree in analytical chemistry, while being a key player in Waters' Core Research Group. Now, a senior research chemist, Jay works on developing and testing the technologies that are still years away from commercialization. He spends considerable time working on large beta collaborations with customers to implement, test, and fine-tune new products before they come to market. One of his most rewarding challenges and accomplishments was helping to design the column for the ionKey/MS™ system, where microflow UPLC is integrated directly into the source of the mass spec.

Jay credits his father, an electrical engineer with the Airforce, for his passion for science. Outside of work, Jay likes to travel, cook, and hike.



Mary E. Lame, M.S.

Motivated by tough problems, Mary's work at Waters focuses on improving pharmaceutical peptide and protein bioanalysis, testing products, and developing applications. Mary's high school chemistry teacher instilled in her the confidence to pursue chemistry as a career. She went on to receive her undergraduate degree in chemistry from Western Connecticut State University and earned her masters degree at Central Connecticut State University. A risk taker, Mary loves working with the latest, cutting edge technology and developing novel methods for peptide and protein therapeutics.

Jim Murphy, PhD

As a principal consultant in Waters' Core Research Group, Jim Murphy has a job many others covet. Jim gets to explore and test novel product ideas years ahead of commercialization. In this role, he is responsible for developing prototypes for some of the most innovative technologies that come to market, including microfluidic and nanofluidic MS platforms. His work at Waters was instrumental in developing the nanoACQUITY UPLC and ionKey/MS systems.

When he's not on the job, Jim loves to coach his kids' lacrosse and baseball teams. He most admires Troy Brown – formerly of the New England Patriots – because, "He was never the most gifted player, but no one ever outworked him."

Jim graduated from Holy Cross University with a degree in biology before earning his PhD in analytical chemistry from the University of Florida.

Paul Rainville, Ph.D.

For 15 years, Paul has led numerous changes and innovations at Waters. He has worked extensively in a number of application areas including biopharmaceuticals, bioanalysis, and was one of the first to do lipid separations with UPLC. Paul's latest role sees him push the ionKey technology further and applying it to the health sciences space. He graduated with a biotechnology degree from the University of Massachusetts and earned his Ph.D. from Kings College London. Outside of work, Paul's passion is gardening and small scale-farming.

Gregory Roman, PhD

As a research scientist in the Core Research Group, Greg Roman is involved in the prototyping and testing of some of Waters' most exciting new technologies – including ionKey/MS and QTof high-resolution mass spec. He has developed many of the new technology applications with a special focus on antibody analysis.

Greg completed his undergraduate work in chemistry at Bard College and his PhD in analytical chemistry (specializing in instrument analysis) at Kansas State University. At Kansas State he built a portable instrument, which measured the effect of ionizing radiation on NASA's KC-135 aircraft (affectionately known as the "Vomit Comet").

He is passionate about developing tools and solutions that fight cancer and has a zeal for constitutional law, gardening, and skiing.

Nikunj Tanna

A senior scientist on Waters' DMPK applications team, Nikunj Tanna recently moved from Berg Health, where he built the regulated bioanalysis lab from nothing to a team of 10, conducting all the analytical and clinical validations for the company's prostate cancer panels.

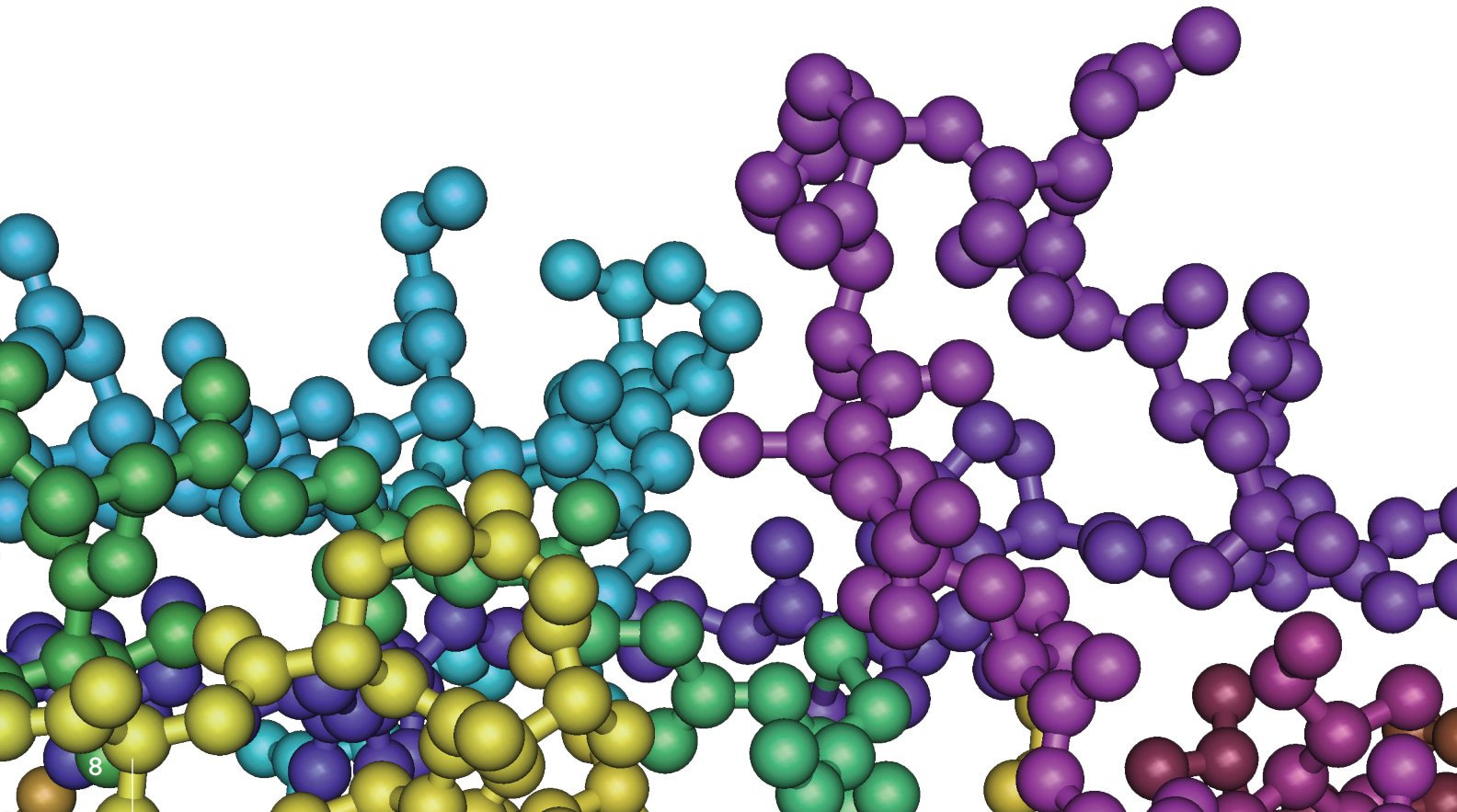
With graduate degrees in bioanalytical sciences and pharmaceutics, Tanna develops new applications focusing on large and small molecule quantitation in regulated environments. He says the most interesting part of his job is the possibility of affecting change. "We hear a lot of the same challenges from customers. One is whether to quantify large molecules via intact proteins or whether to apply a digested method. Helping customers find the right answer is extremely rewarding."

Table of contents

Table of contents (continued)

Table of contents (continued)

Table of contents (continued)

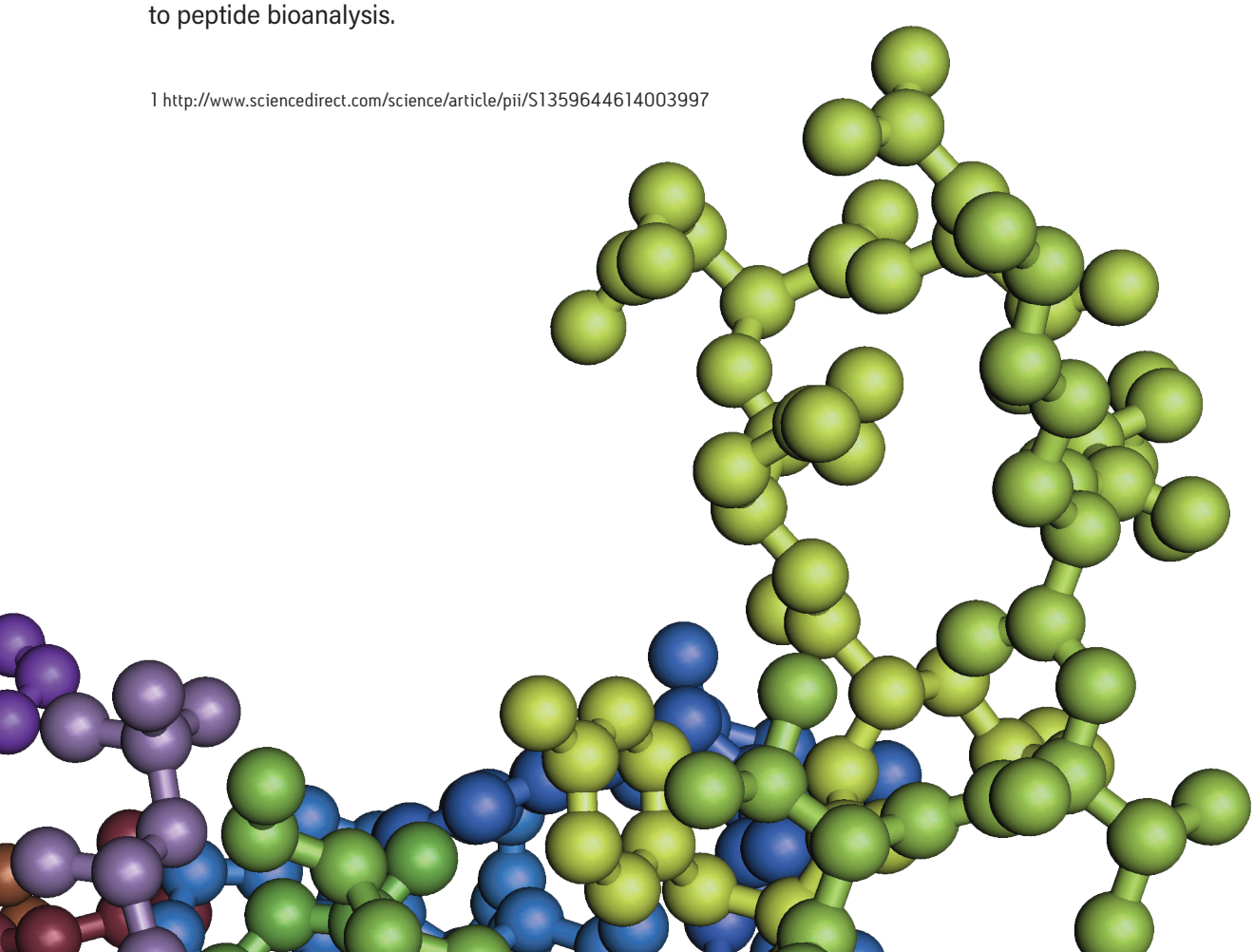


Peptide Bioanalysis

Quantification of peptide therapeutics and endogenous biomarkers is an application area that continues to grow in importance. Approvals for peptide therapeutics have hit an all-time high, with the market predicted to grow by ~80% to $25B by 2018.[1] More than 140 peptide-based therapeutics are in clinical trials, with another 500 in preclinical development. The rapid growth has presented a significant opportunity for drug developers, but this trend, coupled with concomitant interest in biomarkers for safety, efficacy and diagnostic applications, pose enormous challenges for bioanalytical labs.

Among large molecule biotherapeutics, the highest proportion of bioanalytical work is performed on peptides, with LC-MS as the primary platform being used. However, the shift from small molecule to large molecule quantification is rarely seamless. One significant challenge is the high background due to endogenous proteins and peptides. Other challenges include optimization of the LC parameters and multiple reaction monitoring (MRM) methods, dealing with multiple charge states, and more complex fragmentation patterns. The difficulties inherent to large molecule quantification are real, and it is no small task to get up to speed. But this is where Waters' decades of cumulative experience sorting through these challenges alongside pharmaceutical, CRO, and numerous academic collaborators comes into play. The following application notes outline methods, inclusive of sample prep, separations, MS and informatics, which enable scientists to solve the most pressing and challenging analytical problems related to peptide bioanalysis.

1 http://www.sciencedirect.com/science/article/pii/S1359644614003997



Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

SIMPLIFYING PEPTIDE BIOANALYSIS

Erin E. Chambers, Jessalynn P. Wheaton, and Diane M. Diehl
Waters Corporation, Milford, MA, USA

INTRODUCTION

Biomolecules such as proteins and peptides represent a growing number of pharmaceutical drug entities. Drugs in this class – biopharmaceuticals – are often more specialized than small molecules since they typically replace or augment an endogenous molecule known to change a disease or physiological state.[1] Furthermore, since they are analogs to, synthetic versions of, or are endogenous compounds, they tend to be well-tolerated by the body. There are currently more than 40 peptide-based drugs on the market and more than 400 in late-stage clinical trials.[2]

Analytical challenges

Development of bioanalytical methods for the detection of small molecule pharmaceuticals in humans and animals is a challenging and time-consuming process. Regulatory guidelines require methods to be acceptable in terms of linearity, sensitivity, accuracy and precision, selectivity, stability, and carryover. Bioanalytical methods now need to be developed for biopharmaceuticals as well – and many of the same challenges faced in bioanalysis of small molecules also apply to these larger biopharmaceuticals.

Achieving selectivity for analytes in complex biological matrices is the key component of all bioanalytical methods. Sample preparation continues to play a critical role in minimizing matrix effects and reducing variability associated with incurred samples. In the extraction solution we describe here, mixed-mode solid phase extraction (SPE) is employed for this purpose, since it relies on both reversed-phase and ion exchange mechanisms. Mixed-mode SPE can selectively separate the desired analytes from matrix components and has been shown to reduce matrix effects.[3]

Developing bioanalytical methods for peptides is further complicated by several factors. Peptides are zwitterionic, making their retention behavior on SPE sorbents difficult to predict and therefore increasing method development times. Because of their size and charge state distribution, sensitivity by mass spectrometry may be lower for biomolecules than typical small molecules. This often necessitates concentration during sample preparation.

Solution

The Waters Peptide Separation Technology (PST) Method Development Kit was designed to facilitate the development of selective extraction and liquid chromatographic methods for peptides in biological

Figure 1. The PST Method Development Kit.

matrices. The PST Method Development Kit is available for ACQUITY UPLC® (Part #176001835) or HPLC (Part #176001836) analysis. The kit contains a chromatographic column, an Oasis® µElution PST Method Development extraction plate, a simple and straightforward SPE screening protocol, and an LC screening protocol.

In this application note, the kit was used to develop extraction and chromatographic methods for the 12 peptides described in Table 1.

Peptide	PL	MW	# Residues	HPLC Index
Desmopressin	8.60 - 9.10	1069	9	16.8
Vasopressin	9.10	1084	9	7.6
Octreotide	9.30	1019	8	40.8
Bivalirudin	3.87	2180	20	46.2
BNP	12.00	3464	32	15.9
Goserelin	7.30	1270	10	31.7
Enfuvirtide	4.06	4492	36	155.9
Angiotensin I	7.51	1296	10	56.2
Angiotensin II	7.35	1046	8	38.3
Neurotensin	8.93	1673	13	44.4
Somatostatin	10.40	1638	14	52.6
Teriparatide	9.10	4118	34	90.4

Table 1. Summary of names and chemical properties for the set of 12 diverse peptide therapeutics used to evaluate the PST Method Development Kit.

EXPERIMENTAL

Extraction of peptides from human plasma Spiked human plasma samples were pre-treated by diluting 1:1 (v:v) with 4% H_3PO_4. Samples were then extracted using the Oasis® µElution PST Method Development extraction plate according to the basic screening protocol included in the kit. The ACQUITY UPLC System was used to increase the sensitivity of the separation and resolution between analytes and similar endogenous compounds required for a selective assay. Samples were analyzed with the ACQUITY UPLC PST Column included in the kit. PST columns are quality-control tested with peptide standards to ensure consistent performance.

The Xevo® TQ MS tandem quadrupole mass spectrometer was used to detect the peptides in positive ion electrospray. Parent masses were detected for the peptides in their 2+, 3+, or 4+ charge states and fragments were either singly or multiply charged. Since UPLC peak widths are on the order of 2 to 4 seconds wide at base for all peptides, it was important to have a mass spectrometer capable of not only providing enough points across the peaks for accurate and reproducible quantitation, but also one that could maintain this performance under constantly changing collision cell conditions encountered when monitoring multiple peptides at once.

Another key MS requirement for peptide bioanalysis is mass range. The mass range of the first resolving quadrupole should be at least 2000 amu to accommodate the 3+ charge state, which provided the highest sensitivity for certain larger peptides that were tested. The Xevo TQ MS was chosen for its superior performance in these areas.

RESULTS AND DISCUSSION

The screening protocol was developed to streamline the choice of SPE sorbent and to provide initial SPE recovery and matrix effect data for the analytes of interest. For many peptides, analyte recovery and matrix effects resulting from the basic screening protocol were acceptable. Matrix effects, where measured, were less than 11%. (Absolute matrix effects of less than 15% were considered acceptable for this study.) The negligible level of matrix effects observed indicates the specificity of the methodologies.

Recovery for the peptides from human plasma was assessed by the comparison of peak areas from pre-spiked extracted samples to blank plasma extracts spiked post-SPE.

The screening protocol yielded a greater than 82% recovery for nine out of 12 peptides extracted from human plasma. Minor modifications made to the protocol improved recovery for the three other peptides, to more than 83%. Figure 2 summarizes analyte recovery for the diverse set of 12 peptide therapeutics extracted using the screening protocol. The extraction was carried out in a 96-well Oasis µElution plate format to facilitate direct injection of the extracted sample and to obtain the analyte concentration necessary to meet required LOD/LLOQs.

A concentration factor of up to 15X can be achieved without evaporation using the µElution format. It is particularly desirable to eliminate the evaporation step when analyzing peptides as this eliminates any evaporative losses or adsorption to the walls of collection plates. A representative chromatogram for desmopressin extracted from human plasma is shown in Figure 3.

Chromatography was performed using the UPLC® screening method provided in the kit. The chromatographic method has a cycle time of 3.5 minutes, enabling hundreds of samples to be analyzed per day. Sample chromatograms for two of the peptides are shown in Figures 4 and 5.

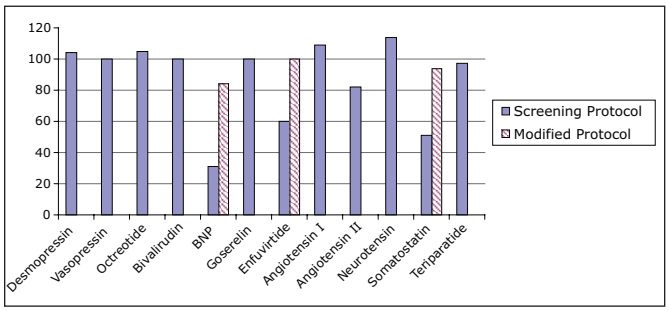


Figure 2. Percent SPE recovery for 12 peptide therapeutics extracted from human plasma using the Oasis PST µElution Method Development plate and protocol.

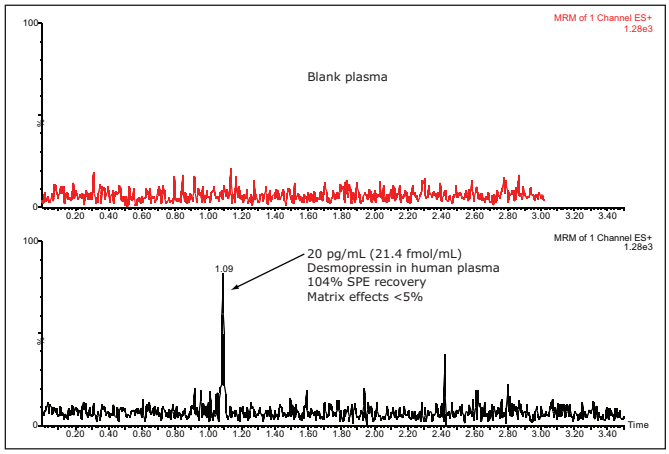


Figure 3. UPLC separation of A) blank plasma and B) a 20 pg/mL sample of desmopressin in human plasma, extracted with the Oasis PST µElution Method Development plate and protocol.

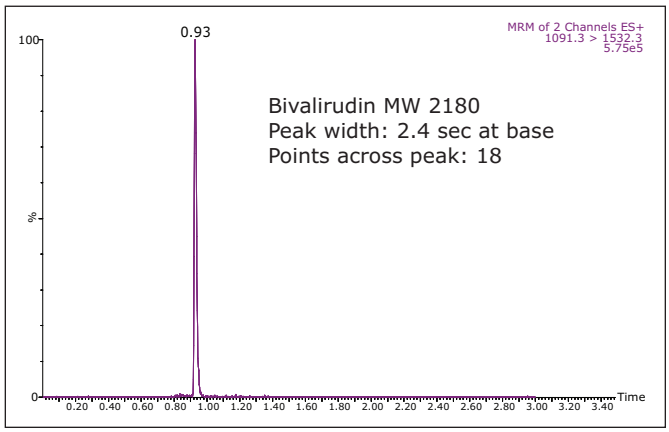


Figure 4. UPLC/MS/MS chromatogram for bivalirudin.

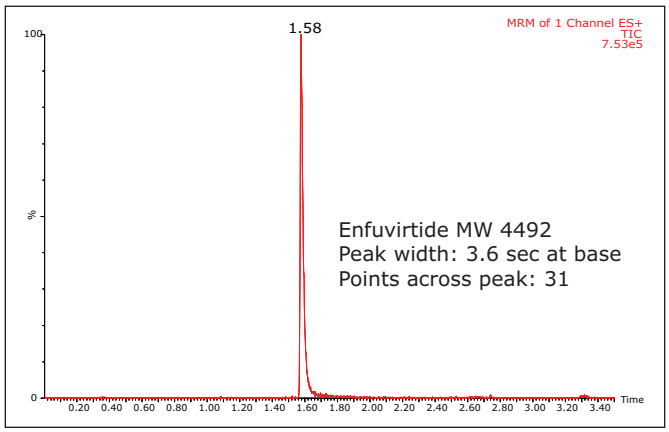


Figure 5. UPLC/MS/MS chromatogram for enfuvirtide.

CONCLUSIONS

The behavior of peptide therapeutics under various sample preparation conditions may be difficult to predict. This has the potential to make development of a selective sample prep method complicated and time consuming. Using the Oasis PST µElution Method Development plate and protocol can greatly simplify extraction method development and rapidly identifies an appropriate SPE sorbent and starting protocol.

In this work, the SPE screening protocol and method development plate were successfully used to develop extraction methods for nine out of 12 peptides tested on a first pass. Minor modifications to the screening protocol resulted in suitable extraction methods for the three other peptides.

Peaks widths on the order of 2 to 4 seconds wide at base, and more than 15 points across the peak, were obtained for all peptides tested using the ACQUITY UPLC Column provided in the kit.

Overall, we have shown that bioanalysis studies for peptide therapeutics are amenable to a platform-based approach to method development. Such standardized approaches for determining optimal SPE recovery and concentration and MRM-based LC/MS analysis should permit laboratories to reduce development timelines and shorten time to market for their peptide drugs.

Ordering information

PST UPLC Method Development Kit: Part# 176001835

PST HPLC Method Development Kit: Part# 176001836

References

1. Bladon C. Pharmaceutical Chemistry: Therapeutic Aspects of Biomolecules. Wiley and Sons. West Sussex, England, 2002.
2. Bioscience Technology. January 2009.
3. Chambers E, Wagrowski-Diehl DM, Lu Z, Mazzeo JR. J. Chromatogr. B 2007; 852 (1-2): 22-34.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

October 2009 720003253EN AG-PDF

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

High Sensitivity Automated Peptide SRM/MRM Development Using Xevo TQ-S MS, MassLynx, and Skyline

GOAL

To harmonize Skyline SRM/MRM software and Xevo® TQ-S MS method development to enable the intuitive, prompt, and seamless development of multiple SRM/MRM peptide transitions and methods.

BACKGROUND

Targeted LC-MS/MS SRM/MRM validation has the potential to bridge protein biomarker discovery and validation. Candidate proteins are typically identified in smaller scale relative quantification discovery studies. Typical bottlenecks observed are the selection of the appropriate proteotypic peptides for SRM/MRM quantitation and the development of the associated LC/MS methods for triple quadrupole mass spectrometers. Software plays a vital role in selecting the correct peptides to be measured, especially in the case of complex experiments, as a substitute for the target protein. This transitions into measurement of target peptides, where optimization for each peptide is typically performed. In this technology brief, we describe the use of the Skyline for peptide and transition selection process and method development for a Waters® Xevo TQ-S Mass Spectrometer, including the results for the analysis allergenic peptide standards in a complex synthetic matrix. Skyline is an open source document editor for creating and analyzing targeted proteomics experiments.[1]

Move quickly from proteomics discovery to quantitative SRM/MRM-based validation with reliable, easy-to-use software for transition selection, automated MS-method building, and intuitive data.

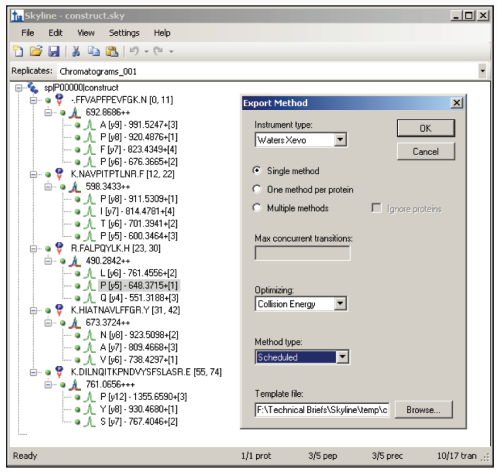


Figure 1. Allergenic proteins of interest were in-silico digested and fragmented generating candidate transitions. Skyline was used to predict product ions, 3 or 4 per peptide, for transitions selection. The final number of transitions for all five peptides = 17. Subsequently, Skyline Export Method Editor was used to produce a Xevo TQ-S method file in which different collision energies were examined to find optimal settings.

THE SOLUTION

Five allergenic peptide standards were spiked in a matrix and the best candidate transitions predicted using Skyline, a software application for building SRM/MRM quantitative methods and analyzing the resulting mass spectrometer data. Skyline is vendor neutral and it was developed for creating and iteratively refining targeted methods for large-scale studies. A scheduled SRM/MRM method was created in order to independently optimize the collision energy for all peptides. The retention times were predicted with the built-in model of Skyline using the observed retention time as input/landmarks for linear retention time prediction and curve optimization/adjustment.

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Figure 1 shows the predicted transitions for the allergenic protein of interest and the use of the Skyline Export Method editor for creating a retention time-scheduled Xevo TQ-S collision energy optimization experiment. A set of 11 collision energies was created, ranging from -5 V to +5 V, versus the optimally predicted collision energy derived from empirical observations for each peptide. The created method can be directly read and edited by the Experiment Setup SRM/MRM method editor of MassLynx™ Software, shown in Figure 2. The dwell times are automatically calculated based on the average chromatographic peak width. After the experiment is run, the results can either be reviewed in MassLynx or Skyline software. Figure 3 overlays the results of 11 SRM/MRM experiment for one of the peptides of interest. In this instance, the optimal collision energy was found to be 2 V lower than the predicted value and in agreement with the MassLynx Software data observed values, which were obtained via infusion of the peptide standards.

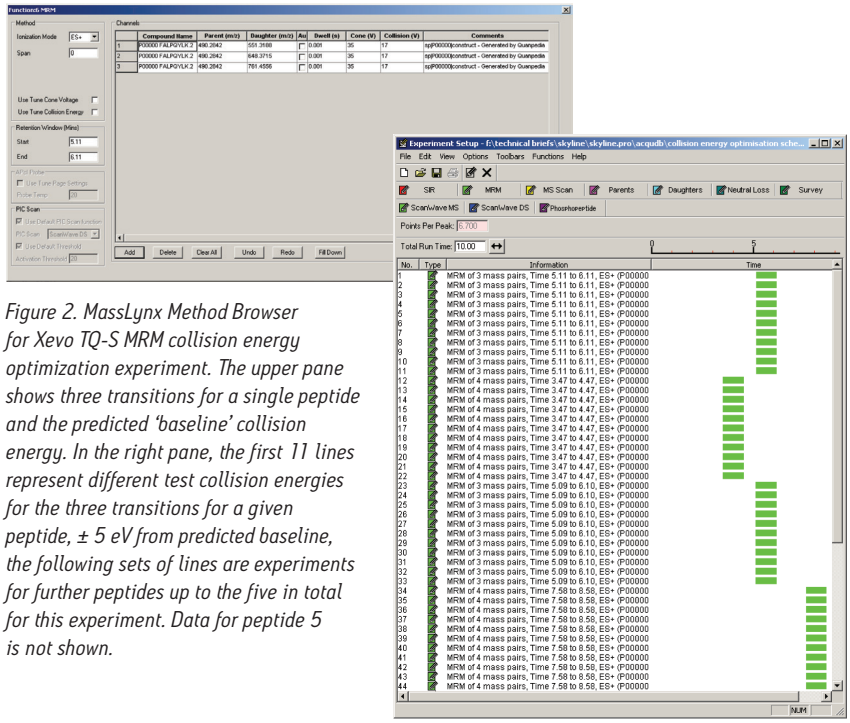


Figure 2. MassLynx Method Browser for Xevo TQ-S MRM collision energy optimization experiment. The upper pane shows three transitions for a single peptide and the predicted 'baseline' collision energy. In the right pane, the first 11 lines represent different test collision energies for the three transitions for a given peptide, ± 5 eV from predicted baseline, the following sets of lines are experiments for further peptides up to the five in total for this experiment. Data for peptide 5 is not shown.

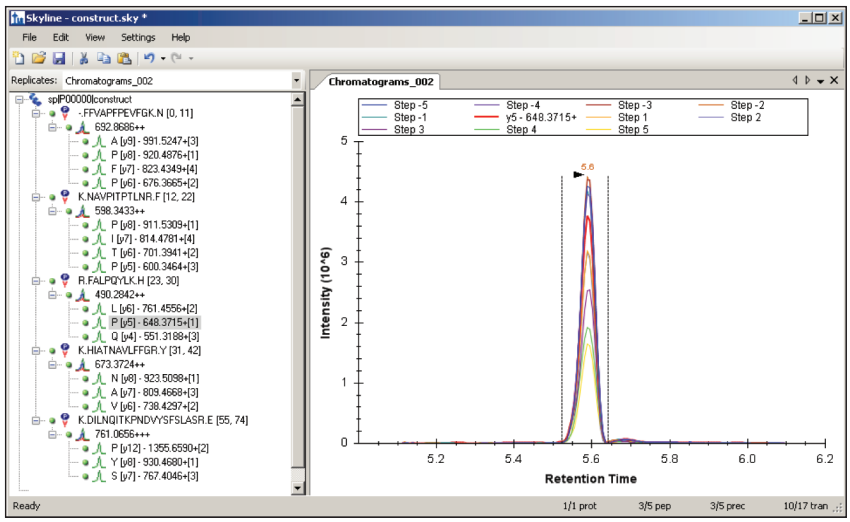


Figure 3. Skyline Transition Evaluation Browser. After collecting data using the method established in Figure 2, the SRM/MRM data can be evaluated to establish which transition and collision energies yielded the best SRM/MRM response. The MRM/SRM chromatogram is shown from one of the transition of interest from a single peptide and the effect on that transition from the 11 different tested collision energies.

SUMMARY

The SRM/MRM method development of five allergenic peptide standards spiked into a complex synthetic background for Xevo TQ-S has been demonstrated using Skyline open-source software.

Reference

1. MacLean *et al.* Bioinformatics. 2010 Apr 1;26(7):966-8.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

High Sensitivity Quantitative Analysis of a Therapeutic Peptide in Plasma using UPLC and Xevo TQ-S

Robert S. Plumb, Gareth Booth, and Paul D. Rainville
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The Xevo TQ-S, a new tandem quadrupole MS system, provides the highest possible sensitivity for peptide bioanalysis. Its fast data capture rate and dual scan MRM capability allows for the accurate quantification of the narrow chromatographic peaks produced by UPLC® as well as confirmation of the peak identity.

WATERS SOLUTIONS

Xevo™ TQ-S

ACQUITY UPLC®

ACQUITY UPLC BEH column chemistry

MassLynx™ with TargetLynx™

KEY WORDS

StepWave™, T-Wave,™ peptide bioanalysis, desmopressin

INTRODUCTION

The use of peptides as therapeutic agents is increasing due to their high tolerability, target receptor selectivity, and high potency. The ability to accurately quantify these therapeutic peptides in biological fluid requires a selective isolation process, a high-resolution chromatography system, and a high-sensitivity detector. A high-resolution chromatography system is required to separate the target analyte from the endogenous peptides in plasma and blood, some of which may be isobaric or form identical fragment ions. As these therapeutic peptides imitate or replace the activity of endogenous peptides, the detection process must be able to differentiate between these endogenous and exogenous compounds.

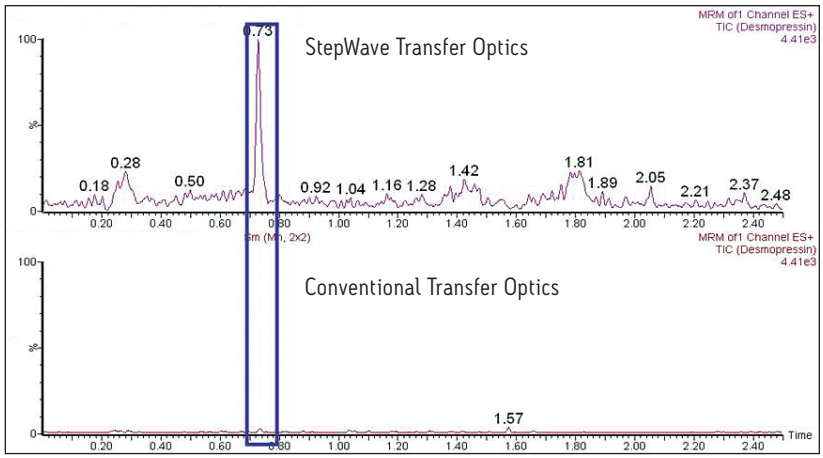
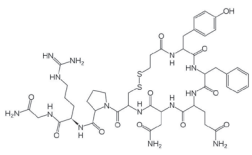


Figure 1. UPLC/MS/MS quantitative analysis of desmopressin in rat plasma extract using MRM mode. The top chromatogram shows the peak response with the new StepWave optics of the Xevo TQ-S; the lower chromatogram shows the peak response with conventional transfer optics.



Chemical structure of desmopressin.

Despite the fact that they are observed as multiply-charged compounds, the high molecular weight of peptides, 1000 to 4000 amu, the detection of these peptides requires a mass spectrometer with an upper mass range in the region of 2000 *m/z* for successful analysis. In this application note, we describe the use of the ACQUITY UPLC System and a new high-sensitivity tandem quadrupole MS, Xevo TQ-S, for high-sensitivity peptide bioanalysis.

EXPERIMENTAL

Chromatography

LC system:	ACQUITY UPLC (binary solvent manager, sample manager, HT column oven)
Column:	ACQUITY UPLC BEH C_{18}, 1.7 µm, 2.1 x 50 mm
Column temp.:	40 °C
Gradient:	Acetonitrile/0.1% Formic acid (Aq.), 5 to 45% ACN over 1.5 min
Flow rate:	450 µL/min
Injection vol.:	10 µL

Mass spectrometry

MS system:	Xevo TQ-S and Xevo TQ operated in electrospray positive mode MRM data acquisition 535 => 328
Voltages:	Capillary, cone, and collision voltage were optimized for each MS as well as cone gas flow
Source temp.:	140 °C
Desolvation temp.:	625 °C
Nebuliser gas flow:	1200 L/hr

Data management

MassLynx 4.1

TargetLynx Application Manager

RESULTS AND DISCUSSION

Peptide therapeutics are extremely potent but are also quickly eliminated from the body either via metabolism or as unchanged drug. In order to accurately characterize the pharmacokinetics (PK) of these medicines, it is necessary to have a high-sensitivity LC/MS/MS system to define the later time points and hence the elimination phase of the PK curve. The Xevo TQ-S is a new tandem quadrupole instrument equipped with a novel source design. This new design significantly improves the efficiency of the ion sampling process allowing more analyte ions to be transferred to the analyzer. This is achieved by the use of a larger sampling orifice and differentially pumped region, and off-axis stacked ring transfer optics to prevent neutral compounds entering analyzer region of the instrument.

To evaluate sensitivity increase for the analysis of peptides in biological fluids, desmopressin was spiked into plasma and extracted via protein precipitation with acetonitrile (2:1). The data obtained for conventional optics and new StepWave optics are shown in Figure 1. Here we can see that the desmopressin peak is barely visible with the conventional transfer optics instrumentation, whereas there is a significant peak with the Xevo TQ-S. The data in Figure 2 compares the data with the baseline expanded for the conventional source. We can clearly see that there is a peak at 0.73 minutes for the desmopressin in the conventional instrumentation chromatogram. This increase in sensitivity was determined to be 25-fold.

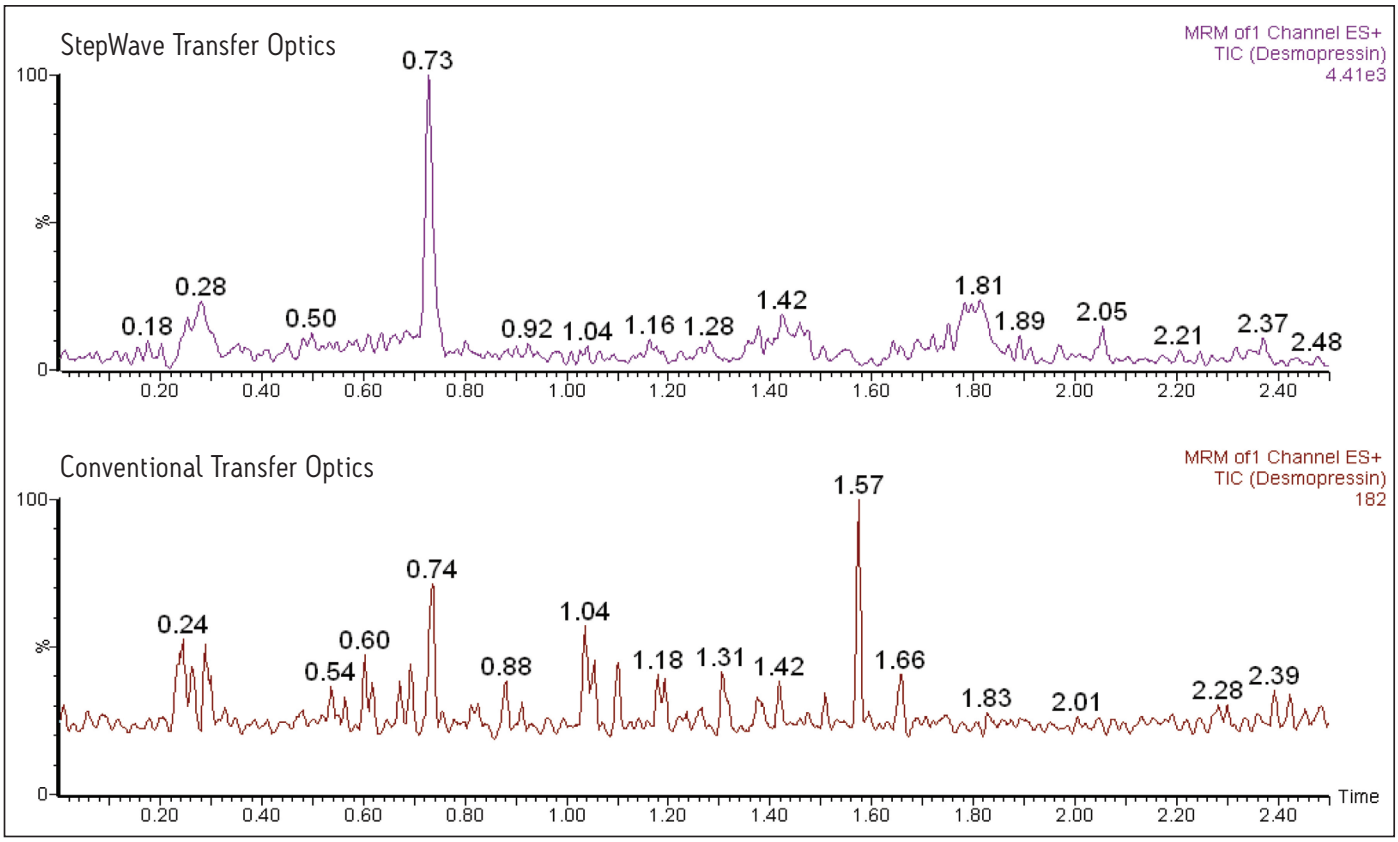


Figure 2. UPLC/MS/MS quantitative analysis of desmopressin in rat plasma extract using MRM mode. The top chromatogram shows the peak response with the new StepWave optics; the lower chromatogram shows the peak response with the conventional transfer optics with the baseline expanded to show the desmopressin peak at 0.74 minutes.

CONCLUSIONS

Peptide therapeutics offer the opportunity to treat new diseases with low risk of side effects, drug-drug interactions, or toxicity. The Xevo TQ-S combined with ACQUITY UPLC provides the ideal platform for the analysis of peptides in biological fluids. This combination offers:

- Highest possible levels of sensitivity
- Fast analysis times
- Resolution from endogenous peptides
- Sufficient mass range in the analyzer to quantify large molecular weight peptides

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

An Improved SPE/LC/MS/MS Platform for the Simultaneous Quantitation of Multiple Amyloid β Peptides in Cerebrospinal Fluid for Preclinical or Biomarker Discovery

Erin E. Chambers[1], Mary E. Lame[2], and Diane M. Diehl[1]
[1]Waters Corporation, 34 Maple St., Milford, MA, [2]Pfizer, Neuroscience Research Unit, Groton, CT USA

APPLICATION BENEFITS

- Improved sensitivity using Xevo TQ-S.
- Reduced sample size required using Xevo TQ-S.
- Fast, flexible platform for peptide quantitation.
- One LC/MS/MS method for quantitation of multiple peptides, reduced reliance on ligand-binding assays in discovery segment.
- Highly selective sample preparation using Oasis® mixed-mode sorbent in µElution format.
- Resolution, sensitivity of ACQUITY UPLC® PST columns for improved separation with shorter run times.

WATERS SOLUTIONS

Xevo TQ-S MS system
ACQUITY UPLC system
Oasis µElution plates
PST columns

KEY WORDS

Biomarker discovery
Peptide quantitation
Amyloid beta
Hydrophobic peptides
LC/MS/MS
Oasis SPE

INTRODUCTION

A previous application note (720003682en) described in detail the development of a fast, flexible SPE/LC/MS/MS platform for the quantification of multiple amyloid beta (aβ) peptides from human or monkey CSF for use in a biomarker or preclinical discovery setting. In this work, the mass spectrometry platform has been updated from the Xevo® TQ MS to the Xevo TQ-S mass spectrometry system. This change facilitated both a 4X reduction in required sample size and a 4-5X increase in assay sensitivity.

Historically, quantification of aβ peptides in biological fluids has relied mainly on the use of immunoassays, such as ELISA. These assays are time consuming and expensive to develop, labor intensive, are subject to cross reactivity, and an individual assay is required for each peptide. In order to meet the throughput requirements and constant flow of demands for new peptide methods in a discovery setting, there is a need for a highly specific yet flexible methodology based on an LC/MS/MS platform. In this work, this platform is coupled with selective sample preparation for the simultaneous quantitation of multiple aβ peptides. This work focuses on methods for the 1-38, 1-40, and 1-42 aβ peptides, in support of preclinical and biomarker discovery studies. Sequence, pI and molecular weight (MW) information for these peptides is shown in Figure 1.

The solid-phase extraction (SPE) sample preparation protocol used to enrich the amyloid beta fraction in CSF is the protocol previously described. However, the sample size required is now only 50 µL instead of 200 µL. The SPE method concentrates the sample to improve detection limits while eliminating matrix interferences and optimizing solubility of the aβ peptides in the mass spectrometer injection solvent.

As strategies emerge for disease modification in Alzheimer's Disease (AD), the quantification of other aβ species (in addition to aβ 38, 40, and 42) that may be linked to AD pathology may be required. The method described herein shows promise for adaptation to quantify those peptides as well.

Amyloid β 1-38
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG
MW 4132 pI 5.2

Amyloid β 1-40
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
MW 4330 pI 5.2

Amyloid β 1-42
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
MW 4516 pI 5.2

Figure 1. Sequence, MW, and pI information for amyloid β peptides.

EXPERIMENTAL

ACQUITY UPLC Conditions

Column:	ACQUITY UPLC BEH C_{18} 300Å, 2.1 x 150 mm, 1.7 µm, Peptide Separation Technology
Part Number:	186003687
Column temp.:	50 °C
Sample temp.:	15 °C
Injection volume:	10.0 µL
Injection mode:	Partial Loop
Flow rate:	0.2 mL/min.
Mobile phase A:	0.3% NH_4OH in H_2O
Mobile phase B:	90/10 ACN/mobile phase A
Strong needle wash:	60:40 ACN:IPA + 10% conc. NH_4OH (600 µL)
Weak needle wash:	90:10 0.3% NH_4OH in H_2O:ACN (400 µL)

Gradient:

Time (min)	Profile %A	%B	Curve
0.0	90	10	6
1.0	90	10	6
6.5	55	45	6
6.7	55	45	6
70	90	10	6

Waters Xevo TQ-S MS Conditions, Electrospray Positive

Capillary Voltage:	2.5 V
Desolvation Temp:	450 °C
Cone Gas Flow:	Not used
Desolvation Gas Flow:	800 L/Hr
Collision Cell Pressure:	2.6 x 10(-3) mbar
MRM transition monitored, ESI+:	See Table 1

Table 1. MRM transitions and MS conditions for the amyloid β peptides and their N15 labeled internal standards.

Peptide Name	Precursor Ion 4+	Product Ion 4+	Product Ion i.d.	Cone Voltage (V)	Collision Energy (eV)
Amyloid β 1-38	1033.5	1000.3	b 36	33	23
Amyloid β 1-38 N15 IS	1046	1012.5		30	22
Amyloid β 1-40	1083	1053.6	b 39	33	25
Amyloid β 1-40 N15 IS	1096	1066.5		35	22
Amyloid β 1-42	1129	1078.5	b 40	28	30
Amyloid β 1-42 N15 IS	1142.5	1091.5		35	28

SPE Conditions

Sample Pre-treatment

50 µL human CSF or spiked artificial CSF + 5% rat plasma was diluted 1:1 with 5 M guanidine HCL and shaken at room temperature for 45 minutes. This was then diluted further with 50 µL 4% H_3PO_4 in H_2O and mixed.

Note: For spiked samples, samples were allowed to equilibrate at room temperature for 30 min after spiking and prior to dilution with guanidine HCl.

Sample Extraction with Oasis MCX

Samples were extracted according to the protocol in Figure 2 below. All solutions are made up by volume. All steps applied to wells of µElution plate containing samples

Oasis MCX µElution Protocol
Part Number: 186001830BA

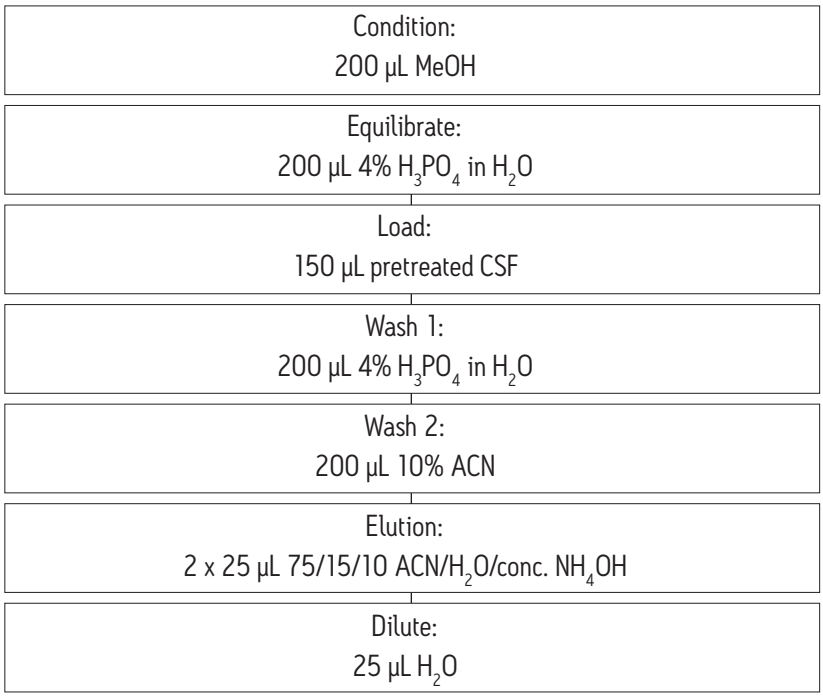


Figure 2. Oasis µElution MCX extraction protocol

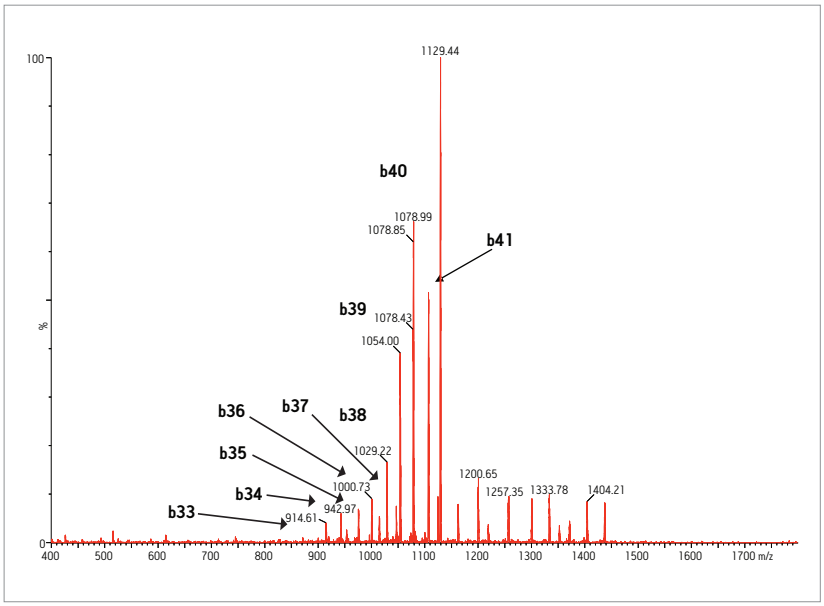


Figure 3. Representative ESI+ MS/MS spectrum for amyloid β 1-42 with fragment sequence ions labeled.

RESULTS AND DISCUSSION

Mass Spectrometry

MS was performed in positive ion mode since CID of the 4+ precursor ion yielded several distinct product ions corresponding to specific b sequence ions (representative spectrum shown in Figure 3.)

The sensitivity increase provided by the Xevo TQ-S facilitated the use of 4X less sample whilst also improving detection limits by approximately 4-5X compared to the previous assay on the Xevo TQ. The lowest QC sample tested was 5X lower in concentration than the low QC when the standard Xevo TQ MS system was used. Earlier work by Rainville and Booth (application note 720003415en) describes the system improvement in more depth and demonstrates a similar sensitivity increase for the therapeutic peptide desmopressin.

Mass range of the instrument was also an important factor in obtaining specificity. The Xevo TQ-S MS has a mass range of 2048 on both quads, easily allowing us to choose a more specific 4+ rather than 5+ precursor and fragment pair.

UPLC Separation

Separation of the three amyloid β peptides is shown in Figure 4. While the exact amount of NH_4OH in the mobile phase was critical for negative ion sensitivity, the signal in ESI positive proved to be more robust to subtle changes in mobile phase composition, providing a minimum of >24 hour LC/autosampler stability. In contrast, 50% or more of the ESI negative signal was lost after 10-12 hours due to the natural change in NH_4OH concentration (volatility) in the mobile phase. This further reinforced the robustness of an ESI positive MS method.

Sample Preparation: SPE

SPE was performed using Oasis MCX, a mixed-mode sorbent, to enhance selectivity of the extraction. The sorbent relies on both reversed-phase and ion-exchange retention mechanisms to selectively separate the aβ fraction from other high abundance polypeptides in complex CSF samples. The Oasis µElution plate (96-well format) provided sample concentration, eliminating the need for evaporation and reconstitution. This has the benefit of saving time and eliminating peptide losses due to adsorption to the walls of the collection plate during dry down.

During initial method development, a high degree of non-specific binding (NSB) was observed when artificial CSF was extracted. Thus, 5% rat plasma (having a different amyloid β sequence) was added to bind to surfaces, eliminating NSB.

The SPE method was one of the more critical aspects of the overall methodology. Very selective isolation of the amyloid fraction coupled with the resolution of analytical-scale flow UPLC,® facilitates analysis of pre-clinical samples without the need for antibodies or time-consuming immuno-precipitation associated with ELISA methods. The increased sensitivity of the Xevo]TQ-S enabled the sample volume to be reduced from 200 µL to 50 µL of CSF, making this method amenable to use in pre-clinical species.

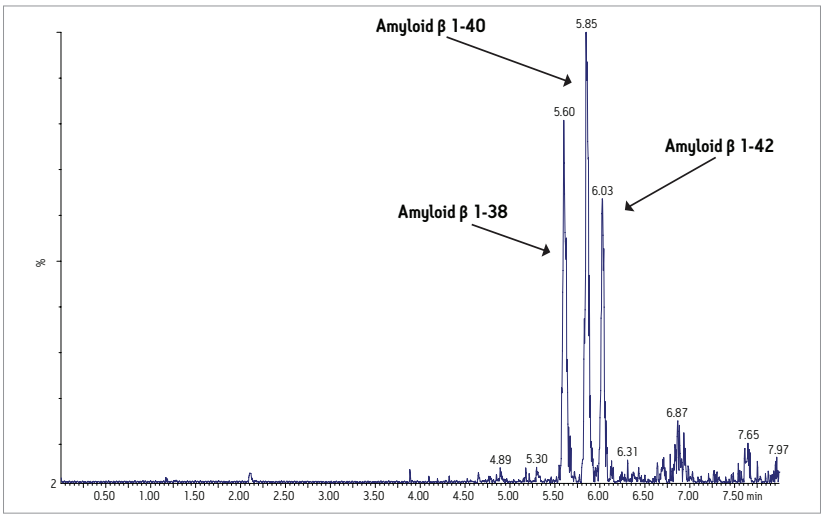


Figure 4. Representative UPLC/MS/MS analysis of amyloid β 1-38, 1-40, and 1-42 peptides extracted from artificial CSF + 5% rat plasma.

Table 2. Comparison of standard curve and QC range using Xevo TQ and TQ-S MS.

Peptide Name	200 µL Sample Xevo TQ	50 µL Sample Xevo TQ-S
Standard Curve Range	0.1-10 ng/mL	0.025 or 0.05-10 ng/mL
QC Range	0.2-6 ng/mL	0.04-6 ng/mL

Name	Type	Std. Conc.	RT	Area	IS Area	Response	Conc.	%Dev
Blank artificial CSF			5.73	19.7	7.0			
50 pg/mL artificial CSF	Standard	0.05	5.71	230.4	3620.5	0.064	0.057	14
100 pg/mL artificial CSF	Standard	0.1	5.71	390.8	3585.1	0.109	0.108	8.1
250 pg/mL artificial CSF	Standard	0.25	5.71	778.3	3737.3	0.208	0.220	-12
350 pg/mL artificial CSF	Standard	0.35	5.71	1267.3	3693.8	0.343	0.372	6.2
500 pg/mL artificial CSF	Standard	0.5	5.71	1494.7	3566.8	0.419	0.457	-8.5
750 pg/mL artificial CSF	Standard	0.75	5.71	2733.5	4152.0	0.658	0.727	-3.1
1 ng/mL artificial CSF	Standard	1	5.71	3166.8	3792.5	0.835	0.926	-7.4
5 ng/mL artificial CSF	Standard	5	5.72	14773.9	3148.3	4.693	5.270	5.4
7.5 ng/mL artificial CSF	Standard	7.5	5.72	24576.9	3877.0	6.339	7.125	-5
10 ng/mL artificial CSF	Standard	10	5.72	33343.3	3662.5	9.104	10.238	2.4

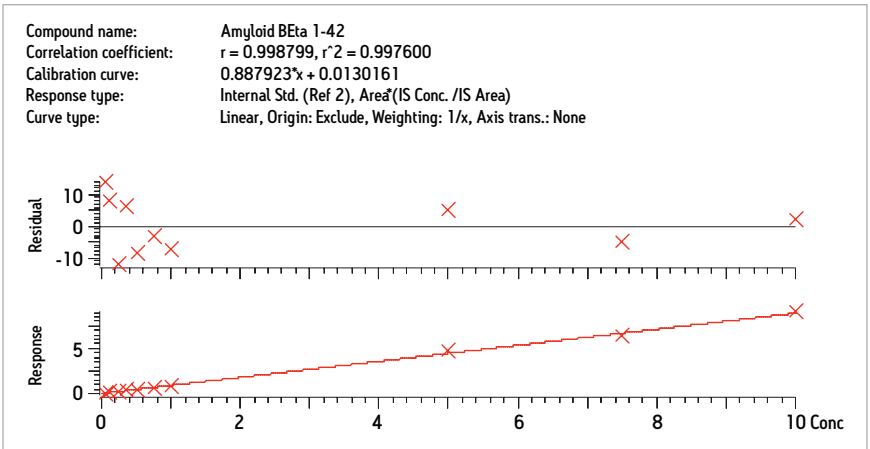


Figure 5. Representative standard curve and statistics from 0.05-10 ng/mL for amyloid β 1-42 extracted from artificial CSF + 5% rat plasma.

Table 3. Baseline levels of amyloid β peptides in 2 sources of pooled human CSF.

	Average Basal Level	% RSD of Basal Level	IS % RSD
Amyloid β 1-38 Human CSF 1	1.396	5.3	6.4
Amyloid β 1-38 Human CSF 2	0.702	1.7	
Amyloid β 1-40 Human CSF 1	5.429	3.3	4.7
Amyloid β 1-40 Human CSF 2	2.611	2.7	
Amyloid β 1-42 Human CSF 1	0.458	5.2	6.6
Amyloid β 1-42 Human CSF 2	0.226	1.9	

Table 4. Average deviation values for all overspike QC samples.

	QC 0.04 ng/mL	QC 0.075 ng/mL	QC 0.15 ng/mL	QC 0.2 ng/mL	QC 0.8 ng/mL	QC 2 ng/mL	QC 6 ng/mL
Amyloid β 1-38 Human CSF 1 and 2	2.3	5.8	-3.2	7.3	14.8	5.1	13.1
Amyloid β 1-40 Human CSF 1 and 2	-0.8	-3.2	-1.9	2.5	-2.6	-4.2	-3.8
Amyloid β 1-42 Human CSF 1 and 2	1.3	13.4	-3.6	5.6	2.0	-0.6	-0.2

RESULTS AND DISCUSSION

- The increased sensitivity of the Xevo TQ-S triple quadrupole mass spectrometer facilitated the use of 4X less sample and a 4-5X improvement in quantification limits.
- An SPE-UPLC/MS/MS bioanalytical method was developed and validated for the simultaneous quantitation of multiple amyloid β peptides in human and monkey CSF.
- The combination of a highly selective extraction method based on mixed-mode SPE in µElution format and the resolution of UPLC chromatography was critical to achieving the accurate, precise and reliable quantitation of 3 major amyloid β peptides in human and monkey CSF.
- The use of positive ion MS/MS and b ion sequence fragments provided the MS specificity required for this application.
- 96 samples can be extracted and ready for injection in <30 minutes, providing the sample prep throughput required for pre-clinical and clinical studies.
- The method described herein eliminates time-consuming immunoassays or immunoprecipitation steps for pre-clinical work.
- This approach also allows one assay for the simultaneous measurement of several different amyloid β peptides from a single sample. This single assay provides a high degree of selectivity and specificity in a high-throughput format while still achieving the high sensitivity required for low level endogenous amyloid β peptides.
- The use of a single UPLC/MS/MS assay represents a significant advantage over an ELISA assay, which would require multiple assays with multiple antibodies to quantify each of the relevant peptides.

ORDERING INFORMATION

Products Used in this Application	Part Number
Oasis MCX µElution Plate	186001830BA
ACQUITY UPLC BEH C_{18}, 300Å, 2.1 x 150 mm, 1.7 µm, Peptide Separation Technology Column	186003687

References

1. T.A. Lanz, J.B. Schachter, Journal of Neuroscience Methods 169 (2008) 16-22.
2. T. Oe et al, Rapid Communications in Mass Spectrometry 20 (2006) 3723-3735.
3. JR Slemmon et al, Journal of Chromatography B 846 (2007) 24-31.
4. NT Ditto et al, Journal of Neuroscience Methods 182 (2009) 260-265.
5. T.A. Lanz, J.B. Schachter, Journal of Neuroscience Methods 157 (2006) 71-81.
6. MJ Ford at al, Journal of Neuroscience Methods 168 (2008) 465-474.
7. E. Portelius et al, Journal of Proteome Research, 6 (2007) 4433-4439.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

ionKey/MS™

NOW YOU CAN START THINKING OF

MICROSCALE SENSITIVITY

IN TERMS OF POSSIBILITIES,

NOT RESTRAINTS.

Discover Waters® ionKey/MS™ System. Open the door to the new world of robust, reliable and reproducible microscale with dramatically enhanced sensitivity and significantly lower solvent consumption compared to 2.1 mm ID chromatography. This plug & play MS system gives your whole lab access to UPLC® chromatography performance and highly sensitive, reliable data. This is where the industry's going. You can get there today. To learn more about what's possible, visit waters.com/ionkey

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

PHARMACEUTICAL • HEALTH SCIENCES • FOOD • ENVIRONMENTAL • CHEMICAL MATERIALS

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

An Improved SPE-LC-MS/MS Method for the Quantification of Bradykinin in Human Plasma Using the ionKey/MS System

Mary E. Lame, Erin E. Chambers, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- 2x reduction in sample and 10x increase in sensitivity facilitates multiple injections of samples for improved accuracy or to meet guidelines for ISR.
- 50x reduction in solvent consumption reduces cost of analysis.
- SPE using mixed-mode SPE reduces matrix interferences and enhances selectivity of the extraction for bradykinin in plasma.
- 96-well µElution™ plate format enables concentration of the sample while maintaining solubility and minimizes peptide losses due to adsorption to reach detection limits of 2.5 pg/mL for brakyinin in plasma.
- Selective, fast SPE extraction (<30 minutes) without time-consuming immunoaffinity purification.

WATERS SOLUTIONS

ionKey/MS™ System
ACQUITY UPLC® M-Class System
ionKey™ Source
Xevo® TQ-S
iKey™ Separation Device
MassLynx® 4.1 Software
Oasis® WCX 96-well µElution Plate
Waters Collection Plate
TargetLynx™ Application Manager

KEY WORDS

bioanalysis, Oasis, sample preparation, peptide quantification, bradykinin, UPLC, 2D Technology, plasma, ionKey/MS, iKey

INTRODUCTION

The need for robust and sensitive analysis of peptide species challenges both chromatographic separation and mass spectrometry. Peptides, in general, are often difficult to analyze by LC-MS/MS, as mass spectrometer (MS) sensitivity is low due to the formation of multiple precursors and poor or overly extensive fragmentation, making liquid chromatography (LC) and sample preparation even more critical. A previous application note (720004833EN) described in detail the development of a fast, flexible analytical scale, SPE-LC-MS/MS method for the quantification of the peptide bradykinin (Figure 1) in human plasma for use as a biomarker in the preclinical or discovery setting.[1] Accurate quantification of bradykinin in plasma is particularly challenging because it is present in low pg/mL levels, is rapidly metabolized, and is also artificially produced during blood sampling and sample preparation via proteolytic processes.[2]

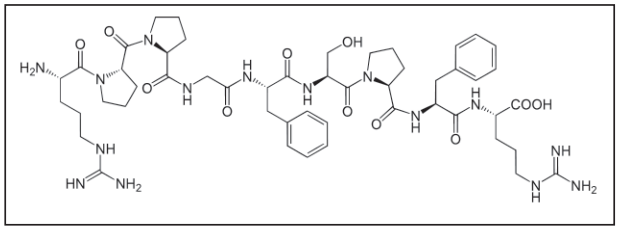


Figure 1. Representative structure and amino acid sequence of bradykinin.

In this work, the LC-MS platform was updated to incorporate the use of the ionKey/MS System which integrates the UPLC® analytical separation directly into the source of the MS (Figure 2). The iKey Separation Device (150 µm I.D.), shown in Figure 3, contains the fluidic channel, electronics, ESI interface, heater, eCord,™ and the chemistry to perform UPLC separations. Additionally, this technology offers significant increases in sensitivity compared to 2.1 mm I.D. chromatography, making it ideal for peptide analyses. Most bioanalytical LC-MS/MS assays often consume high volumes of both solvent and sample, thus increasing the cost of the assay and limiting the number of replicates that can be analyzed. In addition to the sensitivity increase the ionKey/MS System provides over the 2.1 mm diameter scale, it also reduces solvent and sample consumption and provides enough sample to perform multiple injections that may be required to meet incurred sample reanalysis (ISR) guidelines.

EXPERIMENTAL

Method conditions

UPLC conditions

LC system:	ACQUITY UPLC M-Class with 2D Technology configured with optional trap and back flush elution
Separation device:	Key Peptide BEH C_{18} Separation Device, 300Å, 1.7 µm, 150 µm x 50 mm (p/n 186006969)
Trap column:	ACQUITY UPLC M-Class Symmetry® C_{18}, 5 µm, 300 µm x 50 mm (p/n 186007498)
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Loading solvent:	99:1 mobile phase A:B, 25 µL/min for first two minutes, reverse valve
Valve position:	Initial position one (forward loading of trap), switch to position two at two minutes (back flush elute of trap onto the analytical column)
Analytical gradient:	See Table 1
Elution flow rate:	2.5 µL/min
iKey temp.:	75 °C
Sample temp.:	15 °C
Injection vol.:	10 µL
Total run time:	12.0 minutes
Collection plates:	Waters 1 mL collection plates (p/n 186002481)

MS conditions

MS system:	Xevo TQ-S Mass Spectrometer with ionKey Source and iKey Seperation Device
Ionization mode:	ESI positive
Capillary voltage:	3.8 kV
Source temp.:	120 °C
Cone gas flow:	50 L/hr
Collision cell pressure:	3.83 x $10^{(-3)}$ mbar
Collision energy:	Optimized by component, see Table 2
Cone voltage:	Optimized by component, see Table 2

Data management

Chromatography software:	MassLynx 4.1
Quantification software:	TargetLynx

Time (min)	Flow rate (mL/min)	Composition A (%)	Composition B (%)	Curve
0.00	2.5	98.0	2.0	Initial
0.50	2.5	98.0	2.0	6
5.00	2.5	50.0	50.0	6
6.00	2.5	5.0	95.0	6
7.00	2.5	5.0	95.0	6
8.00	2.5	98.0	2.0	6

Table 1. UPLC gradient conditions.

This study utilizes specifically designed blood collection techniques to inhibit bradykinin formation *ex vivo*, takes advantage of mixed mode solid-phase extraction (SPE) and use of the novel and highly efficient ionKey/MS System for selective, sensitive, and robust chromatographic separation, and quantification of the nonopeptide bradykinin. The sensitivity increase that ionKey/MS System provides over the 2.1 mm diameter scale method for bradykinin enables a 2x reduction in plasma and a 7–10x increase in signal-to-noise (S:N). As a result, we can accurately and precisely quantify 2.5 pg/mL of bradykinin above the basal level.

Sample preparation

Blood collection

Human plasma was obtained from one male donor whose blood was collected in BD™ P100, P700, P800, and blood collection tubes containing only K_2EDTA. The various BD P blood collection tubes contain various mixtures of proprietary stabilizers/inhibitors that immediately solubilize during blood collection, and enable preservation of human plasma proteins and peptides.

Sample pretreatment

10 µL of the internal standard (IS), [Lys-des-Arg9]-bradykinin (5 ng/mL) was added to 100 µL of human plasma and mixed. The samples were then diluted 1:1 with 5% NH_4OH in water and mixed.

Sample extraction

Pretreated plasma samples were extracted according to the protocol in Figure 4. All solutions are made up by volume. All extraction steps were applied to all wells of the Oasis WCX 96-well µElution Plate that contained samples.

Figure 2. ionKey/MS System: comprised of the Xevo TQ-S, the ACQUITY UPLC M-Class, the ionKey Source, and the iKey Separation Device.

Figure 3. iKey Separation Device.

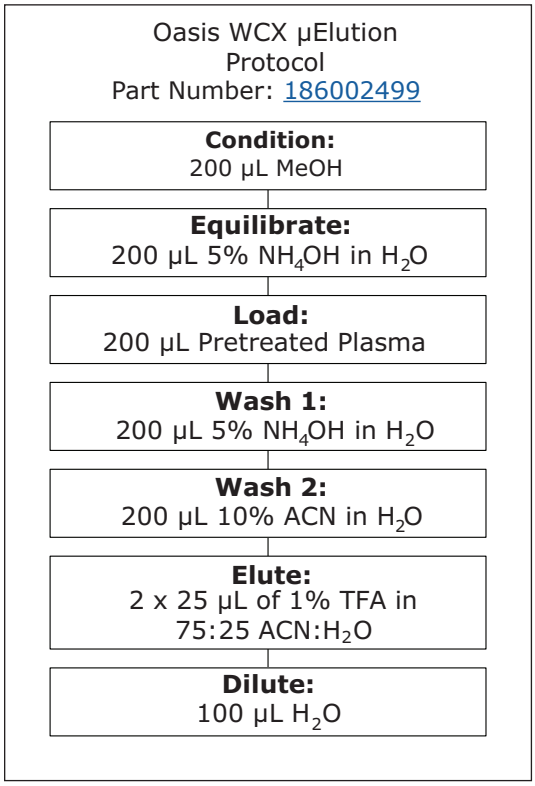


Figure 4. Oasis µElution WCX extraction protocol.

RESULTS AND DISCUSSION

Mass spectrometry

The 3+ precursors of bradykinin (*m/z* 354.18) and IS (344.94) were used for quantitation. The fragment at *m/z* 419.18 $y3^{1+}$ was chosen as the primary fragment for bradykinin quantitative analysis, while the *m/z* 408.18 $b4^{1+}$ fragment was used for confirmatory purposes. For the IS, the fragment at *m/z* 386.03 $b7^{2+}$ was chosen. Optimal MS conditions are shown in Table 2. Although many peptides produce intense fragments below *m/z* 200, these ions (often immonium ions) result in high background in extracted samples due to their lack of specificity. In this assay, the use of highly specific b or y ion fragments with *m/z* values higher than their precursors yielded significantly improved specificity, facilitating the use of simpler LC and SPE methodologies.

Compound	Precursor	MRM transition	Cone voltage (V)	Collision energy (eV)	Production type
Bradykinin	$[M+3H]3^+$	354.18 > 419.18	10	8	$[1H^+]$ 1/γ3
	$[M+3H]3^+$	354.18 > 408.18	10	10	$[1H^+]$ 1/b4
[Lys-des-arg9]-Bradykinin(IS)	$[M+3H]3^+$	344.94 > 386.03	10	10	$[2H^+]$ 1/b7

Table 2. MRM transitions, collision energies, and cone voltages for bradykinin and [Lys-des-Arg9] bradykinin, the internal standard (IS).

Chromatographic separation

Chromatographic separation of bradykinin and its IS was achieved using the novel microfluidic chromatographic iKey Separation Device. The iKey Separation Device has a channel with UPLC-grade, sub-2-µm particles that permits operation at high pressure and results in highly efficient LC separations. By integrating microscale LC components into a single platform design, problems associated with capillary connections, including manual variability, leaks, and excessive dead volume are avoided. Use of the iKey Peptide BEH C18 Separation Device, 300Å, 1.7 µm, 150 µm x 50 mm (p/n 186006969) provided excellent peak shape, increased peak height, and improved S:N compared to the analytical scale (2.1 mm I.D.) LC-MS analysis. Representative chromatograms of bradykinin and the IS using the iKey Separation Device are shown in Figure 5. The use of multidimensional chromatography, specifically a trap and back-elute strategy, provided further sample cleanup and facilitated the loading of 10 µL of the high organic SPE eluate (required to maintain solubility of the peptides) without experiencing analyte break through. Additionally, the ability to inject the larger sample volumes typical for analytical scale LC analysis (e.g. 10 µL) on the iKey Separation Device can provide the substantial gains in sensitivity that are often required to accurately and reliably detect low pg/mL levels of peptide and protein in complex matrices.

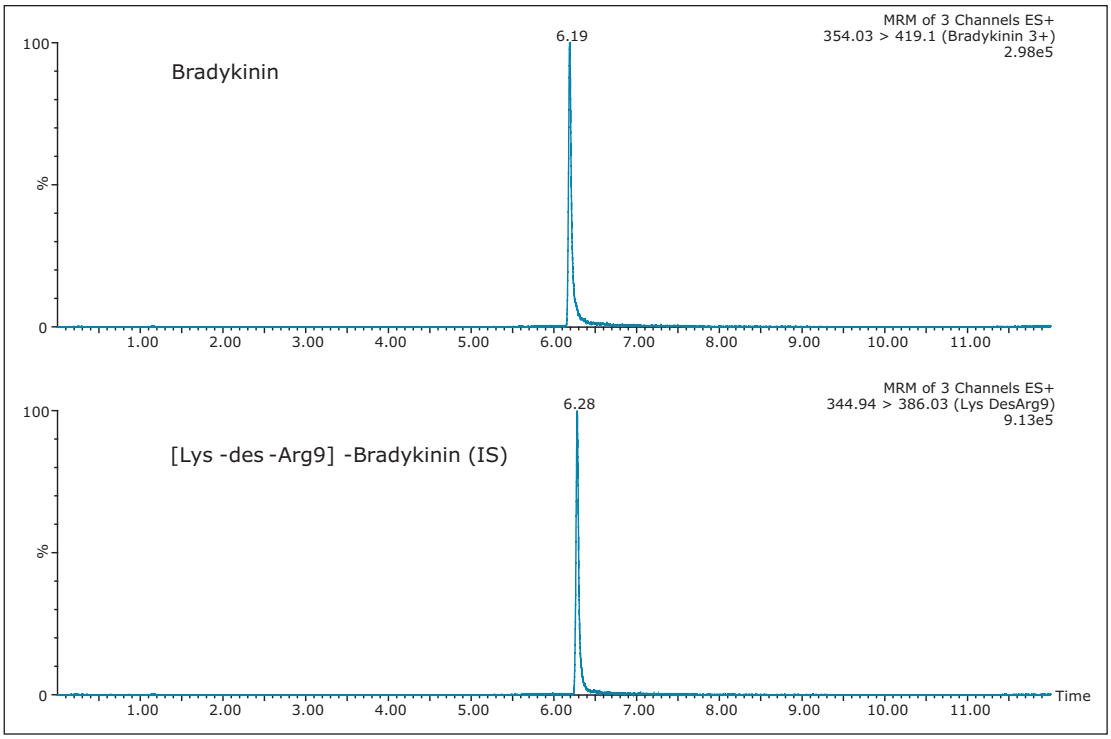


Figure 5. UPLC separation of bradykinin and internal standard, from extracted plasma, using the iKey Peptide BEH C_{18} Separation Device, 300Å, 1.7 µm, 150 µm x 50 mm (p/n 186006969)

Enhanced sensitivity with the use of ionKey/MS System

Use of the ionKey/MS System facilitated the development of a highly efficient LC separation of bradykinin in plasma with significant improvement in sensitivity and S:N over the analytical scale LC-MS using 2.1 mm I.D. chromatography. Initially, samples were extracted using the protocol described in the previous application note (720004833EN). Briefly, 200 µL of plasma was extracted followed by a 1:1 dilution of the eluate with water. A 3 µL injection of this sample on the iKey Separation Device provided a 5x improvement in S:N compared to a 10 µL injection of the same sample analyzed at the 2.1 mm scale, and is shown in Figure 6. The improvement in ionization efficiency and subsequent increase in sensitivity afforded by the iKey Separation Device facilitated this lower injection volume. The ability to obtain comparable or improved sensitivity with smaller injection volumes (1–3 µL) using the ionKey/MS System makes this technology ideal when sample is limited or when multiple injections are required to meet ISR guidelines.

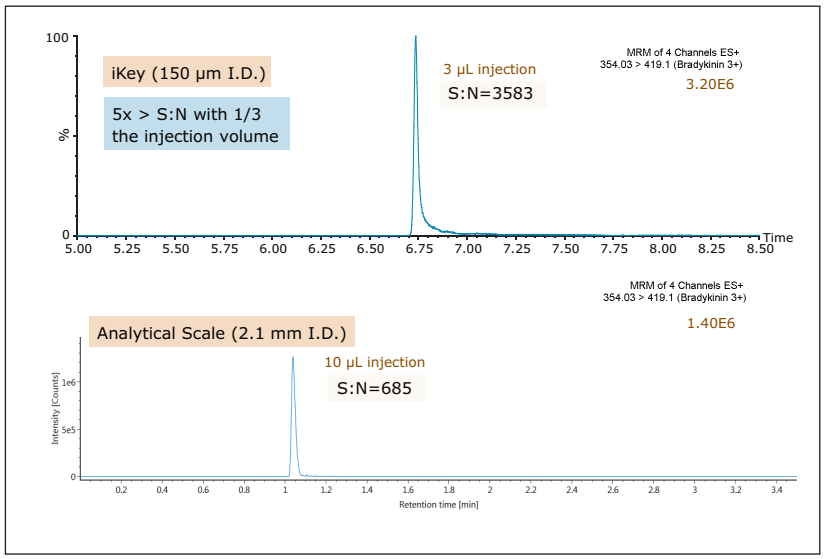


Figure 6. Comparison of 1 ng/mL over-spiked bradykinin extracted from human plasma (200 µL): iKey Separation Device (150 µm I.D.) vs. traditional analytical flow (2.1 mm I.D.).

Method optimization resulted in the reduction of the required plasma sample by half and an increase in eluate dilution to 1:2, both of which minimized matrix interferences. A comparison of a 10 µL injection of extracted plasma (using the optimized method) the ionKey/MS System and a traditional analytical flow system (ACQUITY UPLC and Xevo TQ-S with UNIFI®) resulted in a 10x increase in signal and 7x increase in S:N with the ionKey/MS System. This improvement is illustrated in Figure 7, with a comparison of endogenous levels of bradykinin. Ultimately, the use of the 150 µm iKey Separation Device enabled the development of a low flow quantitative MRM method for bradykinin that achieved a detection limit of 2.5 pg/mL with only 100 µL of plasma.

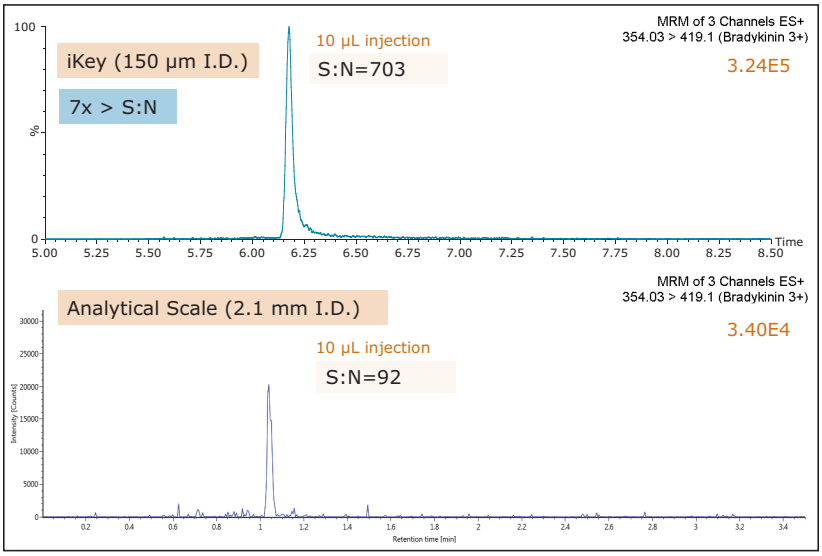


Figure 7. Comparison of endogenous levels of bradykinin extracted from human plasma (100 µL): iKey Separation Device (150 µm I.D.) vs. traditional analytical flow (2.1 mm I.D.).

Sample preparation

The development and optimization of the SPE method was described in detail in the previous application note (720004833EN) and was employed for this study. Use of the Oasis WCX SPE, provided both reversed-phase and ion-exchange modes of retention, enabling greater sample cleanup, selectivity, and ultimate sensitivity for this peptide. Additionally, the Oasis WCX 96-well µElution Plate (p/n 186002499) can be processed manually in under 30 minutes and is compatible with most liquid-handling robotic systems for automation to meet sample throughput requirements. This format also provides the ability to elute in very small sample volumes of only 50 µL, minimizing the potential for peptide losses that might occur during evaporation due to adsorption to the walls of collection plates and/or chemical instability.

Linearity, accuracy, and precision

To generate standard curves, human plasma (derived from blood collected in BD P100 tubes) was fortified with bradykinin at the following final concentrations: 2.5, 5, 10, 20, 40, 60, 100, 600, 1,000, 2,000, 4,000, and 8,000 pg/mL. Each standard level was prepared in duplicate. Quality control (QC) samples were prepared from the same plasma at 15, 30, 50, 150, 300, 800, and 6,000 pg/mL. QC samples at each level were prepared in triplicate. [Lys-des-Arg9]-Bradykinin (final concentration of 0.5 ng/mL) was used as the internal standard (IS). Peak area ratios (PARs) of the analyte peak area to the IS peak were calculated. The calibration curve was constructed using PARs of the calibration samples by applying a one/concentration (1/x) weighted linear regression model. All QC sample concentrations were then calculated from their PARS against the calibration curve. Due to the presence of endogenous bradykinin, standard addition was used. The mean basal level of bradykinin (0.19 ng/mL) in control plasma samples was determined by calculating the x-intercept. The calculated basal level was then added to the spiked concentration for all standard curve and QC samples to enable accurate quantification. Using 1/x regression, bradykinin was linear with an R^2 value of >0.99. A summary of standard curve performance is shown in is shown in Table 3. Results from QC analysis are shown in Table 4. At all levels, QC samples demonstrated very good accuracy and precision, with mean accuracies ranging from 92.7–104.0 and mean %CV's of 1.21–4.31. These results easily meet the recommended FDA acceptance criteria outlined in the white papers describing best practices in bioanalytical method validation for LC-MS/MS assays.[3,4]

Bradykinin overspiked concentration (ng/mL)	Final bradykinin concentration (ng/mL)	Area	IS area	Response	Calculated bradykinin concentration (pg/mL)	Mean accuracy
0.0025	0.1925	8595	20655	0.417	0.1891	98.2
0.0050	0.1950	8369	19474	0.430	0.1956	100.3
0.0100	0.2000	8493	19296	0.441	0.2008	100.4
0.0200	0.2100	8906	19386	0.460	0.2100	100.0
0.0400	0.2300	10287	19462	0.528	0.2432	105.8
0.0600	0.2500	10775	19588	0.551	0.2542	101.7
0.1000	0.2900	11441	19119	0.598	0.2771	95.5
0.3000	0.4900	20435	20694	0.988	0.4656	95.0
0.6000	0.7900	30256	18599	1.628	0.7753	98.2
1.0000	1.1900	54216	20792	2.608	1.2495	105.0
2.0000	2.1900	92974	19438	4.782	2.3018	105.1
4.0000	4.1900	181824	21490	8.454	4.0784	97.4
8.0000	8.1900	349881	20616	16.966	8.1969	0.1

Table 3. Standard curve summary and statistics for bradykinin extracted from human plasma.

Bradykinin overspiked concentration (ng/mL)	Bradykinin QC concentration (ng/mL)	Mean concentration (ng/mL)	SD	%CV	Mean accuracy
0.0000	–	0.1860	0.003	1.62	–
0.0150	0.2050	0.2078	0.003	1.47	101.4
0.0300	0.2200	0.2268	0.003	1.26	103.1
0.0500	0.2400	0.2360	0.010	4.11	98.3
0.1500	0.3400	0.3152	0.014	4.31	92.7
0.3000	0.4900	0.4854	0.010	2.16	99.1
0.8000	0.9900	1.0293	0.031	3.06	104.0
6.0000	6.1900	6.0504	0.073	1.21	97.8

Table 4. QC statistics from bradykinin extracted from human plasma.

Assessment of pre-analytical handling and endogenous bradykinin levels

Accurate quantification of bradykinin in plasma is particularly challenging because it is metabolized rapidly, with a half life of less than 30 seconds, and can be artificially produced during blood sampling and sample preparation, via proteolytic processes.[2,5,6] To assess the best preservation of bradykinin in blood, as well as to prevent the formation of bradykinin *ex vivo*, particular attention was paid to the protocol for blood collection which employed the use of commercially-available blood collection tubes containing proprietary additives that provide enhanced recovery plasma analytes. More specifically, the BD P100, P700, and P800 collection tubes provide a means of preservation of plasma to be used in peptide and protein analysis.[7] The original work presented (720004833EN) only assessed the preservation of bradykinin in P100 blood collection tubes. The BD P100 and P700 blood collection tubes contain proprietary mixtures of additives and inhibitors. The BD P100 collection tubes also contain a mechanical separator that allow for ease of collection and separation of the plasma after blood centrifugation. The P700 tubes contain the same inhibitors as the P100 tubes, with an additional inhibitor for stabilization of Glucagon-Like Peptide I (GLP-1) and contains no mechanical separator. P800 blood collection tubes, like the P100 and P700 blood cllection tubes, contain a proprietray cocktail of inhibitors that provide preservation of bioactive peptide in plasma, and contains no mechanical separator. The P800 blood collection tubes are marketed for assays that require quantitation and measurement of the GLP-1, Glucose-Dependent Insulinotropic Polypeptide (GIP), Glucagon, and Ghrelin.

Mean extracted endogenous plasma bradykinin concentrations, in which the blood was collected with (P100, P700), and without protease inhibitors (K2EDTA only, days 1 and 4) are shown in Table 5. Average CV's of the endogenous bradykinin levels ranged from 0.88–2.18%, indicating a very robust and reproducible method. Representative chromatograms for these results are shown in Figure 8 (panels A-D). Panel A is a representative chromatogram of endogenous plasma bradykinin obtained from blood collected in the P100 tubes, with a mean calculated concentration of 0.1860 ng/mL. P700 blood collection yielded a mean endogenous bradykinin plasma level of 0.0945 ng/mL, and is shown in Panel B. This concentration was approximately half of the concentration determined using the P100 tubes. The artifactual formation of bradykinin in plasma without inhibitor is demonstrated in panels C and D. In these cases, blood was collected in K2EDTA-only blood collection tubes, and the subsequent plasma was brought though 1 freeze/thaw (F/T) cycle. Panel C represents the bradykinin concentration on day 1, where the bradykinin plasma level increased to 0.8107 ng/mL. Panel D represents the bradykinin concentration after 4 days of storage at 10 °C, where bradykinin plasma levels increased to 5.4916 ng/mL. Endogenous levels of bradykinin using the P800 showed relative area counts similar to that of the P700 collection (data not shown), but due to a 10x signal loss of the IS in the P800 tube samples endogenous levels of bradykinin were not calculated for the P800 sample collection. It is assumed that the analogue IS was not protected from metabolism and/or degradation in the P800 tube due to differences in the cocktail of inhibitors. Further, the reduced endogenous bradykinin plasma levels using the P700 collection tube indicated that this cocktail of inhibitors may be more appropriate for stabilization and prevention of ex vivo bradykinin formation. However, another possibility that was not explored was that the presence of the plasma mechanical separator provided a mechanism of bradykinin formation prior or during blood collection and centrifugation. These results further emphasize the need for proper sample collection and storage to accurately quantify endogenous bradykinin plasma levels.

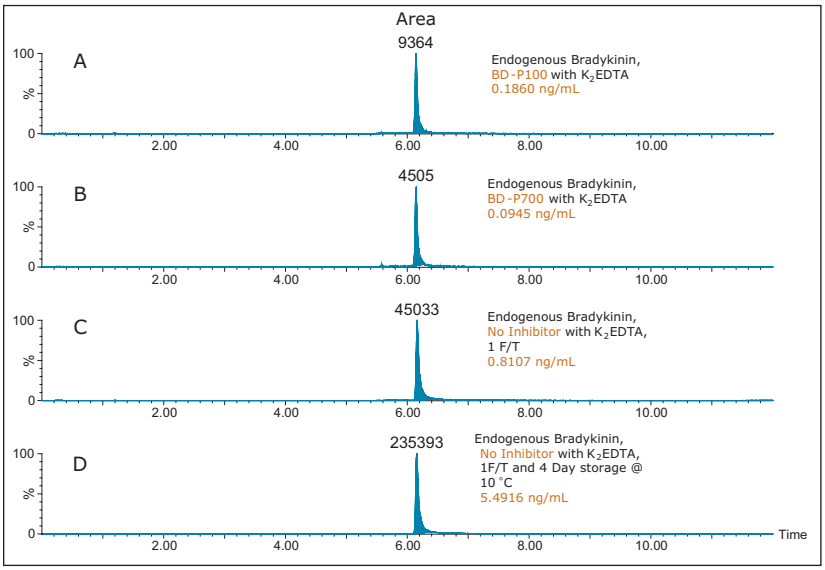


Figure 8. Representative chromatograms of extracted endogenous plasma bradykinin, in which the blood was collected with (P100, P700), and without protease inhibitors (K2EDTA only).

Plasma treatment	Mean concentration (ng/mL)	SD	%CV
BD-P100	0.1860	0.003	1.62
BD-P700	0.0945	0.002	2.18
BD-No inhibitor with K2EDTA, 1F/T	0.8107	0.007	0.88
BD-No inhibitor, K_2EDTA, 1 F/T, 4 days at 10 °C.	5.4916	0.110	2.01

Table 5. Mean extracted endogenous plasma bradykinin, in which the blood was collected with (P100, P700), and without protease inhibitors (K2 EDTA only, days 1 and 4).

CONCLUSIONS

Use of the ionKey/MS System, mixed-mode SPE and higher *m/z* b or y ion MS fragments provided the level of selectivity and sensitivity necessary to accurately quantify bradykinin and distinguish subtle differences in concentrations. The current analysis uses 100 µL of plasma and provides a significant improvement in sensitivity and S:N over the analytical scale LC-MS analysis which uses twice as much sample. The use of the 150 µm iKey Separation Device enabled the development of a highly sensitive, low flow quantitative MRM method for bradykinin that could distinguish a change of 2.5 pg/mL of bradykinin over the basal level. Standard curves were accurate and precise from 2.5–8,000 pg/mL. QC samples at all levels easily met recommended FDA regulatory criteria[4,5] with mean accuracies ranging from 92.7–104.0 and mean %CV's of 1.20–4.31, indicating an accurate, precise, and reproducible method. Furthermore, an injection of the same volume (10 µL) of sample corresponded to a >10x increase in on-column sensitivity as compared to the traditional analytical flow method for this peptide. In addition to the sensitivity increase the ionKey/MS System provides over the 2.1 mm I.D. scale, it also reduces solvent and sample consumption, thereby reducing cost and allowing for multiple injections of samples for improved accuracy or to meet the guidelines for ISR. This study also demonstrates the importance of proper sample collection with appropriate additives for the stabilization/preservation of bradykinin in plasma to accurately represent endogenous levels. This method shows great promise for high sensitivity quantification of bradykinin in patient samples from PK and clinical studies using the ionKey/MS System if further validation was performed.

References

1. Lame ME, Chambers EE, Fountain KJ, Development of a Quantitative SPE LC-MS/MS Assay for Bradykinin in Human Plasma, Waters Application Note 720004833EN. 2013.
2. Murphey LJ, Hachey DL, Oates JA, Morrow JD, and Brown NJ, Metabolism of Bradykinin *In Vivo* in Humans: Identification of BK1-5 as a Stable Plasma Peptide Metabolite *J Pharmacol Exp Ther* July 1, 2000 294:263–269.
3. Viswanathan CT, Bansal S, Booth B, DeStefano AJ, Rose MJ, Sailstad J, Shah VP, Skelly JP, Swann PG, Weiner R, Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays, *Pharm. Res.*, 24 (2007) 1962–1973.
4. Bansal S, DeStefano A, Key elements of bioanalytical method validation for small molecules, *AAPS J.*, 9 (2007) E109-114.
5. Cugno M, Agostoni P, Brunner HR, Gardinali M, Agostoni A, Nussberger. Plasma bradykinin levels in human chronic congestive heart failure *J. Clin Sci (Lond).* 2000 Nov; 99(5):461–6.
6. Nussberger J, Cugno M, Amstutz C, Cicardi M, Pellacani A, Agostoni A. Plasma bradykinin in angio-oedema. *Lancet*. 1998 Jun 6; 351(9117):1693–7.
7. Molecular Diagnostics and Proteomics Blood Collection Systems, BD Diagnostics Corporation. http://www.bd.com/proteomics/pdfs/Molecular_Proteomics_Catalog.pdf

Acknowledgements

We would like to thank:

Jeffrey Widdos and Biological Specialty Corporation for providing excellent service and assistance in the special collection of human plasma used in this work.

David Craft and BD Diagnostics Corporation for providing blood collection tubes for assessment of bradykinin plasma levels, and for the technical discussion regarding the various BD blood collection tubes.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Ultrasensitive Quantification Assay for Oxytocin in Human Plasma Using the ionKey/MS System

Catalin E. Doneanu and Paul Rainville
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Ultrasensitive quantification of a therapeutic peptide in plasma.
- LLOQ equivalent to ELISA assays, but the MRM method is less time-consuming to develop, more reliable, and more precise.
- Protein precipitation followed by SPE clean-up reduces significantly the amount of matrix in the final extract, thus providing increased assay robustness.
- The ionKey/MS System offers significant advantages in terms of operating costs of the assay when compared to analytical-scale chromatographic separations.

WATERS SOLUTIONS

ionKey/MS™ System

ACQUITY UPLC® M-Class System

iKey™ Separation Device

Xevo® TQ-S Mass Spectrometer

Oasis® HLB 96-well Plates

MassLynx® Software

TargetLynx™ Application Manager

ionKey™ Source

*Rapi*Gest™

KEY WORDS

Capillary-flow microfluidic system, LC-MS, LC-MRM, ESI-MS, bioanalysis, quantification, oxytocin, plasma, protein precipitation, SPE, protein binding

INTRODUCTION

Quantitative LC-MRM (multiple reaction monitoring) methods for small molecule drugs are used to provide bioanalytical support in various stages of drug discovery and development. These methods can routinely achieve lower limits of quantification (LLOQ) in the range of 50 to 100 pg/mL, in various biological matrices, using analytical-scale chromatography (e.g., 2.1 mm I.D. UPLC® Columns). In the case of peptide therapeutics, these assays are more challenging because lower LLOQs are often required.

One method that allows significant sensitivity enhancements is to operate the LC-MS system at lower LC flow rates, which provides reduced chromatographic peak volumes and increased ionization efficiency with electrospray ionization mass spectrometry (ESI-MS). However, most "homemade" capillary-flow LC-MS configurations suffer from a lack of robustness and are often not able to provide adequate sample throughput. The ionKey/MS System is an integrated capillary-flow microfluidic system that is designed to operate in the flow range of 1 to 5 µL/min, which can provide a 10- to 20-fold increase in sensitivity for therapeutic peptides when compared to conventional analytical-scale LC-MS platforms.

Oxytocin (OT) is a mammalian, 9-amino-acid cyclic peptide (CYIQNCPLG-NH_2) that acts primarily as a neurotransmitter in the brain. Quantitative measurement of endogenous OT in biological samples is very challenging, because it is present at low pg/ml concentrations in human plasma.[1] ELISA[2-3] and mass spectrometry assays[3-6] have been previously reported for measurement of endogenous OT levels. However, the LLOQ of commercial ELISA assays for OT is above the endogenous level. Several methods using mass spectrometry have been developed recently using affinity capture for OT enrichment,[3] two-dimensional (2D) LC-MS/MS using a tandem quadrupole MS in conjunction with large-volume sample extraction (1.4 mL human plasma),[4] or 2D-LC-MS/MS with large volume injection[5] to achieve the required sensitivity.

Here we report an LC-MRM method developed on the ionKey/MS System that is able to detect very low levels of OT in human plasma, at an LLOQ of 10 pg/mL.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC M-Class System
Separation device:	iKey BEH C_{18} Separation Device, 130Å, 1.7 µm, 150 µm x 100 mm (p/n 186007258)
Mobile phase A:	0.1% Formic acid (FA) in water
Mobile phase B:	0.1% Formic acid in acetonitrile (ACN)
Flow rate and gradient:	See Table 1
iKey temp.:	60 °C
Sample temp.:	10 °C
Injection vol.:	3 µL
Total run time:	8 min

MS conditions

MS system:	Xevo TQ-S with ionKey Source
Ionization mode:	+ESI
ESI voltage:	3.2 kV
Source temp.:	100 °C
Nebulizing gas pressure:	0.2 bar
MRM transitions:	See Table 2
Cone voltage:	See Table 2
Collision energy:	See Table 2

Data management

Chromatography software:	MassLynx Software
Quantification software:	Target Lynx Application Manager

Sample preparation

Oxytocin (Sigma Aldrich, St. Louis, MO, USA) was spiked in 200 µL of K2-EDTA human plasma (Bioreclamation, East Meadow, NY, USA) at the following concentrations: 10, 20, 100, 200, 1,000, 10,000, and 20,000 pg/mL. $^{13}C^{15}N$-isotopically labeled OT (CYIQNCPLG-NH_2, Sigma Aldrich) was added as an internal standard (IS) at 100 pg/mL in all samples. Protein precipitation was performed after adding 200 µL of acetonitrile to achieve 1:1 sample dilution. Samples were vortexed briefly (5–10 sec), and then were spun at 4,000 RPM for 15 minutes (at room temperature) using a 5810R centrifuge (Eppendorf, Hauppauge, NY, USA). The supernatant (200 µL) was diluted with 1.8 mL of 4% H_3PO_4 and sample clean-up was performed using an Oasis HLB 96-well Plate, 5 mg sorbent per well, 30 µm Particle Size (p/n 186000309). The HLB extraction protocol is provided in Figure 1. After 1:1 dilution with 0.1% formic acid in DI water, 3 µL of sample were injected on the ionKey/MS System.

Time (min)	Flow rate (µL/min)	Eluent A (%)	Eluent B (%)
0.0	5.0	100.0	0.0
0.9	5.0	100.0	0.0
1.0	3.0	100.0	0.0
1.2	3.0	80.0	20.0
4.0	3.0	80.0	20.0
4.1	5.0	10.0	90.0
4.5	5.0	10.0	90.0
4.6	5.0	100.0	0.0
8.0	5.0	100.0	0.0

Table 1. Gradient conditions for the OT assay.

Oasis HLB Extraction Protocol P/N 186000309
Conditioning: 500 µL CH_3OH Equilibration: 500 µL 1% H_3PO_4 Sample loading: 2 mL of diluted supernatant from PPT Washing step no 1: 500 µL 1% H_3PO_4 Washing step no 2: 500 µL 5% CH_3OH Elution: 1 x 200 µL 30% ACN, 0.1% FA Dilution: 1:1 with 0.1% FA

Figure 1. Oasis HLB extraction protocol.

RESULTS AND DISCUSSION

One of the first experiments performed when developing an LC-MRM assay for a peptide therapeutic is to record a full scan ESI-MS spectrum of the analyte to establish its most abundant precursor. Figure 2A shows the ESI-MS spectrum of OT (average of 10 scans) recorded during analyte elution at a flow rate of 3 µL/min, after the injection of a standard containing 10 ng/mL OT, on the ionKey/MS System. Surprisingly, the most intense peptide precursor is the singly charged species at *m/z* = 1007.4 and not the expected doubly protonated ion at *m/z* = 504.2. This observation can be explained by the fact that OT contains a disulfide bond that restricts peptide protonation. In a separate LC injection, the MS/MS spectrum of OT (10 average scans) displayed in Figure 2B was produced from the same OT standard. Fragmentation of the singly charged precursor using a collision energy of 25 V produced a very abundant fragment ion at *m/z* = 723.3 assigned to a b6 ion. The best responding MRM transition (1007.4 ⟹ 723.3) was then optimized in terms of cone voltage and collision energy. The optimized parameters for OT and its internal standard are summarized in Table 2.

Peptide sequence	SRM transition	Dwell time (ms)	Cone voltage (V)	Collision energy (V)
CYIQNCPLG-NH_2	1007.4 ⟹ 723.3	50	100	28
CYIQNCPLG-NH_2	1014.4 ⟹ 730.3	50	100	28

Table 2. Optimized MRM transitions.

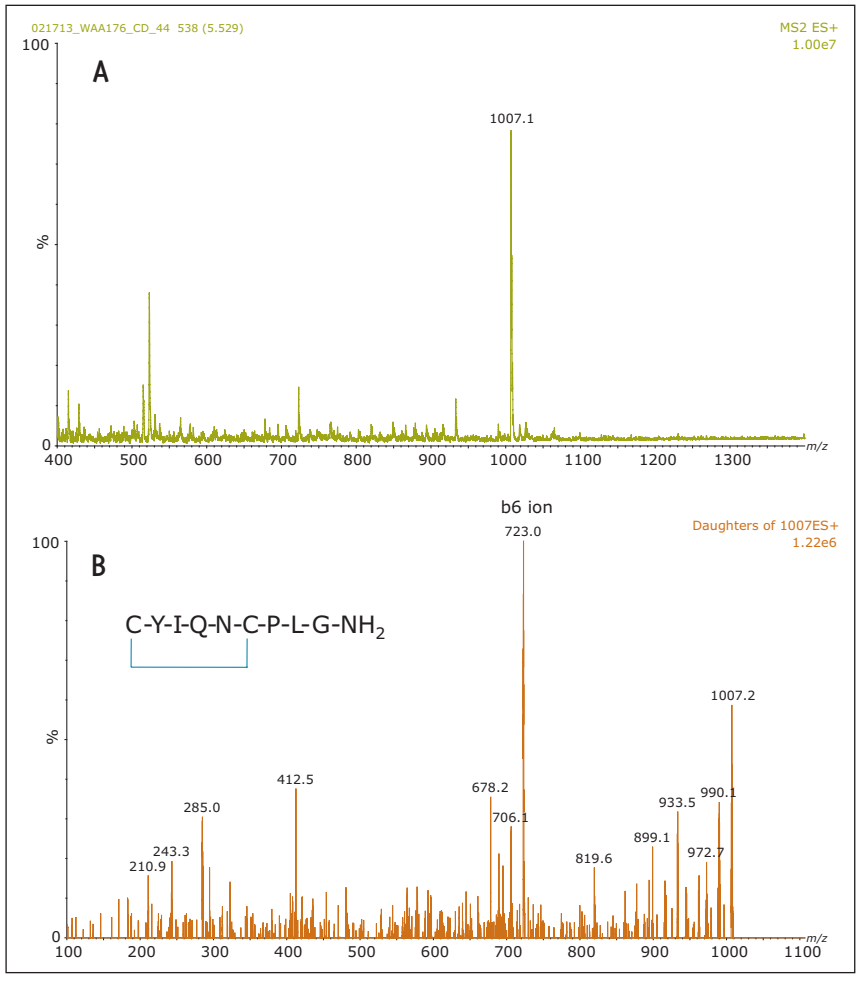


Figure 2A. ESI-MS spectrum of oxytocin (OT).

Figure 2B. ESI-MS/MS spectrum of OT produced by the fragmentation of the singly charged precursor using a CE of 28 V.

The sample preparation protocol was optimized to achieve efficient removal of sample matrix. While SPE is typically used as a one-step sample clean-up in many peptide therapeutic protocols, the amount of residual sample matrix can still be significant and can cause poor chromatography and decreased assay robustness after hundreds of sample injections on capillary-scale chromatography.

Protein precipitation offers an efficient way to decrease the amount of protein component matrix from plasma and it was used here in conjunction with SPE to increase method robustness. In addition, protein precipitation also provides a good opportunity to check for protein binding, as many peptide therapeutics are typical substrates for plasma proteins.

Protein binding can have significant negative effects on the ability of the LC-MRM assay to quantify the therapeutic peptide. Protein binding can be disrupted by surfactants (e.g., *Rapi*Gest) or protein denaturants (e.g., guanidine hydrochloride). In the case of oxytocin, protein binding was evaluated by comparing analyte recovery after protein precipitation in the presence and absence of several protein-binding disrupting reagents. Peak areas of OT, obtained for pre-spiked and post-spiked protein precipitated plasma samples, were used to calculate the OT recovery and the results are summarized in Table 3. With the exception of *Rapi*Gest recoveries, the values presented in this table indicate high analyte recoveries (70% to 85%) regardless of the precipitation protocol. Clearly, OT is not affected by protein binding and protein precipitation can be safely performed in the absence of detergents or protein denaturants.

Reagent concentration	OT Recovery (%)						
added to plasma	Rep01	Rep02	Rep03	Rep04	Rep05	Mean	RSD (%)
No reagent	75.3	81.9	81.9	81.9	76.3	79.5	4.2
0.1% *Rapi*Gest	52.1	61.6	56.1	52.8	49.6	54.4	8.5
8 mM Guanidine HCl	79.6	77.9	83.1	73.7	74.7	77.8	4.9
80 mM Guanidine HCl	77.4	93.1	73.8	92.8	84.5	84.3	10.4
800 mM Guanidine HCl	83.9	74.0	82.6	60.1	68.9	73.9	13.4

Table 3. OT recoveries for protein precipitated samples. RapiGest (0.1%) and guanidine hydrochloride (8 mM, 80 mM and 0.8 mM) were added in plasma before protein precipitation in order to disrupt potential binding of OT to plasma proteins.

OT was spiked in 200 µL of K2-EDTA human plasma at the following concentrations: 10, 20, 100, 200, 1,000, 10,000, and 20,000 pg/mL. $^{13}C^{15}N$-isotopically labeled OT (CYIQNCPLG-NH_2) was added as an IS at 100 pg/mL in all samples. Following protein precipitation, the supernatant was diluted 10-fold with 4% H_3PO_4 and SPE was performed on an Oasis HLB Sorbent to isolate the analyte and the IS. Extracts were diluted 1:1 with 0.1% FA and injected on the ionKey/MS System.

The LLOQ of the OT assay was 10 pg/mL and the MRM chromatograms recorded at the LLOQ level are displayed in Figure 3. The chromatograms shown in Figure 3A represent three successive injections of solvent A blank (0.1% FA), human plasma blank and 10 pg/mL OT spiked in human plasma. The analyte signal detected in the plasma blank was probably produced by the endogenous oxytocin present in human plasma. The OT peak area in the spiked sample is approximately twice the area of the blank signal. Replicate injections at the LLOQ level (Figure 3B) indicate good data reproducibility, with a peak area RSD of 13.2%.

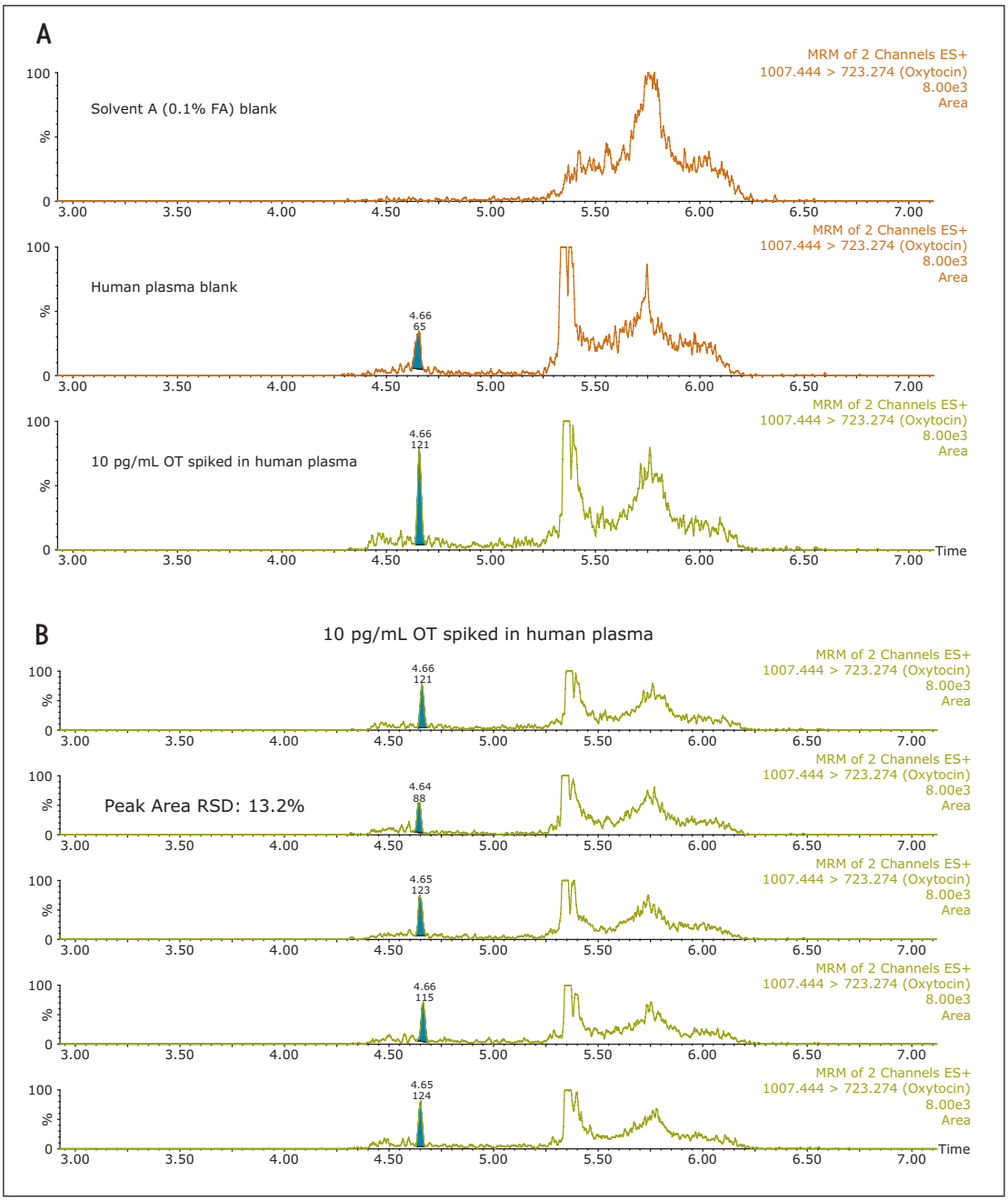


Figure 3A. Three successive injections of solvent A blank (0.1% FA), human plasma blank, and 10 pg/mL OT spiked in human plasma.
Figure 3B. Assay reproducibility.

The assay was tested for dynamic range exceeding three orders of magnitude (10 to 20,000 pg/mL oxytocin in human plasma) and a table containing the peak area ratios (OT area/IS area) along with the corresponding RSDs is presented in Table 4. The TargetLynx calibration curve for the same concentration range is shown in Figure 4 and has very good linearity (r^2=0.998).

Concentration (pg/mL)	Ratio of OT/IS peak area						
	Rep01	Rep02	Rep03	Rep04	Rep05	Mean	RSD (%)
10	0.10	0.09	0.11	0.09	0.11	0.10	9.6
20	0.22	0.21	0.27	0.25	0.23	0.23	10.4
100	1.26	1.24	1.30	1.34	1.29	1.29	3.0
200	2.58	2.42	2.63	2.54	2.63	2.56	3.4
1,000	7.71	7.88	8.17	7.62	8.28	7.93	3.6
10,000	68.49	68.78	65.16	67.69	67.42	67.51	2.1
20,000	123.03	124.94	129.99	126.67	131.95	127.32	2.9

Table 4. Reproducibility of the OT assay in human plasma across the entire concentration range (10 to 20,000 pg/mL).

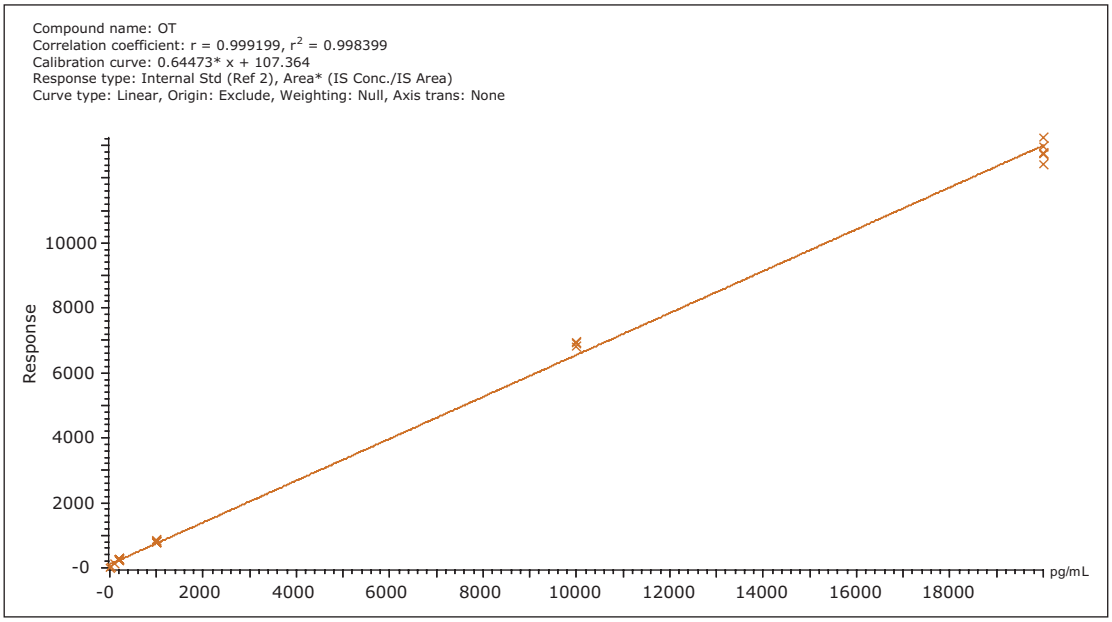


Figure 4. Target Lynx calibration curve for OT spiked in human plasma in the range of 10 to 20,000 pg/mL.

The carryover of the assay was evaluated by injecting a blank (0.1% FA, solvent A) following the injection of the highest concentration spiked sample (20 ng/mL OT spiked in human plasma). According to the data displayed in Figure 5, the analyte carryover was 0.02% and the peak area recorded for the blank sample was approximately two-fold below the peak area at the LLOQ level.

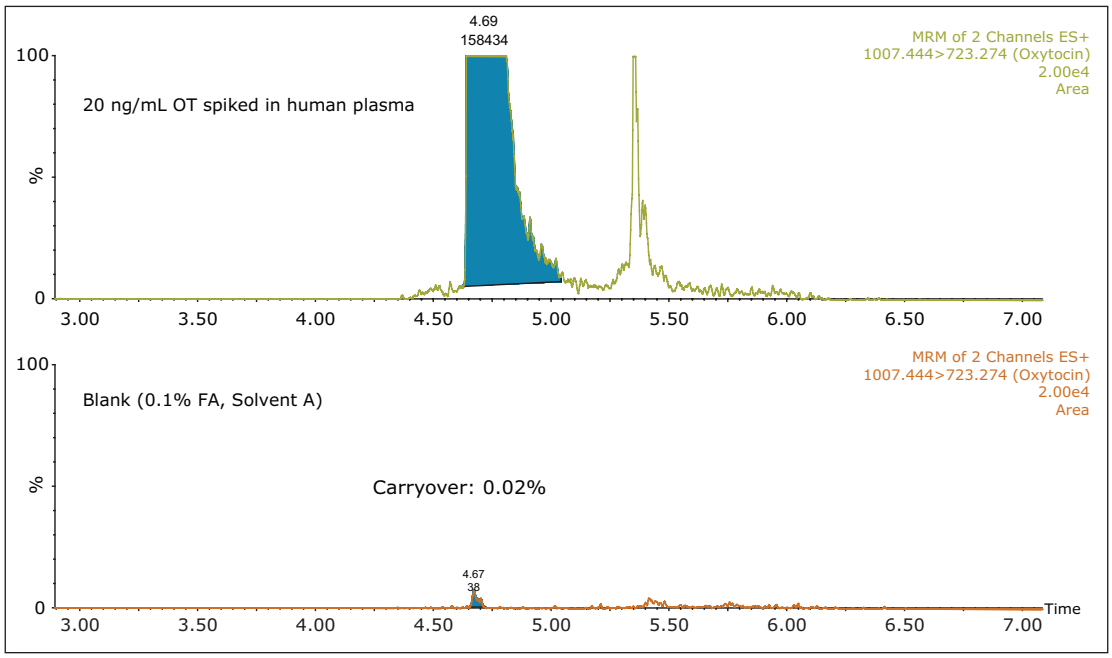


Figure 5. Blank sample injected after the highest concentrated OT sample (20 ng/mL). Analyte carryover is very low (0.02%) for this assay.

The quantification method developed with the ionKey/MS System is simple, specific, robust and has been implemented in a high-throughput (96-well plate) format. In addition, the ionKey/MS System offers significant advantages in terms of operating costs when compared to analytical scale LC-MRM: the cost of mobile phase solvents are typically reduced by 100-fold and sample preparation costs are typically reduced 5- to 10-fold because smaller injection volumes are required (1 to 5 µL).

CONCLUSIONS

- Using the ionKey/MS System, a fast, robust, ultra-sensitive LC-MRM method was developed for the quantification of oxytocin in human plasma.
- An LLOQ of 10 pg/mL was achieved for oxytocin. The analyte signal detected in the plasma blank was probably produced by the endogenous oxytocin present in human plasma.
- Linearity of the assay was maintained over three orders of magnitude (10 to 20,000 pg/mL oxytocin spiked in human plasma).
- Assay reproducibility was better than 15% at all concentrations.
- The carryover of the LC-MRM assay was very low (0.02%).

References

1. Burd JM, Weightman DR, Baylis PH. *J Immunoassay.* 1985;6(3):227–43.
2. Fisher LA, Fernstrom JD. *Life Sci.* 1981 Mar 30;28(13):1471–81.
3. Cool DR, DeBroose D. *J Chromatogr B.* 2003 Jul 25;792(2):375–80.
4. Zhang G, Zhang Y, Fast DM, Lin Z, Steenwyk R. *Anal Biochem.* 2011 Sep 1;416(1):45–52.
5. Sobhi HR, Vatansever B, Wortmann A, Grouzmann E, Rochat B. *J Chromatogr A.* 2011 Nov 25;1218(47):8536–43.
6. Mabrouk OS, Kennedy RT. *J Neurosci Methods.* 2012 Jul 30;209(1):127–33.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Increasing Sensitivity and Minimizing Sample Volume for the Quantification of Therapeutic and Endogenous Cyclic Peptides in Plasma Using ionKey/MS

Mary E. Lame, Erin E. Chambers, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- High sensitivity assay with LOQ of <1 pg/mL with only 100 µL of plasma
- Use of the ionKey/MS™ System facilitates detection limits of 2.5 pg/mL from only 25 µL of plasma
- Use of mixed-mode solid-phase extraction (SPE) reduces matrix interferences and enhances selectivity of the extraction in plasma
- 96-well µElution plate format enables concentration of the sample while maintaining solubility and minimizes peptide loss due to adsorption
- Selective, fast SPE extraction (<30 minutes) without time-consuming immuno-affinity purification
- Reduced solvent consumption (40X) compared to 2.1 mm scale means significant cost savings

WATERS SOLUTIONS

ionKey/MS System

ACQUITY UPLC® M-Class System

ionKey™ Source

Xevo® TQ-S Mass Spectrometer

iKey™ Separation Device

Oasis® WCX 96-well µElution Plate

Waters Collection Plate

MassLynx® 4.1 Software

TargetLynx™ Software

KEY WORDS

bioanalysis, Oasis, sample preparation, peptide quantification, octreotide, desmopressin, vasopressin, UPLC, 2D Technology, plasma, ionKey/MS, iKey, ionKey/MS

INTRODUCTION

The use of peptides and proteins as therapeutic agents has increased significantly in recent years. Thus, the demand for their analysis for toxicokinetic and pharmacokinetic studies is increasing as well.

Historically, biologics have been quantified using ligand binding assays (LBAs). However, with recent advances in mass spectrometry (MS) and liquid chromatography (LC) technologies current approaches towards peptide quantification in biological fluids now include LC-MS/MS. This is in part driven by the fact that LBAs can suffer from significant cross-reactivity issues and lack of standardization. Additionally, LC-MS/MS also has the advantage of greater accuracy and precision, broader dynamic ranges, specificity, and speed of method development. However, accurate quantification of peptides by LC-MS/MS is often not without its own challenges. Peptides have diverse pharmacokinetic profiles, often low circulating plasma levels (pg/mL), generally low MS sensitivity, and require chromatographic resolution from endogenous isobaric matrix interferences.[1] Therefore, to achieve low pg/mL quantification limits, large plasma sample volumes (0.2–1 mL) and sample injection volumes are often required.[2-6] These volumes are often impractical in discovery studies. Thus, the demand for quantitative bioanalytical assays that use decreased sample volumes, while maintaining or improving sensitivity are highly desired.

This application investigates the improved sensitivity and decreased sample volume requirements for the therapeutic and endogenous cyclic peptides: desmopressin, vasopressin and octreotide.[7-9] The general properties of these peptides are shown in Table 1. Using a combination of selective µElution mixed-mode SPE sample preparation, optimal MS precursor and fragment choice, and the ionKey/MS System (source shown in Figure 1), limits of quantification of 1 pg/mL in plasma were achieved. Capitalizing on the attributes of the ionKey/MS System facilitated reducing plasma sample required to 25–100 µL.

EXPERIMENTAL

Method conditions

UPLC conditions

LC system:	ACQUITY UPLC M-Class, configured for trap and back-flush elution
Separation device:	iKey HSS T3, 1.8 µm, 100Å, 150 µm x 100 mm iKey (p/n 186007261)
Trap column:	ACQUITY UPLC M-Class Symmetry® C_{18}, 5 µm, 300 µm x 50 mm (p/n 186007498)
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Loading solvent:	98:2 mobile phase A:B, 25 µL/min for first two minutes, reverse valve
Valve position:	Initial position one (forward loading of trap), switch to position two at two minutes (back flush elute of trap onto the analytical column)
Analytical gradient:	See Table 2
Elution flow rate:	3.0 µL/min
iKey temp.:	75 °C
Sample temp.:	15 °C
Injection volume:	5 µL
Total run time:	12.0 min
Collection plates:	Waters 1 mL Collection Plates

MS conditions

MS system:	Xevo TQ-S
Ionization mode:	ESI positive
Capillary voltage:	3.8 kV
Source temp.:	120 °C
Cone gas flow:	100 L/hr
Collision cell pressure:	5.5 x $10^{(-3)}$ mbar
Collision energy	Optimized by component, see Table 3
Cone voltage:	Optimized by component, see Table 3

Data management

Chromatography software:	MassLynx 4.1
Quantification software:	TargetLynx

Table 1. Peptide chemical properties.

Peptide	Amino acid sequence	MW	pI	HPLC index
Octreotide	8Phe-Cys-Phe-Trp-Lys-Thr-Cys-Thr-ol [Disulfide bridge: 2-7]	1019	9.3	40.8
Desmopressin	Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2 [Disulfide Bridge: 1-6]	1069	8.6	16.8
Vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2 [Disulfide Bridge: 1-6]	1084	9.1	7.6

Figure 1. ionKey Source.

Gradient:	Time (min)	Profile %A	%B	Curve
	0.0	98	2	6
	5.0	50	50	6
	5.5	50	50	6
	7.0	10	90	6
	8.0	10	90	6
	9.0	98	2	6

Table 2. LC gradient conditions.

Sample preparation

Sample pre-treatment

100 µL of human plasma was diluted 1:1 with 4% H_3PO_4 in water and mixed.

Sample extraction with Oasis WCX

Pre-treated plasma samples were extracted according to the protocol in Figure 2. All solutions are made up by volume. All extraction steps were applied to all wells of the µElution plate that contained samples.

Oasis WCX µElution Protocol
Part number: 186002499
Condition: 200 µL MeOH
Equilibrate: 200 µL H_2O
Load: 200 µL Diluted Plasma
Wash 1: 200 µL 5% NH_4OH in H_2O
Wash 2: 200 µL 10% ACN in H_2O
Elute: 2 x 25 µL of 2% FA in 50:50 ACN:H_2O
Dilute: 50 µL H_2O

Figure 2. Oasis µElution WCX extraction protocol.

RESULTS AND DISCUSSION

Mass spectrometry

The 2+ precursors of desmopressin (*m/z* 535.45), vasopressin (*m/z* 542.75), and octreotide (*m/z* 510.30) were used for quantitation. Their corresponding fragments and optimal MS conditions are shown in Table 3. In this assay, the use of highly specific b/y ion specific fragments was more challenging due to the small size and cyclic nature of these peptides. The fragment at *m/z* 328.2, corresponding to a $y3^{1+}$ ion, was chosen for desmopressin and vasopressin. The fragment at *m/z* 120.1, corresponding to the phenylalanine immonium ion, was used for octreotide.

Table 3. MS conditions for cyclic peptides.

Peptide	Precursor	MRM Transition	Cone Voltage (V)	Collision Energy (eV)	Product Ion type
Desmopressin	$[M+2H]^{2+}$	535.4>328.2	40	12	$y3^{(1+)}$
Vasopressin	$[M+2H]^{2+}$	542.7>328.2	40	14	$y3^{(1+)}$
Octreotide	$[M+2H]^{2+}$	510.3>120.1	25	17	immonium ion (Phe)

Chromatographic separation

Chromatographic separation was achieved using the novel microfluidic chromatographic iKey Separation Device. The iKey Separation Device (Figure 3) is packed with UPLC®-grade sub-2-µm particles that permit operation at high pressure and results in highly efficient LC separations. By integrating microscale LC components into a single platform design, problems associated with capillary connections, including manual variability, leaks, and excessive dead volume, are avoided. Use of the iKey HSS T3, 1.8 µm, 100Å, 150 µm x 100 mm (p/n 186007261) provided chromatographic retention, excellent peak shape, narrow peak widths (<4.5 seconds at base), and resolution from endogenous matrix interferences.

Figure 3. iKey Separations Device.

The peptides were eluted using a linear gradient from 2–50% B over 5 minutes, Table 2. Representative chromatograms are shown in Figure 4. The use of a trap and back-flush elution strategy, provided further sample cleanup and facilitated the loading of 5 µL of the high organic SPE eluate (required to maintain solubility of the peptides) without experiencing analyte breakthrough. Additionally, the ability to inject sample volumes typical for 2.1 mm analytical scale LC analysis on the iKey Separation Device can provide the substantial gains in sensitivity that are often required to accurately and reliably detect low pg/mL levels of peptides and proteins in complex matrices.

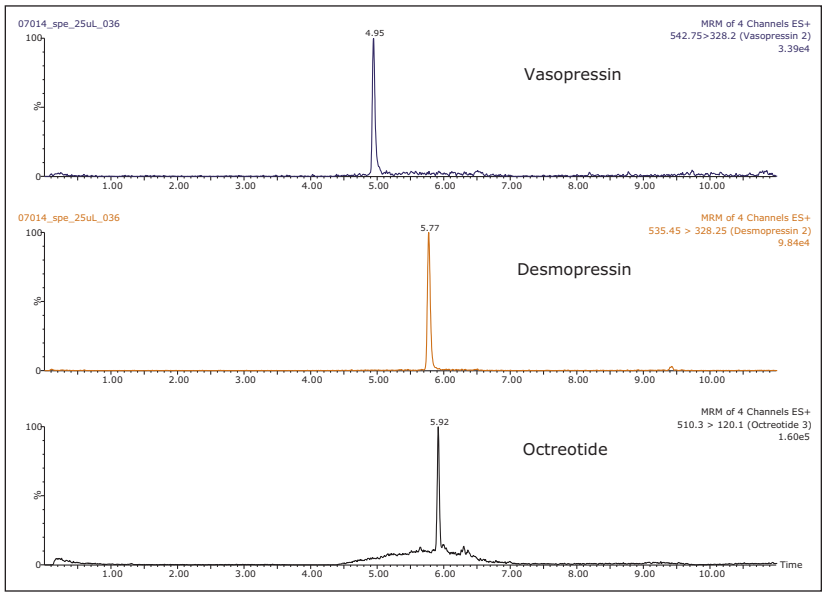


Figure 4. UPLC separation of desmopressin, vasopressin, and octreotide, using the iKey HSS T3, 1.8 µm, 100Å, 150 µm x 100 mm (p/n 186007261).

Enhanced sensitivity with the use of ionKey/MS

Versus analytical scale (2.1 mm I.D.), the ionKey/MS System generally offers increased sensitivity, making it ideal for high sensitivity peptide analysis. This also facilitates the use of smaller sample volumes whilst maintaining or improving sensitivity. In Figures 5 and 6, detection of 2.5 pg/mL of desmopressin and octreotide was easily obtained from extraction of 25, 100, or 200 µL human plasma, using injection volumes ≤10 µL.

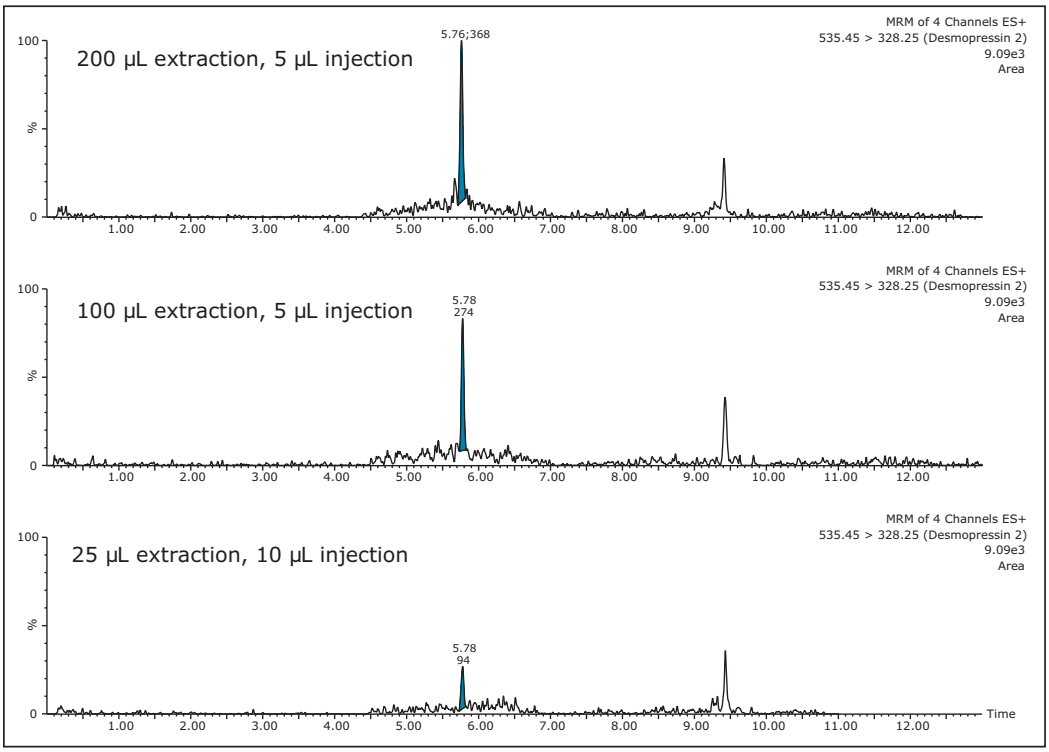


Figure 5. Enhanced sensitivity using the ionKey/MS System: Extraction volume comparison of desmopressin (2.5 pg/mL) from human plasma.

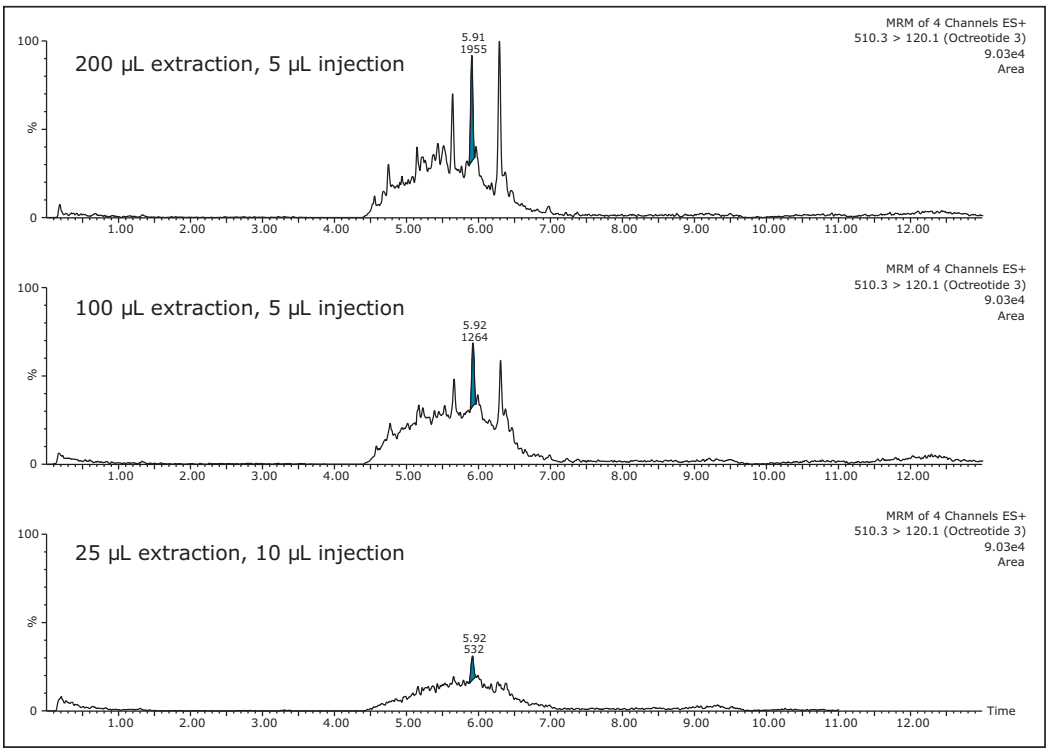


Figure 6. Enhanced sensitivity using the ionKey/MS System: Extraction volume comparison of octreotide (2.5 pg/mL) from human plasma.

Sample preparation

SPE was performed using Oasis WCX, which has both reversed-phase and ion-exchange modes of retention. The orthogonality introduced by the use of mixed-mode sorbents such as these enables greater sample cleanup, improved selectivity, and the sensitivity required for these peptides. Briefly, desmopressin, vasopressin, and octreotide were spiked at various concentrations into the plasma and mixed. These samples were then acidified with 4% H_3PO_4, which helped disrupt protein binding and reduce sample viscosity, improving contact time with the sorbent. Samples were loaded to the SPE device, and washed with 5% NH_4OH followed by 10% acetonitrile. The optimum elution solution was 50% organic, 25% water, with 2% formic acid.

The 96-well Oasis µElution Plate format facilitates fast sample processing (under 30 minutes), and is compatible with automation by most liquid-handling robotic systems, improving sample throughput. Additionally, this format also provides the ability to elute in very small sample volumes, minimizes the potential for adsorptive peptide losses, as well as concentrates the sample for increased sensitivity.

Linearity, accuracy, and precision

To generate standard curves, human plasma was fortified with desmopressin, vasopressin and octreotide at the following final concentrations: 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000, and 2000 pg/mL. SPE of the fortified plasma samples was performed as described above. The calibration curves were constructed using peak areas of the calibration samples by applying a one/concentration (1/x) weighted linear regression model. Using 100 µL of plasma, calibration lines were obtained for each peptide and are shown in Figure 7, panels A, B, C.

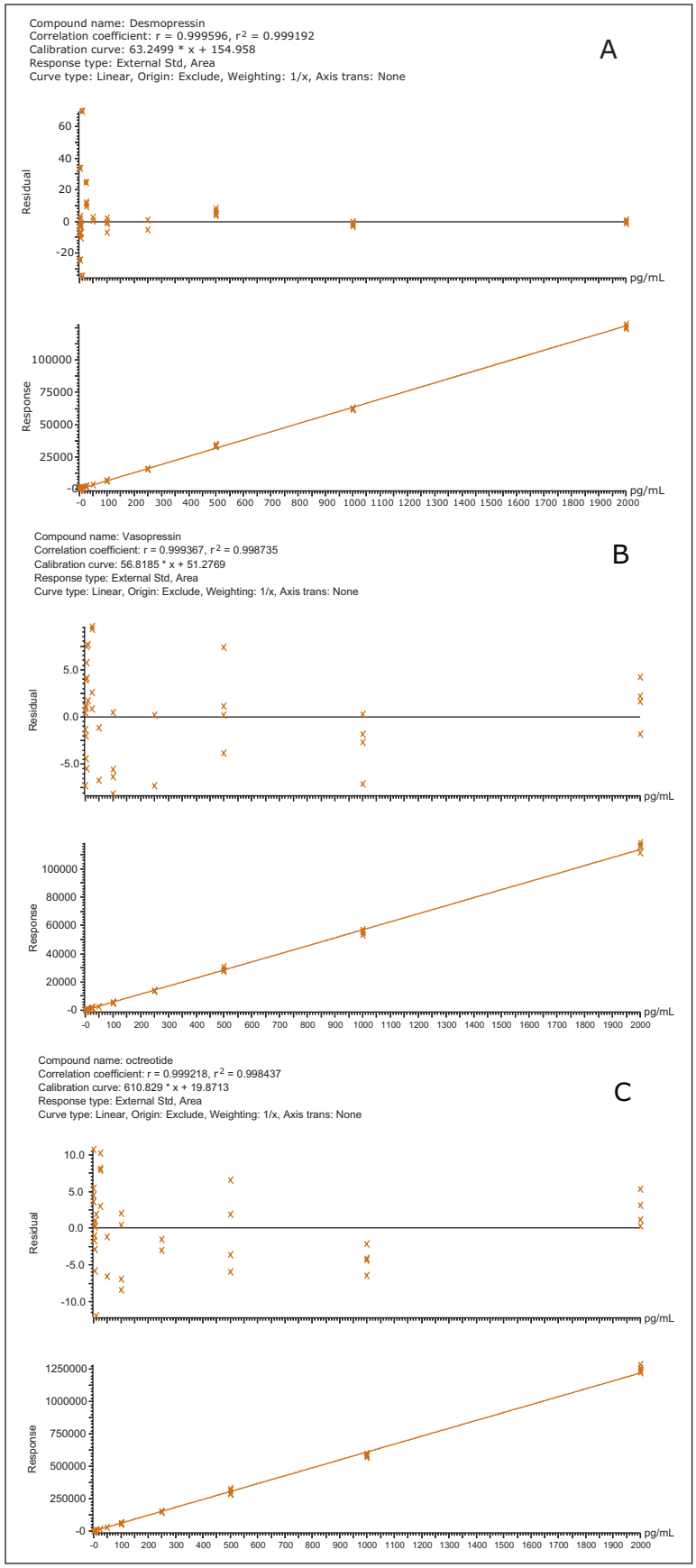


Figure 7. Calibration lines obtained for the quantification of desmopressin (A), vasopressin (B), and octreotide (C) in extracted plasma.

A summary of standard curve performance is shown in is shown in Table 4. Using 1/X regressions, quantification was linear from 1–2,000 pg/mL with R^2 values of >0.99 for all 3 peptides monitored. Representative chromatograms for extracted desmopressin, vasopressin, and octreotide plasma standard samples are shown in Figures 8–10.

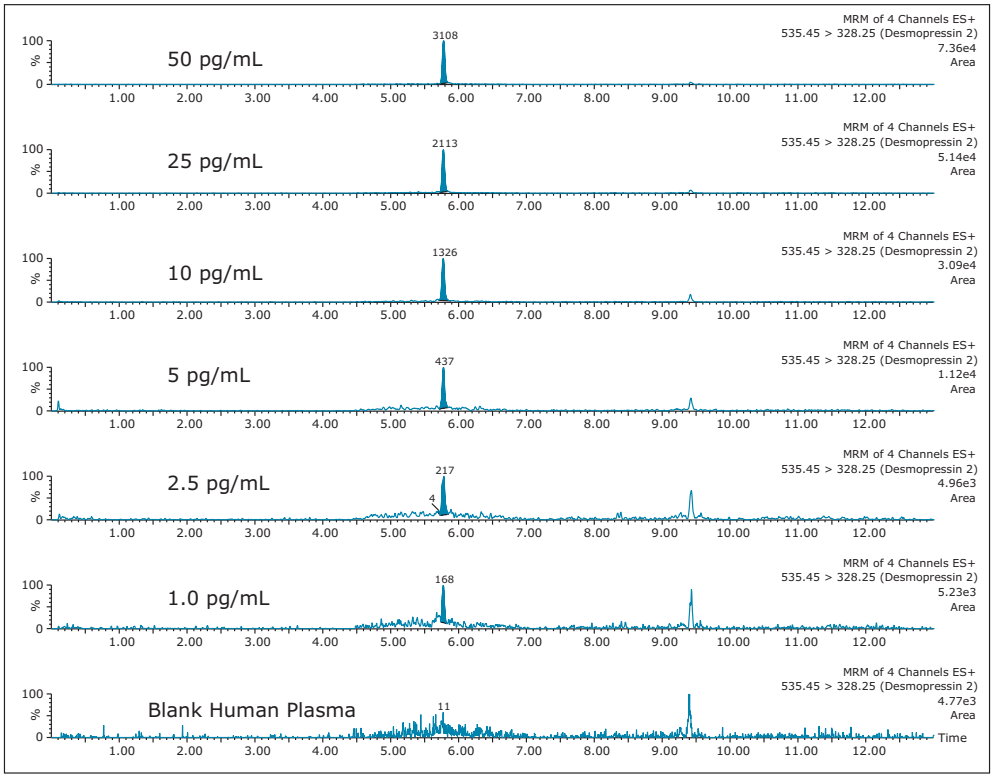


Figure 8. Representative chromatograms from desmopressin extracted from plasma at 1, 2.5, 5, 10, 25, and 50 pg/mL, compared to blank plasma.

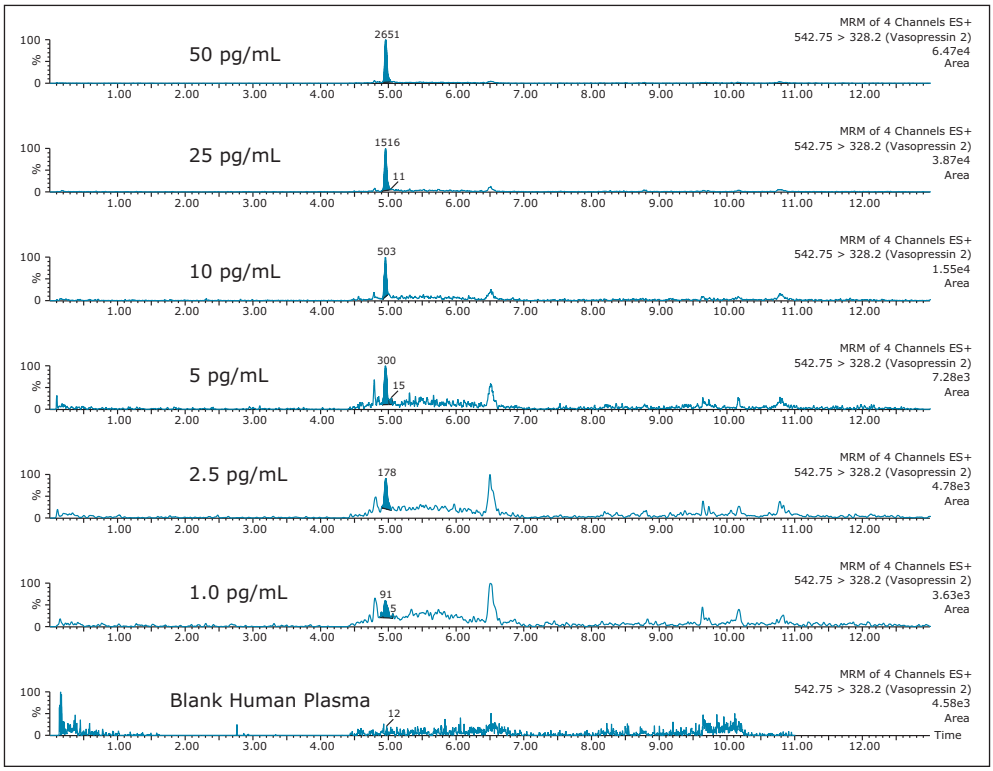


Figure 9. Representative chromatograms from vasopressin extracted from plasma at 1, 2.5, 5, 10, 25, and 50 pg/mL, compared to blank plasma.

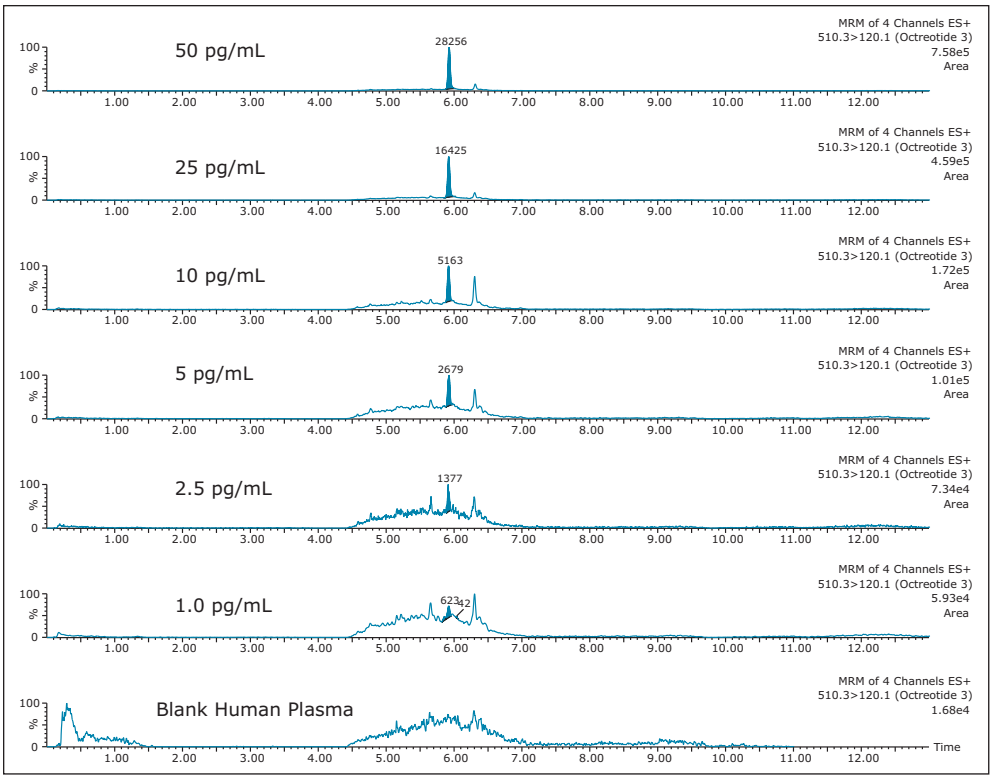


Figure 10. Representative chromatograms from octreotide extracted from plasma at 1, 2.5, 5, 10, 25, and 50 pg/mL, compared to blank plasma.

Table 4. Summary of standard curve performance.

Desmopressin plasma concentration (pg/mL)	Area	Calculated desmopressin concentration (pg/mL)	Mean accuracy	Vasopressin plasma concentration (pg/mL)	Area	Calculated vasopressin concentration (pg/mL)	Mean accuracy	Octreotide plasma concentration (pg/mL)	Area	Calculated octreotide concentration (pg/mL)	Mean accuracy
1.0	215	0.95	94.8	1.0	107	0.98	98.9	1.0	668	1.06	106.0
2.5	319	2.58	103.5	2.5	193	2.50	99.9	2.5	1474	2.38	95.3
5.0	453	4.70	94.3	5.0	344	5.15	103.1	5.0	3017	4.91	98.1
10.0	898	11.75	117.5	10.0	646	10.50	104.8	10.0	5820	9.50	95.0
25.0	1965	28.63	114.5	25.0	1551	26.38	105.6	25.0	16404	26.82	107.3
50.0	3363	50.70	101.5	50.0	2781	48.05	96.1	50.0	29385	48.08	96.2
100.0	6376	98.35	98.4	100.0	5454	95.08	95.1	100.0	59131	96.77	96.8
250.0	15625	244.60	97.9	250.0	13751	241.10	96.5	250.0	149267	244.34	97.8
500.0	33594	528.68	105.7	500.0	28805	506.05	101.2	500.0	304504	498.48	99.7
1000.0	62242	981.60	98.2	1000.0	55252	971.53	97.2	1000.0	584899	957.52	95.8
2000.0	126145	1991.95	99.6	2000.0	115450	2030.98	101.5	2000.0	1251918	2049.51	99.7

CONCLUSIONS

The combination of the ionKey/MS System and mixed-mode µElution SPE provided enhanced selectivity and increased sensitivity, whilst simultaneously significantly reducing sample volume requirements. Use of µElution format SPE eliminated the need for evaporation, reducing peptide losses due to adsorption and non-specific binding. The 150 µm iKey Separation Device enabled the development of a highly sensitive, low flow quantitative MRM method that simultaneously detects vasopressin, desmopressin, and octreotide with LOD <1pg/mL, and dynamic ranges from 1–2,000 pg/mL. The current analysis uses 100 µL of plasma and provides a significant improvement in sensitivity and S:N over analytical scale (2.1 mm I.D.) analysis. Furthermore, detection limits of 2.5 pg/mL are achievable from only 25 µL of plasma. In addition, the ionKey/MS System reduces solvent and sample consumption, thereby reducing cost and allowing for multiple injections of samples for improved accuracy or to meet the guidelines for incurred sample reanalysis (ISR).

References

1. Ewles M, Goodwin L, Bioanalytical approaches to analyzing peptides and proteins by LC-MS/MS. *Bioanalysis* (2011) 3(12), 1379–1397.
2. Jiang Y, Wang J, Wang Y, Du X, Zhang Y, Fawcett JP, Gu J, Determination of long-acting release octreotide, an octapeptide analogue of somatostation, in human plasma by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 2007; 21: 3982–3986.
3. Sidibé J, Varesio E, Hopfgartner G, Quantification of ghrelin and des-acyl ghrelin in human plasma by using cubic-selected reaction-monitoring LCMS. *Bioanalysis* May 2014, Vol. 6, No. 10, Pages 1373–1383.
4. Kim MK, Lee TH, Suh JH, Eom HY, Min JW, Yeom H, Kim U, Jung HJ, Cha KH, Choi YS, Youm JR, Han SB, Development and validation of a liquid chromatography-tandem mass spectrometry method for the determination of goserelin in rabbit plasma. *Journal of Chromatography B*, 878 (2010) 2235–2242.
5. Neudert, L, Zaugg M, Wood S, Struwe P, High Sensitivity Dual Solid Phase Extraction LC-MS/MS Assay for the Determination of the Therapeutic Peptide Desmopressin in Human Plasma. *Celerion eHistory*, accessed July 6, 2014, http://celerion.com/wordpress/wp-content/uploads/2011/09/A-High-Sensitivity-Dual-Solid-Phase-Extract-LC-MSMS-Assay-for-the-Determination-of-the-Therapeutic-Peptide-Desmopressin-in-Human-Plasma_2011.pdf
6. Morin LP, Landry F, Mess JN, Jonakin K, Aiello M, Misonne X, Gary Impey, Cardenas J and Garofolo F, Challenge in Trying to Reach Femtogram per Milliliter (fg/mL) Sensitivity in Plasma for the Quantification of a Cyclic Peptide: Desmopressin. Poster session presented at: 61st Annual Conference of the American Society for Mass Spectrometry; 2013 June 9–13; Minneapolis, MN.
7. JHP (2010) Vasopressin Injection United States drug package insert http://www.parsterileproducts.com/products/PI/00011052013/JHP-Pharmaceuticals-Pitressin-00011052013.pdf
8. Ferring (2008) Desmopressin United States drug package insert. http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/PediatricAdvisoryCommittee/UCM215090.pdf
9. Novartis (1988) Octreotide United States drug package insert http://www.pharma.us.novartis.com/cs/www.pharma.us.novartis.com/product/pi/pdf/sandostatin_lar.pdf

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Simultaneous Analysis of Intact Human Insulin and Five Analogs in Human Plasma Using µElution SPE and a CORTECS UPLC Column

Erin E. Chambers and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- CORTECS™ UPLC® Column for highest sensitivity and narrowest peaks widths
- Selective, fast extraction without time-consuming affinity purification
- Quantitative accuracy; more accurate and precise than traditional LBA methods
- High sensitivity
- Specificity, including differentiation between human insulin and insulin lispro
- Simultaneous quantification of human insulin, glargine, lispro, aspart, glulisine, and detemir
- Quantification without digestion
- Excellent asset utilization; allows the use of current LC/MS instrumentation

WATERS SOLUTIONS

ACQUITY UPLC® I-Class System with 2D Technology

CORTECS UPLC C_{18}+ Columns

Oasis® MAX 96-well µElution Plates

Xevo® TQ-S Mass Spectrometer

MassLynx® Software

KEY WORDS

Non-specific binding, large peptides, Oasis, sample preparation, bioanalysis, quantification, insulin, glargine, lispro, UPLC, CORTECS, plasma

INTRODUCTION

Insulin is perhaps one of the best known and earliest peptide therapeutics. Multiple long and fast-acting analogs have also been developed, and a patient may often be prescribed one of each for diabetes control. Quantification of biologics, such as insulins, has historically been performed using ligand binding assays (LBAs) such as ELISAS. LC/MS/MS, however, has certain advantages over LBAs, such as shorter development times, higher accuracy and precision, the ability to multiplex, no cross-reactivity, and the ability to readily distinguish between closely related insulins. Intact insulins are particularly difficult to analyze by LC/MS/MS, as MS sensitivity is low due to poor transfer into the gas phase, and poor fragmentation patterns exist due to the presence of multiple stabilizing disulfide bonds. In addition, insulin and its analogs suffer from non-specific binding and poor solubility, making LC and sample preparation method development difficult. A few LC/MS/MS methods do exist; however, most of those methods involve time-consuming and laborious immunoaffinity purification and/or nano-flow LC. Distinguishing between human insulin and insulin lispro (Humalog) is a very specific challenge for quantifying insulins, as they differ by a simple reversal in the position of two amino acids. Only a single low-molecular weight fragment differentiates the two, making selective sample preparation and chromatography critical.

This study takes advantage of mixed-mode solid-phase extraction (SPE) and a high-efficiency, solid-core particle column that contains a low-level positive surface charge to eliminate interferences while facilitating high sensitivity quantification. Furthermore, selectivity studies show that the presence of high levels of human insulin, such as one might encounter in type II diabetic patients, does not interfere with quantification of lispro or any of the other analogs. This work provides a single, simple method for the simultaneous, direct quantification of intact human insulin and multiple insulin analogs in human plasma (Figure 1), achieving LODs of 50 to 200 pg/mL for each. Average accuracy for standard curve points was 99% to 100%. Average inter- and intra-day accuracies for QC samples were 98% and 94%, respectively. Average inter- and intra-day precisions for QC samples were 7.5% and 5.3%, respectively. Matrix factors for all analogs were calculated in six sources of human plasma and CVs of matrix factors were <15% in all cases, further supporting the selectivity of the method.

EXPERIMENTAL

Sample preparation

Step 1: Protein precipitation (PPT)

25 µL of bovine insulin (internal standard, final concentration 10 ng/mL) was added to 250 µL human plasma and mixed.

Samples were precipitated with 250 µL 1:1 methanol/acetonitrile containing 1% acetic acid, and centrifuged for 10 minutes at 13,000 rcf.

The supernatant was transferred to a 2-mL 96-well plate containing 900 µL of 5% concentrated NH_4OH in water (v:v) and mixed.

Step 2: SPE using an Oasis MAX µElution 96-well Plate

Condition:	200 µL methanol
Equilibrate:	200 µL water
Load sample:	Entire diluted PPT supernatant was loaded onto the extraction plate in two steps of approximately 700 µL each
Wash:	200 µL 5% NH_4OH in water
Wash:	200 µL 5% methanol + 1% acetic acid
Elute:	2 X 25 µL 60:30:10 methanol/water/acetic acid
Dilute:	50 µL water
Inject:	30 µL

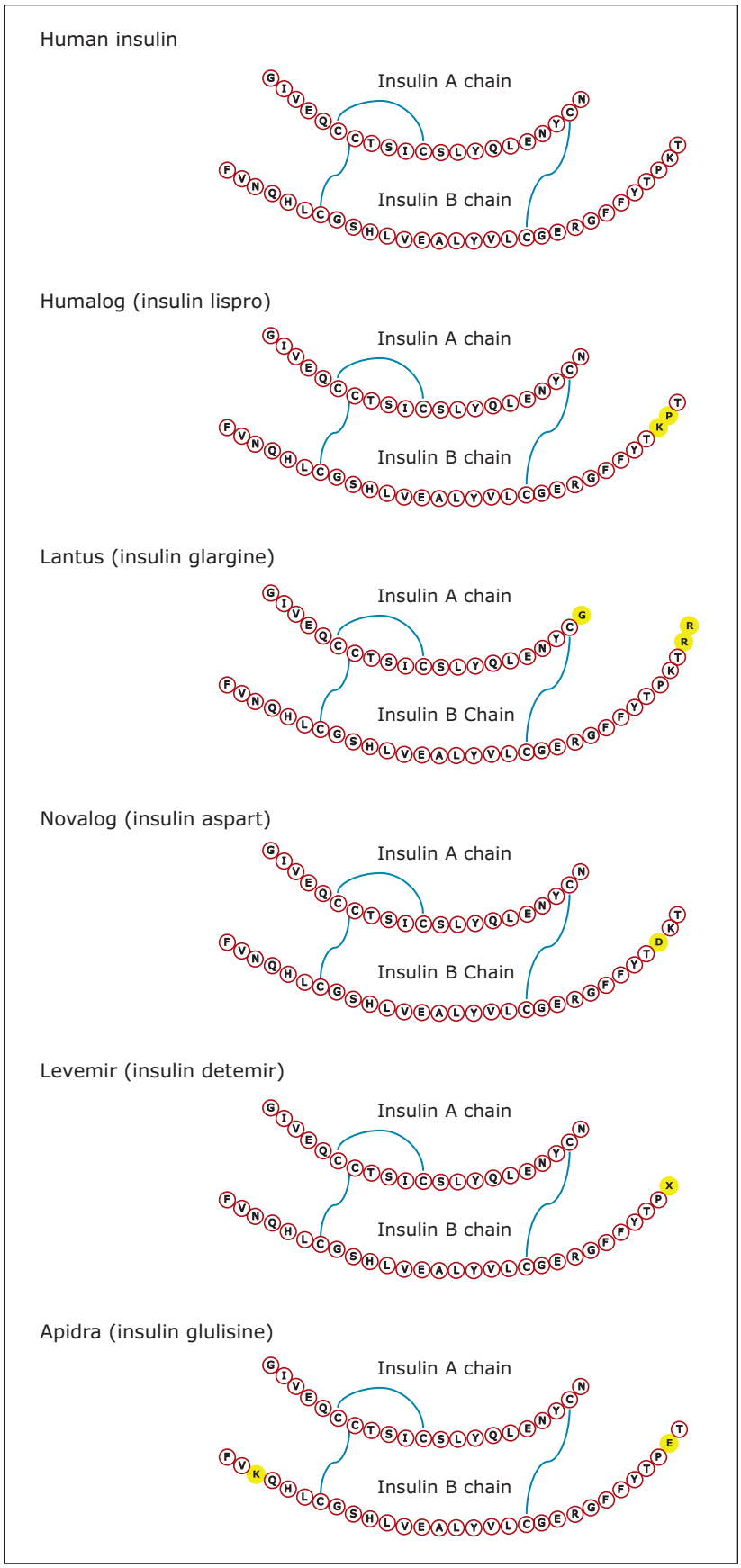


Figure 1. Structures for human insulin and the insulin analogs quantified in this application.

UPLC conditions

System:	ACQUITY UPLC I-Class with 2D Technology, configured for at-column dilution with trap and back elution
Analytical column:	CORTECS UPLC C_{18}+ 1.6 µm, 2.1 x 50 mm (p/n 186007114)
Trap column:	XBridge™ C_{18} *IS*™ 3.5 µm, 2.1 x 20 mm (p/n 186003019)
Elution mobile Phase A:	0.1% HCOOH in water
Elution mobile Phase B:	0.1% HCOOH in acetonitrile
Loading solvent:	85:15 mobile phase A:B; 0.1 mL/min for first two minutes, reverse valve, then clean trap column with ramp from 40% to 90% B
Dilution solvent:	100% mobile phase A, 0.3 mL/min for first two minutes
Valve position:	Initial position one (forward loading of trap), switch to position two at two minutes (back elute of trap onto analytical column), back to position one at 6.5 minutes
Gradient:	Load for two minutes; switch valve and back elute from trap column onto analytical column with a linear gradient from 15% to 40% B over four minutes
Elution flow rate:	0.25 mL/min
Column temp.:	60 °C
Sample temp.:	15 °C
Injection volume:	30 µL
Run time:	8.5 minutes
Collection plates:	Waters® 1-mL ACQUITY collection plates

MS conditions

Mass spectrometer:	Xevo TQ-S
Ionization mode:	ESI positive
Capillary voltage:	3.0 kV
Desolvation temp.:	600 °C
Cone gas flow:	150 L/h
Desolvation gas flow:	1000 L/h
Collision cell pressure:	3.8 X $10^{(-3)}$ mbar
Collision energy:	Optimized by component, see Table 1
Cone voltage:	Optimized by component, see Table 1

Data management

Chromatography software:	MassLynx
Quantification software:	TargetLynx™

Specific insulin	MRM transition	Cone voltage (V)	Collision energy (eV)	Ion type
Glargine	1011 → 1179	60	25	
Lispro	1162 → 217	50	40	
Detemir	1184 → 454.4	60	20	y2
Aspart	971.8 → 660.8	60	18	y11
Glulisine	1165 → 1370	14	22	
Bovine (IS)	956.6 → 1121.2	60	18	
Human insulin	1162 → 226	50	40	

Table 1. MS conditions for human insulin, insulin analogs, and the internal standard bovine insulin.

RESULTS AND DISCUSSION

Mass spectrometry

Several multiple charged precursors were observed for each of the analogs, typically the two most abundant were selected for CID, and either one or two diagnostic fragments monitored during method development. Representative MS/MS spectra at the optimal collision energies for the primary transitions chosen for quantification are shown in Figures 2A and 2B. In several cases, higher intensity MRM transitions existed than the one that was ultimately chosen. Specifically, most insulins will produce very intense immonium ion fragments. Glargine, for example, produces a tyrosine immonium ion fragment at *m/z* 136. However, tests in extracted plasma demonstrated that the use of higher *m/z* precursor and fragment pairs (Table 1) yielded significantly improved specificity in matrix, outweighing any apparent sensitivity difference (Figure 3). This facilitates the use of analytical scale LC and traditional SPE methodologies versus nano-scale chromatography and affinity-based purification schemes previously reported. Human insulin and lispro, however, share almost complete overlap in their fragmentation patterns due to a simple reversal in the positions of amino acids 28 and 29 in the B chain (Figure 1). A single low-molecular weight fragment differentiates the two, with human insulin yielding a fragment at *m/z* 226 and lispro producing a fragment at *m/z* 217 (Figure 2B), both arising from the last two amino acids in the B chain. Without baseline chromatographic separation, the MS must rely on these non-specific fragments for quantification, making both selective sample preparation and high efficiency chromatography crucial.

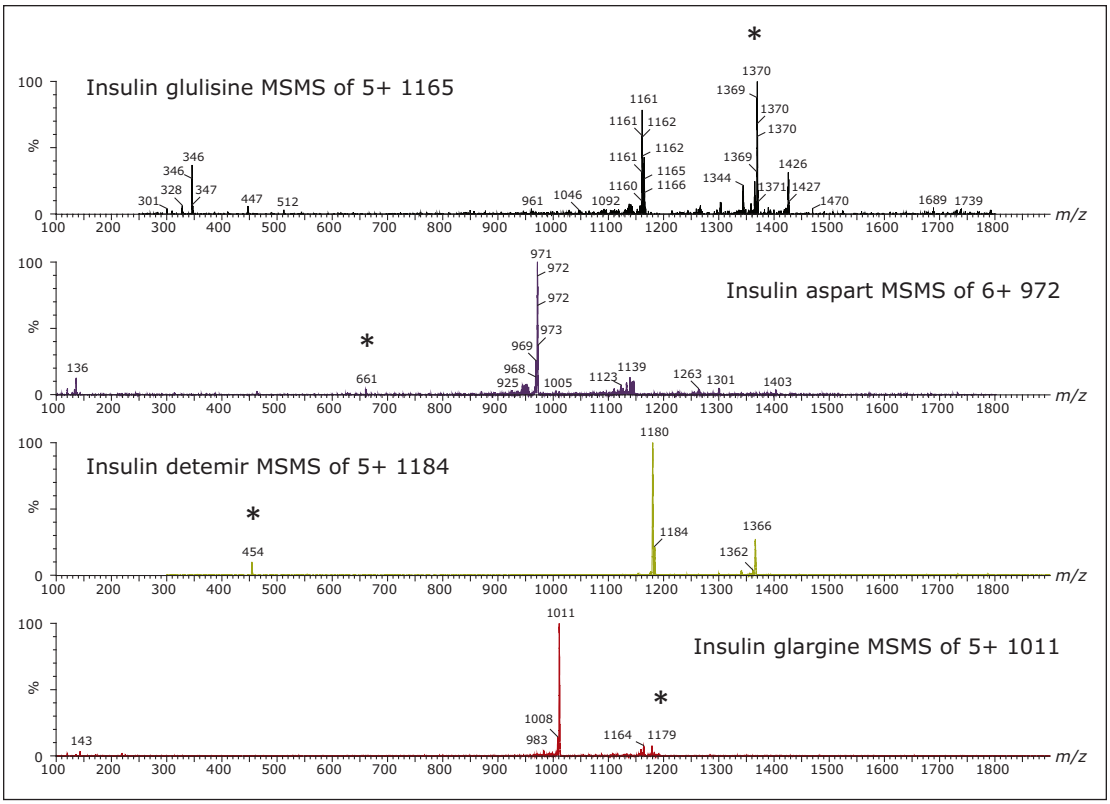


Figure 2A. MS/MS spectra from CID of the optimal precursors for insulin glulisine, aspart, detemir, and glargine. Asterisks indicate the fragments that were chosen for quantification.

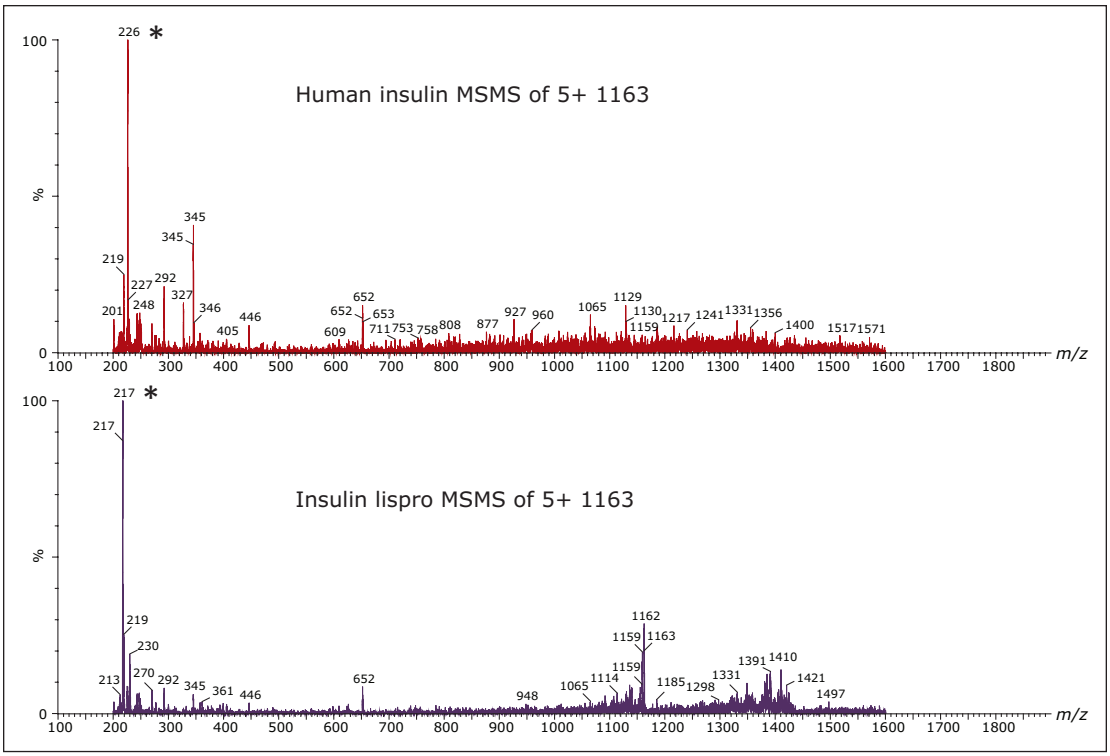


Figure 2B. MS/MS spectra from 5+ precursors of human insulin and insulin lispro. Asterisks indicate the fragments that were chosen for quantification.

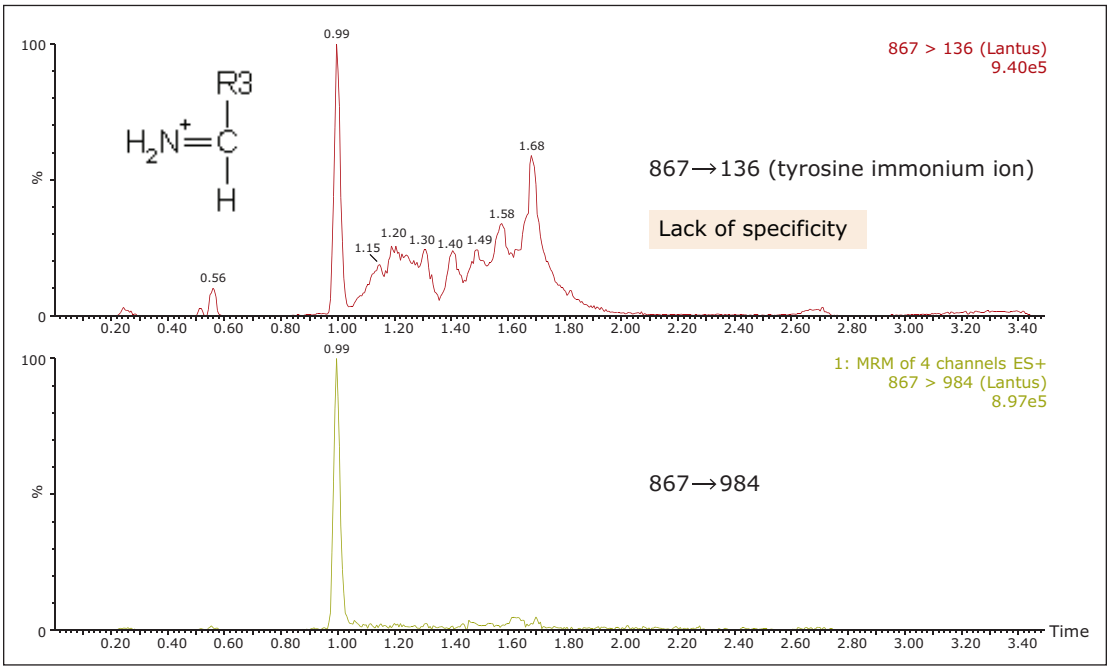


Figure 3. Extracted ion chromatograms from two different transitions for insulin glargine. Use of the immonium ion fragment (top panel) shows a lack of specificity relative to the use of a higher m/z precursor and fragment pair (bottom panel.)

Liquid chromatography

Unlike small molecules, larger peptides and small proteins, *i.e.,* insulins, suffer from poor mass transfer in and out of fully-porous particles. Thus, using a column packed with solid-core particles allows for sharper peak shapes at the higher flow rates typically needed for bioanalytical studies.[1,2] Specifically for insulins, it is documented that using a column packed with particles containing a low-level positive surface charge gives superior peak shape and resolution to other columns.[3] CORTECS C_{18}+ Columns combine the benefits of solid-core particle technology and a low-level positive surface charge to obtain a new level of separation performance for this application. With CORTECS C_{18}+, insulin peaks are typically 4.0 to 4.5 seconds wide with 2X higher area counts than state-of-the-art, fully-porous particle columns. Representative chromatograms are shown in Figure 4.

The use of multidimensional chromatography facilitated higher injection volumes, through the use of at-column dilution (ACD), while providing additional sample cleanup by employing a trap and back-elute strategy. The combination of these chromatographic system elements allowed us to inject up to 40 µL without experiencing insulin breakthrough. Chromatographic breakthrough was evident on a 1D system with injection volumes >10 µL (data not shown).

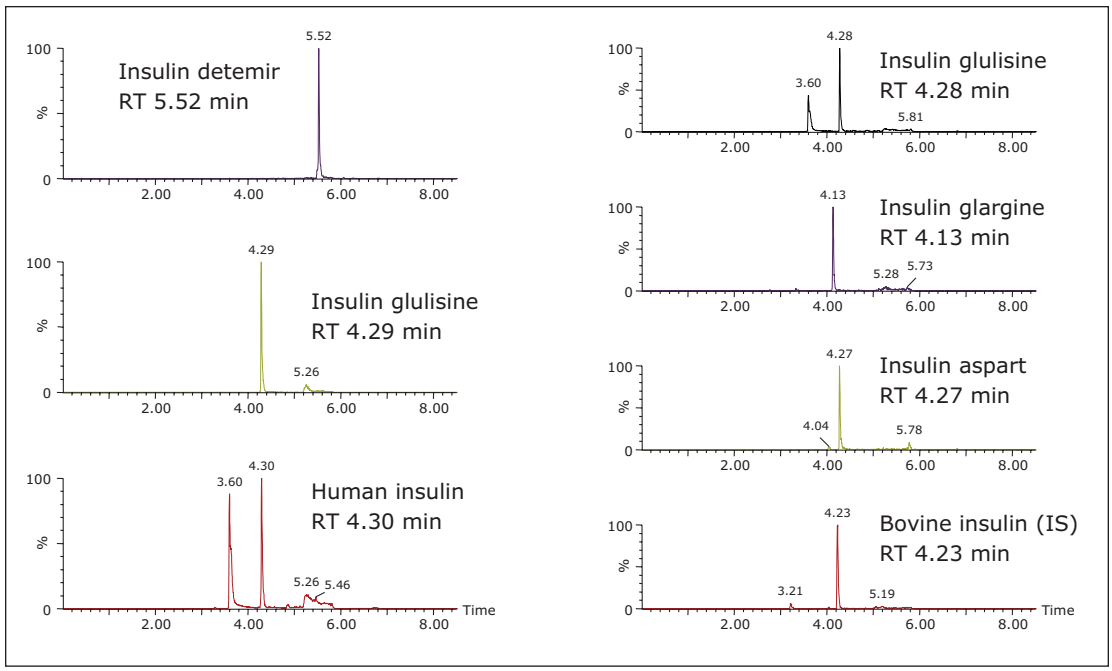


Figure 4. UPLC/MS/MS chromatogram for human insulin, five-insulin analogs, and bovine insulin (IS).

Sample preparation

A proof-of-concept study published earlier in 2013[3] used reversed-phase only SPE to extract four insulin analogs. Although the method clearly demonstrated feasibility, endogenous background was high in the transitions for human insulin and lispro, with average LLOQs of 200 to 500 pg/mL reported for the four insulins, making accurate low-level quantification and distinction between the lispro and human insulin impossible. This application presents a significantly more selective two-step extraction utilizing a protein precipitation (PPT) pre-treatment step and a strong anion-exchange mixed-mode SPE device to effectively reduce endogenous background, as well as improve specificity and detection limits.

During optimization of protein precipitation conditions for insulin, various ratios of organic solvent to plasma, as well as different precipitation solvents were tested. Protein precipitation (PPT) with a 1:1 ratio of various solvents (ACN or ACN modified with one of the following: 5% NH_4OH, 1% acetic acid, or 2% formic acid) resulted in >90% recovery of the six insulins without precipitating the peptides themselves. Protein precipitation with higher ratios of organic resulted in peptide loss due to undesired precipitation of the insulins. The PPT pre-treatment effectively eliminated protein binding and reduced endogenous interferences from large proteins such as albumin.

The PPT supernatants were then diluted with 5% concentrated NH_4OH to bring the pH to ~11. At this pH, insulin and its analogs will carry a net negative charge, putting them in the proper charge state to bind to Oasis MAX by ion exchange. A 2007 study[4] determined that mixed-mode SPE (especially in its ion-exchange elution) was significantly more selective than reversed-phase only SPE. For this assay specifically, where the use of low *m/z* fragments for human insulin and lispro was necessary, it was imperative to have the most selective sample preparation possible.

The diluted supernatant was then applied to conditioned SPE plates, and analytes were well retained on the SPE sorbent during the basic pH load step. Optimization of the elution solution was critical to fully elute the insulins, maintain solubility, and minimize interferences from the plasma matrix. The optimum elution solution was 60% organic with 10% acetic acid. The final eluate was diluted with an equal volume of water.

Linearity, accuracy, and precision

To generate standard curves, human plasma was fortified with a mixture of the insulins at the following final concentrations: 50, 100, 200, 500, 1000, 2000, 5000, and 10,000 pg/mL. Quality control (QC) samples were prepared in human plasma at the following concentrations: 150, 750, 2500, and 7500 pg/mL.

Bovine insulin (final concentration 10 ng/mL) was used as the internal standard (IS) for all insulins. Peak area ratios (PARs) of the analyte peak area to the IS peak were calculated. Calibration curves, prepared in human plasma, were constructed using PARs of the calibration samples by applying a one/concentration weighted linear regression model. All QC sample concentrations were then calculated from their PARs against their respective calibration lines. All curves were linear using the 1/x regression. A summary of standard curve performance for all insulins is shown in Table 2. For human insulin, the basal concentration in pooled or individual control plasma was determined by calculating the x-intercept. The basal level of human insulin (average = 1937 pg/mL) was then added to the spiked concentration for all standard curve and QC samples to enable accurate quantification. A representative standard curve for insulin lispro is shown in Figure 5.

Analyte	Std. curve range pg/mL	Std. curve range fmol/mL	r^2, linear fit, 1/x weighting	Mean % accuracy of all points
Insulin lispro	50 to 10,000	8.6 to 1720	0.998	99.99
Insulin glargine	50 to 10,000	8.3 to 1650	0.996	99.98
Human insulin	50 to 10,000	8.6 to 1720	0.996	100.00
Insulin detemir	200 to 10,000	33.8 to 1690	0.998	96.40
Insulin glulisine	50 to 10,000	8.6 to 1720	0.995	100.00
Insulin aspart	100 to 10,000	17.2 to 1716	0.995	100.00

Table 2. Standard curve ranges, r^2 values, and mean accuracy for curve points for all compounds.

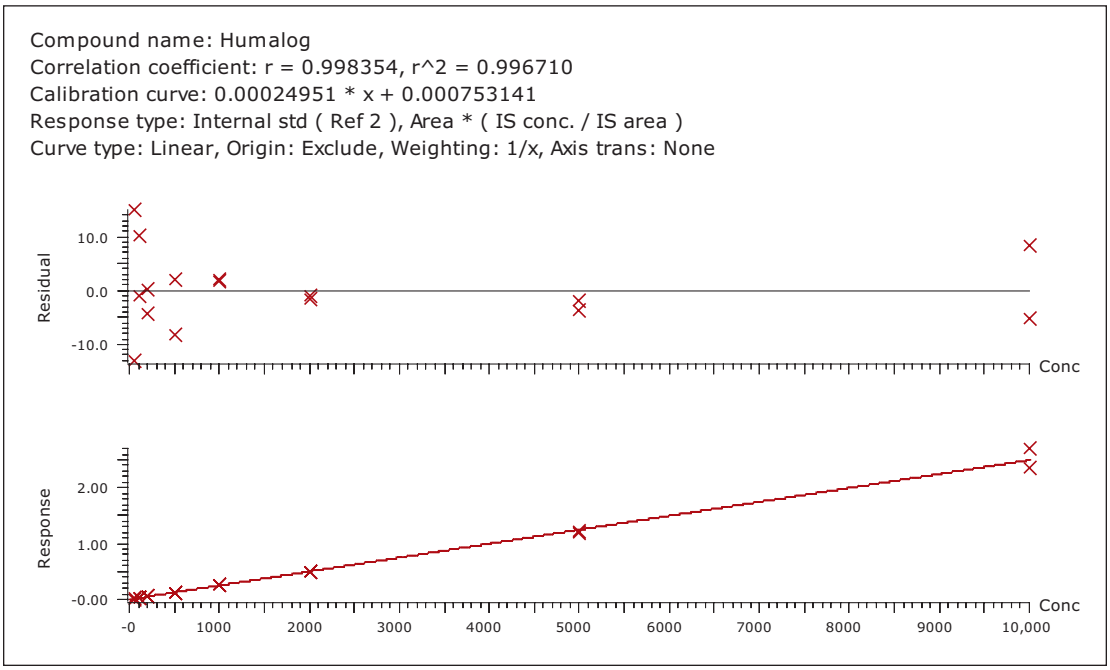


Figure 5. Representative standard curve for insulin lispro.

Inter- and intra-day accuracy and precision were calculated for all QC samples. Representative summary statistics for insulin glargine, lispro, and human insulin can be found in Tables 3, 4, and 5. All other analogs exhibited similar performance.

Insulin Glargine

Inter-day n=9

QC conc. (pg/mL)	Mean calc. conc.	Std dev	% CV	Mean accuracy
150	150.1	18.7	12.4	102.7
750	718.4	47.3	6.6	95.8
2500	2369.3	131.2	5.5	94.8
7500	7648.5	511.3	6.7	102.0

Intra-day n=3

QC conc. (pg/mL)	Mean calc. conc.	Std dev	% CV	Mean accuracy
150	167.4	16.6	9.9	111.6
750	757.7	62.4	8.2	101.1
2500	2378.0	184.9	7.8	95.1
7500	7949.5	257.9	3.2	106.0

Table 3. Inter- and intra-day accuracy and precision for QC samples for insulin glargine.

Insulin Lispro

Inter-day n=9

QC conc. (pg/mL)	Mean calc. conc.	Std dev	% CV	Mean accuracy
150	144.0	17.5	12.2	96.0
750	721.8	32.3	4.5	96.2
2500	2447.1	202.9	8.3	97.9
7500	7697.5	634.8	8.2	102.6

Intra-day n=3

QC conc. (pg/mL)	Mean calc. conc.	Std dev	% CV	Mean accuracy
150	164.6	14.9	9.1	109.8
750	748.2	19.8	2.6	99.8
2500	2417.6	230.4	9.5	96.7
7500	8215.4	243.1	3.0	109.5

Table 4. Inter- and intra-day accuracy and precision for QC samples for insulin lispro.

Human Insulin

Inter-day n=9 (Avg basal level was 1937 pg/mL)

QC conc. (pg/mL)	Mean calc. conc.	Std dev	% CV	Mean accuracy
150	1915.1	125.4	6.5	92.0
750	2542.5	141.0	5.5	94.8
2500	4326.0	146.7	3.4	97.6
7500	9819.0	960.3	9.8	104.0

Intra-day n=3 (Basal level was 1872 pg/mL)

QC conc. (pg/mL)	Mean calc. conc.	Std dev	% CV	Mean accuracy
150	2056.5	16.7	0.8	90.2
750	2506.3	46.6	1.9	99.3
2500	4269.8	206.4	4.8	101.3
7500	10,233.2	265.2	2.6	100.3

Table 5. Inter- and intra-day accuracy and precision for QC samples for human insulin.

Specificity

Matrix factors and CVs of matrix factors for all analogs were calculated in six individual sources of human plasma, as outlined in the 2007 AAPS white paper.[5] The % CV of the matrix factors were 12.3, 11.6, 11.8, 9.0, and 7.7 for insulin detemir, glargine, aspart, glulisine, and lispro, respectively. All easily met the recommended criteria of <15% CV.

In addition, a study was performed to assess the impact of high levels of human insulin, expected to be found in type II diabetic patients, on the assay specificity. This is particularly important for two reasons. First, not all analytes are chromatographically resolved from one and another. Second, due to the use of a nominal mass instrument (triple quadrupole) and the high degree of sequence homology between the analogs themselves and with human insulin, the possibility of MS overlap in some of the transitions exists. For this test, plasma samples were fortified to a final concentration of 5 ng/mL with all of the analogs. A subset of these samples was also spiked with human insulin at 200X higher concentration, to a final concentration of 1 µg/mL human insulin. All samples were then pre-treated, extracted, and quantified. There was no significant change in area counts for any of the analogs when human insulin was present in high concentration.

CONCLUSIONS

Using analytical scale chromatography and simple 96-well SPE sample preparation, this method reaches detection limits for intact insulins, comparable to those previously achieved using immuno-precipitation and nano-scale chromatography.[6-11]

Development of this assay was challenging due to a high degree of non-specific binding (NSB), protein binding, and difficulty maintaining peptide solubility throughout the SPE extraction and elution process. Sample pre-treatment prior to SPE proved to be critical in improving recovery and specificity. Protein precipitation with 1% acetic acid 1:1 acetonitrile/methanol resulted in 80% to 100% recovery without precipitating the peptides themselves. Mixed-mode strong anion SPE provided an additional layer of selectivity, and facilitated the use of the low *m/z* MS fragments that were necessary to distinguish human insulin from lispro.

The use of the CORTECS C_{18}+ Column provided significantly improved sensitivity and peak shape for insulin and its analogs versus charged-surface fully-porous columns and traditional C_{18} solid-core columns. This led to an improvement in detection limits of approximately 2X, when combined with proper MS fragment choice, and selective SPE cleanup enabled quantification limits of 50 to 200 pg/mL for the six insulins.

All FDA criteria[12] for accuracy and precision of the method were easily met. Average accuracies for standard curve points and QC samples were >92%, with most close to 99%. Inter- and intra-day precision for all QC samples was greater than 7.5%. CVs of matrix factors across six lots of human plasma were <15%, further supporting the selectivity of the method.

References

1. Wagner BM, Schuster SA, Boyes BE, Kirkland JJ. Superficially porous silica particles with wide pores for biomacromolecular separations. *Journal of Chromatography A.* 2012; 1264(0):22-30.
2. Kirkland JJ, Schuster SA, Johnson WL, Boyes BE. Fused-Core Particle Technology in High-Performance Liquid Chromatography: An Overview. *Journal of Pharmaceutical Analysis.* http://dx.doi.org/10.1016/j.jpha.2013.02.005
3. Chambers E, Legido-Quigley C, Smith N, Fountain KJ. Development of a fast method for direct analysis of intact synthetic insulins in human plasma: the large peptide challenge. *Bioanalysis.* 2012; 5(1):65-81.
4. Chambers E, Wagrowski-Diehl DM, Lu Z, Mazzeo JR. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *Journal of Chromatography B.* 2007; 852(1-2):22-34.
5. Bansal S, Destefano A. Key elements of bioanalytical method validation for small molecules. *The AAPS Journal.* 2007; 9(1):E109-E114.
6. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schänzer W. Doping Control Analysis of Intact Rapid-Acting Insulin Analogues in Human Urine by Liquid Chromatography-Tandem Mass Spectrometry. *Analytical Chemistry.* 2006; 78(6):1897-1903.
7. Thevis M, Thomas A, Schänzer W. Mass spectrometric determination of insulins and their degradation products in sports drug testing. *Mass Spectrometry Reviews.* 2008; 27(1):35-50.
8. Thevis M, Thomas A, Schänzer W, Östman P, Ojanperä I. Measuring insulin in human vitreous humour using LC-MS/MS. *Drug Testing and Analysis.* 2012; 4(1):53-56.
9. Thomas A, Schänzer W, Delahaut P, Thevis M. Sensitive and fast identification of urinary human, synthetic and animal insulin by means of nano-UPLC coupled with high-resolution/high-accuracy mass spectrometry. *Drug Testing and Analysis.* 2009; 1(5):219-227.
10. Thomas A, Schänzer W, Delahaut P, Thevis M. Immunoaffinity purification of peptide hormones prior to liquid chromatography-mass spectrometry in doping controls. *Methods.* 2012; 56(2):230-235.
11. Thomas A, Thevis M, Delahaut P, Bosseloir A, Schänzer W. Mass Spectrometric Identification of Degradation Products of Insulin and Its Long-Acting Analogues in Human Urine for Doping Control Purposes. *Analytical Chemistry.* 2007; 79(6):2518-2524.
12. Guidance for Industry Bioanalytical Method Validation. 2001.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Reducing Sample Volume and Increasing Sensitivity for the Quantification of Human Insulin and 5 Analogs in Human Plasma Using ionKey/MS

Erin E. Chambers and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- High sensitivity assay with LLOQ of <25–50 pg/mL in human plasma
- Reduced solvent consumption (50X) compared to 2.1 mm scale = significant cost savings
- Use of mixed-mode SPE reduces matrix interferences and enhances selectivity of the extraction for insulins in plasma
- 96-well µElution plate format enables concentration of the sample while maintaining solubility and minimizes peptide losses due to adsorption
- Selective, fast SPE extraction (<30 minutes) without time-consuming immuno-affinity purification
- Versus 2.1 mm scale, proof of concept studies yield greater signal-to-noise from 2.5X less sample and 1/3 injection volume, allowing for greater confidence in results, more tests per sample, and more injections

WATERS SOLUTIONS

ionKey/MS™ System

ACQUITY UPLC® M-Class

ionKey™ Source

Xevo® TQ-S Mass Spectrometer

iKey™ Separation Device

Oasis® MAX 96-well µElution Plate

ACQUITY® Collection Plate

KEY WORDS

bioanalysis, Oasis, sample preparation, peptide quantification, insulin, UPLC, 2D Technology, plasma, ionKey, ionKey/MS,iKey, M-Class, lispro, glargine, detemir, aspart, glulisine

INTRODUCTION

Recombinant human insulin and its analogs (Figure 1) are perhaps the best known and most widely sold biotherapeutics. Historically, such biologics have been quantified using ligand binding assays (LBAs). However, specifically in the case of insulin and analogs, these affinity-based assays lack standardization, are subject to matrix effects, and in some cases lack adequate specificity. Furthermore, multiplexing is desirable as diabetes treatment typically consists of combination dosing with both long and fast acting versions. LBAs do not allow for simultaneous quantification of human insulin and its important analogs. In spite of these short comings, LBAs are incredibly sensitive and consume only minimal sample. Over the past few years, there has been a trend toward the analysis of large molecules by LC-MS/MS. LC-MS/MS has the advantage of shorter development times, greater accuracy and precision, the ability to multiplex, and can readily distinguish between closely related analogues, metabolites, or endogenous interferences. However LC-MS has struggled to achieve the sensitivity of LBAs and often requires significantly more sample. The need for robust and sensitive analysis of peptide species challenges both the chromatographic separation and mass spectrometry.

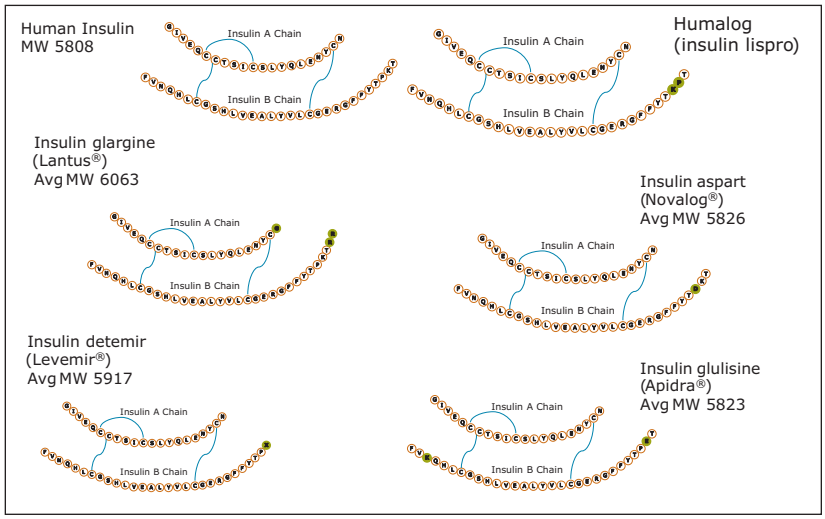


Figure 1. Structures for human insulin and analogs quantified in this application.

EXPERIMENTAL

Sample prepration

Samples were pretreated using protein precipitation (PPT) and extracted on an Oasis MAX 96-well µElution Plate according to a previously published method. [1] For this work, either 100 or 50 µL of human plasma were extracted.

Method conditions

UPLC conditions

System:	ionKey/MS, configured with optional trap and back flush elution
Analytical column:	Peptide BEH C_{18}, 130Å, 1.7 µm, 150 µm x 100 mm iKey (p/n 186006766)
Trap column:	Symmetry C_{18}, 5 µm, 300 µm x 50 mm (p/n 186002590)
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Loading solvent:	85:15 mobile phase A:B, 25 µL/min for first two minutes, reverse valve
Valve position:	Initial position one (forward loading of trap), switch to position two at two minutes (back flush elute of trap onto the analytical column)
Gradient:	25–55% B over 5 minutes
Elution flow rate:	2.5 µL/min
Column temp.:	75°C
Sample temp.:	15°C
Final injection volume:	10 µL
Total run time:	13.5 minutes
Collection plates:	Waters 1 mL ACQUITY Collection Plates

MS conditions

MS system:	ionKey/MS
Ionization mode:	ESI positive
Capillary voltage:	3.6 kV
Source temp.:	120 °C
Cone gas flow:	50 L/hr
Collision cell pressure:	3.83 X $10^{(-3)}$ mbar
Collision energy:	Optimized by component, see Table 1
Cone voltage:	Optimized by component, see Table 1

Data management

Chromatography software:	MassLynx® 4.1
Quantification software:	TargetLynx™

In an earlier publication,[1] we described an ultra-high sensitivity quantitative assay for human insulin and 5 analogs. The method was carefully optimized to maximize sensitivity for the insulins in the following manner: a multi-dimensional LC system was used to enable at-column-dilution and a trap/back elute strategy to increase loading volume and then refocus the analyte band. Mixed-mode SPE and a high-efficiency chromatographic system using a solid-core column with a positively-charged particle surface improved specificity and facilitated the differentiation of human insulin and insulin LisPro. In this earlier method, 250 µL of human plasma were extracted to reach detection limits between 50 and 200 pg/mL with a 30 µL injection volume.

Many of the insulins described in the earlier method have either recently come off patent or are due to shortly. This has resulted in a flurry of research activity aimed at alternate dosing regimes, pediatric extensions, and the development of replacement insulins. In many of these cases, a further decrease in detection limit and reduction in sample volume required were requested.

In this current work, we undertook to a) transfer the original analytical scale method to the ionKey/MS System, and b) to decrease sample volume and further increase sensitivity through the inherent characteristics of ionKey/MS. ionKey/MS integrates the UPLC® analytical separation directly into the source of the mass spectrometer. The iKey Chromatographic Separation Device (150 µm internal diameter), shown in Figure 2, contains the fluidic channel, electronics, ESI interface, heater, eCord,™ and the chemistry to perform UPLC separations. Perhaps most importantly, ionKey/MS can provide increased sensitivity compared to 2.1 mm chromatography with the same injection volume, or equivalent or greater sensitivity with reduced sample consumption, making it ideal for insulin analyses. As previously mentioned, it is common for bioanalytical LC-MS assays to consume high volumes of both solvent and sample, thus increasing the cost of the assay and limiting the number of replicates that can be analyzed. This study combines µElution solid-phase extraction (SPE) and the novel and highly efficient ionKey/MS System to improve a quantitative assay for insulins in human plasma.

Figure 2. iKey Chromatographic Separations Device.

We investigated the potential for increasing sensitivity whilst simultaneously reducing sample volume using ionKey/MS. This study demonstrates a cumulative ~15X benefit over a 2.1 mm ID scale method for all 6 insulins studied. We were able to reduce sample size by 2.5X, reduce injection volume 3X, and increase sensitivity by a minimum of 2X through effective adaptation of the method to ionKey/MS. Specifically, sample volume was decreased to 100 µL and an LLOQ of 25 pg/mL was achieved for most insulins. A human plasma starting volume of 50 µL yielded a 50 pg/mL LLOQ.

RESULTS AND DISCUSSION

Mass Spectrometry

A stock solution of all insulins was infused via an infusion iKey to confirm mass spectrometry conditions previously described for an analytical scale method.[1] Methods in the literature have demonstrated that it is possible to see a shift in relative abundance of multiply-charged peptide precursors at different flow rates.[2] It becomes important, therefore, to evaluate this when adapting a method from analytical LC flow to microflow. In this instance, MS conditions remained the same and are summarized in Table 1.

Specific Insulin	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Glargine	1011.0 → 1179.0	60	25
	867.0 → 984.0	60	18
Lispro	1162.0 → 217.0	50	40
	968.5 → 217.0	50	40
Detemir	1184.0 → 454.4	60	20
	1184.0 → 1366.3	60	20
Aspart	971.8 → 660.8	60	18
	971.8 → 1139.4	12	18
Glulisine	1165.0 → 1370.0	14	22
	1165.0 → 346.2.0	14	22
Bovine (IS)	956.6 → 1121.2	60	18
Human Insulin	1162.0 → 226.0	50	40
	968.5 → 217.0	50	40

Table 1. MRM transitions and MS conditions for human insulin, 5 insulin analogs, and the internal standard bovine insulin; transitions highlighted in blue are the primary quantitative MRMs.

Chromatography

Chromatographic separation of human insulin, 5 key analogs, and the IS was achieved using a novel microfluidic chromatographic separations device (iKey, shown in Figure 2). The iKey has a channel with UPLC grade sub-2-µm particles that permits operation at high pressure and results in highly efficient LC separations. By integrating microscale LC components into a single platform design, problems associated with capillary connections, including manual variability, leaks and excessive dead volume are avoided. Insulin peak widths are 3 to 4.2 seconds wide at base, as shown in chromatograms from extracted human plasma, Figure 3. Interestingly, at analytical scale human insulin and insulin lispro completely co-elute. Normally, one would expect to have to resort to nano-flow, very shallow gradients and unduly long run times to affect separation. However, the two peaks begin to separate under the microflow conditions shown here, within a run time that is compatible with routine bioanalytical assays.

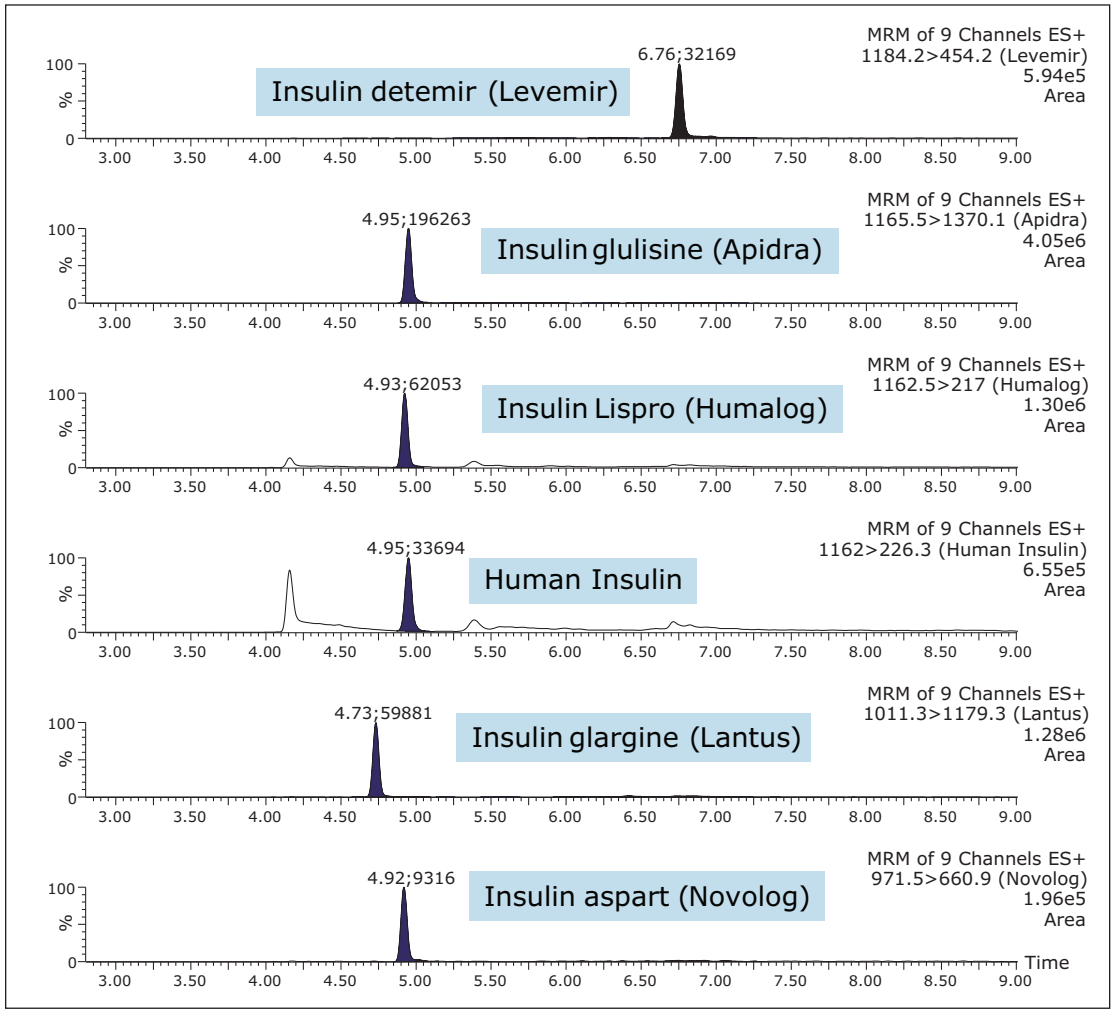


Figure 3. Representative chromatograms of human insulin and analogs extracted from 100 µL human plasma.

The separation was performed using the optional trap valve manger (TVM) configured for single-pump trapping in trap and back elute mode, Figure 4. This configuration facilitates the injection of larger volumes of plasma extracts to improve sensitivity. In this application, the injection volume is 10 µL, which, if properly scaled to a 2.1 mm ID column, would equate to approximately a 2 mL injection. Even at analytical scale, this would normally require some type of trapping to focus the analyte band. Furthermore, the final injection solvent (after SPE of the plasma and dilution of the eluate with water) is 30% methanol and 5% acetic acid. This composition is necessary in order to keep the insulins soluble throughout the chromatographic process. Direct injection without the TVM would result in severe breakthrough due to the organic content which cannot be reduced further without resulting in adsorptive losses and poor solubility. The composition of the injection solvent also plays a key role in eliminating non-column related carry-over. Representative chromatograms of human insulin and analogs extracted from 100 µL human plasma are shown in Figure 3.

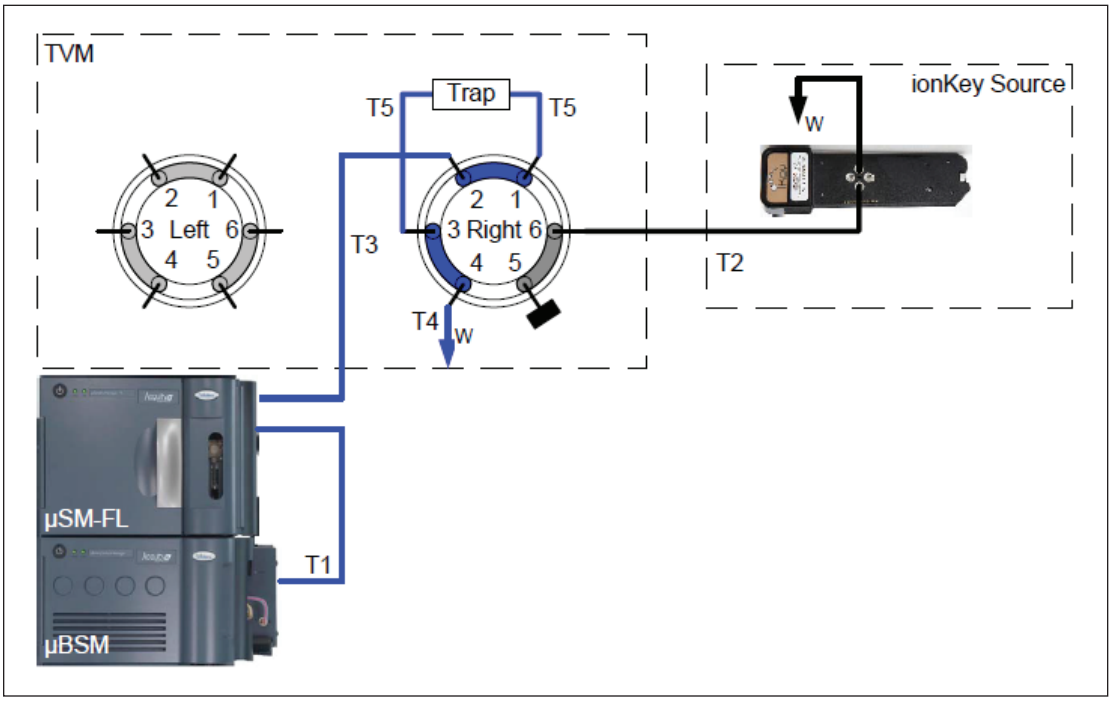


Figure 4. Configuration for single pump trapping on the ionKey/MS System.

Linearity and sensitivity

To demonstrate proof of principle, standard curve points were prepared by fortifying human plasma with human insulin and the 5 analogs at the following final concentrations: 25, 50, 100, 200, 500, 1000, 2000, 5000, and 10,000 pg/mL. Quality control (QC) samples were prepared separately at 150, 750, 2500, and 7500 pg/mL. Bovine insulin was used as the internal standard (IS). Peak area ratios (PARs) of the analyte to the IS peak were calculated. The calibration curve was constructed using PARs of the calibration samples by applying a one/concentration (1/x) weighted linear regression model. All QC sample concentrations were then calculated from their PARS against the calibration curve. For human insulin, the standard addition method was used for quantification. Basal insulin level was determined by calculating the x intercept from the slope of the calibration line. Using 1/x regression, standard curves for all insulins were linear, with all R^2 values greater than 0.99. Representative standard curves are shown in Figure 5. A summary of standard curve performance for all insulins (25–10,000 pg/mL) is shown in Table 2.

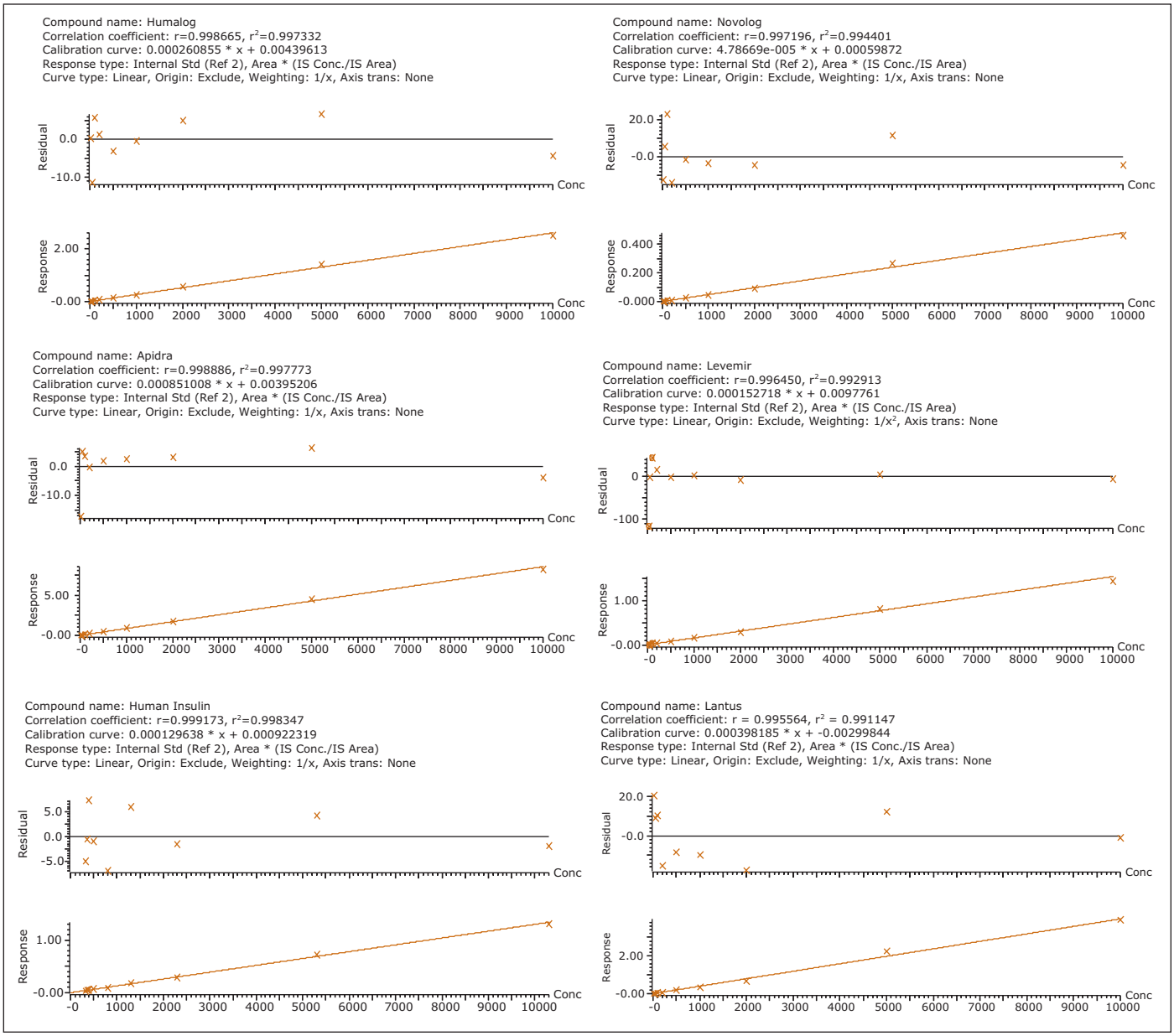


Figure 5. Representative standard curves for human insulin and analogs.

Figure 6 and Figure 7 contain representative chromatograms for insulin glargine and insulin glulisine (respectively) at 25, 50, and 100 pg/mL extracted from 100 µL human plasma, as compared to blank extracted plasma. All QC samples demonstrated very good accuracy and precision, with mean accuracies ranging from 94–109% and mean %CV's of 3.4–8.8%. A summary of QC statistics is shown in Table 3. These results easily meet the recommended FDA acceptance criteria outlined in the white papers describing best practices in bioanalytical method validation for LC-MS/MS assays.[3,4]

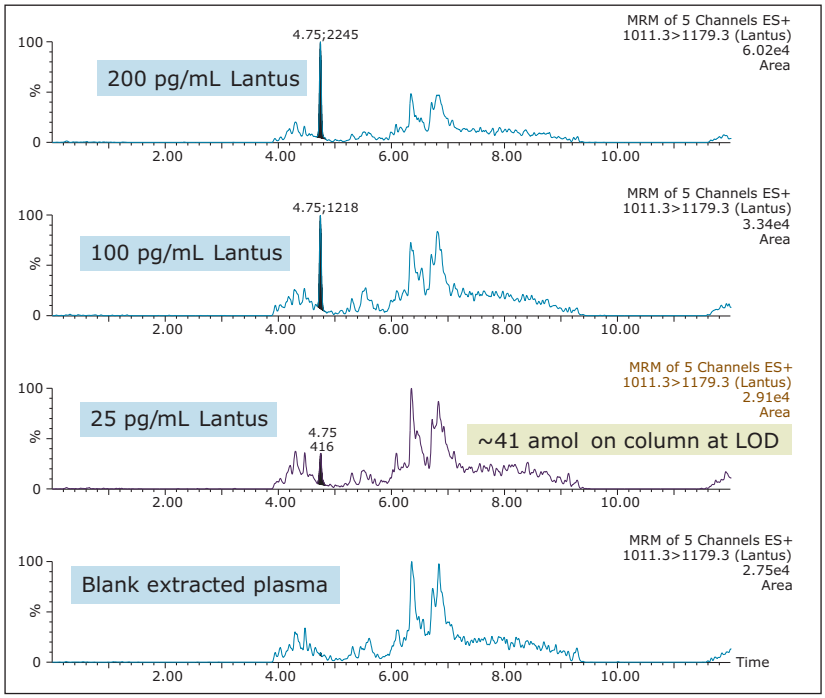


Figure 6. ionKey/MS analysis of insulin glargine (Lantus) from 100 µL human plasma sample, 10 µL injection.

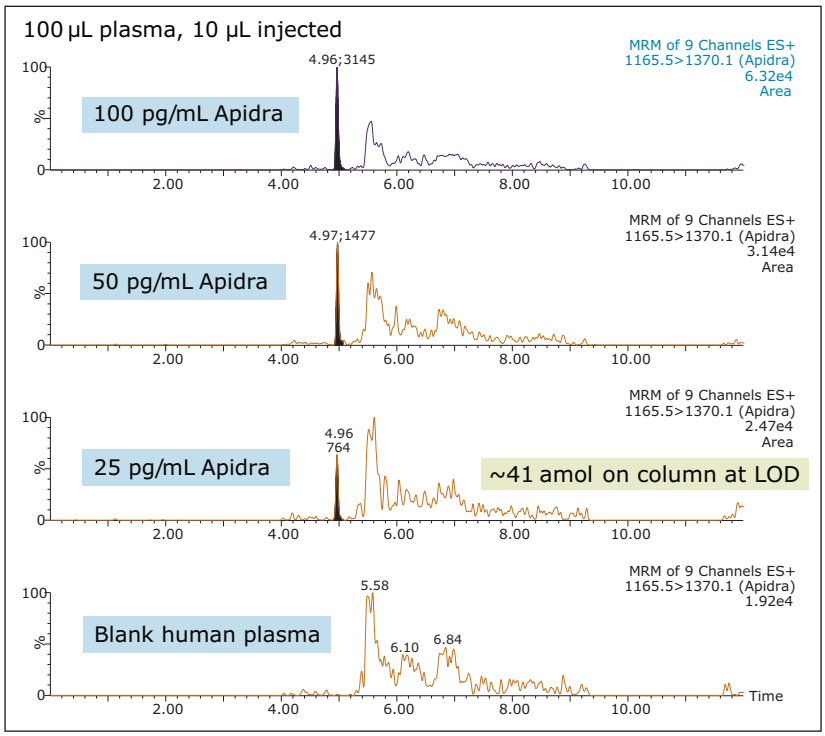


Figure 7. ionKey/MS analysis of insulin glulisine (Apidra) from 100 µL human plasma sample, 10 µL injection.

Insulin Variant	Std Curve Range (pg/mL)	R^2	Avg. % Accuracy	Avg. % CV
Human	25–10,000	0.998	97	2.0
Glargine	25–10,000	0.991	97	9.5
Aspart	25–10,000	0.994	98	8.6
Glulisine	25–10,000	0.998	98	3.5
Detemir	50–10,000	0.993	101	9.7
Lispro	25–10,000	0.997	99	4.0

Table 2. Summary statistics for standard curve performance in a proof of principle study for human insulin and 5 analogs.

Insulin Variant	Avg Accuracy QC 1 (150 pg/mL)	Avg Accuracy QC 2 (750 pg/mL)	Avg Accuracy QC 3 (2,500 pg/mL)	Avg Accuracy QC 4 (7,500 pg/mL)
Human	-1.6	4.1	0.8	-2.9
Glargine	10.1	-0.9	5.1	2.0
Aspart	0.8	6.4	14.4	0.8
Glulisine	6.4	2.3	4.7	-3.5
Detemir	14.6	-10.3	-4.0	0.3
Lispro	11.5	-2.5	2.7	-0.8

Table 3. Summary QC statistics for human insulin and 5 analogs, extracted from human plasma.

When comparing sensitivity, the current ionKey/MS method provides a cumulative benefit of approximately 15X over earlier work[1] from our labs. The ionKey/MS method used 2.5X less sample, injects 3X less, and achieves at least a 2X improvement in detection limit. While the original method had an LOD of ~618 amol on column, the ionKey method has an LLOQ of only 41 amol on column (Figures 6 and 7).

If a further reduction in sample volume is desired, comparable performance (with a slightly higher LLOQ) can be achieved extracting only 50 µL of human plasma. Chromatograms of insulin glargine and insulin glulisine are used as representatives and are shown in Figures 8 and 9.

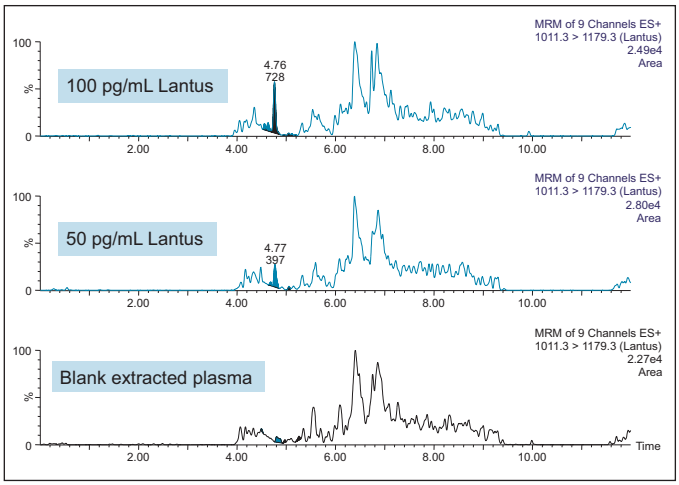


Figure 8. ionKey/MS analysis of insulin glargine (Lantus) from 50 µL human plasma sample, 10 µL injection.

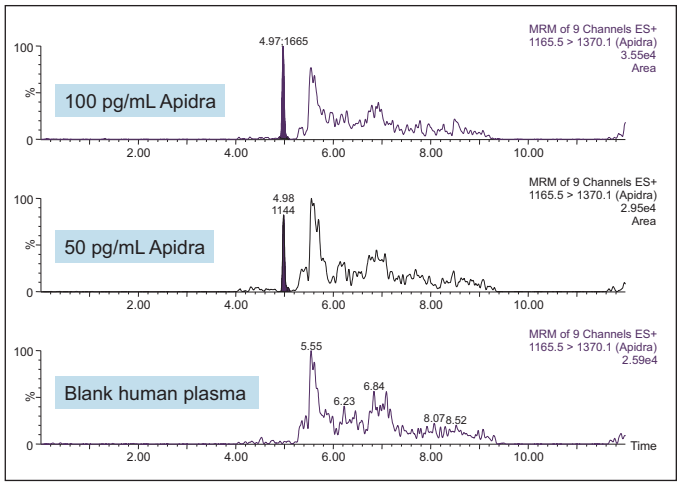


Figure 9. ionKey/MS analysis of insulin glulisine (Apidra) from 50 µL human plasma sample, 10 µL injection.

CONCLUSIONS

The use of ionKey/MS facilitated a reduction in sample volume and concomitant increase in sensitivity for the quantification of human insulin and 5 important analogs. Extraction of 100 µL human plasma yields an LLOQ of 25 pg/mL for insulin glargine, lispro, glulisine, aspart, and endogenous insulin, with a 10 µL injection. Extraction of 50 µL of human plasma yields quantification limits of 50 pg/mL for most insulins. Standard curves were accurate and precise from 25–10,000 pg/mL. QC samples at all levels easily met recommended FDA regulatory criteria with mean accuracies ranging from 94–109% and mean %CVs of 3.4–8.8%, indicating an accurate, precise, and reproducible method. The ionKey/MS method provided a cumulative 15X benefit over an existing analytical scale method by reducing sample required by 2.5 to 5X, reducing injection volume 3X, all whilst increasing sensitivity >2X. In addition, ionKey/MS also reduces solvent consumption by approximately 60X, thereby reducing cost. The reduction in sample volume required for this analysis allows for multiple injections of samples for improved accuracy, more tests per sample, or to meet the guidelines for ISR. This method shows great promise for high sensitivity quantification of intact insulins in patient samples from PK studies using the ionKey/MS System if further validation was performed.

References

1. Chambers, E., *et al.*, Multidimensional LC-MS/MS Enables Simultaneous Quantification of Intact Human Insulin and 5 Recombinant Analogs in Human Plasma. *Analytical Chemistry*, 2014. 86(1): p. 694–702.
2. Chambers, E.E., *et al.*, Development of a fast method for direct analysis of intact synthetic insulins in human plasma: the large peptide challenge. *Bioanalysis*, 2012. 5(1): p. 65–81.
3. *Guidance for Industry Bioanalytical Method Validation*, C. FDA, CVM, Editor 2001: Rockville, MD.
4. Bansal, S. and DeStafano, A. Key elements of bioanalytical method validation for small molecules. *The AAPS Journal*, 2007. 9(1): p. E109–E114.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Development of a High Sensitivity SPE-LC-MS/MS Assay for the Quantification of Glucagon in Human Plasma Using the ionKey/MS System

Mary E. Lame, Erin E. Chambers, Sukhdev S. Bangar, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- High sensitivity assay with LOD of 12.5 pg/mL in human plasma.
- Reduced solvent consumption (50X) compared to 2.1 mm scale means significant cost savings.
- Use of mixed-mode solid-phase extraction (SPE) reduces matrix interferences and enhances selectivity of the extraction.
- 96-well µElution plate format enables concentration of the sample while maintaining solubility and minimizes peptide loss due to adsorption.
- Selective, fast SPE extraction (<30 minutes) without time-consuming immunoaffinity purification.
- The ionKey/MS™ System yielded 4X greater S:N and a 10X improvement in sensitivity over 2.1 mm scale allowing for greater confidence in results, more tests per sample, and more injections.

WATERS SOLUTIONS

ionKey/MS System
ACQUITY UPLC® M-Class System
ionKey™ Source
Xevo® TQ-S Mass Spectrometer
iKey™ Separation Device
Oasis® MAX 96-well µElution Plate
MassLynx® 4.1 Software
Waters® Collection Plate

KEY WORDS

Oasis, sample preparation, bioanalysis, peptide quantification, Glucagon, UPLC, plasma, ionKey/MS, iKey

INTRODUCTION

Glucagon for Injection (rDNA origin) is a polypeptide hormone identical to human glucagon and is used to treat severe hypoglycemia (low blood sugar).[1] It is a single chain polypeptide that contains 29 amino acids residues with a molecular weight of 3483 (Figure 1). As a research tool, accurate quantification of glucagon from biological matrices can help us to better understand diabetes as a function of disease progression and/or drug treatment. Many assays, using different methodologies exist for glucagon analysis in biological samples.[2-7] Glucagon, like other biologics, has historically been quantified using ligand binding assays (LBAs).[2-5] With advances in MS and chromatography technologies over the past few years there has been a trend toward the analysis of large molecules by LC-MS/MS. This is, in part, driven by the fact that LBAs can suffer from significant cross-reactivity issues and lack of standardization. Additionally, LC-MS/MS also has the advantage of shorter development times, greater accuracy and precision, the ability to multiplex, and can readily distinguish between closely related analogues, metabolites or endogenous interferences. Large peptides, such as glucagon, are particularly difficult to analyze by LC-MS/MS as MS sensitivity is low due to poor transfer into the gas phase and poor fragmentation. In addition, glucagon suffers from significant non-specific binding, poor solubility, and must be properly stabilized in biological matrices during collection and sample preparation,[6-8] making LC and sample preparation method development challenging.

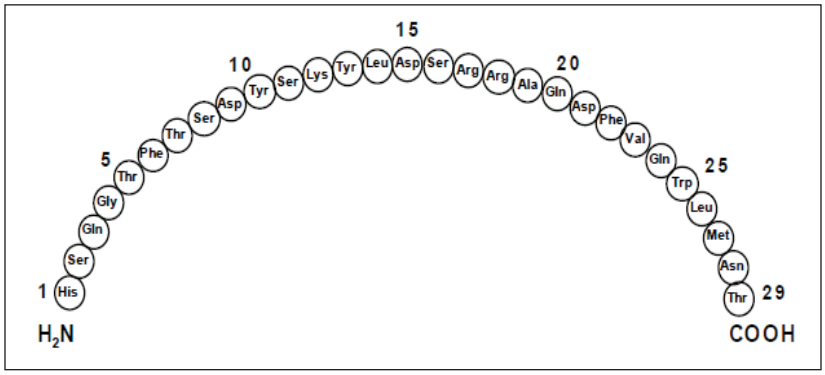


Figure 1. Representative amino acid sequence of glucagon.

EXPERIMENTAL

Sample prepration

Step 1: Pretreatment

Commercially available plasma was treated with a protease inhibitor cocktail (1:100). Plasma was then spiked with various concentrations of glucagon and mixed. These samples (200 µL) were acidified with acetic acid (0.5% final concentration) and vortexed, followed by dilution with 200 µL 5% ammonium hydroxide in water.

Step 2: SPE using a Oasis® MAX µElution 96-well Plate (p/n 186001829)

CONDITION: 200 µL methanol
EQUILIBRATE: 200 µL water
LOAD SAMPLE: Entire diluted plasma sample (400 µL) was loaded onto the extraction plate
WASH 1: 200 µL 5% ammonium hydroxide in water
WASH 2: 200 µL 10% acetonitrile in water
ELUTE: 2 X 25 µL 65:25:10 acetonitrile:water: acetic acid
DILUTE: 50 µL water
INJECT: 5 µL

Method conditions

UPLC conditions

LC System:	ACQUITY UPLC M-Class, configured for trap and back-flush elution
Separation device:	iKey Peptide BEH C_{18}, 130Å, 1.7 µm, 150 µm x 100 mm (p/n 186006766)
Trap column:	ACQUITY UPLC M-Class Symmetry C_{18} Trap Column, 100Å, 5 µm, 300 µm x 50 mm (p/n 186007498)
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Loading solvent:	85:15 mobile phase A:B, 25 µL/min for first two minutes, reverse valve
Valve position:	Initial position one (forward loading of trap), switch to position two at two minutes (back flush elute of trap onto the analytical column)
Analytical gradient:	see Table 1
Elution flow rate:	2.0 µL/min
Column temp.:	75 °C
Sample temp.:	15 °C
Injection volume:	5 µL
Total run time:	14.5 min
Collection plates:	Waters 1 mL Collection Plates

Gradient:

Time (min)	Profile %A	%B	Curve
0	85	15	6
6	55	45	6
6.5	15	85	6
8.5	15	85	6
9.5	85	15	6

Table 1. LC gradient conditions.

MS conditions

MS system:	Xevo TQ-S
Ionization mode:	ESI positive
Capillary voltage:	3.8 kV
Source temp.:	120 °C
Cone gas flow:	100 L/hr
Collision cell pressure:	5.5 x $10^{(-3)}$ mbar
Collision energy:	Optimized by component, see Table 2
Cone voltage:	Optimized by component, see Table 2

Data management

Chromatography software:	MassLynx 4.1
Quantification software:	TargetLynx™

The pharmacokinetic profile of administered exogenous glucagon is characterized by a rapid absorption and elimination with a half-life of <20 minutes, resulting in a total duration of exposure to the peptide of ~2 hours. At the practical clinical dose, 0.25–2.0 ng/mL, maximum glucagon levels of ~8 ng/mL are reached in 20 minutes. Endogenous glucagon in plasma is present in low pg/mL levels (<100 pg/mL), which makes detection by LC-MS/MS even more difficult.

The work described herein uses a combination of selective µElution mixed-mode SPE sample preparation, optimal MS precursor and fragment choice, and the ionKey/MS System (Figure 2) for the highly selective and sensitive quantification of glucagon in human plasma. Detection limits of 12.5 pg/mL using only 200 µL of plasma were achieved with a linear dynamic range from 12.5 to 1,000 pg/mL. This work also capitalizes on the attributes of the ionKey/MS System enabling a 5X reduction in injection volume, a 10X increase in sensitivity, and 4X increase in signal-to-noise (S:N) compared to 2.1 mm I.D. analytical scale method.

Figure 2. ionKey Source.

RESULTS AND DISCUSSION

Mass spectrometry

The 4+(*m/z* 871.5) and 5+(*m/z* 697.1) multiply charged precursors were observed for glucagon; MSMS spectra for these precursors, obtained at their optimal collision energies, are shown in Figure 3. The fragments at *m/z* 693.5 and 940.2 of the 5+ precursor, and 1040.2 of the 4+ precursor were chosen for quantification (Table 2). Although many peptides produce intense fragments below *m/z* 200, these ions (often immonium ions) result in high background in extracted samples due to their lack of specificity. In this assay, the use of highly specific b/y fragments yielded significantly improved specificity, facilitating the use of simpler LC and SPE methodologies.

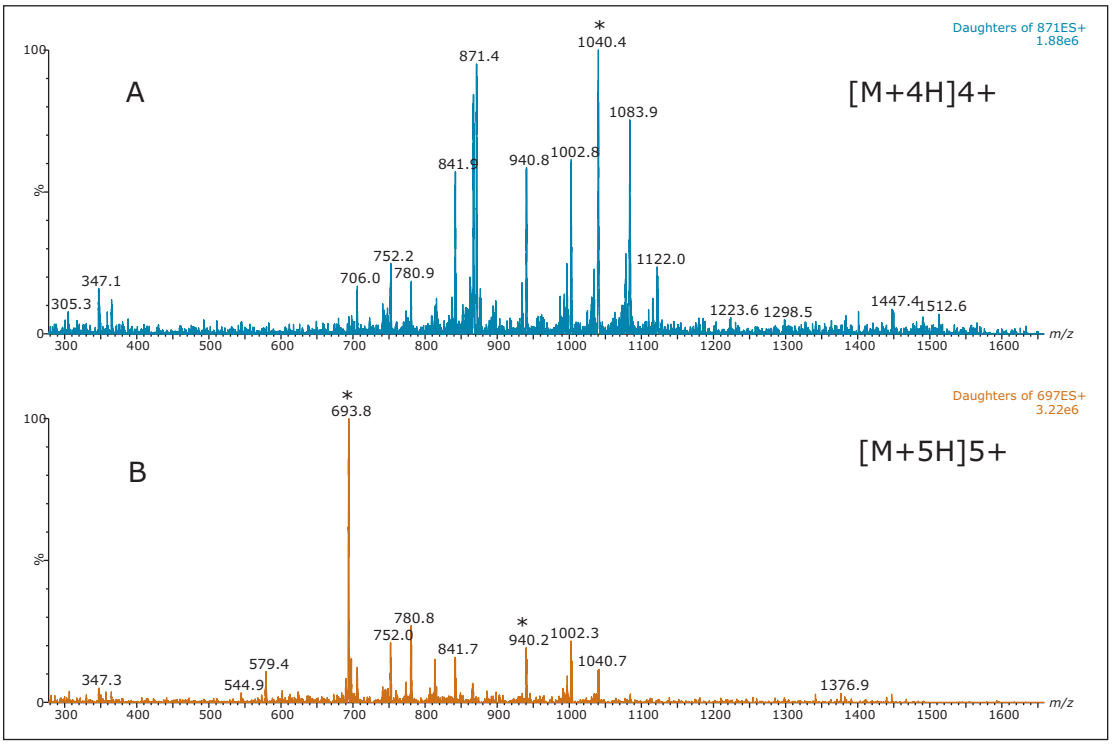


Figure 3. MSMS spectra of the 4+ (A) and 5+ (B) precursors of glucagon. Fragments chosen for quantitation are indicated by asterisks (*).

Precursor	MRM transition	Cone voltage (V)	Collision energy (eV)	Product ion type
$[M+5H]^{5+}$	697.1>693.5	40	12	Ammonia loss(5+)
$[M+5H]^{5+}$	697.1>940.2	40	20	b24(3+)
$[M+4H]^{4+}$	871.5>1040.2	40	26	b26(3+)

Table 2. MS Conditions for glucagon.

Liquid chromatography

Chromatographic separation of glucagon was achieved using the novel microfluidic chromatographic iKey Separation Device. The iKey Separation Device (Figure 4) is packed with UPLC®-grade sub-2-µm particles that permits operation at high pressure and results in highly efficient LC separations. By integrating microscale LC components into a single platform design, problems associated with capillary connections, including manual variability, leaks, and excessive dead volume, are avoided. Use of the iKey Separation Device provided excellent peak shape, narrow peak widths (<4.0 secs at base), and resolution from endogenous matrix interferences.

Glucagon was eluted using a linear gradient from 15–45% B over 6 minutes. Representative chromatograms are shown in Figure 5. The use of multidimensional chromatography, specifically a trap and back-flush elution strategy, provided further sample cleanup and facilitated the loading of 5 µL of the high organic SPE eluate (required to maintain solubility of the peptides) without experiencing analyte breakthrough. Additionally, the ability to inject sample volumes typical for analytical scale LC analysis on the iKey Separation Device can provide the substantial gains in sensitivity that are often required to accurately and reliably detect low pg/mL levels of peptides and proteins in complex matrices.

Figure 4. iKey Separations Device.

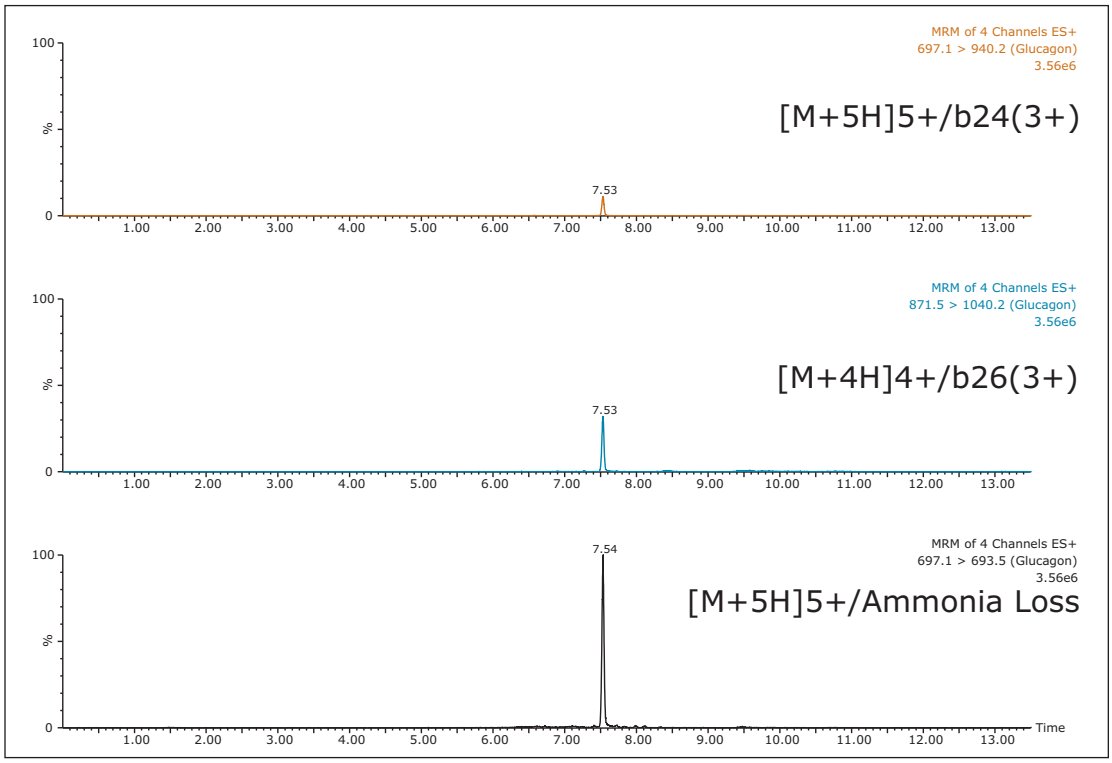


Figure 5. UPLC separation of glucagon from extracted plasma, using the iKey Peptide BEH C_{18}, 130Å 1.7 µm, 150 µm x 100 mm (p/n 186006766).

Enhanced sensitivity with the use of the ionKey/MS System

Versus analytical scale (2.1 mm I.D.), the ionKey/MS System generally offers increased sensitivity, making it ideal for glucagon analysis. This also facilitates the use of smaller sample volumes whilst maintaining or improving sensitivity. For a 250 pg/mL plasma extracted sample, the same injection volume (5 µL) on the ionKey/MS System yielded 4X greater S:N and a 10X improvement in sensitivity versus 2.1 mm scale (Figure 6). Using the 150 µm iKey Separation Device, low pg/mL levels of glucagon were detected in extracted plasma. Figure 7 demonstrates the improvements the ionKey/MS System provides for a 25 pg/mL extracted plasma sample. Versus 2.1 mm scale (25 µL injection), a 5 µL injection on the ionKey/MS System yields 5X greater sensitivity and a 3X improvement in S:N. Ultimately, the use of the ionKey/MS System enabled the development of a low flow quantitative MRM method for glucagon that achieved detection limits of 12.5 pg/mL from only 200 µL of plasma.

Sample preparation

Development of this assay was challenging due to a high degree of non-specific binding (NSB) and difficulty maintaining peptide solubility throughout the SPE extraction and elution process. SPE was performed using Oasis MAX, a mixed-mode sorbent, to enhance selectivity. To ensure glucagon stability during sample preparation and extraction, human plasma was treated with protease inhibitor cocktail. Glucagon was then spiked at various concentrations into the plasma and mixed. These samples were then acidified with acetic acid (0.5% final concentration). Acidification helped disrupt protein binding. Plasma samples were then pre-treated with 5% NH_4OH in water to adjust pH prior to SPE. The diluted plasma (pH >10) samples were then applied to conditioned SPE plates. Glucagon was well retained on the SPE sorbent during the load step, with no break-through occurring. At this basic pH, glucagon will carry a net negative charge, putting it in the proper charge state to bind to Oasis MAX (quaternary amine) by ion exchange. Optimization of the elution solution was critical to maximize recovery, maintain its solubility, and minimize interferences from the plasma matrix. The optimum elution solution was 65% organic, 25% water, with 10% acetic acid.

The enhanced selectivity of the Oasis MAX SPE extraction was imperative to accurately detect and quantify low pg/mL levels of glucagon in plasma. This is especially important where the use of the less specific 5+ precursor and ammonia loss fragment MRM transition might be necessary to achieve low limits of detection. During method development, use of reversed-phase (RP) only SPE was assessed. RP SPE yielded 10–15% greater recovery than the strong anion-exchange mixed-mode SPE (MAX). However, endogenous background was higher and yielded greater matrix effects (data not shown). In particular, matrix effects were >30% for the ammonia loss MRM transition when RP only extraction was employed. This greatly limited its use for robust quantification. Alternatively, the enhanced selectivity of the Oasis MAX SPE device greatly reduced matrix effects (<15%) and facilitated use of the less specific ammonia loss fragment for accurate quantification. In addition, the 96-well Oasis µElution Plate can be processed manually in under 30 minutes and is compatible with most liquid-handling robotic systems for automation to meet sample throughput requirements. This format also provides the ability to elute in very small sample volumes, minimizes the potential for adsorptive peptide losses and chemical instability, as well as concentrates the sample for increased sensitivity.

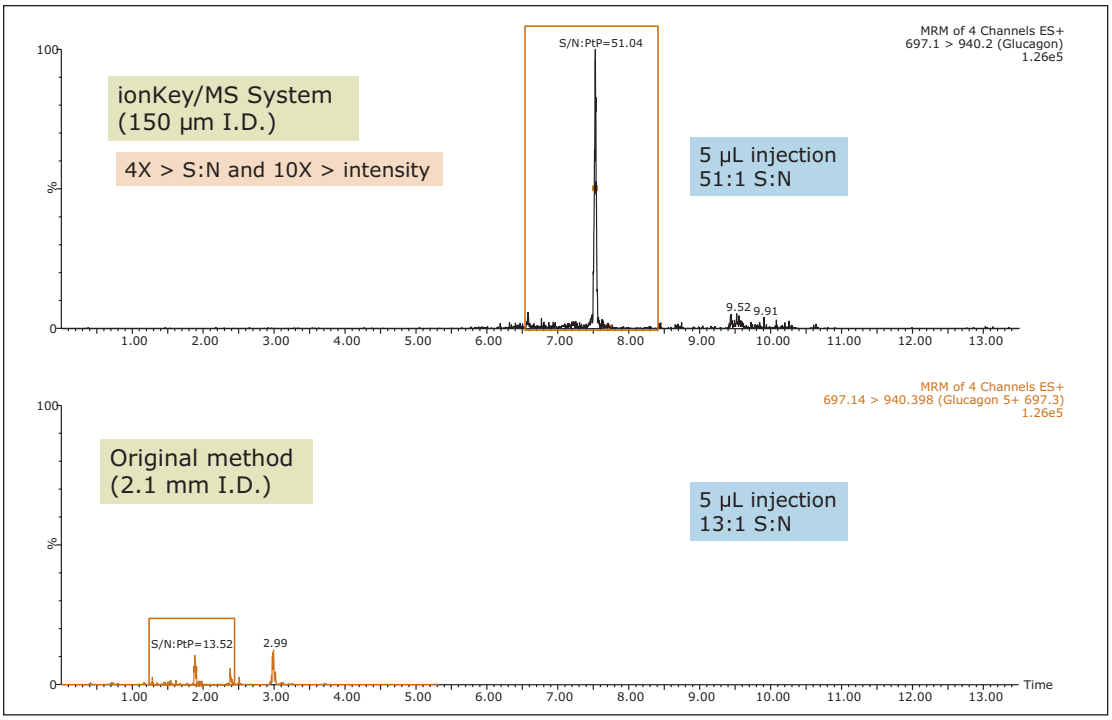


Figure 6. Comparison of 250 pg/mL glucagon extracted from human plasma (200 µL): iKey Separation Device (150 µm I.D.) vs. traditional analytical flow (2.1 mm I.D.).

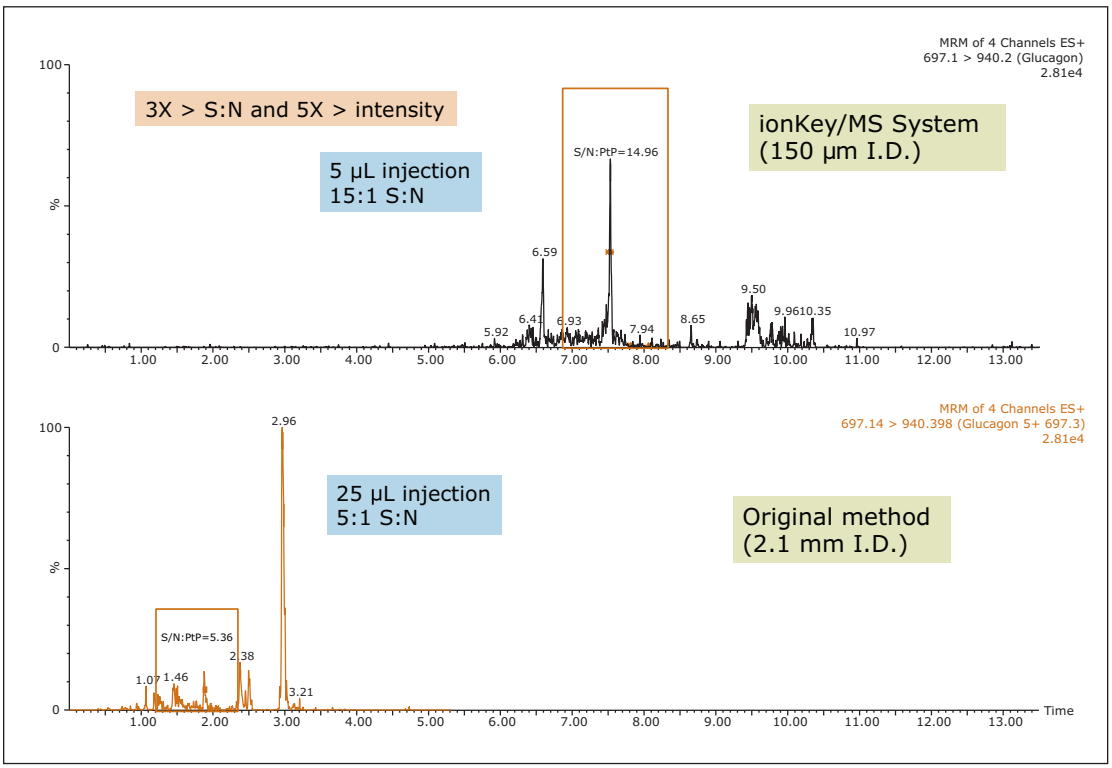


Figure 7. Comparison of 25 pg/mL glucagon extracted from human plasma: iKey Separation Device (5 µL injection) vs. traditional analytical flow (25 µL injection).

Specificity vs. sensitivity

Triple quadrupole mass spectrometers, when operated in MRM mode, offer a unique combination of sensitivity, specificity, and dynamic range. However, in biological matrices, the task of achieving analyte specificity can be difficult, particularly with large peptides due to the high abundance of other endogenous proteins and peptides. Thus, the challenge of improving specificity lies not only in the sample preparation, and chromatography, but also selective choice of MRM transition for analysis. In the case of glucagon, the 697.1/693.5 MRM transition, corresponding to the 5+ precursor and ammonia loss fragment, resulted in a 10X greater signal than any of the other selective precursor/fragment transitions when tested in neat standard solution (data not shown) and in extracted plasma. However, in extracted plasma samples it was not as specific and resulted in higher background noise. Figure 8 shows a 500 pg/mL extracted plasma sample, and demonstrates the increased sensitivity and lack of specificity of the ammonia loss transition compared to the selective b-ion transitions.

Std. conc (pg/mL)	Area	Calc. conc. (pg/mL)	%Dev	Accuracy
Blank	–	–	–	–
Blank	–	–	–	–
12.5	469	13.8	10.5	89.5
12.5	461	13.6	9.0	91.0
25	982	25.8	3.3	96.7
25	959	25.3	1.1	98.9
50	2005	49.8	-0.4	100.4
50	2080	53.5	6.9	93.1
100	3958	95.6	-4.4	104.4
100	3733	90.3	-9.7	109.7
250	10142	240.5	-3.8	103.8
250	9481	225.0	-10.0	110.0
500	20893	492.4	-1.5	101.5
500	20184	475.8	-4.8	104.8
1000	44244	1039.5	4.0	96.0
1000	44094	1036.0	3.6	96.4

Table 3. Glucagon standard curve summary statistics from 12.5–1,000.0 pg/mL extracted from human plasma.

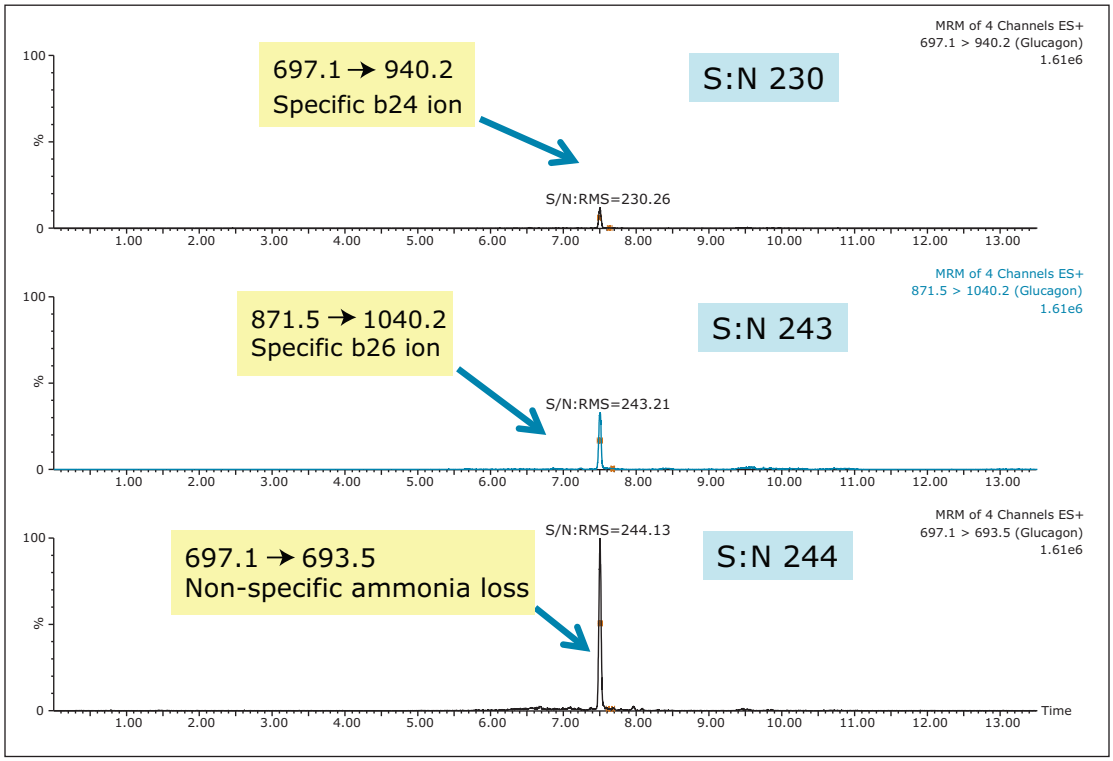


Figure 8. Comparison of glucagon MRM transition sensitivity and specificity in a 500 pg/mL extracted human plasma sample.

Although the intensity using the 697.1/ 693.5 MRM transition is 10X greater than the b-specific ion transitions (697.1>940.2 and 871.5 >1040.2), its sensitivity is mitigated by the accompanying higher background signal, as is demonstrated by the equivalent S:N ratios of all 3 MRM transitions. Additionally, measured matrix effects for the b-specific ion transitions were less than 10% in plasma, while the ammonia loss transition resulted in matrix effects between 10–15%. A summary of standard curve performance is shown in Table 3, and is illustrated in Figure 9. Using a 1/X regression, glucagon was linear from 12.5–1,000.0 pg/mL with R^2 values of >0.99 for all 3 MRM transitions monitored. Representative chromatograms for extracted glucagon plasma standard samples are shown in Figure 10.

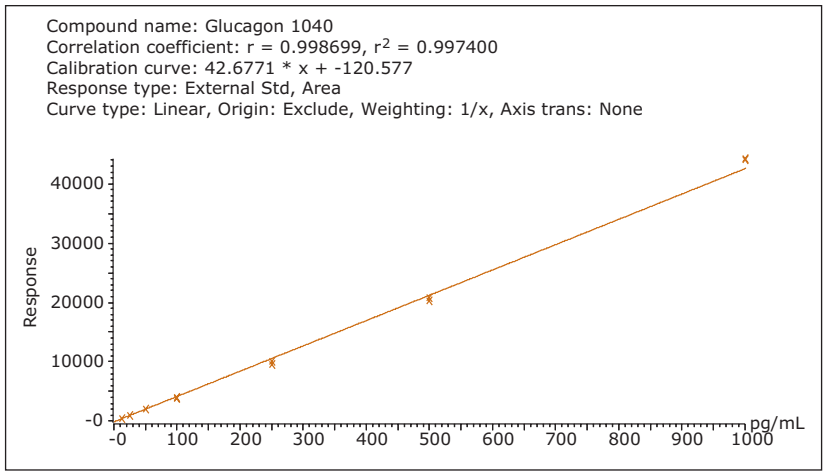


Figure 9. Representative standard curve in human plasma, from 12.5–1,000.0 pg/mL.

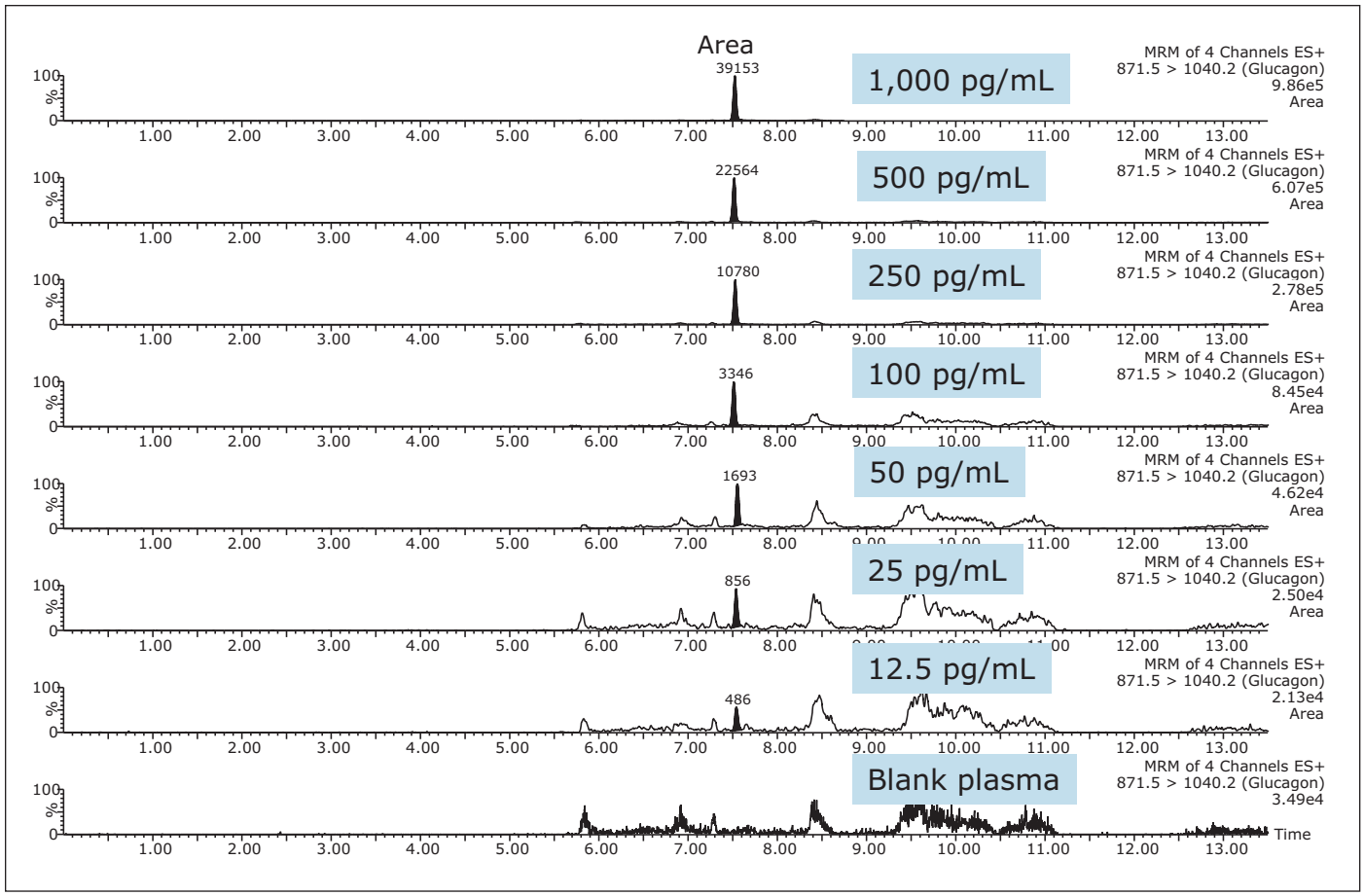


Figure 10. Representative chromatograms from glucagon extracted from plasma at 12.5, 25, 50, 100, 250, 500, and 1,000 pg/mL, compared to blank plasma.

CONCLUSIONS

The combination of the ionKey/MS System, mixed-mode µElution SPE, and higher *m/z* b or y ion MS fragments provided the level of selectivity and sensitivity necessary to accurately quantify low pg/mL concentrations of glucagon in extracted plasma. Use of µElution format SPE eliminated the need for evaporation, reducing glucagon losses due to adsorption and non-specific binding. The 150 µm iKey Separation Device enabled the development of a highly sensitive, low flow quantitative MRM method for glucagon with an LOD of 12.5 pg/mL and a dynamic range from 12.5–1,000.0 pg/mL. The current analysis uses 200 µL of plasma and provides a significant improvement in sensitivity and S:N over the analytical scale (2.1 mm I.D.) analysis using 1/5th the sample injection volume. Furthermore, an injection of the same volume (5 µL) of sample corresponded to a 10X increase in on-column sensitivity allowing for greater confidence in results, as compared to the traditional analytical method for this peptide. In addition, the ionKey/MS System reduces solvent and sample consumption, thereby reducing cost and allowing for multiple injections of samples for improved accuracy or to meet the guidelines for incurred sample reanalysis (ISR).

References

1. Eli Lilly and Company Glucagon United States package insert. http://pi.lilly.com/us/rglucagon-pi.pdf
2. R&D Systems, Inc. Quantikine® ELISA Glucagon Immunoassay, Catalog Number DGCG0. For the Quantitative Determination of Glucagon Concentrations in Cell Culture Supernates, Serum, and Plasma. R&D Systems, Inc., Minneapolis, MN, USA (2011).
3. EMD Millipore Corporation. Glucagon Chemiluminescent ELISA Kit, 96-Well Plate (Cat # EZGLU-30K). EMD Millipore Corporation, St. Charles, MO, USA (2012).
4. Millipore. Glucagon RIA Kit, 250 Tubes (Cat. # GL-32K). Millipore, St. Charles, MO, USA (2011).
5. ALPCO Diagnostics. Glucagon RIA: For the Quantitative Determination of Glucagon in Plasma. ALPCO Diagnostics, Salem, NH, USA (2011).
6. Lapko V, Brown P, Nachi R et al. Exploring quantification of peptides: measurement of glucagon in human plasma by LC-MS/MS. Presented at: EBF 3rd Annual Open Symposium: From Challenges to Solutions. Barcelona, Spain, 1–3 December 2010.
7. Lapko VN, Miller PS, Brown GP, Islam R et al. Sensitive glucagon quantification by immunochemical and LC-MS/MS methods. *Bioanalysis* (2013) 5(23).
8. Gerich JE, Lorenzi M, Bier DM et al., Effects of physiologic levels of glucagon and growth hormone on human carbohydrate and lipid metabolism. Studies involving administration of exogenous hormone during suppression of endogenous hormone secretion with somatostatin. *J.Clin Invest.* 1976 Apr; 57(4):875–84.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

DO YOU HAVE REAL WORLD

SENSITIVITY?

There's sensitivity you can get in ideal conditions. Then there's the rugged sensitivity you can get in any conditions. That's the power of Xevo® G2-XS, the next generation of QTof. To bring extraordinary possibilities to your everyday analysis, visit waters.com/XEVOG2XS

XEVO G2-XS QTof

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

PHARMACEUTICAL ▪ HEALTH SCIENCES ▪ FOOD ▪ ENVIRONMENTAL ▪ CHEMICAL MATERIALS

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Application of High- and Low-resolution Mass Spectrometers Combined with Nanoscale LC and Microfluidic LC Platforms for the Quantitative Analysis of Peptide Biomarkers in Human Serum

Billy J. Molloy, Chris J. Hughes, Johannes P.C. Vissers, and James I. Langridge
Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

- Comparison of eight distinct LC-MS platforms for the quantitative analysis of multiple proteolytic peptides from a non-fractionated, undepleted, tryptically-digested serum sample
- Low attomole levels of sensitivity
- Increased throughput and usability for large sample cohorts in translational research studies
- Excellent linear dynamic range and reproducibility

WATERS SOLUTIONS

Xevo® TQ-S, Xevo TQ-S micro, Xevo G2-XS QTof

SYNAPT® G2-S*i*

ionKey/MS™ System

ACQUITY UPLC® M-Class System

*Rapi*Gest™ SF Surfactant

KEYWORDS

Proteomics, peptides, tandem quadrupole, time-of-flight, nanoscale LC, MRM, biomarkers, translational research

INTRODUCTION

Targeted LC-MS-based methodologies are increasingly applied in the post-discovery proteomics area with an emphasis on validation, which is the first of many phases in translational research.[1] These types of experiments are technologically challenging, because they require the analysis of large sample cohorts with high throughput, high sensitivity, large linear dynamic range, and excellent selectivity. Multiple Reaction Monitoring- (MRM) based methods have the potential to provide the performance required to improve biomarker acceptance.

This application note presents the comprehensive comparison of both tandem quadrupole and time-of-flight mass spectrometers combined with nanoscale and microfluidics (ionKey) liquid chromatography platforms for the quantitative MRM-based analysis of peptides in a tryptically-digested, non-fractionated, undepleted human serum sample. The LC and MS platforms were combined to form eight distinct LC-MS configurations. Moreover, these platforms were compared in terms of throughput, sensitivity, linearity, and reproducibility in order to demonstrate their suitability for the analysis of large sample cohorts in translational research studies.

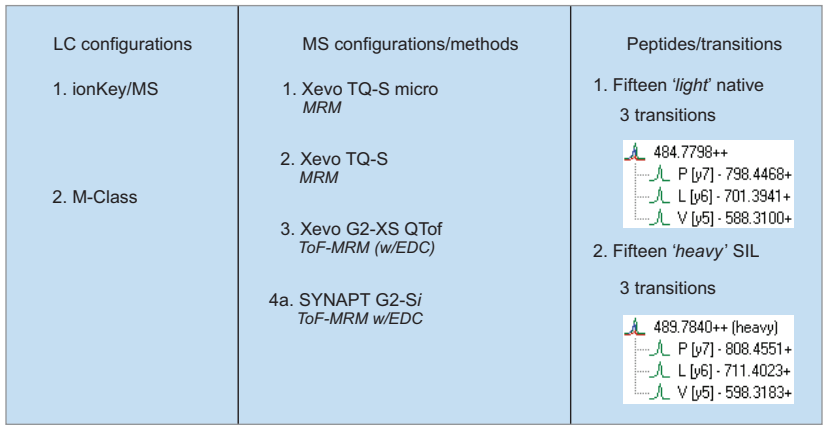


Figure 1. Experimental design for comparison of LC-MS platforms.

EXPERIMENTAL

Fifteen stable isotope-labeled (SIL) peptides – representing putative cardiovascular disease protein biomarkers – were obtained from PepScan (Lelystad, Netherlands). The peptides were simultaneously spiked as a dilution series from 6.25 amol to 12.5 fmol/µL in a non-fractionated, undepleted, tryptically-digested human serum sample.

HUMAN SERUM SAMPLE PREPARATION

20 µL of non-fractionated, undepleted human serum was diluted with 80 µL of 50 mM ammonium bicarbonate$_{(aq)}$, and denatured in the presence of 10 µL of 1% *Rapi*Gest detergent solution at 80 °C for 45 minutes. The plasma proteins were reduced in the presence of 5 µL of 100 mM dithiothreitol at 60 °C for 30 minutes, and alkylated in the dark in the presence of 6 µL of 200 mM iodoacetamide at ambient temperature for 30 minutes. Proteolytic digestion was initiated by adding 40 µL of 1 µg/µL sequencing grade, modified trypsin, and then incubated overnight at 37 °C. Breakdown of the acid-labile detergent was achieved in the presence of 1% TFA at 37 °C for 45 minutes. The peptide solutions were centrifuged at 13,000 rpm for 10 minutes, and the supernatants collected. The resulting solution was diluted with water prior to use in order to give a serum digest on-column amount of 200 ng (assuming a total protein concentration of 100 g/L).

LC conditions for nanoscale separation

LC system:	ACQUITY UPLC M-Class
Trap column:	ACQUITY UPLC M-Class Symmetry® C_{18}, 5 µm, 180 µm x 20 mm (P/N 186007496)
Analytical column:	ACQUITY UPLC M-Class HSS T3 C_{18}, 1.8 µm, 75 µm x 250 mm (P/N 186007474)
Column temp.:	35 °C for analytical column
Sample temp.:	8 °C
Injection volume:	1 µL
Flow rate:	300 nL/min
Mobile phase A:	0.1% formic acid
Mobile phase B:	Acetonitrile containing 0.1% formic acid
Gradient:	3–40% mobile phase B over 90 min

1 µL of sample was transferred with 0.1% formic acid$_{(aq)}$ to the trap column at a flow rate of 5 µL/min for 3 minutes. Mobile phase A was 0.1% formic acid$_{(aq)}$ and mobile phase B was acetonitrile containing 0.1% formic acid. After desalting and pre-concentration, the peptides were eluted from the trap column onto the analytical column, and separated with a gradient of 3 to 40% mobile phase B over 90 minutes at a flow rate of 300 nL/min, followed by a 2 minute column wash at 85% mobile phase B. The columns were then re-equilibrated to initial conditions for 20 minutes. The analytical column temperature was maintained at 35 °C.

LC conditions for microfluidic separation

LC system:	MRM using Xevo TQ-S, Xevo TQ-S micro, Xevo G2-XS QTof, and SYNAPT G2-S*i*
Device:	iKey™ Peptide BEH C_{18}, 1.7 µm, 150 µm x 100 mm (P/N 186006766)
Column temp.:	35 °C
Sample temp.:	8 °C
Injection volume:	1 µL
Flow rate:	1 µL/min
Mobile phase A:	0.1% formic acid
Mobile phase B:	Acetonitrile containing 0.1% formic acid
Gradient:	3–40% mobile phase B over 45 min

1 µL of sample was loaded directly onto the iKey Separation Device. Peptides were separated with a gradient of 3 to 40% mobile phase B over 45 minutes at a flow rate of 1 µL/min followed by a 6 minute column wash with 85% mobile phase B. The ionKey was then re-equilibrated to initial conditions for 9 minutes. The analytical column temperature was maintained at 35 °C.

MS conditions

MS system:	MRM using Xevo TQ-S, Xevo TQ-S micro, Xevo G2-XS QTof, and SYNAPT G2-S*i*
Ionization mode:	ESI+
Ion source temp.:	100 °C
Capillary voltage:	3.4 kV
Cone voltage:	30 V
Cone gas flow:	35 L/h
Nanoflow gas pressure:	0.2 bar

MRM analyses were performed using two tandem quadrupole mass spectrometers (Xevo TQ-S and Xevo TQ-S micro), and two hybrid quadrupole orthogonal acceleration time-of-flight (oa-Tof) mass spectrometers (Xevo G2-XS QTof and SYNAPT G2-S*i*). Endogenous and labeled peptides were targeted by at least three MRM transitions with a minimum of 10 data points over a chromatographic peak. Tandem quadrupole dwell and interscan delay times were automatically calculated by the operating software based on a minimum number of data points specified at half height across a chromatographic peak. Collision energies were set at fixed values for the tandem quadrupole instruments and ramped for the time-of-flight instruments. In addition, for the time-of-flight-based MRM acquisitions, integration and interscan delay times were manually set. Collision energies were ramped and initially calculated using the following regression equation, (0.034 x *m/z*) + 3.314 eV, and further optimized by CID fragmentation evaluation obtained by repeat injections of SIL peptides in the absence of matrix.

Table 1. MRM transition overview tandem quadrupole and oa-Tof MRM experiments.

peptide sequence	precursor *m/z* [charge]	fragment *m/z* [fragment ion type]					EDC *m/z**
		1	2	3	4	5	
LVNEVTEFAK	575.3 [2+]	595.3 [y5]	694.4 [y6]	823.4 [y7]	937.5 [y8]		937.5
LVNEVTEFA[K]	579.3 [2+]	603.3 [y5]	702.4 [y6]	831.4 [y7]	945.5 [y8]		945.5
ATEHLSTLSEK	608.3 [2+]	664.4 [y6]	777.4 [y7]	914.5 [y8]			777.4
ATEHLSTLSE[K]	612.3 [2+]	672.4 [y6]	785.4 [y7]	922.5 [y8]			785.5
TGLQEVEVK	501.8 [2+]	603.3 [y5]	731.4 [y6]	844.5 [y7]	901.5 [y8]		731.4
TGLQEVEV[K]	505.8 [2+]	611.3 [y5]	739.4 [y6]	852.5 [y7]	909.5 [y8]		739.4
FPEVDVLTK	524.3 [2+]	575.3 [y5]	674.4 [y6]	803.5 [y7]	900.5 [y8]		674.4
FPEVDVLT[K]	528.3 [2+]	583.4 [y5]	682.4 [y6]	811.5 [y7]	908.5 [y8]		682.4
FQPTLLTLPR	593.4 [2+]	599.4 [y5]	712.5 [y6]	813.5 [y7]	910.6 [y8]		910.6
FQPTLLTLP[R]	598.4 [2+]	609.4 [y5]	722.5 [y6]	823.5 [y7]	920.6 [y8]		920.6
TAAQNLYEK	519.3 [2+]	552.3 [y4]	666.3 [y5]	794.4 [y6]	865.4 [y7]		865.4
TAAQNLYE[K]	523.3 [2+]	560.3 [y4]	674.4 [y5]	802.4 [y6]	873.5 [y7]		873.5
LGPLVEQGR	484.8 [2+]	489.2 [y4]	588.3 [y5]	701.4 [y6]	798.4 [y7]	855.5 [y8]	701.4
LGPLVEQG[R]	489.8 [2+]	499.2 [y4]	598.3 [y5]	711.4 [y6]	808.5 [y7]	865.5 [y8]	711.4
AAAATGTIFTFR	613.8 [2+]	683.4 [y5]	784.4 [y6]	841.5 [y7]	942.5 [y8]	1013.6 [y9]	942.5
AAAATGTIFTF[R]	618.8 [2+]	693.4 [y5]	794.4 [y6]	851.5 [y7]	952.5 [y8]	1023.6 [y9]	952.5
EANYIGSDK	498.7 [2+]	519.3 [y5]	682.3 [y6]	796.4 [y7]			682.3
EANYIGSD[K]	502.7 [2+]	527.3 [y5]	690.4 [y6]	804.4 [y7]			690.4
ESDTSYVSLK	564.8 [2+]	609.4 [y5]	696.4 [y6]	797.4 [y7]	912.5 [y8]		696.4
ESDTSYVSL[K]	568.8 [2+]	617.4 [y5]	704.4 [y6]	805.5 [y7]	920.5 [y8]		704.4
GYSIFSYATK	568.8 [2+]	569.3 [y5]	716.4 [y6]	829.4 [y7]	916.5 [y8]		916.5
GYSIFSYAT[K]	572.8 [2+]	577.3 [y5]	724.4 [y6]	837.5 [y7]	924.5 [y8]		924.5
GFYFNKPTGYGSSSR	834.4 [2+]	713.3 [y7]†	814.4 [y8] †	911.4 [y9]			911.4
GFYFNKPTGYGSSS[R]	839.4 [2+]	723.3 [y7]†	824.4 [y8] †	921.4 [y9]			921.4
LVNVVLGAHNVR	645.9 [2+]	653.3 [y6]	766.4 [y7]	865.5 [y8]	964.6 [y9]	1078.6 [y10]	766.4
LVNVVLGAHNV[R]	650.9 [2+]	663.4 [y6]	776.4 [y7]	875.5 [y8]	974.6 [y9]	1088.6 [y10]	776.4
ITLYGR	361.7 [2+]	395.2 [y3]	508.3 [y4]	609.3 [y5]			609.3
ITLYG[R]	366.7 [2+]	405.2 [y3]	518.3 [y4]	619.3 [y5]			619.3
SYPGLTSYLVR	628.3 [2+]	637.4 [y5]	738.4 [y6]	851.5 [y7]	908.5 [y8]	1005.6 [y9]	908.5
SYPGLTSYLV[R]	633.3 [2+]	647.4 [y5]	748.4 [y6]	861.5 [y7]	918.5 [y8]	1015.6 [y9]	918.5

[K] = $^{13}C_6{}^{15}N_4$ labeled; [R] = $^{13}C_6{}^{15}N_2$ labelled; *oa-Tof MRM based acquisitions only; † <10% most abundant MRM fragment ion.

RESULTS

THROUGHPUT

The fastest gradient separations possible were employed without introducing isobaric interferences for the fifteen SIL peptides monitored for both LC platforms. The microfluidics interface operates at a higher flow rate with a reduced number of connections compared to the nanoscale LC setup. Because of this, system volumes are more rapidly cleared, achieving faster gradient delivery and column conditioning. Consequently, extra column volumes – which normally can lead to band broadening – will be less critical. The complete experimental injection-to-injection cycle times were 1 and 2 hours for the microfluidics and nanoscale LC-based separations, respectively – providing a 2-fold increase in throughput for the microfluidics experiments. Figure 2 demonstrates this increase in throughput for one of the SIL peptides. Peak capacities under these gradient conditions were found to be similar for both LC configurations (data not shown).

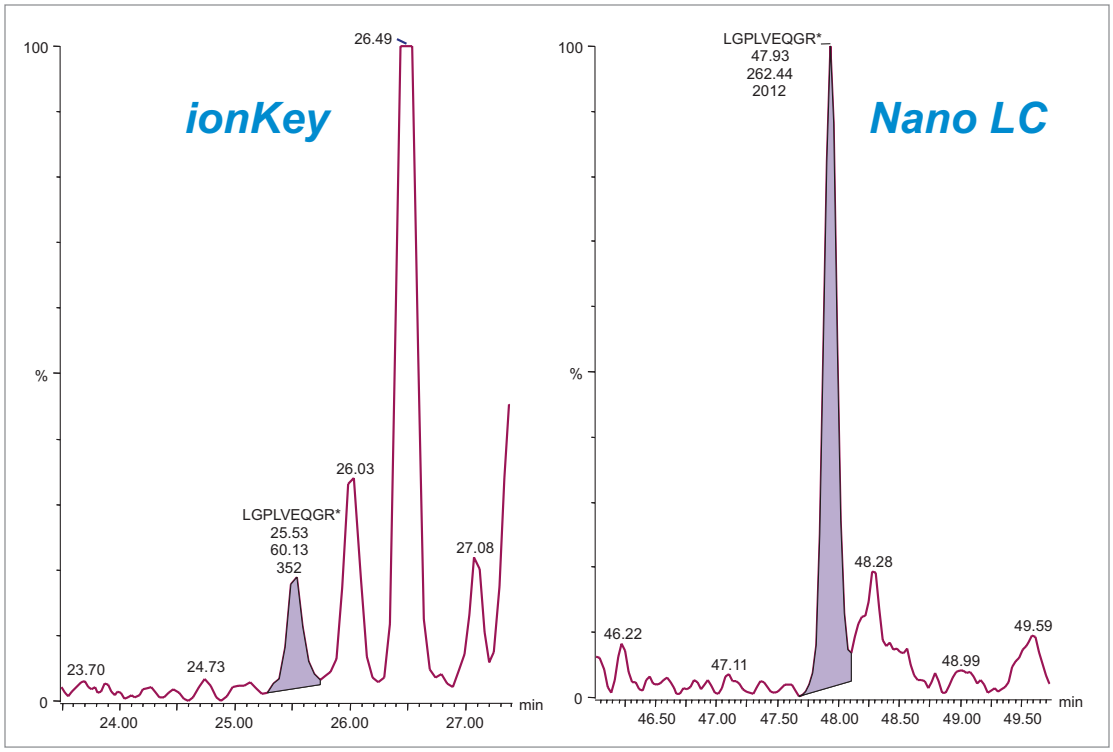


*Figure 2. ionKey/MS and nanoscale LC detection for LGPLVEQG**R** at 12.5 amol on-column in serum matrix on Xevo G2-XS QTof.*

SENSITIVITY

The peak-to-peak, signal-to-noise ratio values measured at the 12.5 amol level for the nanoscale LC-MS platforms, and 125 amol level for the microfluidics LC-MS platforms, were found to be comparable on average across the various SIL peptides on all four MS platforms. Figure 3 demonstrates the similar sensitivity observed when using the microfluidic LC platform along with tandem quadrupole and time-of-flight mass MS platforms. Peak-to-peak, signal-to-noise values ranged from 2 to 30 for the nanoscale LC-MS platforms, and 2 to 100 for the microfluidics LC-MS platforms at the two levels described above. The median and mean lower limit of detection (LLOD) values across all MS platforms were 5 and 8 amol for the nanoscale LC-MS platforms, and 20 and 39 amol for the microfluidics LC-MS platforms, demonstrating nanoscale LC to be approximately 4 times the sensitivity of microfluidics LC. Figure 5 shows the average sensitivity across all SIL peptides for the four nanoscale LC-MS platforms in terms of both signal-to-noise ratio and limit of detection.

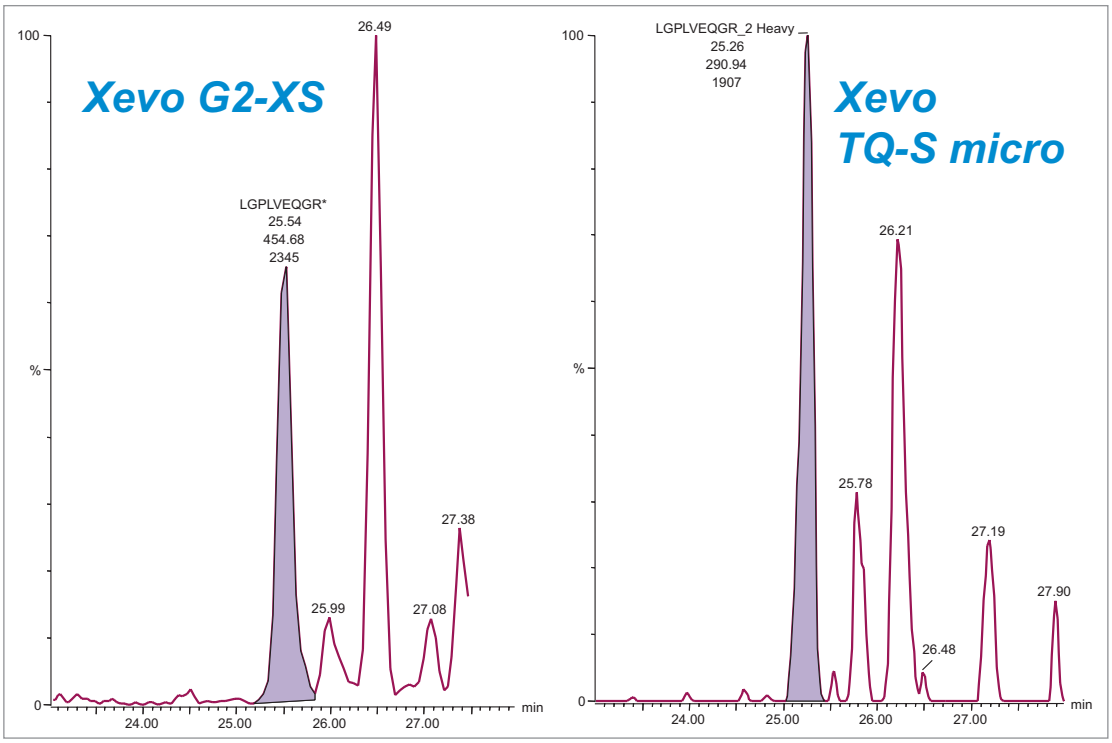


*Figure 3. Xevo G2-XS QTof and Xevo TQ-S micro responses for LGPLVEQG**R** at 62.5 amol on-column in serum matrix using ionKey/MS.*

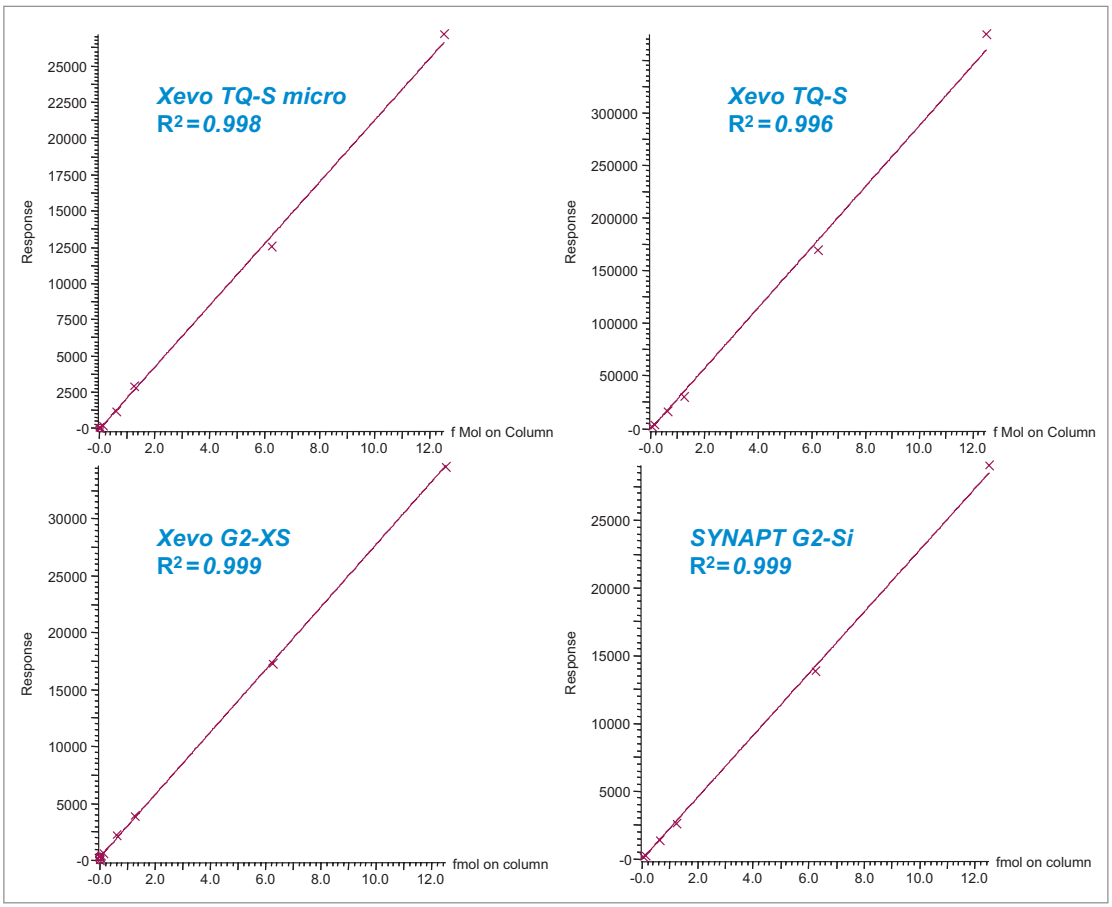


*Figure 4. Comparison of the linearity achieved for four different MS configurations using ionKey/MS (TGLQEVEV**K**).*

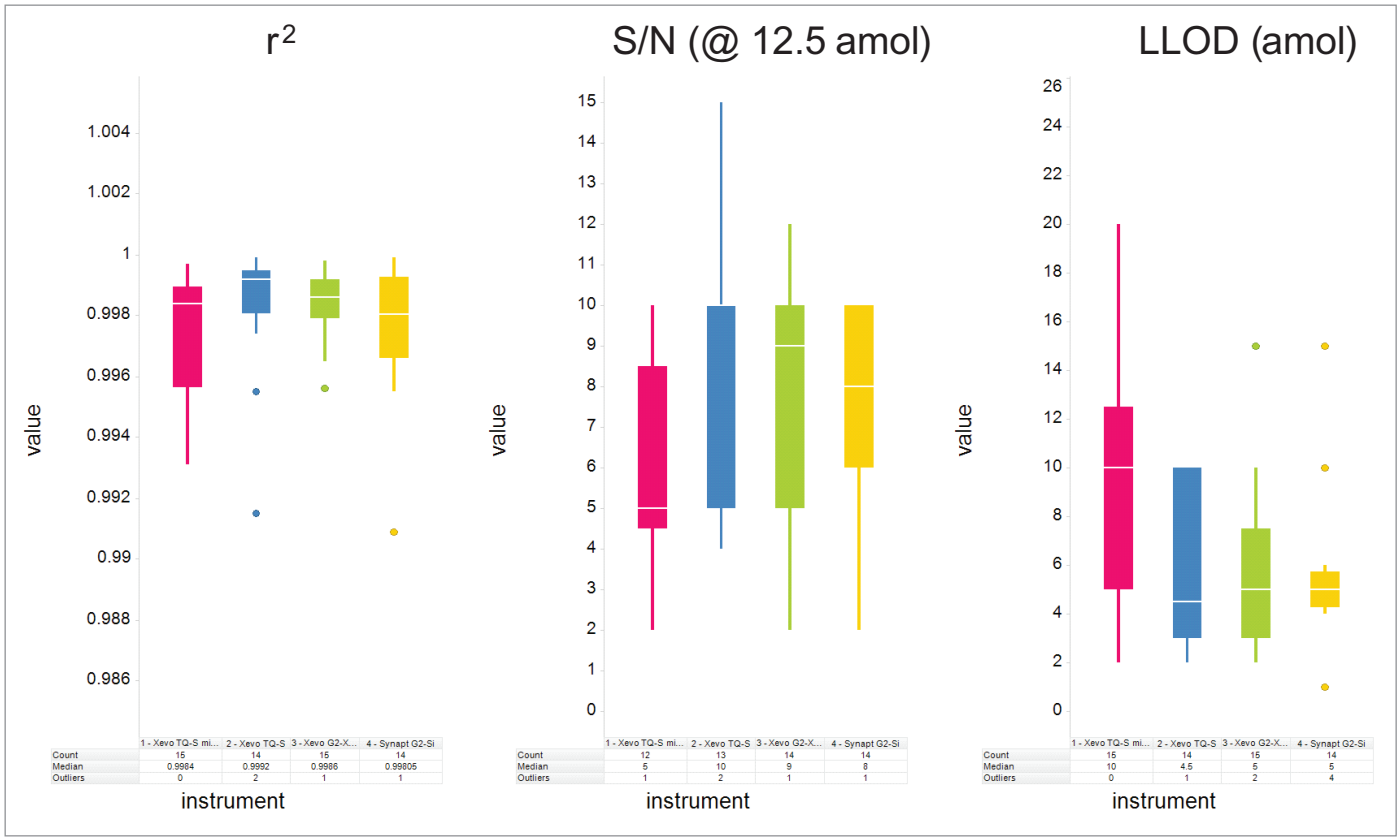


Figure 5. Average linearity and sensitivity of MS-MS platforms using Nano LC.™

LINEARITY

All eight LC-MS platforms demonstrated 1/x weighted linear behavior with r^2 regression correlation coefficient values of 0.986 or greater for all SIL peptides over the tested ranges. Figure 4 demonstrates the linearity of all four MS platforms for one of the SIL peptides using the microfluidics LC platform. Figure 5 graphically summarizes the average r^2 regression correlation coefficient values observed across all SIL peptides on all four nanoscale LC-MS platforms, as well as the S/N at 12.5 amol injected on-column and the estimated LLOD.

SENSITIVITY AND REPRODUCIBILITY

As the fifteen SIL peptides were spiked in a non-fractionated, undepleted, tryptically-digested human serum matrix, the quantitation of the endogenous peptides present in the matrix, within the measured linear dynamic range, was possible. The average concentration and relative standard deviation (%RSD) were calculated for each of the peptides that were present within this range across all eight LC-MS platforms. Figure 7 illustrates the overall average sensitivity/reproducibility relationship for the eight LC-MS configurations studied, showing the earlier mentioned 4-fold sensitivity difference between LC platforms and improved reproducibility for the tandem quadruple mass spectrometers. The results plotted in Figure 6 show the individually-determined average concentrations as well as the average relative standard deviation (%RSD) for each LC-MS configuration for these peptides.

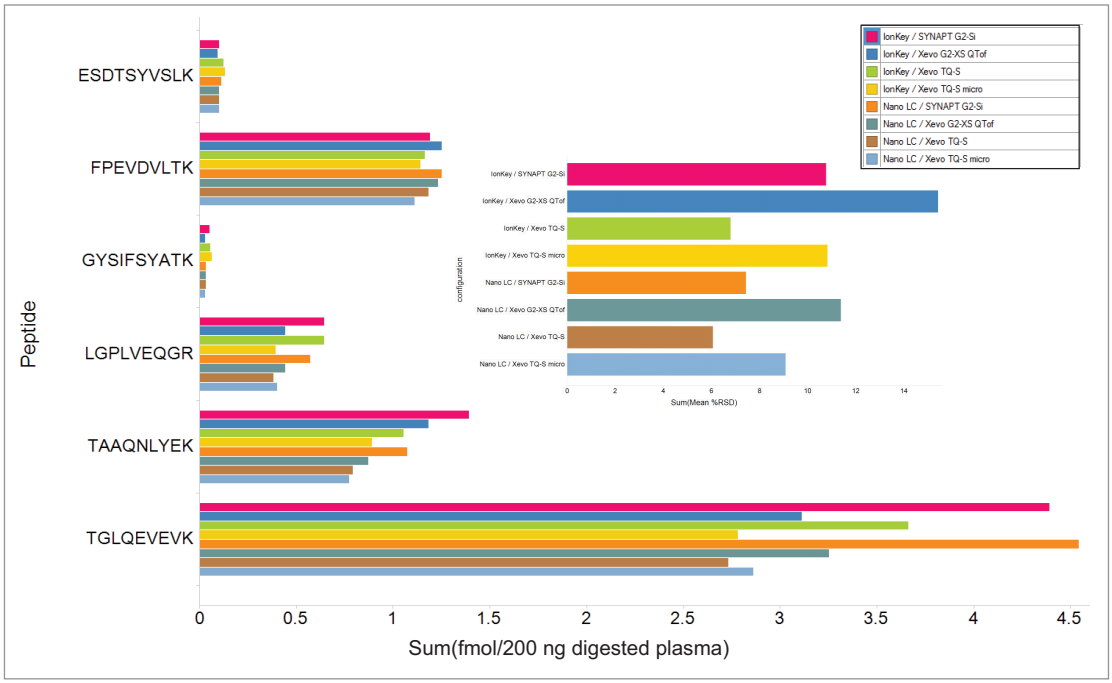


Figure 6. Quantitative results summary of the levels of six candidate peptide biomarkers (fmol/200 ng of matrix) using standard MRM settings on various LC-MS platforms. Inset comparison of reproducibility in terms of the mean %RSD values obtained for the analysis on various LC-MS platforms.

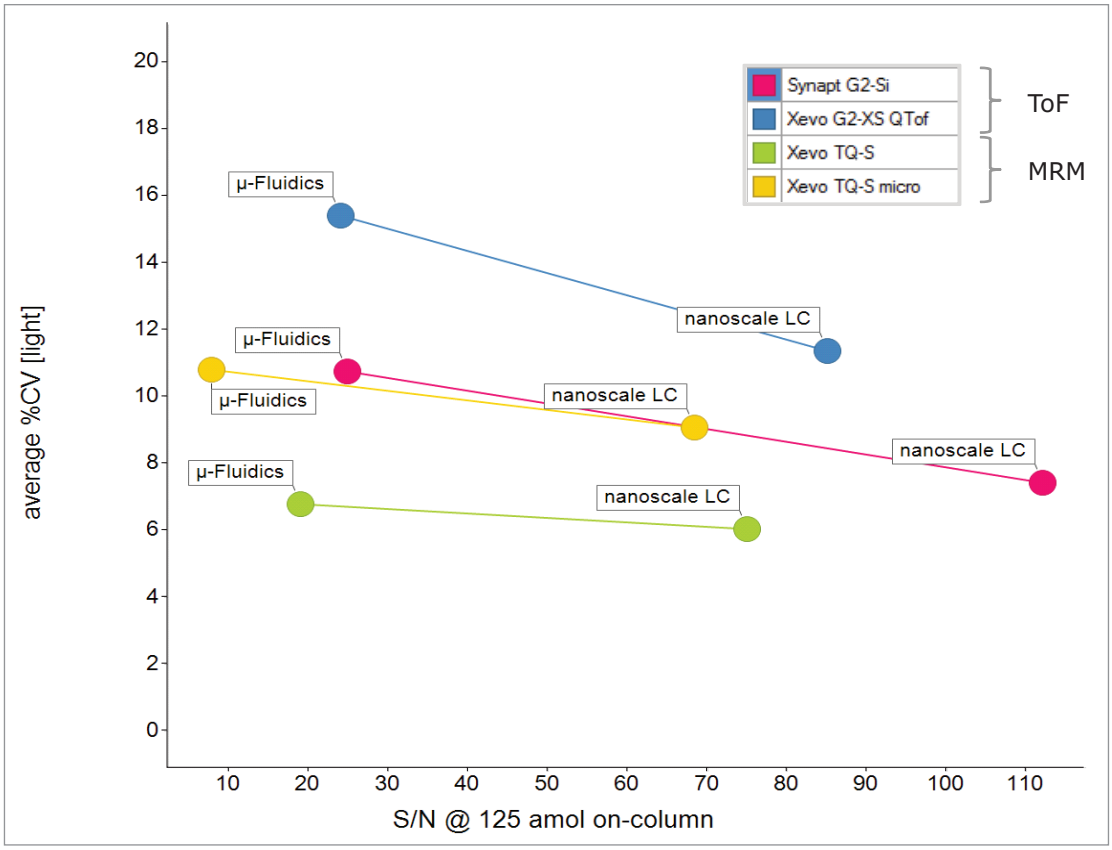


Figure 7. Average experiment-wide endogenous peptide %CV and S/N showing comparison of LC-MS configurations.

CONCLUSIONS

Throughput is increased when using the microfluidic LC-MS platforms when compared to nanoscale LC-MS platforms. However, this must be balanced against the increased sensitivity of the nanoscale LC-MS platforms. The limits of detection possible using the microfluidic LC-MS platforms were within the range of 3 to 250 amol, depending on the peptide and LC-MS platform used. The linearity of all eight LC-MS platforms were shown to be excellent across the ranges measured. In terms of reproducibility, all eight LC-MS platforms demonstrated %RSD values below 16%, with the tandem quadrupole LC-MS platforms showing increased reproducibility when compared to the high-resolution time-of flight platforms, especially the Xevo TQ-S platform.

When all of these factors are combined and considered, the increased throughput, usability, and reproducibility – balanced against a relatively small drop in sensitivity – make the microfluidic LC platform combined with the Xevo TQ-S tandem quadrupole mass spectrometer the ideal LC-MS system for translational research studies where low level quantitation of tryptic peptides is required.

References

1. Mbasu, et al. Advances in Quadrupole and Time-of-Flight Mass Spectrometry for Peptide MRM based Translational Research Analysis. *Proteomics*, in press. May 23, 2016. doi: 10.1002/pmic.201500500

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Improving a High Sensitivity Assay for the Quantification of Teriparatide in Human Plasma Using the ionKey/MS System

Mary E. Lame and Erin E. Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- High sensitivity assay with LOD of 10 pg/mL in human plasma
- Reduced solvent consumption (50X) compared to 2.1 mm scale means significant cost savings
- Use of solid-phase extraction (SPE) reduces matrix interferences and enhances selectivity of the extraction for teriparatide in plasma
- 96-well µElution plate format enables concentration of the sample while maintaining solubility and minimizes peptide losses due to adsorption
- Selective, fast SPE extraction (<30 minutes) without time-consuming immuno-affinity purification
- Compared to 2.1 mm scale, proof of concept studies yield 4X greater S:N from 4X less sample and half the injection volume allowing for greater confidence in results, more tests per sample, and more injections

WATERS SOLUTIONS

ionKey/MS™ System
ACQUITY UPLC® M-Class System
ionKey™ Source
Xevo® TQ-S
iKey™ Separation Device
Oasis® HLB 96-well µElution Plate
Waters Collection Plate
MassLynx® Software
TargetLynx™

KEY WORDS

bioanalysis, Oasis, sample preparation, peptide quantification, teriparatide, UPLC, 2D Technology, plasma, ionKey/MS, iKey

INTRODUCTION

Teriparatide (FORTEO®), Figure 1, is a recombinant form of a fragment of human parathyroid hormone, used in the treatment of osteoporosis. Osteoporosis is responsible for 1.5 million bone fractures a year and teriparatide is the first treatment that stimulates new bone formation. It is an anabolic drug that acts to build up bones and has the potential to improve skeletal micro architecture and increase bone density. Teriparatide is the first 34 amino acids (the biologically active region) of the 84-amino acid human parathyroid hormone (PTH), and is also referred to as, rhPTH (1–34).[1]

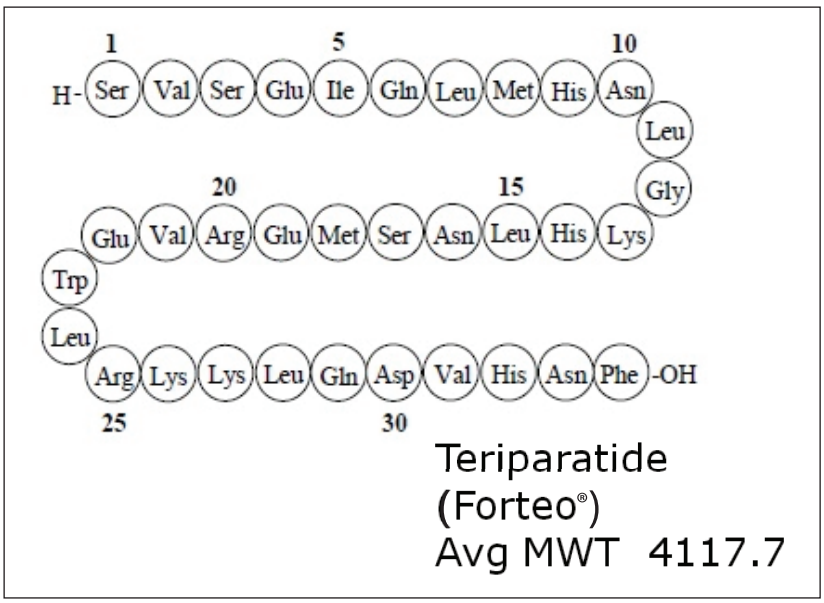


Figure 1. Representative structure and amino acid sequence of teriparatide.

Although biologics have historically been quantified using ligand binding assays (LBAs), over the past few years, there has been a trend toward the analysis of large molecules by LC-MS/MS. This is, in part, driven by the fact that LBAs can suffer from significant cross-reactivity issues and lack of standardization. LC-MS/MS has the advantage of shorter development times, greater accuracy and precision, the ability to multiplex, and can readily distinguish between closely related analogues, metabolites or endogenous interferences.

(Continued on page 96)

EXPERIMENTAL

Sample prepration

Samples were pretreated using protein precipitation (PPT) and extracted on an Oasis HLB 96-well µElution Plate, 2 mg Sorbent per Well, 30 µm (p/n 186001828BA) particle size according to a previously published method.[3]

Method conditions

UPLC conditions

LC system:	ACQUITY UPLC M-Class with 2D Technology configured with optional trap and back flush elution
Separation device:	iKey Peptide BEH C_{18} Separation Device, 130Å, 1.7 µm, 150 µm x 50 mm (p/n 186006764)
Trap column:	ACQUITY UPLC M-Class Symmetry C_{18} Trap Column, 100Å, 5 µm, 300 µm x 50 mm (p/n 186007498)
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Loading solvent:	95:5 mobile phase A:B, 35 µL/min for first two minutes, reverse valve
Valve position:	Initial position one (forward loading of trap), switch to position two at two minutes (back flush elute of trap onto the analytical column)

Final optimized analytical gradient: See Table 1

Elution flow rate:	2.0 µL/min
iKey temp.:	75 °C
Sample temp.:	15 °C
Final injection vol.:	15 µL
Total run time:	13.0 minutes
Collection plates:	Waters 1 mL Collection Plates (p/n 600001043)

MS conditions

MS system:	Xevo TQ-S
Ionization mode:	ESI positive
Capillary voltage:	3.6 kV
Source temp.:	120 °C
Cone gas flow:	50 L/hr
Collision cell pressure:	$3.83 \times 10^{(-3)}$ mbar
Collision energy:	Optimized by component, see Table 2
Cone voltage:	Optimized by component, see Table 2

Data management

Chromatography software:	MassLynx 4.1
Quantification software:	TargetLynx

The need for robust and sensitive analysis of peptide species challenges both chromatographic separation and mass spectrometry. Peptides, in general, are often difficult to analyze by LC-MS/MS, as MS sensitivity is low due to the formation of multiple precursors and poor or overly extensive fragmentation, making LC and sample preparation even more critical. In addition, teriparatide also suffers from significant non-specific binding and poor solubility, making LC and sample preparation method development challenging.

The pharmacokinetics of teriparatide are characterized by rapid absorption within 30 minutes and rapid elimination with a half-life of 1 hour, resulting in a total duration of exposure (to the peptide) of approximately 4 hours.[1,2] At the practical clinical dose of 20 µg the typical teriparatide levels are ~50 pg/mL, which makes detection by traditional LC-MS/MS even more difficult.

Through a combination of selective sample preparation, optimal MS precursor and fragment choice, and UPLC® separation on a charged surface column, we developed and published an analytical scale method for accurate, precise, teriparatide quantification with a detection limit of 15 pg/mL.[3] In this current work however, we undertook to a) transfer this method to the ionKey/MS System (phase 1), and b) further improve the method through the inherent characteristics of the ionKey/MS System (phase 2). This technology integrates the UPLC analytical separation directly into the source of the mass spectrometer (Figure 2). The iKey Separation Device (150 µm I.D.), shown in Figure 3, contains the fluidic channel, electronics, ESI interface, heater, eCord,™ and the chemistry to perform UPLC separations. Additionally, the ionKey/MS System can provide increased sensitivity compared to 2.1 mm I.D. chromatography with the same injection volume, or equivalent or greater sensitivity with reduced sample consumption, making it ideal for peptide analyses. It is common for bioanalytical LC-MS assays to consume high volumes of both solvent and sample, thus increasing the cost of the assay and limiting the number of replicates that can be analyzed. This study combines µElution SPE and the novel and highly efficient ionKey/MS System to improve a quantitative assay for teriparatide in human plasma. In phase 1, we will demonstrate the effective transfer of the previously developed analytical method using a 200 µL sample size to the ionKey/MS System. Results will show that we can readily achieve a limit of detection (LOD) of 10 pg/mL with a linear dynamic range of 10–3,000 pg/mL in human plasma, using 1/3 of the injection volume. Mean accuracy and precision of quality control samples were 102.9 and 3.5%, respectively. In phase 2, we show proof of concept for further method improvement, fully capitalizing on the attributes of the ionKey/MS System to reduce the sample volume by 4X, reduce injection volume by half, and increase signal-to-noise (S:N) by 4X over the 2.1 mm I.D. scale.

Time (min)	Flow rate (µL/min)	Composition A (%)	Composition B (%)	Curve
0.00	2.0	85	15	Initial
5.00	2.0	55	45	6
6.00	2.0	5	95	6
8.00	2.0	5	95	6
9.00	2.0	85	15	6

Table 1. UPLC gradient conditions.

Figure 2. ionKey/MS System: comprised of the Xevo TQ-S, the ACQUITY UPLC M-Class, the ionKey Source, and the iKey Separation Device.

Figure 3. iKey Separation Device.

RESULTS AND DISCUSSION

Mass spectrometry

Several multiple-charged precursors were observed for teriparatide and rhPTH (1-38). The 6+ charge state of teriparatide at *m/z* 687.05 was determined to be the most intense and yielded a selective fragment at *m/z* 787.26 for quantitative analysis. The 7+ precursor at *m/z* 589 was also intense, but did not yield any useable fragments. CID of the 5+ precursor at *m/z* 824.25 produced fragment ions of sufficient intensity to be used for confirmatory purposes. The 6+ charge state of the IS [rhPTH(1-38)] at *m/z* 637.58 and its fragment ion at *m/z* 712.51 was used for quantitation. Although many peptides produce intense fragments below *m/z* 200, these ions (often immonium ions) result in high background in extracted samples due to their lack of specificity. In this assay, the use of highly specific y ion fragments above *m/z* 700 yielded significantly improved specificity, facilitating the use of simpler SPE methodologies.

Phase I. Initial chromatographic separation

Chromatographic separation of teriparatide and its IS was achieved using the novel microfluidic chromatographic iKey Separation Device. The iKey Separation Device has a channel with UPLC-grade, sub-2-µm particles that permits operation at high pressure and results in highly efficient LC separations. By integrating microscale LC components into a single platform design, problems associated with capillary connections, including manual variability, leaks, and excessive dead volume are avoided. iKey Peptide BEH C18 Separation Device, 130Å, 1.7 µm, 150 µm x 50 mm (p/n 186006764) provided excellent peak shape, narrow peak widths (<2.5 secs at base), and resolution from endogenous matrix interferences.

Representative chromatograms of teriparatide and the IS, eluted using an initial linear gradient from 8 to 65% B over 5 minutes, on an iKey Peptide BEH C_{18} Separation Device, 130Å, 1.7 µm, 150 µm x 50 mm (p/n 186006764), are shown in Figure 4. These samples were extracted from 200 µL of sample and 10 µL was injected. This corresponded to injecting 1/3 of the sample required for the 2.1 mm I.D. scale, but extracting the same sample volume. The use of multidimensional chromatography, specifically a trap and back-elute strategy, provided further sample cleanup and facilitated the loading of 10–15 µL of the high organic SPE eluate (required to maintain solubility of the peptides) without experiencing analyte break through. Additionally, the ability to inject the larger sample volumes typical for analytical scale LC analysis on the iKey Separation Device can provide the substantial gains in sensitivity that are often required to accurately and reliably detect low pg/mL levels of peptide and protein in complex matrices.

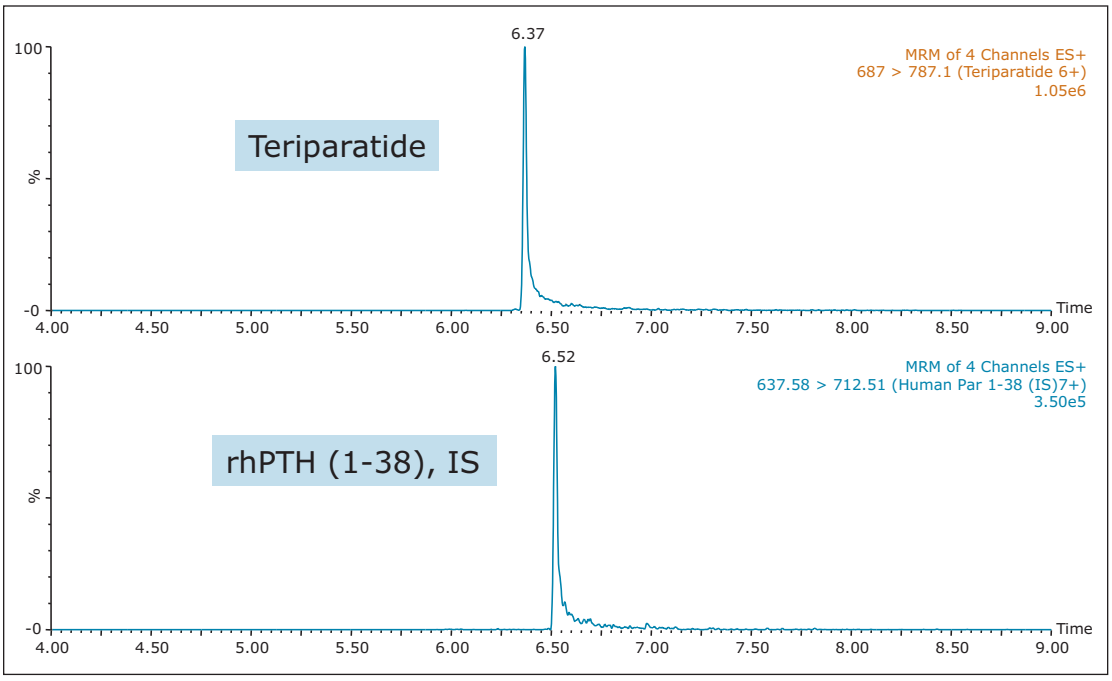


Figure 4. UPLC separation of teriparatide and IS, from extracted plasma, using the iKey Peptide BEH C_{18} Separation Device, 130Å, 1.7 µm, 150 µm x 50 mm (p/n 186006764).

Sample preparation

Development of this assay was challenging due to non-specific binding (NSB) and maintenance of peptide solubility throughout the SPE extraction and elution process. Sample pretreatment prior to SPE proved to be critical in improving recovery and specificity. Protein precipitation (1:1) with 5% NH_4OH in acetonitrile resulted in 80–100% recovery without precipitating the peptide itself. Use of Oasis HLB SPE provided a reversed-phase mode of retention, enabling sample cleanup, selectivity, concentration of the sample, and ultimate sensitivity for this peptide. Teriparatide and the IS were well retained on this SPE sorbent during the basic pH load step, with no break through occurring. Optimization of the elution solution was critical to fully elute teriparatide, maintain its solubility and minimize interferences from the plasma matrix. The optimum elution solution was 60% organic, with 1% trifluoroacetic acid, and 5% trifluoroethanol (TFE), the latter being added to maintain solubility of the compound. Additionally, the Oasis HLB 96-well µElution Plate can be processed manually in under 30 minutes and is compatible with most liquid-handling robotic systems for automation to meet sample throughput requirements. This format also provides the ability to concentrate the sample and elute in very small sample volumes, minimizing the potential for peptide losses that might occur during evaporation due to adsorption to the walls of collection plates and/or chemical instability.

Linearity, accuracy, and precision

To generate standard curves, human plasma was fortified with teriparatide at the following final concentrations: 10, 20, 40, 60, 100, 300, 600, 1,000, and 3,000 pg/mL. Each standard level was prepared in duplicate. Quality control (QC) samples (N=5) were prepared from the same plasma at 25, 50, 80, 200, and 500 pg/mL. Human parathyroid hormone 1-38 [rhPTH (1-38)] was used as the internal standard (IS). Peak area ratios (PARs) of the analyte peak area to the IS peak were calculated. The calibration curve was constructed using PARs of the calibration samples by applying a one/concentration (1/x) weighted linear regression model. Using 1/x regression, teriparatide was linear with an R^2 value of >0.99. A summary of standard curve performance (10–3,000 pg/mL) is shown in Table 3. All QC sample concentrations were then calculated from their PARS against the calibration curve. Results from QC analysis are shown in Table 4. Figure 5 contains representative chromatograms for QC samples containing teriparatide at 25, 50, 80, 200, and 500 pg/mL extracted from 200 µL human plasma as compared to blank extracted plasma. At all levels, QC samples demonstrated very good accuracy and precision, with mean accuracies ranging from 101.2-104.9 and mean %CV's of 2.56–5.09. These results easily meet the recommended FDA acceptance criteria outlined in the white papers describing best practices in bioanalytical method validation for LC-MS/MS assays.[4,5]

Peptide	MRM transition	Cone voltage (V)	Collision energy (eV)
Teriparatide	687.05 > 787.26	45	18
	824.25 > 983.79	45	25
Human Parathyroid 1-38 (ISTD)	637.58 > 712.61	45	11
	892.22 > 854.80	45	21

Table 2. MRM transitions, collision energies, and cone voltages for teriparatide and human parathyroid hormone 1-38 [rhPTH (1-38)], the IS.

Teriparatide concentration (pg/mL)	Teriparatide/IS ratio response	Calculated teriparatide concentration (pg/mL)	Mean accuracy
10.00	0.07	10.56	105.63
20.00	0.14	20.53	102.63
40.00	0.29	38.99	97.58
60.00	0.43	57.58	95.97
100.00	0.73	97.00	97.00
300.00	2.17	286.39	95.50
600.00	4.75	626.81	104.45
1,000.00	8.05	1061.49	106.15
3,000.00	22.31	2937.14	97.95

Table 3. Standard curve summary and statistics from 10–3,000 pg/mL for teriparatide extracted from human plasma.

Teriparatide QC concentration (pg/mL)	Mean (N = 5) calculated concentration (pg/mL)	SD	%CV	Mean accuracy
25	25.8887	1.32	5.09	103.6
50	51.4236	1.91	3.72	102.8
80	83.8803	2.15	2.56	104.9
200	202.3569	6.49	3.20	101.2
500	511.1018	15.23	2.98	102.2

Table 4. QC statistics from teriparatide extracted from human plasma.

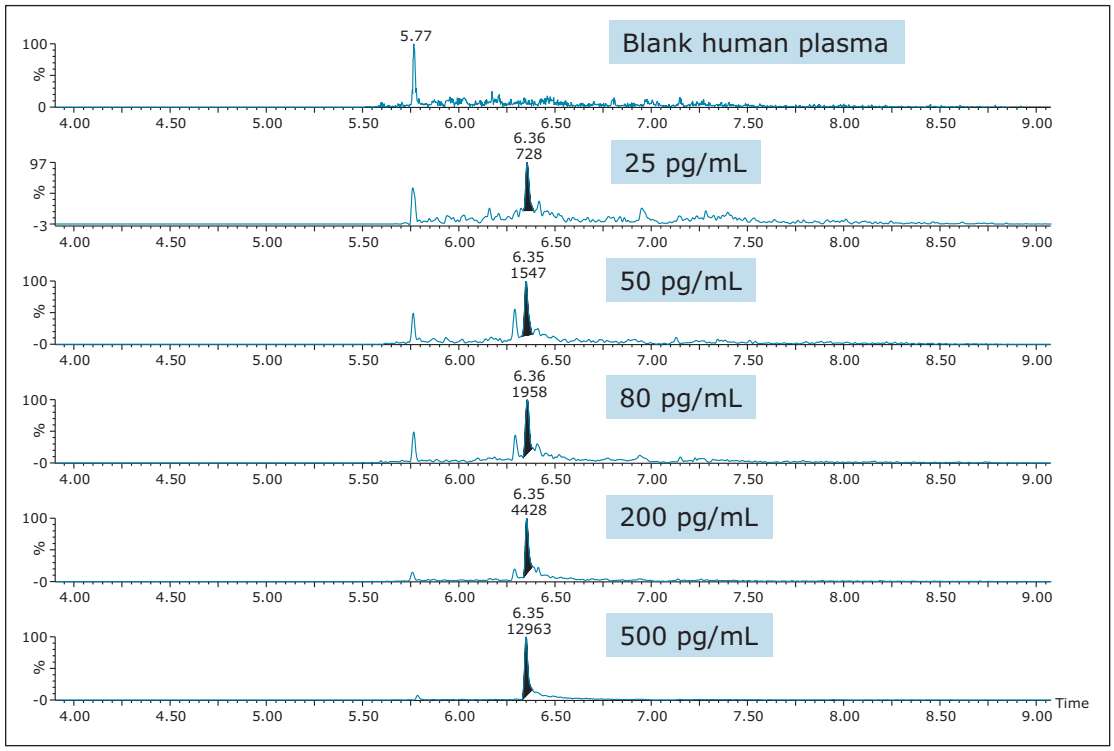


Figure 5. Representative QC chromatograms of teriparatide extracted from 200 µL of human plasma at 25, 50, 80, 200, and 500 pg/mL compared to extracted blank plasma.

Phase II. Minimizing sample requirements and reducing injection volume using the ionKey/MS System

Initial validation of the method using the ionKey/MS System demonstrated increased sensitivity over the 2.1 mm I.D. scale, which allowed us to make significant further improvements. Two of the key benefits of integrated microscale separations are the ability to maintain or improve sensitivity using smaller sample volumes, and lower injection volumes. This obviously has the advantage of preserving precious study samples or allowing one to gain more information from each sample, especially if initial volume is limited (*i.e.* rat or mouse samples.) Following the original validation using 200 µL of sample, further chromatographic refinements were made and a proof of concept study was performed where only 50 µL of sample were extracted. Representative chromatograms from these QC samples containing teriparatide at 15, 25, and 50 pg/mL, as compared to blank extracted plasma, are shown in Figure 6. The linearity of the method (R^2 = 0.998) extracting 50 µL sample and injecting 15 µL, is shown in Figure 7. Finally, S:N for a 20 pg/mL extracted sample comparing the 2.1 mm published method to the ionKey/MS System proof of concept work is shown in Figure 8. While S:N is approximately 11:1 at the 2.1 mm I.D. scale, it is 45:1 using the ionKey/MS System with 4X less sample, and half the injection volume. The cumulative sensitivity and sample reduction benefits are particularly significant for labs where ultra-high sensitivity is required, especially from small samples.

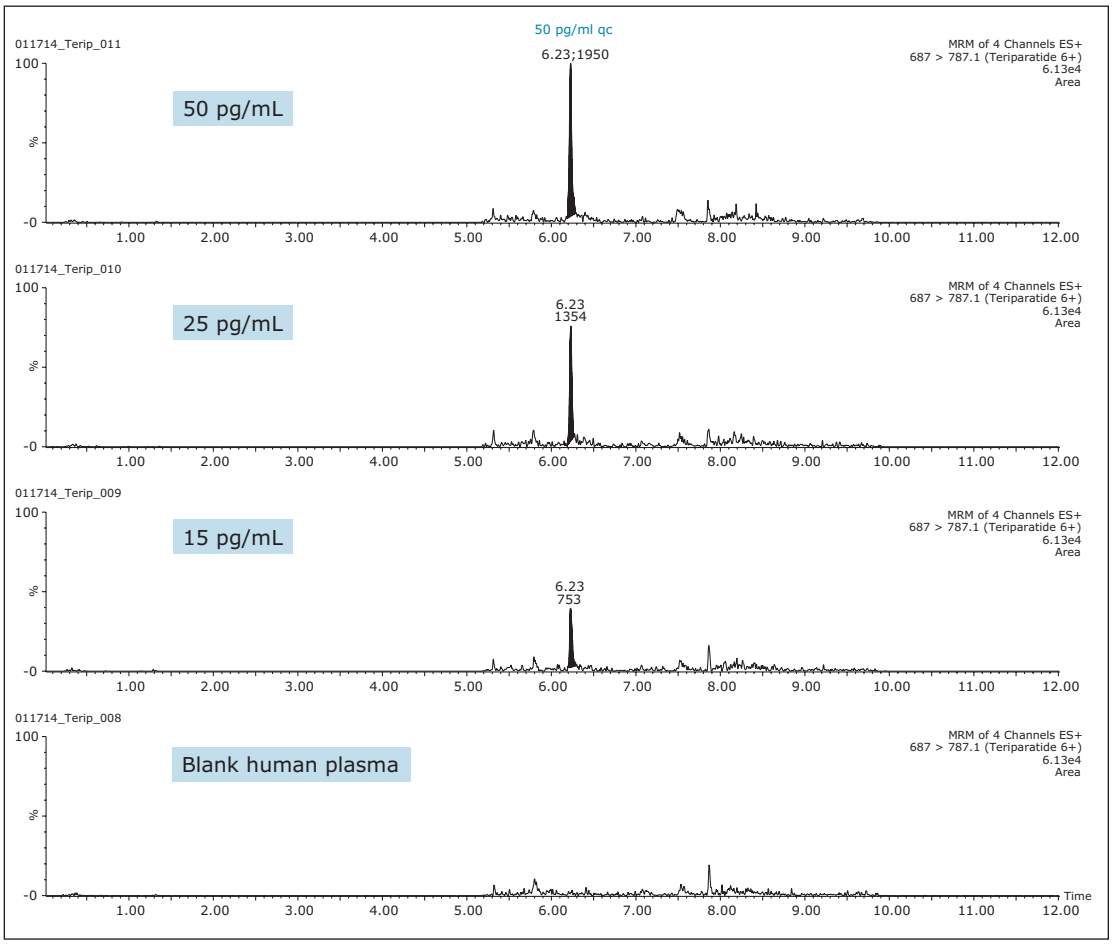


Figure 6. Representative QC chromatograms of teriparatide extracted from 50 µL human plasma at 15, 25, and 50 pg/mL compared to extracted blank plasma.

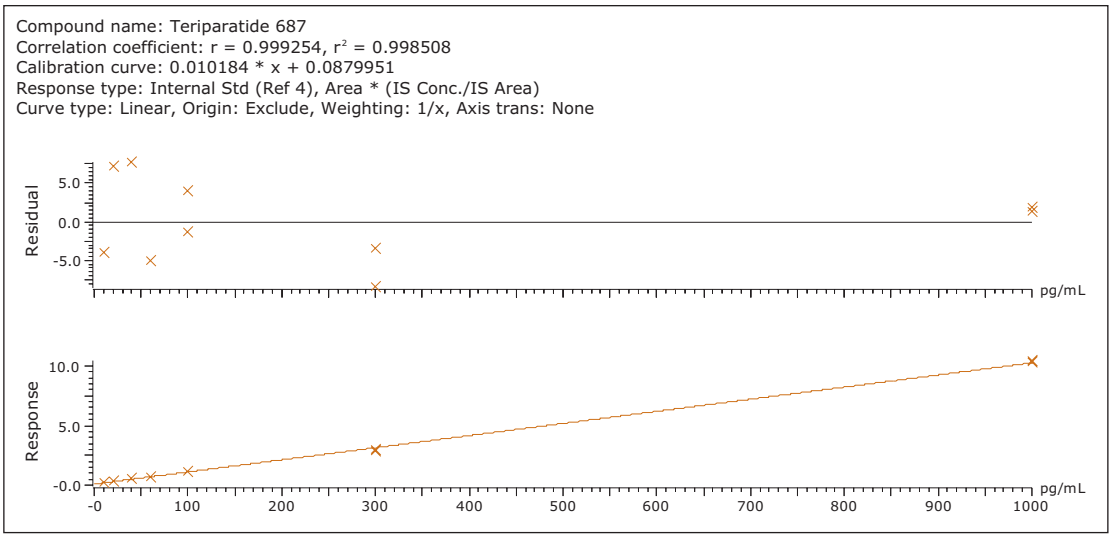


Figure 7. Linearity of the optimized ionKey/MS System assay.

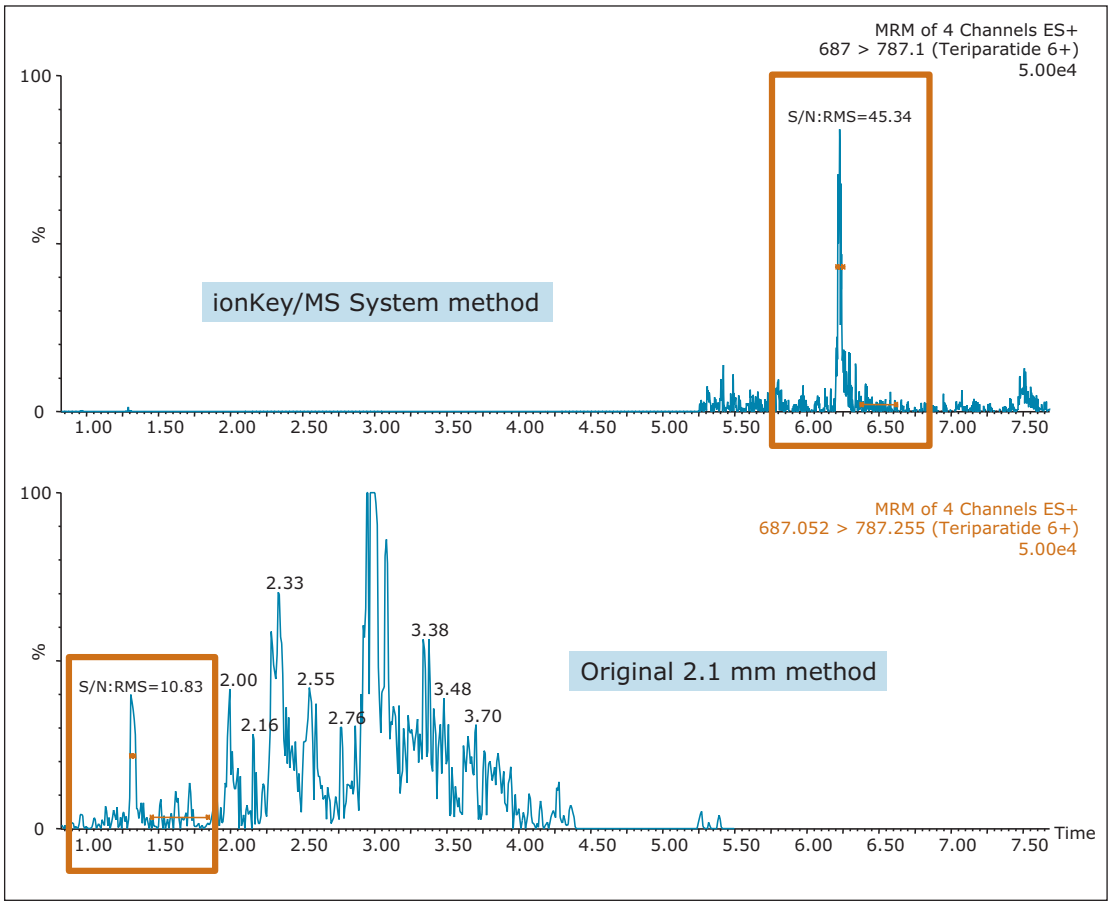


Figure 8. A comparison of 20 pg/mL teriparatide extracted from human plasma using the optimized ionKey/MS System method and an optimized 2.1 mm scale method. 50 µL of plasma were extracted for the ionKey/MS System and 200 µL plasma for 2.1 mm scale.

CONCLUSIONS

The combination of the ionKey/MS System, µElution reversed-phase SPE, and higher *m/z* b or y ion MS fragments provided the level of selectivity and sensitivity necessary to accurately quantify low pg/mL concentrations of teriparatide in extracted plasma. Use of the µElution format SPE plate eliminated the need for evaporation, reducing teriparatide losses due to adsorption and non-specific binding. The use of the 150 µm iKey Separation Device enabled the development of a highly sensitive, quantitative MRM method for teriparatide with an LOD of 10 pg/mL from only 200 µL of plasma with a 10 µL injection of sample. Standard curves were accurate and precise from 10–3,000 pg/mL. QC samples at all levels easily met recommended FDA regulatory criteria[4,5] with mean accuracies ranging from 101.2–104.9 and mean %CV ranges of 2.56–5.09, indicating an accurate, precise, and reproducible method. The ionKey/MS System method was further optimized to provide a 4X improvement in S:N over the 2.1 mm I.D. scale using 4X less sample and half the injection volume. In addition, the ionKey/MS System also reduces solvent and sample consumption, thereby reducing cost and allowing for multiple injections of samples for improved accuracy or to meet the guidelines for ISR. This method shows great promise for high sensitivity quantification of teriparatide in patient samples from PK and clinical studies using the ionKey/MS System if further validation was performed.

References

1. Eli Lilly and Company (2009) Teriparatide [rhPTH(1–34)] (Forteo) United States package insert.
2. Satterwhite J, Heathman M, Miller PD, Marin F, Glass EV, Dobnig H. Pharmacokinetics of teriparatide (rhPTH[1–34]) and calcium pharmacodynamics in postmenopausal women with osteoporosis. *Calcif Tissue Int* 2010 Dec;87(6):485–92.
3. Chambers E, Lame M, *et al., Journal of Chromatography B,* 938 (2013) 96–104.
4. Viswanathan CT, Bansal S, Booth B, DeStefano AJ, Rose MJ, Sailstad J, Shah VP, Skelly JP, Swann PG, Weiner R, Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays, *Pharm. Res.,* 24 (2007) 1962–1973.
5. Bansal S, DeStefano A, Key elements of bioanalytical method validation for small molecules, *AAPS J.*, 9 (2007) E109–114.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

A Rapid and Sensitive Method Estimation of Octreotide in Human Plasma Using UPLC and Xevo TQ-S

Veeranjaneyulu P., Thirupatheswara Rao B., Sudarshan Mantha, and Gopal Vaidyanathan
Waters India MS Application Laboratory, Bangalore, India

APPLICATION BENEFITS

A unique optimization of this Waters system for bioanalysis, from sample preparation to UPLC® chromatography to tandem quadrupole MS, enables the determination and quantification of octreotide at the 5 pg/mL level.

WATERS SOLUTIONS

Oasis® SPE

ACQUITY UPLC®

Xevo® TQ-S

KEY WORDS

Octreotide, octapeptide

INTRODUCTION

Octreotide is an octapeptide (Figure 1) that mimics natural somatostatin pharmacologically, and compared to natural hormone is a more potent inhibitor of growth hormone, glucagon, and insulin. Octreotide has been used to treat malignant bowel obstruction. Octreotide is also used in the treatment of acromegaly, diarrhea, and flushing episodes associated with carcinoid syndrome. In addition, octreotide has been used with varying degrees of success in infants to decrease insulin hypersecretion, and investigated for patients with pain from chronic pancreatitis.

Despite the wide therapeutic use of octreotide, it is a challenge to identify the fate of this drug and its metabolites in plasma because of a lack of analytical instruments and technologies that can monitor drug concentrations at very low pg/mL levels. Although an LC/MS/MS method has been previously reported[1] for the quantitative analysis of octreotide in human plasma, the sample preparation procedure included a manual protein precipitation step followed by liquid-liquid extraction step. Such steps are not well suited for high-throughput applications.

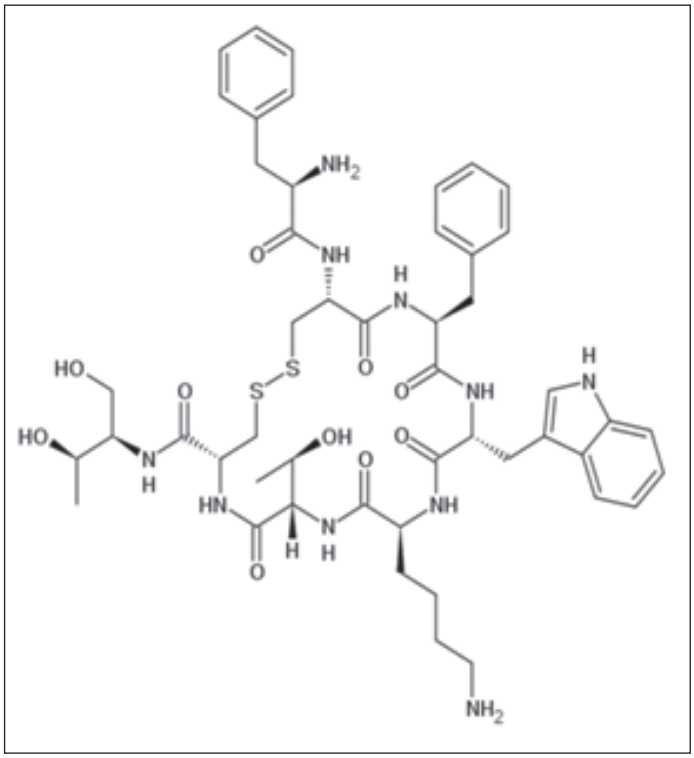


Figure 1. Octreotide structure.

EXPERIMENTAL

All the dilutions for octreotide were made in 50% (v/v) methanol in water to obtained required spiking solutions. The plasma was then spiked with diluted octreotide solutions and calibration standards ranging from 5 to 640 pg/mL were prepared. Leuprolide was used as an internal standard (IS) at a concentration of 2 ng/mL, also prepared with 50% (v/v) methanol in water. The samples were isolated by solid phase extraction (SPE) utilizing a Waters Oasis WCX cartridge. A 500 µL aliquot of plasma with 50 µL of IS was diluted with an aqueous acidic solution and loaded onto the SPE cartridge previously condition with organic solvent and water. The plasma solution was then washed with a basic solution, eluted in solvent, evaporated to dryness and reconstituted in mobile phase for analysis by LC/MS/MS.

LC conditions

LC system:	ACQUITY UPLC System equipped with a Binary Solvent Manager, Column Manager, and Sample Manager
LC column:	ACQUITY UPLC BEH 300 C_{18}, 2.1 x 100 mm, 1.7 µm
Gradient:	Reversed-phase chromatography with an acidic aqueous buffer solution and acetonitrile as the organic modifier
Elution:	20-80% organic gradient over 3.25 min
LC gradient run:	5.0 min starting with 90% acid solution and 10% acetonitrile
Column temp.:	50 °C

MS conditions

MS system:	Xevo TQ-S
MS mode:	Positive ion electrospray MS/MS
MS transition:	510.54 ⇒ 120.10

In this study, we demonstrate detection of octreotide in human plasma at very low concentrations using a Waters ACQUITY UPLC System coupled with a Xevo TQ-S tandem quadrupole mass spectrometer. When compared to an HPLC, UPLC helps a user achieve faster separations with lower consumption of mobile phase. In addition, UPLC offers short run times with low dispersion resulting in better separation of signals from unwanted signals from plasma, phospholipids, and other endogenous materials.

This study will demonstrate the robustness of the method that was developed to analyze octreotide for a range of 5 to 640 pg/mL.

RESULTS AND DISCUSSION

Octreotide is a cyclic peptide with molecular weight of 1019.24 while its IS, leuprolide, is a single, non-glycosylated polypeptide chain with a molecular weight of 1209.4. The octreotide analyte eluted with a retention time of 1.39 min. The chromatogram in Figure 2 shows excellent symmetrical peak shape and resolution from endogenous interferences. In addition, all the six chromatograms at the 5 pg/mL lower level of quantitation (LLOQ) level for octreotide showed signal-to-noise ratio greater than 20 (Figure 2).

The quality of the peak shape and resolution can be attributed to an optimized combination of the ACQUITY UPLC System and its ACQUITY UPLC BEH 300 C_{18} Column. Also, the results showed excellent reproducibility at the LLOQ levels (Table 1), which provides a unique ability to quantify octreotide at the LLOQ concentration.

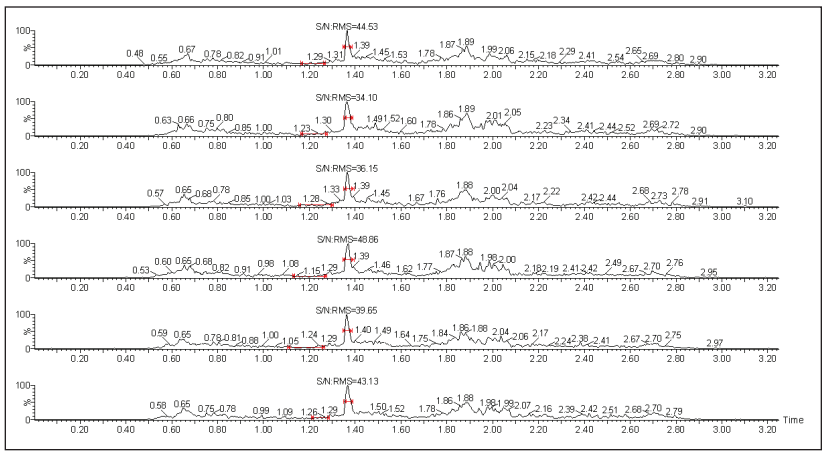


Figure 2. Signal-to-noise ratio observed for six replicate injections of octreotide at different LLOQ levels.

The high quality data obtained for octreotide at the LLOQ level can also be attributed to the high sensitivity detection capabilities provided by the Xevo TQ-S System. The co-joined, off-axis StepWave™ ion guide in the Xevo TQ-S provides superior levels of sensitivity while maintaining the robustness and cleanliness of the source and ion optics. This enables the Xevo TQ-S to increase the ion flux entering the mass spectrometer, resulting in the highest levels of sensitivity. The assay reported in this study was demonstrated to be linear over the range of 5 to 640 pg/mL in both organic solvents and human plasma samples.

The data obtained for octreotide showed %RSD to the order of about 3% and did not vary significantly within three different concentration ranges (Figure 3 and Appendix, Table 1). In addition, the accuracy and %RSD observed at the LLOQ level was in good agreement with that observed for the three other concentration ranges (Figure 3 and Appendix, Table 1). The accuracy and precision at different concentration ranges of octreotide were in excellent agreement within two different batches of samples that were analyzed (Figure 4 and Appendix, Table 2).

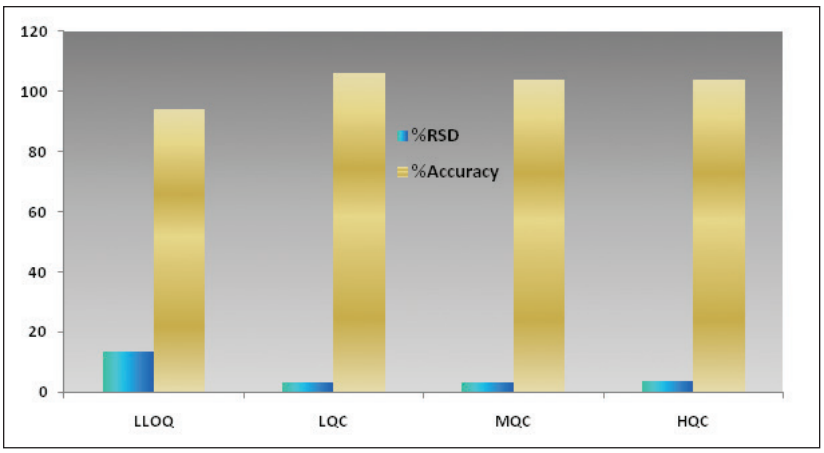


Figure 3. Bar graph showing %RSD and %Accuracy of one batch of six octreotide samples at four different concentration levels including LLOQ, LQC, MQC, and HQC.

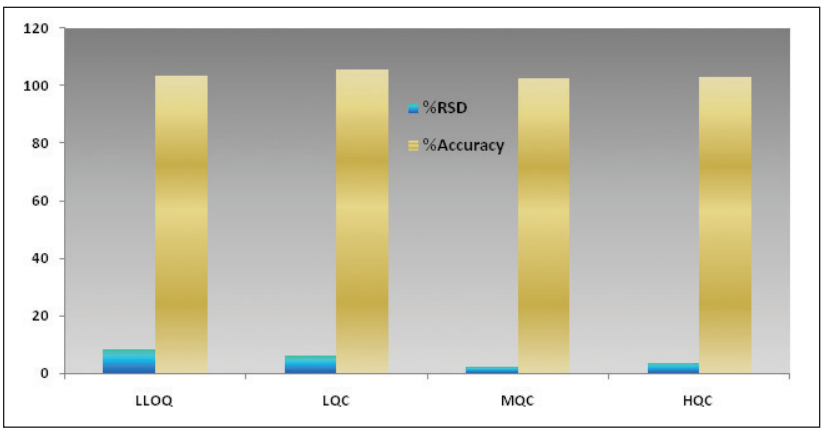


Figure 4. Bar graph showing %RSD and %Accuracy of a second batch of six octreotide samples at four different concentration levels including LLOQ, LQC, MQC, and HQC.

In addition, the global precision did not vary significantly between the three different concentration ranges and the accuracy of the results was within acceptable range (Figure 5 and Appendix, Table 3). Such high degree of precision and accuracy and low %RSD can be attributed to the robustness, reproducibility, and sensitivity of the combination of Waters technologies including best in class sample preparation capability, ACQUITY UPLC, and Xevo TQ-S.

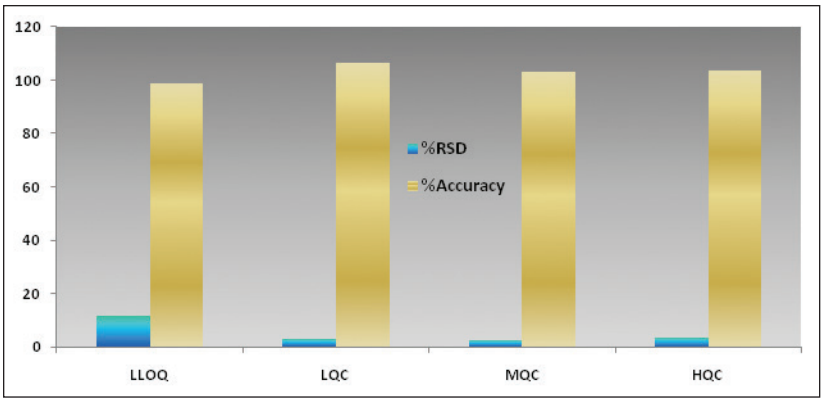
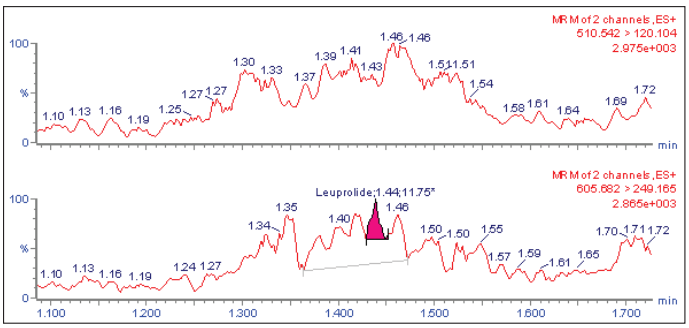


Figure 5. Bar graph showing global %RSD and %Accuracy of six octreotide samples at four different concentration levels including LLOQ, LQC, MQC, and HQC.

The UPLC/MS/MS chromatogram of the blank plasma sample and that of the octreotide at LLOQ level showed little interference from the endogenous materials (Figures 6A and Figure 6B). In addition, the phospholipid elution was checked by injecting an extracted standard with an MS scan at 184 ⇒ 184 *m/z*, a unique MRM transition for typical phospholipids (Figure 7). No significant phospholipid elution was observed at the retention time of either the octreotide analyte or its IS, leuprolide.

Such excellent separation of analyte of interest from any endogenous materials helps address some major regulatory issues, such as estimation of matrix interference. Once again, such abilities are achieved with an optimized combination of Waters sample preparation technology, chemistry, ACQUITY UPLC, and Xevo TQ-S.



Figures 6A. MRM of blank plasma and blank plasma spiked with IS, leuprolide.

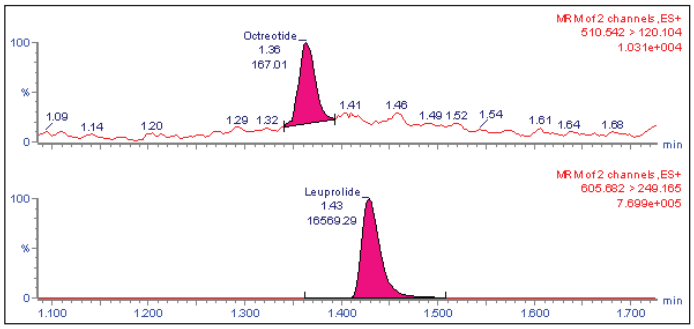


Figure 6B. MRM of octreotide and its IS, leuprolide.

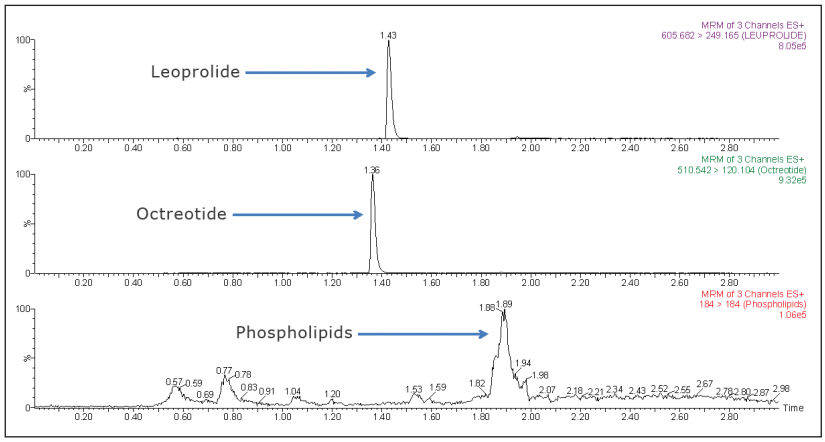


Figure 7. Comparison of phospholipid elution with respect to leuprolide (IS) and octreotide.

CONCLUSION

The low circulating concentration levels of octreotide, a cyclic octapeptide that is used for treating cancer patients, requires a highly sensitive assay for accurate determination of the pharmacokinetics. However, such a highly sensitive analytical method with proper sample preparation protocols was not known for octreotide and hence its analysis in the low pg/mL concentration level was not previously achievable.

This study demonstrates that the combination of Waters Oasis SPE, an ACQUITY UPLC System with ACQUITY UPLC BEH 300 C_{18} Column, and the Xevo TQ-S Mass Spectrometer enables the development of an assay for octreotide with an LLOQ of 5 pg/mL in human plasma.

UPLC chromatograms not only demonstrate better sensitivity, but also better separation compared to any conventional HPLC instruments. The data in this study exhibit low %RSD, a high degree of accuracy, and excellent batch-to-batch reproducibility and, therefore, demonstrate the benefits of sensitivity, robustness, and reproducibility of this integrated bioanalytical system solution.

Reference

1. Jiang Y., Wang J., Wang Y., Du X., Zhang Y., Fawcett J.P., Gu J. Rapid Comm. Mass Spectrom., 2007, 21(24), 3982-3986.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

APPENDIX

Conc. Level	Nominal	Calcd	%Accuracy
LLOQQC-1	5.000	4.187	83.74
LLOQQC-2	5.000	5.046	100.92
LLOQQC-3	5.000	4.228	84.56
LLOQQC-4	5.000	5.449	108.98
LLOQQC-5	5.000	5.329	106.58
LLOQQC-6	5.000	4.049	80.98
Mean		**4.715**	
Std. Deviation		**0.6301**	
%RSD		**13.36**	
%Accuracy (avg)		**94.29**	
LQC-7	15.000	16.721	111.47
LQC-8	15.000	15.521	103.47
LQC-9	15.000	15.734	104.89
LQC-10	15.000	15.601	104.01
LQC-11	15.000	15.752	105.01
LQC-12	15.000	16.364	109.09
Mean		**15.949**	
Std. Deviation		**0.4812**	
%RSD		**3.02**	
%Accuracy (avg)		**106.33**	
MQC-7	135.000	141.152	104.56
MQC-8	135.000	132.637	98.25
MQC-9	135.000	142.856	105.82
MQC-10	135.000	144.552	107.08
MQC-11	135.000	135.931	100.69
MQC-12	135.000	143.576	106.35
Mean		**140.117**	
Std. Deviation		**4.7682**	
%RSD		**3.40**	
%Accuracy (avg)		**103.79**	
HQC-7	575.000	570.461	99.21
HQC-8	575.000	606.594	105.49
HQC-9	575.000	626.871	109.02
HQC-10	575.000	575.275	100.05
HQC-11	575.000	614.687	106.90
HQC-12	575.000	592.949	103.12
Mean		**597.806**	
Std. Deviation		**22.2978**	
%RSD		**3.73**	
%Accuracy (avg)		**103.97**	

Table 1. Data showing %RSD and %Accuracy of one batch of six samples of Octreotide.

Conc. Level	Nominal	Calcd	%Deviation
LLOQQC-1	5.000	5.000	5.547
LLOQQC-2	5.000	5.000	4.631
LLOQQC-3	5.000	5.000	5.384
LLOQQC-4	5.000	5.000	4.664
LLOQQC-5	5.000	5.000	5.184
LLOQQC-6	5.000	5.000	5.636
Mean		**5.174**	
Std. Deviation		**0.4363**	
%RSD		**8.43**	
%Accuracy (avg)		**103.49**	
LQC-7	15.000	16.522	110.15
LQC-8	15.000	13.97	93.13
LQC-9	15.000	16.116	107.44
LQC-10	15.000	16.429	109.53
LQC-11	15.000	16.418	109.45
LQC-12	15.000	15.688	104.59
Mean		**15.857**	
Std. Deviation		**0.9737**	
%RSD		**6.14**	
%Accuracy (avg)		**105.71**	
MQC-7	135.000	140.928	104.39
MQC-8	135.000	136.911	101.42
MQC-9	135.000	140.231	103.87
MQC-10	135.000	141.513	104.82
MQC-11	135.000	136.375	101.02
MQC-12	135.000	134.219	99.42
Mean		**138.363**	
Std. Deviation		**2.9402**	
%RSD		**2.13**	
%Accuracy (avg)		**102.49**	
HQC-7	575.000	580.126	100.89
HQC-8	575.000	610.287	106.14
HQC-9	575.000	581.714	101.17
HQC-10	575.000	557.94	97.03
HQC-11	575.000	613.298	106.66
HQC-12	575.000	604.334	105.10
Mean		**591.283**	
Std. Deviation		**21.615**	
%RSD		**3.66**	
%Accuracy (avg)		**102.83**	

Table 2. Data showing %RSD and %Accuracy of second batch of six samples of Octreotide.

Conc. Level	Nominal	Calcd	%Deviation
LLOQQC-1	5.000	4.187	83.74
LLOQQC-2	5.000	5.046	100.92
LLOQQC-3	5.000	4.228	84.56
LLOQQC-4	5.000	5.449	108.98
LLOQQC-5	5.000	5.329	106.58
LLOQQC-6	5.000	4.049	80.98
LLOQQC-7	5.000	5.000	5.547
LLOQQC-8	5.000	5.000	4.631
LLOQQC-9	5.000	5.000	5.384
LLOQQC-10	5.000	5.000	4.664
LLOQQC-11	5.000	5.000	5.184
LLOQQC-12	5.000	5.000	5.636
Mean		**4.945**	
Std. Deviation		**0.5697**	
%RSD		**11.52**	
%Accuracy (avg)		**98.89**	
LQC-1	15.000	16.721	111.47
LQC-2	15.000	15.521	103.47
LQC-3	15.000	15.734	104.89
LQC-4	15.000	15.601	104.01
LQC-5	15.000	15.752	105.01
LQC-6	15.000	16.364	109.09
LQC-7	15.000	16.522	110.15
LQC-8	15.000	13.97	93.13
LQC-9	15.000	16.116	107.44
LQC-10	15.000	16.429	109.53
LQC-11	15.000	16.418	109.45
LQC-12	15.000	15.688	104.59
Mean		**15.949**	
Std. Deviation		**0.4812**	
%RSD		**3.02**	
%Accuracy (avg)		**106.33**	
MQC-1	135.000	141.152	104.56
MQC-2	135.000	132.637	98.25
MQC-3	135.000	142.856	105.82
MQC-4	135.000	144.552	107.08
MQC-5	135.000	135.931	100.69
MQC-6	135.000	143.576	106.35
MQC-7	135.000	140.928	104.39
MQC-8	135.000	136.911	101.42
MQC-9	135.000	140.231	103.87
MQC-10	135.000	141.513	104.82
MQC-11	135.000	136.375	101.02
MQC-12	135.000	134.219	99.42
Mean		**139.240**	
Std. Deviation		**3.8863**	
%RSD		**2.79**	
%Accuracy (avg)		**103.14**	
HQC-1	575.000	570.461	99.21
HQC-2	575.000	606.594	105.49
HQC-3	575.000	626.871	109.02
HQC-4	575.000	575.275	100.05
HQC-5	575.000	614.687	106.90
HQC-6	575.000	592.949	103.12
HQC-7	575.000	580.126	100.89
HQC-8	575.000	610.287	106.14
HQC-9	575.000	581.714	101.17
HQC-10	575.000	557.94	97.03
HQC-11	575.000	613.298	106.66
HQC-12	575.000	604.334	105.10
Mean		**594.545**	
Std. Deviation		**21.2294**	
%RSD		**3.57**	
%Accuracy (avg)		**103.40**	

Table 3. Data showing %RSD and %Accuracy of 12 samples of Octreotide (global).

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

A High-Sensitivity UPLC/MS/MS Method for the Quantification of Desmopressin in Rat Plasma

P.M.N. Rajesh and Gopal Vaidyanathan
Waters MS Applications Laboratory, Bangalore, India

APPLICATION BENEFITS

The low circulating levels of desmopressin requires a high-sensitivity assay to accurately define its pharmacokinetics in humans, especially pediatrics. The combination of solid-phase extraction methodology, UPLC chromatography, and Xevo TQ-S mass spectrometer has facilitated the development of an assay for desmopressin with a LLOQ of 1 pg/mL in plasma.

WATERS SOLUTIONS

Oasis® Sample Extraction Products

ACQUITY UPLC® System

ACQUITY UPLC HSS T3 Column

Xevo® TQ-S

KEY WORDS

Desmopressin, low-level quantification

INTRODUCTION

Desmopressin (*1-desamino-8-D-arginine vasopressin*), Figure 1, (trade names: DDAVP, Stimate, Minirin) is a peptide containing nine amino acids, a form of the normal human hormone arginine vasopressin that reduces urine production. It may be taken nasally, intravenously, or as a tablet. Desmopressin is most commonly prescribed for the treatment of diabetes insipidus or nocturnal enuresis.

Compared to vasopressin, desmopressin's first amino acid has been deaminated, and the arginine at the eighth position is in the *dextro* rather than the *levo* form. Desmopressin works by limiting the amount of water that is eliminated in the urine. Desmopressin binds to V2 receptors in renal collecting ducts, increasing water reabsorption. It also stimulates release of von Willebrand factor from endothelial cells due to stimulation of the V1a receptor.

Desmopressin is degraded more slowly than recombinant vasopressin, and requires less frequent administration. In addition, it has little effect on blood pressure, while vasopressin may cause arterial hypertension.

Desmopressin is typically dosed at level of 0.3 mcg DDAVP/kg body weight and is mainly excreted in the urine with a terminal half-life from three hours in normal healthy patients to nine hours in patients with severe renal impairment. The peak plasma concentration levels are observed at one to one-and-a-half hours after dosing by nasal administration. This dosing level and rapid clearance results in a circulating level in the low pg/mL range.

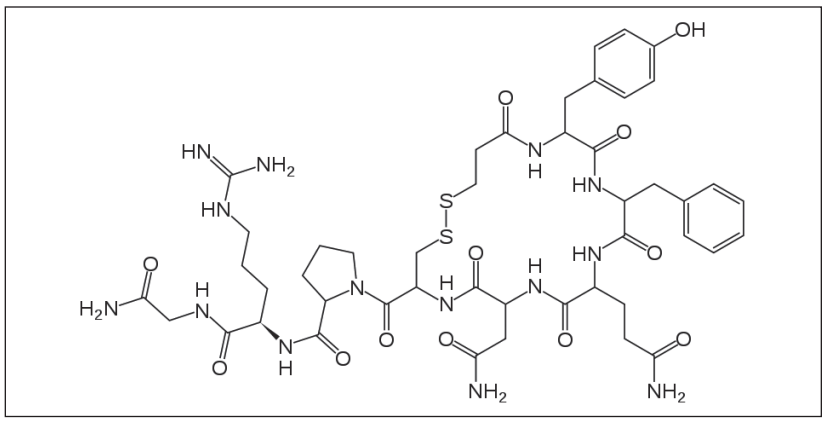


Figure 1. Desmopressin.

EXPERIMENTAL

The samples were isolated using SPE employing a Waters Oasis WCX cartridge. A 500 µL aliquot of plasma was diluted with an aqueous acidic solution and loaded onto the SPE cartridge previously conditioned with organic solvent and water. The plasma solution was then washed with a basic solution, eluted in solvent, evaporated to dryness, and reconstituted in mobile phase for analysis by LC/MS/MS. The extracted samples were analysed by reversed phase gradient chromatography employing an acidic aqueous buffer and acetonitrile as the organic modifier.

LC conditions

LC system:	ACQUITY UPLC System with Binary Solvent Manager, Column Manager, and Sample Manager
LC column:	ACQUITY UPLC HSS T3 C_{18}, 1.8 µm, 2.1 x 100 mm
Column temp.:	45 °C
Gradient:	30 to 95% organic over 3.5 min

MS conditions

MS system:	Xevo TQ-S
MS mode:	Positive ion electrospray MS/MS
MS transition :	535 ⟹ 328

In order to accurately determine the pharmacokinetics of desmospressin, it is necessary to have an assay with a limit of detection at least 20 to 100 times lower than the C_{max} value, in this case on the order of 1 to 2 pg/mL. As this compound is an analogue of a naturally occurring peptide, the development of a high-sensitivity, robust, and reliable assay requires selective sample isolation and separation from the endogenous materials in blood products as well as a highly specific and sensitive mechanism of detection.

In this application note we describe a highly specific and sensitive methodology for the quantification of desmopressin in plasma using solid phase extraction (SPE) followed by reversed-phase UPLC® coupled to tandem quadrupole mass spectrometry.

RESULTS AND DISCUSSION

The chromatogram shown in Figure 2 shows the 47.5 pg/mL mid-level QC for desmopressin extracted from plasma, with the desmopressin analyte eluting with a retention time of 2.55 min. We can see from this data that the desmopressin peak shows excellent symmetrical peak shape and resolution from endogenous interferences. This peak shape and chromatographic resolution are due to the high resolution provided by the ACQUITY UPLC System and HSS T3 column combination.

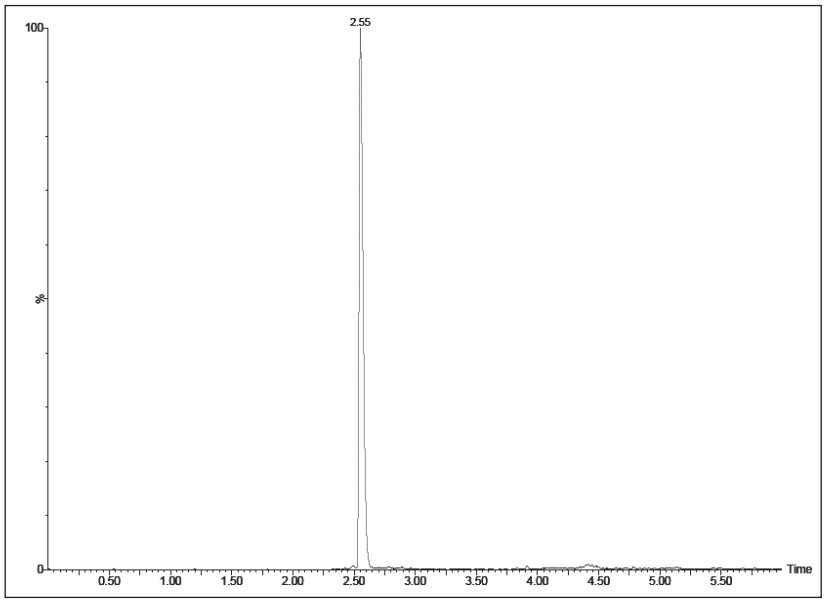


Figure 2. LC/MS/MS chromatogram of the desmopressin Mid-Level QC.

The lower level of quantification was determined to be 1 pg/mL, with a signal-to-noise of 14:1. The data displayed in Figure 3 shows the UPLC/MS/MS chromatogram illustrating the typical chromatogram from a 1 pg/mL standard of desmopressin in plasma. The peak area generated by the 1 pg/mL LLOQ standard was 181, which was nearly five times that obtained from the extraction of blank plasma.

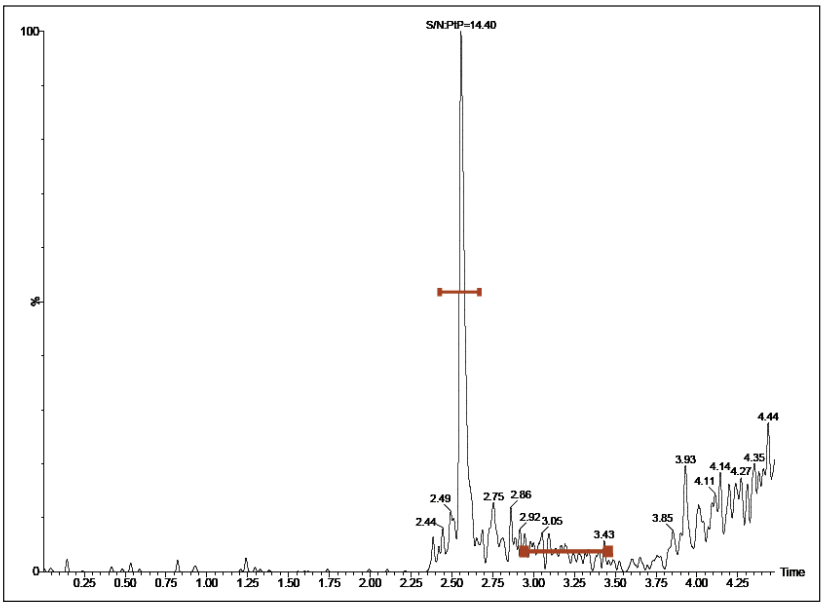


Figure 3. LC/MS/MS chromatogram of the desmopressin LLOQ at 1 pg/mL.

The low level of quantification obtained for this analysis was due, in part, to the high sensitivity detection provided by the Xevo TQ-S tandem quadrupole mass spectrometer. The co-joined off-axis StepWave™ ion guide in the Xevo TQ-S provides a high level of sensitivity while maintaining the robustness and cleanliness of the source and ion optics. This enables the Xevo TQ-S to increase the ion-flux entering the mass spectrometer and hence the sensitivity while maintaining usability of the instrument in terms of maximum up time. The assay was demonstrated to be linear over the range of 1 to 100 pg/mL; a typical calibration line is shown in Figure 4.

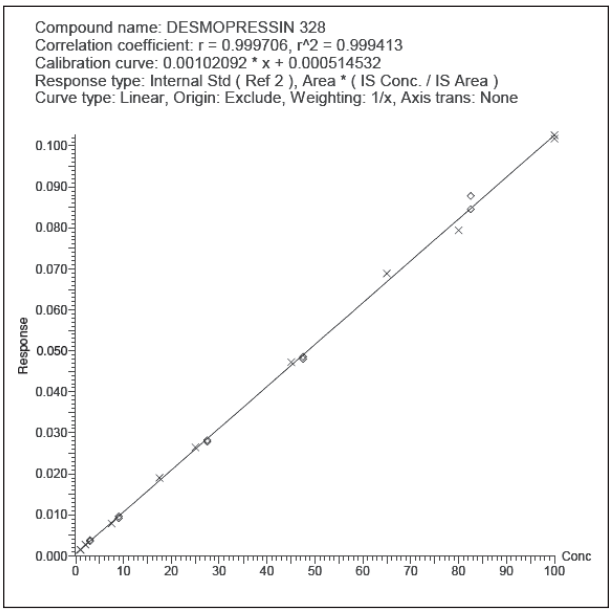


Figure 4. Typical calibration line obtained for the quantification of desmopressin in plasma.

CONCLUSION

The low circulating levels of desmopressin, analogue of the human hormone vasopressin, requires a high-sensitivity assay to accurately define its pharmacokinetics in humans, especially pediatrics. The combination of Oasis SPE products, the ACQUITY UPLC System, and Xevo TQ-S Mass Spectrometer has facilitated the development of an assay for desmopressin with a LLOQ of 1 pg/mL in plasma. The assay showed excellent reproducibility, specificity, and robustness. Despite the complex nature of this analytical challenge, the overall analytical cycle time was just 6 minutes injection to injection.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

A Sensitive and Rapid Estimation of Leuprolide in Human Plasma Using UPLC and Xevo TQ-S

Veeranjaneyulu P., Thirupatheswara Rao B., Sudarshan Mantha, and Gopal Vaidyanathan
Waters India MS Application Laboratory, Bangalore, India

APPLICATION BENEFITS

A unique optimization of this Waters system for bioanalysis, from sample preparation to UPLC® chromatography to tandem quadrupole MS, enables the determination and quantification of leuprolide at the 5 pg/mL level.

WATERS SOLUTIONS

Oasis® SPE

ACQUITY UPLC®

Xevo® TQ-S

KEY WORDS

Leuprolide, nonapeptide

Leuprolide is a synthetic nonapeptide that acts as an agonist at pituitary GnRH receptors. Leuprolide down-regulates the secretion of gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by interrupting the normal pulsatile stimulation and the desensitization of the GnRH receptors.

An LH-RH (GnRH) analog, leuprolide (Figure 1) may be used in the treatment of hormone-responsive cancers such as prostate cancer or breast cancer, estrogen-dependent conditions (such as endometriosis or uterine fibroids) to treat precocious puberty, and also to control ovarian stimulation in in vitro fertilization (IVF). It is also considered a possible treatment for paraphilias.

Leuprolide received its first approval for treatment of advanced prostate cancer in 1985 and received several other approvals for treating prostate cancer at several dosage levels. However, monitoring leuprolide and its analytes in its low-dosage concentration had not been possible because analytical techniques were not able to offer the necessary sensitivity.

In this study, we demonstrate detection of leuprolide in human plasma at very low concentrations using a Waters ACQUITY UPLC and Xevo TQ-S tandem quadrupole system, which is able to deliver the required sensitivity for this challenging assay.

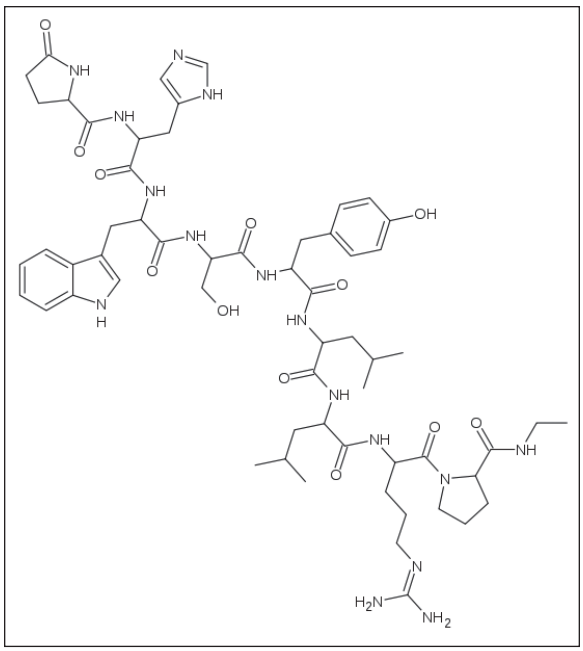


Figure 1.
Leuprolide structure.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC System equipped with a Binary Solvent Manager, Column Manager, and Sample Manager
LC column:	ACQUITY UPLC BEH 300 C_{18}, 2.1 x 100 mm, 1.7 µm
Gradient:	Reversed-phase chromatography with an acidic aqueous buffer solution and acetonitrile as the organic modifier
Elution:	30-95% organic gradient over 3.5 min
LC gradient run:	5.0 min starting with 90% acid solution and 10% acetonitrile
Column temp.:	50 °C

MS conditions

MS system:	Xevo TQ-S
MS mode:	Positive ion electrospray MS/MS
MS transition:	605.69 ⇒ 249.11

When compared to an HPLC, UPLC® Technology enables a user to achieve faster separations with lower consumption of the mobile phase. UPLC also offers short run times with low dispersion, resulting in better separation of signals from unwanted signals arising from plasma, phospholipids, and other endogenous materials. In addition, the ACQUITY UPLC BEH 300 Column used in this study offers significantly better separation of peptides when compared to traditional reversed phase columns.

For mass spectrometry, the Xevo TQ-S offers the advantage of high sensitivity and its ZSpray™ Technology provides added stability to the analysis. The combined effect of the outstanding UPLC column chemistry and chromatographic system along with the Xevo TQ-S provides a powerful tool for the estimation of a variety of molecules, from small molecules to large peptides, in very low concentrations.

RESULTS AND DISCUSSION

The leuprolide analyte eluted with a retention time of 1.75 min. As can be observed from Figure 2, the leuprolide signal shows excellent symmetrical peak shape and resolution from endogenous interferences. Such excellent peak shape and chromatographic resolution can be attributed to an optimized combination of the ACQUITY UPLC System and BEH 300 C_{18} Column.

The LLOQ for leuprolide was determined to be 5 pg/mL with the signal-to-noise ratio being at least 20:1 (Figure 2). In addition, the results showed excellent reproducibility at LLOQ levels (Table 1). Such excellent reproducibility and sensitivity provides the ability to quantify leuprolide at the LLOQ level.

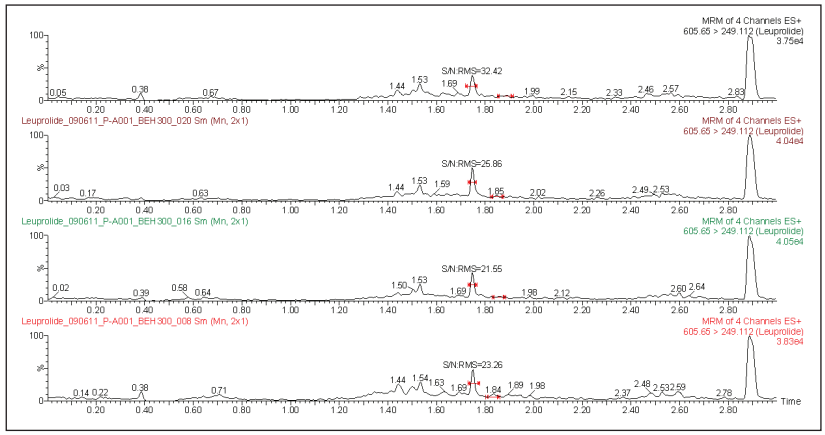


Figure 2. UPLC/MS/MS chromatogram of leuprolide showing signal-to-noise ratio of greater than 20 at the LLOQ level.

Sample Name	Standard Conc.	Calculated Conc.	% Deviation	% Recovery
Reference	650	610.1	-6.1	93.9
CC 1	5	5	-0.2	99.8
CC 2	10	10.2	2.3	102.3
CC 3	25	23.5	-5.9	94.1
CC 4	50	50.9	1.9	101.9
CC 5	100	107.6	7.6	107.6
CC 6	150	136.4	-9.1	90.9
CC 7	325	319.8	-1.6	98.4
CC 8	650	683.3	5.1	105.1
LLOQQC-1	6	6.4	6.9	106.9
LQC-1	15	15.7	4.5	104.5
MQC-1	120	115.4	-3.9	96.1
HQC-1	475	536.2	12.9	112.9
LLOQQC-2	6	6.9	14.6	114.6
LQC-2	15	14.9	-0.5	99.5
MQC-2	120	104.9	-12.6	87.4
HQC-2	475	462.8	-2.6	97.4
LLOQQC-3	6	5.6	-6.6	93.4
LQC-3	15	15.3	2	102
MQC-3	120	114.8	-4.4	95.6
HQC-3	475	511.9	7.8	107.8
LLOQQC-4	6	5.3	-11.4	88.6
LQC-4	15	16.3	8.9	108.9
MQC-4	120	103.6	-13.6	86.4
HQC-4	475	471.3	-0.8	99.2
LLOQQC-5	6	6.7	10.9	110.9
LQC-5	15	15.9	5.7	105.7
MQC-5	120	109.8	-8.5	91.5
HQC-5	475	536.8	13	113
LLOQQC-6	6	6	0.4	100.4
LQC-6	15	13.9	-7.1	92.9
MQC-6	120	104.3	-13	87
HQC-6	475	470.5	-1	99

Table 1. Comparison of calculated with standard concentration, percent deviation, and percent recovery for leuprolide samples at various concentration levels using UPLC and Xevo TQ-S.

The LLOQ obtained for this analysis was due, in part, to the high-sensitivity detection provided by the Xevo TQ-S System. The co-joined, off-axis StepWave™ ion guide in the Xevo TQ-S provides superior levels of sensitivity while maintaining the robustness and cleanliness of the source and ion optics. This enables the Xevo TQ-S to increase the ion flux entering the mass spectrometer, resulting in the highest levels of sensitivity. The assay reported in this study was demonstrated to be linear over the range of 5 to 650 pg/mL in both organic solvents and human plasma samples.

As observed from Table 1, the %RSD varied from 4.887 to 6.874 within a range of concentration. Although the inter-day precision varied between 2.130 and 9.475 for the lowest level of concentration, the medium, high-concentration ranges and the LLOQQC showed a variation within the range of 5.128 to 9.927. In addition, the percent recovery of samples that was conducted by comparing the area under the curve of the extracted sample with that of the neat sample, was found to be about 60% at all concentration levels.

The LC/MS/MS chromatogram of the blank plasma sample and that of the leuprolide at LLOQ level showed little interference from the endogenous materials (Figure 3 and Figure 4). In addition, phospholipid elution was checked by injecting an extracted standard with an MS scan at 184 ⟹ 184 *m/z*, a unique MRM transition for typical phospholipids. As can be observed from Figure 5, no significant phospholipid elution was observed at the retention time of either the leuprolide analyte (1.75 min) or the internal standard, octreotide (1.63 min).

Matrix interference, which is considered to be one of the major regulatory challenges in the pharmaceutical world, is automatically calculated by MassLynx™ Software. A comparison of the post-spiked samples with those of the neat samples in the same concentration level reveals that the matrix interference for the Leuprolide samples was not more than 2.051%. Such a value is well within the acceptable range indicated by the regulatory agencies.

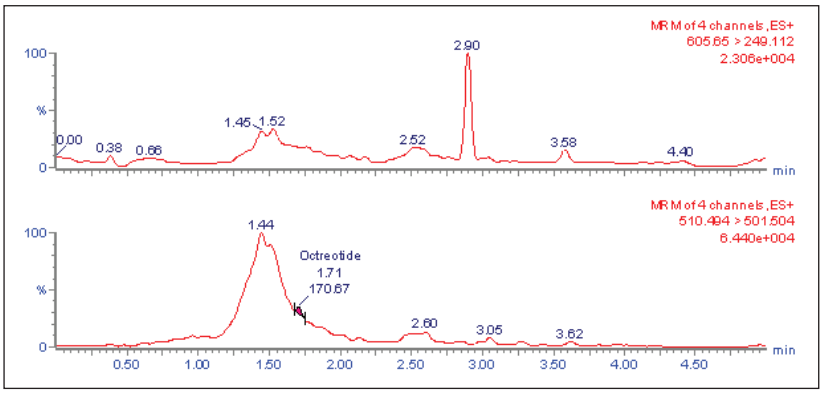


Figure 3. Representative chromatogram of blank in leuprolide channel.

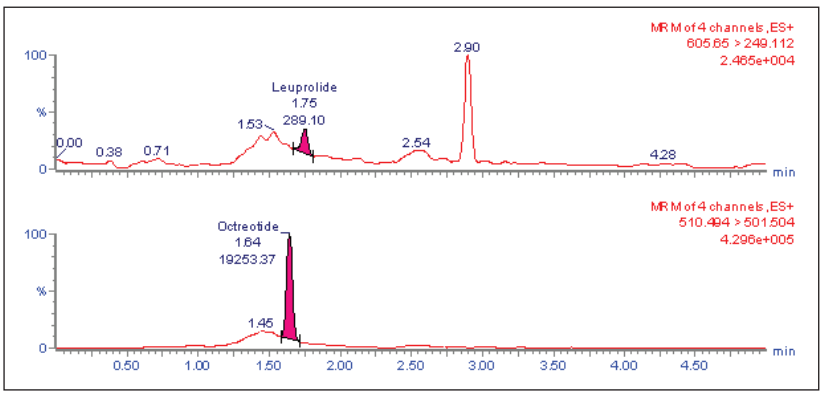


Figure 4. Representative chromatogram of leuprolide at LLOQ.

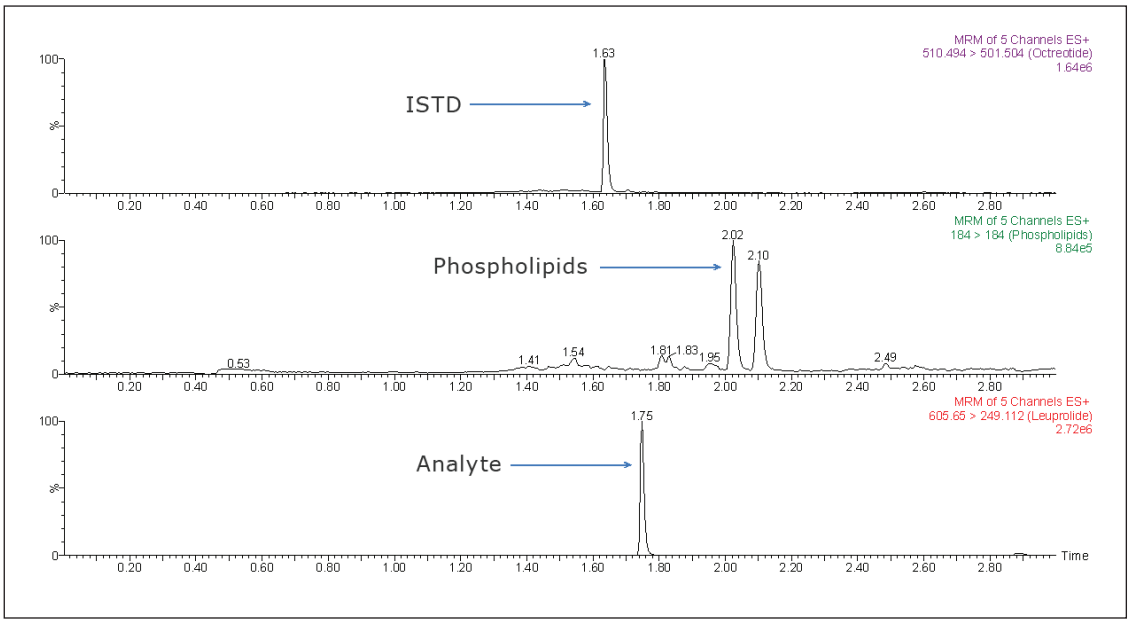


Figure 5. Comparsion of phospholipid elution with respect to leuprolide (analyte) and its IS, octreotide.

CONCLUSION

The low circulating concentration levels of leuprolide, a synthetic nonapeptide that acts as an agonist at pituitary GnRH receptors, requires a highly sensitive assay for accurate determination of the pharmacokinetics in humans, especially in prostate cancer patients.

This study demonstrates that the combination of Oasis SPE Technology, the ACQUITY UPLC System with a BEH 300 C_{18} column, and the Xevo TQ-S Mass Spectrometer combine to enable the development of an assay for Leuprolide with an LLOQ of 5 pg/mL in human plasma.

The UPLC chromatograms demonstrate not only better chromatography, but also better resolution compared to the data obtained from any conventional HPLC systems. The assay reported in this study showed excellent reproducibility, specificity, and robustness. Despite the complicated nature of this analytical challenge, the overall cycle time for the UPLC/MS/MS experiments reported in this study was about 3 minutes.

References

1. MacNeill R, Evans E. Evaluation of acetone as organic modifier in SPE for bioanalytical quantitative LC-MS/MS. Bioanalysis. 2011 Feb; 3(3):291-300.
2. Adjei A, Doyle R, Pratt M, Finley R, Johnson E. Bioavailability of leuprolide following intratracheal administration to beagle dogs. International Journal of Pharmaceutics. Vol 61, Issues 1-2, 11 June 1990: 135-144.
3. Zhan Y, Chen X, Zhao X, Zhong D. Rapid and sensitive liquid chromatography–tandem mass spectrometry method for the determination of leuprolide in human serum. J Chromatogr B Analyt Technol Biomed Life Sci. 2009 Oct 1;877(27):3194-200. Epub 2009 Aug 21.
4. Bhatia S, Neely EK, Wilson DM. Serum Luteinizing Hormone Rises Within Minutes After Depot Leuprolide Injection: Implications for Monitoring Therapy. Pediatrics Vol. 109 No. 2 February 2002. http://www.pediatrics.org/cgi/content/full/109/2/e30.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

A Sensitive and Robust Method for the Quantification of Goserelin in Plasma Using Micro-Elution Plates

Tirupateswara B. Rao, P. Veeranjaneyulu, Sudarshan Mantha, and Dr. Gopal Vaidyanathan
Waters Corporation, Bangalore, India

APPLICATION BENEFITS

In today's world of regulated and non-regulated bioanalysis, one of the biggest challenges is addressing upcoming analytical demands, such as the ability to perform LC/MS analysis of large molecules (peptide, protein, oligonucleotides, etc.) with accuracy, reproducibility, and desired sensitivity. This application note demonstrates the benefits of Waters® Regulated Bioanalysis System Solution for quantification of large molecules, such as peptides. The peptide, goserelin, exhibits multiply-charged parent masses. The reported application methodology in this study achieves the desired sensitivity and robustness, while retaining the necessary high throughput value and accuracy. Oasis® WCX Ion-Exchange Micro-Elution Plates, the ACQUITY UPLC® System, and the Xevo® TQ-S Mass Spectrometer were used in this study for the development of an LC/MS method to analyze pg/mL concentration of goserelin in plasma.

WATERS SOLUTIONS

ACQUITY UPLC System

Xevo TQ-S Mass Spectrometer

ACQUITY UPLC BEH300 C_{18} Column

Oasis WCX Micro-Elution extraction products

KEY WORDS

Goserelin, WCX micro-elution plates, high sensitivity, high throughput

INTRODUCTION

Goserelin is a synthetic hormone analogue.[1] Structurally it is a decapeptide, as shown in Figure 1, which is used to suppress the production of the sex hormones (testosterone and estrogen). Goserelin is particularly used in the treatment of breast and prostate cancer. For identification and quantification of goserelin in plasma, a method has been reported in rabbit plasma[2] with an LLOQ of 100 pg/mL with an overall runtime of 10 min. Other methods include radio-immunoassay,[3] capillary zone electrophoresis (CZE)-UV/MS,[4] multiple-injection CZE (MICZE),[5] CE hydrogen deuterium exchange-MS (CE-H/D-MS),[6] and fast atom bombardment-MS (FAB-MS)[7] for crude peptide mixtures only. However, none of these methods address the challenge of analyzing and quantifying goserelin at LLOQ concentrations. In this application note, we report an LC/MS method to determine and quantify goserelin at an LLOQ of 2.5 pg/mL with an overall runtime of 3.5 min in plasma. These results demonstrate the capability of Waters ACQUITY UPLC, Xevo TQ-S, sample preparation and column chemistries to address several bioanalytical challenges, such as achieving the desired sensitivity, addressing upcoming analytical challenges, and regulatory concerns while maintaining high throughput and desired robustness.

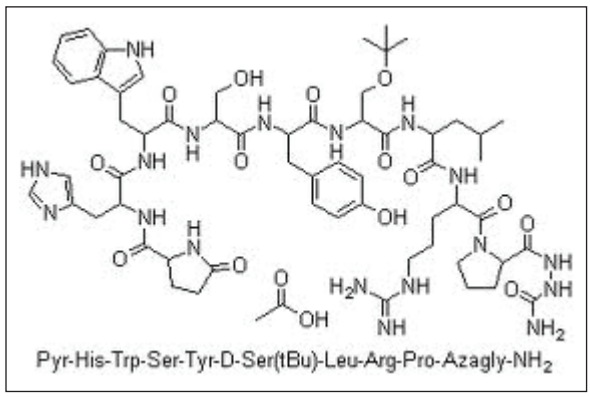


Figure 1. Molecular structure of goserelin and its sequence.

EXPERIMENTAL

Samples were extracted using solid phase extraction (SPE) employing Oasis WCX Micro-Elution 96-Well Plate. An aliquot of plasma was diluted with acidic water, and loaded onto the plate previously conditioned with organic solvent and water. The plasma solution was then washed with water in basic conditions followed by an organic wash, and then eluted using acidified organic elution solvent. The eluted samples were then mixed with Milli-Q water, and injected on to the system. Triptorelin, a decapeptide (M.W. 1311.5), which is a gonadotropin-releasing hormone agonist, was used as an internal standard (IS).

RESULTS AND DISCUSSION

Goserelin and triptorelin (IS) eluted at 1.43 min with a peak width of about 5 s, as shown in Figure 2. The data shown below illustrates the blank signal, shown in Figure 3. The signal obtained from the lower limit of quantification (LLOQ) of goserelin in human plasma is also, shown in Figure 3.

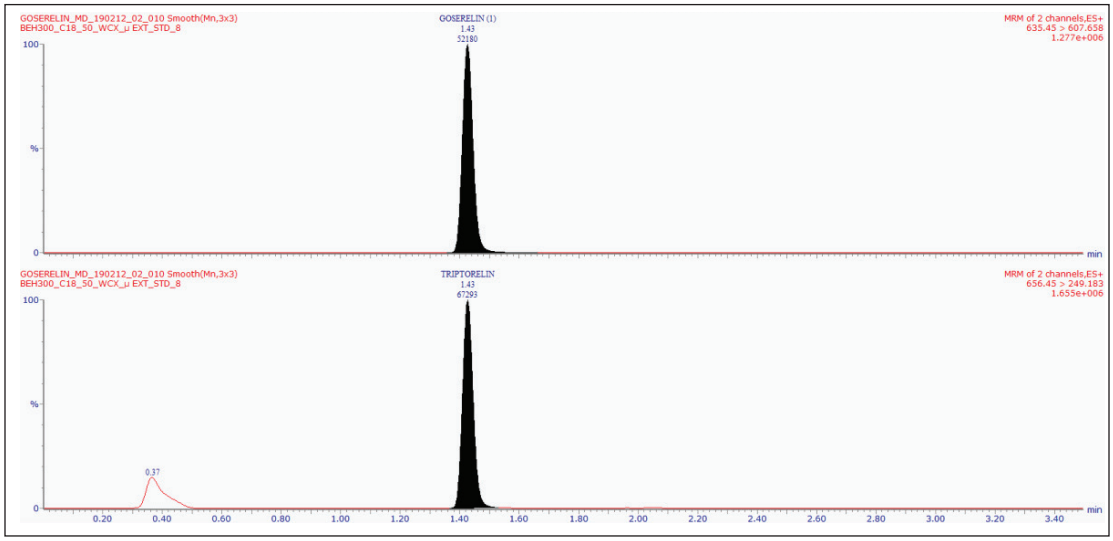


Figure 2. Elution pattern of goserelin and triptorelin.

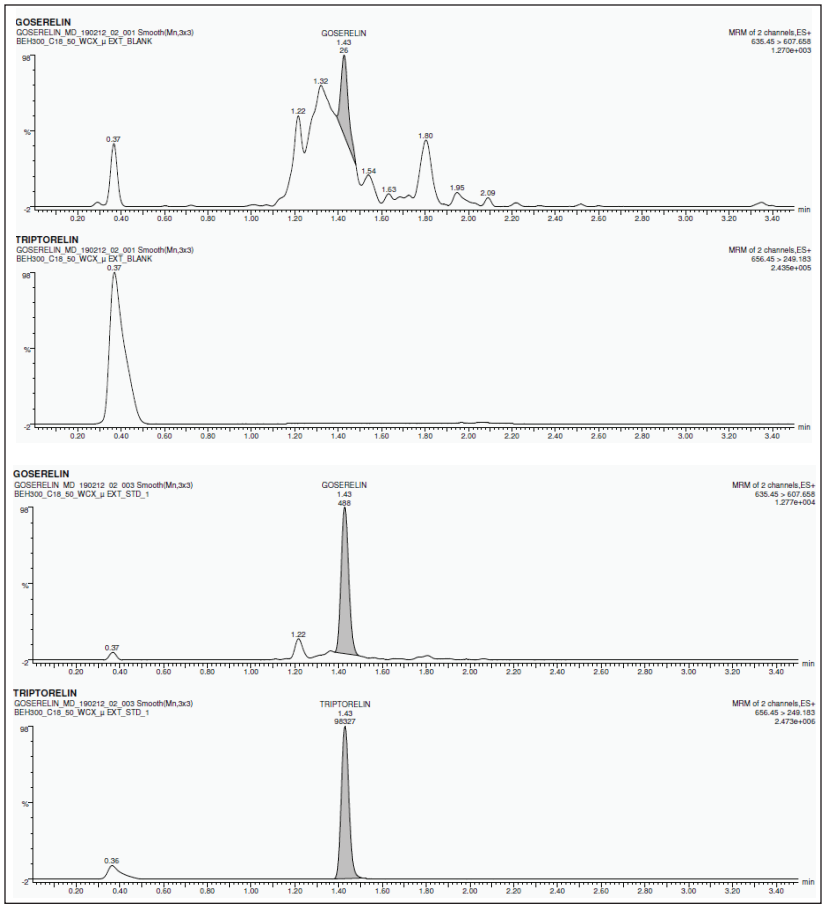
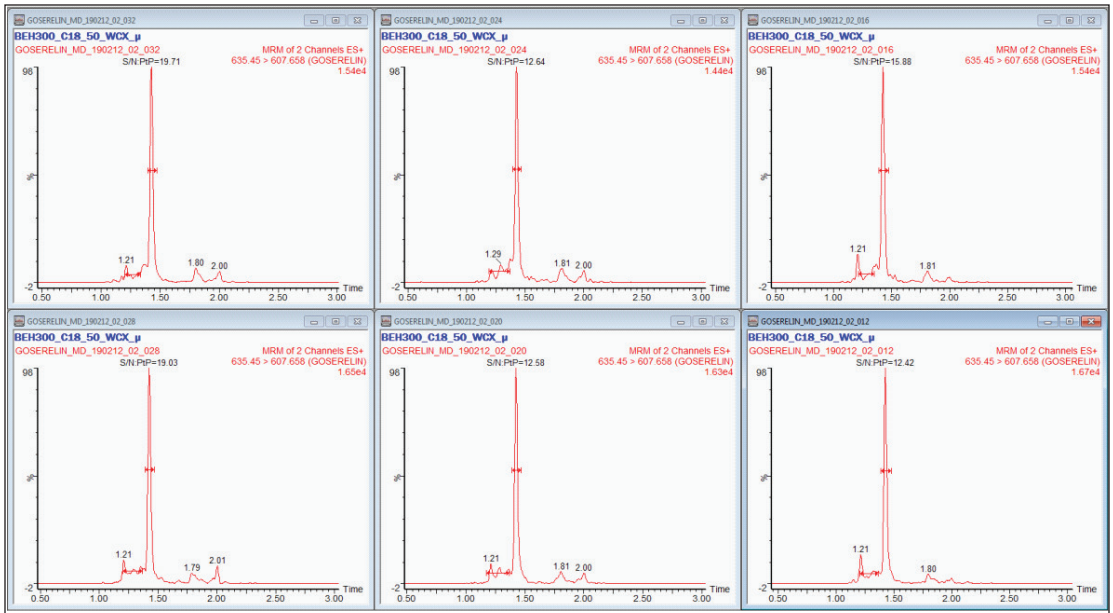


Figure 3. Chromatogram of blank and goserelin at the LLOQ concentration of 2.5 pg/mL.



Signal-to-Noise Ratio of Six Replicates of LLOQ						
LLOQ1	LLOQ2	LLOQ3	LLOQ4	LLOQ5	LLOQ6	AVERAGE
19.71	12.64	15.88	19.03	12.58	12.42	15.38

Figure 4. Chromatogram of LLOQ concentration (2.5 pg/mL) of goserelin with a signal-to-noise ratio of about 16 obtained using ACQUITY UPLC and Xevo TQ-S.

No significant interference(s) were observed in the retention time (1.43 min) where goserelin elutes, shown in Figure 3. In addition, the signal-to-noise (S/N) ratio of ~16:1 was observed at the LLOQ concentration of 2.5 pg/mL for an average of six replicates of LLOQ samples, as shown in Figure 4.

The assay in this report showed linear calibration over the range of 2.5 to 320.0 pg/mL with an excellent r^2 value of 0.99, shown in Table 1 and Figure 5. This assay was performed with a 3.5 min injection-to-injection time scale highlighting the capability of ACQUITY UPLC in delivering fast gradients with desired sensitivity, high throughput value, and precision.

Sample	Type	Nominal Conc.	Area	IS Area	Area ratio	Calculated Conc.	Accuracy
EXT_BLK	BLANK		26	4	6.7080	3131.97	
EXT_BLK_IS	ZERO		17	100773	0.0002	0.29	
EXT_CC_1	STD-1	2.5	488	98327	0.0050	2.53	101.2
EXT_CC_2	STD-2	5.0	1032	95435	0.0108	5.26	105.2
EXT_CC_3	STD-3	10.0	1560	85355	0.0183	8.74	87.4
EXT_CC_4	STD-4	20.0	3428	82765	0.0414	19.54	97.7
EXT_CC_5	STD-5	40.0	6866	87965	0.0780	36.65	91.6
EXT_CC_6	STD-6	80.0	13368	77597	0.1723	80.64	100.8
EXT_CC_7	STD-7	160.0	24608	68792	0.3577	167.22	104.5
EXT_CC_8	STD-8	320.0	51096	66860	0.7642	357.00	111.6

Table.1. Calibration data of goserelin over the range of 2.5 to 320.0 pg/mL.

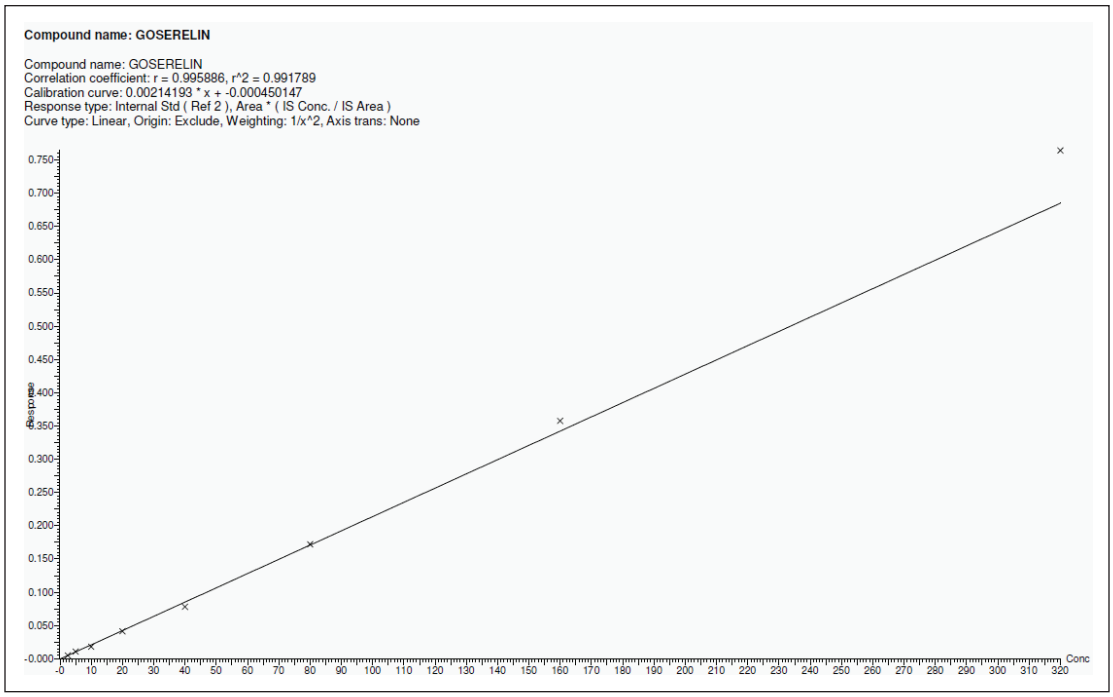


Figure 5. Calibration curve of goserelin.

Recovery of the analyte and internal standard (IS) was calculated by comparing the extracted QC samples against six post-extracted samples and was found to be approximately 66% for the analyte, shown in Tables 2 and 3.

	LQC		MQC		HQC	
S.No	Extracted	Post Spiked	Extracted	Post Spiked	Extracted	Post Spiked
1	1614	2320	14692	20194	24151	41005
2	1706	2479	14493	20253	29566	39894
3	1200	2275	13655	20279	26617	40463
4	1295	2519	14091	20419	27363	42486
5	1562	2448	15043	20797	27176	40887
6	1634	2509	14328	21014	27844	40560
Mean	1502	2425	14384	20493	27120	40883
%Recovery	61.9		70.2		66.3	

Table 2. Analyte recoveries (area under the curve) from six samples of goserelin at LQC, MQC, and HQC concentrations.

LQC	MQC	HQC
61.9	70.2	66.3
Mean analyte recovery (%) = 66.2		

Table 3. Mean analyte recovery (%) of goserelin at LQC, MQC, and HQC levels.

For a comparison of samples within the global batches, four separate batches were analyzed with six samples in each batch at the LLOQQC, LQC, MQC, and HQC concentration levels. The data showed excellent agreement between the six samples in all the four batches, as shown in Table 4. The mean accuracy obtained for all the sample levels was found to be >97% for every concentration, as shown in Table 4. The %CV for repeat batches was found to be within 8% of LLOQQC and varied between 3% and 6% for all QC levels. This variability is well within the acceptable limits in the regulated bioanalysis world.

	LLOQQC	LQC	MQC	HQC
Nominal Conc. (pg/mL)	2.50	10.00	80.00	160.00
P&A-1	2.65	8.84	78.61	158.75
	2.46	9.29	78.31	166.63
	2.65	8.77	78.46	161.05
	2.48	8.95	80.16	168.64
	2.65	9.07	77.98	162.95
	2.59	9.33	78.21	172.10
P&A-2	2.84	10.40	78.24	167.24
	2.82	9.81	77.82	168.72
	2.84	9.74	78.43	168.79
	2.96	9.52	79.76	168.75
	2.72	9.66	78.50	169.23
	2.89	10.09	77.96	167.95
P&A-3	2.67	10.31	77.70	175.98
	2.60	9.93	85.20	164.26
	2.65	10.50	76.41	173.98
	2.79	9.64	83.39	163.49
	2.58	10.47	77.90	173.95
	2.96	9.76	85.75	165.24
P&A-4	2.50	9.79	81.48	151.65
	2.24	9.45	80.66	148.97
	2.50	10.34	78.63	177.09
	2.42	10.50	77.79	155.26
	2.32	9.95	80.72	160.85
	2.33	9.84	85.95	133.38
Mean	2.630	9.748	79.751	164.371
SD	0.1999	0.5263	2.7127	9.7066
%CV	7.6	5.4	3.4	5.9
% Nominal	105.2	97.5	99.7	102.7

Table 4. Comparison of the four separate batches, each containing six goserelin samples at the LLOQQC, LQC, MQC, and HQC concentrations.

CONCLUSIONS

Goserelin is a synthetic hormone analogue and it is specifically used in the treatment of breast and prostate cancer. An LC/MS method to determine and quantify goserelin in low concentrations in plasma has not previously been developed. In this application note, we report a rapid, robust, and sensitive method that was developed for the determination of decapeptide, goserelin from human plasma by using the ACQUITY UPLC System and Xevo TQ-S Mass Spectrometer. Goserelin and triptorelin (IS) were well separated within 3.5 min having good peak shapes, under the optimized conditions of UPLC® and MS/MS. Finally, the established method is suitable for the determination of goserelin from human plasma. This application note successfully addresses the bioanalytical challenge of upcoming analytical demands while addressing several other challenges such as sensitivity, robustness, and regulatory concerns.

References

1. AstraZeneca official Zoladex website.
2. Min KyungKima, TaeHoLeea, JoonHyukSuha, HanYoungEoma, JungWonMina, Hyesun Yeoma, UnyongKima, HyungJoonJungb, KyungHoiChab, YongSeokChoic, Jeong-Rok Youma, SangBeomHana. J Chromatogr B Analyt Technol Biomed Life Sci. 2010 Aug 15;878 (24):2235-42, 20655813.
3. T J Perren, R N Clayton, G Blackledge, L C Bailey, G Holder, S S Lynch, D G Arkell, J Cottam, D Farrar, C H Young. Cancer Chemother Pharmacol. 18 (1986) 39.
4. M A Hoitink, E Hop, J H Beijnen, A Bult, J J KettenesvandenBosch, W JM Underberg. J Chromatogr A. 776 (1997) 319.
5. H Loden, A Amini. Electrophoresis. 28 (2007) 1548.
6. S S Lau, N M Stainforth, D G Watson, G G Skellern, S A Wren, J N Tettey. Electrophoresis 29 (2008) 393.
7. M A Hoitink, J H Beijnen, M U S Boschma, A Bult, E Hop, J Nijholt, C Versluis, G Wiese, W J M Underberg. Anal Chem. 69 (1997) 4972.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Quantification of Exenatide Using Xevo TQD

Joanne Mather, Erin Chambers, Robert Plumb, and Debadeep Bhattacharya
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

Bioanalysis of biotherapeutics has attained increased interest in the bioanalysis community in recent years. As required for all drug candidates, determination and quantification of peptide therapeutics should be performed at low concentrations while addressing all regulatory concerns, ensuring reproducibility, and maintaining method robustness with high throughput value. In addition, it is advisable for these studies to be conducted with minimal financial and other resource investment.

This application note showcases the capabilities of the Xevo® TQD Mass Spectrometer, ACQUITY UPLC® H-Class System, sample preparation, and column chemistries, to achieve a sensitive LC/MS/MS method for the detection and quantification of exenatide in plasma.

WATERS SOLUTIONS

Oasis® MAX micro-elution plates

ACQUITY UPLC H-Class System

ACQUITY UPLC BEH300 C_{18} Column

Xevo TQD Mass Spectrometer

KEY WORDS

Exenatide, sensitivity, robust, high throughput, diabetes

INTRODUCTION

The use of peptides and proteins as therapeutic agents has increased significantly in recent years. The bulk of the drug pipeline in most major pharmaceutical companies is now comprised of peptides and proteins. With increased focus on biotherapeutics, the use of LC/MS for the quantitative analysis of proteins and peptides has gained substantial interest due to its accuracy, dynamic range capability, and speed of method development.

Exenatide, shown in Figure 1, is a large therapeutic peptide that is a synthetic version of Exendin-4, a hormone found in the saliva of the Gila monster. It is a 39-amino-acid peptide with a molecular weight of 4186.6 Da. Exenatide has been approved for the treatment of diabetes mellitus type 2 as an adjunctive therapy marketed as Byetta (Amylin Pharmaceuticals). Exenatide enhances glucose-dependent insulin secretion by the pancreatic beta-cell; thereby, regulating glucose metabolism and insulin secretion. It slows the emptying of the gastric system, increases satiety, reduces appetite, and lowers liver fat content.

Traditionally, the plasma concentrations of exenatide have been measured by ligand-binding assays, such as immunoenzymetric assays used for pharmacokinetic studies. Developing antibodies and assays for ligand-binding assays is very time-consuming, cost prohibitive, and lacks the precision and accuracy of chromatographic assays.

The ability to accurately quantify therapeutic peptides in biological fluids requires a selective isolation process, a high-resolution chromatography system (LC), and a high-sensitivity detector (MS). As these therapeutic peptides imitate and/or replace the activity of endogenous peptides, it is desirable for the detection process to differentiate the endogenous and exogenous compounds. Although therapeutic peptides exhibit multiple charged states, the more specific high molecular weights typically observed for such peptides benefit from a mass spectrometer with an upper mass range in the region of 2000 Da on both quadrupoles, for the successful analysis of higher mass precursor or product ions.

EXPERIMENTAL

LC conditions

System:	ACQUITY UPLC H-Class including the High Temperature Column Heater
Column:	ACQUITY UPLC BEH300 C_{18} 2.1 x 50 mm, 1.7 µm
Column temp.:	50 °C
Solvent:	0.2% formic acid in H_2O (A), and 0.2% formic acid in acetonitrile (B)
Gradient:	20% A to 85% A/2 min
Flow rate:	200 µL/min
Injection vol.:	10 µL
Run time:	5 min

Mass Spectrometry Conditions

MS Detector:	Xevo TQD
MRM data acquisition:	838 → 948; 838 → 396
Ion mode:	ESI positive
Capillary voltage:	3.00 kV
Desolvation gas flow:	800 L/h
Source temp.:	150 °C
Desolvation temp.:	350 °C

In this application note, we describe the successful implementation of sample preparation and column chemistries using Waters' ACQUITY UPLC H-Class System, and Xevo TQD Mass Spectrometer to develop an accurate, robust, and specific bioanalytical method for the quantification of exenatide in plasma.

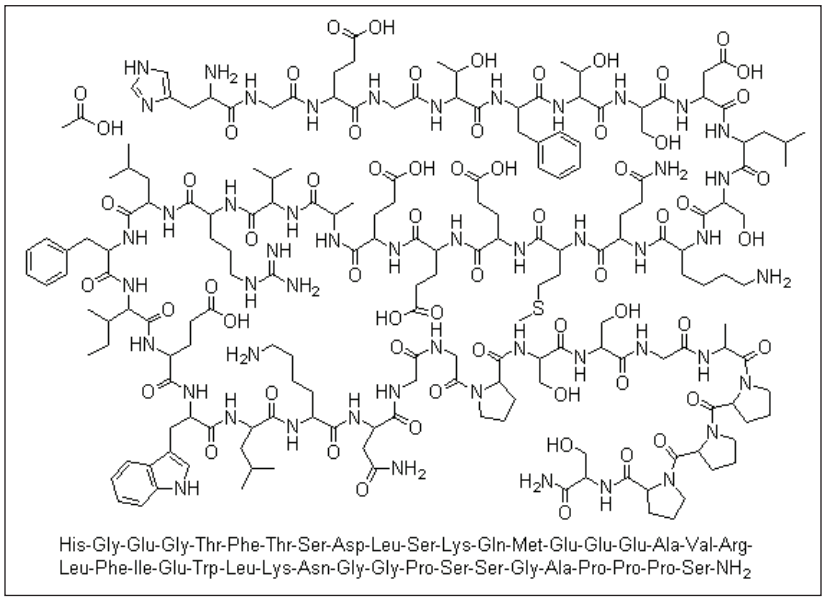


Figure 1. Chemical structure and amino acid sequence of exenatide.

Sample Preparation Protocol

Exenatide was spiked into plasma, and extracted using an Oasis MAX µElution plate with 375 µL of plasma diluted 1:1 with 5% NH_4OH. The samples were washed with 200 µL of 5% NH_4OH, followed by 200 µL of 20% acetonitrile, and eluted with 2x 25 µL of 50:40:5:5 Acetronitrile/IPA/ H_2O/Formic acid. The resulting samples were diluted with 50 µL of H_2O containing the internal standard glucagon-like peptide.

A 10-µL aliquot of the sample was injected onto the column.

Data Management

Data was recorded and processed using MassLynx™ with TargetLynx™ Application Manager.

RESULTS AND DISCUSSION

Peptide therapeutics, unlike small molecules, exhibit less toxicity; however, they also exhibit high clearance and potency. Therefore, it is essential for the analytical technique of choice to have a fast, robust, reproducible, and sensitive method that would ensure determination and quantification of the peptide therapeutics and/or its metabolites in biological samples.

LC/MS/MS systems enable the user to accurately characterize the pharmacokinetics (PK) of such therapeutic peptides, especially for the low concentration of drugs after the Tmax.

The Xevo TQD Mass Spectrometer is equipped with the proven ZSpray™ ionization source, maximizing sensitivity and robustness by the unique use of dual orthogonal geometry to enable efficient transmission of ions into the analyzer while, simultaneously, providing robust removal of non-ionized materials (neutrals).

Of the two MRM transitions mentioned in the Experimental section of this application note, the transition 838 ⟶ 946 was monitored for optimal resolution and sensitivity. Exenatide eluted with a retention time of 3.28 min, as shown in Figure 2. As can be seen from this data, the peak produced by the chromatography system is very symmetrical and has a width at the base of 8 s. The peak sharpness and its symmetrical nature allow for efficient processing and peak integration with good resolution from any endogenous interference.

The limit of quantitation (LOQ) for the assay was determined to be 1 ng/mL of exenatide in plasma, with a signal to noise ratio of ~16:1. Figure 2 also illustrates the injection of an extracted plasma blank injection, immediately following the analysis of the 1000 ng/mL standard. This data shows that there is no discernable carryover in the blank chromatogram. The extremely low carryover exhibited by the ACQUITY UPLC H-Class System allows the full sensitivity of the Xevo TQD Mass Spectrometer to be exploited.

A calibration line obtained for the assay of exenatide is shown in Figure 3, with a correlation coefficient of 0.9993 using a 1/x weighting linear regression.

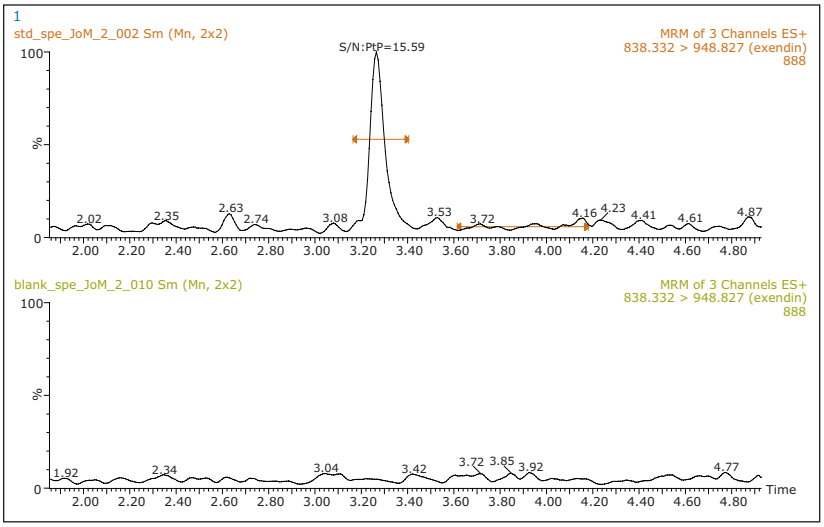


Figure 2. MRM transition (838 > 948) of 1 ng/mL exenatide (top) and blank plasma (bottom) samples.

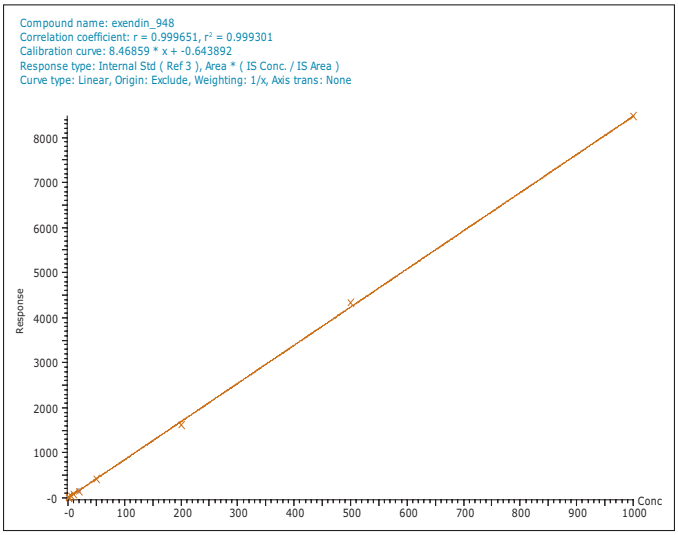


Figure 3. Calibration line for exenatide from 1 ng/mL to 1000 ng/mL.

CONCLUSIONS

- A sensitive method has been tested for the analysis of exenatide in plasma.
- Level of quantification was determined to be 1 ng/mL.
- No significant carryover was detected following the injection of 1 ng/mL sample of exenatide.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

JOURNAL OF APPLIED BIOANALYSIS, October 2015, p. 128-135.
http://dx.doi.org/10.17145/jab.15.021 (ISSN 2405-710X)
Vol. 1, No. 4

OPEN ACCESS

APPLICATION NOTE

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

High throughput analysis at microscale: performance of ionKey/MS with Xevo G2-XS QTof under rapid gradient conditions

Yun Wang Alelyunas, Gregory T. Roman, Jay Johnson, Catalin Doneanu, Mark D. Wrona

Waters Corporation, Milford, MA, USA

In this paper, high throughput analysis with 3 minute, rapid gradient conditions is described using the ionKey/MS™ System with an integrated ACQUITY UPLC® M-Class System and the Xevo® G2-XS QTof Mass Spectrometer. Extensive testing of representative small molecules and a peptide shows that the system is well-tolerated and exhibits excellent reproducibility and linear response. The iKey™ HSS T3 Separation Device used is robust, withstanding ~2200 injections of prepared human plasma with excellent peak shape and system pressure profile. A 99% solvent savings was realized when compared with an analytical system using a 2.1 mm column with flow rate ranging from 0.6 mL/min to 1.5 mL/min. These data, coupled with examples from the literature, illustrate that the ionKey/MS System with Xevo G2-XS QTof can be used as a full service platform for high throughput analysis and high sensitivity analysis to support all phases of drug discovery and development.

Introduction

High throughput LC-MS analysis typically refers to conditions of using a rapid gradient from 1 to 2 minutes followed by column washing, and column (re)equilibration, for a total gradient/cycle time of 2-5 minutes. This high throughput operation is important in drug discovery and bioanalysis settings due to the vast number of compounds that have gone through various in vitro and in vivo tests, where compound concentrations need to be quantified by LC-MS techniques. For these laboratories, short cycle times can be equally important to instrument sensitivity. Although microscale LC-MS is advantageous for high sensitivity analysis and reduced solvent consumption, it has not historically been used for high throughput analysis. This is largely due to previous limitations of long cycle time and poor reproducibility. These barriers have been overcome with the integrated and user-friendly ionKey/MS System [1]. In addition, the choice of MS platforms for bioanalysis is evolving, with high resolution mass spectrometry (HRMS) technologies gaining momentum, particularly in the area of biotherapeutic quantitation requiring high sensitivity [2]. This changing landscape can be best demonstrated through integration of the microfluidic iKey Separation Device with the Xevo G2-XS HRMS Mass Spectrometer. The signature attributes of this system include high sensitivity, high speed, solvent savings, and ease of use. Sensitivity and throughput of the system can be optimized by adjusting the system flow rate. To date, most applications have been carried out at or near a flow rate of 3 µL/min, with approximately 5-10 minute cycle times. The operating pressure using a 150 µm x 50 mm iKey is generally ~3000-3400 psi (200-227 bar) which is well under the iKey tolerance of 10000

***Correspondence:**
34 Maple Street Milford, MA 01757-3696 USA. Phone +1 508.478.2000 & +1.800.252.4752; Fax +1 508.872.1990. http://www.waters.com

psi. The ACQUITY UPLC M-Class Binary Pump can also deliver consistent flow rates up to 100 µL/min. The full capabilities for pressure and flow rate of the system have not yet been fully exploited. In this study, we investigated the performance of the system at higher flow rates and pressure conditions to perform high throughput analysis. System performance, including autosampler carryover, reproducibility, linear response, and iKey robustness were assessed using buspirone, a relatively polar small molecule drug, clopidogrel, a relatively non-polar compound, and oxytocin, a cyclic peptide hormone with a molecular weight of ~1000 Dalton.

Experimental

Samples description

Human plasma was treated via protein precipitation by the addition of acetonitrile (ACN) using a volume ratio of 3:1 (ACN:plasma). The solution was centrifuged at 13000 relative centrifugal force (RCF), and the supernatant was transferred to a new vial. The supernatant was then diluted with water containing 0.1% formic acid to a percentage of ACN that was specific for each of the three compounds. Test compounds: buspirone, buspirone-d8, clopidogrel, clopidogrel-d4, oxytocin, and oxytocin (Ile-$^{13}C_6$,^{15}N) (Sigma Aldrich) were spiked into protein-precipitated human plasma. Final buspirone and clopidogrel samples contained 20% ACN, whereas the oxytocin samples contain 5% ACN. Other sample details are explained in the **Results and Discussion** section.

LC-MS Conditions

The analytical LC-MS experiments were performed using the ionKey/MS System with the ACQUITY UPLC M-Class System, and the Xevo G2-XS QTof Mass Spectrometer. The ACQUITY UPLC M-Class System was configured with direct injection using an iKey HSS T3 Separation Device, 100Å, 1.8 µm, 150 µm x 50 mm (P/N: 186007260). The iKey temperature was maintained at 65°C. Flow rate was 7 µL/min. Mobile phase A consisted of 0.1% formic acid in H_2O. Mobile phase B was 90% ACN/10% MeOH containing 0.1% formic acid. The injection volume was 1 µL. Sample manager temperature was 10°C. Weak wash solvent was 10% ACN/90% H_2O. Strong wash solvent was 25% ACN/25% IPA (isopropyl alcohol)/25% MeOH/25% H_2O. Generic or compund-specific gradient conditions are described in the **Results and Discussion** section. The total run time was 3 minutes. Data was acquired using sensitivity mode with resolution >30000 FWHM under positive electrospray ionization. Acquisition range was 50-1200 *m/z*. Capillary voltage was 3.5 kV. Cone voltage was 60 V for the small molecules and 100 V for the peptide. The source temperature was 120°C, cone gas flow 50 L/hr, and nano gas flow was 0.1 L/hr. Scan times were either 0.1 s or 0.036 s and are detailed further in the **Results and Discussion** section. Tof MRM transitions for each of the compounds are as follows: buspirone, 386.3>122.0438, 394.3>122.0438 (IS), CE=30; clopidogrel, 322.1>212.0669, 326.1>216.0669 (IS), CE=16; and Oxytocin, 1007.4>1007.4454, 1014.4>1014.4454 (IS), CE=6. MassLynx® Software was used for data acquisition and TargetLynx™ Application Manager was used for data processing.

Results and discussion

Gradient condition and performance of the iKey

Generic or compound-specific conditions were used for each compound and are summarized in **Table 1**. For the generic condition, a ballistic gradient with in-

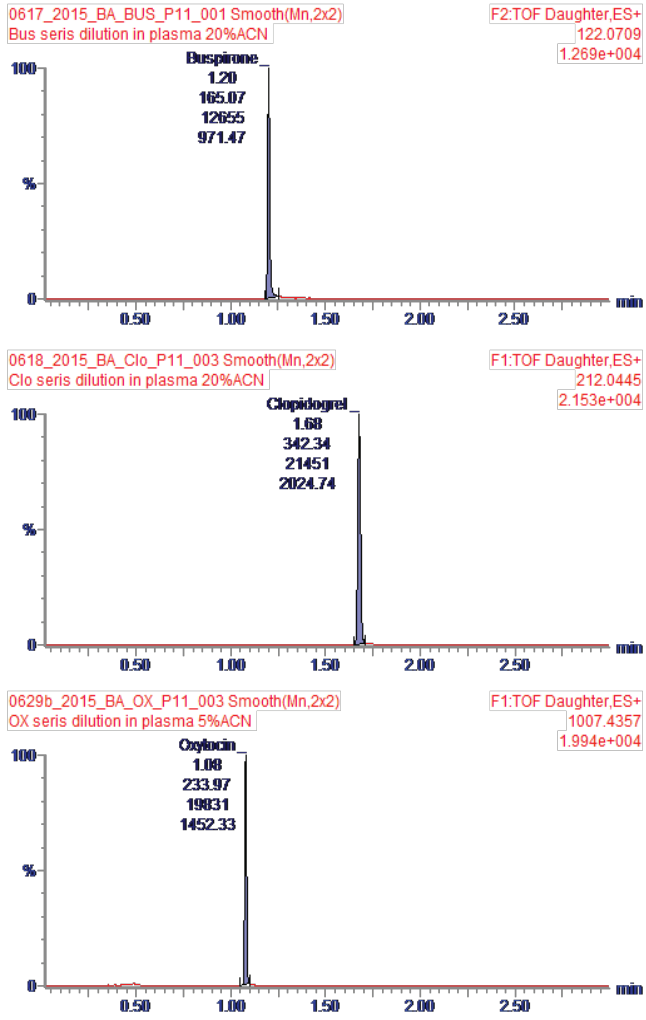


Figure 1. Chromatograms of test compounds in human plasma using generic gradient elution. Top: buspirone, middle: clopidogrel, and bottom: oxytocin

Table 1. Summary of gradient conditions used

Gradient time (min)	Flow rate µL/min	Generic gradient		Buspirone		Clopidogrel		Oxytocin	
		%A	%B	%A	%B	%A	%B	%A	%B
0.0	7	98	2	98	2	80	20	98	2
1.0	7			65	35	35	65	70	30
1.5	7	5	95	5	95	5	95	5	95
2.0	7	5	95	5	95	5	95	5	95
2.5	7	98	2	98	2	98	2	98	2
3.0	7	98	2	98	2	98	2	98	2

creasing %B from 2% to 95% in 1.5 min was used. After holding at 95% B for 0.5 min, the %B was changed back to initial and held for 0.5 min (**Table 1**). The total gradient time was 3 minutes. Sample injection time was used to achieve complete iKey equilibration. The total cycle time, including sample injection, was approximately 4 minutes. Chromatograms for each of the three compounds studied are shown in **Figure 1**. For the compound-specific gradient conditions, a brief method development was carried out where the percent mobile phase was adjusted for the first minute to enable the compound eluting at approximately 1.5 min. The subsequent ramping to 95 %B, holding and return back to the initial condition were the same as the generic gradient. Using a 1 µL sample loop, the system's theoretical delay volume was calculated to be 3.65 µL. The increase of flow rate to 7 µL/min corresponds to a theoretical delay time of 0.52 min. The performance of BEH and HSS T3 iKeys were tested. Both iKeys are packed with C18 for reversed phase applications.

The HSS T3 iKey is packed with high strength silica with tri-functional C18 alkyl phase bonding, which enables the column to withstand high pressure and promote polar compound retention and aqueous phase compatibility. The pressure traces shown in **Figure 2** show that both iKeys yielded highly reproducible profiles. The pressure for the BEH iKey was found to cycle between 4500 and 8500 psi (300-567 bar), and the HSS T3 between 3400 and 6400 psi (227-426 bar). The iKey packed with 1.8 µm HSS T3 particles exhibited a lower maximum system pressure profile vs. the 1.7 µm BEH particles and was used in subsequent studies reported in this application note.

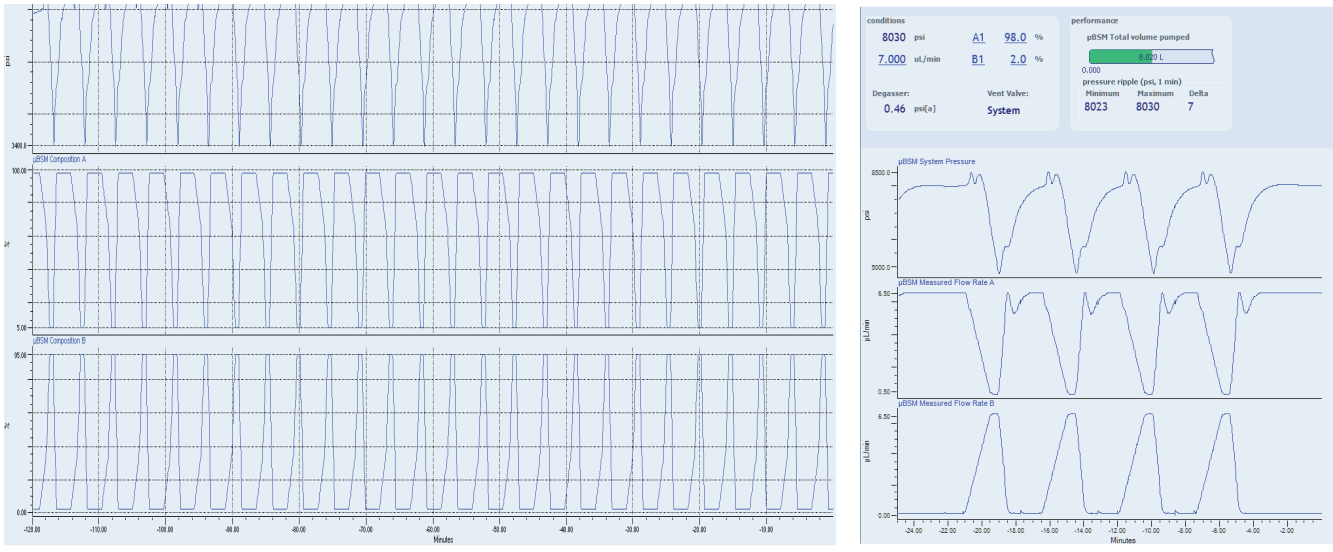


Figure 2. A screen capture of the ACQUITY UPLC M-Class Binary Solvent Manager showing system pressure change during sample analysis. On the left is the pressure history using the HSS T3 iKey in a two hour period, and on the right the system pressure using the BEH iKey for four sequential injections. In each graph, the top trace is the system pressure, and the middle and bottom traces are composition changes in channel A or B, respectively.

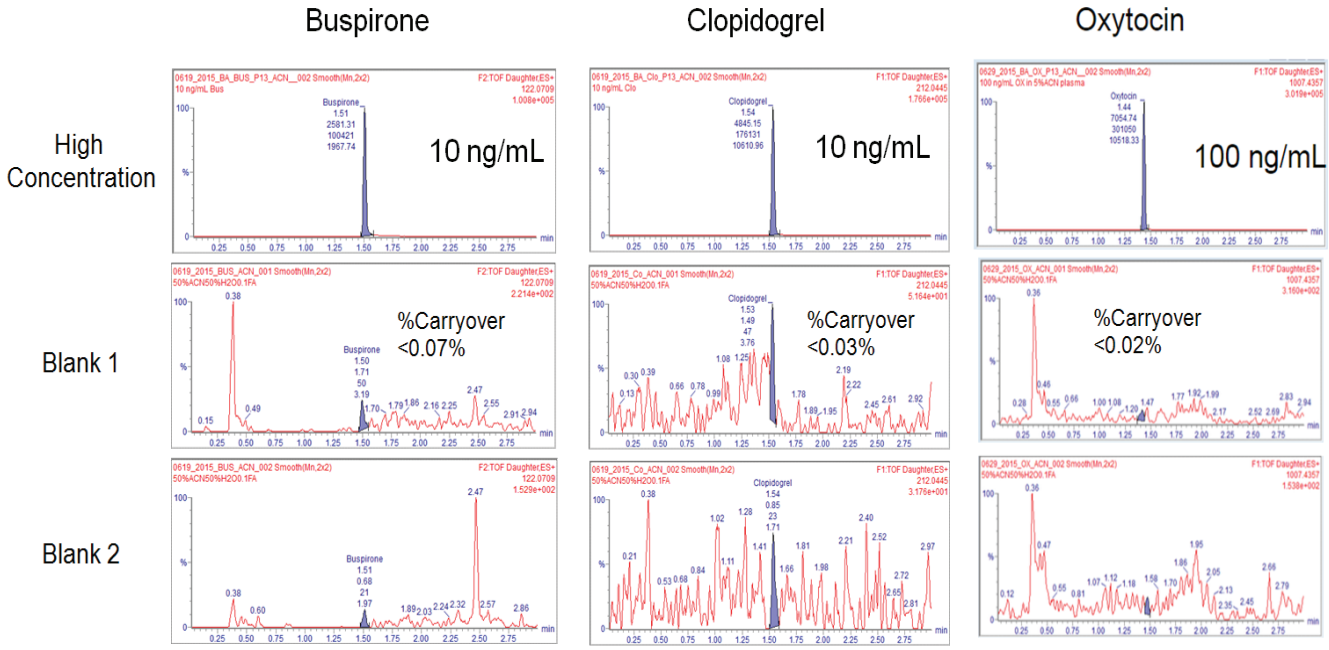


Figure 3. Chromatograms from the carryover study of the three compounds. The top chromatograms for each compound are from sample injection, followed immediately by blank injections reflected in the middle and lower chromatograms. Compound-specific gradient conditions were used.

System carryover

System carryover was measured by injecting a highly concentrated solution of each compound followed by blank solutions consisting of 50% ACN/50% H_2O. The high concentration used was 10 ng/mL for buspirone and clopidogrel, and 100 ng/mL for oxytocin. These concentrations are near the saturation level of the Xevo G2-XS QTof for these compounds. **Figure 3** shows chromatograms of the sample and the two blanks injected immediately afterwards. Percentage carryover was calculated based on the peak area at the retention time of the sample. The % carryover calculated for the blank injection immediately after the sample is 0.07% for buspirone, 0.03% for clopidogrel, and 0.02% for oxytocin. These results suggest that there is minimal or no sample carryover using the ionKey/MS with Xevo G2-XS under these conditions.

Reproducibility

The reproducibility was tested by injecting the same sample and internal standard solution 100 times using either generic or compound-specific gradient conditions.

Figure 4 shows a representative plot of peak area versus injection numbers. Percent RSD was calculated as 4.8% for buspirone, 1.7% for clopidogrel, and 4.6% for oxytocin. The data suggest good reproducibility for the ionKey/MS System under high throughput analysis conditions.

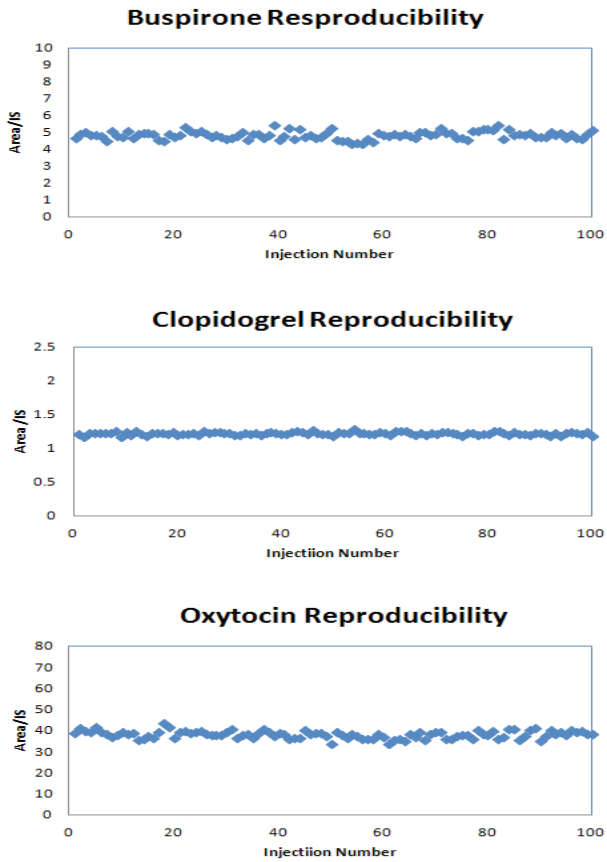


Figure 4. Plot of peak area/IS ratio versus injection number for 100 injections of corresponding compound in human plasma.

Linear response

The linear response was determined for serial diluted solutions in human plasma ranging from 1 pg/mL to 10 ng/mL for buspirone and clopidogrel, and from 10 pg/mL to 100 ng/mL for oxytocin. Each compound solution was injected in triplicate and analyzed using both generic and compound-specific conditions. The Tof MRM scan rate was 0.1 second for the compound-specific gradient, and 0.036 seconds for the generic gradient. The faster scan time at the generic gradient condition ensured that a minimum of 10 data points across the peak were collected for both the compound and internal standard when they eluted early with narrower peak width in the gradient time window. **Figure 5** shows the linear response curve using compound-specific conditions.

Figure 6 shows the linear response curve using generic gradient conditions. Results show excellent linear response under all conditions and scan rates, with R^2 >0.995 for all three compounds tested. For buspirone and clopidogrel, the linear range extended from 10 pg/mL to 10 ng/mL, and for oxytocin, the range was 100 pg/mL to 100 ng/mL using both generic and compound-specific gradients. The data show that the ionKey/MS System with Xevo G2-XS QTof can be used to perform quantitative analysis under high throughput conditions.

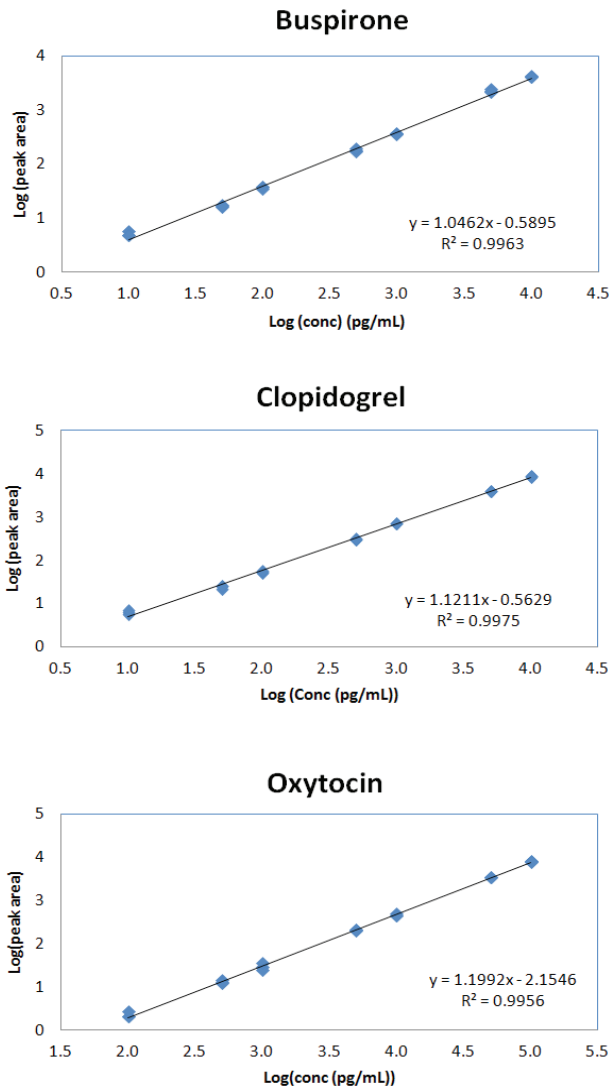


Figure 5. Plot of peak area versus concentration for a serially diluted solution of test compounds in human plasma. Each concentration was injected in triplicate and plotted. A compound-specific gradient and scan rate of 0.1 s were used.

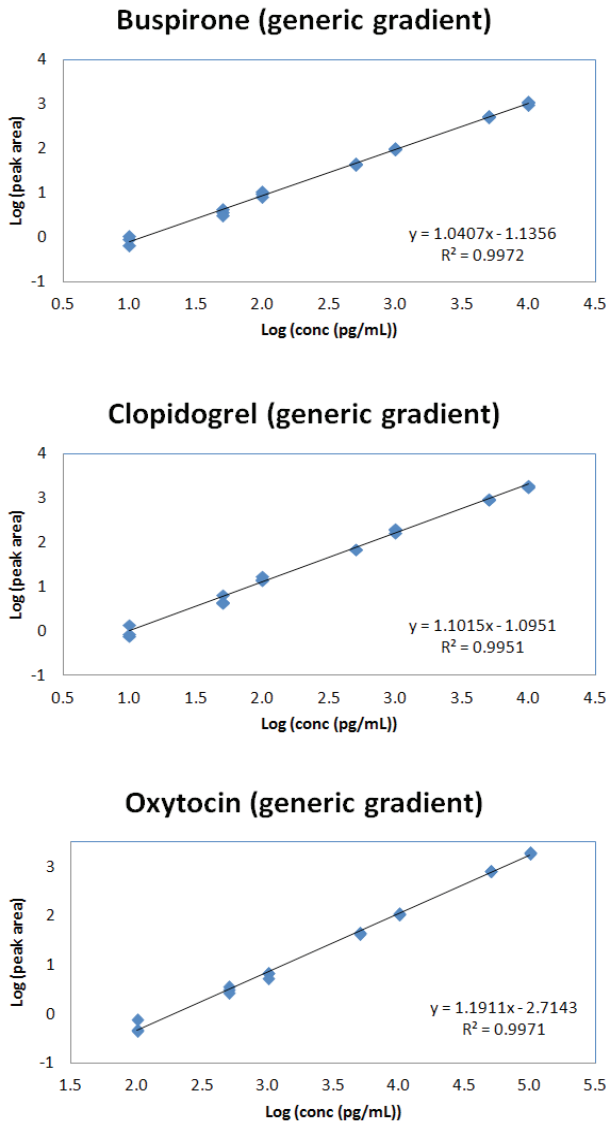


Figure 6. Plot of peak area versus concentration for serially diluted solutions of test compounds in human plasma. Each concentration was injected in triplicate and plotted. Generic gradient conditions and a scan rate of 0.036 s were used for all compounds.

Table 2. Gradient time tables for scaling flow rate from 7 µL/min to a lower flow rate; %mobile phase composition remains the same at each time point.

Flow rate (uL/min)	Initial gradient time (min)					
7	1.0	1.50	2.00	2.50	3.00	4.20
6	1.17	1.75	2.33	2.92	3.50	4.90
5	1.40	2.10	2.80	3.50	4.20	5.88
4	1.75	2.63	3.50	4.38	5.25	7.35
3	2.33	3.50	4.67	5.83	7.00	9.80
2	3.50	5.25	7.00	8.75	10.50	14.70

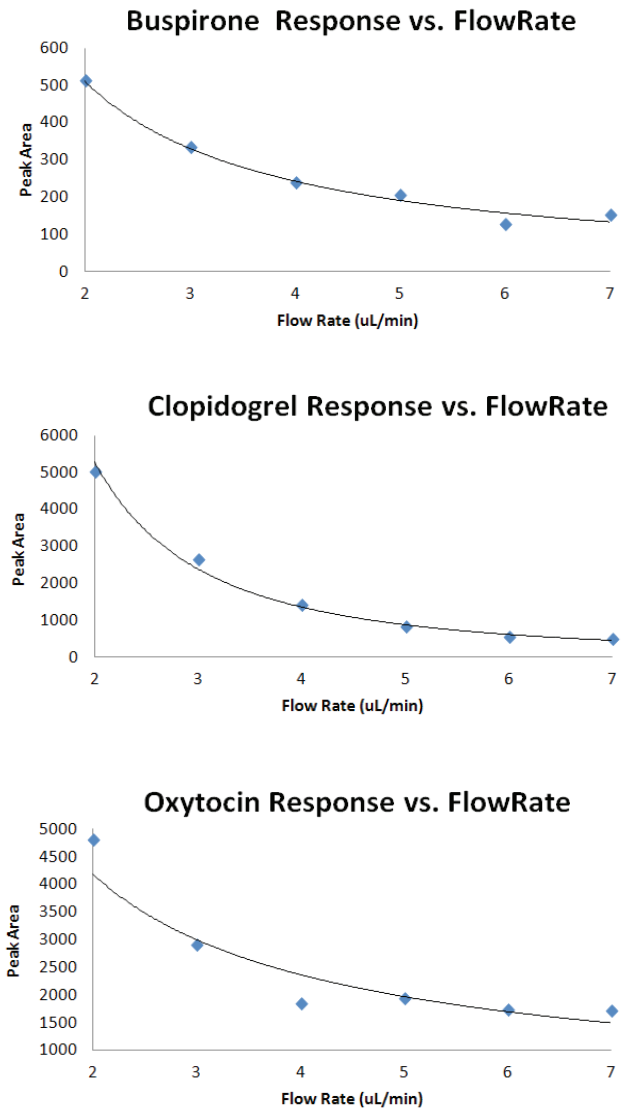


Figure 7. Plot of MS peak area versus flow rate showing response increase with decreasing flow rate from 7 µL/min to 2 µL/min.

Effect of flow rate on MS response

Available literature suggests that MS response is a function of flow rate in micro flow systems. In general, MS response increases with decreasing flow rate as the result of reduced droplet size and increases in ionization efficiency and MS sampling [3]. This effect is also measured in the present study by collecting data at flow rates from 7 µL/min to 2 µL/min. **Table 2** is a summary of the gradient times for each of the flow rates used. The time for each segment of the gradient was scaled based on column volume. **Figure 7** is a plot of MS response versus flow rate. Results show that with flow rate decreasing from 7 µL/min to 2 µL/min, there is an exponential increase in MS response for all three compounds used in the present study, which is consistent with the reported literature [3]. This provides a facile path to transition from high throughput analysis to extreme high sensitivity analysis by simply modulating flow rate. Incorporating trap-and-elute ionKey configurations has also been employed to further enhance the overall system sensitivity and has been described elsewhere [4].

iKey robustness

The data presented here were collected using a single HSS T3 iKey, and by the end of the study ~2200 injections of human plasma had been injected, most of which were collected at the 7 µL/min flow rate. The iKey remained viable beyond these ~2200 injections. **Figure 8** is a screen capture of the iKey history as monitored and stored by MassLynx Software. The history shows that with the first injections of the iKey, the maximum pressure is approximately 6400-6500 psi and is consistent to the end of the study. The peak shape also remains excellent.

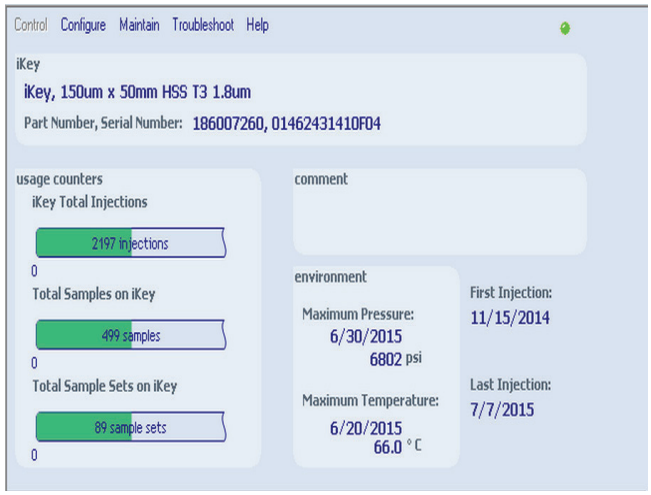


Figure 8. Screen capture of the HSS T3 iKey history, showing the number of injections (2197) and maximum operating pressure.

Figure 9. Comparison of relative solvent consumption of ionKey running at 7 µL/min flow rate vs. analytical scale chromatography running at 1 mL/min flow rate is displayed. The figure shows that consuming 1 L solvent on the ionKey/MS System running continuously at 7 µL/min for 99 days or 35,700 injections at 4 min run time, is equivalent to thirty six 1-gallon bottles using an analytical scale system.

Solvent usage

Solvent consumption and cost continues to be a concern in DMPK discovery labs. Due to the large amount of solvent used, many labs have also invested in expensive systems that can dispense solvent from larger solvent containers which need to follow strict safety guidelines. The 7 µL/min flow rate used in the present study represents a 98.8% to 99.5% solvent savings, assuming analytical scale chromatography with a flow rate ranging from 0.6 mL/min to 1.5 mL/min. To put this into perspective, Figure 9 shows that consuming 1 L of solvent on the ionKey/MS System at a flow rate of 7 µL/min is equivalent to consuming thirty-six 1-gallon bottles at a flow rate of 1 mL/min. Lower solvent requirements also translates to reduced time and labor required for preparation. The reduced solvent usage and consequential reduced waste disposal is increasingly important in making companies and laboratories more "green" and environmental friendly.

Conclusion

The ionKey/MS System with Xevo G2-XS QTof HRMS is a high performance platform that can be used for highly sensitive detection of small molecules and peptides (and other biomolecules). The present application shows that the ionKey/MS can also be operated under high throughput conditions with a 3 minute full gradient and a 7 µL/min flow rate. Using representative small molecules and a peptide, extensive testing of the platform shows that it is well-suited for routine analysis with high reproducibility, excellent linear response and minimal sample carryover. The HSS T3 iKey is also shown to be robust under high throughput conditions, after ~2200 injections of human plasma, with minimal impact on the system's maximum pressure or peak shape. In summary, the present data, coupled with the growing body of literature on the utility and robustness of next generation microfluidics, suggests the ionKey/MS with Xevo G2-XS QTof can be used as a full service platform for high throughput and high sensitivity analysis to support all phases of drug discovery and development.

Acknowledgement

We'd like to acknowledge the following from Waters Corp for the review of the manuscript: Kelly Doering, Pete Claise, Nigel Ewing, Larry Hines, and Sylvia Vollering.

References

1. Rainville PD, Langridge JI, Wrona MD, Wilson ID, Plumb RS. Integration of microfluidic LC with HRMS for the analysis of analytes in biofluids: past, present and future. Bioanalysis. 7(11), 1397-411 (2015).
2. Ramanathan R, Jemal M, Ramagiri, Xia SY, WG Humpreys WG, T Olah, WA Korfmacher. It is time for a paradigm shift in drug discovery bioanalysis: From SRM to HRMS. J Mass spectrom. 46(6), 595-601 (2011).
3. Murphy JP, Johnson J, Rainville PD. Enhancing Mass Spectrometry Sensitivity by Reducing Chromatographic Flow Rates with ionKey/MS, Waters

White Paper (2014). (P/N 720004967EN).
4. Alelyunas YW, Wrona MD, Murphy J, Doneanu A, Roman G, Rainville P. Exploring Extra Sensitivity Using ionKey/MS with the Xevo G2-XS Q-Tof HRMS for Small Molecule Pharmaceutical Analysis in Human Plasma. Waters Application Note (2015). (P/N 720005371EN)

Appendix

Recommendations for start up and end of run conditions are described below. These practices will help prolong the lifetime of the iKey under high throughput conditions.

A. Start up from idle.

It is recommended to slowly bring the iKey from idle, which could be mostly an organic mobile phase at no or slow flow rate, to the initial gradient condition of mostly aqueous mobile phase and high flow rate. A gradient method is shown in the table below, where the solvent composition was changed from idle condition, followed

	Time (min)	Flow (µL/min)	%A	%B	Curve
1	Initial	3.000	5.0	95.0	Initial
2	2.00	3.000	95.0	5.0	6
3	3.00	3.000	95.0	5.0	6
4	5.00	7.000	95.0	5.0	6

B. Idle conditions for iKey post analysis

It is recommended to add the following gradient conditions at the end of an LC method. This gradient will ensure the iKey is maintained at low flow and high organic composition when in idle. The high organic mobile phase will help with washing out late eluting components that were not completely eluted during sample analysis. With this portion added to a LC method, after 30 min, if there is no new injection which will reset the time, the flow rate will decrease to 1 µL/min and the mobile phase changes to 95%B, and will be maintained at this condition throughout idle.

	Time (min)	Flow (µL/min)	%A	%B	Curve
7	30.00	7.000	80.0	20.0	6
8	31.00	1.000	80.0	20.0	6
9	35.00	1.000	5.0	95.0	6

[TECHNOLOGY BRIEF]

Performing Peptide Bioanalysis Using High Resolution Mass Spectrometry with Target Enhancement MRM Acquisition

Yun Wang Alelyunas, Mark D. Wrona, and Nick Tomczyk
Waters Corporation, Milford, MA, USA

GOAL

To demonstrate Tof MRM acquisition of commercially available peptide mixture MSQC1 using ionKey™/Xevo® G2-XS QTof platform for peptide bioanalysis.

BACKGROUND

The rise of biotherapeutics in the drug discovery and development pipeline has resulted in the increasing demand for protein/peptide bioanalysis by LC-MS techniques. This has led to a renewed interest in high resolution mass spectrometry (HRMS) platforms for use in quantitation because they enable both a larger mass range and additional options for selectivity. HRMS platforms retain the ability to quickly switch back to characterization mode to elucidate or resolve qualitative, underlying matrix issues with the assay. In this way, both peptide mapping and quantitation can be performed on the same platform.

In addition to full scan data acquisition, newer targeted HRMS modes enable faster tandem-like throughput, while avoiding some of the complexity of full scan approaches. Currently, quantitation through a surrogate peptide approach remains the most popular practice for large molecule bioanalysis. In this approach, proteins and peptides are prepared through enzyme digestion. The resulting mixture is analyzed first to map out peptides thus formed and to search for signature peptides. Informatics tools such as Skyline are tailored for developing quantitative methods.

Quantitation for serial diluted samples shows ionKey/Xevo G2-XS is well suited for peptide characterization and quantitation with excellent sensitivity and linear response.

Once the signature peptides are identified, subsequent peptide quantitation is carried out using either tandem quadrupoles or high resolution mass spectrometers.

In this tech brief, peptide quantitation using HRMS is demonstrated using ionKey/MS™ and Xevo G2-XS with Tof MRM mode for data acquisition (although the acquisition/data processing is amenable to all inlets available at Waters®). Tof MRM, or target enhancement mode of acquisition, provides a selectivity and sensitivity boost for enhanced HRMS quantitation. Microfluidics such as ionKey also produce enhanced selectivity and sensitivity across small and large molecules, particularly with intact molecule characterization for both surrogate and intact quantitation.

A commercially available peptide mixture called MSQC1 (Sigma Aldrich) is used in the present study. Peptide composition of the mixture, its sequence, and corresponding light/heavy (L/H) stable isotope ratios are summarized in Table 1. The mixture is frequently used by labs as QC standards before running peptides of interest. Table 2 is a summary of the first four peptides which are used to describe the process of creating Tof MRM methods and building a targeted Tof MRM HRMS method.

Table 1. Protein digest and stable isotope labeled peptide composition of MSQC1 mix

Protein (UniProt accession number)	Calc'd MW (Da)	Approx. protein per vial		Corresponding SIL peptide sequences	SIL peptide content per vial (pmol)	Theoretical ratio light : heavy (Protein : SIL peptide)
		pmol	µg			
Carbonic Anhydrase I (P00915)	28739	100	2.9	GGPFSDSY[R]	100	1
				VLDALQAI[K]	50	2
Carbonic Anhydrase II (P00918)	29115	100	2.9	AVQQPDGLAVLGIFL[K]	10	10
				SADFTNFDP[R]	2	50
NAD(P)H dehydrogenase (P15559)	30736	20	0.62	EGHLSPDIVAEQ[K]	20	1
				ALIVLAHSE[R]	10	2
C-reactive Protein (P02741)	23047	20	0.46	ESDTSYVSL[K]	2	10
				GYSIFSYAT[K]	0.4	50
Peptidyl-prolyl cis-trans isomerase A (P62937)	20176	4	0.08	FEDENFIL[K]	8	0.5
				VSFELFAD[K]	4	1
				TAENF[R]	2	2
Catalase (P04040)	59625	4	0.24	GAGAFGYFEVTHDIT[K]	20	0.2
				FSTVAGESGSADTV[R]	0.4	10
				NLSVEDAA[R]	0.08	50

Table 2. A summary of peptide sequence, charge, and transition for the first four peptides (Sigma Aldrich)

Protein (UniProt accession number)	Peptide	MH+ (mono)	z (Q1)	Q1 *m/z*	Q3 *m/z*	Fragment ion type	CE	DP	CXP	EP
Carbonic Anhydrase I (P00915)	GGPFSDSYR	985.44	2	493.2	871.4	y7	21	80	12	10
			2	493.2	627.3	y5	27	80	12	10
			2	493.2	774.3	y6	33	80	12	10
	GGPFSDSY[R]	995.44	2	498.2	881.4	y7	21	80	12	10
			2	498.2	637.3	y5	27	80	12	10
			2	498.2	784.3	y6	33	80	12	10
	VLDALQAIK	970.59	2	485.8	643.4	y6	26	80	12	10
			2	485.8	758.4	y7	26	80	12	10
			2	485.8	871.5	y8	20	80	12	10
	VLDALQAI[K]	978.60	2	489.8	651.4	y6	26	80	12	10
			2	489.8	766.4	y7	26	80	12	10
			2	489.8	879.5	y8	20	80	12	10
Carbonic Anhydrase II (P00918)	AVQQPDGLAVLGIFLK	1668.97	2	835.0	1242.8	y12	36	80	12	10
			2	835.0	1030.7	y10	42	80	12	10
			2	835.0	973.6	y9	42	80	12	10
	AVQQPDGLAVLGIFL[K]	1676.97	2	839.0	1250.8	y12	36	80	12	10
			2	839.0	1038.7	y10	42	80	12	10
			2	839.0	981.6	y9	42	80	12	10
	SADFTNFDPR	1169.52	2	585.3	896.4	y7	25	80	12	10
			2	585.3	749.4	y6	25	80	12	10
			2	585.3	1011.5	y8	25	80	12	10
	SADFTNFDP[R]	1179.52	2	590.3	906.4	y7	25	80	12	10
			2	590.3	759.4	y6	25	80	12	10
			2	590.3	1021.5	y8	25	80	12	10

RESULTS

Results are described in three sections:

I – Setting up a Tof MRM experiment in MassLynx®

II – Developing a Tof MRM scouting experiment to identify the best peptide transitions and also the best conditions (CE) to monitor the peptide

III – Quantification using TargetLynx™

I. Setting up TofMRM data acquisition in MassLynx

Step 1. Open MS method editor and choose "Tof-MRM" function. A function line appears in the function table as shown in Figure 1.

Step 2. Click on the function to open a new window/ dialog. In the first tab, "Acquisition," enter acquisition time, polarity, etc. as shown in Figure 2a. Next, click on the "TOF MS" tab and enter mass range and scan time as shown in Figure 2b. Mass ranges from 50 to 1200 or 50 to 2000 *m/z* and scan time of 0.1 s are typical. Scan time should be adjusted based on run time and peak width to ensure a minimum of 10 data points base-to-base are collected across the peaks for the number of MRMs scanning concurrently.

Compound information is entered in the third tab called "MRM" as shown in Figure 3. In this tab, choosing the "Add" button opens the MRM editor. Enter the time window and set mass, CE, CV, and "Target Enhancement *m/z*" to the appropriate cells shown. Additional fragments may be entered for monitoring, but typically only one is chosen for maximum sensitivity and entered in fragment box 1. "Set Mass" and "Target enhancement *m/z*" are critical for data acquisition. Repeat this process for all the transitions of interest. In this example four peptides along with their internal standards are being monitored. A time window is entered for each peptide providing maximum signal for each pair. For the time window, ending at 1.0 min and starting next at 1.0 min is considered overlapping, which results in two traces of data. Try to start the second window at 1.01 min to produce one data trace.

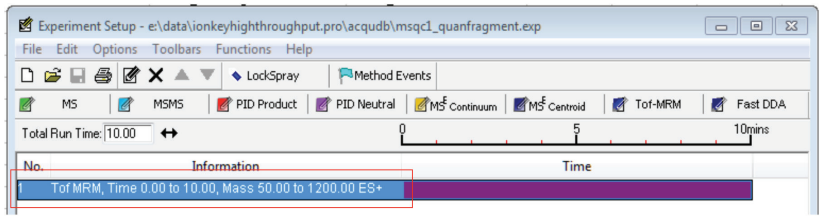


Figure 1. A snapshot of the user interface after opening the method editor and choosing "Tof MRM."

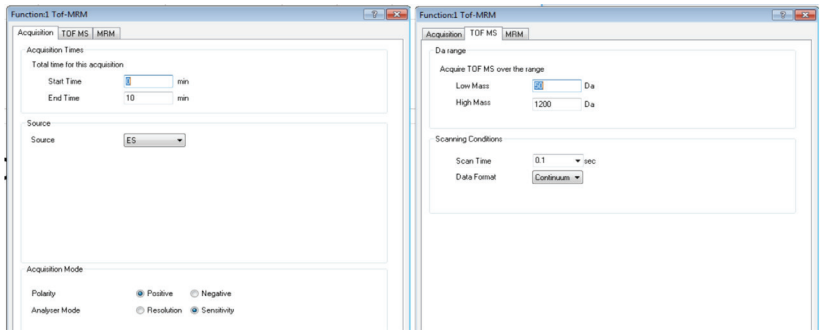


Figure 2. (a) Acquisition, (b) "TOF MS" tab of the MS method.

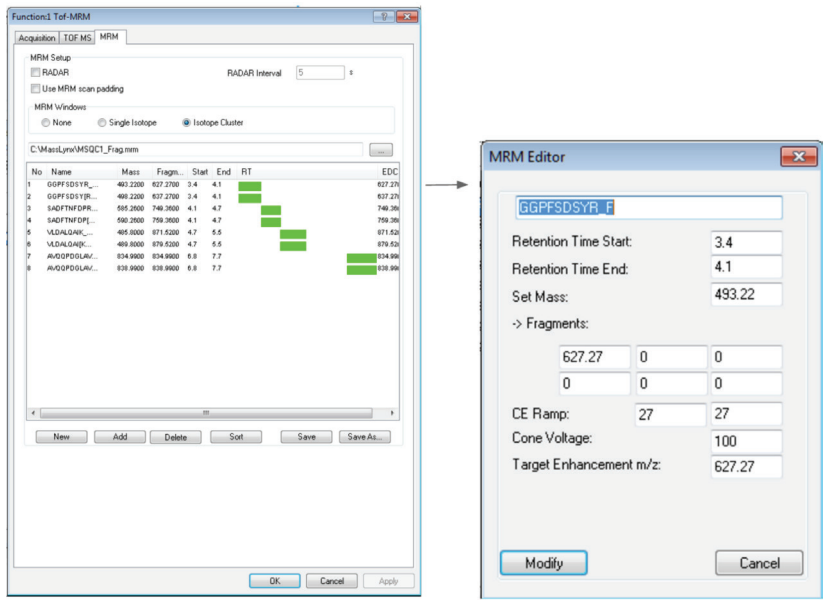
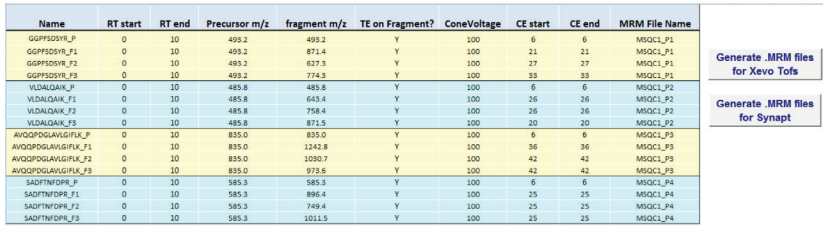


Figure 3. MRM table and MRM editor shows an example of Precursor ([M+2H2+]= 493.22 m/z) >Fragment ([M+H+] = 627.27 m/z) transition (middle), and a tuneless Precursor>Precursor transition (right).

Once all information is entered, click "Save" to store the MRM table as a ".mrm" file. A .mrm file is a collection of compounds, time window, and other information shown in the MRM editor page. Once it is created, it can be imported to easily create the MRM function. An Excel Macro file to help generate .mrm files is freely available from Waters upon request.

Because data acquisition reads from the .mrm file, if the "Save" button is not clicked, any modified information is not used. If one also wants to collect full scan spectrum simultaneously, the "RADAR" box should be checked (note: this reduces the duty cycle and is recommended for method development, but not final assay method). MRM window choices describe the tolerance of data shown with the target enhancement region of about 50 Da around the target mass. "None" indicates the entire 50 Da range centered around ion of interest; "single isotope" only retains the single isotope (1 Da), the "isotope cluster" retains a 5 Da window. In all cases, the data can be further extracted during the XIC creation to an HRMS mDa tolerance, thus further increasing specificity of the extracted ion chromatogram (XIC).



Name	RT start	RT end	Precursor m/z	fragment m/z	TE on Fragment?	ConeVoltage	CE start	CE end	MRM File Name
GGPFSDSYR_P	0	10	493.2	493.2	Y	100	6	6	MSQC1_P1
GGPFSDSYR_F1	0	10	493.2	871.4	Y	100	21	21	MSQC1_P1
GGPFSDSYR_F2	0	10	493.2	627.3	Y	100	27	27	MSQC1_P1
GGPFSDSYR_F3	0	10	493.2	774.3	Y	100	33	33	MSQC1_P1
VLDALQAIK_P	0	10	485.8	485.8	Y	100	6	6	MSQC1_P2
VLDALQAIK_F1	0	10	485.8	643.4	Y	100	26	26	MSQC1_P2
VLDALQAIK_F2	0	10	485.8	758.4	Y	100	26	26	MSQC1_P2
VLDALQAIK_F3	0	10	485.8	871.5	Y	100	20	20	MSQC1_P2
AVQQPDGLAVLGIFLK_P	0	10	835.0	835.0	Y	100	6	6	MSQC1_P3
AVQQPDGLAVLGIFLK_F1	0	10	835.0	1242.8	Y	100	36	36	MSQC1_P3
AVQQPDGLAVLGIFLK_F2	0	10	835.0	1030.7	Y	100	42	42	MSQC1_P3
AVQQPDGLAVLGIFLK_F3	0	10	835.0	973.6	Y	100	42	42	MSQC1_P3
SADFTNFDPR_P	0	10	585.3	585.3	Y	100	6	6	MSQC1_P4
SADFTNFDPR_F1	0	10	585.3	886.4	Y	100	25	25	MSQC1_P4
SADFTNFDPR_F2	0	10	585.3	749.4	Y	100	25	25	MSQC1_P4
SADFTNFDPR_F3	0	10	585.3	1011.5	Y	100	25	25	MSQC1_P4

Generate .MRM files for Xevo Tofs

Generate .MRM files for Synapt

Figure 4. Excel macro display for creation of .mrm files.

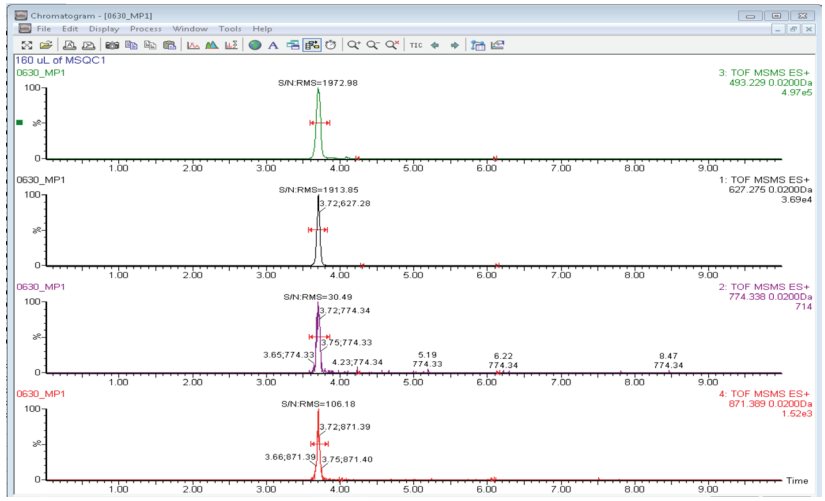


Figure 5. Overlaid extracted ion chromatograms for peptide 1 scouting results from the four transitions.

II. Performing scouting experiments for MSQC1

The first set of MRM experiments is carried out to select the best transitions for eventual quantitation for each of the peptides. The transitions tested are the three precursor>fragment transitions provided in the catalog (Table 2), and a fourth transition based on precursor>precursor without fragmentation (tuneless transition). A spreadsheet containing the transitions is shown (Figure 4) and when translated to a .mrm file it can be imported or transcribed into the Tof MRM method editor.

A resulting XIC for each of the peptides is then overlaid. Peptide 1 and peptide 2 are shown in Figure 5 and Figure 6 as an example. As expected, the precursor>precursor transition produces the highest signal for all four peptides. In this example the precursor>precursor transition and one of the precursor>fragment transitions with the highest response for subsequent quantitation and instrument performance check are chosen.

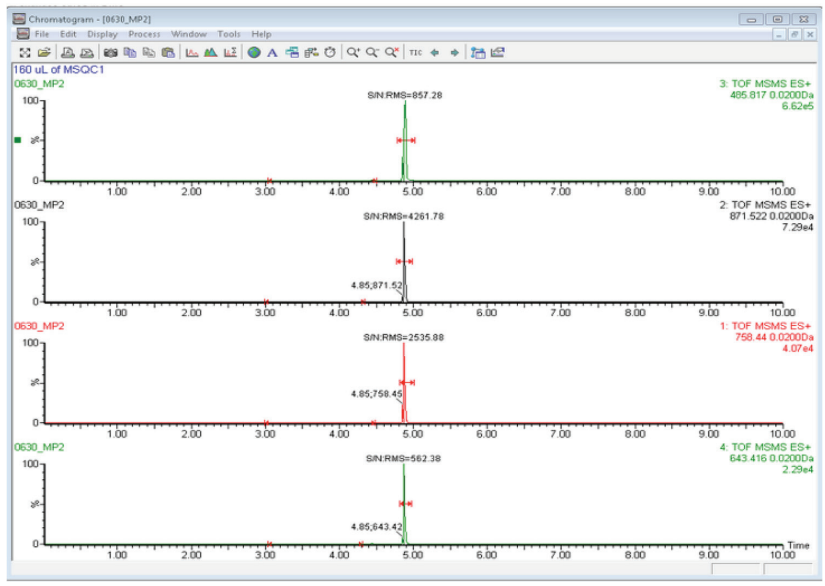


Figure 6. Overlaid extracted ion chromatograms for peptide 2 scouting results from the four transitions.

III. Quantitation

A standard curve is prepared for MSQC1 by successive 1:2 dilutions based on instruction from Sigma Aldrich. The range of the dilution starts with 1:16 down to 1:4096 fold. Four peptides are monitored simultaneously according to the time window shown in Figure 3. A sample chromatogram is shown in Figure 7. Gradient conditions for ionKey are shown in Figure 8.

Figure 9 shows TargetLynx results of Peptide 4 based on precursor>precursor transition. A good linear range from 1:64 to 1:4096 fold dilution is obtained with R^2 = 0.99. Above 1:64 fold, the signal suggests saturation of the mass detector. At the lowest prepared concentration of 1:4096 fold, the average signal-to-noise ratio is 310, indicating low detection limit has not been reached and the mass spec is capable of detecting sample at lower concentration.

Figure 10 shows TargetLynx results of Peptide 4 based on isotope labeled precursor>precursor transition. As expected, the lower abundance of the labeled peptide produces a lower response. The linear range now extends to all concentrations with no signal saturation at higher concentration. An R^2 of 0.99 is also obtained. The average signal-to-noise ratio at 1:4096 fold dilution is 25 which is expected based on lower response observed.

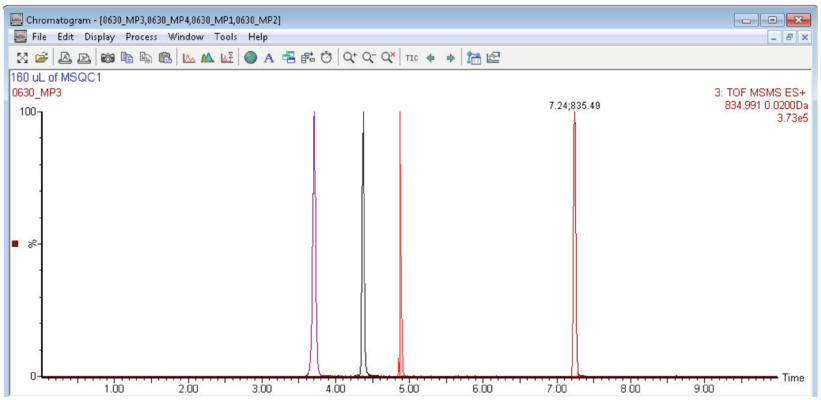


Figure 7. Overlaid extracted mass chromatogram of the four peptides monitored. The iKey™ Separation Device used is the iKey HSS T3, 150 µm x 50 mm (P/N 186007260). Injection volume is 1 µL and iKey temperature is 45 °C. Scan time is 0.1 s.

Gradient:

	Time (min)	Flow (µL/min)	%A	%B	Curve
1	Initial	3.000	99.0	1.0	Initial
2	7.00	3.000	55.0	45.0	6
3	7.50	3.000	5.0	95.0	6
4	8.50	3.000	5.0	95.0	6
5	9.00	3.000	98.0	2.0	6
6	20.00	3.000	98.0	2.0	6
7	21.00	1.000	5.0	95.0	6

Figure 8. Gradient conditions used in the present analysis.

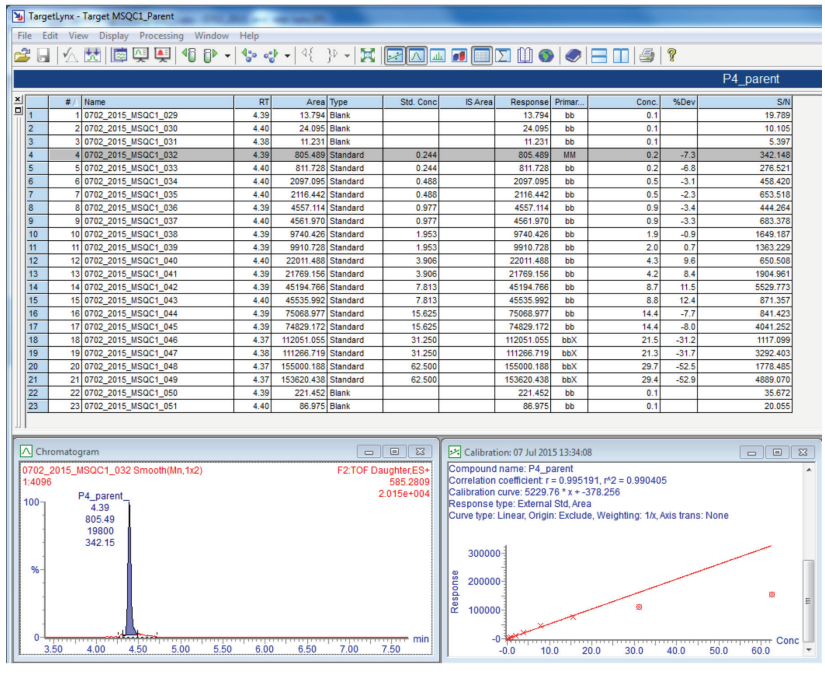
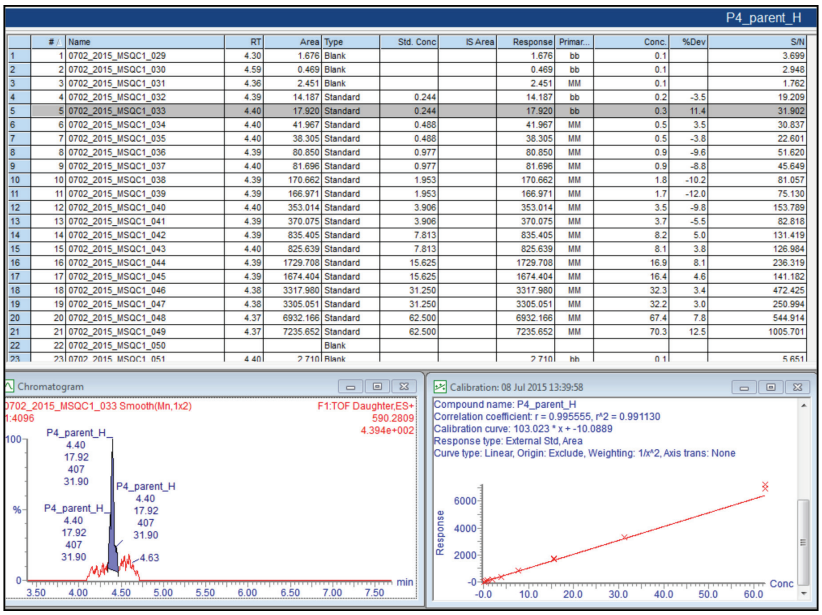


Figure 9. TargetLynx results of P4 peptide showing summary data, chromatogram of 1:4096 fold of dilution, and plot of response versus concentration.



	#	Name	RT	Area	Type	Std. Conc	IS Area	Response	Primar...	Conc.	%Dev	S/N
1	1	0702_2015_MSQC1_029	4.30	1.676	Blank			1.676	bb	0.1		3.699
2	2	0702_2015_MSQC1_030	4.59	0.469	Blank			0.469	bb	0.1		2.948
3	3	0702_2015_MSQC1_031	4.36	2.451	Blank			2.451	MM	0.1		1.762
4	4	0702_2015_MSQC1_032	4.39	14.187	Standard	0.244		14.187	bb	0.2	-3.5	19.209
5	5	0702_2015_MSQC1_033	4.40	17.920	Standard	0.244		17.920	bb	0.3	11.4	31.902
6	6	0702_2015_MSQC1_034	4.40	41.967	Standard	0.488		41.967	MM	0.5	3.5	30.837
7	7	0702_2015_MSQC1_035	4.40	38.305	Standard	0.488		38.305	MM	0.5	-3.8	22.601
8	8	0702_2015_MSQC1_036	4.39	80.850	Standard	0.977		80.850	MM	0.9	-9.6	51.620
9	9	0702_2015_MSQC1_037	4.40	81.696	Standard	0.977		81.696	MM	0.9	-8.8	45.649
10	10	0702_2015_MSQC1_038	4.39	170.662	Standard	1.953		170.662	MM	1.8	-10.2	81.057
11	11	0702_2015_MSQC1_039	4.39	166.971	Standard	1.953		166.971	MM	1.7	-12.0	75.130
12	12	0702_2015_MSQC1_040	4.40	353.014	Standard	3.906		353.014	MM	3.5	-9.8	153.789
13	13	0702_2015_MSQC1_041	4.39	370.075	Standard	3.906		370.075	MM	3.7	-5.5	82.818
14	14	0702_2015_MSQC1_042	4.39	835.405	Standard	7.813		835.405	MM	8.2	5.0	131.419
15	15	0702_2015_MSQC1_043	4.40	825.639	Standard	7.813		825.639	MM	8.1	3.8	126.984
16	16	0702_2015_MSQC1_044	4.39	1729.708	Standard	15.625		1729.708	MM	16.9	8.1	236.319
17	17	0702_2015_MSQC1_045	4.39	1674.404	Standard	15.625		1674.404	MM	16.4	4.6	141.182
18	18	0702_2015_MSQC1_046	4.38	3317.980	Standard	31.250		3317.980	MM	32.3	3.4	472.425
19	19	0702_2015_MSQC1_047	4.38	3305.051	Standard	31.250		3305.051	MM	32.2	3.0	250.994
20	20	0702_2015_MSQC1_048	4.37	6932.166	Standard	62.500		6932.166	MM	67.4	7.8	544.914
21	21	0702_2015_MSQC1_049	4.37	7235.652	Standard	62.500		7235.652	MM	70.3	12.5	1005.701
22	22	0702_2015_MSQC1_050			Blank							
23	23	0702_2015_MSQC1_051	4.40	2.710	Blank			2.710	bb	0.1		5.651

Figure 10. TargetLynx results of isotope labeled P4 peptide showing summary data, chromatogram of 1:4096 fold of dilution, and plot of response versus concentration.

SUMMARY

Tof MRM mode of data acquisition provides an effective approach for analyte quantitation using HRMS. Setting up the Tof MRM MS method is straightforward. In the present example, Tof MRM mode of data acquisition is used for the identification of the monitoring mass of a representative peptide in MSQC1. Subsequent quantitation for serial diluted samples shows that the ionKey/Xevo G2-XS is well suited for the peptide characterization and quantitation with excellent sensitivity and linear response. In this way, one can go from characterization to high performance quantitative method using a single MS platform. The use of a simple turnkey microfluidics technology such as ionKey offers high sensitivity. In summary, the coupling of targeted HRMS (Tof MRM with target enhancement) on the Xevo G2-XS HRMS QTof with ionKey microfluidics, offers a powerful, simple workflow for HRMS peptide bioanalysis.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

[POSTER NOTE]

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

EVALUATION OF A NOVEL TANDEM QUADRUPOLE MASS SPECTROMETER FOR THE MULTI-POINT INTERNAL CALIBRATION ANALYSIS OF PEPTIDES

Billy J Molloy[1], Liam M Heaney[2,3], Donald JL Jones[2,3], Johannes PC Vissers[1], Donald P Cooper[1] and James I Langridge[1]
1 Waters Corporation, Wilmslow, United Kingdom, 2 Department of Cancer Studies, 3 Cardiovascular Research Centre, Glenfield Hospital, Leicester, United Kingdom

OVERVIEW

- **Evaluation of a novel tandem quadrupole mass spectrometer for peptide MRM analysis and comparison with other platforms**
- **Quantitative analysis of tryptic peptides using a novel Multi-Point Internal Calibration method**
- **Application to translational analysis of putative protein biomarkers for heart failure**

INTRODUCTION

Translational and biomarker verification studies are challenged in that they not only require the analysis of large sample cohorts with high-throughput, but also demand high sensitivity, high resolution and selectivity over a large dynamic range. Targeted LC-MS/MS based assays afford analyte quantification with the reproducibility and throughput required in order to rapidly assess biomarker performance. Multiple Reaction Monitoring (MRM), using tandem quadrupole mass spectrometry, is an enabling technology that provides speed and selectivity, whilst miniaturized LC systems offer additional improved sensitivity. Here, the application of micro-fluidics (IonKey) coupled to a novel tandem quadrupole MS/MS system (Xevo TQ-XS), using a Multi-Point Internal Calibration (MPIC) methodology for the quantitation of peptides and proteins, is demonstrated.

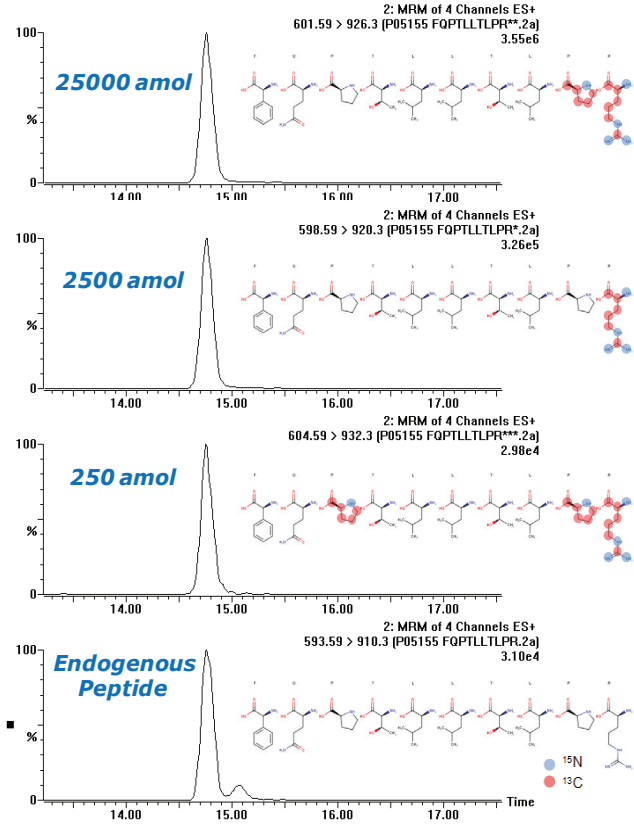


Figure 1. A three point MPIC curve is constructed from 3 differentially labeled versions of the analyte of interest, that are spiked into the sample at three different levels.

TO DOWNLOAD A COPY OF THIS POSTER NOTE, VISIT WWW.WATERS.COM/POSTERS

METHODS

Sample preparation

- Stable Isotope Labeled (SIL) peptides, whose analogues are putative biomarkers for cardiovascular disease (CVD) [1], were spiked at various levels into diluted, non-depleted, un-fractionated, tryptically digested human serum (20 - 1000 ng/µL of matrix).
- Blood samples were collected from a cohort of 20 healthy donors, 20 HFPEF patients, and 20 HFREF patients. All serum samples were mixed with NH_4HCO_3 in the presence of RapiGest, DDT reduced, IAA alkylated and trypsin digested overnight.

LC-MS system

IonKey integrated micro-fluidics

- ACQUITY M-class system
- Mobile phase A - water + 0.1% FA, mobile phase B - acetonitrile + 0.1% FA
- Gradient - 3-25% mobile phase B in 11 min
- 150 µm x 100 mm BEH C18 130 Å 1.7 µm
- Flowrate - 1.0 µL/min
- IonKey temperature - 35˚C
- Injection volume - 1 µL

Mass spectrometry

- Xevo TQ-XS tandem quadrupole system
- Capillary voltage - 3.5 kV
- Source temperature - 100˚C
- Resolution - unit mass resolution

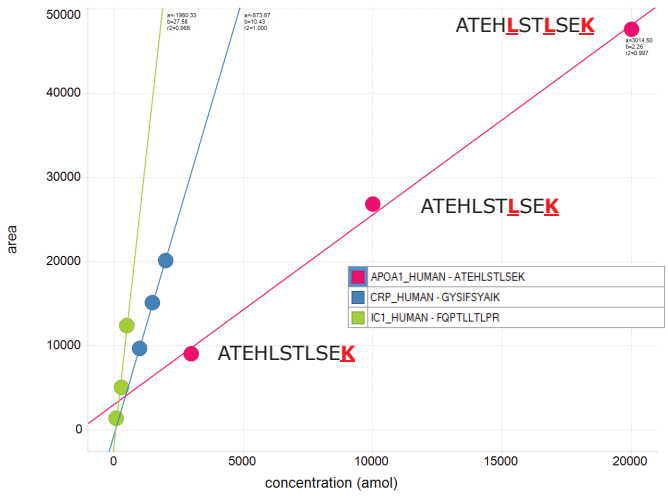


Figure 2. MPIC can be used for multiple analytes simultaneously. Here, the quantification principle of 3 proteins in human serum that are present over 3 different ranges is illustrated.

RESULTS AND DISCUSSION

Experimental design

SIL peptides were spiked into diluted, non-depleted, digested human serum matrix (200 ng/µL) at 12.5 fmol/µL and serially diluted in matrix to various levels over the range 0.00625 - 12.5 fmol/µL. This experiment had previously been replicated eight times using all possible combinations of LC and MS/MS platforms shown in Figure 3 using various MRM acquisition modes [2]. This analysis was replicated using the Xevo TQ-XS tandem quadrupole mass spectrometer combined with integrated micro-fluidics. The sensitivity achieved with this new instrument was compared to the other platforms.

Calibration methods

A subset of these samples were then selected and re-analysed (20 ng/µL of matrix) using a new, novel MPIC methodology as follows. Three different SIL variants of the same peptides were sourced (PepScan, Lelystad, The Netherlands) for three different peptides. These differentially labeled SIL peptides were then used to spike a calibration line within each of the samples selected from the previous study. This concept is demonstrated in Figures 1 and 2.

The endogenous level of two of the peptides, representing potential CVD biomarkers, were quantified by calibration against this internal calibration curve. The third peptide, not endogenously present, was used to perform a comparison of the internal calibration approach to the more widely used technique of quantifying against a separate external calibration line.

This comparison experiment was performed over a more limited linear range to replicate a "real world" experiment where the analyte of interest (biomarker) may be present over a much smaller reference range, and was performed using a higher level of matrix (1000 ng/µL) to replicate a high sensitivity biomarker assay.

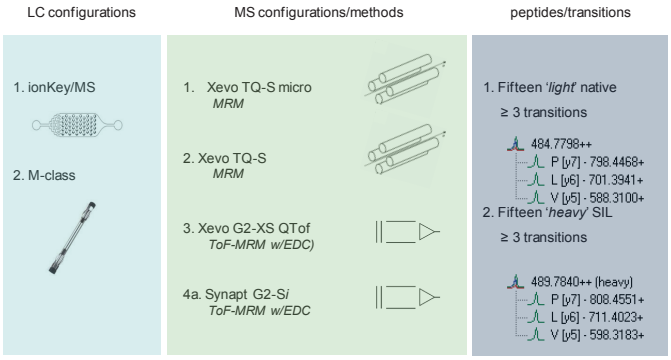


Figure 3. LC-MS configurations and MRM methods.

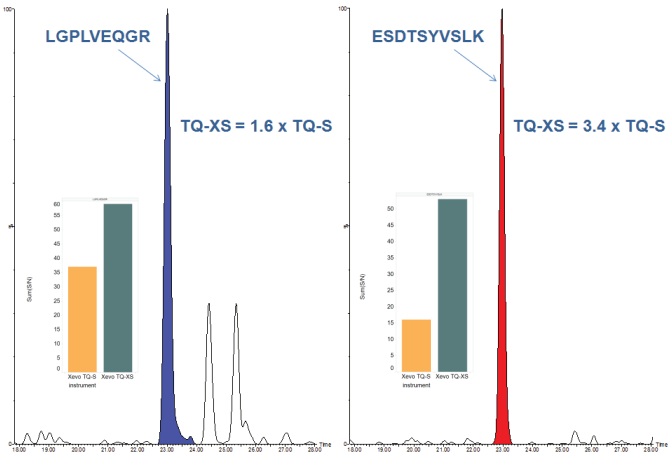


Figure 4. Chromatograms showing response of two tryptic peptides on Xevo TQ-XS using IonKey for 125 amol of peptide injected on column, and shown inset comparative analysis vs. Xevo TQ-S in terms of S/N (yellow = Xevo TQ-S; green = Xevo TQ-XS).

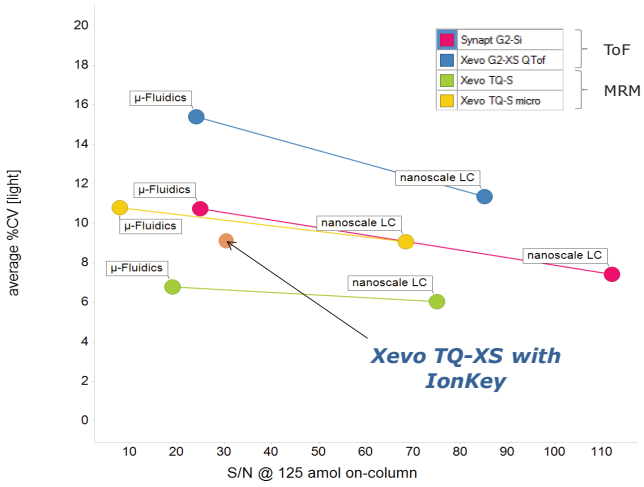


Figure 5. Average performance metrics for data acquired using the new TQ-XS mass spectrometer when compared to other MS platforms used in a previous study [2].

Sensitivity

The sensitivity of the Xevo TQ-XS mass spectrometer was compared directly to the previously used mass spectrometry platforms in terms of signal to noise ratio (S/N) at 125 amol on column. Figure 4 shows the response for two of the SIL peptides analysed. The increase in sensitivity observed for the new MS platform compared to the Xevo TQ-S platform varied from no increase to a 3.4 times increase in S/N. When the results from all of the peptides analysed (nine) were averaged, the mean increase was 1.6 times. Figure 5 shows how the LC-MS platform compared overall in comparison to the platforms used in the previous study [2].

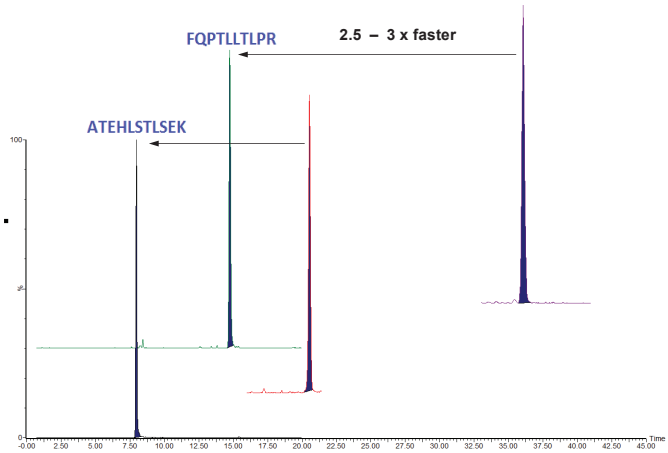


Figure 6. Response for the SIL form of the two endogenous peptides analysed in 20 ng/µL of matrix, using faster (2.5 - 3 times) IonKey LC conditions (20 min cycle time).

Multi-Point Internal Calibration (MPIC)

The MPIC method was used to analyse eighteen HF patient samples, comprising six samples from each of the three groups. A faster gradient was used with an increased flow rate because of reduced matrix consumption, resulting in improved throughput and sensitivity (LoD < 10 amol). Figure 6 shows the chromatographic performance for the two endogenous peptides analysed using this methodology.

As an example, Figure 7 shows the two trends observed for one of these CVD biomarkers (Plasma Protease C1 Inhibitor, FQPTLLTLPR) studied in this and a previous study [2]. Even with the different mass spectrometer, calibration method, LC conditions, matrix level and number of samples used, the same trends were observed for both biomarkers.

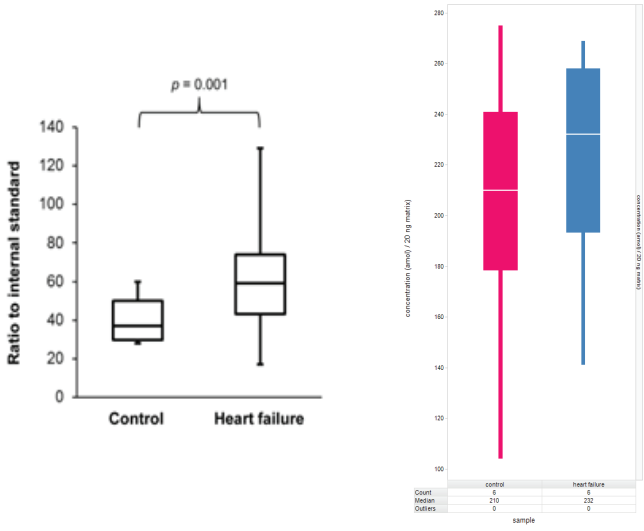


Figure 7. Comparison of data obtained in a previous study (left) [2], n=60), to data collected using the multi-point internal calibration methodology (right, n=18) for Plasma Protease C1 Inhibitor (FQPTLLTLPR).

A more detailed statistical evaluation of the MPIC method is shown in Figure 8 comparing the quantitative results within the experiments of a single point, relative quantitation type of approach (SIL) used in the previously mentioned study [2] and those obtained with the absolute MPIC method (InX). Good correlation was obtained between both methods with Spearman's ρ coefficients of 0.84 and 0.76 for the peptides of Apolipoprotein A1 and Plasma Protease C1 Inhibitor (SERPING1), respectively.

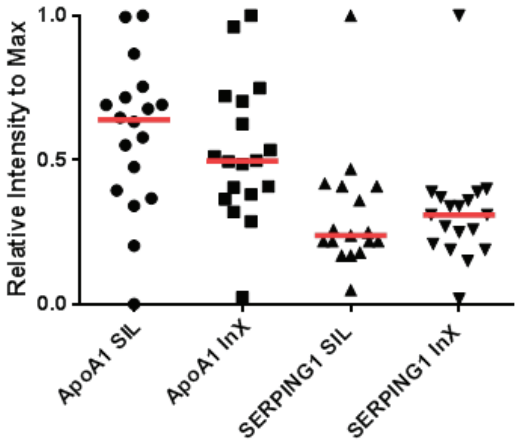


Figure 8. Normalised abundances measured with a single AQUA peptide (SIL) and MPIC (InX). . Data were normalised and scaled to the maximum. Red line = median relative normalised abundance.

Nine patient samples were randomly selected and spiked with 500 amol/µL of the exogenous GYSIFSYAIK, and analysed using the internal multi-point method (200 - 1000 amol/µL), as well as with an external calibration line. This experiment was performed at 1000 ng/µL of matrix to replicate a high sensitivity biomarker assay. Figure 9 shows the improvements in precision seen due to the improved compensation for matrix effects seen with MPIC methodology.

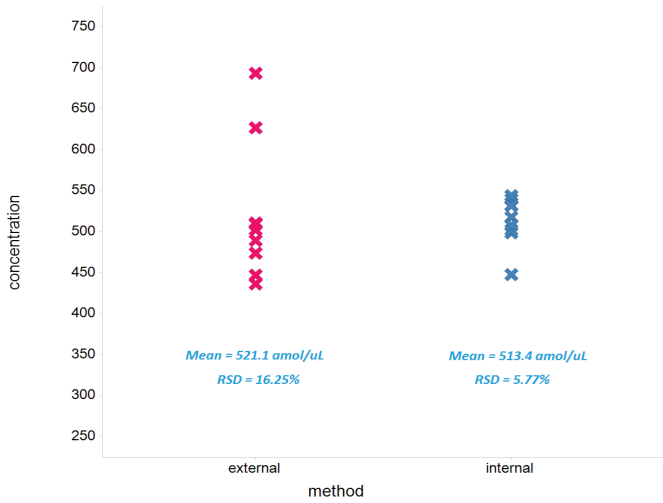


Figure 9. Analysis precision for nine matrix (1000 ng/µL) samples, spiked with an exogenous peptide (500 amol/µL), using external and internal multi-point calibration methods.

CONCLUSION

- **A new and novel tandem quadrupole mass spectrometer was successfully utilised to quantitatively analyse non-depleted, tryptically digested human serum samples for putative peptide biomarkers using novel multi-point internal calibration methodology**
- **The Xevo TQ-XS was shown to be on average 1.6 times more sensitive than the Xevo TQ-S MS platform when using IonKey integrated micro-fluidics for tryptic peptide analysis**
- **The increased sensitivity allowed for faster gradients to be used due to the reduced level of sample required for analysis, increasing throughput**
- **The multi-point internal calibration method also increased throughput further as no external calibrators are required to assess linearity**
- **The multi-point internal calibration methodology was also shown to be superior to an external calibration in terms of compensation for matrix effects**

References

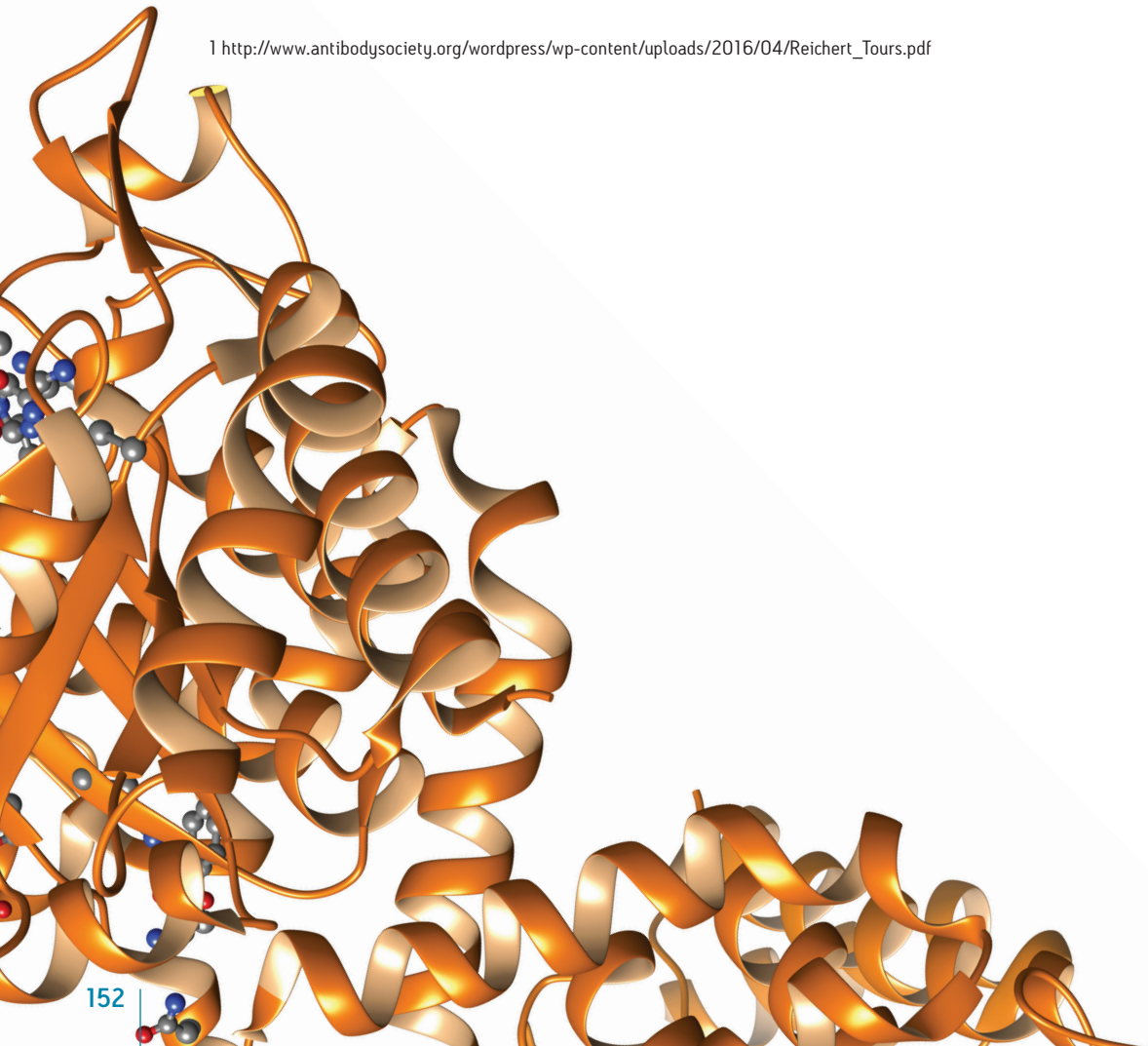
1. MRM-based multiplexed quantitation of 67 putative cardiovascular disease biomarkers in human plasma. *Domanski et al.* Proteomics. 2012 Apr;12(8):1222-43.
2. Advances in Quadrupole and Time-of-Flight Mass Spectrometry for Peptide MRM based Translational Research Analysis. *Mbasu et al*. Proteomics. 2016 May 23. doi: 10.1002/pmic.201500500. [Epub ahead of print]

Protein Bioanalysis

Biopharmaceutical drug development continues to experience rapid growth, with more than 600 biologics, including 50 monoclonal antibodies (mAbs) approved by the FDA and EU. There are no signs that this trend will abate, evidenced by the >480 mAbs in clinical trials.[1] Mastering peptide bioanalysis certainly prepares the analyst for moving into quantification of larger molecules like protein biomarkers and therapeutics because many of the same challenges observed in peptide analysis relative to small molecule quantification remain, such as multiple precursor charge states, increased fragmentation, and higher background noise due to endogenous peptides and proteins. When tackling proteins >10kD, new considerations arise, starting with the optimization of all the permutations of reagents, temperature, and digestion time.

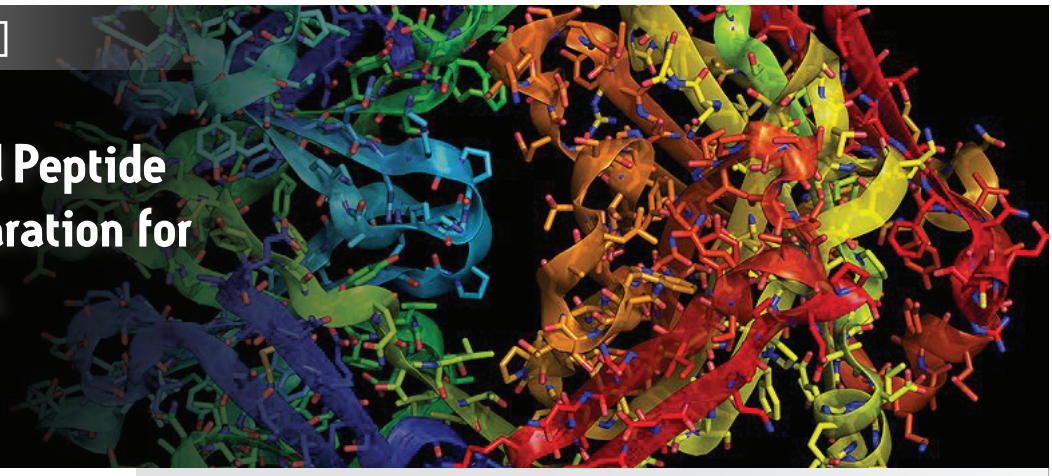
Additional aspects to consider include the mass range of the quadrupole, how to optimize and standardize quantification using the surrogate peptide approach, and options for sample clean-up at the protein and peptide levels. The following section contains a comprehensive collection of application notes focused on the quantification of proteins, many of which are specific to the surrogate peptide method for a range of monoclonal antibody therapeutics currently on the market, and some of which are coming off-patent. These methods draw on Waters scientists' extensive awareness of the key challenges that drug developers and bioanalysts face today.

1 http://www.antibodysociety.org/wordpress/wp-content/uploads/2016/04/Reichert_Tours.pdf



[TECHNOLOGY BRIEF]

Generic Protein and Peptide Level Sample Preparation for Protein Bioanalysis



GOAL

To evaluate the impact of various degrees of sample preparation on the quantification of therapeutic or diagnostic antibodies in plasma.

BACKGROUND

Increasingly, new drugs in development are based on large biomolecules such as peptides and proteins as opposed to traditional small molecule entities. This shift to therapeutic "biopharmaceuticals" has led to an increased need for appropriate bioanalytical strategies. Traditional ligand binding assays (LBA) such as ELISA require individual reagents that can take up to six months to develop, often at significant expense. By contrast, LC-MS/MS assays have the advantage of short development times, the ability to multiplex different analytes, and are based upon a well established technology with which many investigators are comfortable. A significant challenge, however, is the complexity of the plasma proteome and the fact that many biotherapeutics are present at concentrations many orders of magnitude lower than the most abundant, endogenous plasma proteins. These analytical challenges make the need for appropriate sample preparation paramount. Figure 1 shows the typical steps in common protein bioanalysis workflows, including cleanup at the protein level using techniques such as affinity purification or depletion plates, and post-digest peptide level cleanup options such as SPE.

Answers these questions: What is the industry pressure that requires this application? Why is this specific analytical capability needed?

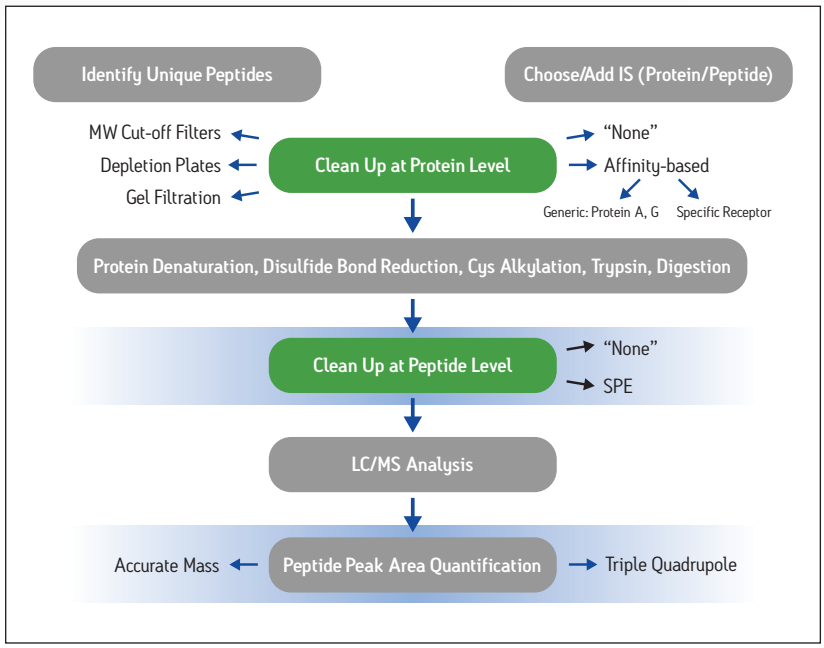


Figure 1. Common steps in the protein bioanalysis workflow. For this specific example, protein A cleanup was followed by trypsin digestion and mixed mode SPE cleanup.

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

THE SOLUTION

To demonstrate the advantages of appropriate sample preparation, the therapeutic monoclonal antibody trastuzumab was analyzed in human plasma. Two parallel strategies were used. In one scheme, 96-well protein A purification plates were used to isolate the IgG fraction (including trastuzumab) from plasma samples. In a second scheme, no protein level cleanup was performed. Each set of samples was then denatured with *Rapi*Gest™, reduced, alkylated and digested with trypsin to yield peptide fragments suitable for LC-MS/MS analysis. These resulting digests were then split into two aliquots. One aliquot was directly analyzed by LC-MS/MS while a second was further cleaned up using Oasis® MCX µElution plates prior to LC-MS/MS analysis.

Figure 2 demonstrates the differences and incremental benefits achieved through various degrees of sample preparation. The sample in Figure 2A was a direct digestion of the plasma, followed by direct injection of the subsequent digest. There was no sample cleanup performed at either the protein or peptide level. The resultant chromatogram yields a poor intensity peak which co-elutes with closely related interferences, rendering accurate quantification challenging and yielding a poor LLOQ. The sample in Figure 2B was cleaned up at the protein level only using protein A 96-well plates. This type of sample prep is generic enough to be applied in any workflow where the drug is an IgG-based antibody, making it ideal for a discovery setting. Furthermore, the 96-well format is also compatible with high-throughput analysis. The increase in peak area for the signature peptide relative to no protein-level isolation (Figure 2A) is nearly 5 fold. The chromatogram in Figure 2C represents a sample which has been cleaned up both at the protein level with protein A and at the peptide level with mixed-mode cation exchange SPE. The incremental benefit over protein A cleanup alone (Figure 2B) is additional 2 fold increase in peak area and an elimination of adjacent peaks, increasing specificity of the assay. Mixed-mode SPE cleanup not only removes interfering peptides, but also removes digest reagents, buffers, and other plasma components such as phospholipids, improving instrument robustness and data quality. Protein A sample preparation also eliminates high abundance plasma proteins such as albumin and transferrin, which reduces the required SPE capacity and facilitates the use of the µElution plate format. The major benefit of this SPE format is concentration of the sample without evaporation, thus minimizing peptide losses due to adsorption. Although not tested here, mixed-mode SPE can also be used to purify signature peptides from direct plasma digests (without protein-level cleanup). However, a larger bed mass 96-well plate may be required to accommodate the same sample volume due to the increased concentration of undesired peptides. An additional advantage to the use of generic protein level isolation, such as protein A, is that it reduces the required amount of trypsin by almost 10X, significantly reducing the assay cost.

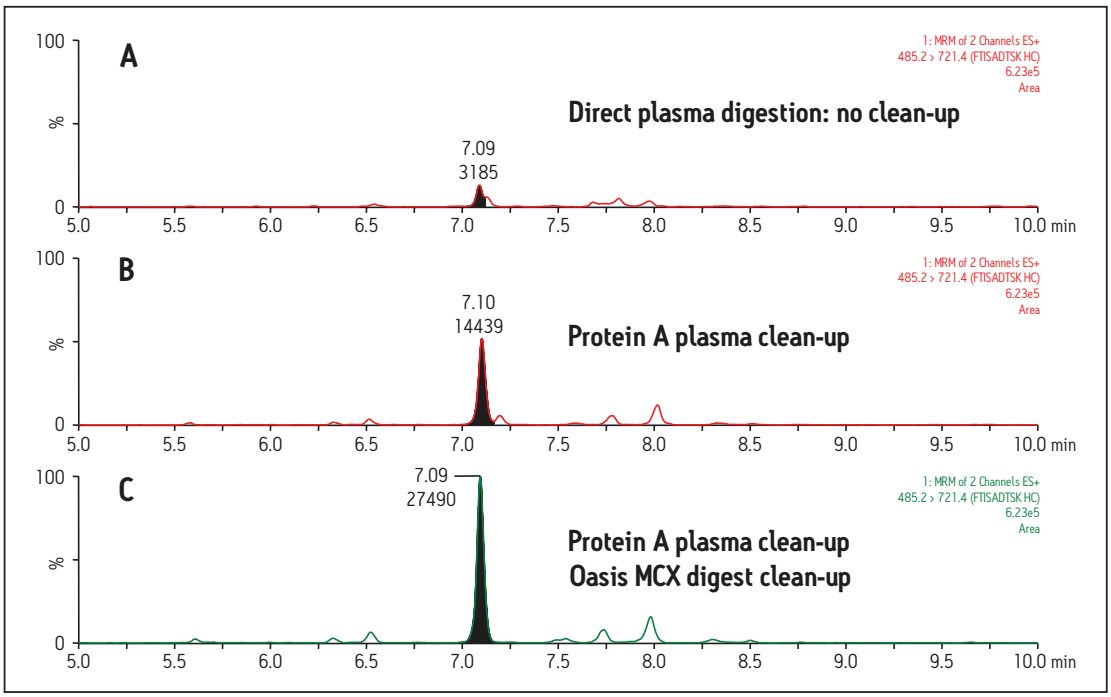


Figure 2. Comparison of sample preparation options for protein quantification.

SUMMARY

- Generic protein-level cleanup, such as protein A, significantly reduces sample complexity by eliminating nearly 85% of endogenous proteins, improving detection limits by 5X in this assay. Protein A fractionation also minimizes assay cost by reducing the amount of trypsin required.
- Oasis mixed mode MCX µElution plates can be used to further improve sensitivity, specificity, and assay and system robustness for the bioanalysis of therapeutic proteins.
  - The mixed-mode sorbent and µElution format enables the selective cleanup and concentration of specific signature peptides without evaporation, minimizing potential losses of desired peptides due to adsorption.
  - SPE of protein digests also eliminates digest reagents, buffers, and other plasma components such as phospholipids.
  - In conjunction with other Oasis sorbents (WCX, MAX, WAX, and HLB), the technique is generic enough to enable cleanup of a wide variety of therapeutic or diagnostic proteins after digestion in both the drug discovery and development settings.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Produced in the U.S.A. August 2012 720004442EN IH-PDF

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Advantages of Online Two-Dimensional Chromatography for MRM Quantification of Therapeutic Monoclonal Antibodies in Serum

Catalin Doneanu, Paul Rainville, and Robert S. Plumb
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The 2D LC/MRM assay can provide up to a three-fold increase in sensitivity compared to 1D LC/MRM. High pH/low pH RP/RP 2D LC separations can significantly reduce analyte suppression in protein bioanalysis.

WATERS SOLUTIONS

ACQUITY UPLC® System with 2D Technology

Oasis® MCX SPE 96-well Plate

ACQUITY UPLC BEH130 C_{18} Column

Xevo® TQ-S Mass Spectrometer

KEY WORDS

Trastuzumab, 2D LC/MRM, high pH/low pH RP/RP separation, two-dimensional chromatography, serum digest

INTRODUCTION

Quantification of therapeutic proteins in serum without analyte pre-fractionation can offer some advantages in terms of reducing the assay costs and simplifying the sample preparation workflow. Analyte isolation (typically performed by immunoaffinity) requires additional purification steps and uses expensive isotopically labeled protein standards to account for analyte recovery.

An alternative approach is the use of an LC system with greater chromatographic resolution, such as multi-dimensional LC. The multiple reaction monitoring (MRM) assays designed for measuring protein therapeutics in complex serum digests produced without analyte fractionation can be enhanced by multi-dimensional chromatography.

In particular, two-dimensional reversed phase/reversed phase (RP/RP) chromatography has been of significant interest in the bioanalysis community in recent years.[1-5] The major driving force behind the adoption of 2D-chromatographic methods is demonstrated improvement in the separation of the analyte of interest from other sample components in order to reduce suppression of the analyte signal. 2D-chromatographic techniques are responsible for increased sensitivities compared to one-dimensional LC methods using the same amount of sample.

Trastuzumab (herceptin) is a humanized IgG1 *kappa* monoclonal antibody (mAb). The antibody was obtained through genetic engineering[6,7] by joining the constant regions of the human monoclonal antibody with the complementarity-determining regions (CDRs) of a mouse monoclonal antibody able to bind human epidermal growth factor receptor 2 proteins (HER2) receptors. These HER2 receptors belong to a family of human oncoproteins expressed in approximately 25% of invasive breast cancers. Trastuzumab was approved in 1998 by the U.S. Food and Drug Administration (FDA) for the treatment of HER2-overexpressing breast cancers. Trastuzumab is administered by intravenous infusions in clinical doses to produce saturation of the HER2 receptor. For a conventional 4 mg/kg loading dose, followed by 2 mg/kg weekly doses, the mean maximum concentration of trastuzumab in plasma of 22 patients was approximately 70 µg/mL.[8]

In this application note, we report the development of a highly sensitive 2D LC/MRM assay for trastuzumab in human serum, employing gradient separation at pH 10.0 in the first RP chromatographic dimension, followed by gradient RP separation at pH 2.5 in the second dimension. We demonstrate that two-dimensional high pH/low pH RP/RP chromatography is able to significantly reduce ion suppression in protein bioanalysis.

EXPERIMENTAL

Sample Description

A stock solution of trastuzumab (150 kDa monoclonal antibody) was spiked with the internal standard ($^{13}C^{15}N$-isotopically labeled extended peptide GRFTISADTSK), and digested using trypsin to produce a stock solution containing 5 µM of digested trastuzumab and 10 µM of internal standard peptide (FTISADTSK). In parallel, 400 µL of human serum was dispensed in 10 Eppendorf vials (40-µL serum/vial), and digested using trypsin following the same procedure, to produce 200 µL of human serum digest in each vial. The digestion protocol involved sample denaturation (with 0.05% RapiGest at 80 °C for 10 min), disulfide bond reduction (in the presence of 20 mM dithiothreitol (DTT) for 60 min at 60 °C), cysteine alkylation (10 mM iodoacetamide (IAM) for 30 min at room temperature in the dark), and overnight digestion using porcine trypsin (25:1 (w/w) protein to enzyme ratio). Following digestion, half of the sample (100 µL of peptide digest) was diluted 1:1 with a solution containing 4% H_3PO_4, then loaded onto an Oasis mixed-mode SPE Elution Plate (10 mg/well of 30 µm MCX particles p/n 186000259). Digests were washed with 500 µL of 2% FA and 500 µL of 5% methanol before being eluted with 2 x 200 µL aliquots of 25% ACN in 1.5% NH_4OH (pH 10).

Matrix-free digests were prepared by diluting the 5-µM trastuzumab digest with 20 mM ammonium formate (pH 10) to prepare the following concentrations: 0.1, 1.0, 5.0, and 50.0 nM.

The same trastuzumab concentrations (0.1, 1.0, 5.0, and 50.0 nM) were prepared in 20 mM ammonium formate (pH 10) by diluting the stock trastuzumab digest in the human serum digest (using more than 80% of the serum digest matrix for each dilution).

LC/MS conditions

1D LC/MRM

An ACQUITY UPLC I-Class System equipped with an ACQUITY UPLC BEH300 C_{18} 2.1 x 150 mm, 1.7 µm Column (p/n 180003687) was used. The column temperature was maintained at 35 °C, and the flow rate was 0.3 mL/min. Mobile phases contained 0.1% (v/v) formic acid (FA) in water (A) and 0.1% (v/v) FA in acetonitrile (B). Peptides were eluted with a linear gradient from 0% to 35% B in 10 min.

2D LC/MRM

An ACQUITY UPLC H-Class System with 2D Technology configured in the heart-cutting mode was used for two-dimensional chromatography. Peptide separations were performed by RP/RP chromatography using the pH of the mobile phases to change the selectivity of the separation.[9,10] A diagram of the 2D-LC system is shown in Figure 1. The first dimension separation was performed on a 1.0 x 50 mm column packed with 2.5-µm XBridge™ $C_{18\ pa}$ particles, kept at 35 °C, and operated at 100 µL/min. Mobile phases contained 20 mM ammonium formate in water, pH 10.0 (Solvent A), and 20 mM ammonium formate in 90% ACN (Solvent B). Peptides were eluted with a linear gradient from 0% to 40% B in 5 min, as shown in Figure 1A. The analyte of interest (FTISADTSK peptide from trastuzumab), eluting between 6.6 and 6.9 min in the first chromatographic dimension, was transferred to the second chromatographic dimension using a switching valve, as shown in Figure 1B. Shortly after analyte transfer (~ 0.1 min later), the gradient for the second chromatographic separation began, as shown in Figure 1C. This separation was performed on a 2.1 x 50 mm column, packed with BEH300 1.7-µm particles, maintained at 35 °C, and operated at 300 µL/min. Mobile phases for low pH separations contained 0.1% formic acid (FA) in water (Eluent A) and 0.1% FA in ACN (Eluent B). Peptides were eluted with a linear gradient from 0% to 30% B in 5 min (gradient started 7 min after sample injection on the first dimension). The total run time for the entire 2D LC method was 15 min.

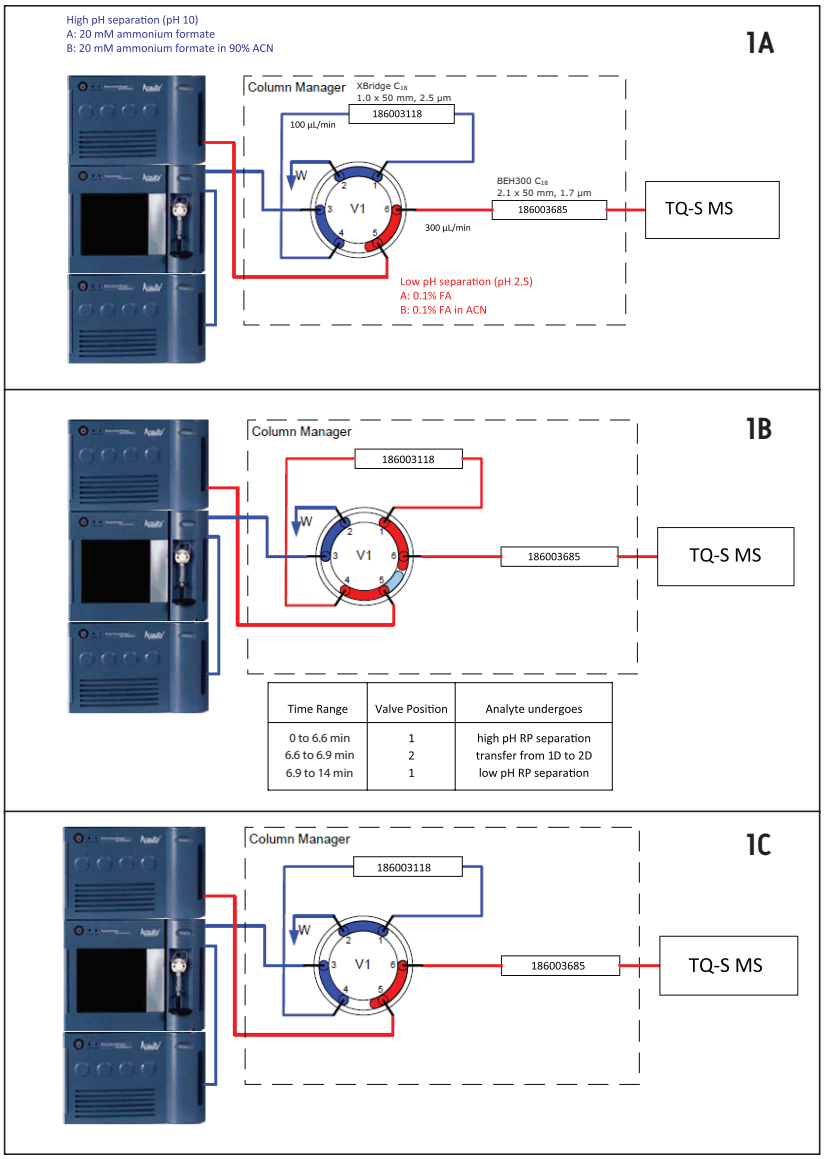


Figure 1. Heart-cutting configuration for two-dimensional chromatography: (A) Sample loading and first dimension separation under basic conditions (pH 10.0); (B) Analyte transfer from the first dimension to the second dimension; (C) Separation in the second dimension under acidic conditions at pH 2.5.

Mass Spectrometry

Assays were performed on a Xevo TQ-S Mass Spectrometer operated in MRM positive ion electrospray mode. The operating parameters were as follows: ESI potential 3.5 kV, CV 28 V, source temperature 120 °C, MS1/MS2 isolation window 0.75 Da (FWHM), 28 eV collision energy. Four MRM transitions were monitored continuously throughout the 2D LC/MRM assay using a dwell time of 100 ms: two MRMs monitored the endogenous signature peptide FTISADTSK from trastuzumab (485.2 → 721.4 for peptide quantification; 485.2 → 608.3 for peptide confirmation), while the other two MRM channels monitored the corresponding 13C15N-isotopically labeled internal standard peptide FTISADTSK (489.2 → 729.4 for peptide quantification; 489.2 → 616.3 for peptide confirmation). In addition to the assays designed for MRM monitoring only, several experiments were performed with RADAR and MRM monitoring. The scans for RADAR monitoring were performed in positive ESI mode over a mass range of m/z = 400 to 1400 with 1 s scans.

RESULTS AND DISCUSSION

Figure 2 shows the 2D LC/MRM chromatograms of FTISADTSK, the signature peptide from trastuzumab, recorded at four digest concentrations in the range of 0.1 to 50.0 nM. The digest was diluted with 20 mM ammonium formate (pH=10.0) in the absence of serum digest matrix. The assay linearity over the dynamic range investigated (500-fold) is clearly demonstrated by the peak areas displayed in Figure 2. The reproducibility of the 2D assay is shown in Figure 3A where peak areas were recorded for replicate injections (n=4) of the digest containing 5 nM trastuzumab and 10 nN $^{13}C^{15}N$-isotopically labeled peptide standard. The overall assay reproducibility was also very good, with the average peak area RSD for all concentrations tested greater than 2%, when four replicate injections were performed for each trastuzumab concentration.

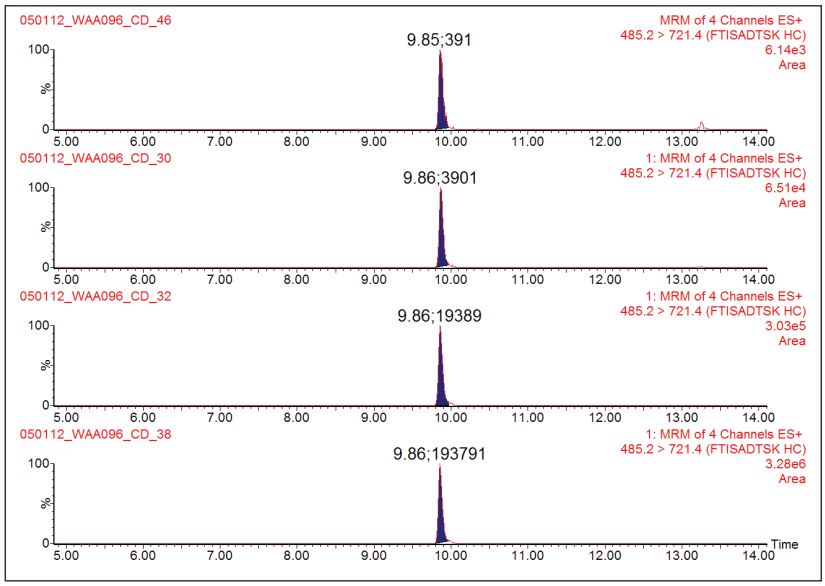


Figure 2. 2D LC/MRM chromatograms of the trastuzumab peptide FTISADTSK showing the linearity of the assay. Four digest concentrations covering a dynamic range of 500 fold (0.1 to 50.0 nM trastuzumab) were prepared in 20 mM ammonium formate (pH 10) and analyzed in replicate (n=4). The RSD for this experiment was greater than 2%.

The 2D LC/MRM assay, in the absence of the serum digest matrix, is very sensitive, with the lowest detected trastuzumab concentration determined to be 0.1 nM or 15.0 ng/mL, which is at minimum three orders of magnitude lower than the mean maximum trastuzumab concentration of 70 µg/mL measured in patients' plasma.[8]

The sensitivity of the MRM assay was also investigated in the presence of the complex serum digest matrix. Trastuzumab digests were spiked in SPE-cleaned human serum digests and analyzed by 2D LC/MRM, as shown in Figure 3B. This figure reveals that the signals corresponding to the native FTISADTSK peptide, as well as the signals produced by its isotopicallly labeled analogue, were clearly suppressed by co-eluting compounds from serum digest. However, the ratio between the native peptide and the isotopically labeled peptide was not affected by the complex matrix. This is an important observation, as the quantification method is based on comparing the peak area obtained for an unknown trastuzumab concentration to the peak area produced by the IS peptide spiked at a known concentration in the serum sample. Peak area RSDs were greater than 5%, as shown in these chromatograms in Figure 3B. In conclusion, in the 2D LC/MRM experiment, the analyte/IS suppression due to the complex serum digest background was only ~25%.

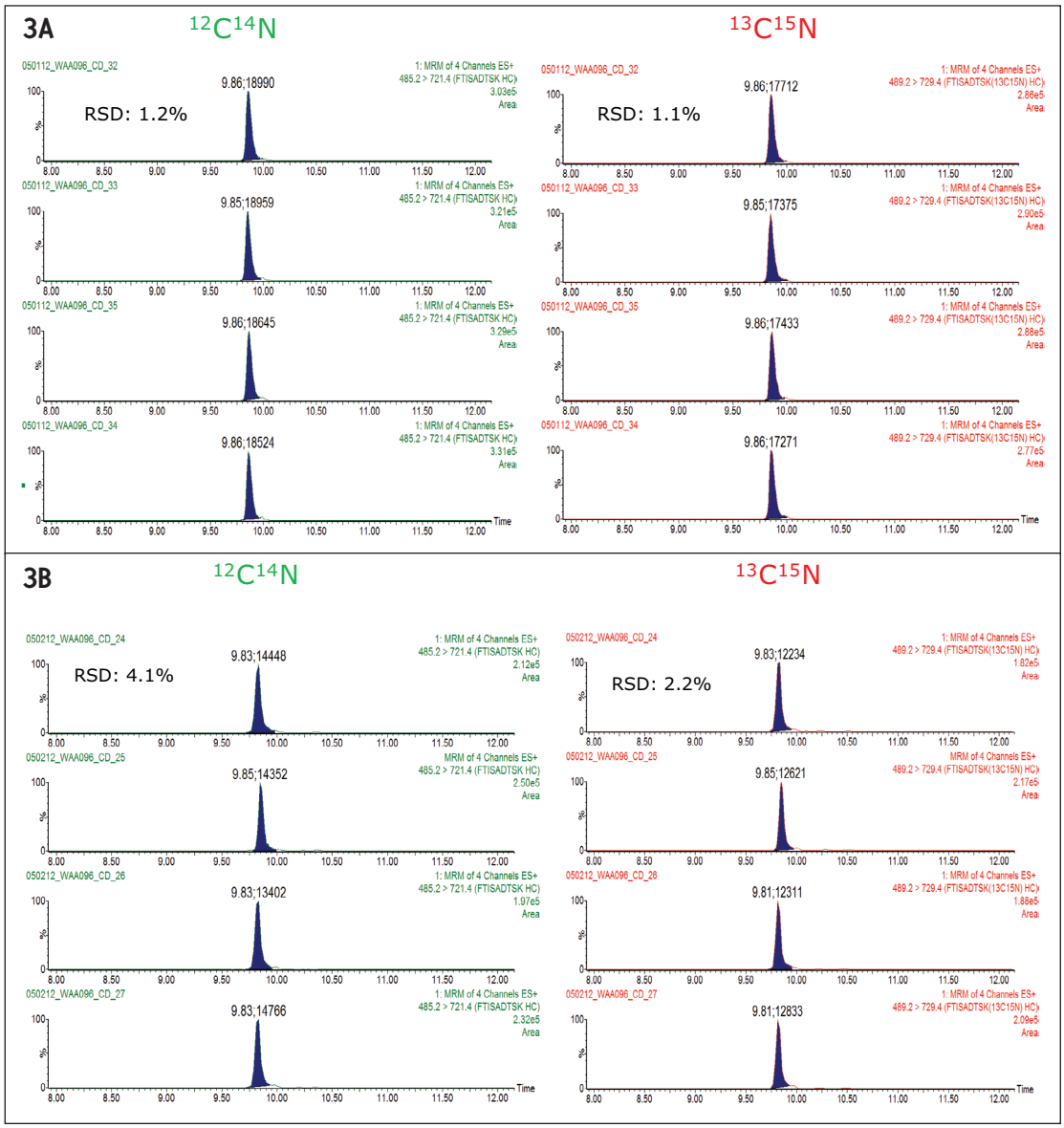


Figure 3. Reproducibility of the 2D LC/MRM assay: (A) in the absence of the serum digest background; (B) in the presence of the serum digest matrix. Figure 3A shows a sample containing 5 nM trastuzumab and 10 nM $^{13}C^{15}N$ labeled peptide prepared in 20 mM ammonium formate (pH 10). Figure 3B shows the sample containing 5 nM trastuzumab and 10 nM $^{13}C^{15}N$ peptide spiked into SPE-cleaned human serum digest.

When the trastuzumab digests spiked in SPE-cleaned human serum were analyzed by 1D LC/MRM, the analyte suppression was four-fold higher, as illustrated by the graphs shown Figure 4. The average peak areas of the native and isotopically labeled peptide IS are displayed in this figure for 1D- and 2D-MRM experiments performed for 5 nM trastuzumab digest prepared in neat solvent (20 mM ammonium formate, pH=10.0), as well as in SPE-cleaned human serum. These results clearly indicate that, relative to the quantification of therapeutic proteins in serum, the 2D LC/MRM method can provide up to a three-fold increase in sensitivity compared to conventional 1D LC/MRM.

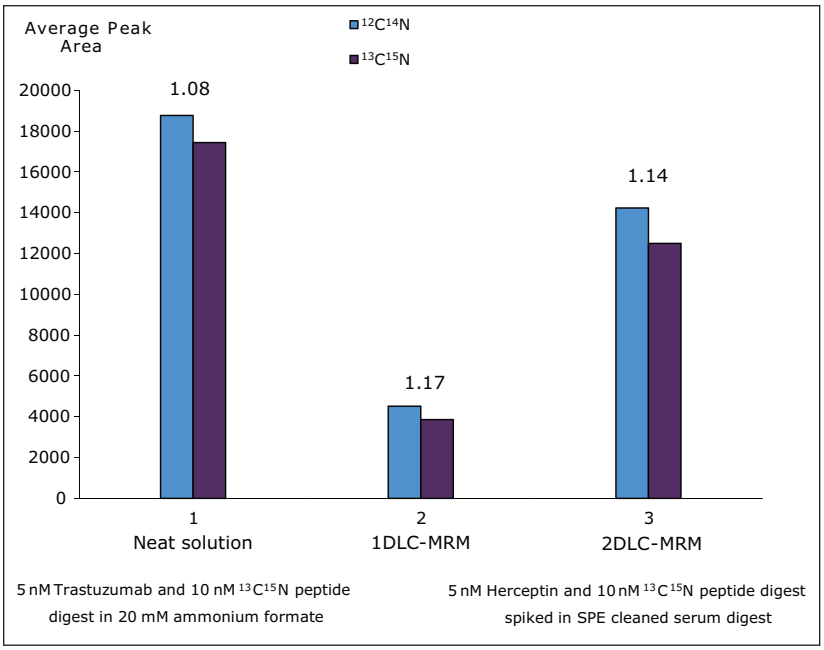


Figure 4. Evaluation of signal suppression. Comparison of average peak areas recorded for native and isotopically labeled trastuzumab peptide FTISADTSK in the absence and presence of the serum digest matrix. A sample containing 5 nM trastuzumab digest and 10 nM $^{13}C^{15}N$ labeled peptide was spiked into SPE-cleaned human serum digest.

The increase in sensitivity of the 2D LC/MRM method is usually explained by the ability of extensive chromatographic separations that remove some of the co-eluting compounds responsible for analyte/IS suppression. To verify this hypothesis, the presence of background peptides from the serum digest was monitored by full scan MS scans (RADAR scans) during 1D- and 2D-MRM separations. These RADAR scans were scheduled throughout the chromatographic run (1 s scans), and they were performed at the end of each MRM cycle containing the four transitions (50 ms each) used for trastuzumab quantification. The data collected during these experiments are illustrated in Figure 5. During the 1D LC/MRM run, the chromatogram recorded with full scan MS acquisitions (RADAR chromatogram), shown in Figure 5A, reveals the true complexity of the serum digest sample. The inset shows the MRM chromatograms corresponding to the native/IS peptides. The signals of these peptides are significantly reduced (~four-fold) by the presence of the "heavy" peptide background contained in the SPE-cleaned sample. Figure 5B shows the RADAR chromatogram produced by the

2D high pH/low pH RP/RP chromatography with heart-cutting. The MRM chromatograms, shown in the inset, indicate that most of the background components that were co-eluting with the analyte in 1D chromatography no longer co-elute after two-dimensional chromatography. Data in Figure 5 provide a clear explanation for the increase in assay sensitivity observed in the 2D-MRM method.

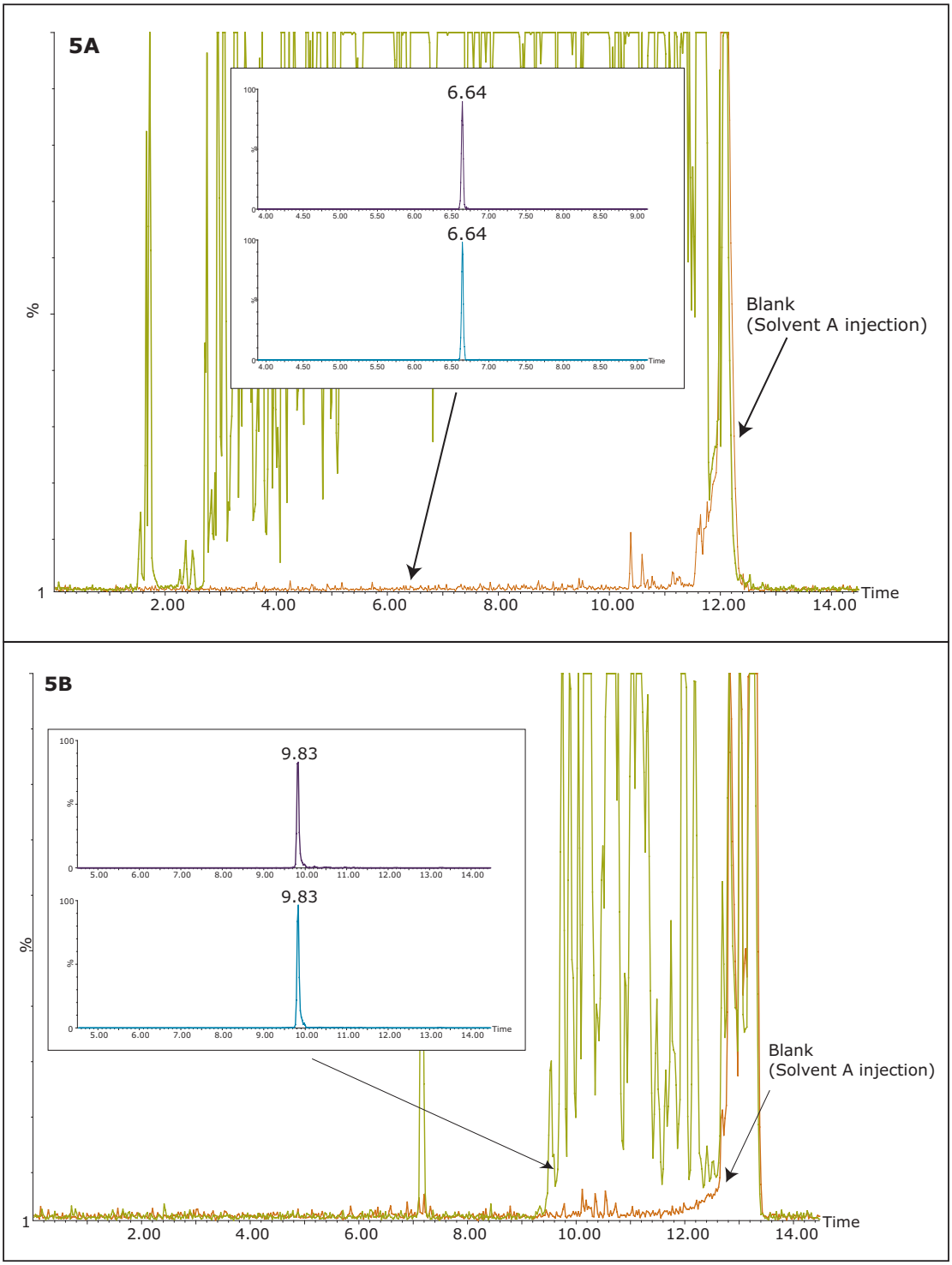


Figure 5. RADAR monitoring of SPE-cleaned samples containing 5 nM trastuzumab digest: (A) 1D LC/MRM separation under acidic conditions (pH 2.5); (B) 2D LC/MRM separation with heart cutting. The sample was prepared by spiking a trastuzumab digest (5 nM trastuzumab and 10 nM $^{13}C^{15}N$ labeled peptide) into SPE-cleaned human serum digest.

CONCLUSIONS

- A high sensitivity 2D LC/MRM method has been tested for the analysis of trastuzumab in human plasma digest using a signature peptide.
- The ratio of 12C/13C peptides was not significantly affected by the serum matrix.
- High pH/low pH RP/RP 2D LC separation can significantly reduce analyte suppression in protein bioanalysis.
- The 2D LC/MRM assay can provide up to a three-fold increase in sensitivity compared to 1D LC/MRM.

References

1. Rogatsky E, Tomuta V, Cruikshank G, Vele L, Jayatillake H, Stein D. Direct sensitive quantitative lC/MS analysis of C-peptide from human urine by two dimensional reverse phase/reverse phase high-performance liquid chromatography. *J Sep Sci.* 2006; 29(4):529-37.
2. Dowell JA, Heyden WV, Li L. Rat neuropeptidomics by LC-MS/MS and MALDI-FTMS: Enhanced dissection and extraction techniques coupled with 2D RP-RP HPLC. *J Proteome Res.* 2006; 5(12): 3368-75.
3. Zhang G, Zhang Y, Fast DM, Lin Z, Steenwyk R. Ultra sensitive quantitation of endogenous oxytocin in rat and human plasma using a two-dimensional liquid chromatography-tandem mass spectrometry assay. *Anal Biochem.* 2011;416(1): 45-52.
4. Rao RN, Kumar KN, Shinde DD. Determination of rat plasma levels of sertraline enantiomers using direct injection with achiral-chiral column switching by LC-ESI/MS/MS. *J Pharm Biomed Anal.* 2010; 52(3): 398-405.
5. Rao RN, Vali RM, Shinde DD. On-line 2D-LC-ESI/MS/MS determination of rifaximin in rat serum. *Biomed Chromatogr.* 2009; 23(11): 1145-50.
6. Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME, Shepard HM. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci USA.* 1992;15:89(10): 4285-9.
7. Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA, Ullrich A. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. *Cancer Res.* 1990; 50(5):1550-8.
8. Storniolo AM, Pegram MD, Overmoyer B, *et al*. Phase I dose escalation and pharmacokinetic study of lapatinib in combination with trastuzumab in patients with advanced ErbB2-positive breast cancer. *J Clin Oncol.* 2008;10:26(20): 3317-23.
9. Gilar M, Olivova P, Daly AE, Gebler JC. Two-dimensional separation of peptides using RP-RP-HPLC system with different pH in first and second separation dimensions. *J Sep Sci.* 2005; 28(14):1694-703.
10. Doneanu CE, Xenopoulos A, Fadgen K, Murphy J, Skilton SJ, Prentice H, Stapels M, Chen W. Analysis of host-cell proteins in biotherapeutic proteins by comprehensive online two-dimensional liquid chromatography/mass spectrometry. *mAbs Journal.* 2012; 4(1): 24-44.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Optimization of Trypsin Digestion for MRM Quantification of Therapeutic Proteins in Serum

Catalin Doneanu, Hua Yang, Paul Rainville, Ed Bouvier, and Robert Plumb
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The analytical workflow of trypsin digestion can be optimized to develop a successful multiple reaction monitoring (MRM) assay. Results include higher assay sensitivity, reduced sample preparation time, and higher assay reproducibility.

WATERS SOLUTIONS

ACQUITY UPLC® I-Class System

ACQUITY UPLC BEH130 C_{18} Column

Oasis® MCX SPE 96-Well Plate

Xevo® TQ-S Mass Spectrometer

KEY WORDS

Trastuzumab, LC-MRM, trypsin digestion, serum digest

INTRODUCTION

Mass spectrometry-based quantification of therapeutic proteins uses a surrogate peptide as a stoechiometric representative of the protein which is cleaved. One of the challenges encountered in the quantification of therapeutic proteins in biological fluids (for example serum, plasma, and urine) is to find a suitable internal standard for each protein analyte. Isotopically labeled protein standards have been used successfully for quantification of mAbs,[1-3] but these standards are expensive and time-consuming to produce.

Since protein quantification is performed at the peptide level, another alternative is to use relatively inexpensive isotopically labeled peptides as peptide IS.[4-7] To account for trypsin digestion efficiency, $^{13}C^{15}N$-isotopically labeled cleavable peptides (extended peptides) were introduced in 2004.[5]

Regardless of the nature of the IS, both quantification approaches rely on the digestion of the protein sample with a specific enzyme (such as trypsin, Lys C). Protein digestion has been recognized as the major source of variability in the analytical workflow[4-9] which has to be carefully optimized. In addition, when the whole digest approach is implemented, using peptide IS for quantification, there is an additional concern related to the ability of the digestion enzyme to cleave with the same efficiency as the therapeutic protein as well as the isotopically labeled peptide IS, in the presence of the biological matrix.

In this application, trypsin digestion optimization is required for developing a successful MRM assay. Generally, some of the benefits of trypsin digestion optimization include higher assay sensitivity, shorter sample preparation times, and higher assay reproducibility.

Here, we used a therapeutic mAb to investigate the trypsin digestion efficiency relative to the whole digest quantification method. Two digestion parameters, protein to trypsin ratio and digestion time, were optimized. Additionally, the reproducibility of the entire sample preparation protocol was assessed.

EXPERIMENTAL

LC Conditions

System:	ACQUITY UPLC I-Class
Column:	BEH300 C_{18} 2.1 x 150 mm, 1.7 µm
Column temp.:	35 °C
Flow rate:	0.3 mL/min
Mobile phase A:	0.1% (v/v) formic acid (FA) in water
Mobile phase B:	0.1% (v/v) FA in acetonitrile
Linear gradient:	0% to 35% B in 10 min
Run time:	15 min

MS Conditions

Mass Spectrometer:	Xevo TQ-S
Mode:	MRM positive ion electrospray
ESI potential:	3.5 kV
Cone voltage:	28 V
Source temp.:	120 °C
MS1/MS2 isolation window:	0.75 Da (FWHM)
Collision energy:	28 eV

Four MRM transitions were monitored continuously throughout the LC-MRM assay using a dwell time of 50 ms: two MRMs monitored the endogenous signature peptide FTISADTSK from trastuzumab (485.2 → 721.4 for peptide quantification; 485.2 → 608.3 for peptide confirmation), while the other two MRM channels monitored the corresponding $^{13}C^{15}N$-isotopically labeled internal standard peptide FTISADTSK (489.2 → 729.4 for peptide quantification; 489.2 → 616.3 for peptide confirmation).

Trastuzumab (herceptin) is a humanized IgG1 *kappa* monoclonal antibody (mAb). The antibody was produced through genetic engineering[10,11] by joining the constant regions of the human monoclonal antibody with the complementarity-determining regions (CDRs) of a mouse monoclonal antibody able to bind human epidermal growth factor receptor 2 proteins (HER2) receptors. These HER2 receptors belong to a family of human oncoproteins expressed in approximately 25% of invasive breast cancers. Trastuzumab was approved in 1998 by the U.S. Food and Drug Administration (FDA) for the treatment of HER2-overexpressing breast cancers.

In this application note, we optimized the trypsin digestion step for the development of a sensitive LC-MRM assay for trastuzumab in human serum.

Sample Description

Aliquots containing 40 µL of human serum were dispensed in Low-Bind Eppendorf vials and digested with trypsin, following the procedure described below, to produce 200 µL of human serum digest per vial. As shown in Figure 1, the digestion protocol involved sample denaturation (with 0.05% RapiGest at 80 °C for 10 min), disulfide bond reduction (in the presence of 20 mM dithiothreitol (DTT) for 60 min at 60 °C), and cysteine alkylation (with 10 mM iodoacetamide (IAM) for 30 min at room temperature in the dark). After adding the internal standard peptide (the $^{13}C^{15}N$-isotopically labeled extended peptide GRFTISADTSK), samples were digested with porcine trypsin (Sigma catalog no T-6567) under different experimental conditions. In one experiment, the amount of trypsin was varied, so that the ratio between the substrate (total serum proteins) and the digestion enzyme could be altered. Five digestion ratios (10:1, 20:1, 30:1, 50:1, and 100:1) were tested. In another experiment, the substrate to enzyme ratio was kept constant (30:1), and the digestion time was varied. Aliquots were taken after 15 min, 30 min, 1 h, 3 h, 6 h, and 16 h (overnight). The digestion was stopped by adding TFA (2 µL), and samples were incubated for 30 min at 37 °C to decompose RapiGest. The supernatant was recovered from each sample, following centrifugation at 12,000 rpm (10 min), and 10 µL of sample was injected onto the LC/MS instrument.

RESULTS AND DISCUSSION

Figure 1 shows a general analytical workflow with no analyte pre-fractionation, developed for the quantification of therapeutic proteins in serum, and tested for the quantification of trastuzumab in human serum. After spiking the mAb in serum, the protein mixture was denatured, reduced, and alkylated, as indicated in steps 2 through 4 in Figure 1 (also see the Experimental section). The extended $^{13}C^{15}N$-isotopically labeled peptide GRFTISADTSK was spiked at a concentration of 100 nM, and digested with trypsin. The sample was diluted four-fold during digestion, making the final concentration of the peptide IS 25 nM. Trypsin is the preferred digestion enzyme for protein bioanalysis applications for several reasons including: it is easily available, well characterized, and has very reproducible cleavage sites (K/R).

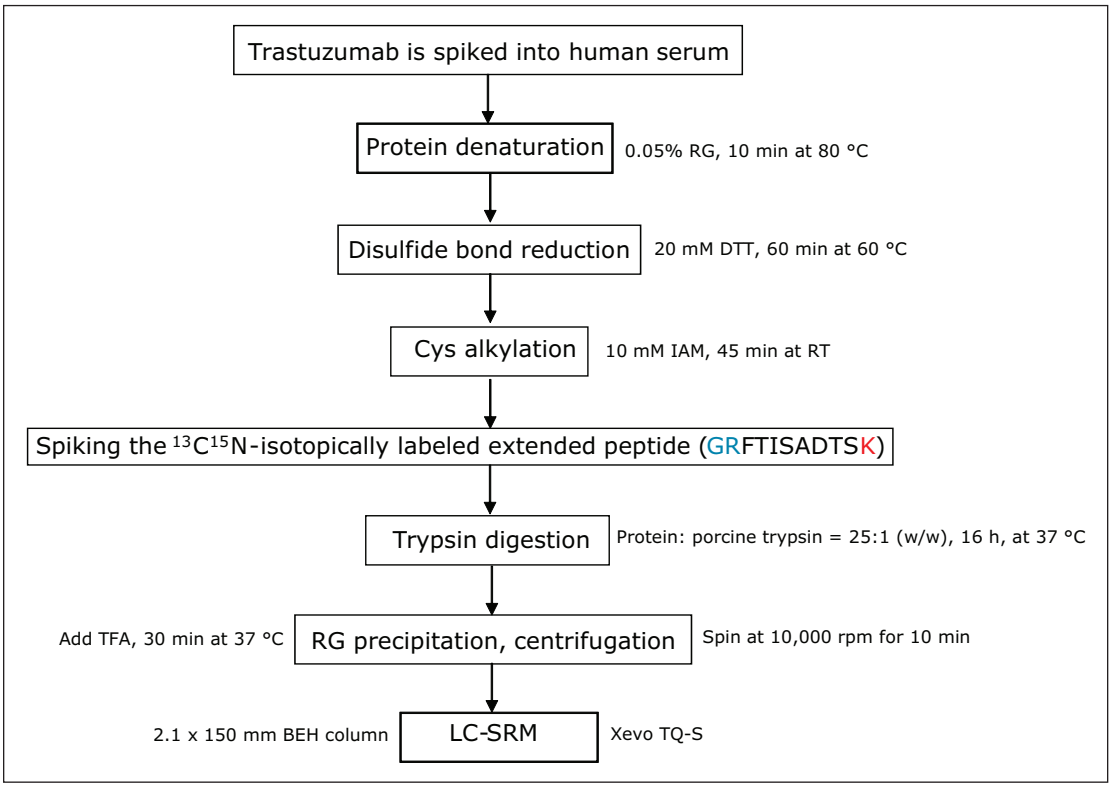


Figure 1. Analytical workflow for the quantification of therapeutic proteins involving no analyte fractionation (the whole digest approach).

Two digestion parameters were investigated including: protein to trypsin ratio and digestion time. In one experiment, the amount of trypsin was varied so that the ratio between the substrate (total serum proteins) and the digestion enzyme could be altered. Five digestion ratios (10:1, 20:1, 30:1, 50:1, and 100:1) were investigated with the results shown in Figure 2. Digestion efficiency was calculated from the normalized averaged peak areas (n=4) obtained from the MRM chromatograms of the native FTISADTSK peptide (originating from spiked trastuzumab, MRM transition: 485.2 → 721.4), and the corresponding isotopically labeled analogue (FTISADTSK, 489.2 → 729.4).

According to the data shown in Figure 2, for substrate to enzyme ratio of up to 30:1, there are no significant differences between the digestion of the analyte (trastuzumab) and the digestion of the peptide substrate (extended peptide IS). However, at ratios above 30, trypsin becomes more efficient at digesting the smaller substrate, to the detriment of the therapeutic protein. Clearly, ratios lower than 30:1 should be used to avoid quantification errors due to the incomplete digestion of the analytical target. A decrease in trypsin efficiency at low substrate to trypsin ratios (for example 10:1) is typically associated with a higher amount of trypsin auto-cleavage at the expense of protein substrate cleavage.

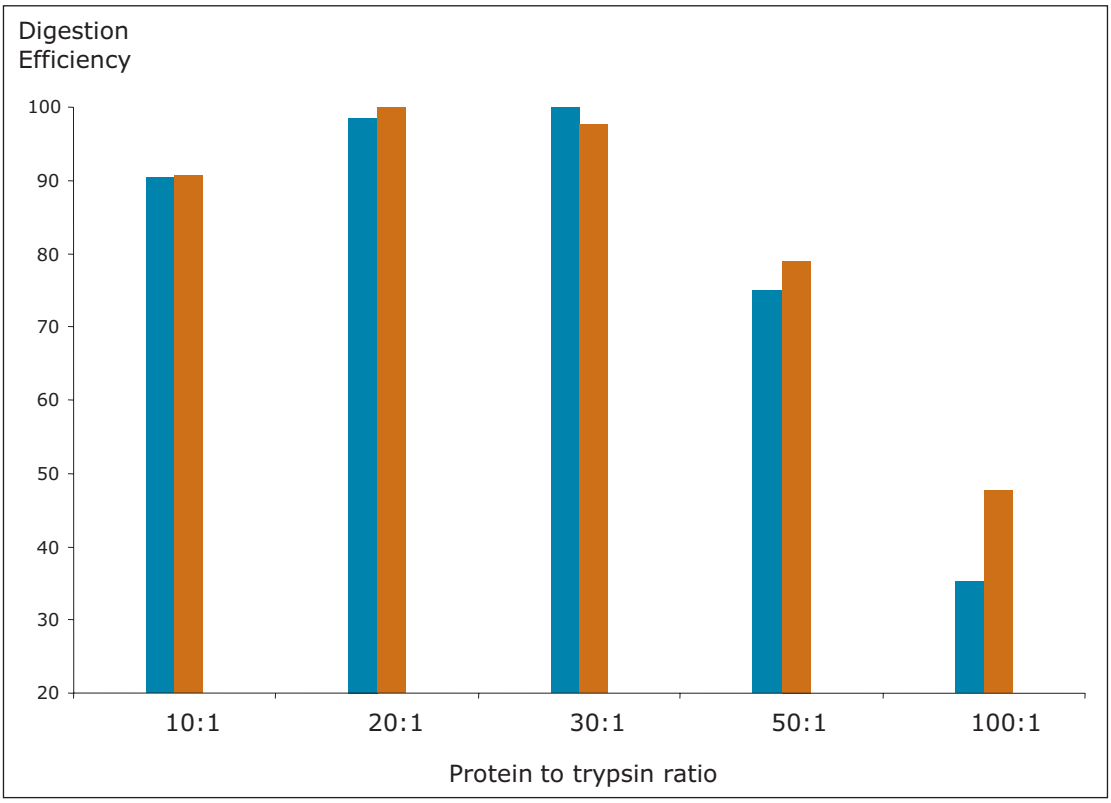


Figure 2. Protein to trypsin ratio optimization. Five digestion ratios (10:1, 20:1, 30:1, 50:1, and 100:1) were investigated for 25 nM spiked trastuzumab and 25 nM $^{13}C^{15}N$-isotopically labeled peptide digested in human serum. Samples were digested overnight (16 h) with porcine trypsin at 37 °C. Digestion efficiency was calculated from the normalized averaged peak areas (n=4) obtained from the MRM chromatograms of the native FTISADTSK peptide (originating from spiked trastuzumab, MRM transition: 485.2 → 721.4) and the corresponding isotopically labeled analogue (FTISADTSK, 489.2 → 729.4).

In addition to the substrate to trypsin ratio, the digestion time was evaluated. The substrate to enzyme ratio was kept constant (30:1) while the digestion time was varied. Aliquots were taken after certain digestion times [15 min, 30 min, 1 h, 3 h, 6 h, and 16 h (overnight)], and the digestion was stopped by adding TFA. Digestion efficiency was again calculated from the normalized averaged peak areas of FTISADTSK peptides, as shown in Figure 3. The optimum digestion time was 3 h with no signal increase observed after that point in time.

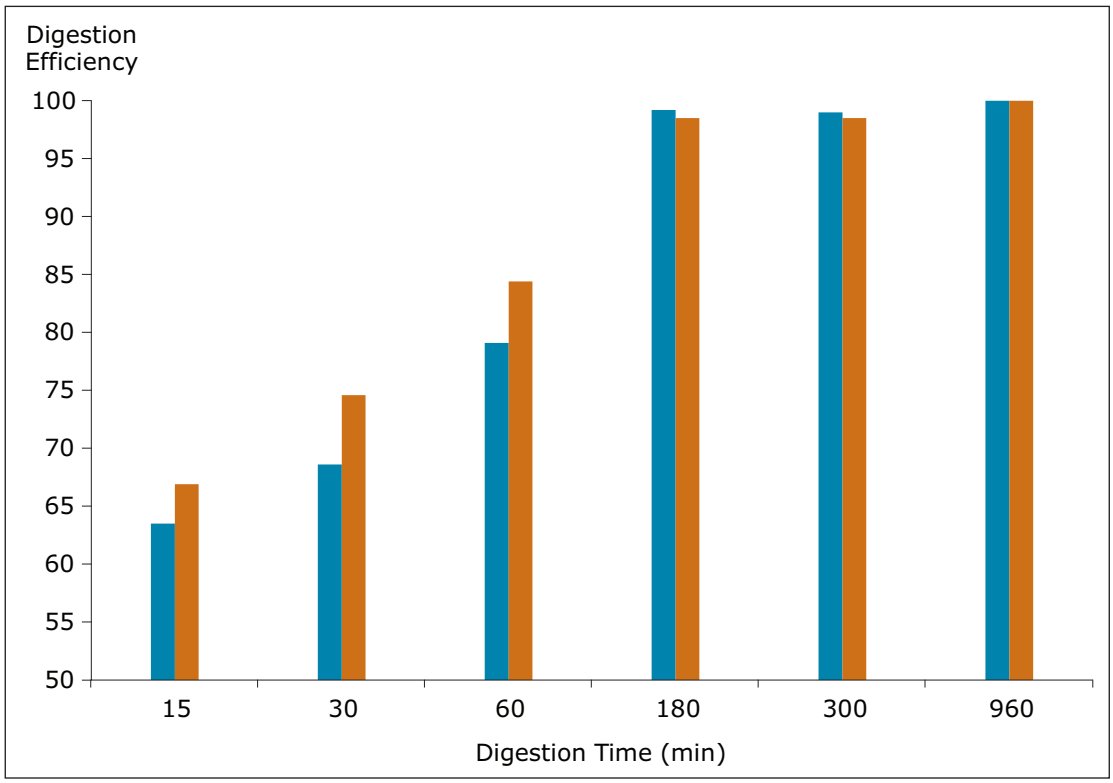


Figure 3. Effect of incubation time on digestion efficiency. Trastuzumab (25 nM) and $^{13}C^{15}N$-isotopically labeled peptide (25 nM) were digested in serum for 15 min, 30 min, 1 h, 3 h, 6 h, and 16 h (overnight) with porcine trypsin at 37 °C. The protein to trypsin ratio was 1:30. Digestion efficiency was calculated from the normalized averaged peak areas (n=4) obtained from the MRM chromatograms of the native FTISADTSK peptide (originating from spiked trastuzumab, MRM transition: 485.2 ⟶ 721.4), and the corresponding isotopically labeled analogue (FTISADTSK, 489.2 ⟶ 729.4).

As shown in Figure 3, the reproducibility of the entire analytical workflow was tested at a lower concentration: 5 nM trastuzumab spiked in human serum with 5 nM $^{13}C^{15}N$-isotopically labeled extended peptide added as an IS. Figure 4 shows the average peak areas obtained for five replicate digestions. While the peak area RSD was approximately 10% for individual MRM traces, the %RSD for the $^{12}C/^{13}C$ peptide ratio (used for quantification) was greater than 6%.

Even though the analyte trastuzumab and the peptide IS were spiked into human serum at the same concentration (5 nM), the ratio between the peak areas of the native peptide and the isotopically labeled peptide was approximately 2:1 because the therapeutic protein generates two peptide molecules for each molecule of digested mAb. Since the calculated $^{12}C/^{13}C$ peptide ratio is close to the expected value (2:1), it is clear that the trypsin digestion efficiency was minimally affected by the type of the substrate (150 kDa therapeutic mAb versus 1 kDa peptide IS). The whole digest approach can be employed for quantification of therapeutic proteins in serum.

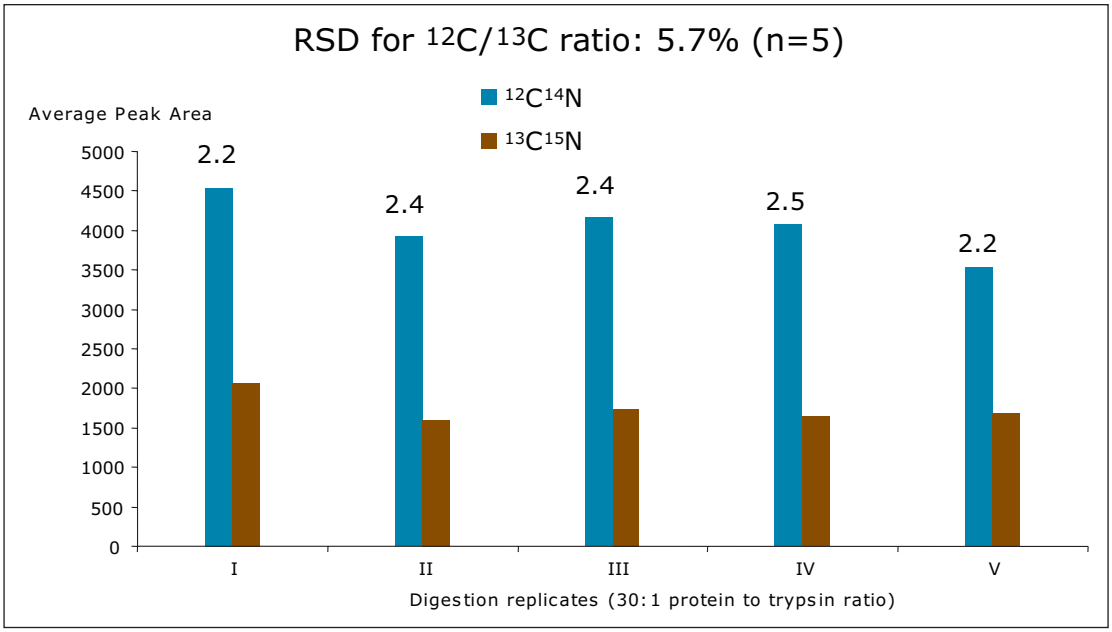


Figure 4. Reproducibility of trypsin digestion. Serum was spiked with 5 nM trastuzumab and 5 nM $^{13}C^{15}N$-isotopically labeled peptides, and digested overnight (16 h) with porcine trypsin at 37 °C. The %RSD for the $^{12}C/^{13}C$ peptide ratio was greater than 6%.

CONCLUSIONS

- A general workflow for the quantification of therapeutic proteins in serum without analyte pre-fractionation was tested to determine the quantification of trastuzumab in human serum.
- Trypsin digestion optimization can provide increased sensitivity, shorter sample preparation protocols, and higher assay reproducibility.
- The reproducibility of the entire sample preparation protocol was greater than 10%.
- Digestion efficiency was not significantly affected by the type of substrate (150 kDa therapeutic mAb versus peptide IS).

References

1. Heudi O, Barteau S, Zimmer D, Schmidt J, Bill K, Lehman N, Bauer C, Kretz O. Towards absolute quantification of therapeutic monoclonal antibody in serum by LC-MS/MS using isotope-labeled antibody standard and protein cleavage isotope dilution mass spectrometry. *Anal Chem.* 2008;80:4200.
2. Liu H, Manuilov A, Chumsae C, Babineau M, Tarcsa E. Quantitation of a recombinant monoclonal antibody in monkey serum by liquid chromatography-mass spectrometry. *Anal Biochem.* 2011;414:2011.
3. Li H, Ortiz R, Tran L, Hall M, Spahl C, Walker K, Laudemann J, Miller S, Salimi-Moosavi H, Lee JW. General LC-MS/MS method approach to quantify therapeutic monoclonal antibodies using a common whole antibody internal standard with application to preclinical studies. *Anal Chem.* 2012;84:1267.
4. Barr JR, Maggio VL, Paterson Jr DG, Cooper GR, Henderson LO, Turner WE, Smith SJ, Hannon WH, Needham LL, Sampson EJ. Isotope dilution – mass spectrometric quantification of specific proteins: model application with apolipoprotein A-I. *Clin Chem.* 1996;42:1676.
5. Barnidge DR, Hall GD, Stocker JL, Muddiman DC. Evaluation of a cleavable stable isotope labeled synthetic peptide for absolute protein quantification using LC-MS/MS. *J Proteome Res.* 2004;3:658.
6. Arsene C, Ohlendorf R, Burkitt W, Pritchard C, Henrion A, O'Connor D, Bunk D, Guttler B. Protein quantification by isotope dilution mass spectrometry of proteolytic fragments: cleavage rate and accuracy. *Anal Chem.* 2008;80:4154.
7. Hagman C, Ricke D, Ewert S, Bek S, Falchetto R, Bitsch F. Absolute quantification of monoclonal antibodies in biofluids by liquid chromatography-tandem mass spectrometry. *Anal Chem.* 2008;80:1290.
8. Lesur A, Varesio E, Hopfgartner. Accelerated tryptic digestion for the analysis of biopharmaceutical monoclonal antibodies in plasma by liquid chromatography with tandem mass spectrometric detection. *J Chrom A.* 2010;1217:57.
9. Xu Y, Mehl JT, Bakhtiar R, Woolf EJ. Immunoaffinity purification using anti-PEG antibody followed by two-dimensional liquid chromatography/tandem mass spectrometry for the quantification of a PEGylated therapeutic peptide in human plasma. *Anal Chem.* 2010;82:6877.
10. Carter P, Presta L, Gorman CM, et al. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci U S A.* 1992;89:4285.
11. Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA, Ullrich A. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. *Cancer Res.* 1990;50:1550.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

UPLC/MS/MS Method for the Quantification of Trastuzumab in Human Serum at the 5-nM Level Using Xevo TQD MS and ACQUITY UPLC H-Class System

Catalin Doneanu and Robert Plumb
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

This work demonstrates the capability of Xevo® TQD to conduct bioanalysis of large protein molecules, as trastuzumab, a monoclonal antibody. Oasis® micro-elution plates, the ACQUITY UPLC® H-Class System, and an advanced tandem quadrupole mass spectrometer (Xevo TQD) were used for the development of a sensitive method for the quantification of trastuzumab in human serum digest. This application note addresses some critical challenges in the world of bioanalysis, such as developing a method to quantify protein molecules with desired sensitivity in a robust, reproducible, high-throughput LC/MS/MS system.

WATERS SOLUTIONS

Oasis MCX micro-elution plates

ACQUITY UPLC H-Class System

ACQUITY® BEH130 C_{18} column

Xevo TQD Mass Spectrometer

KEY WORDS

Trastuzumab, high-sensitivity, robust, high-throughput, breast cancer, HER2 receptor

INTRODUCTION

Monoclonal antibodies (mAbs) comprise a new group of therapeutic compounds, which are used to treat cancer, auto-immune diseases, and systemic infections.[1] HER2/neu receptor is over-expressed in about 25% of all breast cancer patients.[2,3] Trastuzumab, as shown in Figure 1, directed against the HER2/neu receptor, is primarily used to treat certain breast cancers. It selectively binds with high affinity to the extracellular domain of the human epidermal growth factor receptor 2 protein, HER2. Trastuzumab is produced by recombinant DNA technology in a mammalian cell (Chinese hamster ovary) culture containing the gentamicin antibiotic. Trastuzumab is administered by intravenous infusions of 10 to 500 mg once every week. The compound demonstrated dose-dependent pharmacokinetics, with an average half-life of two and 12 days at the 10- and 500-mg dose levels, respectively. The volume of distribution of trastuzumab was approximately that of serum volume (44 mL/kg). At the highest weekly dose studied (500 mg), mean peak serum concentrations were 377 µg/mL.[4]

Mass spectrometry is the analytical tool of choice for qualitative identification of mAbs. Quantification of mAbs in biological matrices utilizes enzyme-linked immune sorbent assay (ELISA), the most widely applied technique. However, LC/MS exhibits better specificity, and generates a better protein-specific charge envelope. In addition, structural changes in the molecule can be detected by the mass spectrometer, which is helpful in obtaining more insight in degradation patterns of mAbs, or to detect post-translational modifications. ELISA generates a result based on antibody binding properties, and does not reveal changes in the structure of mAbs. In this application note, we report the development of a sensitive LC/MS/MS assay for the analysis of the signature peptide of trastuzumab with an assay sensitivity of 5 nM trastuzumab (0.75 µg/mL) in human serum.

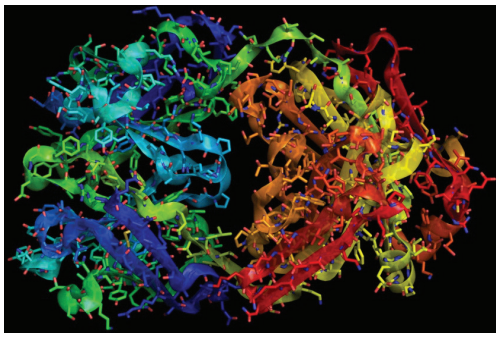


Figure 1. Molecular structure of trastuzumab.

EXPERIMENTAL

Sample Description

A stock solution of trastuzumab (a 150 kDa monoclonal antibody) was spiked with the internal standard (the $^{13}C^{15}N$-isotopically labeled extended peptide *GR*FTISADTS*K*) and digested with trypsin to produce a stock solution containing 5 µM digested trastuzumab and 5 µM internal standard peptide (FTISADTS*K*). In parallel, 400 µL of human serum was dispensed in 10 Eppendorf vials (40-µL serum/vial) and digested with trypsin following the same procedure, to produce 400 uL of human serum digest in each vial. The digestion protocol involved sample denaturation (with 0.05% RapiGest at 80 °C for 10 min), disulfide bond reduction (in the presence of 20 mM dithiothreitol (DTT) at 60 °C for 60 min), cysteine alkylation (with 10 mM iodoacetamide (IAM) at room temperature for 30 min in the dark), and overnight digestion with porcine trypsin (25:1 (w/w) protein/ enzyme). Following digestion, 100 µL of digested peptides were diluted 1:1 with a solution containing 4% H_3PO_4 and loaded onto an Oasis MCX mixed-mode µElution plate (P/N 186001830BA). Digests were washed with 200 µL of 2% FA and 200 µL of 5% methanol before being eluted with 2 x 50 µL aliquots of 25% ACN in 2% NH_4OH (pH 10). The trastuzumab digest was spiked into the human serum digest (using more than 90% of the serum digest matrix for each dilution) at the following concentrations: 5, 10, 20, 50, 100, 200, and 500 nM trastuzumab.

LC-SRM Method Conditions

The analysis was performed on an ACQUITY UPLC H-Class System. A 10-µL aliquot of the sample was injected onto an ACQUITY BEH130 C_{18} 2.1 x 150 mm, 1.7 µm column. The column temperature was maintained at 35 °C, with a flow rate of 0.3 mL/min. Mobile phases contained 0.1 % (v/v) formic acid (FA) in water (A), and 0.1% (v/v) FA in acetonitrile (B). Peptides were eluted with a linear gradient from 0% to 30% B over 10 min. The column effluent was monitored using a Xevo TQD Mass Spectrometer operated in selected reaction monitoring (SRM) positive ion electrospray mode. Four SRM transitions were monitored continuously throughout the LC-SRM assay using a dwell time of 40 ms: two SRMs monitored the endogenous signature peptide FTISADTS*K* from trastuzumab (485.2 → 721.4 for peptide quantification; 485.2 → 608.3 for peptide confirmation), while the other two SRM channels monitored the corresponding $^{13}C^{15}N$-isotopically labeled internal standard peptide FTISADTS*K* (489.2 → 729.4 for peptide quantification; 489.2 → 616.3 for peptide confirmation).

RESULTS AND DISCUSSION

Trastuzumab signature peptide (FTISADTS*K*) eluted with a retention time of 7.55 minutes, as shown in Figure 2. The data illustrates the injection of a 5 nM trastuzumab standard spiked in the human serum digest immediately following the analysis of human serum digest blank. This data shows very symmetrical peaks produced by the chromatography system that have a width at the base of approximately 4 seconds. The lower limit of quantification (LLOQ) for the assay was determined to be 5 nM (or 0.75 µg/mL) of trastuzumab in human serum, with an RMS signal to noise ratio of 27:1. A typical calibration obtained for the assay for the FTISADTS*K* signature peptide of trastuzumab is shown in Figure 3, with a correlation coefficient of 0.99326 using a 1/x weighting linear regression.

Figure 4 shows the SRM chromatograms of a Solvent A blank run, following the injection of the highest standard tested (500 nM trastuzumab in human serum digest). We can see from this data that there is no discernable carryover in the blank chromatogram (where the baseline has been magnified) for the FTISADTS*K* peptide. The extremely low carryover exhibited by the ACQUITY UPLC H-Class System allows the full sensitivity of the Xevo TQD Mass Spectrometer to be exploited.

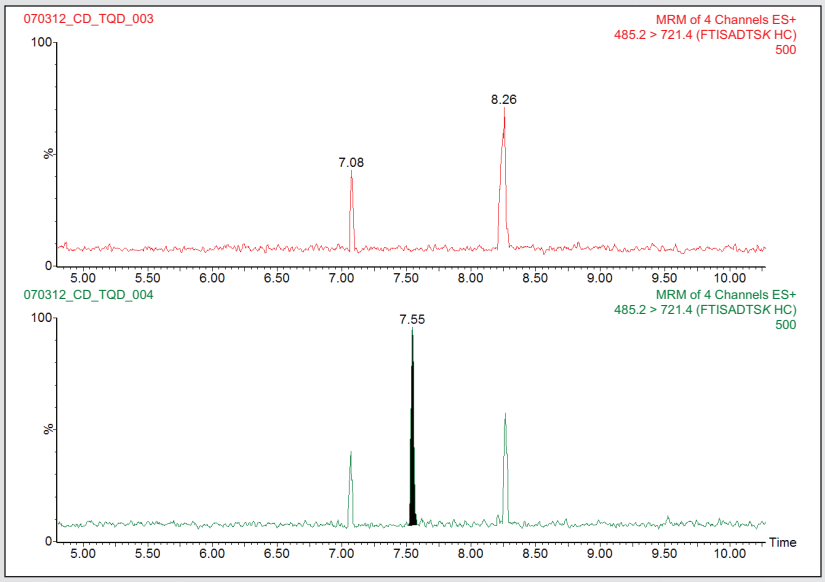


Figure 2. LC-SRM chromatogram of the 5-nM trastuzumab standard and the corresponding serum blank.

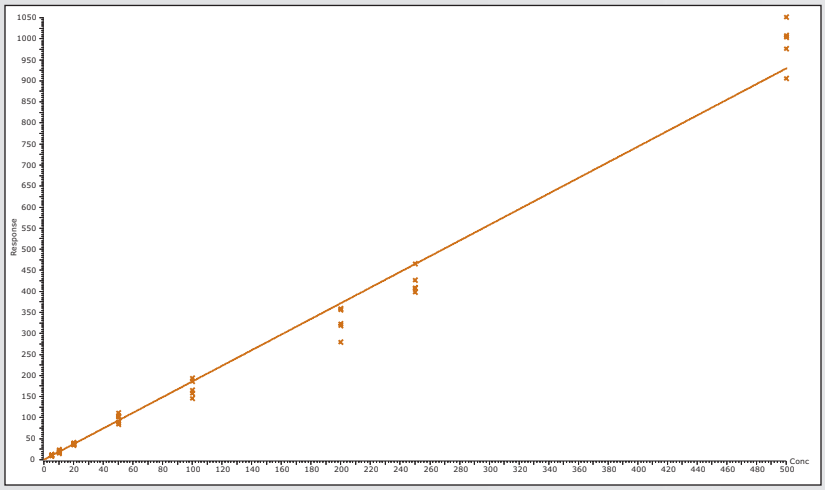


Figure 3. Representative calibration line for the LC-SRM quantification of trastuzumab.

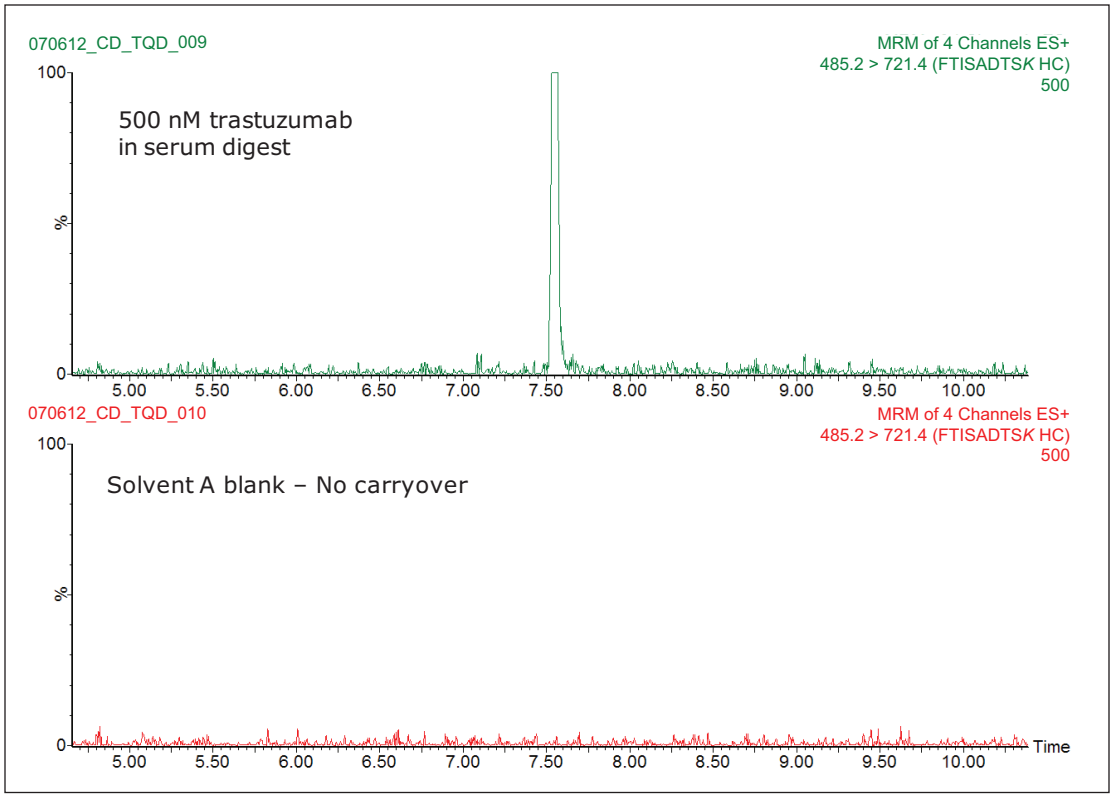


Figure 4. Carryover evaluation of the ACQUITY UPLC H-Class System.

CONCLUSIONS

- A high-sensitivity method has been tested for the analysis of trastuzumab (150 kDa protein) in human serum digest using a signature peptide.
- The level of quantification was determined to be 5 nM (0.75 µg/mL).
- No significant carryover was detected, following the injection of the highest concentration standard tested (500 nM trastuzumab in human serum).

References

1. Brekke OH, Sandlie I. *Nat. Rev. Drug Discov.* 2003; 2, 52-62.
2. Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Knotts C, Carver ME, Shepard HM. *Proc. Natl. Acad. Sci.* USA. 1992; 89, 4285-4289.
3. Hudis CA. *N. Engl. J. Med.* 2007; 357, 39-51.
4. Baughman TM, Graham RA, Wells-Knecht K, Silver IS, Tyler LO, Wells-Knecht M, Zhao Z. *Drug Metabolism and Disposition.* 2005; 33, 733-738.

Waters

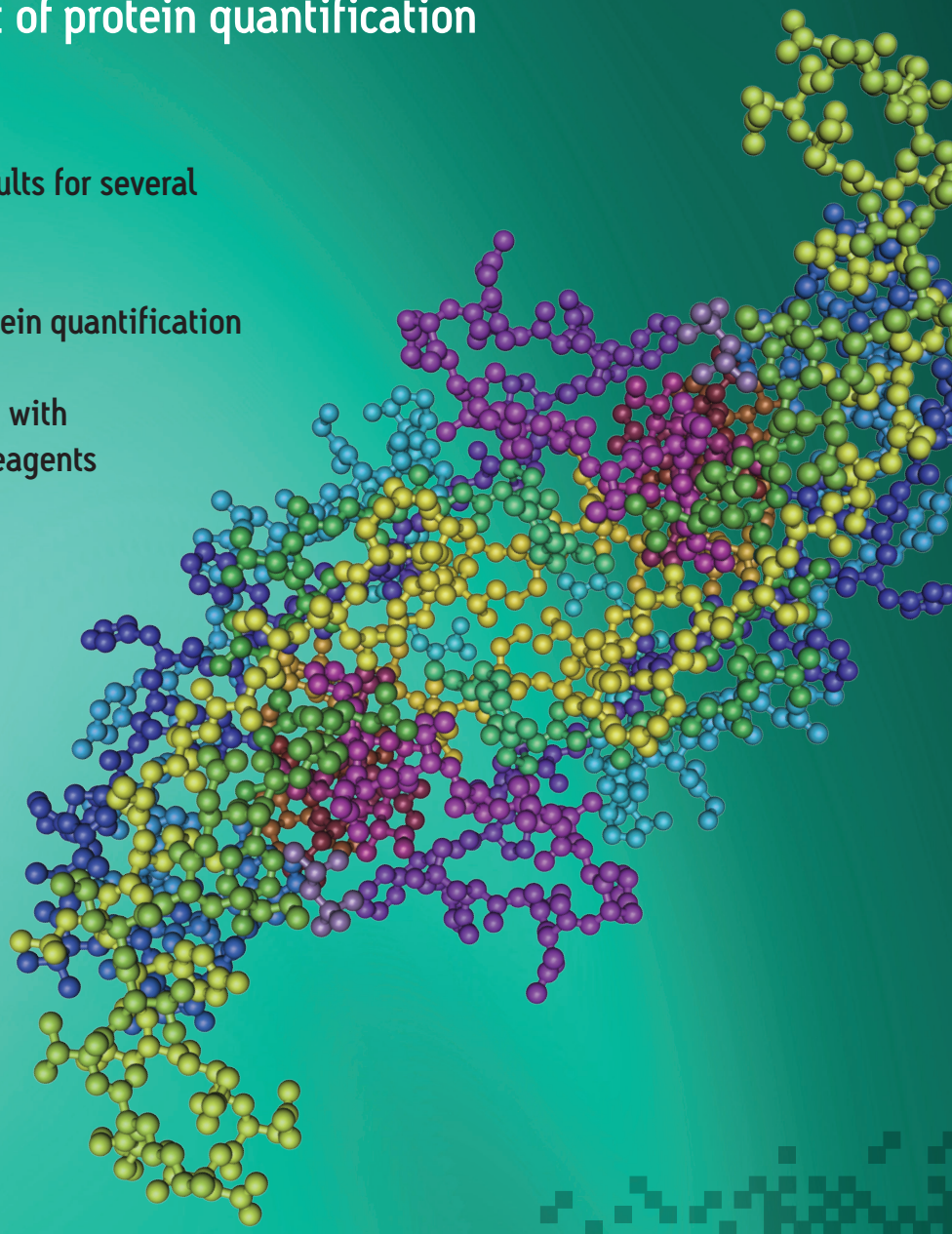
THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Introducing ProteinWorks™

Take the complexity out of protein quantification

- Reproducible and accurate results for several monoclonal antibodies
- Standardized approach to protein quantification
- Simplified sample preparation with pre-measured, lot-traceable reagents
- Reduced time to results and cost of analysis



waters.com/proteinworks

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

PHARMACEUTICAL ▪ HEALTH SCIENCES ▪ FOOD ▪ ENVIRONMENTAL ▪ CHEMICAL MATERIALS

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

High Sensitivity Quantification of Infliximab in Rat Plasma Using a Fast, Standardized Kit-Based Approach

Mary Lame, Hua Yang, Sherri Naughton, and Erin Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Simple, standardized approach to protein quantification; broadly applicable optimized digest kit eliminates method development for discovery studies; samples are ready for LC-MS analysis in 4–6 hours; high sensitivity detection limit of 10 ng/mL for infliximab was achieved.

WATERS SOLUTIONS

ProteinWorks™ eXpress Digest Kit (p/n 176003689)

Intact mAb Check Standard (p/n 186006552)

ACQUITY UPLC® Peptide BEH C_{18}, 300Å 1.7 µm, 2.1 mm x 150 mm, Column (p/n 186003687)

ACQUITY UPLC

Xevo® TQ-S Mass Spectrometer

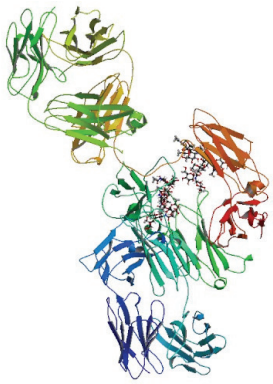
ProteinWorks µElution SPE Clean-up Kit (p/n 186008304)

KEY WORDS

monoclonal antibody, infliximab, Remicade, protein quantification, eXpress Digest

INTRODUCTION

As more drug development efforts focus on large molecules such as antibodies or ADC's, traditional "small molecule" scientists find themselves challenged not only by the complexity and time consuming nature but also the multitude of potential workflows that exist for protein quantification by LC-MS. This is also true for researchers investigating protein biomarkers where the use of ELISA's and other immuno-affinity (IA) methods are commonplace. While IA methods are sensitive and simple to execute, poor reagent reproducibility, lack of standardization, cross-reactivity, limited linear dynamic range, and other short-comings have led the drive to convert to LC-MS, especially for discovery and early development/pre-clinical studies. LC-MS workflows, however, encompass a multitude of sub-segments, each having many steps. Decisions about specific reagents, as well as the time, temperature, and concentration of the reagents or steps can all affect sensitivity, making it difficult to quickly arrive at a method which produces the desired detection limits. This application note describes the fast, sensitive quantification of infliximab (Figure 1) from rat plasma using the ProteinWorks eXpress Digest Kit and Protocol. Using a single universal sample prep method with pre-weighed, lot-traceable reagents and a set of carefully developed, yet generic set of simple step-wise instructions, an LLOQ of 10 ng/mL infliximab was achieved.



Formula: $C_{6428}H_{9912}N_{1694}O_{1987}S_{46}$
Molecular Weight: ~ 149.1 kD

http://www.drugbank.ca/drugs/DB00065

Figure 1. Infliximab (Remicade) protein structure.

EXPERIMENTAL

Sample description

Infliximab was first immuno-purified from 35 µL rat plasma using a 96-well Protein A agarose-based plate. Samples were then prepared for LC-MS analysis using the ProteinWorks eXpress Digest Kit and Protocol. Finally, signature peptides were cleaned-up using the ProteinWorks µElution SPE Clean-up Kit and Protocol.

Method conditions

LC system:	ACQUITY UPLC
Detection:	Xevo TQ-S Mass Spectrometer, ESI+
Column:	ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7 µm, 2.1 mm x 150 mm
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile

Gradient:

Flow rate (mL/min)	Time (min)	Profile %A	Profile % B	Curve
0.3	0.0	100	0	6
0.3	1.0	100	0	6
0.3	7.0	50	50	6
0.3	8.0	10	90	6

Data management: MassLynx® (v4.1)

MS conditions

Capillary (kv):	3
Cone (V):	30
Source offset (V):	50
Source temp. (°C):	150
Desolvation temp. (°C):	600
Cone gas flow (L/hr):	150
Collision gas flow (mL/min):	0.15
Nebulizer gas flow (Bar):	7

Peptide	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
DILLTQSPAILSVSPGER*	633.10>731.80	31	21
SINSATHYAESVK*	469.6>603.80	40	10
DSTYSLSSTLTLSK	751.88>836.47	31	24
SVSELPIMHQDWLNGK (ISTD)	618.64>834.41	16	12

*Unique Signature Peptide

Table 1. MRM conditions for infliximab peptides and internal standard peptide.

RESULTS AND DISCUSSION

With the infliximab US patent expiration date of 2017 drawing ever closer,[1] the focus on this important drug in CRO's as well as biosimilar research labs has increased. However, typical workflows are incredibly complex, with a multitude of choices and options. This makes the development of high sensitivity methods challenging. In this application note, we have used the ProteinWorks eXpress Digest Kit to simplify and streamline the process. Infliximab samples were affinity purified, digested, and peptides extracted using SPE in under 6 hours total. This enabled data to begin to be acquired the same day, with several 96-well plates being run by the following morning. Multiple unique signature peptides as well as a generic human peptide were simultaneously monitored for use in quantification. The best sensitivity was achieved using the unique peptide SINSATHYAESVK from the heavy chain, while additional unique (DILLTQSPAILSVSPGER, light chain) and generic (DSTYSLSSTLTLSK, light chain) infliximab peptides were monitored for confirmation. A unique peptide (SVSELPIMHQDWLNGK) from a common murine mAb standard (p/n 186006552) was used as the internal standard.

Using the optimized protocol and reagents provided in the kit, only 35 µL of plasma was needed to achieve a detection limit of 10 ng/mL for infliximab (Figure 2). Linearity and accuracy of the standard curves arising from each peptide are summarized in Table 2. The primary, and most sensitive quantitative peptide, SINSATHYAESVK, was linear over 4 orders of magnitude with a mean accuracy of >98% for all points on the curve. The additional two peptides were linear over 3.5 orders of magnitude with average accuracies >99% for all curve points.

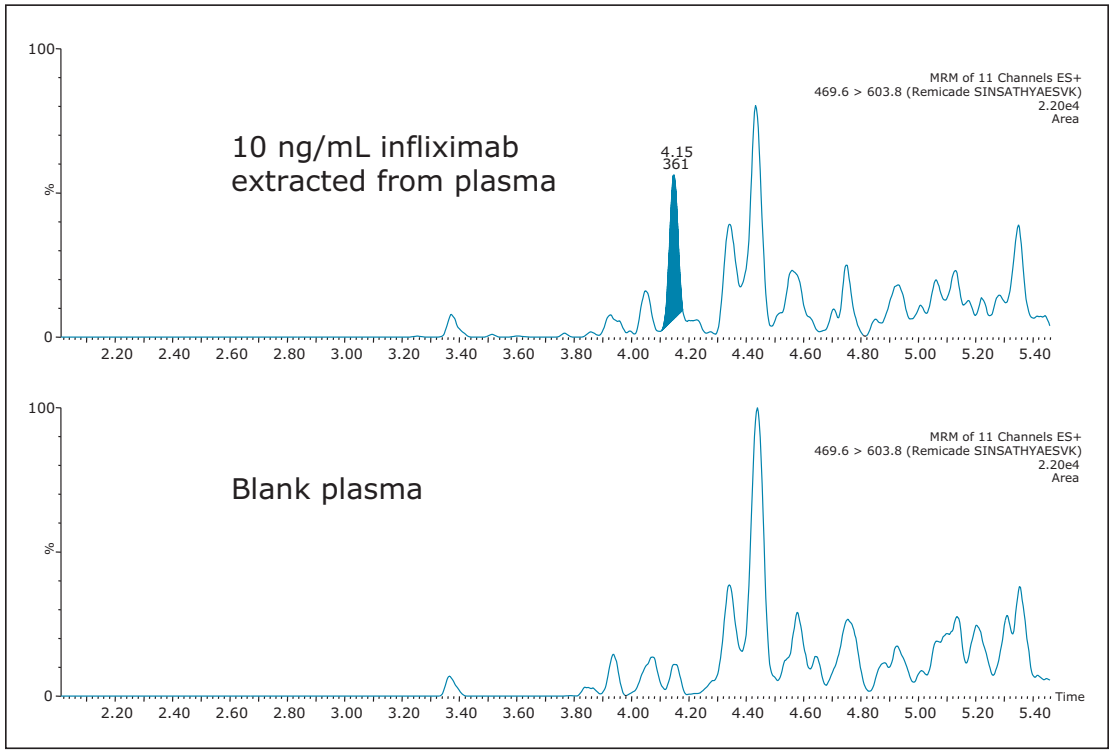


Figure 2. Chromatogram showing 10 ng/mL of infliximab in rat plasma, as compared to blank rat plasma. Infliximab is quantified using the unique peptide SINSATHYAESVK.

Peptide	Std. curve range (µg/mL)	Weighting	Linear fit (r^2)	Mean % accuracy of all points
DILLTQSPAILSVSPGER*	0.05–250	1/X	0.998	100.00
SINSATHYAESVK*	0.01–100	$1/X^2$	0.995	98.47
DSTYSLSSTLTLSK	0.10–500	$1/X^2$	0.997	99.34

*Unique signature peptide.

Table 2. Linear dynamic range and standard curve statistics for signature peptides used to quantify infliximab in rat plasma.

In addition, the accuracy and precision for the QC samples was excellent with %CVs all <6%. This is summarized in Table 3. In fact, the average %CV for QC samples from the SINSATHYAESVK peptide was <3%.

Peptide	QC conc (µg/mL)	Mean cal. conc (µg/mL)	Std. dev.	%CV	Mean accuracy
SINSATHYAESVK*	0.035	0.036	0.001	2.78	103.1
	0.350	0.331	0.003	0.80	94.5
	3.500	3.330	0.105	3.15	95.1
	35.000	38.287	1.168	3.05	109.4
	350.000	–	–	–	–
Peptide	**QC conc (µg/mL)**	**Mean cal. conc (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
DILLTQSPAILSVSPGER*	0.035	–	–	–	–
	0.350	0.359	0.015	4.10	102.6
	3.500	3.210	0.026	0.81	91.7
	35.000	37.054	0.581	1.57	105.9
	350.000	327.304	13.672	4.18	93.5
Peptide	**QC conc (µg/mL)**	**Mean cal. conc (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
DSTYSLSSTLTLSK	0.035	–	–	–	–
	0.350	0.333	0.010	2.85	95.3
	3.500	3.271	0.186	5.70	93.5
	35.000	36.256	1.999	5.51	103.6
	350.000	369.975	7.432	2.01	105.7

*Unique signature peptide.

Table 3. Statistics for QC samples from all infliximab peptides used for quantification.

From an assessment of the chromatographic data, it is clear that the quality of the data in terms of peak width and separation from residual endogenous components facilitated both the low level detection and the very high accuracy and precision that were achieved. This can be observed and is highlighted in the QC chromatograms from all signature peptides in Figures 3–5.

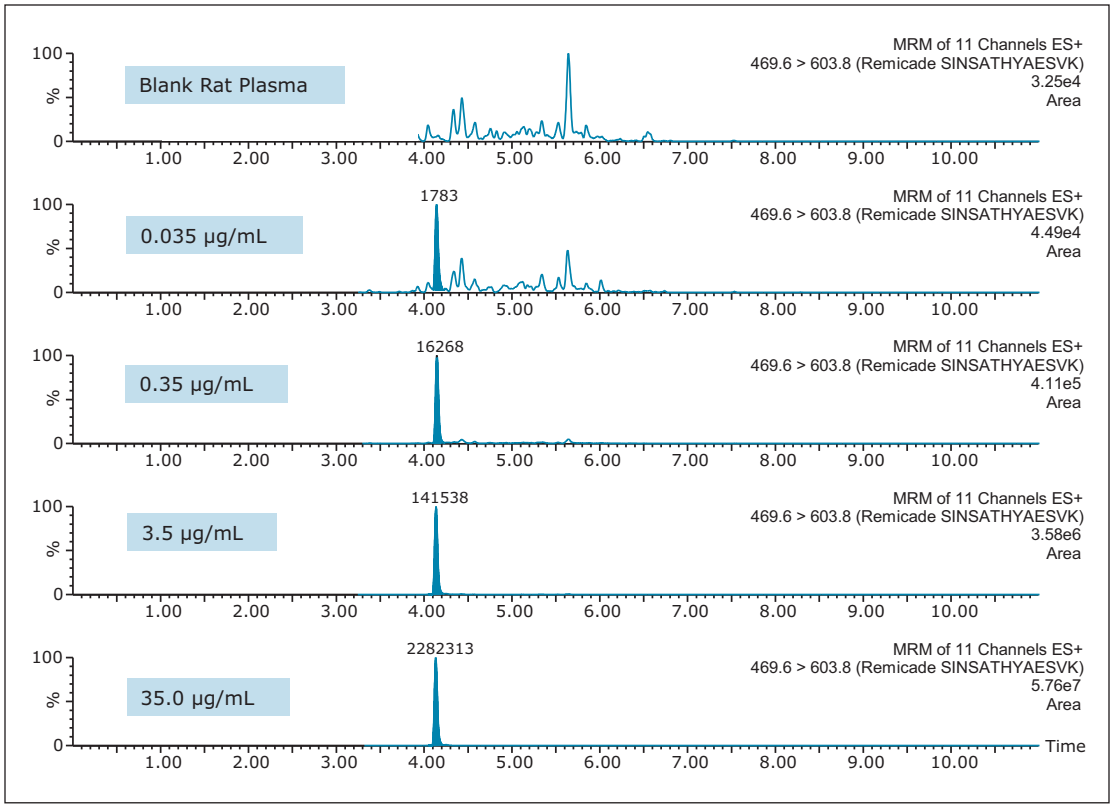


Figure 3. Infliximab QC chromatograms for the SINSATHYAESVK Unique Signature Peptide.

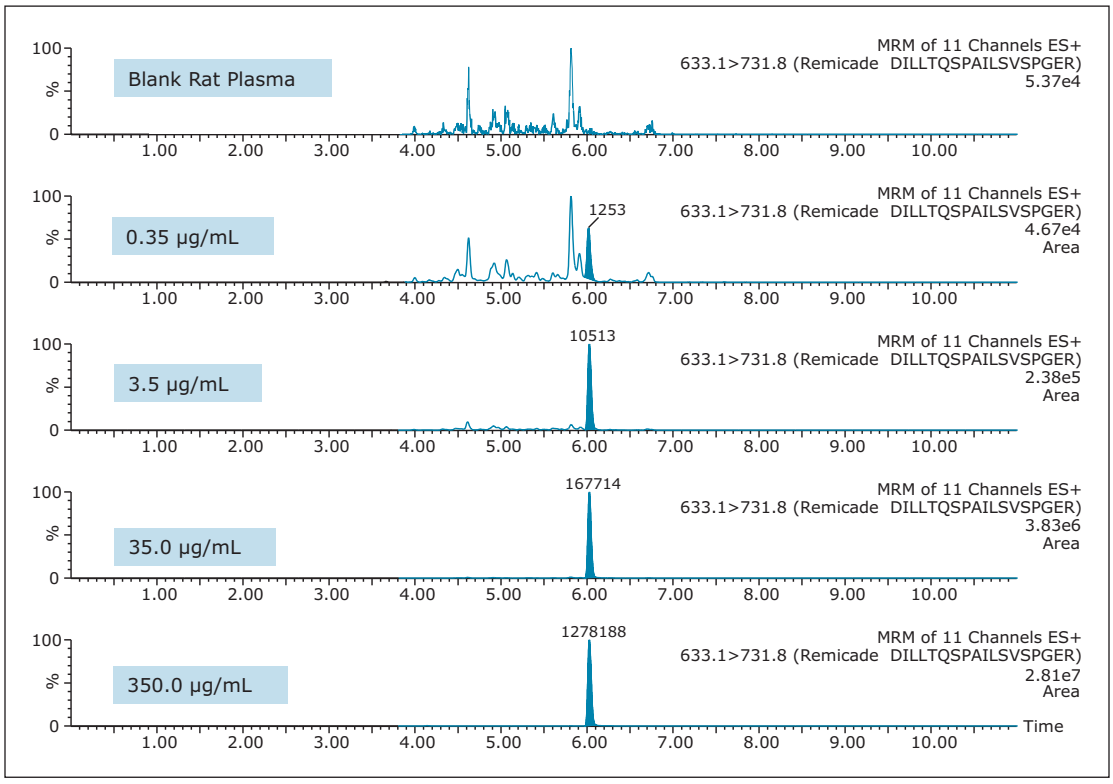


Figure 4. Infliximab QC chromatograms for the DILLTQSPAILSVSPGER Unique Signature Peptide.

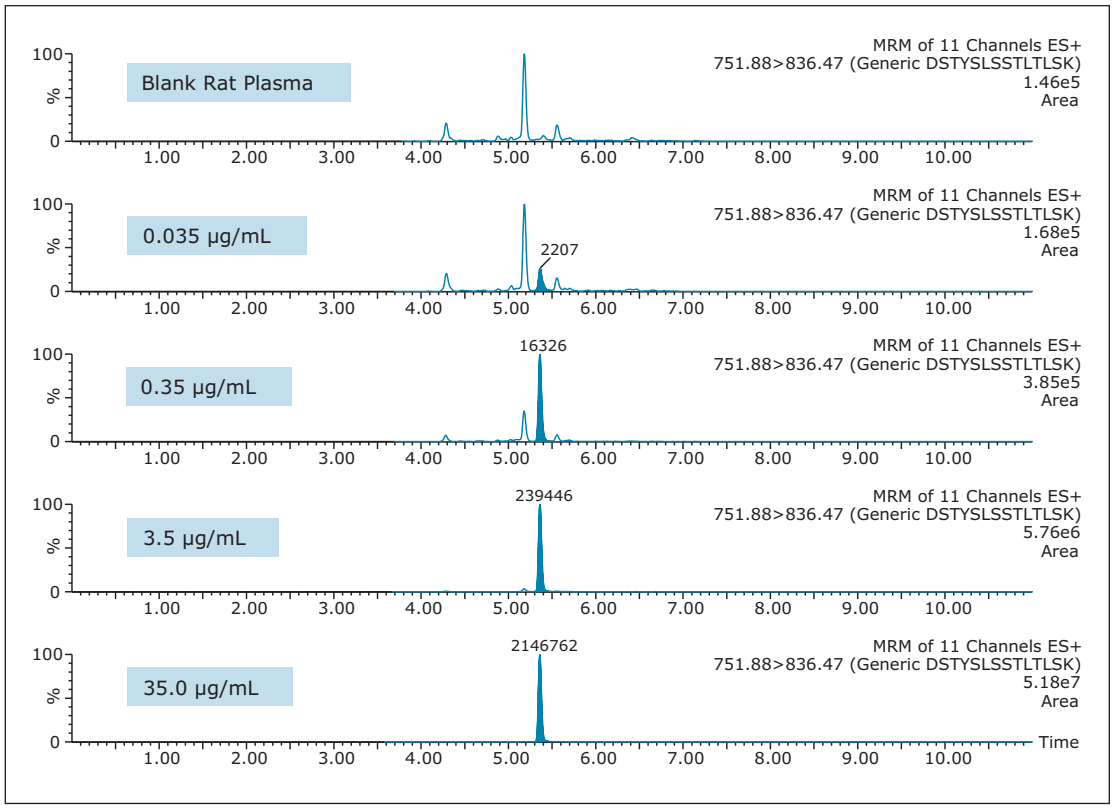


Figure 5. Infliximab QC Chromatograms for the DSTYSLSSTLTLSK Generic Signature Peptide.

CONCLUSIONS

The ProteinWorks eXpress Digest Kit was successfully used to purify infliximab from a typical set of standard curve and QC samples in rat plasma. A limit of quantification of 10 ng/mL was readily achieved, while maintaining excellent linearity and single digit precision. The total sample prep time including an affinity purification step was under 6 hours. The total digest prep time was just over 2 hours. The universal, kit-based approach allows novice users to achieve ultra-low detection limits with a simple step-wise protocol and a set of standardized, pre-measured reagents, ensuring both the sensitivity required and the transferability desired of such methods.

References

1. McKinsey and Company; Data Source: Evaluate Pharma, US Patent Expiration Dates.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

A Generic Kit-Based Approach for Quantifying Monoclonal Antibody Drugs Through Direct Digestion of Discovery Study Samples

Mary Lame, Hua Yang, Sherri Naughton, and Erin Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

Simple, standardized approach to protein quantification; broadly applicable optimized digest kit eliminates method development; samples are ready for LC-MS analysis in 4–6 hours

WATERS SOLUTIONS

ProteinWorks eXpress Direct Digest Kit (p/n 186003688)

Intact mAb Check Standard (p/n 186006552)

ACQUITY UPLC® Peptide BEH C_{18}, 300Å 1.7 µm, 2.1 mm x 150 mm Column (p/n 186003687)

ACQUITY UPLC

Xevo® TQ-S Mass Spectrometer

ProteinWorks µelution SPE Clean-up Kit (p/n 186008304)

KEY WORDS

Monoclonal antibody, infliximab, Remicade, protein quantification, eXpress Direct Digest, ProteinWorks, adalimumab, Humira, bevacizumab, Avastin, trastuzumab, Herceptin

INTRODUCTION

Over the past 5–10 years, there has been a significant shift towards a greater % of biologics in pharmaceutical pipelines.[1] However, the industry finds itself in the middle of patent expiry for many of the critical monoclonal antibody and other protein-based drugs, with patent expiration dates ranging from ~2012–2020.[2] This has resulted in a focus on protein quantification in bioanalytical labs, innovator pharma and CRO's as well as biomarker research labs. While immunoassay (IA) methods are sensitive and simple to execute, poor reagent reproducibility, lack of standardization, cross-reactivity, limited linear dynamic range, and other short-comings have led the drive to convert to LC-MS. These LC-MS workflows however, encompass a multitude of sub-segments, each having many steps. Those that are common to most workflows may include affinity purification, denaturation, reduction, alkylation, digestion, and SPE clean-up (each requiring optimization). Such traditional protein quantification protocols often require as much as a day and half for completion. Furthermore, the margin and possibility of error is significant within each individual step. There is a strong need for simpler, more standardized workflows which enable scientists to complete sample preparation and start an analytical run by mid-day. At the same time, ideally using generic, kitted methods, assay sensitivity must be high enough to accurately and precisely quantify low enough levels of the target protein to make critical decisions in discovery. The typical workflow complexity as shown in Figure 1, often leads to errors and poor reproducibility or sensitivity. In this application note, we have used the ProteinWorks™ eXpress Direct Digest Kit to simplify and streamline the workflow process using the same universal protocol and reagents for all monoclonal antibody drugs tested. Infliximab, bevacizumab, trastuzumab, and adalimumab (Figures 2–5) in plasma were directly digested, and peptides extracted using SPE in under 4 hours total time. This enabled data to begin to be acquired the same day, with several 96-well plates being run by the following morning.

EXPERIMENTAL

Sample description

Infliximab, adalimumab, bevacizumab, or trastuzumab were spiked into human plasma. 35 µL plasma samples were then prepared for LC-MS analysis using the ProteinWorks eXpress Direct Digest Kit and Protocol. After digestion, signature peptides were cleaned-up using the ProteinWorks µElution SPE Clean-up Kit and Protocol.

Method conditions

LC System:	ACQUITY UPLC
Detection:	Xevo TQ-S Mass Spectrometer, ESI+
Column:	ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7 µm, 2.1 mm x 150 mm
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile

Gradient:

Gradient:	Flow rate (mL/min)	Time (min)	Profile %A	%B	Curve
	0.3	0.0	100	0	6
	0.3	1.0	100	0	6
	0.3	7.0	50	50	6
	0.3	8.0	10	90	6

Capillary (kv):	3
Cone (V):	30
Source offset (V):	50
Source temp. (°C):	150
Desolvation temp. (°C):	600
Cone gas flow (L/hr):	150
Desolvation gas flow (L/hr):	1000
Collision gas flow (mL/min):	0.15
Nebulizer gas flow (Bar):	7

Protein	Peptide	MRM transition	Cone voltage (V)	Collision energy (eV)
Infliximab	SINSATHYAESVK	469.60>603.80	40	10
Bevacizumab	FTFSLDTSK	523.30>797.48	16	14
Adalimumab	APYTFGQGTK	535.30>901.44	40	24
Trastuzumab	FTISADTSK	485.20>721.40	28	20
murine mAb	MNSLQTDDTAK (ISTD)	612.30>978.56	20	20

Table 1. MRM conditions for infliximab, adalimumab, trastuzumab, bevacizumab, and the murine mAb internal standard.

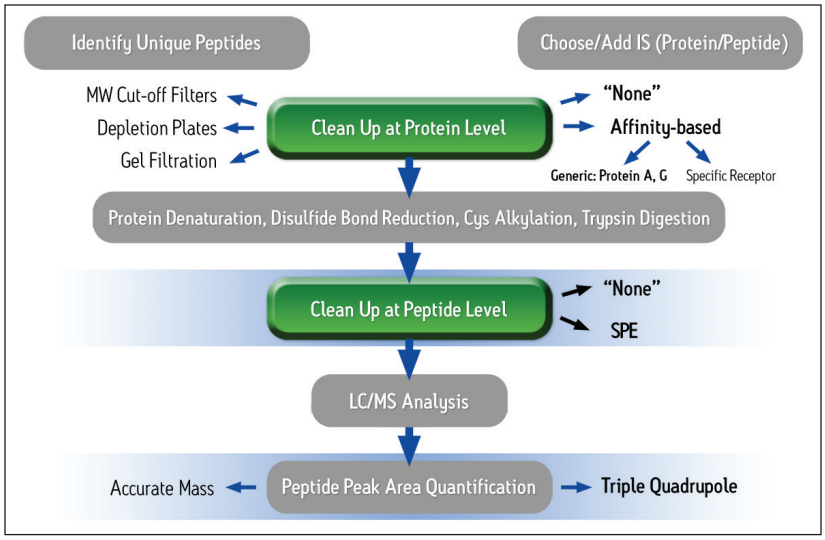


Figure 1. Typical protein bioanalysis workflow.

RESULTS AND DISCUSSION

In a pre-clinical setting, there is an emphasis on simple, broadly applicable, generic protocols as method development time and expertise are at a premium. Multiple signature peptides were used to quantify 4 different monoclonal antibody drugs in human plasma using direct digestion and the ProteinWorks eXpress Direct Digest Kit. For each protein, sensitivity, linearity, accuracy and precision data all met typical method validation requirements using the same broadly applicable ProteinWorks Kit. Through a direct digest of a 35 µL plasma sample, quantification limits ranged from 250 ng/mL–2.5 µg/mL for the 4 monoclonal antibody-based drugs. Standard curves were linear over 3.5–4 orders of magnitude with the average accuracies for standard curve points typically within 95–105%. Summary statistics from standard curves for infliximab, adalimumab, trastuzumab, and bevacizumab are shown in Table 2 below.

Protein	Peptide	Std. curve range (µg/mL)	Weighting	Linear fit (r^2)	Mean % accuracy of all points
Infliximab	SINSATHYAESVK	0.25–250	1/X	0.996	101.74
Bevacizumab	FTFSLDTSK	0.50–500	1/X	0.999	100.00
Adalimumab	APYTFGQGTK	2.50–500	$1/X^2$	0.997	99.99
Trastuzumab	FTISADTSK	2.50–500	$1/X^2$	0.997	100.01

Table 2. Linear dynamic range, weighting, and average accuracy for standard curves for infliximab, adalimumab, trastuzumab, and bevacizumab in plasma digested and extracted using the ProteinWorks eXpress Direct Digest Kit.

Remicade Light chain [2]:

DILLTQSPAILSVSPGERVSFSCRASQFVGSSIHWYQQRTNGSPRLLIKYASESMSGIPSRFSGSGSGTDFTLSINTVESEDIADYYCQQSH
SWPFTFGSGTNLEVKTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD
YEKHKVYACEVTHQGLSSPVTKSFNRGEC

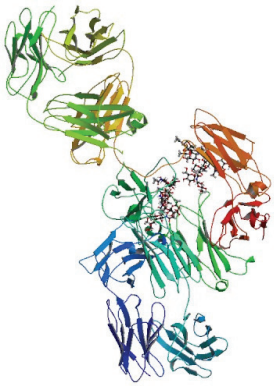
Remicade Heavy chain [2]:

EVKLEESGGGLVQPGGSMKLSCVASGFIFSNHWMNWVRQSPEKGLEWVAEIRSKSINSATHYAESVKGRFTISRDDSKSAVYLQMNSLRTED
TGVYYCSRNYYGSTYDYGQGTTLTVSXASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL
SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQV
SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Conserved region: blue
variable regions: red
CDR regions: green

Unique signature
Generic signature

Van Dongen et al. 61st ASMS, MP525 Minneapolis, Minnesota, USA 9-13 June 2013.



Formula: $C_{6428}H_{9912}N_{1694}O_{1987}S_{46}$
Molecular Weight: ~ 149.1 kD

http://www.drugbank.ca/drugs/DB00065

Figure 2. Structures of the monoclonal antibody-based drug infliximab (Remicade).

Conserved region Surrogate Peptides

Anti-HER2 Light chain DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPS
RFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

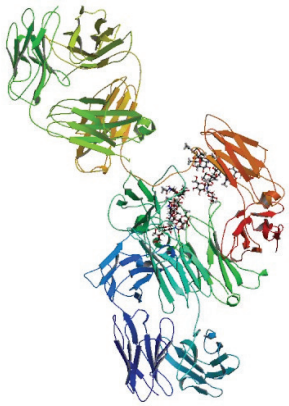
Anti-HER2 Heavy chain EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRY
ADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSS
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPPKSCDKTHTCPPCPAPELLG
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD
ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Unique Signature
Generic Signature

Formula: $C_{6470}H_{10012}N_{1726}O_{2013}S_{42}$
Molecular Weight: ~ 145.5 kDa (claims are 148 package insert)

http://www.drugbank.ca/drugs/DB00072

Figure 3. Structures of the monoclonal antibody-based drug trastuzumab (Herceptin).



Bevacizumab light chain
DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN
ALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
Bevacizumab heavy chain
EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSK
STAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTALGCLVK
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKVEPKSCDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS
REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGK

Unique Signature
Generic Signature

Drug Bank

Formula:
$C_{6538}H_{10034}N_{1716}O_{2033}S_{44}$
Molecular Weight: ~ 149.0 kD

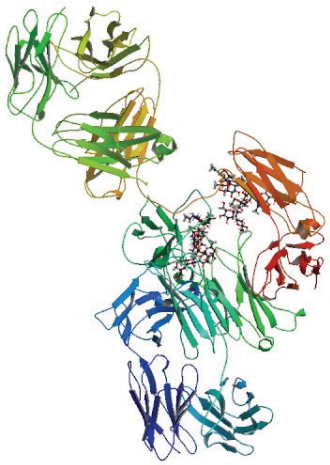
http://www.drugbank.ca/drugs/DB00065

Figure 4. Structures of the monoclonal antibody-based drug bevacizumab (Avastin).

Light chain: DIQMTQSPSSLSASVGDRVTITCRASQGIRNYLAWYQQKPGKAPKLLIYAASTLQSGVPS
RFSGSGSGTDFTLTISSLQPEDVATYYCQRYNRAPYTFGQGTKVEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK
ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Heavy chain: EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSAITWNSGHIDY
ADSVEGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAKVSYLSTASSLDYWGQGTLVTVS
SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS
SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC DKTHTCPPCPAPELLG
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD
ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Unique Signature
Generic Signature



$C_{6428}H_{9912}N_{1694}O_{1987}S_{46}$
Protein average weight 144190.3000

Drug Bank
http://www.drugbank.ca/drugs/DB00051

Figure 5. Structures of the monoclonal antibody-based drug adalimumab (Humira).

At the same time, QC statistics (summarized in Table 3 below) also easily met regulatory guidelines,[3] with average precision values well under 15%, and averaging in the single digits.

For a typical discovery study, detection limits ~1 µg/mL are common for monoclonal antibody type drugs. Using the ProteinWorks eXpress Direct Digest kit, these limits are easily obtained for all 4 drugs evaluated. Low QC chromatograms are shown in Figure 6 which demonstrate that adequate sensitivity is achieved with a single universal protocol and the kit.

In this study, the single universal digest protocol and SPE method designed for tryptic peptides eliminated the need for discovery-stage method development. The fact that the kit was able to accurately and precisely quantify 4 monoclonal antibody drugs in plasma without the need for optimization demonstrates its broad applicability and utility in an environment where time is critical and experience with protein bioanalysis may be limited. Furthermore, the application of a kit with lot-traceable, pre-measured reagents ensures that methods may be seamlessly transferred across sites and labs, or analysts.

Protein	Peptide	QC conc (µg/mL)	Mean cal. conc (µg/mL)	Std. dev.	%CV	Mean accuracy
Infliximab	SINSATHYAESVK	0.350	0.333	0.010	3.10	95.0
		3.500	3.816	0.098	2.56	109.0
		35.000	36.075	0.576	1.60	103.1
		350.000	359.301	19.892	5.54	102.6
		QC conc (µg/mL)	**Mean cal. conc (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
Bevacizumab	FTFSLDTSK	0.350	0.356	0.004	1.08	101.7
		3.500	3.393	0.196	5.78	96.9
		35.000	38.461	1.282	3.33	109.9
		350.000	369.788	28.066	7.59	105.6
		QC conc (µg/mL)	**Mean cal. conc (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
Adalimumab	APYTFGQGTK	0.350	–	–	–	–
		3.500	3.978	0.570	14.34	113.7
		35.000	36.567	1.023	2.80	104.5
		350.000	380.963	18.143	4.76	108.8
		QC conc (µg/mL)	**Mean cal. conc (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
Trastuzumab	FTISADTSK	0.350	–	–	–	–
		3.500	3.663	0.067	1.82	104.7
		35.000	39.182	2.389	6.10	112.0
		350.000	374.080	14.010	3.75	106.9

Table 3. Statistics for QC samples of infliximab, adalimumab, trastuzumab, and bevacizumab in plasma digested and extracted using the ProteinWorks eXpress Direct Digest Kit.

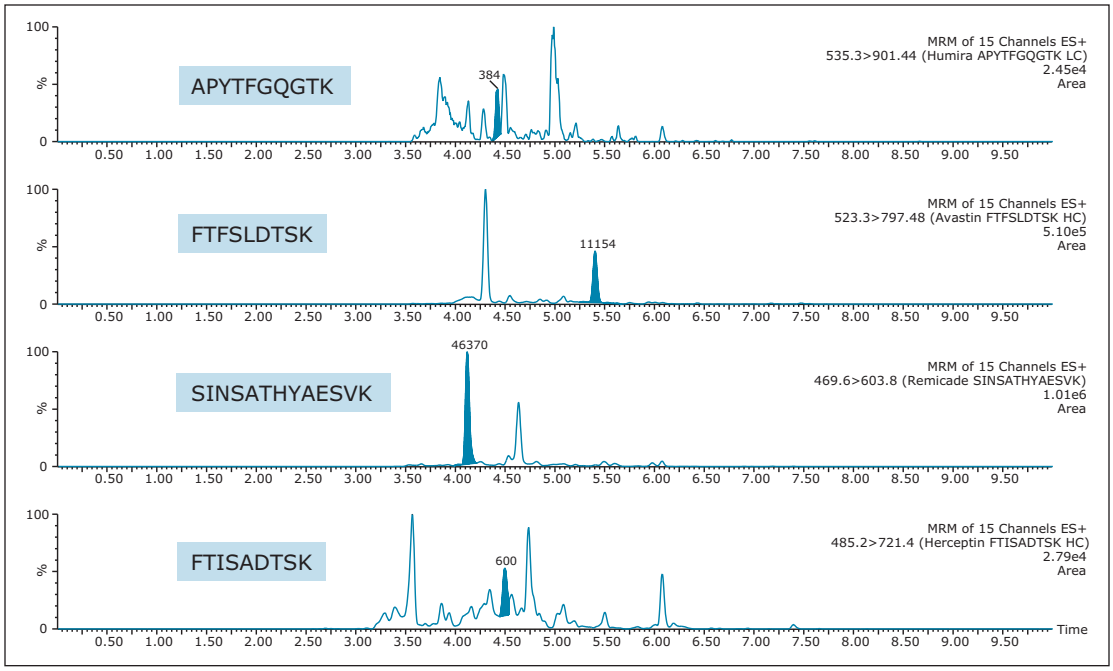


Figure 6. Low QC chromatograms (3.5 µg/mL) for adalimumab, bevacizumab, infliximab, and trastuzumab.

CONCLUSIONS

The ProteinWorks eXpress Direct Digest Kit was successfully used to purify and simultaneously quantify infliximab, adalimumab, bevacizumab, and trastuzumab from a typical set of standard curve and QC samples in human plasma. Quantification limits of 250 ng/mL to 2.5 µg/mL for each antibody were readily achieved, while maintaining excellent linearity and precision. The total sample prep time including digestion and SPE was just over 3 hours. The standardized, kit-based approach enables inexperienced users to immediately obtain meaningful data in discovery studies in order to make time sensitive and critical project decisions.

References

1. Dalzell, Managed Care, October 2013.
2. McKinsey and Company; Data Source: Evaluate Pharma, US Patent Expiration Dates.
3. FDA Guidance for Industry for Bioanalytical Method Validation, CDER.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Single Digit Reproducibility Using ProteinWorks eXpress Digest Kits

Mary Lame, Paula Orens, Sherri Naughton, and Erin Chambers
Waters Corporation, Milford, MA, USA

GOAL

To demonstrate the high reproducibility of ProteinWorks™ eXpress Digest Kits resulting from a standardized protocol and pre-weighed, lot-traceable reagents.

BACKGROUND

In any bioanalytical assay, one of the greatest sources of variability arises from the sample preparation. This is especially true for protein quantification workflows which often contain many segments-each with multiple steps capable of introducing variability. This can be of particular concern when assays are transferred from sponsor-to-CRO or from lab-to-lab within the same company, or across sites for example. Furthermore, given the multitude of possible options within a typical workflow, method development time and the expertise required are significant. The difficulty in implementing these assays is further aggravated by the complexity of the troubleshooting required when analytical goals are not met. At the same time, an assay is expected to meet acceptable accuracy and precision guidelines and provide reliable, reproducible results to make critical research and discovery-stage decisions. Thus, there is a strong need for simpler, more standardized workflows. These would ideally employ generic, kitted methods that provide a "recipe" and the reagents necessary to streamline the workflow, reduce variability and allow for implementation by less experienced scientists.

Protein quantification workflows are not only time consuming and complex, but due to their elaborate, multi-segment nature, the margin for error and the potential variability across sites, analysts, and even day-to-day can be very high. Poor reproducibility in protein quantification analytical data and a general lack of expertise strongly support the requirement for a standardized, kit-based approach.

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

THE SOLUTION

ProteinWorks eXpress Digest Kits are flexible, broadly applicable, sample preparation kits containing pre-measured, lot traceable reagents optimized for the accurate, precise, reproducible and robust LC-MS quantification of proteins via the surrogate peptide approach.

Reproducibility within an assay (intra-kit) and between assays (inter-kit) was evaluated with two (2) analysts, on different days, using a total of five (5) unique lots of kits, and six (6) technical replicates per kit. Both the ProteinWorks eXpress Direct Digest Kit (p/n 176003688), for direct digestion of whole plasma, and ProteinWorks eXpress Digest Kit (p/n 176003689), for digestion of affinity-purified plasma, were employed. Using the included generic protocol, several signature peptides (a combination of both generic and unique), resulting from the digestion of the monoclonal antibody drug bevacizumab (Avastin) in plasma, were evaluated and analyzed by LC-MS. The coefficient of variation (CV), also known as relative standard deviation (RSD), was used to evaluate reproducibility, as high CV values are indicative of poor reproducibility and precision. Raw area counts for multiple tryptic peptides from the aforementioned protein were used to make the assessment.

For the two types of kits, both intra-kit and inter-kit % CV's on average were ≤10. Figures 1A and 2A summarize intra and inter-kit reproducibility data from direct digestion of whole plasma using ProteinWorks eXpress Direct Digest Kits. Figures 1B and 2B summarize intra and inter-kit reproducibility data from affinity purified plasma using ProteinWorks eXpress Digest Kits. Additionally, using two (2) analysts, on two (2) separate days, the calculated mAb concentrations of the multiple tryptic peptides, when compared, were within 10% of each other, with % CV's across the six (6) technical replicates ≤15. Data from direct plasma digests are shown in Figure 3A, while data from affinity purified plasma digests are shown in Figure 3B.

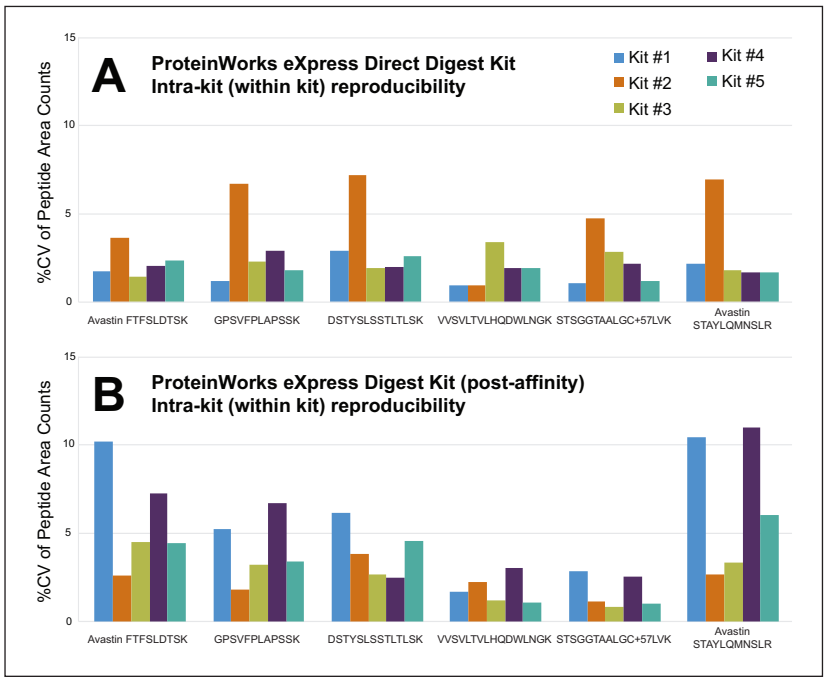


Figure 1. Intra-kit (N=5) % CV's of raw area counts for unique and generic signature peptides from bevacizumab; Panel A: ProteinWorks eXpress Direct Digest Kit (direct digestion of whole plasma) and Panel B: ProteinWorks eXpress Digest Kit (from affinity purified plasma).

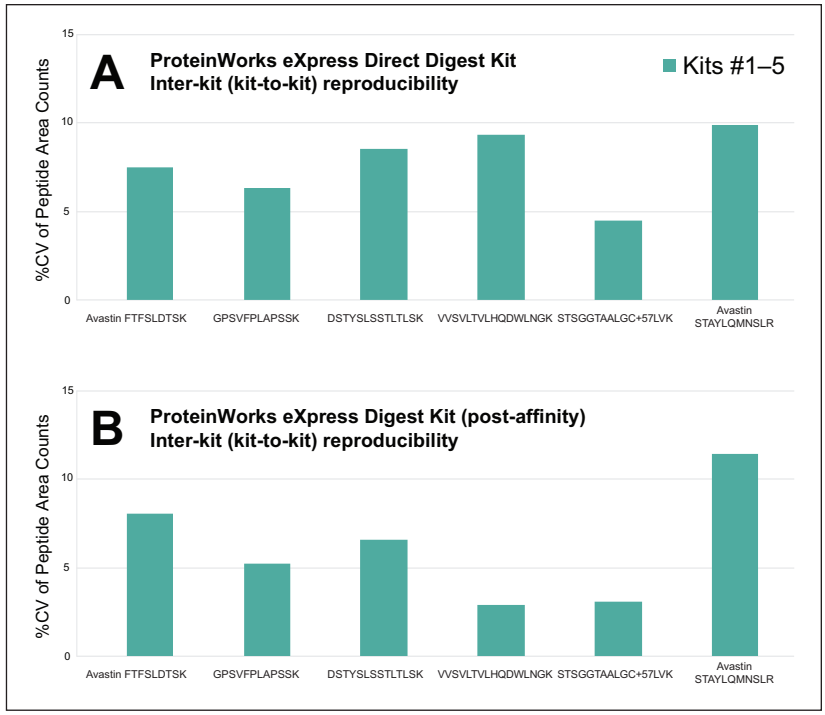


Figure 2. Inter-kit (N=5) % CV's of raw area counts for unique and generic signature peptides from bevacizumab; Panel A: ProteinWorks eXpress Direct Digest Kit (direct digestion of whole plasma) and Panel B: ProteinWorks eXpress Digest Kit (from affinity purified plasma).

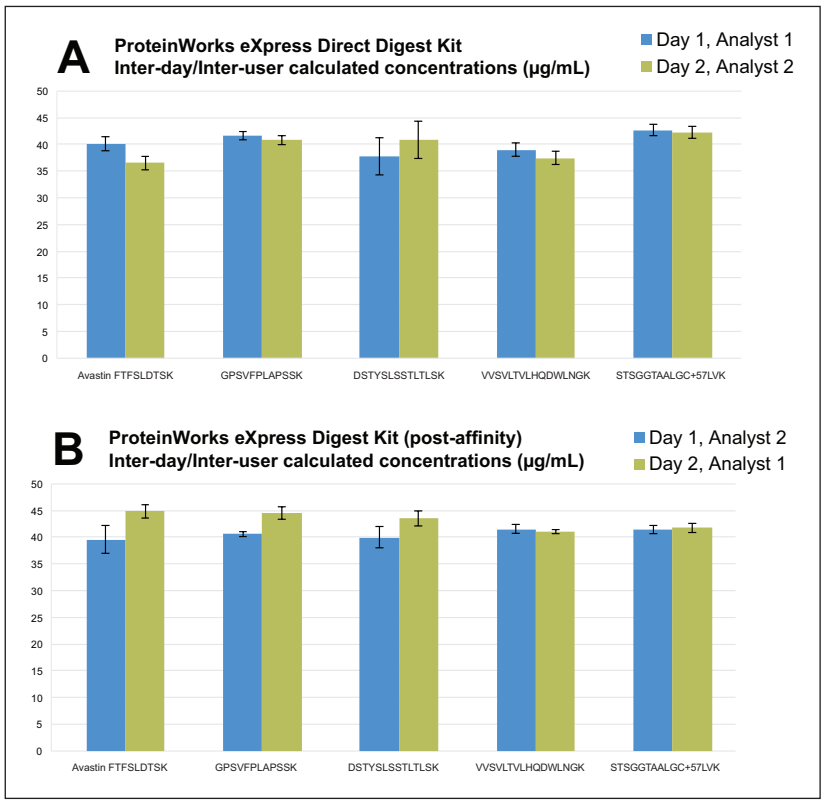


Figure 3. Inter-day/Inter-user calculated concentrations, ProteinWorks eXpress Direct Digest Kit (Panel A) and ProteinWorks eXpress Digest Kit (Panel B), for unique and generic signature peptides from a 40 µg/mL bevacizumab plasma sample.

SUMMARY

This work demonstrates that single digit reproducibility, accuracy and precision is achievable using this kit-based approach to protein quantification. These data suggest that a high degree of standardization can be achieved across analysts and sites implementing the kit strategy outlined herein.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Simple, Standardized, and Sensitive Quantification of Bevacizumab (Avastin) Using ProteinWorks eXpress Digest Kits

Mary Lame, Hua Yang, Sherri Naughton, and Erin Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Simple, standardized approach to protein quantification; broadly applicable optimized digest kit eliminates method development for discovery studies; samples are ready for LC-MS analysis in 4–6 hours; high sensitivity detection limit of 100 ng/mL for bevacizumab was achieved

WATERS SOLUTIONS

ProteinWorks™ eXpress Digest Kit (p/n 176003689)

Intact mAb Check Standard (p/n 186006552)

ACQUITY UPLC® Peptide BEH C_{18}, 300Å, 1.7 µm, Column (p/n 186003687)

ACQUITY UPLC System

Xevo® TQ-S Mass Spectrometer

ProteinWorks µElution SPE Clean-up Kit (p/n 186008304)

KEY WORDS

monoclonal antibody, bevacizumab, Avastin, protein quantification, eXpress Digest

INTRODUCTION

During the period of 2013-2020, many of the world's top selling antibody-based drugs come off patent,[1] including bevacizumab, an almost $6 billion dollar drug, expiring in 2020. In addition, as of 2013, there were 338 new monoclonal antibody drugs in development.[2] This represents the largest class of biologics in the pipelines. As patents for bevacizumab and other important antibody therapies expire and additional antibody drugs are developed, the need for streamlined LC-MS protein bioanalysis methods and approaches continues to grow. This is particularly true as bioanalysis studies using LC-MS were historically dominated by small molecule therapies with far simpler and more straightforward sample prep and analysis methods. Bevacizumab (Avastin, Genentech/Roche) is a tumor angiogenesis inhibitor, which selectively binds to and neutralizes the biologic activity of human vascular endothelial growth factor (VEGF) and is an adjunct IV therapy for colorectal, lung, cervical and kidney cancer, amongst others.[3,4] Typical dosing is in the 5–15 mg/kg range every 2–3 weeks, depending on indication. It was reported that doses >1 mg/kg produced serum levels of bevacizumab ≥10 µg/ml for at least 14 days.[5] This information suggests that a quantification method with a detection limit of ≥100 ng/mL and an upper limit of quantification (ULOQ) of several hundred µg/mL would be more than sufficient. While this would be a trivial exercise for a small molecule bioanalyst, the lack of expertise in biological molecule handling and the techniques associated with protein quantification via the surrogate peptide approach make it challenging for those same individuals to readily obtain high quality bioanalytical data in support of antibody drug programs. A generic, yet standardized approach to protein bioanalysis using LC-MS which is broadly applicable to large molecule drug development would enable novice scientists to successfully support discovery studies. In addition, one such universal proven methodology could facilitate transfer of methods between sites and ensure reproducibility of results. This application note describes the fast, sensitive quantification of bevacizumab (Figure 1) from rat plasma using the ProteinWorks eXpress Digest Kit and Protocol. A single universal sample prep method using pre-weighed, lot-traceable reagents and a carefully developed, yet generic set of simple step-wise instructions produced an LLOQ of 100 ng/mL bevacizumab from 35 µL of rat plasma.

EXPERIMENTAL

Sample description

Bevacizumab was first immuno-purified from 35 µL rat plasma using a 96-well Protein A agarose-based plate. Samples were then prepared for LC-MS analysis using the ProteinWorks eXpress Digest Kit and protocol. Finally, signature peptides were cleaned-up using the ProteinWorks µElution SPE Clean-up Kit and Protocol.

Method conditions

LC system:	ACQUITY UPLC
Detection:	Xevo TQ-S Mass Spectrometer, ESI+
Column:	ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7µm, 2.1 mm x 150 mm
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Gradient:	

Gradient:

Flow rate (mL/min)	Time (min)	Profile %A	Profile %B	Curve
0.3	0.0	100	0	6
0.3	1.0	100	0	6
0.3	7.0	50	50	6
0.3	8.0	10	90	6

Data management: MassLynx® (v4.1)

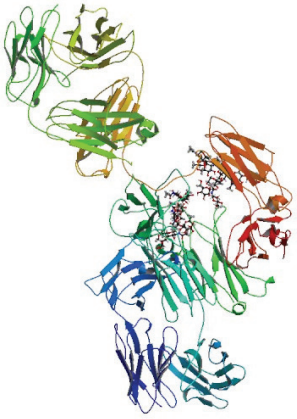
MS conditions

Capillary (kv):	3
Cone (V):	30
Source offset (V):	50
Source temp. (°C):	150
Desolvation temp. (°C):	600
Cone gas flow (L/hr):	150
Desolvation gas flow (L/hr):	1000
Collision gas flow (mL/min):	0.15
Nebuliser gas flow (Bar):	7

Peptide	MRM transition	Cone voltage (V)	Collision energy (eV)
FTFSLDTSK*	523.30>797.48	16	14
STAYLQMNSLR*	642.30>748.45	36	20
DSTYSLSSTLTLSK	751.88>836.47	31	24
STSGGTAALGC[+57.0]LVK	661.34>576.32	31	25
NTQPIMDTDGSYFVYSK (ISTD)	983.95>397.21	32	26

*Unique Signature Peptide

Table 1. MRM conditions for bevacizumab peptides and the internal standard peptide.



Bevacizumab light chain
DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCQQYSTVPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN
ALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Bevacizumab heavy chain
EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSK
STAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTALGCLVK
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKVEPKSCDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS

REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGK

Unique Signature

Generic Signature

Drug Bank

Formula: $C_{6538}H_{10034}N_{1716}O_{2033}S_{44}$
Molecular Weight: ~ 149.0 kD

http://www.drugbank.ca/drugs/DB00065

Figure 1. Bevacizumab (Avastin) Protein Structure.

RESULTS AND DISCUSSION

With the bevacizumab US patent expiration date of 2020 drawing ever closer, the focus on this important drug in CRO's as well as biosimilar research labs has increased. However, typical workflows are incredibly complex, with a multitude of choices and options. This makes the development of high sensitivity LC-MS methods for this and other monoclonal antibody-based drugs particularly challenging. In this application note, we have used the ProteinWorks eXpress Digest Kit to simplify and streamline the process. Bevacizumab samples were affinity purified, digested, and peptides extracted using SPE in under 6 hours total. This enabled same day data acquisition, with several 96-well plates being run by the following morning. Multiple unique signature peptides as well as a generic human peptide were simultaneously monitored for use in quantification. The best sensitivity and specificity were achieved using the unique peptide FTFSLDTSK from the variable region of the heavy chain, while additional generic (STSGGTAALGC+57LVK, heavy chain) and specific (STAYLQMNSLR, heavy chain) bevacizumab peptides were monitored for confirmation. A signature peptide (NTQPIMDTDGSYFVYSK) from a common murine mAb standard (p/n 186006552) was used as the internal standard.

Using the optimized protocol and reagents provided in the kit, only 35 µL of plasma was needed to achieve a quantification limit of 100 ng/mL for bevacizumab (Figure 2). Linearity and accuracy of the standard curves arising from each peptide are summarized in Table 2. The primary, and most sensitive and specific quantitative peptide, FTFSLDTSK, was linear over 3.5 orders of magnitude with a mean accuracy of 100% for all points on the curve. The additional three peptides were linear over 3–3.5 orders of magnitude with average accuracies >99% for all curve points.

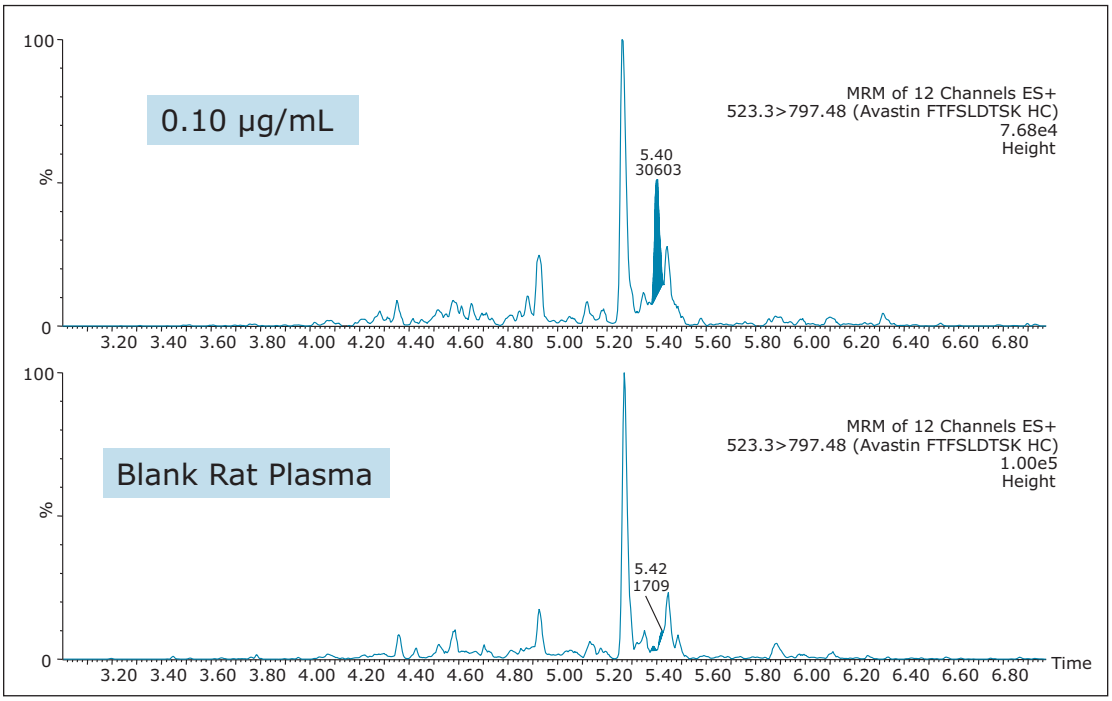


Figure 2. Chromatogram showing 100 ng/mL of bevacizumab in rat plasma, as compared to blank rat plasma. The unique peptide FTFSLDTSK is shown.

Peptide	Std. curve range (µg/mL)	Weighting	Linear fit (r^2)	Mean % accuracy of all points
FTFSLDTSK*	0.05–250	1/X	0.998	100.00
STAYQMNSLR*	0.50–500	$1/X^2$	0.997	100.02
DSTYSLSSTLTLSK	0.25–500	$1/X^2$	0.996	100.00
STSGGTAALGC+57LVK	0.05–250	$1/X^2$	0.996	100.60

*Unique signature peptide

Table 2. Linear dynamic range and standard curve statistics for signature peptides used to quantify bevacizumab in rat plasma.

In addition, the accuracy and precision for the QC samples was excellent with %CVs all ≤7%. This is summarized in Table 3. In fact, the average %CV for QC samples from the FTFSLDTSK peptide was 4%. Similarly, the average %CV for QC samples from the STAYLQMNSLR peptide was only 3%. Furthermore, the precision of the low QC across all peptides was 3%. Mean accuracies for all peptides hovered close to 100%.

Peptide	QC conc (µg/mL)	Mean cal. conc (µg/mL)	Std. dev.	%CV	Mean accuracy
FTFSLDTSK*	0.035	–	–	–	–
	0.350	0.342	0.013	3.82	97.7
	3.500	3.553	0.126	3.56	101.5
	35.000	32.386	0.611	1.89	92.5
	350.000	290.135	21.024	7.25	82.9
Peptide	**QC conc (µg/mL)**	**Mean cal. conc (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
STAYLQMNSLR*	0.035	–	–	–	–
	0.350	0.345	0.004	1.12	98.6
	3.500	3.819	0.110	2.89	108.3
	35.000	35.065	1.262	3.60	100.2
	350.000	335.347	15.208	4.54	95.8
Peptide	**QC conc (µg/mL)**	**Mean cal. conc (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
DSTYSLSSTLTLSK	0.035	–	–	–	–
	0.350	0.351	0.016	4.61	100.3
	3.500	3.397	0.045	1.32	97.1
	35.000	32.082	0.469	1.46	91.7
	350.000	320.836	9.141	2.85	91.7
Peptide	**QC conc (µg/mL)**	**Mean cal. conc (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
STSGGTALGC+57LVK	0.035	–	–	–	–
	0.350	0.366	0.012	3.36	104.4
	3.500	3.509	0.063	1.79	100.2
	35.000	33.015	1.406	4.26	94.3
	350.000	305.140	3.259	1.07	87.2

Table 3. Statistics for QC samples from all bevacizumab peptides used for quantification.

We consistently achieved single digit accuracy and precision during bevacizumab analysis through a combination of three primary factors: high fidelity chromatographic data, an optimized, well controlled protocol, and the use of a set of standardized, pre-measured reagents. These three critical components were realized through application of the ProteinWorks eXpress Digest Kit, which facilitated low level detection, separation from residual endogenous interferences, and the very high accuracy and precision. This performance is highlighted in the QC chromatograms from representative signature peptides in Figures 3 and 4.

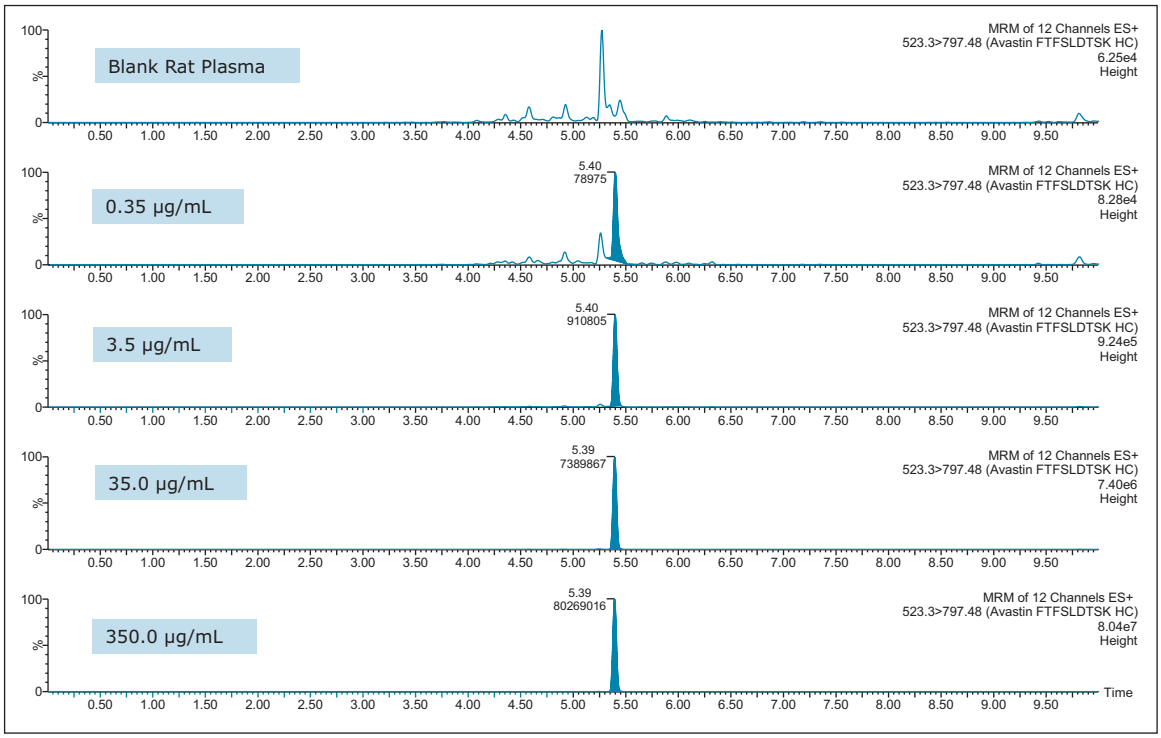


Figure 3. Bevacizumab QC Chromatograms for the FTFSLDTSK Unique Signature Peptide.

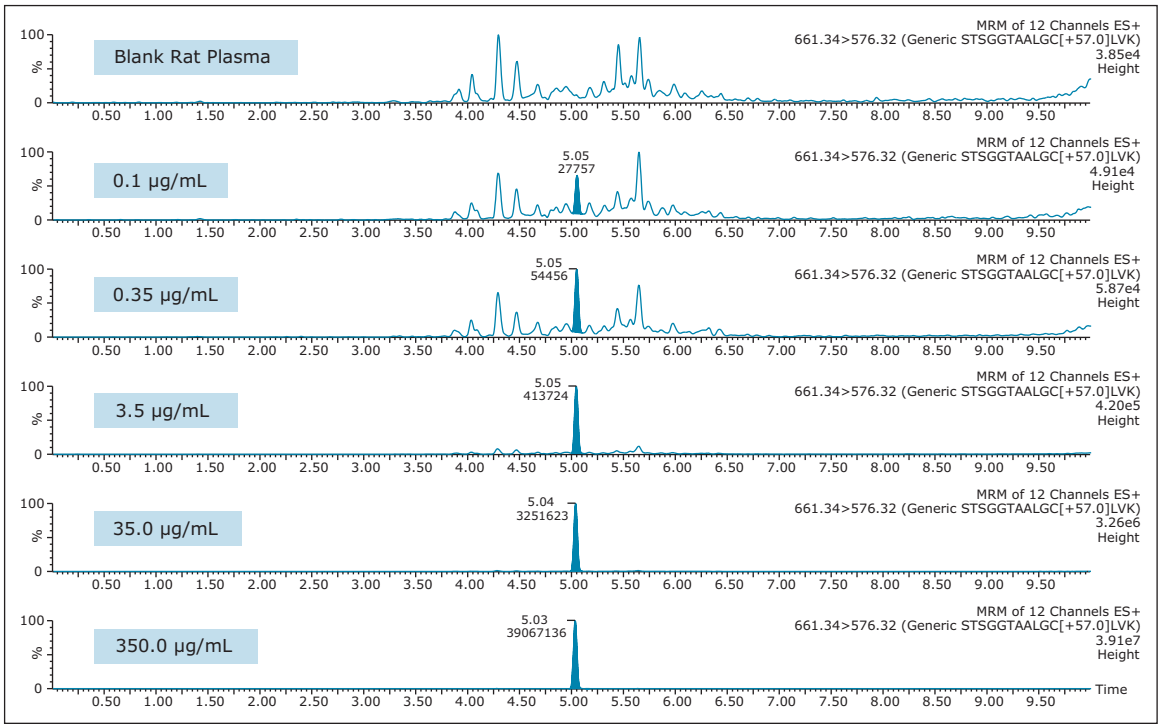


Figure 4. Bevacizumab QC Chromatograms for the STSGGTAALGC+57LVK Generic Signature Peptide.

CONCLUSIONS

The ProteinWorks eXpress Digest Kit was successfully used to purify bevacizumab from a typical set of standard curve and QC samples in rat plasma. A limit of quantification of 100 ng/mL was readily achieved from only 35 µL rat plasma, while maintaining excellent linearity and single digit precision. The total sample prep time including an affinity purification step was under 6 hours. The total digest prep time was just over 2 hours. The universal, kit-based approach allows novice users to achieve low detection limits with a simple step-wise protocol and a set of standardized, pre-measured reagents, ensuring both the sensitivity required and the transferability desired of such methods.

In addition, the kit is optimized and flexible enough to enable simultaneous, sensitive quantification of both unique and generic signature peptides from monoclonal antibodies. This is important as confirmatory data from multiple peptides is critical in supporting the confident use of LC-MS for protein bioanalysis workflows.

References

1. McKinsey and Company; Data Source: Evaluate Pharma, US Patent Expiration Dates.
2. Medicines in Development: *Biologics*, PhRMA, phrma.org report 2013.
3. Avastin product insert, *Roche*
4. Genentech site for Health Care Professionals, www.avastin-hcp.com
5. Emily Bergsland and Maura N. Dickler, *The Oncologist*, June 2004 vol. 9 Supplement 1 36–42.

Waters

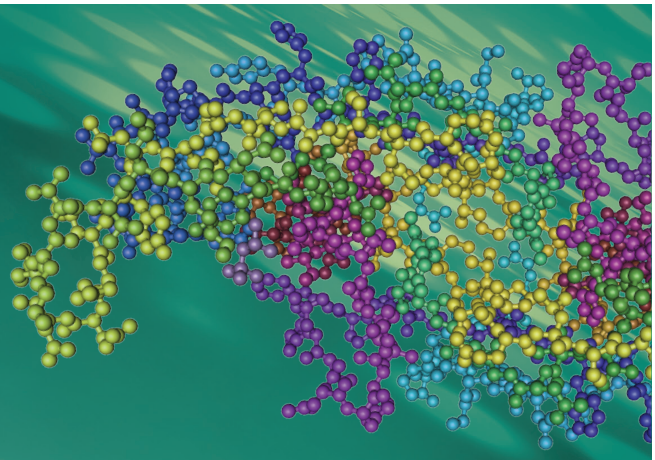
THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

[TECHNOLOGY BRIEF]

A Universal, Optimized SPE Protocol for Clean-up of Tryptic Peptides in Protein Bioanalysis

Mary Lame, Hua Yang, Sherri Naughton, and Erin Chambers



GOAL

To demonstrate the performance of an optimized method for tryptic peptide clean-up in protein bioanalysis.

BACKGROUND

During a typical protein bioanalytical workflow, it is common to either directly digest an unpurified plasma/serum sample or to perform affinity purification followed by digestion. An affinity step may be included to isolate a class of proteins (for example, the use of Protein A/G which isolates an IgG fraction) or a specific target from the plasma/serum sample. Depending on the degree of protein-level clean-up, which ranges from none (direct plasma digestion) to significant (specific affinity capture of a target) the concentration of resultant peptides can be extremely high. In small molecule SPE the majority of proteins (undigested, of course) pass through the extraction device on the load step. In contrast, in a protein bioanalysis workflow, 10's of thousands of digested peptides from as high as 50–60 mg/mL starting protein concentration, are all now capable of binding to the SPE sorbent bed. This creates a significant capacity challenge. A possible solution that might occur to a "small molecule" scientist would be to use a larger sorbent bed size, for example 10, 30 or even 60 mg sorbent beds. However, these larger bed sizes require larger volumes to elute, which must then be dried down and reconstituted. The same practices, when applied in large molecule quantification, could result

As of 2013, there were 338 new monoclonal antibody drugs in development,[1] representing the largest class of biologics in drug development pipelines. Recently, LC-MS has been gaining ground as the technique of choice over ligand binding assays (LBAs) for the support of biologic drug development programs. An important component of these LC-MS workflows is the post-digest purification of tryptic peptides. While SPE is commonly used in small molecule sample preparation, the same methods and guidelines do not apply to peptide extraction. Knowledge of peptide handling concerns as well as the ability to efficiently manipulate wash and elution conditions for tryptic peptides have become critical capabilities in bioanalytical labs. Historically, these labs have been dominated by work on small molecule therapies with far simpler and more straightforward sample prep and analysis methods. The expertise in peptide and protein bioanalysis, if it exists, tends to be a rare and valuable skill, held by only a few individuals.

in significant peptide/protein losses due to adsorption during evaporation. Therefore, it is critical to avoid the dry-down step commonly associated with small molecule SPE methods.

In addition to the requirement for clear capacity guidelines for loading protein digests, a method tailored to targeted elution of tryptic peptides is also needed. A broadly applicable protocol which effectively removes excess digest reagents, digestion buffer, phospholipids, and other plasma/serum components could not only increase sensitivity by reducing matrix effects, but would also improve system robustness.

THE SOLUTION

A total of 17 signature peptides (a combination of both generic and unique) were used in the development and verification of an SPE protocol, as well as an optimal device format, which specifically targets tryptic peptide clean-up during protein bioanalysis experiments. The result of this research is the ProteinWorks™ µElution SPE Clean-up Kit (p/n 186008304). Key attributes of the kit include: the ability to concentrate digests without evaporation, orthogonal retention mechanism for maximum specificity, and a protocol designed expressly for efficient purification of tryptic peptides. Oasis® MCX was chosen as the ideal sorbent due to its strong ion exchange binding to the positively charged termini produced through cleavage at the basic residues arginine and lysine during trypsin digestion. In addition, very polar peptides are more efficiently trapped by ion exchange on the Oasis MCX plate than they would be on a traditional reversed-phase only sorbent. The µElution 96-well device format is characterized by elution in as little as 25 µL, which enables significant concentration of extracts without evaporation. The entire 96-well plate can be processed in under 20 minutes using either vacuum or positive pressure, and is amenable to automation.

Performance of the SPE Kit was evaluated using a broad range of chemically diverse representative peptides. Key peptide characteristics are summarized in Table 1.

Through extensive testing and optimization, recovery was maximized for all peptides and sample loading guidelines were created. The comprehensive guidelines, which are related to starting protein content, address both direct plasma/serum digest samples and extracts resulting from affinity purification of the target protein or class of proteins. Recovery of the various peptides using the optimized protocol in the SPE Kit is summarized in Figure 1. The average peptide recovery for the antibody drugs is 104%. Peptides from the generic murine mAb IS average 84% recovery.

Table 1. Relevant characteristics of signature peptides tested during the development and verification of the ProteinWorks µElution SPE Clean-up Kit

Protein Name	Peptide	MW	pI	# of Residues	HPLC Index
Herceptin	FTISADTSK	969	6.2	9	26.5
Humira	APYTFGQGTK	1069	9.1	10	39.3
Herceptin	DTYIHWVR	1089	7.4	8	31.1
Generic Human IgG	GPSVFPLAPSSK	1186	9.4	12	54.7
Murine	MNSLQTDDTAK	1223	4.1	11	21.5
Murine	VNSAAFPAPIEK	1243	6.4	12	47.4
Avastin	STAYLQMNSLR	1283	9.2	11	48.4
Avastin	FTFSLDTSK	1285	7.3	11	40.5
Generic Human IgG	STSGGTAALGC[+57.0]LVK	1322	9.4	14	50.8
Humira	NSLYLQMNSLR	1339	9.2	11	54.6
Remicade	SINSATHYAESVK	1407	7.4	13	7.1
Humira	NYLAWYQQKPGK	1496	9.7	12	52.2
Generic Human IgG	DSTYSLSSTLTLSK	1503	6.2	14	47.8
Generic Human IgG	VVSVLTVLHQDWLNGK	1808	7.4	16	77.7
Murine	SVSELPIMHQDWLNGK	1854	5.5	16	52.5
Remicade	DILLTQSPAILSVSPGER	1869	4.3	18	74.2
Murine	NTQPIMDTDGSYFVYSK	1966	4.1	17	35.1

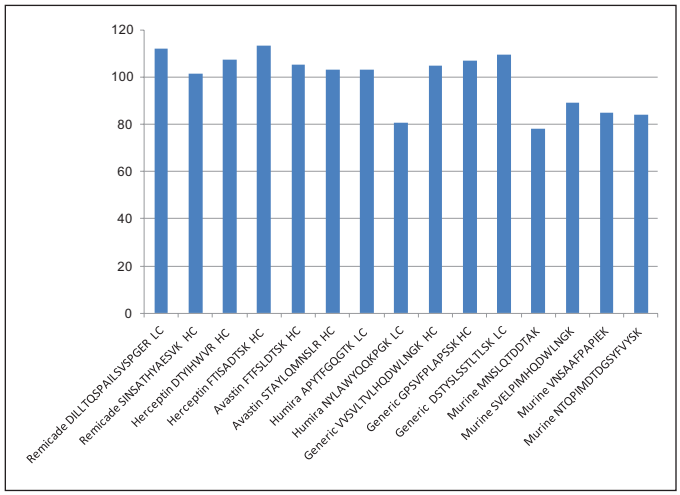


Figure 1. MCX µElution SPE recovery of unique and generic signature peptides from infliximab, bevacizumab, adalimumab, trastuzumab, and a generic murine mAb internal standard.

It is recommended that the total protein load in the digested sample on the Oasis MCX µElution plate not exceed 1.25 mg. In addition, for best performance, the minimum SPE loading volume range is 15–25 µL. The sample loading guidelines, summarized in Table 2, were designed to produce the highest sensitivity by identifying the maximum digest volume which should be loaded.

Table 2. Recommended maximum digest loading volumes for the ProteinWorks µElution SPE Clean-up Kit

Oasis MCX 96-well µElution Plate Recommended Maximum Digest Loading Volumes		
	SPE Loading Volume (µL)	
Starting Plasma/Serum Volume (µL)*	Direct Digest**	Post-Generic Affinity Digestion†
15	110-200	Total Digest Supernatant
25	70-140	Total Digest Supernatant
35	50-100	Total Digest Supernatant
50	35-70	170-200
70	25-50	120-200

** Starting volume of plasma added for protein digestion or affinity purification using the ProteinWorks eXpress Digest Kit and Protocols, with a final digestion volume of 200 µL.*

***Based on a total protein content of 75 mg/mL in whole plasma/serum.*

† Based on a total protein content 15 mg/mL post-generic affinity purified plasma (Protein A/G) and assuming all Affinity captured sample is used for digestion.

Plasma was spiked with infliximab, trastuzumab, adalimumab, bevacizumab, and a common murine mAb IS and then digested. The resultant tryptic peptides were isolated from digest reagents (which are washed off during loading and wash steps), phospholipids (which remain stuck to the SPE plate after peptide elution), and concentrated using the ProteinWorks SPE Clean-up Kit. Chromatographic purity of the signature peptides extracted from the plasma digest sample is demonstrated in Figure 2.

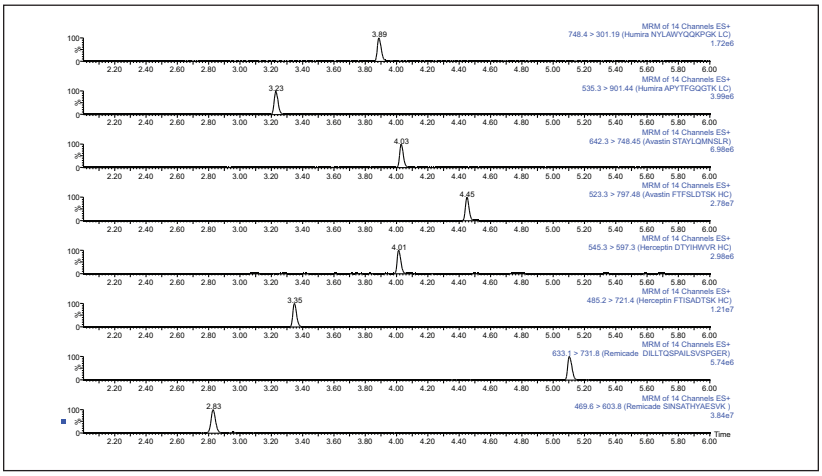


Figure 2. LC-MS chromatograms for representative unique signature peptides from a plasma digest containing adalimumab, bevacizumab, trastuzumab, and infliximab.

SUMMARY

The ProteinWorks µElution SPE Clean-up Kit was successfully used to isolate peptides resulting from plasma digests of bevacizumab, trastuzumab, adalimumab, and infliximab as well as a common murine mAb internal standard. The simple 96-well µElution format enabled clean-up and concentration of digest samples in <20 minutes, without the need for evaporation and reconstitution, preserving low levels of precious tryptic peptides. Furthermore, the kit takes advantage of the orthogonal nature of mixed-mode SPE (binding tryptic peptides by ion-exchange) to provide the degree of specificity required for low-level protein quantification studies. Finally, the specific sample loading guidelines and an optimized kit-based approach allow novice users to quickly, easily, and effectively clean-up protein digests. This strategy generates data with the best possible reproducibility and highest peptide recovery for challenging protein bioanalysis studies.

References

1. Medicines in Development: Biologics, PhRMA, phrma.org report 2013.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

[TECHNOLOGY BRIEF]

Antibody Quantification Using ProteinWorks eXpress Digest Kits and Multiple Plasma Volumes

Paula Orens, Mary Lame, Sherri Naughton, and Erin Chambers
Waters Corporation, Milford, MA, USA

GOAL

To demonstrate the broad applicability across a range of sample volumes, and the inherent flexibility of the ProteinWorks™ eXpress Direct Digest Kits for the quantification of monoclonal antibody (mAb) drugs by LC-MS/MS.

BACKGROUND

Over the past decade, MS platform technologies have steadily increased in sensitivity and ease of use. At the same time, this increase in sensitivity has facilitated the use of smaller sample volumes. As such, microsampling techniques have become common place. These techniques allow for collection from pre-clinical species such as rodent, and minimize animal use. Additionally, as biological drug development increases, LC-MS assays to quantify both therapeutic and endogenous peptides and proteins are growing. The desire to use small sample volumes presents an even greater challenge for protein and peptide quantification analyses due to the complexity of the workflow and their inherent lower MS sensitivity, relative to small molecules. While some studies are dominated by the need to use sample volumes <50 µL, others require more sample to increase sensitivity. Therefore, there is a need for a simple, kit-based approach that accommodates a range of plasma volumes and is easily implemented by scientists unfamiliar with the workflows.

Recently there has been a trend towards LC-MS for the bioanalytical quantification of peptide and protein therapeutics due to the many benefits it affords (multiplexing, improved specificity, broader dynamic range and fast method development time). However, protein quantification challenges still exist with regard to assay sensitivity, small sample volume requirements, and laborious and often complex workflows. This is especially in contrast to ligand binding assays. Thus, there is a strong need for simpler and more broadly applicable standardized workflows, ideal for low sample volume assays (<50 µL), for the quantification of proteins.

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

THE SOLUTION

LC-MS for quantification of therapeutic and endogenous proteins is steadily gaining in its use, and unlike small molecule LC-MS analysis, it presents many new and unique challenges. Typically one needs to perform an enzymatic digestion (most commonly trypsin) in plasma, followed by quantification of one or multiple representative tryptic peptides using multiple reaction monitoring (MRM). There is no single standard work flow for this task, and it is often traditional "small molecule" scientists faced with learning and implementing these unfamiliar workflows, which can be complex and laborious. Required sample volumes will vary depending on assay or sensitivity requirements. This can make it difficult to know where to start, particularly if one considers the diversity of proteins, as they can vary greatly in their size, structure, and amino acid sequence. For this reason, we have developed a fully flexible yet generic, kit-based sample preparation strategy using the ProteinWorks eXpress Direct Digest Kit (p/n 176003688) for the simultaneous quantification of the monoclonal antibody drugs: infliximab, adalimumab, bevacizumab, and trastuzumab with plasma volumes ≤70 µL. In short, the mAb's were spiked into plasma at concentrations between 5–50 µg/mL. Sample aliquots of 15, 35, and 70 µL of the mAb spiked plasma were then directly digested with the ProteinWorks eXpress Direct Digest Kit using the generic protocol provided. For all plasma sample volumes, and using the ProteinWorks kits for digestion, standard curves for all peptides, from all mAb drugs, were linear with R^2 ≥0.99, using 1/x weighting. Mean % accuracies of the standard curve points were >99% (Table 1). Standard curves arising from a range of starting plasma volumes are shown in Figure 1, Panels A–C. The representative infliximab tryptic peptide (DILLTQSPAILSVSPGER) is shown as an example. The chromatographic performance is highlighted in Figure 2, panels A–D. Multiple calibration levels from representative signature peptides of infliximab, bevacizumab, trastuzumab, and adalimumab are shown.

Table 1. Linear dynamic range and standard curve statistics for the various plasma digestion volumes, for infliximab, adalimumab, trastuzumab, and bevacizumab using the ProteinWorks eXpress Direct Digest Kit.

Protein	Peptide	Linear fit (r^2) with 1/x weighting			Mean % accuracy		
Standard curve range 5.0–50.0 (µg/mL)		15 µL plasma	35 µL plasma	70 µL plasma	15 µL plasma	35 µL plasma	70 µL plasma
Infliximab	SINSATHYAESVK	0.999	0.999	0.997	100.00	99.99	99.99
	DILLTQSPAILSVSPGER	0.999	0.998	0.994	99.99	100.00	100.01
Trastuzumab	FTISADTSK	0.997	0.993	0.998	100.00	100.01	99.99
	DTYIHWVR	0.995	0.995	0.996	100.00	100.02	100.02
	IYPTNGYTR	0.998	0.996	0.991	99.98	99.98	98.79
Bevacizumab	STAYLQMNSLR	0.999	0.998	0.995	100.00	100.01	99.99
	FTFSLDTSK	0.999	0.999	0.993	100.02	100.00	100.00
Adalimumab	APYTFGQGTK	0.994	0.997	0.995	99.99	99.99	99.99
	NYLAWYQQKPGK	0.997	0.998	0.999	99.99	100.02	100.01

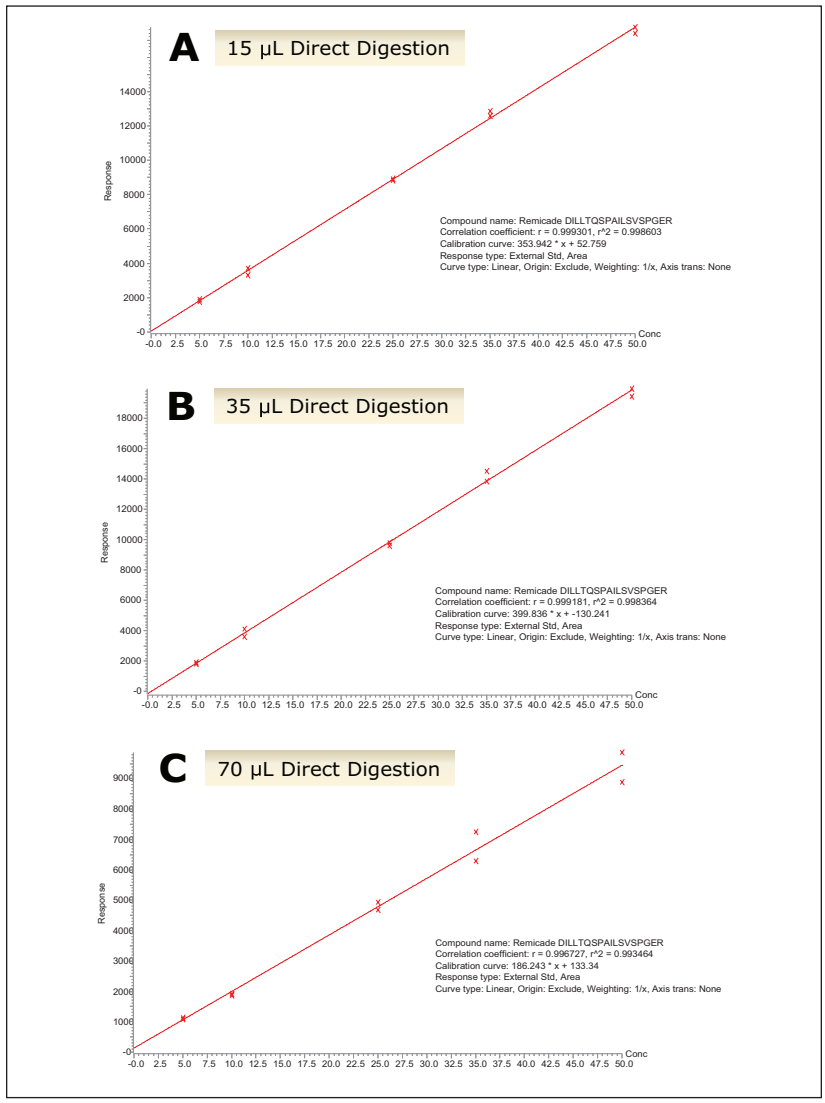


Figure 1. Representative calibration curves arising from digestion of 15, 35, and 70 µL of plasma. The infliximab signature peptide DILLTQSPAILSVSPGER is shown as an example.

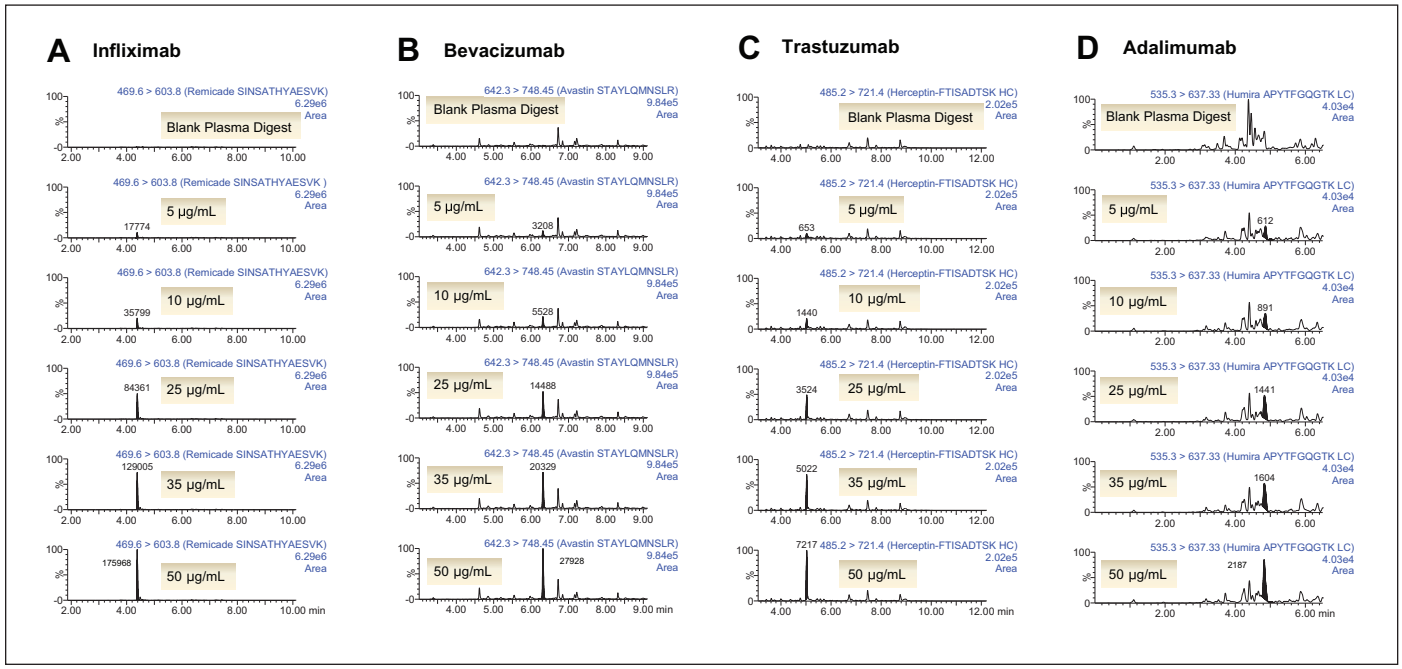


Figure 2. Chromatograms from calibration levels (5–50 µg/mL) of infliximab, bevacizumab, trastuzumab, and adalimumab (15 µL starting plasma) are shown in panels A–D, respectively.

SUMMARY

In this study, a flexible 'kit-based' approach, using a single protocol which accommodates a range of sample volumes (15–70 µL) eliminated the need for method development in discovery studies, and facilitated the accurate quantification of 4 monoclonal antibody drugs.

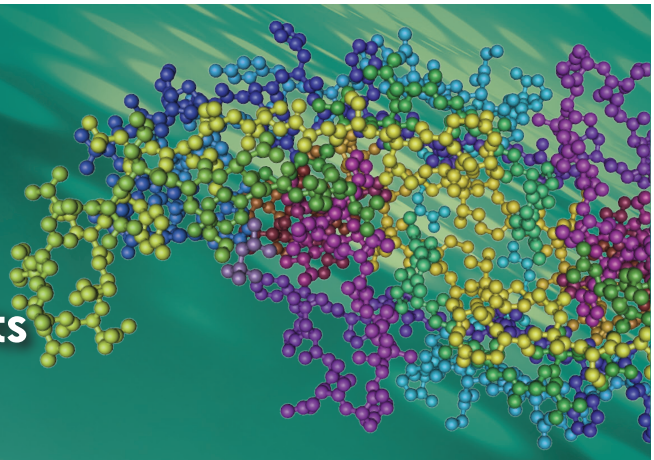
Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

[TECHNOLOGY BRIEF]

LC-MS Quantification of Trastuzumab Using Pellet Digestion and ProteinWorks eXpress Direct Digest Kits



Mary Lame, Paula Orens, Sherri Naughton, and Erin Chambers
Waters Corporation, Milford, MA, USA

GOAL

To demonstrate the compatibility of ProteinWorks™ Kits with pellet digestion and to show improved sensitivity/selectivity for the monoclonal antibody (mAb) trastuzumab (Herceptin) by combining pellet digestion and ProteinWorks eXpress Direct Digest Kits.

BACKGROUND

Due to the similarity of therapeutic proteins with each other and other plasma/serum proteins, there are often significant matrix and isobaric interferences which can severely limit selectivity and sensitivity of a quantitative LC-MS assay for antibody and other protein-based drugs. One of the major culprits in this respect is serum albumin. Present at 35–50 mg/mL, albumin not only interferes significantly and causes ion suppression chromatographically, but its presence can also reduce digestion efficiency, and at the very least, increases the amount of enzyme required, adding significant cost to the assay. For this reason, additional clean-up strategies (e.g., protein precipitation, immuno-capture with Protein A/G or specific capture reagents, solid phase extraction, and molecular weight cut-off filters) are often employed to reduce sample complexity and to impart additional specificity and sensitivity. Of these techniques, protein precipitation (PPT) with organic solvents prior to protein digestion is the most attractive. PPT results in an aqueous/organic layer containing small molecules, phospholipids,

Protein quantification workflows are often complex and laborious, and extensive sample clean-up to achieve specificity and sensitivity for accurate quantification from complex biological matrices is required. While affinity purification at the protein level can significantly increase the ultimate sensitivity of the assay, the cost and time necessary are not realistic for discovery studies. Identification and implementation of a simpler pre-fractionation step, such as protein precipitation, can improve sensitivity, while a standardized, kit-based approach minimizes method development. Both provide a common generic option for inexperienced analysts to achieve sensitive, accurate and robust quantification of proteins.

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

salts, and some proteins. The pellet typically contains larger precipitated proteins. Depending on the choice of organic and the ratio of organic to plasma, it is possible to precipitate proteins such as antibodies, while maintaining solubility of the majority of albumins in the liquid layer so that they may be removed. This has the advantage of being a simple, inexpensive, and fast way to enrich antibodies and other large proteins while removing the majority of interfering albumins, detergents, small proteins, phospholipids and other endogenous components of biological matrices. Ultimately, incorporating this option into a generic, yet standardized LC-MS approach for protein bioanalysis enables novice scientists to more successfully support discovery studies.

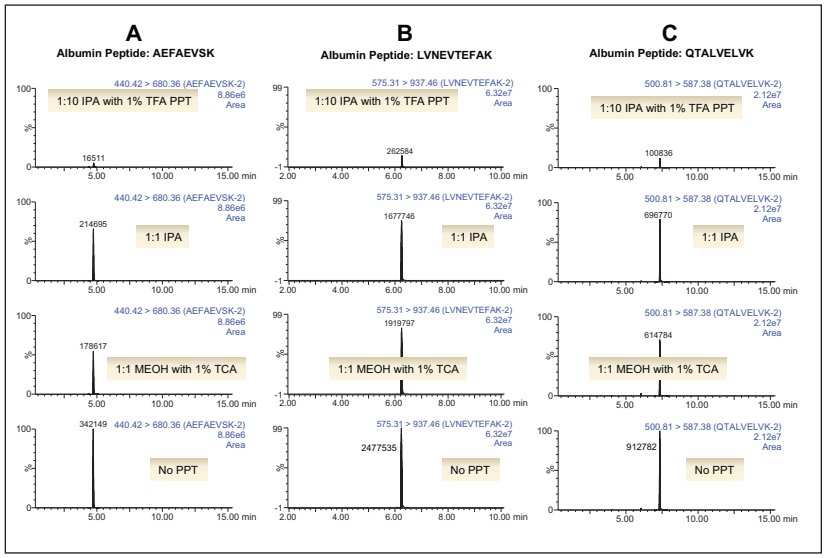


Figure 1. Chromatograms showing albumin depletion (three representative peptides) following PPT pre-treatment and digestion using the ProteinWorks eXpress Direct Digest Kit. Results of the three PPT pre-treatments, compared to no PPT, and are shown in panels A–C, respectively.

THE SOLUTION

Preliminary experiments were conducted using a series of PPT conditions such as varying the starting plasma volume, ratio of organic to plasma, nature of organic, and time and rate of centrifugation. To measure performance, raw area counts from tryptic peptides of both human serum albumin (HSA) and trastuzumab were monitored. Conditions which produced the greatest removal in albumin (as represented by a reduction in albumin peptide area counts) while maintaining or improving trastuzumab sensitivity/recovery (as represented by increased trastuzumab peptide area counts) were progressed for further study.

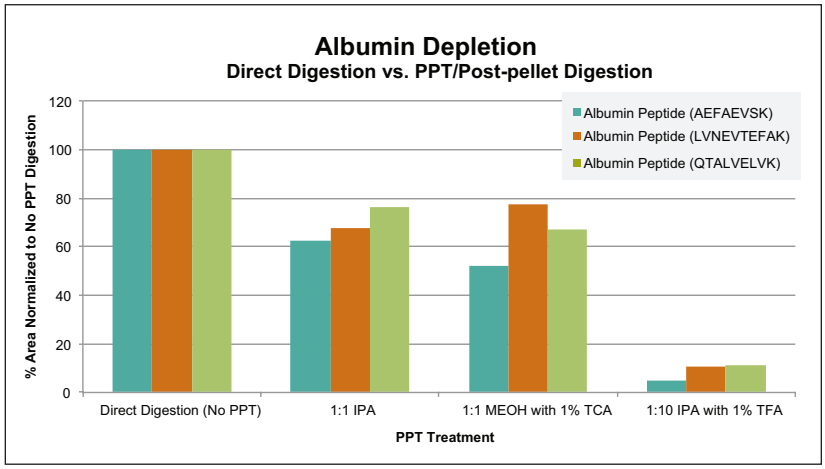


Figure 2. Bar Graph demonstrating reduction of representative albumin peptides (AEFAEVSK, LVNEVTEFAK, and QTALVELVK), resulting from preparation using ProteinWorks eXpress Direct Digest Kits with and without PPT pre-treatment.

Following PPT of plasma samples (15 µL), pellets were re-suspended with buffer and subsequently digested using the ProteinWorks eXpress Direct Digest kit and protocol. Using a targeted UPLC-MS/MS method for the representative tryptic peptides (mAb and albumin), digested plasma samples with and without the PPT pre-treatment were analyzed. Of the various plasma PPT pre-treatments, three PPT conditions (1:1 isopropanol, 1:1 methanol containing 1% trichloroacetic acid, and 1:10 isopropanol containing 1% trifluoroacetic acid) were chosen based on sensitivity increases in the mAb tryptic peptides (area count) and reduction of several human

serum albumin peptides (area count) when compared to non-PPT treated plasma samples. The reduction of 3 particular albumin peptides (AEFAEVSK, LVNEVTEFAK, and QTALVELVK) with the various PPT treatments is demonstrated in Figure 1, panels A–C. While all 3 treatments provided some degree of albumin removal, PPT with isopropanol (IPA) with 1% trifluoroacetic acid (TFA), at a ratio of 1:10, provided the most effective removal of the albumin peptides (>90%), as shown in Figure 2. Area counts for all 3 unique tryptic peptides from trastuzumab increased significantly using any of the top three PPT conditions (Figure 3). In fact, for the three best PPT pre-treatments, peptide area counts increased 2–8x on average, as is demonstrated in Figure 4.

Standard curves were then prepared in plasma. 15 µL aliquots were subjected to the different PPT conditions, followed by the generic ProteinWorks eXpress Direct Digest Kit and Protocol. Three unique trastuzumab peptides were assessed for linearity and accuracy as an initial gauge of performance. For all PPT treatments, and using the ProteinWorks kits for digestion, standard curves for all peptides were linear with R^2 ≥0.99, using 1/x weighting. Mean % accuracies of the standard curve points were >99% (Table 1). Representative calibration curves for the three trastuzumab tryptic peptides (DTYIHWVR, IYPTNGYTR, and FTISADTSK) prepared using PPT pre-treatment (1:10 IPA containing 1% TFA) of the plasma samples and pellet digestion with ProteinWorks eXpress Direct Digest Kits, are illustrated in Figure 5, panels A–C.

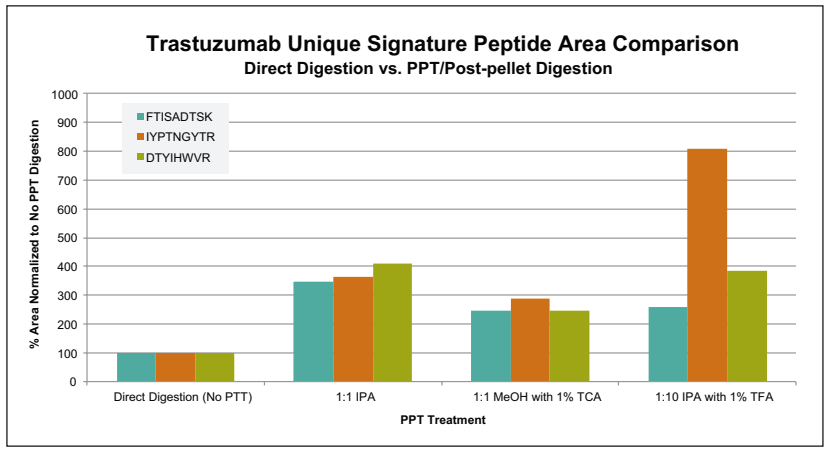


Figure 3. Bar Graph demonstrating improved area counts for 3 unique tryptic peptides of trastuzumab, resulting from preparation using ProteinWorks eXpress Direct Digest Kits with and without PPT pre-treatment.

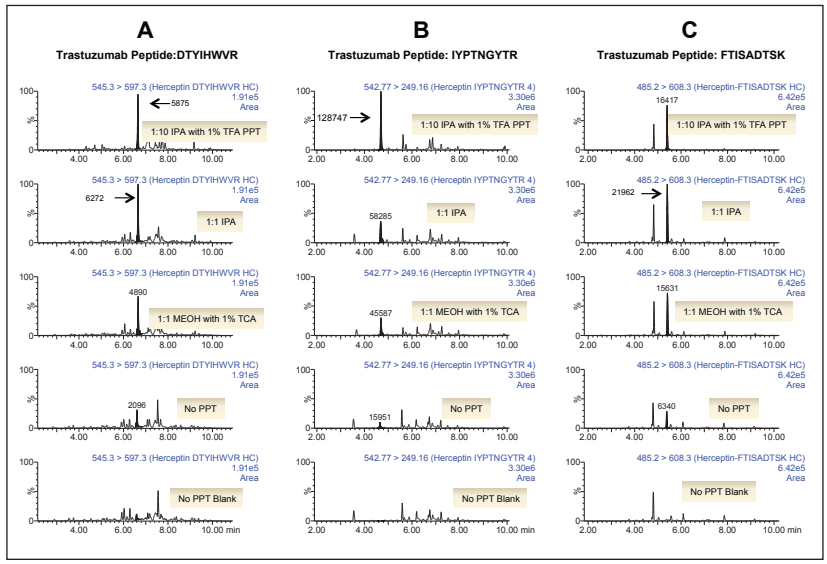


Figure 4. Chromatograms demonstrating improved chromatographic performance and intensity of 3 unique tryptic peptides of trastuzumab, resulting from preparation using ProteinWorks eXpress Direct Digest Kits with and without PPT pre-treatment.

Table 1. Linear dynamic range and standard curve statistics for signature peptides used to quantify trastuzumab in plasma following a PPT and pellet digestion with ProteinWorks eXpress Direct Digest Kits

Protein	Peptide	Linear fit (r^2) with 1/x weighting			Mean % accuracy		
Standard curve range 5.0-50.0 (µg/mL)		15 µL plasma IPA with 1% TFA (1:10)	15 µL plasma IPA (1:1)	15 µL plasma MeOH with 1% TCA (1:1)	15 µL plasma IPA with 1% TFA (1:10)	15 µL plasma IPA (1:1)	15 µL plasma MeOH with 1% TCA (1:1)
Trastuzumab	FTISADTSK	0.996	0.997	0.992	99.98	99.99	98.64
	DTYIHWVR	0.994	0.995	0.993	100.00	100.01	98.73
	IYPTNGYTR	0.999	0.997	0.995	100.00	100.01	99.31

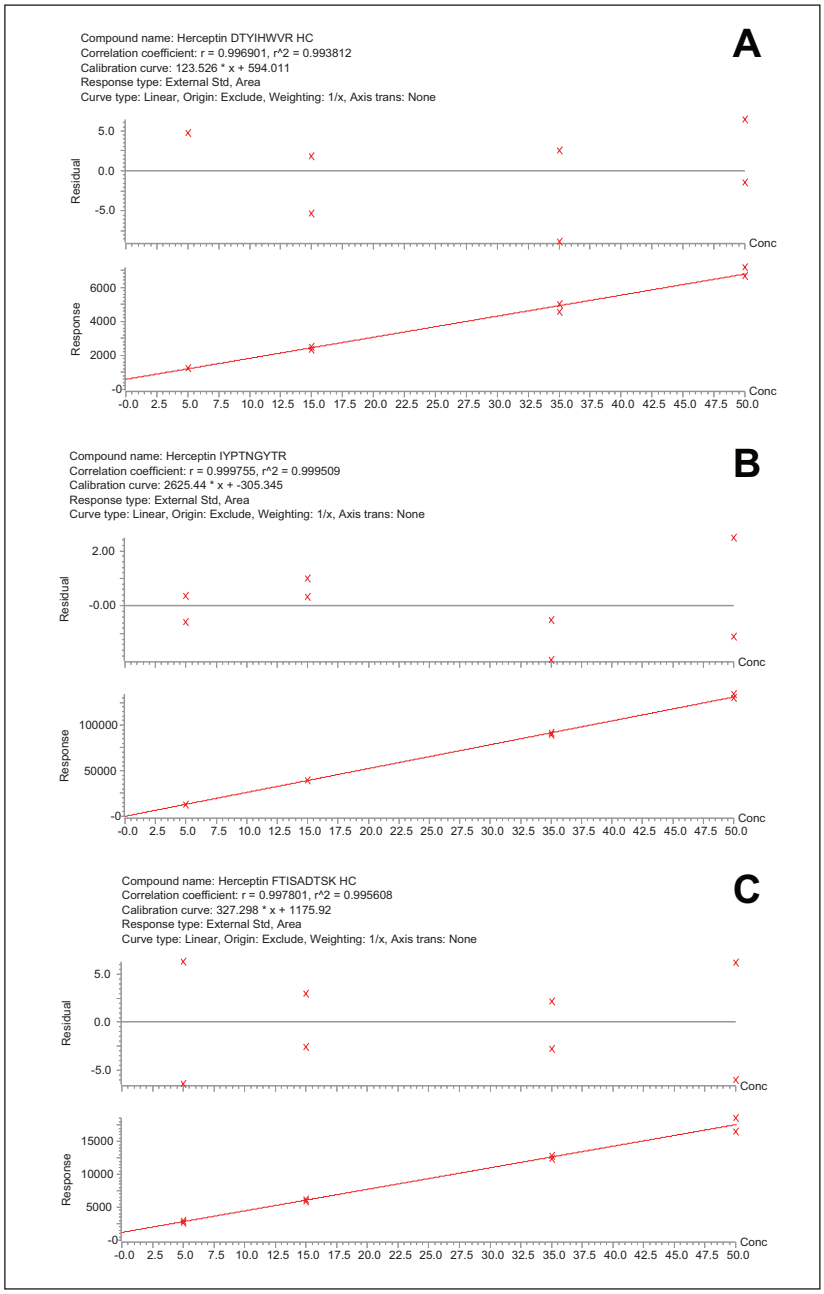


Figure 5. Representative calibration curves for the three tryptic peptides (DTYIHWVR, IYPTNGYTR, and FTISADTSK) of trastuzumab in plasma following PPT pre-treatment (1:10 isopropanol containing 1% trifluoroacetic acid) and pellet digestion with ProteinWorks eXpress Direct Digest Kits, are shown in panels A-C, respectively.

SUMMARY

We describe here a robust and reproducible pellet digestion and LC-MS/MS methodology to quantify the mAb trastuzumab. A simple PPT clean-up and post-pellet digestion using the ProteinWorks eXpress Direct Digest Kit yields accurate, precise and robust LC-MS quantification of trastuzumab via the surrogate peptide approach. Area counts for trastuzumab peptides increased 2–8x using the above strategy, significantly improving sensitivity and specificity, whilst achieving accurate and reproducible quantitative results.

Waters

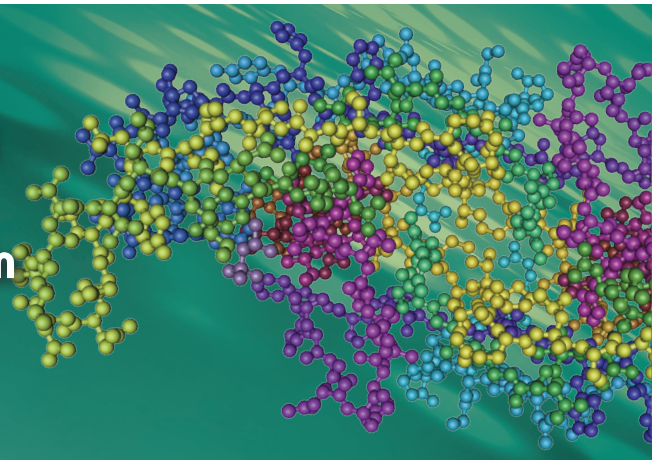
THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

[TECHNOLOGY BRIEF]

An Intact Murine Monoclonal Antibody for Use as a Generic Internal Standard and Workflow Check Standard in Protein Bioanalysis Studies

Authors: Mary Lame, Hua Yang, Sherri Naughton, and Erin Chambers



GOAL

To demonstrate the value of an intact murine monoclonal antibody standard as both a generic internal standard and as a workflow check/system health standard in protein bioanalysis.

BACKGROUND

For those small molecule bioanalytical scientists now faced with performing bioanalysis studies on proteins, the task may be a difficult one. One of the first challenges encountered is the choice of internal standard (IS). While small molecule workflows have become commonplace and are simple and straightforward, protein quantification involves many unfamiliar processes such as digestion and affinity purification. In order to monitor the efficiency of these steps, an appropriate internal standard must be used. Options such as labeled and/or extended tag surrogate/signature peptides are not effective in compensating for or identifying changes in digestion efficiency or upfront, protein-level pull-down. The optimal IS for protein quantification via the surrogate peptide approach would naturally be a protein. While labeled antibody IS's exist such as SILu™Mab, they can be prohibitively expensive.

In addition, protein bioanalysis workflows not only contain many segments and multiple steps within each segment, but a significant fraction of the workflow is comprised of techniques which are new to typical small molecule

Protein quantification workflows are complex and replete with challenges, not the least of which are choice of internal standard and the ability to demonstrate analyst capability. While ligand binding assays (LBAs) have long been the gold standard for large molecule quantification, LC-MS has risen to the top as a technique of choice due to its improved specificity, improved accuracy and precision, and elimination of long development timelines for often poorly standardized biological reagents. In spite of its obvious advantages, LC-MS for protein bioanalysis is relatively new and is often carried out by scientists with traditional small molecule training. For this reason, we have developed a murine monoclonal antibody for use as both a generic internal standard for discovery studies involving antibody-based drugs as well as for use in evaluating staff proficiency with these new workflows.

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

bioanalysts. It is the unfamiliar nature of the steps, coupled with the preparation of numerous specialty (some biological) reagents that creates opportunities for error, imprecision, and poor reproducibility. A well characterized workflow test standard with an accompanying "kit" and protocol recipe would be an ideal way to not only learn and validate the process, but also to demonstrate competence.

THE SOLUTION

We have developed and verified an intact murine (mouse) antibody (p/n 186006552) for use as both a generic internal standard and as a tool, in conjunction with ProteinWorks™ eXpress Digest Kits, to both learn and then test aptitude in protein bioanalysis workflows. This standard can also be used on a periodic basis to confirm system readiness/health for these same workflows. The murine antibody is an ideal internal standard for antibody quantification via the surrogate peptide approach as it is added to the sample prior to any type of sample preparation and, subsequently, efficiently and effectively compensates for changes and or sample spillage throughout every step of these complex multi-stage workflows. This includes its ability to adjust for efficiency, recovery, or other changes in the affinity purification (if used) of the protein drug from plasma. It is also ideally suited to compensate for changes in digestion efficiency or volume as it is essentially an analog of any antibody-based drug, thus making it a suitable generic IS for discovery studies. For use as an IS, we recommend a final concentration in plasma of 20–50 µg/mL.

In order to broaden its utility, we have identified several signature peptides from this murine mAb. Depending on the signature peptide used, one can obtain specificity in human, rat, or other species. For example, while the SVSELPIMHQDWLNGK and VNSAAFPAPIEK peptides from the heavy chain are specific in rat, human and mouse plasma, the MNSLQTDDTAK peptide is specific in human

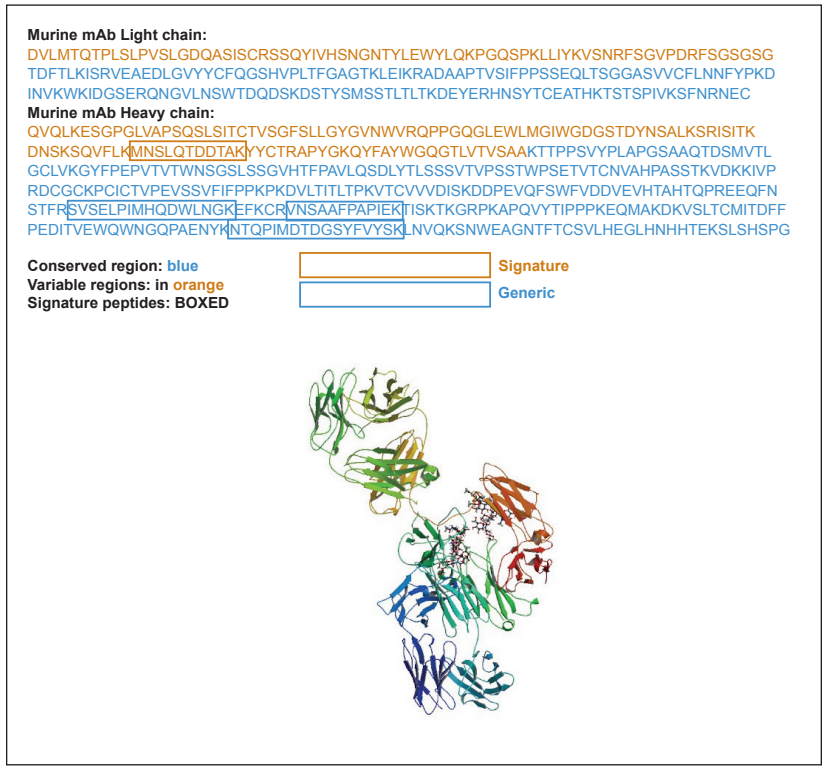
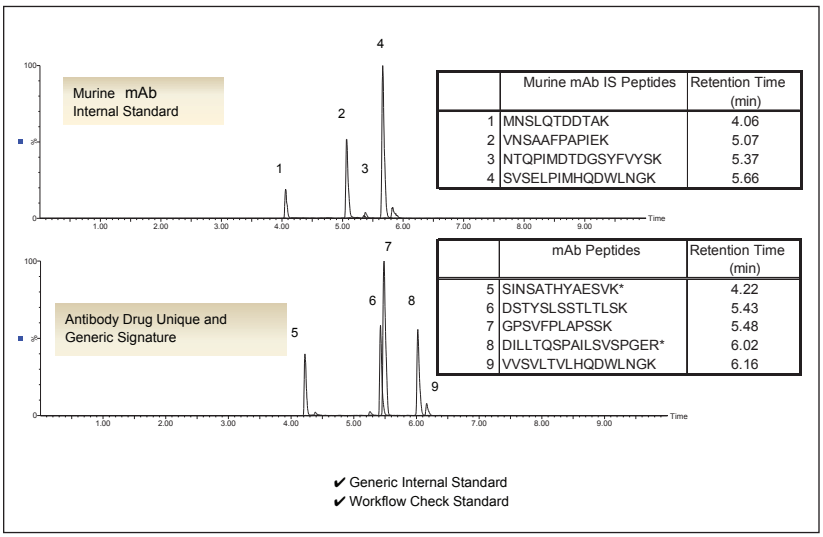


Figure 1. Sequence and signature peptide options for the intact murine antibody standard.

and mouse, but not rat plasma. The final peptide, NTQPIMDTDGSYFVYSK , is also specific in human and mouse plasma, but not necessarily in rat. MRM and chromatographic test conditions are provided in order to confirm and monitor the workflow performance. The sequence and available signature peptides from the intact murine mAb IS/check standard are shown in Figure 1. When the murine mAb is used with and according to the directions of the ProteinWorks eXpress Digest Kits, its signature peptides elute in the same gradient window as both unique and generic signature peptides from humanized monoclonal antibody-type drugs. For example, Figure 2 demonstrates the elution profile of the murine peptides as compared to multiple representative unique and generic signature peptides from mAb therapeutics such as trastuzumab, infliximab, adalimumab, and bevacizumab, following the recommended chromatographic conditions from the ProteinWorks eXpress Digest Kit. Furthermore, as an internal standard, its performance enabled single digit accuracy and precision and a quantification limit of 10 ng/mL for infliximab in Waters Application Note 720005535EN.

SUMMARY

An intact murine (mouse) antibody has been developed and is available for use in protein bioanalysis workflows as either a generic IS or as a workflow test/system health standard, in combination with the specified MRM and LC conditions. In conjunction with a universal, proven protocol and the pre-measured, lot-traceable reagents in ProteinWorks eXpress Digest Kits, one has a recipe which simplifies protein bioanalysis and enables novice users to quickly generate high quality data in discovery studies. In addition, this generic commercially available antibody can be used to evaluate analyst proficiency and to monitor LC-MS system performance.



	Murine mAb IS Peptides	Retention Time (min)
1	MNSLQTDDTAK	4.06
2	VNSAAFPAPIEK	5.07
3	NTQPIMDTDGSYFVYSK	5.37
4	SVSELPIMHQDWLNGK	5.66

	mAb Peptides	Retention Time (min)
5	SINSATHYAESVK*	4.22
6	DSTYSLSSTLTLSK	5.43
7	GPSVFPLAPSSK	5.48
8	DILLTQSPAILSVSPGER*	6.02
9	VVSVLTVLHQDWLNGK	6.16

Figure 2. Chromatographic elution profile for signature peptides from the intact murine mAb check standard (top pane) as compared to signature peptides from various mAb therapeutics (bottom pane).

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Rapid Digestion and Reproducible LC-MS Quantification of Cytochrome C: A Potential Biomarker for Apoptosis

Paula Orens, Mary Lame, and Erin Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

Rapid digestion and quantification of a protein biomarker, demonstrating the flexibility, speed, and reproducibility of a generic kit-based approach.

WATERS SOLUTIONS

ProteinWorks™ eXpress Direct Digest Kit

ProteinWorks µElution SPE Clean-up Kit

CORTECS® UPLC® C_{18+} Column, 1.6 µm, 2.1 mm x 50 mm

ACQUITY UPLC® System

Xevo® TQ-S Mass Spectrometer

KEYWORDS

cytochrome C, biomarker, protein quantification, ProteinWorks, eXpress Direct Digest, µElution SPE, Oasis® MCX, CORTECS, CORTECS UPLC C_{18+}

INTRODUCTION

Cytochrome C (Figure 1),[1] is a mitochondrial protein (~13kDa) which plays important roles in oxidative phosphorylation and apoptosis, or programmed cell death.[2] Elevated plasma concentrations (~2 µg/mL) of circulating cytochrome C have been reported in patients with conditions associated with mitochondrial damage.[3] As a result, the ability to accurately quantify cytochrome C as a potential biomarker is of high interest. Historically, cytochrome C has been quantified using ligand binding assays (LBAs) or western blot analysis. However, use of LC-MS analysis for protein quantification has become more popular in the past few years due to the many benefits it offers (e.g., multiplexing, improved specificity, broader linear dynamic range, and faster method development times). For protein quantification by LC-MS, the bottom up approach using enzymatic digestion (usually trypsin) and analysis of resulting tryptic peptides is often employed. However, these protein digestion workflows are complex and time consuming, with enzymatic digestions often taking upwards of 24 hours to achieve sensitive and accurate quantification from complex biological matrices. Thus, there is a strong need for simpler, more standardized LC-MS workflows. In this application note, we describe a fast (10-minute) digestion using the ProteinWorks eXpress Direct Digest Kit and post digest peptide clean-up using ProteinWorks µElution SPE Clean-up Kit for the accurate quantification of cytochrome C in plasma.

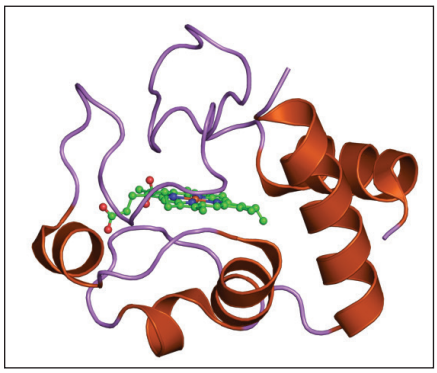


Figure 1. Cytochrome C protein structure.

EXPERIMENTAL

Sample description

To prepare standards and quality control (QC) samples, cytochrome C (derived from bovine heart) was spiked into plasma at various concentrations (0.5–250.0 µg/mL). Plasma samples (35 µL) were digested for 10 minutes using the ProteinWorks eXpress Direct Digest kit. Post digestion purification of signature peptides was completed using the ProteinWorks µElution SPE Clean-up Kit and supplied protocol.

Method conditions

LC system:	ACQUITY UPLC
Detection:	Xevo TQ-S Mass Spectrometer, ESI+
Column:	CORTECS UPLC C_{18+} Column, 1.6 µm, 2.1 mm x 50 mm
Temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Mobile phases:	A: 0.1% Formic Acid in H_2O
	B: 0.1% Formic Acid in ACN

Gradient

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.400	98.00	2.00	6
0.50	0.400	98.00	2.00	6
3.75	0.400	60.00	40.00	6
3.80	0.400	10.00	90.00	6
4.35	0.400	10.00	90.00	6
4.40	0.400	98.00	2.00	6
5.00	0.400	98.00	2.00	6

MS conditions

Capillary (kV):	3
Cone (V):	30
Source offset (V):	50
Source temp. (°C):	150
Desolvation temp. (°C):	600
Cone gas flow (L/Hr):	150
Desolvation gas flow (L/hr):	1000
Collision gas flow (mL/min):	0.15
Nebulizer gas flow (Bar):	7

RESULTS AND DISCUSSION

Mass spectrometry

Preliminary digestion experiments in buffer were performed to identify tryptic peptides and corresponding MRM transitions for cytochrome C quantification. Four unique signature tryptic peptides were identified: TGPNLHGLFGR, MIFAGIK, EDLIAYLK, and GITWGEETLMEYLENPKK. The amino acid sequence of cytochrome C and unique signature peptides (highlighted in orange) can be seen in Figure 2.[4] With the exception of the GITW tryptic peptide, where the triply charged precursor was used, the doubly charged precursors were determined to be the most intense and generated highly specific y ion fragments. MS conditions are summarized in Table 1.

Peptide	MW	pI	Precursor charge state	MRM transition	Cone voltage (V)	Collision energy (eV)	Product ion identification
TGPNLHGLFGR	1168.32	10.2	$[M+2H]^{2+}$	584.82>505.90	30	15	[2H+]1/y9
MIFAGIK	779.01	9.4	$[M+2H]^{2+}$	390.23>534.60	30	10	[1H+]1/y5
EDLIAYLK	964.13	4.3	$[M+2H]^{2+}$	482.77>494.30	30	15	[1H+]1/y4
GITWGEETLMEYLENPKK	2138.43	4.5	$[M+3H]^{3+}$	713.35>840.50	30	15	[2H+]1/y14

Table 1. Final MS conditions for cytochrome C tryptic peptides, including precursor and fragment ions and other physiochemical properties (MWT and pI).

Chromatographic separation

Chromatographic separation of cytochrome C tryptic peptides was achieved using a CORTECS UPLC C_{18+}, 1.6 µm, 2.1 mm x 50 mm Column. CORTECS C_{18+} Columns combine the benefits of solid-core particle technology and a low-level positive surface charge, which provides excellent peak shape, narrow peak widths (<3 secs at base), and resolution from matrix interferences. Representative chromatograms for the four cytochrome C tryptic peptides are shown in Figure 3. Here you can also see that the TGPN peptide (2.12 minutes) elutes immediately before the MIFA peptide (2.16 minutes).

MGDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFSYTDANKN
KGITWGEETLMEYLENPKKYIPGTKMIFAGIKKKGEREDLIAYLKKATNE

Figure 2. Amino acid sequence and structure of cytochrome C (derived from bovine heart). Tryptic peptides used for quantification are highlighted in orange.

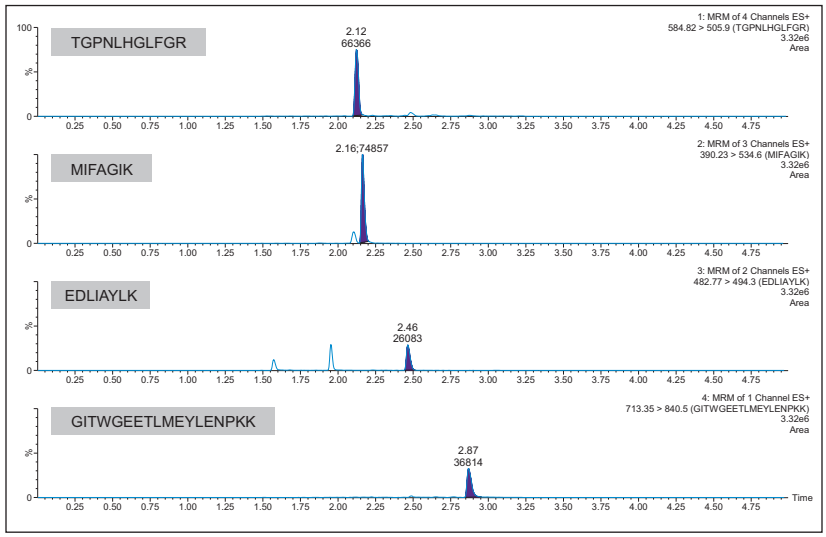


Figure 3. UPLC chromatographic separation of cytochrome C tryptic peptides (mid QC samples), using the CORTECS UPLC C_{18}+ 90Å, 1.6 µm, 2.1 mm x 50 mm Column.

These two peptides share a common precursor and fragment pair (390.23>534.6) derived from different charge states and fragment ions. This common MRM pair corresponds to the 3+ precursor and [2H+]1/y10 fragment for the TGPN peptide and the 2+ precursor and [1H+]1/y5 fragment for the MIFA peptide. Use of the CORTECS UPLC C_{18+} Column ensured chromatographic separation of these peaks that was not obtainable using a BEH C_{18} 300Å (also 2.1 x 50 mm) Column. This is illustrated in Figure 4.

Sample preparation

For tryptic peptide purification, use of a mixed-mode sorbent (reversed-phase and ion-exchange retention) provided enhanced specificity, while the µElution plate format minimized peptide loss (eliminating evaporation and reconstitution). This combination provided excellent recoveries (≥90%) for all four cytochrome C tryptic peptides. A comparison of raw area counts for the MIFA (Panel A) and GITW (Panel B) cytochrome C tryptic peptides with and without SPE clean up is shown in Figure 5. For both peptides, the SPE samples exhibited a peptide area increase of more than 2X, without any form of concentration (SPE sample load volume of 100 µL and final SPE eluate volume of 100 µL). This increase could be the result of eliminating salts or phospholipids not seen in the specific transition.

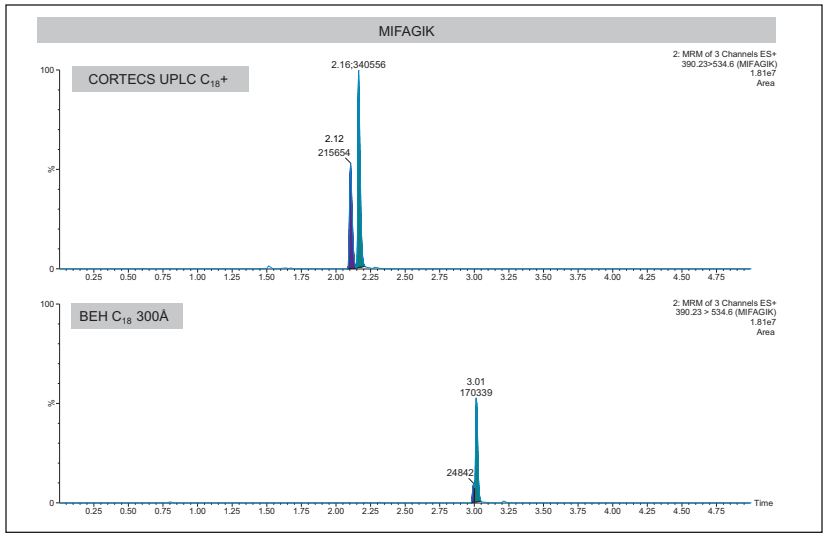


Figure 4. Representative chromatograms demonstrating improved specificity and resolution from interference (RT 2.12) using the CORTECS UPLC C_{18+} for the MIFAGIK (RT 2.16) cytochrome C tryptic peptide.

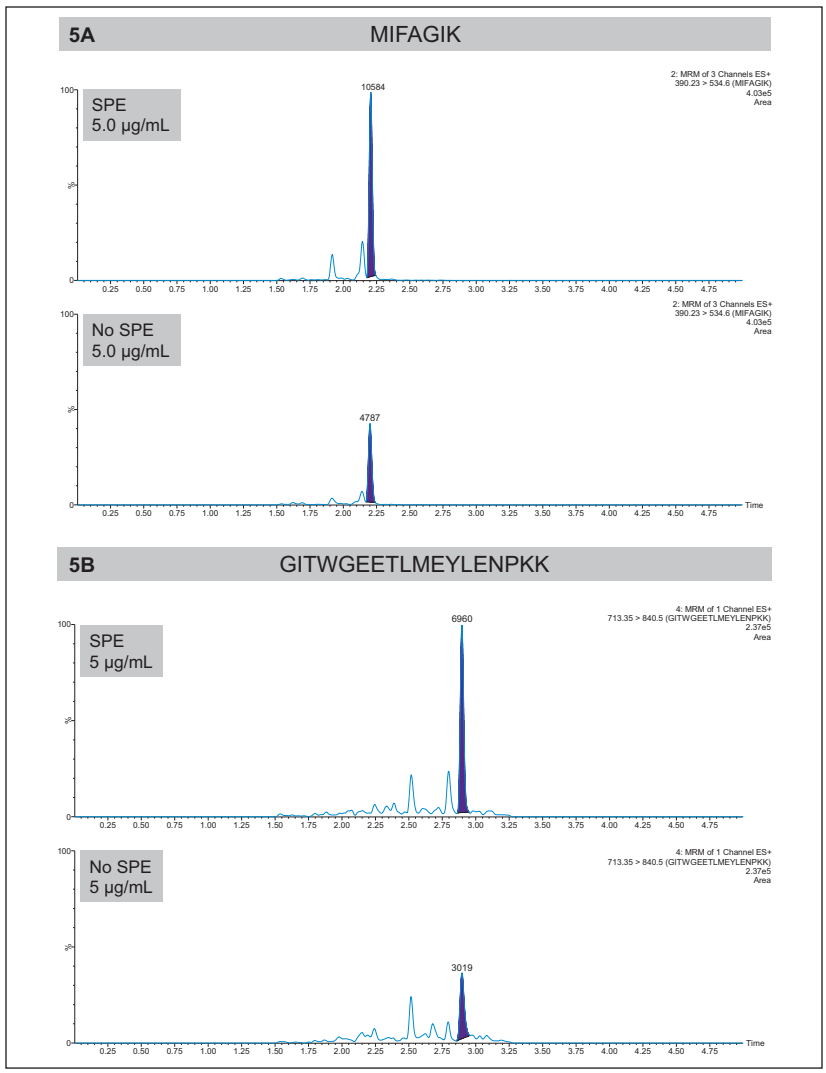


Figure 5. Chromatograms showing the improved sensitivity of the MIFA (A) and GITW (B) peptides following purification with µElution SPE Kit.

Linearity, precision, and accuracy

Following digestion of 35 µL of plasma and subsequent SPE clean-up, excellent linearity and single digit RSDs (relative standard deviation) for duplicate calibration curves were readily achieved. Using 1/x2 regression, the curves from all 4 tryptic peptides were linear with R^2 values >0.99. A summary of standard curve performance is shown in Table 2. Results from QC analysis are shown in Table 3. For all four peptides, at all QC levels, samples demonstrated excellent accuracy and precision with an average %CV of 2.5%. Figure 6, panels A and B, demonstrate sensitivity for plasma samples at 0.5 and 2.0 µg/mL compared to the blank plasma for the EDLI and GITW peptides. Generally, the limit of quantification (LOQ) and limit of detection (LOD) are the concentrations which yield peak area counts ≥5X and 3X of the blank matrix sample, respectively. In the case of the EDLI and GITW peptides at 2.0 µg/mL, the reported LOQ peptide peak areas were 25X and 31X that of the blank plasma sample. The lowest concentration assessed was 0.5 µg/mL and was reported as the LOD. At this concentration the peptide peak areas were 7.5X and 8.3X that of the blank plasma sample. If one were to extrapolate based on the aforementioned criteria, the approximate LOD would be ~0.2 µg/mL.

Peptide	Std. curve range (µg/mL)	Limit of detection (LoD)	Weighting	Linear fit (r^2)	Mean % accuracy of all points
TGPNLHGLFGR	2.0-250	2.0	1/x2	0.996	100.01
MIFAGIK	2.0-250	0.5	1/x2	0.998	100.86
EDLIAYLK	2.0-250	0.5	1/x2	0.999	99.99
GITWGEETLMEYLENPKK	2.0-250	0.5	1/x2	0.999	100.00

Table 2. Linear dynamic range and standard curve statistics for the cytochrome C tryptic peptides used for quantification. Plasma samples were digested and extracted using ProteinWorks eXpress Direct Digest and µElution SPE Clean-Up Kits.

Peptide	QC conc. (µg/mL)	Mean QC conc. (µg/mL)	Std. dev.	%CV	Mean accuracy
TGPNLHGLFGR	3.000	2.850	0.058	2.03	93.93
	15.000	15.800	0.503	3.19	105.60
	75.000	79.500	1.457	1.83	104.83
	200.000	186.200	2.858	1.54	93.90
MIFAGIK	3.000	2.900	0.058	1.99	95.50
	15.000	15.150	1.136	7.50	102.63
	75.000	81.450	1.513	1.86	108.30
	200.000	194.050	6.058	3.12	98.03
EDLIAYLK	3.000	2.700	0.000	0.00	90.00
	15.000	14.400	0.503	3.50	97.70
	75.000	76.150	0.874	1.15	101.37
	200.000	184.300	6.306	3.42	93.83
GITWGEETLMEYLENPKK	3.000	2.750	0.058	2.10	91.67
	15.000	14.500	0.346	2.39	98.03
	75.000	77.200	1.069	1.39	102.47
	200.000	187.300	6.080	3.25	95.27

Table 3. QC sample statistics for cytochrome C tryptic peptides used to quantify cytochrome C in plasma.

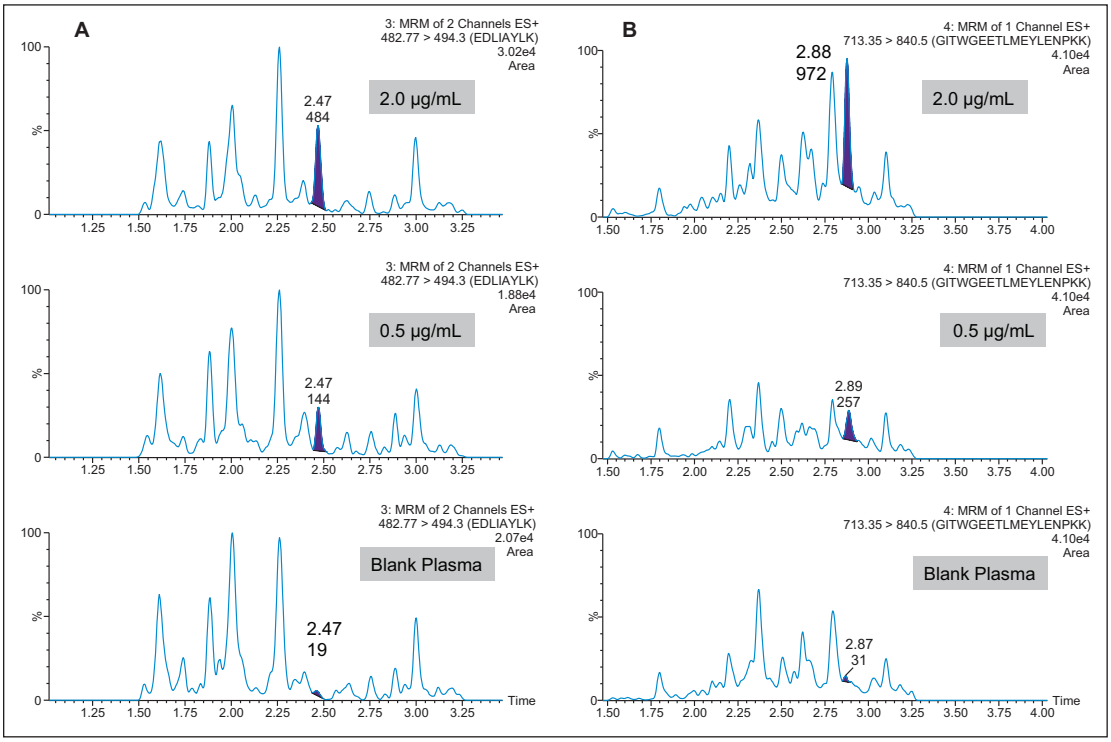


Figure 6. Chromatograms demonstrating sensitivity of the MIFA (A) and GITW (B) peptides, at the LOQ (2.0 µg/mL) and LOD (0.5 µg/mL), respectively.

CONCLUSION

This work demonstrates proof of concept for the rapid, accurate and precise quantification of the protein biomarker, cytochrome C. The combination of the ProteinWorks eXpress Digest Kit (10 minute sample digestion) and µElution SPE Clean-up Kit (15 minute SPE) allows sample preparation to be completed in ~40 minutes, while a fast, 5 minute LC-MS method allows analysis of a full 96-well plate in 8 hours. Using this kitted approach for protein quantification eliminates the need for method development and allows both inexperienced and experienced bioanalytical labs to quickly generate robust, accurate and precise data while also achieving low, single digit reproducibility.

References

1. Cytochrome C Structure (n.d.). In Wikipedia. Retrieved April 14, 2015, from https://en.wikipedia.org/wiki/Cytochrome_c
2. Alberts B, Johnson A, Lewis J, et al. *Molecular Biology of the Cell. 4th edition.* New York: Garland Science; 2002. Programmed Cell Death (Apoptosis) available from: http://www.ncbi.nlm.nih.gov/books/NBK26873/
3. Radhakrishnan J, Wang S, Ayoub IM, Kolarova JD, Levine RF, Gazmuri RJ. Circulating Levels of Cytochrome C after Resuscitation from Cardiac Arrest: A Marker of Mitochondrial Injury and Predictor of Survival. *American journal of physiology heart and circulatory physiology*. 2007;292(2):H767-H775. doi:10.1152/ajpheart.00468.2006.
4. UniPro, (23Jan2007). UniProtKB – 96284 (CYC_BOVIN). Primary accession number:P62894. Retrieved from http://www.uniprot.org/uniprot/P62894

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

A Rapid, Kit-based Approach for LC-MS/MS Quantification of Apolipoprotein A1 in Plasma

Hua Yang, Mary Lame, Sherri Naughton, and Erin Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Rapid digestion time (<30 mins); Total sample preparation time under 1.5 hrs
- Simple and standardized approach to biomarker/endogenous protein quantification
- Excellent linearity, accuracy, and precision

INTRODUCTION

Apolipoprotein A1 (Apo A1) protein is a major component of High Density Lipoprotein (HDL) in plasma. Along with other lipoproteins, Apo A1 plays a key role in lipid metabolism.[1] While typical levels of Apo A1 are >1.2 mg/mL for males and >1.4 mg/mL for females,[2] reduced plasma concentrations of Apo A1 have been reported in patients at risk for certain types of heart disease.[3–6] In addition, in Tangier Disease, which is characterized by a severe reduction in the amount of HDL, Apo A1 plasma concentration is only ~ 1% of those in normal subjects,[7] that is, in the tens of microgram per mL range. Thus, measurements of Apo A1, and other lipoproteins are of high interest as potential biomarkers for cardiovascular disease. Traditionally, proteins have been quantified using ligand binding assays (LBAs). However, these immunoassays suffer from problems such as cross-reactivity, long antibody development time, reagent reliability, etc.

Advances in MS and bioanalytical methods have led to a rapid expansion in the area of MS-based biomarker quantification.[8–10] In addition, LC-MS avoids common LBA shortcomings. In this application note, we showed a fast and standardized kit-based approach that is capable of quantifying Apolipoprotein A1 accurately and reproducibly in plasma.

WATERS SOLUTIONS

ProteinWorks™ eXpress Direct Digest Kits (p/n 176003688)

Acquity UPLC® Peptide BEH C_{18}, 300Å, 1.7 µm, 2.1 mm x 150 mm Column (p/n 186003687)

Acquity UPLC System

Xevo® TQ-S Mass Spectrometer

KEYWORDS

ProteinWorks eXpress Digest, Apolipoprotein A1, protein quantification, biomarker quantification, endogenous protein quantification

EXPERIMENTAL

Sample preparation

Apolipoprotein A1 digestion time course studies:
Human Apo A1 was spiked into rat plasma at a concentration of 1 mg/mL. 15 µL aliquots of the Apo A1 plasma samples were then digested using the ProteinWorks eXpress Direct Digest Kit and 3-step protocol (no reduction and alkylation). Several digestion time points in plasma (5–60 min) were taken to assess digestion performance for the various signature peptides of Apo A1. Subsequent samples were then analyzed by LC-MS.

Quantification of Apo A1 in plasma:
To prepare calibration standards and quality control (QC) samples, various concentrations of human Apo A1 were spiked into rat or human plasma. Stable isotopically labeled Apolipoprotein A1 was used as internal standard (IS). Plasma samples (15 µL) were directly digested (30 min) using the ProteinWorks eXpress Direct Digest kit and the 3-step protocol (no reduction and alkylation).

Method conditions

LC system:	ACQUITY UPLC System
Detection:	Xevo TQ-S Mass Spectrometer, ESI+

LC conditions

Column:	ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7 µm, 2.1 mm x 150 mm
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile

Gradient:

Flow rate (mL/min)	Time (min)	Profile %A	%B	Curve
0.3	0.0	100	0	6
0.3	1.0	100	0	6
0.3	7.0	50	50	6
0.3	8.0	10	90	6
0.3	8.8	10	90	6
0.3	9.0	100	0	6
0.3	15.0	100	0	6

MS conditions

Capillary:	3 kV
Cone (V):	Optimized for individual peptides, see Table 1
Source offset):	50 V
Source temp. (°C):	150 °C
Desolvation temp.:	600 °C
Cone gas flow:	150 L/hr
Desolvation gas flow:	1,000 L/hr
Collision gas flow:	0.15 mL/min
Nebuliser gas flow:	7 Bar
Data management:	MassLynx® (v4.1)

RESULTS AND DISCUSSION

Apolipoprotein A1 (Apo A1), a 28 kDa protein, (Figure 1),[11] has become an important biomarker for predicting cardiovascular risk.[2–7] As a result, there is growing interest in developing and implementing improved tools for its quantification. Using the ProteinWorks Direct Digestion Kit and protocol, unpurified plasma (15 µL) containing Apo A1 was directly digested. LC-MS/MS quantification of signature peptides was performed using a Xevo TQ-S triple quadrupole MS. MRM transitions of the four Apo A1 tryptic peptides are listed in Table 1. These peptides were chosen based on the literature[8] and were optimized for their signal intensity and specificity. Chromatographic separation of Apo A1 peptides was achieved using an ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7 µm, 2.1 mm x 150 mm Column. Representative chromatograms in rat and human plasma are illustrated in Figure 2, Panels A and B, respectively. Due to high endogenous Apo A1 concentration in human plasma, the peptide signal is much higher than the signal in rat plasma. The corresponding internal standards elute at the same retention time as the native peptides (data not shown).

To evaluate Apo A1 digestion efficiency over time, plasma samples (15 µL) containing 1 mg/mL of human Apo A1 were digested using the ProteinWorks eXpress Direct Digest Kit and 3-step protocol. At various time points, an aliquot of sample was quenched and the resulting Apo A1 tryptic peptides were analyzed by LC-MS. The tryptic peptide signals were normalized to the 5 minute time point.

MKAAVLTLAVLFLTGSQARHFWQQDEPPQSPWDRVKDLATVYVDVLKDSGRDYVSQFEG
ALGKQLNLKLLDNWDSVTSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAK
VQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAH
DALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQ
GLLPVLESFKVSFLSALEEYTKKLNTQ

Figure 1. Amino acid sequence of human apolipoprotein A1. Tryptic peptides used for quantification are highlighted in blue.

Peptide	MRM transition	Cone voltage (V)	Collision energy (eV)
ATEHLSTLSEK	608.3>664.4	40	*24*
DLATVYVDVLK	618.3>736.4	12	*12*
VQPYLDDFQK	626.8>1025.5	32	*12*
LLDNWDSVTSTFSK	806.9>971.5	28	*26*

Table 1. Final MS conditions for apolipoprotein A1 tryptic peptides used for quantification.

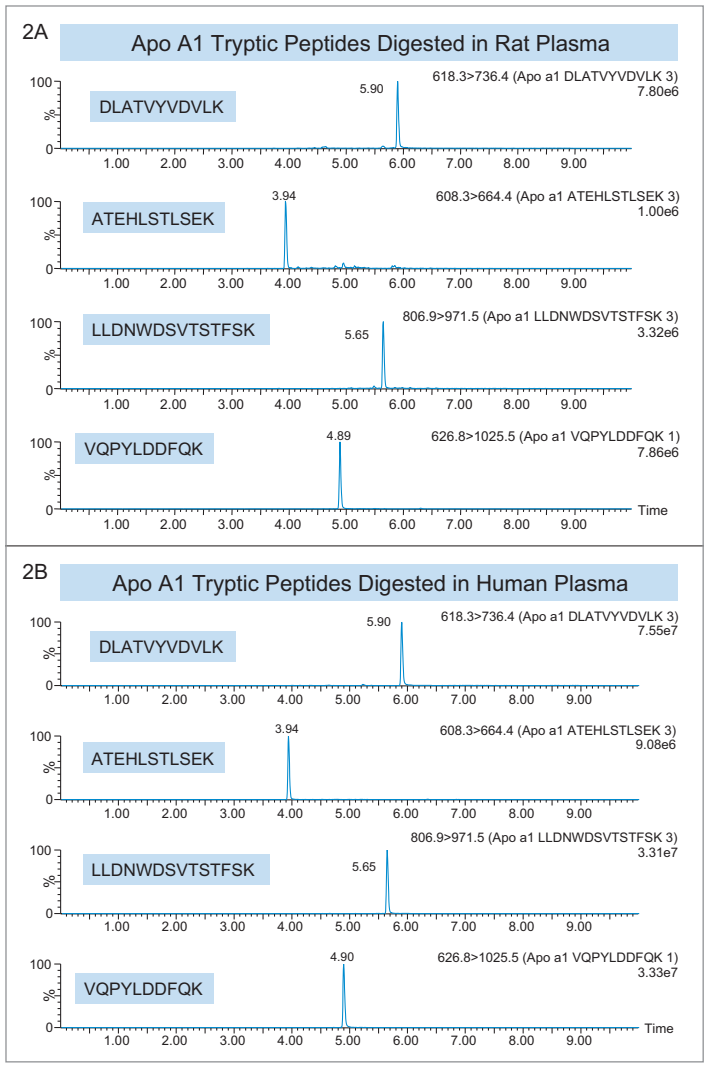


Figure 2. Representative chromatograms of the Apo A1 tryptic peptides in rat (A) and human (B) plasma using the ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7 µm, 2.1 mm x 150 mm Column.

For all 4 signature peptides, the analyte peak areas stabilized within 15 minutes (Figure 3), indicating quantification of Apo A1 can be done by digesting the protein for a very short period of time (~15–30 minutes).

For quantification, calibration and QC samples were prepared by fortifying rat or human plasma with human Apo A1 protein at various concentrations and IS was then added to each sample. Each calibration standard level was prepared in duplicate, while each QC was prepared in triplicate (rat plasma) or in quadruplicate (human plasma). Plasma samples were then digested (30 minutes) using the ProteinWorks eXpress Direct digest kit according to the generic 3-step protocol included in the kit. Finally, digested samples were analyzed by LC-MS. Peak area ratios (PARs) of the analyte peak area to the IS peak were calculated and calibration curves for the four tryptic peptides were constructed using PARs of the calibration samples by applying a one/concentration^2 ($1/x^2$) weighted linear regression model. All QC sample concentrations were then calculated from their PARS against the calibration curve. For all 4 tryptic peptides, Apo A1 calibration curves in rat and human plasma were linear with R^2 values of >0.98, and mean accuracy of the data points >99%. A summary of standard curve performance in rat and human plasma is shown in Tables 2, Panels A and B, respectively.

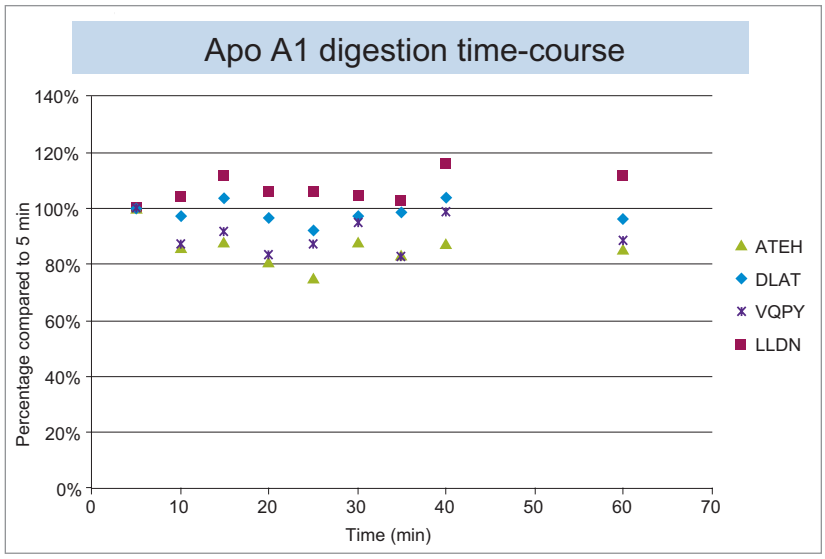


Figure 3. Apo A1 digestion time-course. Responses for Apo A1 tryptic peptides are plotted against the length of digestion time in rat plasma. For all 4 signature peptides, the signals stabilized within 15 minutes.

2A

Peptide	Std. curve range (µg/mL)	Weighting	Linear fit (r^2)	Mean % accuracy of all points
DLATVYVDVLK	5–1500	$1/x^2$	0.99	100.01
ATEHLSTLSEK	5–1500	$1/x^2$	0.98	99.99
LLDNWDSVTSTFSK	5–1500	$1/x^2$	0.99	99.99
VQPYLDDFQK	5–1500	$1/x^2$	0.98	99.99

2B

Peptide	Std. curve range (µg/mL)	Weighting	Linear fit (r^2)	Mean % accuracy of all points
DLATVYVDVLK	100–1000	$1/x^2$	0.99	99.98
ATEHLSTLSEK	100–1000	$1/x^2$	0.98	99.99
LLDNWDSVTSTFSK	100–1000	$1/x^2$	0.99	99.99
VQPYLDDFQK	100–1000	$1/x^2$	0.98	99.99

Table 2. Linear dynamic range and standard curve statistics for the Apo A1 tryptic peptides in rat plasma (A) and human plasma (B) used for quantification. Plasma samples were digested and extracted using ProteinWorks eXpress Direct Digest Kit.

Due to the presence of endogenous Apo A1 in human plasma, the method of standard addition was used for quantification. In this case, the slope and y-intercept from the individual tryptic peptide calibration curves were used to calculate the endogenous Apo A1 concentrations (x-intercept). The calculated endogenous concentration of Apo A1 was then added onto the spiked concentration of Apo A1 in the standard and QC samples to enable accurate assessment of Apo A1 in the human plasma. For all four tryptic peptides, standard curves in plasma were accurate and precise from 5–1500 µg/mL (rat) and 100–1000 µg/mL (human). This is illustrated in Tables 2A and 2B, respectively. The detection limit is estimated to be in the 2.5–5 µg/mL range. At all QC levels, in both rat and human plasma, QC samples demonstrated accuracy and precision with CVs ≤15%. QC performance for the DLAT tryptic peptide in rat and human plasma is highlighted in Tables 3 and 4, respectively, and is illustrated in Figure 4, Panels A and B, respectively. For the blank rat plasma digest, there is no detection of the human Apo A1 peptide (rat Apo A1 amino acid sequence is different than human Apo A1 sequence); while in human plasma blank digest, there is a strong signal of the human Apo A1 peptide, due to the high concentration of endogenous Apo A1 in human plasma. For the DLAT, ATEH, LLDN and VQPY tryptic peptides, mean endogenous levels of Apo A1 in human plasma were determined to be 589.02, 603.74, 549.72, and 552.99 µg/mL, respectively (Table 5). These values are lower than the typical reported levels (>1.2 mg/ml for males and >1.4 mg/ml for females).[2] Sub-optimal pre-analytical collection and storage of the plasma samples could be a plausible explanation for the lower endogenous plasma concentrations observed herein. It has been reported that pre-analytical sample collection and storage can affect the stability of Apo A1 in plasma. For example, Pasella et al. found that Apo A1 decreases in abundance when stored at 4 °C for 13 days.[12] The fact that the commercially available plasma that was used in this application was stored at 4 °C for more than 2 weeks supports the above hypothesis. Regardless, our method demonstrates excellent accuracy and precision for Apo A1 quantification, with a dynamic range which is well-suited for measuring Apo A1 levels in both normal and disease conditions.

Peptide	**Human** Apo A1 over-spiked QC conc. (µg/mL)	Mean Cal. **Human** Apo A1 conc. in **Rat** plasma (µg/mL)	Std. dev.	%CV	Mean %accuracy	# of QCs passed
DLATVYVDVLK	18	15.46	0.12	0.78%	85.90%	3 out of 3
	180	166.35	7.34	4.41%	92.43%	3 out of 3
	1200	1268.65	71.12	5.61%	105.70%	2 out of 3

Table 3. QC sample statistics for the DLAT tryptic peptides used to quantify Apo A1 in rat plasma.

Peptide	**Human** Apo A1 overspiked QC conc. (µg/mL)	Mean cal. **Human** Apo A1 conc. in **Human** plasma (µg/mL)	Mean cal. conc. (µg/mL)	Std. dev.	%CV	Mean %accuracy	# of QCs passed
DLATVYVDVLK	0	601.48 (endogenous)	589.02	12.77	2.17%	97.92%	6 out of 6
	75	676.48	723.58	26.85	3.71%	106.98%	4 out of 4
	150	751.48	777.14	39.39	5.07%	103.43%	4 out of 4
	350	951.48	924.38	33.31	3.60%	97.15%	4 out of 4
	750	1351.48	1360.39	85.60	6.29%	100.68%	4 out of 4

Table 4. QC sample statistics for the DLAT tryptic peptides used to quantify Apo A1 in human plasma.

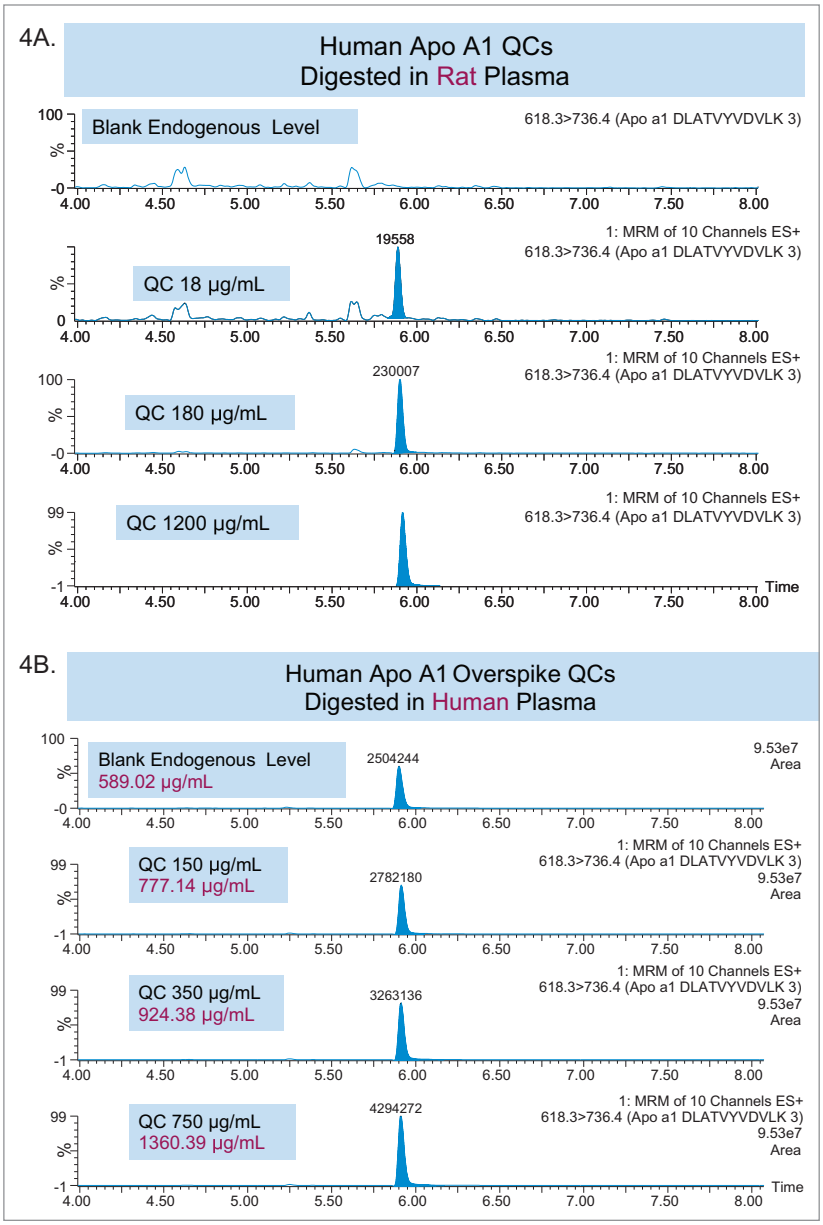


Figure 4. QC chromatograms for the DLAT tryptic peptide of Apo A1 in rat (A) and human (B) plasma.

Peptide	Mean (N=6) Calculated Endogenous Human Apo A1 Concentration (µg/mL)
DLATVYVDVLK	589.02
ATEHLSTLSEK	603.74
LLDNWDSVTSTFSK	549.72
VQPYLDDFQK	552.99

Table 5. Calculated endogenous Apo A1 concentrations in human plasma for the various tryptic peptides of Apo A1.

CONCLUSIONS

This work demonstrates the accurate and precise quantification of the endogenous protein, Apolipoprotein A1 in plasma. Using the ProteinWorks eXpress Direct Digest Kit with 3-step protocol, the total sample preparation time was under 1.5 hrs. Through direct digestion of 15 µL of plasma, quantification limits of 5–1500 µg/mL were achieved, while maintaining linearity, precision and accuracy. This method easily distinguishes 5 µg/mL changes in Apo A1 in rat plasma and changes of 75 µg/mL over the endogenous level in human plasma. We've demonstrated that using a simple kit-based approach can eliminate the need for method development and allow bioanalytical labs with limited experience to quickly generate robust, accurate and precise data.

References

1. Wasan et al., *Nature Reviews Drug Discovery*, 7(1), 84–99, 2008.
2. Apolipoprotein A1, plasma values. May 25, 2016 from http://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/80309
3. Genest et al., *JACC*, 1992, Vol. 19, No.4, 792–802.
4. Dawar et al., *International J. Pharma and Bio Sciences*, 2010, V1(2), Biological Science, No. 43.
5. Ashmaig et al., *J Vasc Bras.* 2011, 10(4): 293–297.
6. Shilpasree et al. *J Clin Diagn Res*., 2013, 7(7): 1303–1306.
7. Schaefer et al., *N Engl J Med*, 1978, 299,:905–910.
8. Wang et al., *Bioanalysis*, 2015, Vol. 7, No. 22, 2895–2911.
9. Lassman et al., *Rapid Commun. Mass Spectro.* 2012, 26, 101–108.
10. Agger et al., *Clin. Chem*., 2010, 56(12), 1804–1813.
11. UniPro, (23Jan2007). UniProtKB – P02647 (APOA1_HUMAN). Primary accession number:P02647. Retrieved from http://www.uniprot.org/uniprot/P02647
12. Pasella et al., *Proteome Science* 2013, 11:10.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Improving the Detection of Thyroglobulin in Human Plasma for Clinical Research by Combining SISCAPA Enrichment and Microflow LC-MS

Jay S. Johnson,[1] Morteza Razavi,[2] James Murphy,[1] Selena Larkin,[2] and Paul Rainville[1]
[1]Waters Corporation, Milford, MA, USA; [2]SISCAPA Assay Technologies, Washington, DC, USA

APPLICATION BENEFITS

- ionKey/MS™ configured for dual-pump trapping is well suited to analyze SISCAPA eluents
- Sub 1 ng/mL quantitation level of thyroglobulin is achieved using 10x less plasma than the comparable standard flow method
- Accuracy is highly correlated with the values obtained from the standard flow method but offers higher levels of precision LC over 4 replicates
- Dual-pump trapping significantly reduces cycle times to under 7 minutes allowing a similar number of samples to be run in the same time frame as the best in literature standard flow method
- Microflow is a viable and attractive solution for clinical research

WATERS SOLUTIONS

ACQUITY UPLC® M-Class System

ionKey/MS System

Xevo® TQ-S

iKey™ Separation Device

MassLynx® Software

TargetLynx™ Application Manager

KEY WORDS

Thyroglobulin, SISCAPA, UPLC, MRM, TQ-S, ionKey/MS, dual-pump trapping, health sciences, high throughput, iKey Separation Device

INTRODUCTION

Current research immunoassays for Thyroglobulin (Tg) may be subject to high false negative rates in a significant portion of the sample population due to the presence of endogenous anti-Tg autoantibodies (Tg-AAbs) that block the binding epitope resulting in the reporting of a negative result in the immunoassay. The prevalence of these negative results has lead researchers to look for alternative analytical approaches that can improve the quality of the result.

Stable isotope standards and capture by anti-peptide antibodies (SISCAPA) enrichment for Tg combined with standard flow LC-MS has been implemented as an alternative approach in clinical research labs. The high analytical selectivity and specificity of the capture step using anti-peptide antibodies specific for a proteolytic peptide unique to Tg greatly enhances the detection and quantitiation of Tg down to levels of approximately 1 ng/mL or 1.52 amol/µL. However, standard flow LC-MS requires 200–400 µL of plasma to reach these relevant LOQ levels, a very large volume of sample.

Microflow LC-MS, exemplified by the ionKey/MS System, operating at 10's of µL/min offers substantial analytical sensitivity benefits over standard flow using less starting plasma in sample-limited applications.[1] Accordingly, we investigate here if the ionKey/MS System operating in a dual-pump trapping configuration can provide reductions in LLOQ levels for Tg, using less plasma while maintaining the requisite accuracy, precision, and throughput exemplified by published standard flow LC-MS assays. The dual-pump trapping configuration was explicitly chosen due to the ability of the set-up to handle relatively large injection volumes compared to iKey Separation Device volume, reduce carryover coming from the sample loop and trap column, and decrease cycle time by affording load ahead capability on the trap column and independent washing and equilibration of the trap column and the iKey Separation Device.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC M-Class
Analytical column:	iKey Peptide BEH C_{18} Separation Device, 130Å, 1.7 µm, 150 µm x 50 mm (p/n 186006764)
Trap column:	ACQUITY UPLC M-Class Symmetry C_{18} Trap Column, 100Å, 5 µm, 300 µm x 50 mm (p/n 186007498)
iKey temp.:	45 °C
Sample temp.:	12 °C
Injection volume:	20 µL partial loop in a 22.8 µL loop
Flow rate:	3 µL/min
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
WNW:	0.1% formic acid in water
SNW:	2% formic acid in 25/25/25/25 water/acetonitrile/isopropanol/methanol
Gradient:	9.9% B to 27.5% B in 2.2 min
Trap loading:	99.5% A at 50 µL/min for 0.8 min
Total cycle time:	6.75 min injection to injection

MS conditions

MS system:	Xevo TQ-S operating in MRM Mode with Unit Mass Resolution
Ionization mode:	ESI Positive
Capillary voltage:	Optimized through infusion of analyte of interest
Source temp.:	100 °C
Cone gas flow:	50 L/Hr
Nano gas flow:	Off
Collision energy:	Optimized through infusion of analyte of interest, see Table 1
Cone voltage:	Optimized through infusion of analyte of interest, see Table 1

Data management

Chromatography software:	MassLynx v4.1
Quantification software:	TargetLynx

Sample preparation

The generic SISCAPA enrichment workflow coupled with ionKey/MS is detailed in Figure 1. The sample preparation detailed in this application note was performed by SISCAPA Assay Technologies following their recommended procedures.

1. Plasma sample is digested using trypsin
   Any potential auto Tg antibodies are digested along with the target, Tg, to their corresponding peptides.
2. A highly selective and specific antibody against a proteotypic peptide unique to Tg with the amino acid sequence FSPDDSAGASALLR (FSP) is conjugated to a magnetic bead support.
3. A stable isotope standard (SIS) of the FSP peptide and the bead-conjugated antibody is added to the plasma digest.
4. The FSP peptide and SIS are selectively enriched by the anti-peptide antibody bead complex in an automated fashion in the 96-well plate format.
5. The beads are then washed to remove unbound matrix material and the bound peptides are released using acid elution.
6. The resulting eluent is subjected to microflow LC-MS using the conditions described below.

Peptide	Precursor	Product	CE	Cone voltage
FSP.light	708.8	768.5	27	30
FSP.heavy	703.8	758.5	27	30
FSP.light	708.8	697.4	27	30
FSP.heavy	703.8	687.4	27	30
FSP.light	**708.8**	**591.8**	**21**	**30**
FSP.heavy	**703.8**	**586.8**	**21**	**30**

Table 1. Optimized MRM transition parameters for the heavy and light versions of FSP. The qualifier MRMs are shown in bold. These parameters were optimized thru infusion using the onboard fluidics of the Xevo TQ-S and an infusion iKey.

Instrumental set-up

In attempts to decrease cycle time and allow more samples to be run on the ionKey/MS System, the configuration chosen was a dual-pump trapping configuration as shown in Figure 2. In dual-pump trapping a dedicated binary solvent manager plumbed with larger I.D. transfer lines handles the loading of the trap column. A second binary solvent manager is dedicated for gradient elution of the analyte of interest off of the trap column to the iKey Separation Device. Due to the fact that the loading pump is plumbed with larger I.D. transfer lines, this loading step can occur at a faster flow rate without reaching the pressure limit of the system. The optimized loading flow rate in this method was found to be 50 µL/min, however, flow rates of up to 70 µL/min are possible. Furthermore as we employ two dedicated pumps, the loading of the trap column by the loading pump can be overlapped with the equilibration of the iKey Separation Device by the gradient pump, effectively cancelling out the sample loading time from the total cycle time, resulting in considerable analytical time savings. After the set loading time, the valve is switched to the elution configuration as seen in Figure 2 and 3, and the gradient pump forms a gradient that back flushes the analyte off the trap column to the iKey Separation Device. During this elution step the loading pump is in line with the sample loop and can be used to flush the loop at a high flow rate with any mixture of mobile phase which should help manage carryover.

The dual-pump trapping configuration also allows heart cutting type experiments in which the trap is decoupled from the analytical iKey just after the last analyte of interest elutes by switching the trapping valve back into the loading configuration. Decoupling is beneficial from an analytical column and MS optics cleanliness standpoint as any later eluting matrix components such as proteins and phospholipids will be directed to waste.

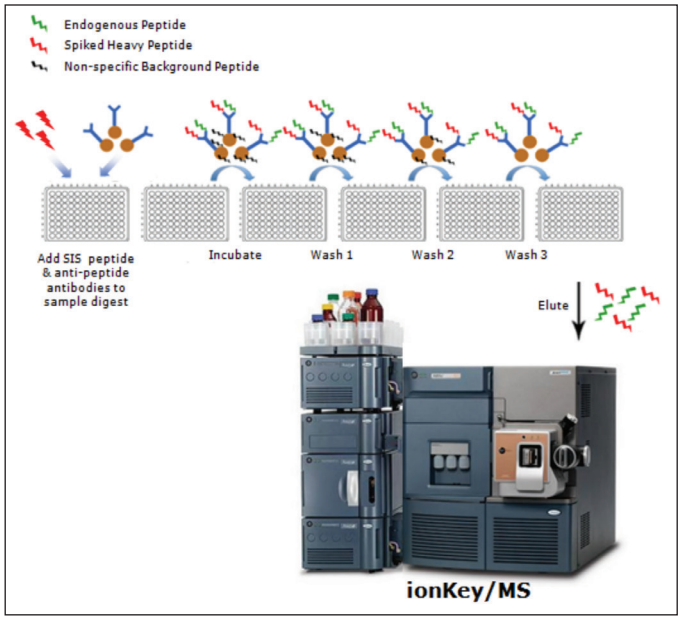
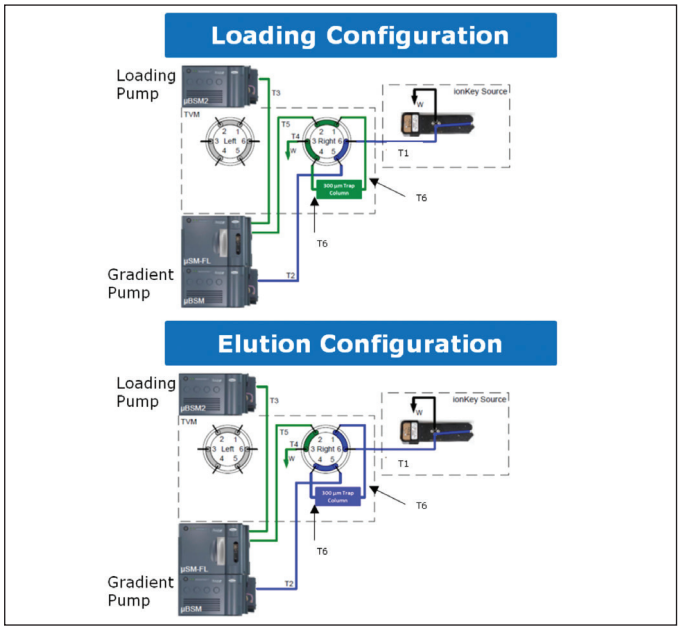


Figure 1. Analytical workflow employed combining SISCAPA enrichment and microflow LC-MS using the ionKey/MS System for the sensitive detection of Tg.

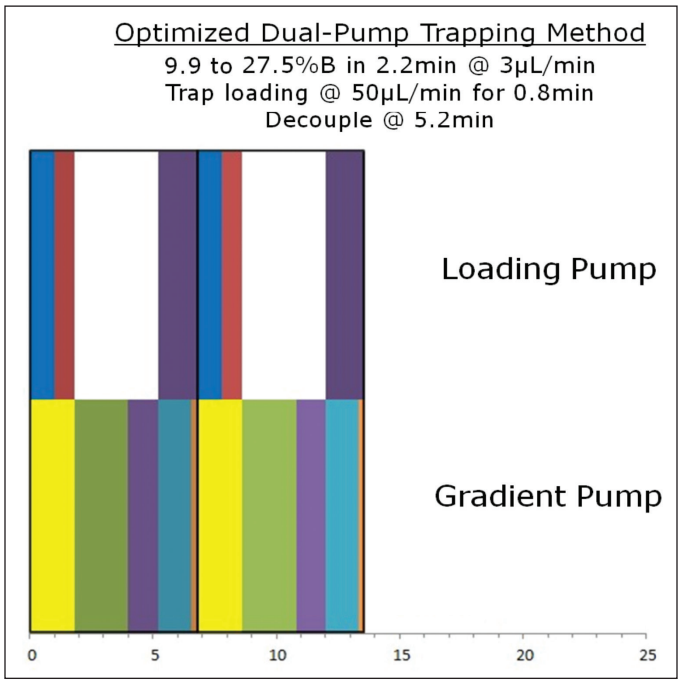


#	Length	ID	Fittings	Part #	Order #
T1	40"	40 µm	V-F	430004188	700010399
T2	30"	25 µm	M-V	430003619	700009872
T3	30"	40 µm	M-V	430003620	700009873
T4	31"	.01"	PEEK	430004090	700009892
T5	26"	40 µm	V-V	430003658	700009881
T6	6"	40 µm	V-V	430003649	700009878

Figure 2. The dual-pump trapping set-up contains a dedicated binary solvent manager for fast sample loading onto the loop and trap column and a dedicated gradient elution pump. In this set-up line T3 has a 40 µm I.D. which affords a loading flow rate of 50 µL/min. The gradient transfer line, T2, remains a 25 µm I.D.

RESULTS AND DISCUSSION

The analytical sensitivity of the ionKey/MS System in the dual-pump trapping configuration, using the parameters defined previously, was first evaluated using synthetic standards of the light and heavy FSP peptides. A 6 point calibration curve was created comprising a concentration range of 2,000 amol/µL down to 0.64 amol/µL utilizing a 1 in 5 dilution with 3% acetonitrile in 0.1% formic acid. Each calibration point was run in triplicate and we observed an excellent linear response and reproducibility over the calibration range as detailed in Figure 4 with the 13 amol level having a coefficient of variation (CV) of approximately 16%. This data reinforces the ability of the platform and method described to analyze synthetic standards of FSP.



Load pump events	Time (min)	Gradient pump events	Time (min)
Trap equilibration/sample loading onto loop	0.95	iKey equilibration	1.75
Trap loading	0.8		
Wash loop	3.4	Gradient	2.2
Wash trap	1.6	Wash iKey	1.2
		Delay volume	1.35
		Housekeeping	0.25
Cycle time	6.75	Cycle time	6.75

Figure 3. Schedule of events in the optimized dual-pump trapping workflow.

amol on column	Avg light area	CV	Avg heavy area	CV
40000	1343852.3	4.9%	1181870.0	4.4%
8000	267515.0	1.1%	234463.0	0.8%
1600	43533.7	4.8%	39211.7	1.5%
320	8312.3	2.0%	7147.7	2.6%
64	1681.0	4.9%	1614.0	1.3%
12.8	246.0	16.3%	254.0	16.4%

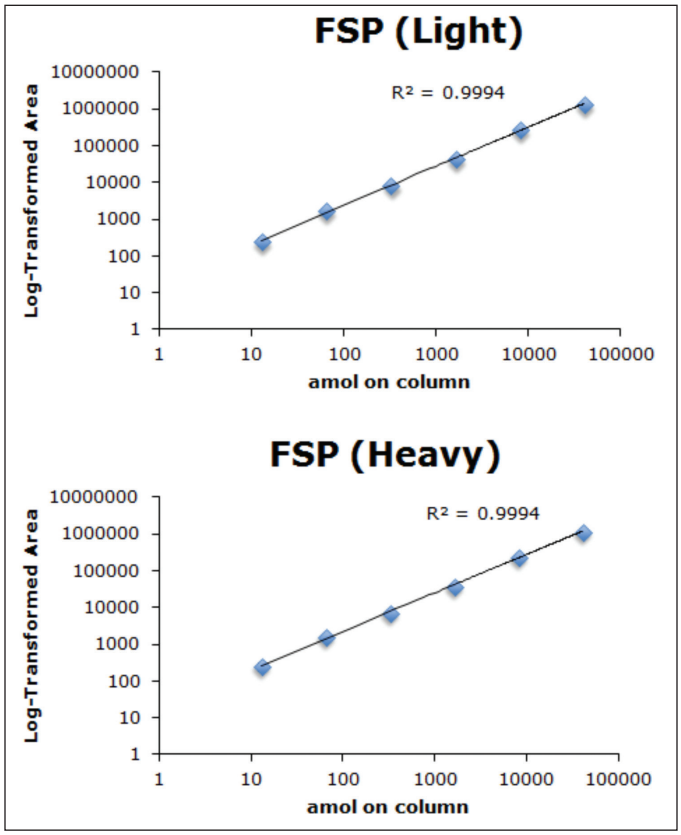


Figure 4. Calibration curve expressed in amol on column for the analysis of the synthetic standards of both the heavy and light versions of FSP. We observed an excellent linear response and reproducibility when working with these standard variants of the peptide.

Next, to demonstrate that the platform is compatible with SISCAPA eluates and can actually detect endogenous Tg in human plasma, we performed a plasma titration experiment where varying amounts of pooled human plasma were digested followed by SISCAPA enrichment of the FSP peptide. Accordingly, it is expected that the PAR value, or the ratio of the endogenous light FSP to the heavy SIS FSP added after digestion at a consistent concentration, should increase linearly when plotted against pooled human plasma amount. The results shown in Figure 5 show the expected linear response was achieved for human plasma amounts down to 40 µL, with no observed backpressure fluctuations in the iKey Separation Device. Accordingly, it can be concluded that the platform is compatible, robust, and analytically sensitive for endogenous FSP in human plasma enriched using the SISCAPA workflow. Furthermore, as a positive control, the experiment was replicated on an Agilent 1290/6490 QqQ instrument operating in the standard flow regime and utilizing the recommended method parameters for the instrument. A high linear correlation of $R^2 = 0.998$ as seen in Figure 6 was achieved between the two platforms confirming the accuracy of the PAR values as measured on the ionKey/MS System. Additional evidence of the agreement between the PAR values obtained on the standard flow and ionKey/MS can be visualized in the Bland-Altman plot seen in Figure 7. Agreement between all measurements is within the 95% confidence interval. Furthermore, the ionKey/MS System showed better precision across 4 replicates than the standard flow system. This suggests microflow offers tangible improvements in the precision of measurement of FSP while maintaining the accuracy expected of the conventional standard flow approach.

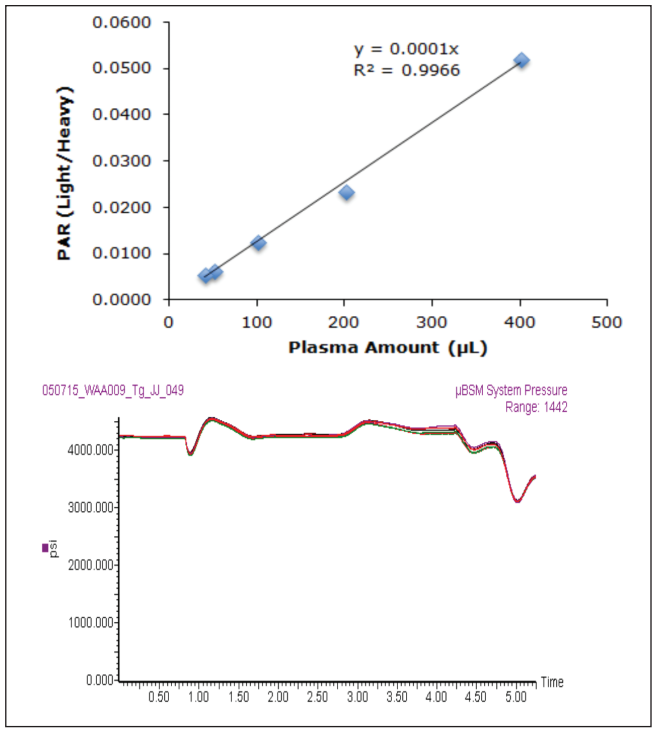
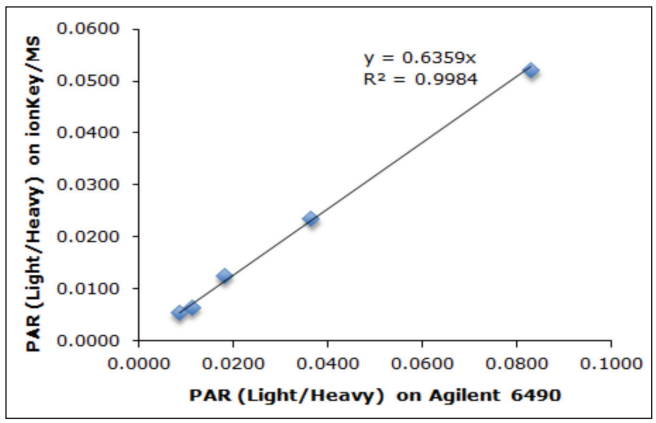


Figure 5. A linear response was achieved for pooled human plasma amounts down to 40 µL with no observed backpressure fluctuations demonstrating the ionKey/MS System as described is compatible, robust, and sensitive for SISCAPA eluates.



Plasma (µL)	ionKey/MS CV	Agilent 6490 CV
400	6.8%	17.2%
200	6.5%	9.7%
100	9.6%	12.5%
50	13.9%	17.7%
40	6.0%	36.2%

Figure 6. The experiment above was replicated on a standard flow Agilent System as a positive control and an excellent correlation was obtained. The high correlation and better precision across 4 replicates proves that microflow offers tangible benefits in the analysis of Tg over the conventional standard flow approach.

To further study the analytical sensitivity of the platform in terms of LLOD and LLOQ, a reverse curve was generated by titrating the heavy FSP peptide from 5,000 amol/µL down to 0.75 amol/µL and spiking synthetic light peptide at a constant level in human plasma. The LLOD, defined in this work as the point below which the CV is consistently above 30% for FSP, was determined to be 15 amol on column. The LLOQ, defined in this work as the point below which the CV is consistently greater than 20%, was 45 amol column. Representative chromatograms for the LLOD and LLOQ levels along with the reverse curve can be seen in Figure 8. The LLOQ of 45 amol is slightly higher than that estimated in the synthetic standard FSP work as one would expect due to the influence of the matrix.

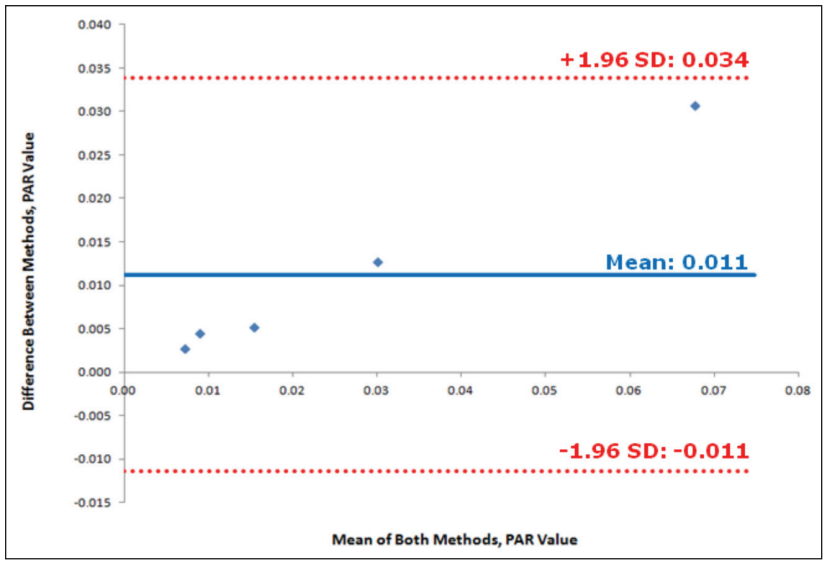


Figure 7. Bland-Altman Plot showing all differences between the standard flow Agilent method and the microflow method measurements of the FSP PAR value lie within the upper and lower confidence intervals. Agreement is therefore expected for 95% of the samples.

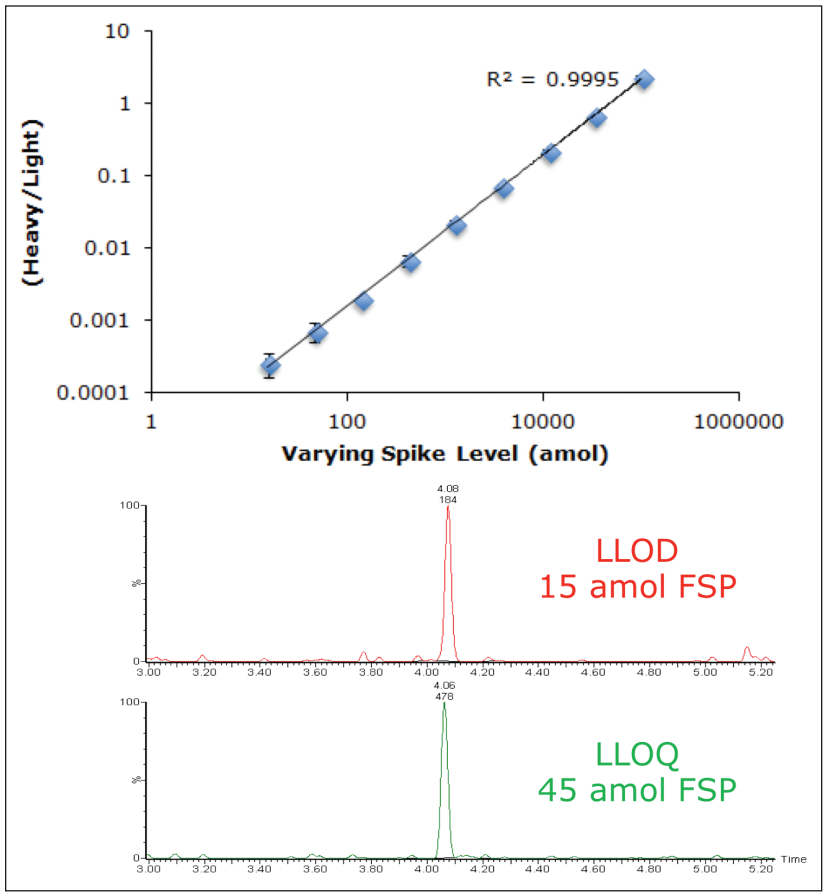


Figure 8. A reverse curve was generated to determine the approximate LLOD and LLOQ of the peptide measurement. The LLOD is 15 amol FSP on column. The LLOQ is 45 amol FSP on column.

A final curve was generated by titrating purified Tg protein in bovine plasma known to be deficient in Tg from 152 to 0.152 amol/µL followed by SISCAPA enrichment in attempts to get an estimated LLOQ value for the entire assay including the digestion step. The LLOQ of the method using only 50 µL of plasma as shown in Figure 9 is estimated to be approximately 1.18 amol/µL (0.78 ng/mL). Accordingly, the method achieves quantitation levels of Tg in bovine serum of 1.52 amol/µL (1 ng/mL) with ease.

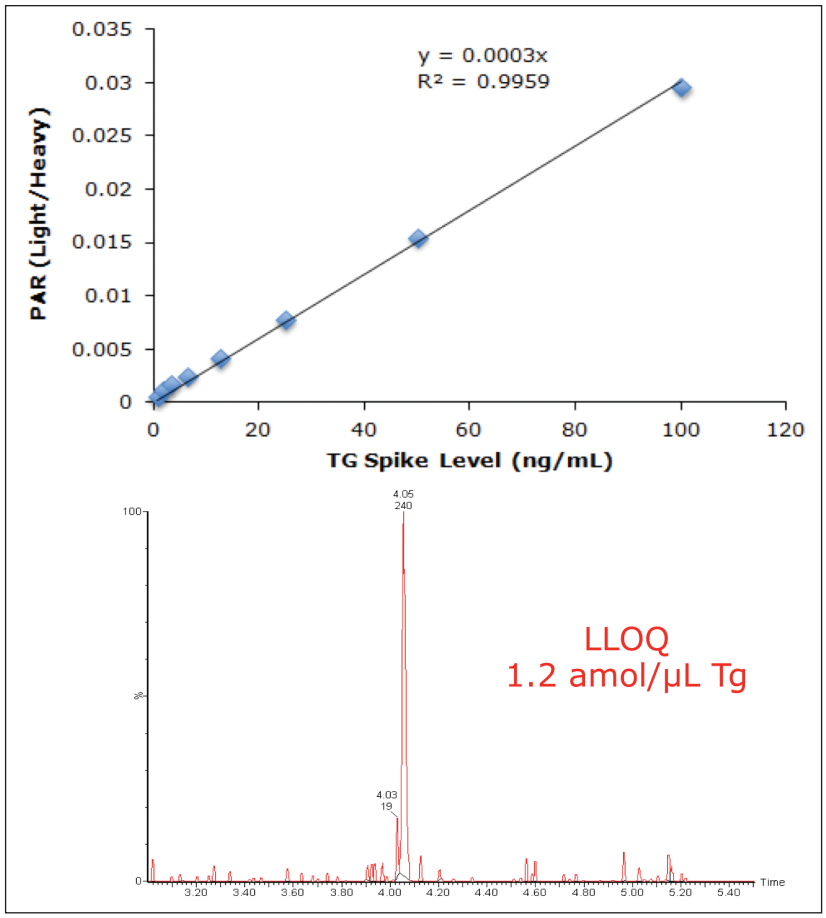


Figure 9. A curve was generated by titrating purified Tg in bovine plasma from 152 to 0.152 amol/µL. The LLOQ of the assay including the digestion step using 50 µL of sample is estimated to be 1.2 amol/µL (0.78 ng/mL).

CONCLUSIONS

Use of an optimized SISCAPA enrichment that is highly specific for a signature peptide of Tg combined with LC-MS using a vetted dual-pump trapping ionKey/MS System provides a sub 1.52 amol/µL (1 ng/mL) quantification limit of Tg protein with a cycle time of 6.75 min. This quantification limit is comparable with the best in literature for standard flow. However, the ionKey/MS System methodology outlined above also offers a few tangible benefits to the standard flow method including; a simplified enrichment procedure, the use of ten times less starting plasma prior to enrichment, an injection volume that is two times smaller, and significantly less solvent consumption. Additionally, evidence is provided in a head-to-head comparison with standard flow in which the microflow approach offers highly correlated PAR measurements while being significantly more precise across 4 replicate measures. Furthermore, the cycle time on the microflow system is only 0.25 min longer than the standard flow method outlined in the literature allowing a similar number of samples to be run in the same time frame but with higher analytical sensitivity and lower sample volumes.

We therefore conclude that the ionKey/MS System operating in the dual-pump trapping configuration does provide acceptable LLOQ levels for Tg using significantly less plasma while maintaining the requisite accuracy, better precision, and throughput levels exemplified by standard flow LC-MS methods. Accordingly, microflow is a viable and attractive solution for clinical research.

References

1. Lame, M.E., Chambers, E.E., Improving a High Sensitivity Assay for the Quantification of Teriparatide in Human Plasma Using the ionKey/MS System, Waters Application Note 720004948EN, 2015.

For Research Use Only. Not for Use in Diagnostic Procedures.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Absolute MRM Biomarker Quantification Using AQUA Stable Isotopes and Chemically Equivalent Internal Standards

GOAL

The targeted quantitative analysis of protein mixtures requires proteolytic digestion coupled with a strategy that can accurately quantify proteins with high specificity, sensitivity, dynamic range, reproducibility, and robustness. The Xevo® TQ-S System has seen substantial improvements in instrument performance, enabling highly accurate and sensitive protein quantification, using stable isotope peptides as internal standards, which will be presented here.

BACKGROUND

Multiplexed multiple reaction monitoring (MRM)/selected reaction monitoring (SRM)-based quantification assays are an attractive alternative to biochemical-based protein quantification methods, such as ELISA, in terms of speed and cost savings during the early and late validation/verification stages of protein biomarkers. The requirements for such studies place a demand on the performance of the analytical LC/MS system, requiring the highest sensitivity, specificity, speed, and robustness possible to quantify the proteins of interest present within a sample. As such, technology improvements are indispensable. The use of Xevo TQ-S and TRIZAIC UPLC® systems and the results for the quantitative analysis of pre-eclampsia related calcyclin peptides in formalin-fixed paraffin embedded placenta are described.

Detect and quantify potential protein biomarkers with maximum specificity and sensitivity using Xevo TQ-S.

THE SOLUTION

The suitability of AQUA stable isotopes and chemically equivalent internal standards for protein quantification in solution, synthetic matrix, and formalin-fixed paraffin tissue samples was investigated. Limit of detection (LOD) studies were conducted by means of experiments where calcyclin peptides with an extra glycine inserted in the middle of the amino acid sequence were spiked into a synthetic matrix. MRM assay linearity and protein concentrations were determined with AQUA stable isotope peptides as the internal standard. The peptides were separated and quantified using a Waters® TRIZAIC UPLC System with nanoTile™ Technology coupled with a Xevo TQ-S Mass Spectrometer. The data were acquired in time-scheduled MRM mode of acquisition. Quantification was conducted with TargetLynx™ Application Manager.

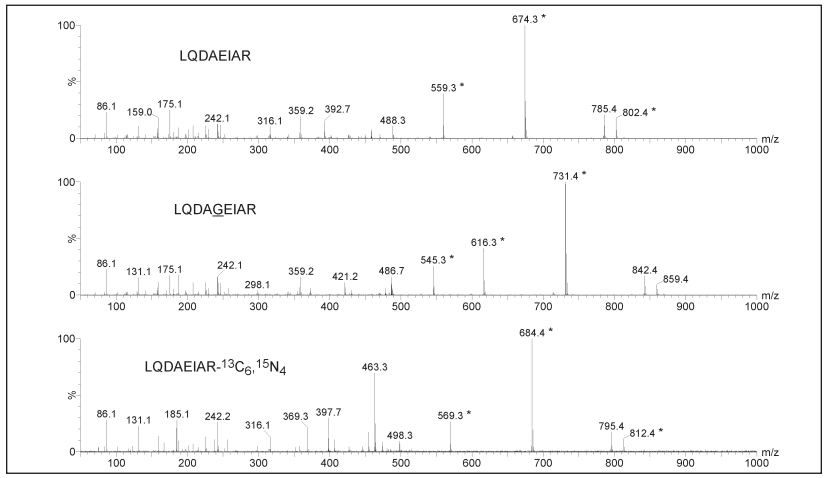


Figure 1. Product ion spectra of LQDAEIAR, glycine inserted chemical equivalent LQDAGEIAR and AQUA standard LQDAEIAR-13C6,15N4.

** Fragment ion candidates for MRM quantification.*

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Figure 1 shows the product ion spectra of one of the calcyclin peptide candidates for MRM quantification and two possible internal standard equivalents. The chemically synthesized glycine inserted equivalent illustrates similar ionization, fragmentation, and chromatographic characteristics as the native peptides of interest. It was spiked together with native standards into a synthetic matrix, shown in Figure 2, to access the limits of detection of the MRM assay, suggesting that low attomole on-column quantification should be achievable.

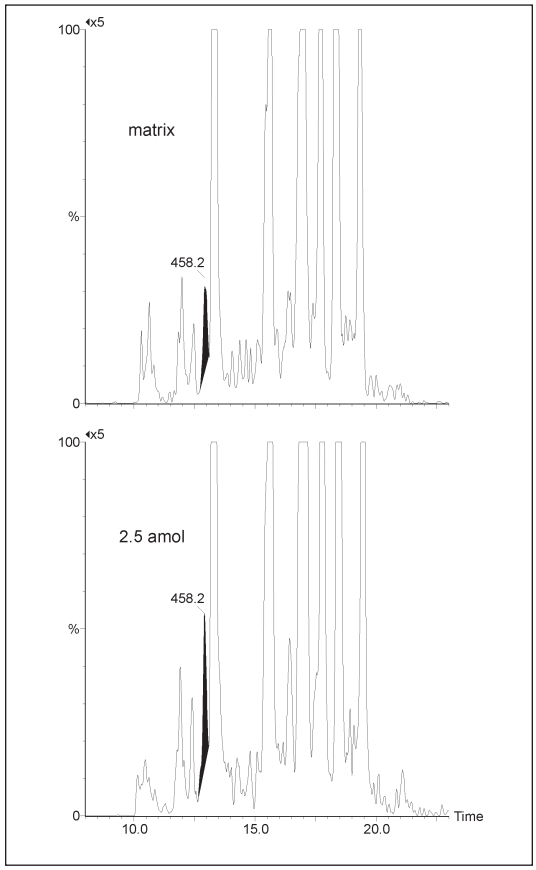


Figure 2. Summed total ion current of three MRM transitions (458.2 > 559.3, 458.2 > 674.3 and 458.2 > 802.4) from LQDAEIAR spiked at 2.5 attomole in a synthetic matrix vs. blank matrix.

Figure 3 illustrates an MRM quantification curve for one of the AQUA stable isotope peptides, covering at least four orders of linear quantification dynamic range. Enzymatic digests of approximately 300 microdissected trophoblast and stroma cells were spiked with two stable isotope AQUA peptides in order to determine calcyclin levels. The MRM channels of one of the native peptides and the corresponding AQUA stable isotope internal standards are shown inset of Figure 3. The determined amounts were approximately 0.3 and 7.0 attomole per trophoblast and stroma cell, respectively, corroborating previously reported results.[1]

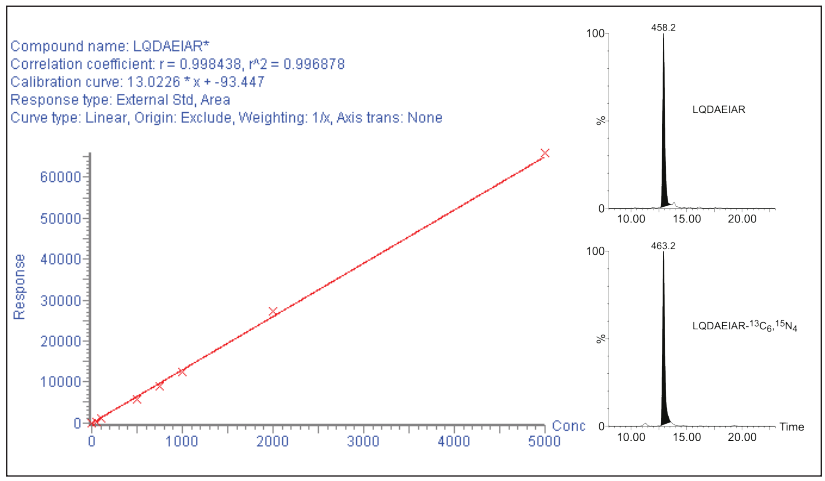


Figure 3. MRM calibration curve and regression curve metrics for LQDAEIAR-$^{13}C_6$,$^{15}N_4$ ranging from 0.5 attomole to 5.0 femtomole loaded on column. The inset shows the summed total ion current transitions of LQDAEIAR and LQDAEIAR-$^{13}C_6$,$^{15}N_4$ from approximately 300 enzymatically digested trophoblast cells loaded on-column.

SUMMARY

The absolute quantification of clusterin – a potential biomarker for pre-eclamsia formalin-fixed paraffin embedded placenta using AQUA stable isotopes and chemically equivalent peptides has been illustrated. The additional sensitivity of the Xevo TQ-S affords the detection and quantification of calcyclin in a smaller number of microdissected placental cells.[1] The MRM-based validation of clusterin in plasma on a larger patient cohort is currently under study.

Reference

1. Güzel C, Ursem NT, Dekker LJ, Derkx P, Joore J, van Dijk E, Ligtvoet G, Steegers EA, Luider TM. Multiple reaction monitoring assay for pre-eclampsia related calcyclin peptides in formalin fixed paraffin embedded placenta. J Proteome Res. 2011;10(7):3274-82.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

High-Throughput LC/MS MRM Disease Protein Marker Verification Using the ionKey/MS System

Chris Hughes, Lee Gethings, Hans Vissers, and Jim Langridge
Waters Corporation, Manchester, United Kingdom

APPLICATION BENEFITS

The ionKey/MS™ System is a robust, novel microfluidics platform that eliminates manual chromatographic connections and enables high throughput and robust validation analysis.

WATERS SOLUTIONS

ionKey/MS System

Xevo® TQ-S System

iKey™ Separation Device, BEH C_{18}

nanoACQUITY UPLC® System

KEY WORDS

Biomarkers, verification, validation, proteomics, peptides, microfluidics, MRM

INTRODUCTION

Biomarker discovery and validation are the first two steps in understanding disease and drug development. Validation is technology-challenged since it requires analyzing a large number of samples with high-throughput, but nevertheless requires high sensitivity, high resolution, large dynamic range and excellent selectivity.

Targeted LC/MS based assays afford protein quantification with the reproducibility and throughput required to improve marker acceptance. Multiple reaction monitoring (MRM) using tandem quadrupole mass spectrometers is one of the enabling technologies applied in targeted LC/MS approaches.[1] Overall, MRM-based methods compare favorably with antibody-based techniques, such as ELISAs or protein arrays, in that MRM-based methods are less expensive and can be developed more rapidly. However, one of the major challenges using MRM for candidate marker verification in mammalian body fluids is the required sensitivity for the quantification of low-abundance proteins, especially in the case of sample limited conditions.

Miniaturized LC systems offer improved mass sensitivity but often lack the required throughput, robustness, and reproducibility. The application of a novel microfluidics platform for the quantification of marker peptides and proteins is presented, considering speed, sensitivity, and selectivity.

EXPERIMENTAL

LC conditions

LC system:	nanoACQUITY UPLC System
Sample loop:	5 µL
Column:	iKey BEH C_{18} 130, 1.7 µm, 150 µm x 100 mm
Column temperature:	40 °C
Flow rate:	1.2 µL/min
Mobile phase A:	98.9:1:0.1% v/v water/ acetonitrile/formic acid
Mobile phase B:	98.9:1:0.1% v/v acetonitrile/water/formic acid in water
Volume injected:	0.1 to 1 µL

Gradient:

Time (min)	% A	% B	curve
Initial	98	2	Initial
1.0	98	2	6
45	60	40	6
46	15	85	6
47	15	85	6
48	98	2	6

MS conditions

Mass spectrometer:	Xevo TQ-S
Acquisition mode:	MRM
Quadrupole resolution:	0.4 Da or 0.7 Da
Ionization mode:	ESI positive
Capillary voltage:	3.0 kV
Source temperature:	100 °C

Software processing

MassLynx® raw data were analyzed using Skyline[2] and visualized using Spotfire Decisionsite (Tibco Spotfire, Boston, MA).

Materials

MS Qual/Quant QC Mix was obtained from Sigma-Aldrich (St. Louis, MO, USA). MassPREP™ *E. Coli* Digest Standard was from Waters Corporation (Milford, MA, USA).

The MS Qual/ Quant mixture was spiked into the *E. Coli* background such that loads for a 1 µL injection ranged from 32 amol to 40 fmol peptides in the presence of 100 ng *E. Coli*. The sample was injected three times at four different loadings (0.1, 0.2, 0.5, and 1 µL).

RESULTS AND DISCUSSION

The MS Qual/Quant QC mixture consists of 14 peptide species present as light and heavy labeled analogues and at varying amounts to give an in-sample dynamic range of 1.25e3, Table 1. The peptide mixture was spiked into a complex background matrix of an *E.Coli* tryptic digest to represent a high-throughput validation study and access quantitative precision and accuracy.

Protein	Peptide sequence*	On-column amount (attomoles)**	Expected light to heavy ratio***
Carbonic Anhydrase I	GGPFSDSY[R]	4000	1.02
Carbonic Anhydrase I	VLDALQAI[K]	2000	2.04
Carbonic Anhydrase II	AVQQPDGLAVLGIFL[K]	400	8.4
Carbonic Anhydrase II	SADFTNFDP[R]	80	55
NAD(P)H dehydrogenase	EGHLSPDIVAEQ[K]	800	0.92
NAD(P)H dehydrogenase	ALIVLAHSE[R]	400	0.93
C-reactive Protein	ESDTSYVSL[K]	80	9.4
C-reactive Protein	GYSIFSYAT[K]	16	38
Cyclophilin A	FEDENFIL[K]	320	0.29
Cyclophilin A	VSFELFAD[K]	160	0.54
Cyclophilin A	TAENF[R]	80	0.92
Catalase	GAGAFGYFEVTHDIT[K]	800	0.161
Catalase	FSTVAGESGSADTV[R]	16	4.9
Catalase	NLSVEDAA[R]	3.2	49

Table 1. Protein digest and stable isotope labeled (SIL) peptide composition of MS Qual/Quant QC Mix.

* *Amino acid in [brackets] denotes site of label incorporation for heavy SIL peptides: [K] $^{13}C_6{}^{15}N_2$, [R] $^{13}C_6{}^{15}N_4$*

** *heavy labeled on-column amount for 0.1 µL injection*

*** *Certificate of Analysis*

An MRM method containing 28 functions was programmed to monitor three transitions from each of the light and heavy peptide species, and the sample was analyzed using the ionKey/MS System with the nanoACQUITY UPLC System and the Xevo TQ-S Mass Spectrometer. Figure 1 shows the extracted MRM chromatograms for each peptide species resulting from a 0.5 µL injection and exhibits i) peptides that are resolved from the background matrix and ii) the dynamic range present within the sample. The on-column amounts displayed here range from 16 amol for the peptide NLSVEDAA[R] to 20 fmol for the peptide GGPFSDSYR.

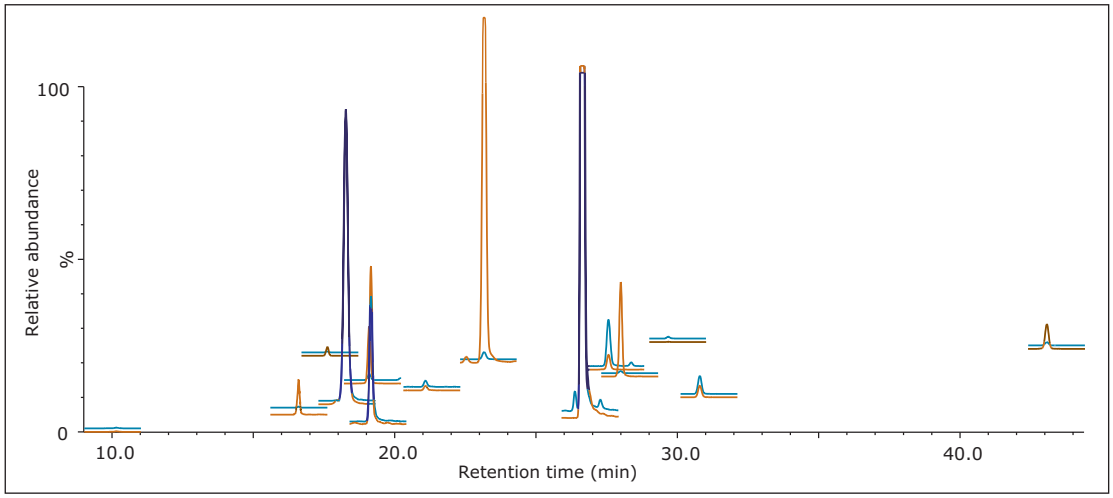


Figure 1. Overlay of extracted MRM chromatograms for each of the light and heavy stable isotope labeled peptides in the mixture for a 0.5 µL injection in the presence of E. Coli background matrix. Red indicates the light and blue the heavy labeled analogue.

MRM transitions were inspected using Skyline, ensuring that a minimum of three peptides per protein and three transitions per peptide were detected and analyzed. Figure 2 represents the technical reproducibility of three transitions for an example light/heavy peptide, *i.e.* fragment ions y5, y6, and y7 for peptide GGPFSDSYR. Further Skyline interrogation shows that excellent quantitative measurement consistency, even without normalization, between technical replicates is readily achieved, Figure 3. The example illustrates mass chromatograms and ratio measurements for peptide AVQQPDGLAVLGIFLK from Carbonic Anhydrase II, which is present in the mixture at elevated levels for the light analogue.

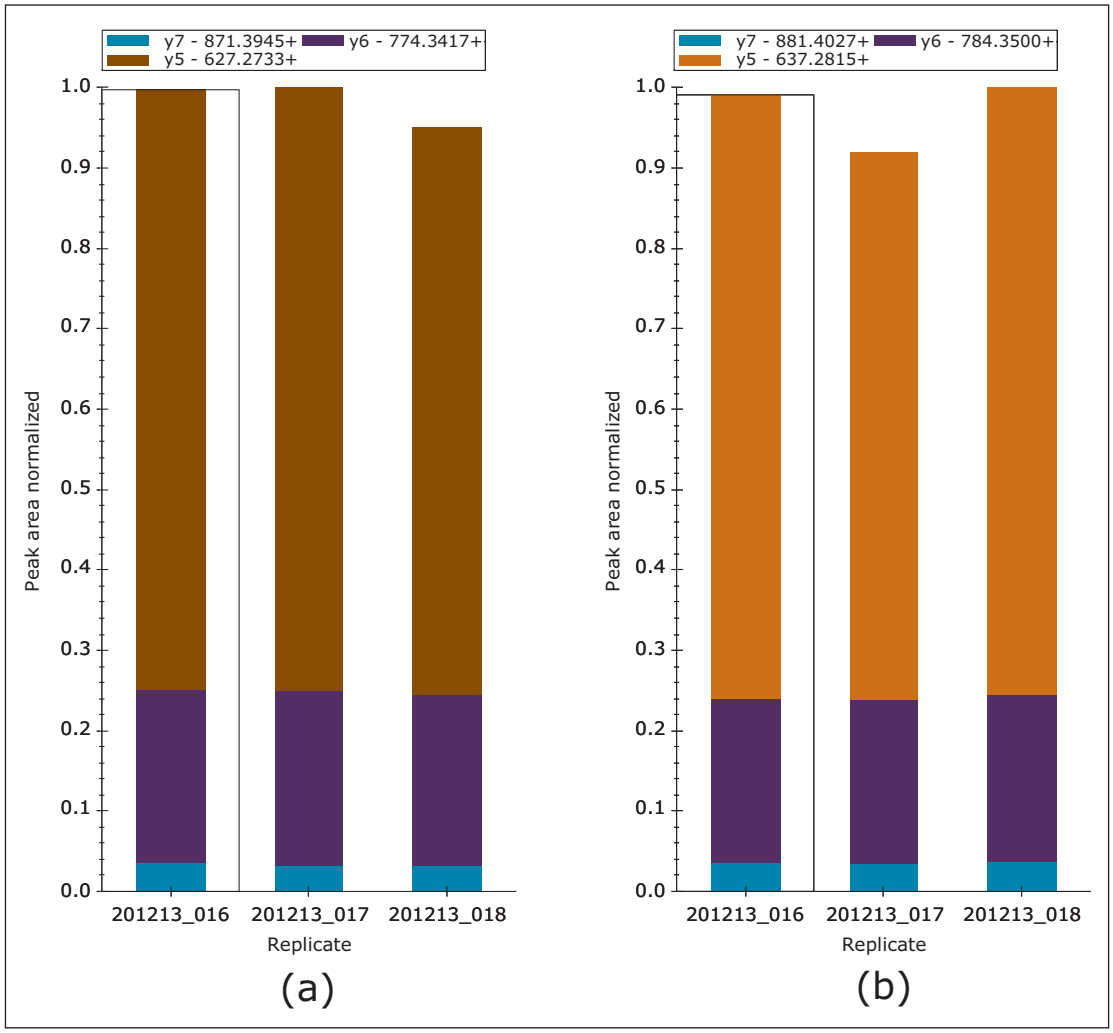


Figure 2. Reproducibility of raw MRM transitions;
a) 493.2 ⟹ 627.3 (y5), ⟹ 774.3 (y6) and 871.4 (y7) for light GGPFSDSYR
and b) 498.2 ⟹ 637.3 (y5), ⟹ 784.3 (y6) and 881.4 (y7) for heavy GGPFSDSY[R].

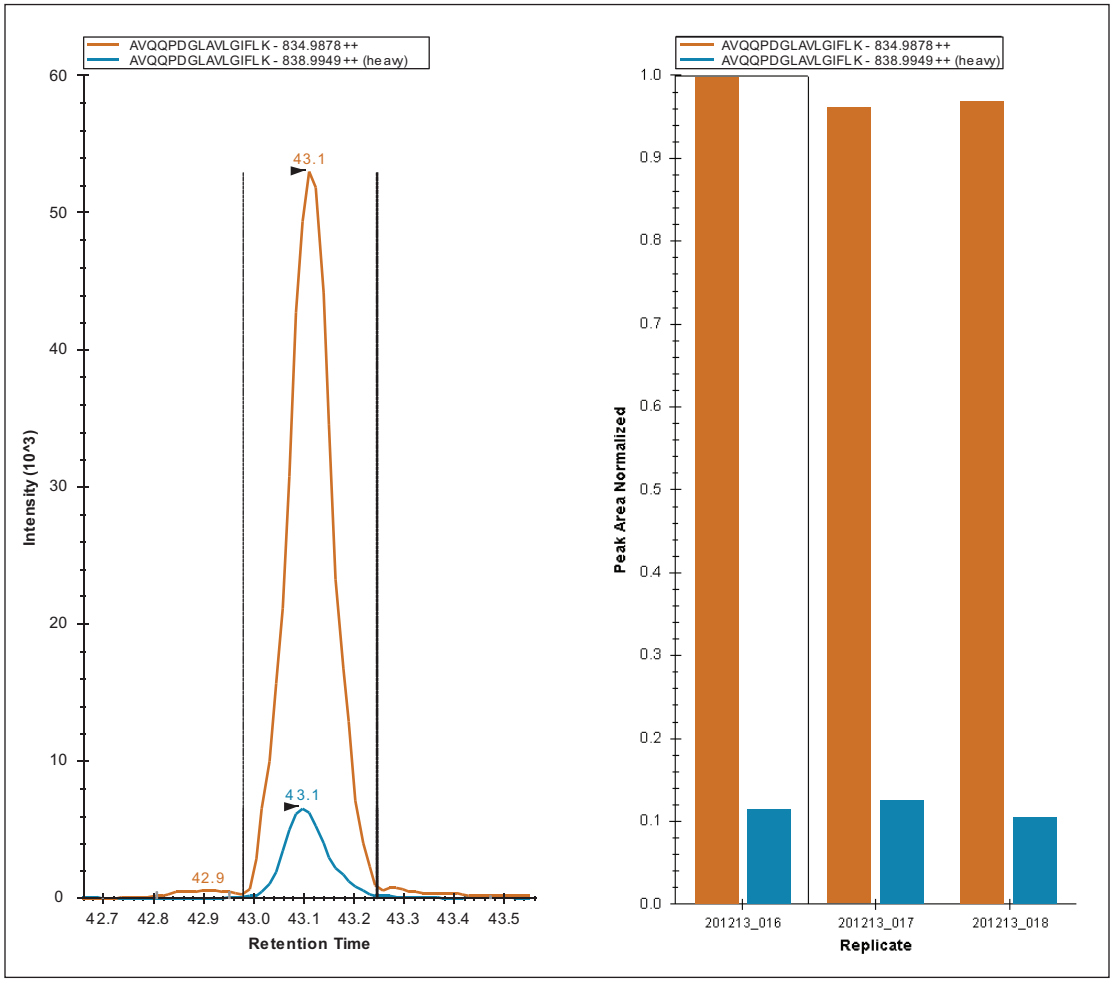
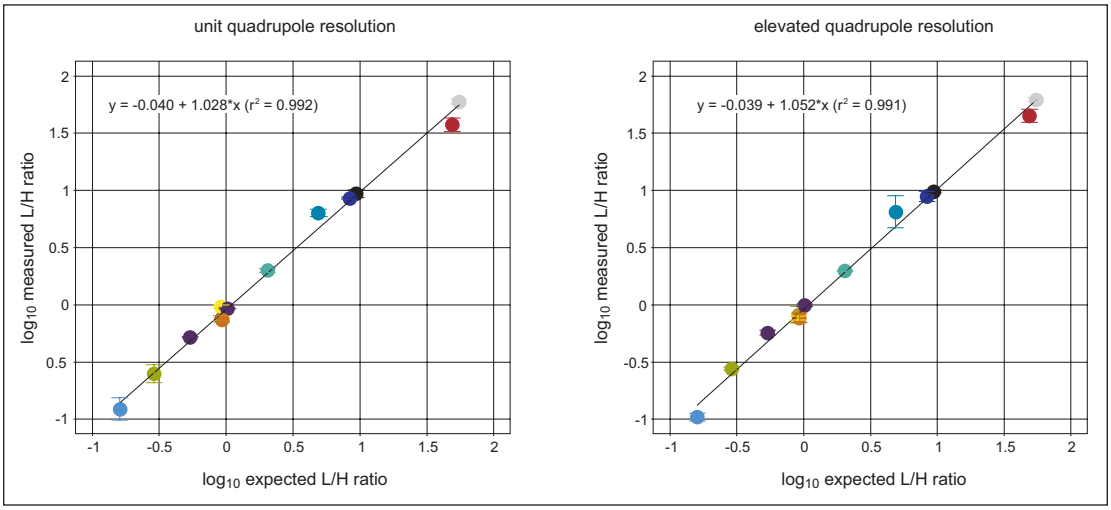


Figure 3. Quantitative measurement consistency for peptide AVQQPDGLAVLGIFLK, where brown indicates the light analogue. The expected light/heavy (L/H) ratio equals 8.4.

In Figure 4, the measured light-to-heavy ratios for every injection, 12 for each quadrupole setting, and every peptide in the mixture, are plotted against the expected ratios from the supplier certificate of analysis at unit and elevated quadrupole resolution settings. The results clearly show that excellent ratio measurements are achieved over the range of expected ratios for both mass spectrometer quadrupole resolution settings.
For this application, unit quadrupole resolution afforded sufficient quantitative accuracy. The results presented are the average relative quantification results for four different injection volumes, ranging from 0.1 to 1 µL. This covers a sample load from 3.2 amol for NLSVEDAA[R] to 40 fmol for GGPFSDSY[R] and VLDALQAIK, spanning four orders of concentration dynamic range. As can be observed by the error measurement values, precision was not noticeably affected by quadrupole resolution. As an indication of the limits of detection that can be achieved, shown in Figure 5 is the chromatogram for the peptide NLSVEDAA[R] from Catalase. This chromatogram was generated from the lowest sample injection amount, 0.1 µL and so equates to 3.2 amol on column.



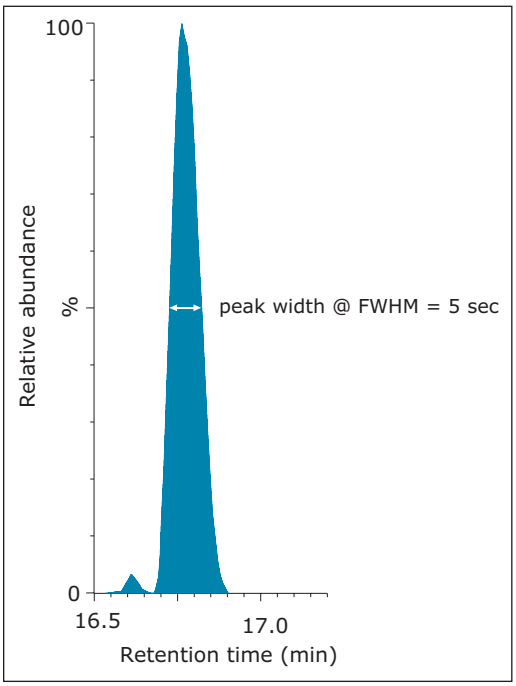


Figure 5. Typical chromatogram representing 3.2 amol on-column of the 'heavy' peptide variant of NLSVEDAA[R] from catalase.

CONCLUSIONS

This application note has demonstrated the utility of the novel ionKey/MS System for rapid and robust discovery validation experiments. Quantification measurements for light to heavy stable isotope labeled peptides have shown excellent consistency and are in agreement with expected values. The limit of detection has been demonstrated down to at least 3.2 amol on-column.

References

1. Addona *et al*. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat Biotechnol.* 2009 Jul;27(7):633-41.
2. MacLean *et al*. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*. 2010 Apr 1;26(7):966-8.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

MRM Analysis of a Parkinson's Disease Protein Signature

Tiziana Alberio,[1,2] Kelly McMahon,[3] Manuela Cuccurullo,[4] Lee A Gethings,[3] Craig Lawless,[5] Maurizio Zibetti,[6]
Johannes PC Vissers,[3] Leonardo Lopiano,[6] Mauro Fasano[1,2]
[1] Division of Biomedical Sciences, Department of Theoretical and Applied Sciences, University of Insubria, Italy;
[2] Center of Neuroscience, University of Insubria, Italy; [3] Waters Corporation, United Kingdom; [4] Waters Corporation, Italy;
[5] Faculty of Life Sciences, University of Manchester, United Kingdom; [6] Department of Neuroscience, University of Torino, Italy

APPLICATION BENEFITS

This work demonstrates the combination of nanoscale separations with quantitative tandem quadrupole mass spectrometry to accurately determine relative peptide and protein abundances. Statistical analysis of MRM data sets determined peptide abundances, which allowed for the differentiation of Parkinson's disease in patients and control subjects.

WATERS SOLUTIONS

nanoACQUITY UPLC® System

Symmetry® Column

ACQUITY UPLC® BEH Column

Xevo® TQ-S MS

MassLynx® Software

TargetLynx™ Application Manager

KEY WORDS

Parkinson's disease, nanoscale UPLC, MRM quantification, biomarkers, peptide abundance

INTRODUCTION

Parkinson's disease (PD) is a degenerative disorder of the central nervous system. PD is currently assessed by the clinical evaluation of extrapyramidal symptoms, such as tremor, rigidity, and bradykinesia, which appear when the degeneration of dopaminergic nigral neurons has raised over 70%.[1] The identification of specific biomarkers is critical for the early diagnosis of PD, as well as for monitoring disease progression and treatment efficacy.

A Parkinson's disease protein signature/panel was quantitatively evaluated in T-lymphocytes and peripheral blood mononuclear cells from a cohort of nine patients with Parkinson's disease and nine controls utilizing multiple reaction monitoring (MRM) LC/MS. A previously determined and proposed[2,3] discriminant function was applied to the measured signature, assigning seven controls out of nine as true negatives and nine patients out of nine as true positives.

EXPERIMENTAL

Sample preparation and digestion protocol

Peripheral blood mononuclear cells and T-lymphocytes were isolated as previously described.[2] Cell pellets were re-suspended in a PBS/RapiGest™ solution and centrifuged. The supernatants were collected, NH_4HCO_3 solution added, and samples denatured. Each sample was reduced, alkylated, and digested with trypsin overnight. Prior to MRM measurement, TFA was added, peptides collected, and samples further diluted with an aqueous formic acid solution and spiked with pre-digested Chaperone protein ClpB (*E. coli*) standard for relative peptide quantification.

LC/MS conditions

Time-scheduled MRM experiments were conducted with a nanoACQUITY UPLC System interfaced to a Xevo TQ-S[4] tandem quadrupole mass spectrometer. A 60-min reversed-phase gradient from 3 to 40% acetonitrile (0.1% formic acid) was employed using a vented trap configuration comprising a 5-µm Symmetry C_{18} 2 cm x 180 µm trap column and a 1.7-µm ACQUITY UPLC BEH C_{18} 15 cm x 75 µm analytical column. Unit quadrupole resolution settings were employed. Three technical replicates were acquired in random order.

Peptide quantification

Peptide MRM methods were created with Skyline Software (MacCoss Lab, University of Washington, Seattle, Washington, U.S.),[5] which interfaces directly with MassLynx Software. Peak area integration, regression analysis, and sample quantification was performed using TargetLynx Application Manager. Relative quantitation was achieved using the exogenous CLPB protein digest spike, applying linear regression analysis. At least two non-interfered MRM transitions per peptide were required for quantitation. The resultant quantitative LC/MS peptide data were analyzed with mProphet (Biognosys AG, Schlieren, Switzerland).[6]

Statistical analysis

Relative calculated MRM amounts were normalized to a peptide of a housekeeping protein (beta-actin). Fold-change values were obtained for each peptide by normalizing the complete data set against a single control subject. Relative quantities were analyzed using the Wilcoxon test. Nine peptides, selected from a previously reported protein panel,[2] were selected for analysis. Parameters for a classification function were obtained by linear discriminant analysis of relative 2-DE amounts and applied to the MRM results.

RESULTS AND DISCUSSION

MRM transitions were designed iteratively starting with *in silico* prediction, followed by experimental validation and comprehensive MRM testing of all of the candidate peptides. In short, Cys, Met, and RP/KP containing peptides were excluded, as well as missed cleaved and modified peptides. The minimum and maximum amino acid lengths were 8 and 25 amino acids, respectively. Verify^{E} Software was employed as a filter to exclude non-proteotypic, interfered transitions using 2 Th precursor and product ion tolerances and a 2-min retention time window. A pooled sample comprising equal amounts of all patient and control subject samples was used to examine the usability of the predicted final transitions in terms of response and interference. The retained transitions were manually inspected and curated using TargetLynx, and analyzed and scored with mProphet.

The resulting MRM chromatograms are shown in Figure 1, illustrating both good chromatographic performance and separation, as well as adequate MRM quantitation response for the majority of the proteins from the signature panel with a mere 100 ng of material loaded on-column. The relative intensities of the individual MRM transitions for one of the peptides of interest are illustrated in Figure 2. Throughout the complete experiment, comprising 147 LC/MS runs in total, including blanks and calibration standard runs, the relative areas of the individual transitions were constant, as shown by the inset of Figure 2 for LLGELLQDNAK. The average relative areas were 0.59 ± 0.01, 0.18 ± 0.02, and 0.23 ± 0.01 for y9, y7, and y6, respectively.

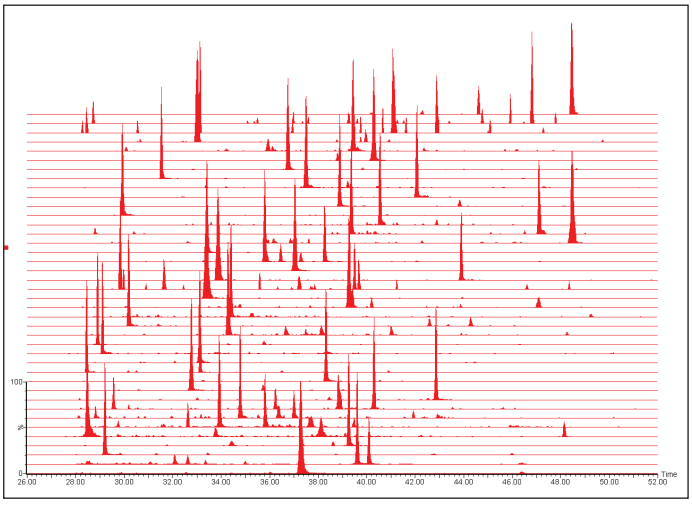
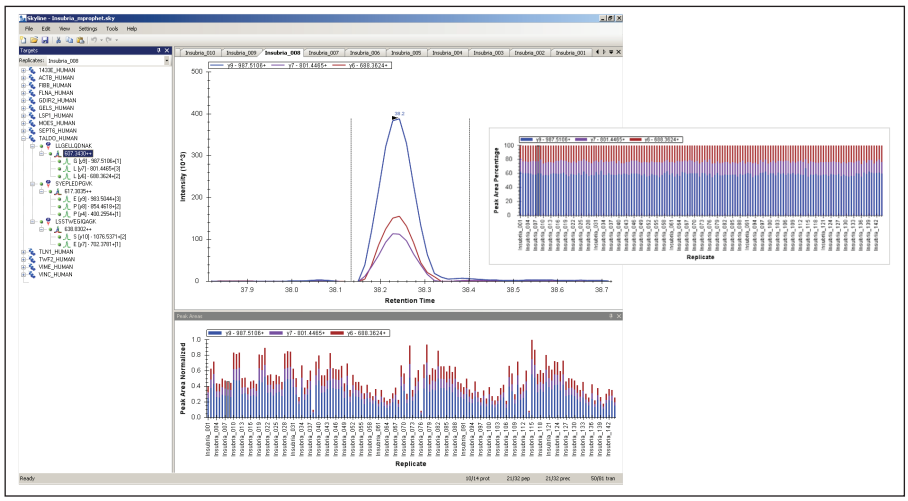


Figure 1. MRM chromatograms.



The transitions were manually inspected and curated using Skyline and TargetLynx and analyzed and scored with mProphet. An example of the manual evaluation/curation process and analysis of the MRM transitions is shown in Figure 3. The left-hand panel of Figure 3 illustrates the tree transitions of LLGELLQDNAK, corresponding to the earlier mentioned fragments, respectively, illustrating similar chromatographic properties, *i.e.*, retention time and peak shape/profile, and negligible apparent interference. The right-hand panel graphically overviews the mProphet result, which calculated error rates for the identification and provides scoring of the targeted peptides. The computational analysis confirmed manual curation in all instances, excluding objective or incorrect transition selection for the peptides of the protein signature.

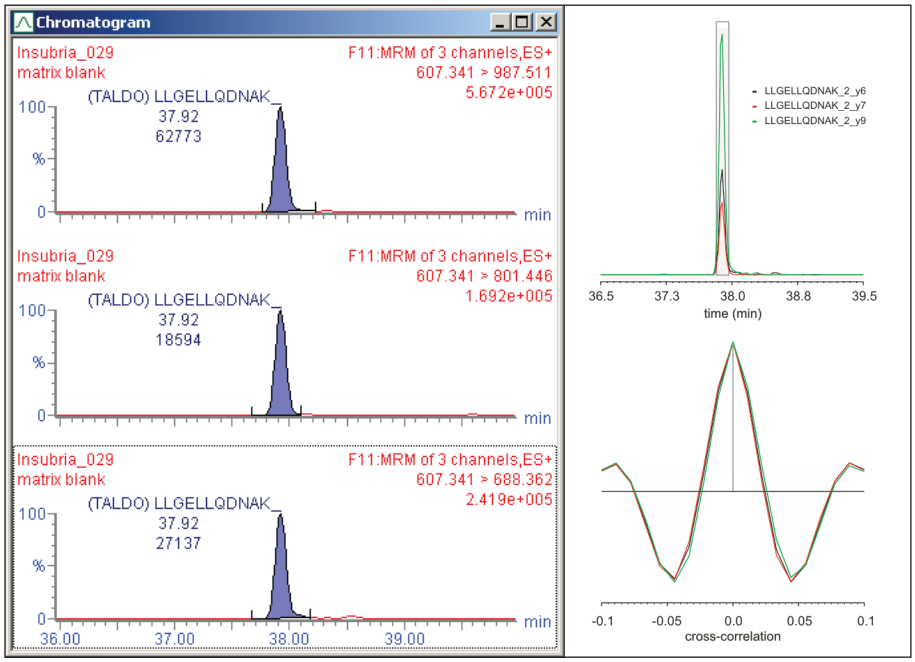


Figure 3. Use of TargetLynx and mProphet for the evaluation and curation of the MRM transitions.

Selected peptides were used to quantify the signature proteins and classify the samples. Prior to relative quantification, the complete data set was normalized to a peptide of a housekeeping protein from one of the control samples.[7] In the instance of transaldolase, three peptides were measured by means of MRM. The results shown in Figure 4 contrast the relative abundances of the peptides for the control (CO) and PD group samples. As can be seen, the regulation trend was in agreement for all peptides measured; moreover, the magnitude of the relative fold change was similar and the difference statistically significant in all instances. A subset of the measured peptides was used for classification.

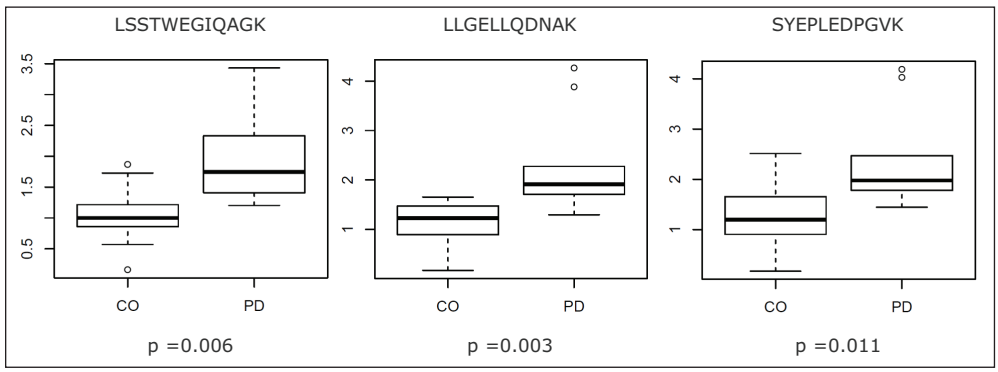


Figure 4. Relative abundances of peptides for the control (CO) and Parkinson's disease (PD) group samples.

The procedure afforded PD likelihood scores, which are summarized in Figure 5. As shown, the scores do not significantly overlap, illustrating that MRM quantification of the selected transitions provides a robust tool for the classification of subjects based on a protein signature identified from the analysis of proteomics discovery data obtained by a different technique such as two-dimensional electrophoresis.

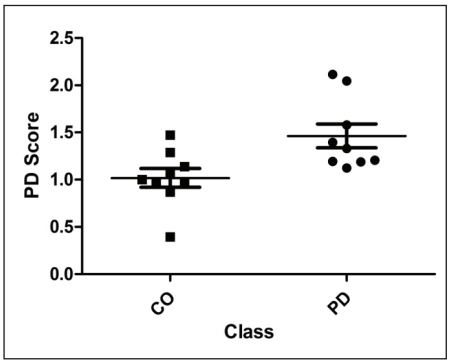


Figure 5. Scoring and distinct classification of subjects based on the protein signature.

CONCLUSIONS

- Manual and computational validation of protein signature MRM transitions were complementary and in agreement
- Accurate determination of relative peptide and protein abundances using nanoscale LC/MS and MRM analysis
- Statistical analysis of MRM determined peptide abundances allowed for the differentiation of Parkinson's disease patients and control subjects

Acknowledgement

The authors kindly acknowledge the volunteers who donated blood for the present study.

References

1. Parkinson's disease: genetics and pathogenesis. Shulman *et al. Annu Rev Pathol.* 2011; 6:193-222.
2. Discovery and verification of panels of T-lymphocyte proteins as biomarkers of Parkinson's disease. Alberio *et al. Sci Rep.* 2012; 2:953.
3. Method for the in vitro diagnosis of Parkinson's disease, Alberio *et al.* WIPO Patent No. 2013098786, 2013, July 4.
4. A new conjoined RF ion guide for enhanced ion transmission, Giles and Gordon, ASMS 2010 poster (described in Travelling wave ion mobility, Giles, *Int J Ion Mobility Spectrom*, 2013; 16(1):1-3).
5. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. MacLean *et al. Bioinformatics*. 2010 Apr 1;26(7):966-8.
6. mProphet: automated data processing and statistical validation for large-scale SRM experiments. Reiter *et al. Nat Methods.* 2011 May; 8(5):430-5.
7. Multiple-reaction monitoring-mass spectrometric assays can accurately measure the relative protein abundance in complex mixtures. Hoofnagle *et al. Clin Chem.* 2012 Apr; 58(4):777-81

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

High Sensitivity Intact Mass Analysis of Antibodies (IgG1) Using ionKey/MS

Gregory T. Roman, Henry Shion, James P. Murphy, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Improved sensitivity for monoclonal antibody analysis enabling 1 ng on-column detection limits. Ideal for sample limited environments, or preservation of precious samples.
- Rugged and easy to use ionKey/MS™ greatly facilitates the utilization of micro-LC-MS for highly sensitive intact mass analysis.
- Integrated ESI, column, and fluidic fittings enable rapid setup and ease of operation.
- Significantly reduced solvent consumption over existing ACQUITY UPLC® methods by at least ten fold.

WATERS SOLUTIONS

ionKey/MS System

iKey™ BEH300 C4 Separation Device

ACQUITY UPLC M-Class System

Xevo® G2-XS QTof Mass Spectrometer

MassLynx® Software

KEY WORDS

ionKey/MS, antibody, antibody drug conjugate, microfluidic, intact protein, biotherapeutic characterization

INTRODUCTION

LC-MS has emerged as a powerful and robust tool for the characterization of intact proteins. This approach has been widely applied to heterogeneous therapeutic monoclonal antibodies, antibody drug conjugates, bispecific mAb, antibody-antigen complexes, and antibody mixtures, to name a few. The ability to also separate the proteins from impurities, small molecules, and dissociated light and heavy chains, allows for cleaner spectra with less ion adducts. It also allows for the ability to discern glycoform variants, post translational modifications, and genetic modifications, for example. The data from such experiments is a critical component to the biopharmaceutical drug development process.

ionKey/MS offers a number of advantages for therapeutic monoclonal antibody analysis, including increased ESI efficiency leading to improved MS sensitivity, reduced sample overheads, and reduced solvent consumption for high throughput analysis. The iKey Separation Device contains the fluidic connections, electronics, ESI interface, column heater, eCord,™ and chemistry to perform UPLC® separations in the source of the mass spectrometer. It provides ease-of-use that has historically not been present with microflow LC-MS systems. Specifically, integrated microfluidics combined with clamp-on connections allows for zero dead volume connections in seconds. Alternative chemistries and maintenance can be performed rapidly with minimal system downtime. In addition, integrated heating elements, memory, and ESI tips provide easy programming, control of LC-gradients and ESI spray in a customized environment.

The ionKey/MS System consisting of the ACQUITY UPLC M-Class System, ionKey Source, and the Xevo G2-XS QTof provides high sensitivity for biotherapeutic applications. Data from this configuration can be processed in UNIFI® Scientific Information System to provide an automated solution that reduces human error in data workup.

Results from an intact IgG1 mAb mass analysis were used to illustrate the sensitivity capabilities that can help biopharmaceutical laboratories extend precious samples, and detect low level glycoforms.

EXPERIMENTAL

UPLC conditions

LC system:	ACQUITY UPLC M-Class
Column:	iKey Protein BEH C4 Separation Device, 300Å, 1.7 µm, 150 µm x 50 mm (p/n 186006765)
Column temp.:	80 °C
Loop size:	1 µL
Injection volume:	Full loop mode
Flow rate:	5.0 µL/min
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	Acetonitrile with 0.1% formic acid
Weak needle wash:	Water with 0.1% formic acid
Strong needle wash:	50% acetonitrile, 25% methanol, 25% water
Seal wash:	90:10 water:acetonitrile

Gradient:

Time (min)	Flow (µL/min)	%A	%B	Curve
initial	5.0	97.0	3.0	initial
1.0	5.0	97.0	3.0	6
4.5	5.0	3.0	97.0	6
9.0	5.0	3.0	97.0	6
9.5	5.0	97.0	3.0	6
13.0	5.0	97.0	3.0	6

MS conditions

MS system:	Xevo G2-XS QTof with ionKey/MS
Ionization mode:	ESI +
Capillary voltage:	3.5 kV
Source temp.:	150 °C
Cone voltage:	190 V
Source offset:	150 V
Cone gas:	50 L/h
Nano Flow gas:	0.10 Bar
Quad profile	Auto
Scan:	500–4000 Da, 1 second
Data format:	Continuum
Analyzer mode:	Sensitivity
RF settings:	RF Amplitude: Collision 400 V Gain 10 V

Sample preparation and analytical conditions

A Waters glycosylated mAb check mass standard (p/n 186006552) was constituted in 3% acetonitrile and 0.1% formic acid, sonicated for 5 min, and vortexed prior to insertion into the sample manager at 10 °C. New samples were prepared daily.

RESULTS AND DISCUSSION

Detection and quantification using ionKey/MS with the Xevo G2-XS QTof

The Waters mass check mAb standard was serially diluted and injected with the on-column mass noted in Figure 1, followed by a blank. The results illustrate an integrated total ion chromatogram (TIC), and show the total charge envelope of the mAb (left) and a single charge state (52+) selected from this envelope (right). Glycoform variants were clearly detected down to 1 ng (on-column) including (G0F/G0F, G0F/G1F, G1F/G1F, G1F/G2F, and G2F/G2F).

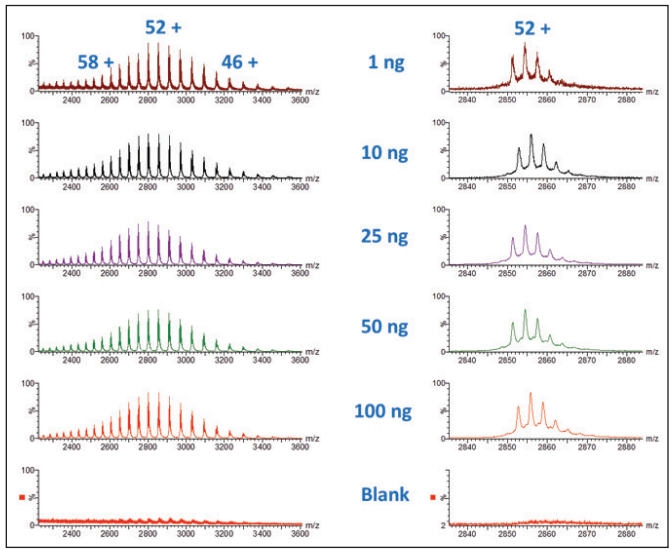


Figure 1. Overlay showing intact IgG spectra of a serially diluted Waters Mass Check mAb standard, and solvent blank. (Left) Total charge state envelope of IgG. (Right) a single charge state selected from the charge envelope.

Figure 2 demonstrates a deconvoluted spectrum from both the 1 and 50 ng (on-column) loads. Deconvoluted spectrum was a result of TIC integration followed by a MaxEnt deconvoluted MS spectrum that corresponds to the summed spectra under the detected peak. The spectrum was within 2 ppm, illustrating no significant deterioration of mass accuracy at the 1 ng detection limit of the Waters mass check mAb standard. Accurate mass of the glycoforms were achievable between 1–100 ng on-column.

Reproducibility studies were performed over a 100 replicate injections for the Waters standard. Figure 3 demonstrates the 100 replicate for the Waters standard with 1ng on-column loading. Glycoform variants were robustly measured over this range with good retention time reproducibility and peak height reproducibility, as shown in Figure 4.

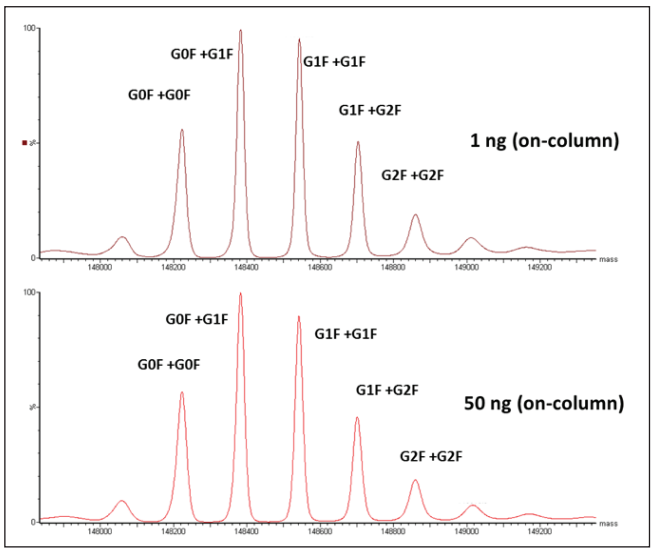


Figure 2. Deconvoluted spectra from the analysis of either 1 ng (top pane) or 50 ng (bottom pane) on-column loads, respectively.

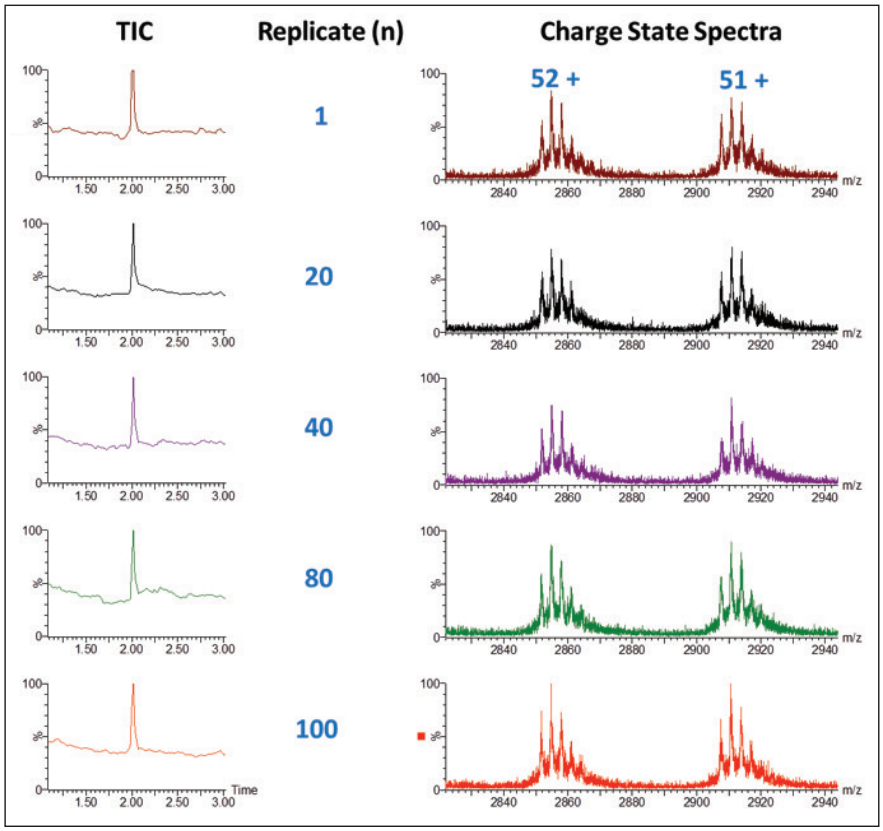


Figure 3. Peak height reproducibility and charge state spectra for 100 replicate injections of 1 ng (on-column).

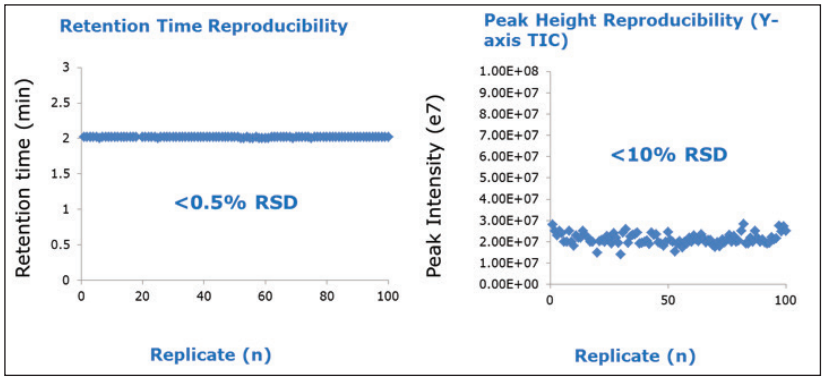


Figure 4. Retention time reproducibility and peak height reproducibility for mAb mass check standard.

CONCLUSIONS

- The ionKey/MS system with the Xevo G2-XS QTof can perform rugged and easy to use microflow LC-MS analysis of monoclonal antibodies at improved sensitivity when compared to 2.1 mm I.D. columns.
- Integrated iKey separation device enables high reproducibility at the microflow-LC scale, this was demonstrated in both peak height intensity reproducibility and retention time for standard mAb.
- Improvement in sensitivity enabling customers to further detect intact mAb at 1 ng (on-column).
- Low flow rates of 5.0 µL/min allow for 10 fold savings in solvent consumption and costly hazardous waste disposal charges to improve a laboratories bottom line.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Robustness of the ionKey/MS System in the Analysis of Pharmaceutical Compounds in Biological Fluids

Paul D. Rainville and Catalin Doneanu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

This work illustrates the robustness of the ionKey/MS™ System when analyzing complex biological samples produced by common bioanalytical sample preparation techniques.

WATERS SOLUTIONS

ionKey/MS System

ACQUITY UPLC® M-Class System

ionKey™ Source

Xevo® TQ-S

iKey™ Separation Device

MassLynx® 4.1 Software

KEY WORDS

TQ-S, iKey, ionKey, Bioanalysis, robustness

INTRODUCTION

The robustness and reliability of pharmacokinetic (PK) data is an essential part of bioanalysis. LC-MS is the technique of choice in quantitative bioanalysis due to the high selectivity and sensitivity the technique offers. The use of reduced-bore chromatographic column dimensions such as 180–300 µm I.D. for the analysis of biological fluids can be traced back to the late 1990's by Fraser et al. and has been shown as a means to increase assay sensitivity as well as reduce the amount of sample required to perform drug metabolism and pharmacokinetic (DMPK) analysis.[1,2] However, the implementation of these scales of chromatography require: specialized equipment, smaller tubing I.D.s, and connections-related dead volumes in order to achieve the best chromatographic performance. Plugging of the small scale chromatographic components by the relative dirtiness of biological samples can be a major concern when implementing this scale of chromatography. In this work, we present the robustness in the utilization of the novel ionKey/MS System for analysis of plasma samples prepared by crude protein precipitation (PPT), liquid-liquid extraction (LLE), and by immunoaffinity isolation followed by trypsin digestion (IA/TD).

EXPERIMENTAL

UPLC conditions

LC system:	ACQUITY UPLC M-Class
Separation device:	iKey Peptide BEH 130Å, 1.7 µm, 150 µm x 50 mm (p/n 186006764)
iKey temp.:	40 °C
Sample temp.:	4 °C
Injection vol.:	1 µL
Flow rate:	4 µL/min
Mobile phase A:	0.1 % Formic acid in water
Mobile phase B:	0.1% Formic acid in Acetonitrile
Gradient (PPT):	5% B to 95% B over 5 minutes
Gradient (LLE):	5% B to 95% B over 5 minutes
Gradient (IA/TD):	0% B to 30% B over 5 minutes

MS conditions

MS system:	Xevo TQ-S
Ionization:	Positive ESI
Acquisition mode:	MRM
Capillary voltage:	3.2 kV

Cone voltage and collision energies were optimized for each compound.

Data management

Chromatography software:	MassLynx 4.1

Sample preparation

Protein precipitation (PPT)

Human plasma was prepared by the addition of acetonitrile in a ratio of 2:1 (acetonitrile:plasma). The plasma sample was then vortex mixed for one minute and subsequently centrifuged at 5,000 relative centrifugal force (RCF) for five minutes. The supernatant was then removed, pipetted into an LC vial, and injected onto the LC-MS system. At regular intervals of fifty injections, a QC standard, consisting of dextromethorphan and propranolol, was monitored to access chromatographic performance over the test period.

Liquid-liquid extraction (LLE)

Human plasma was prepared by the addition of hexane in a ratio of 10:1. The plasma sample was then vortex mixed for one minute and subsequently centrifuged at 5,000 RCF for five minutes. The supernatant was then removed into a new vial. The sample was then dried down and reconstituted in one fifth the initial volume, and injected onto the LC-MS system. At regular intervals of twenty injections, a QC standard, consisting of dextromethorphan and propranolol, was monitored to access chromatographic performance over the test period.

Immunoaffinity isolation and tryptic digestion of a monoclonal antibody (IA/TD)

Samples were kindly obtained from Bristol Myers Squibb (BMS). Human plasma was spiked with a therapeutic monoclonal antibody (mAb) and immunoaffinity isolation, implemented in the magnetic bead format, was used for the isolation of the mAb from plasma. After denaturation, the mAb was digested with trypsin. Over 1,000 injections of the mAb digest were performed and two signature peptides were monitored in each LC-MS run to evaluate the chromatographic performance over the test period.

RESULTS AND DISCUSSION

Routine analysis within a bioanalytical laboratory usually consists of a batch of two to four 96-well sample plates that have been prepared by the bench chemist using protein precipitation, liquid-liquid extraction, or in the case of biopharmaceuticals such as monoclonal antibodies, immunoaffinity isolation followed by proteolytic digestion. Of these techniques, protein precipitation is the most commonly utilized due to the speed and relative low cost of the technique for the analysis of small molecules.[3] However this technique is also the crudest in its ability to produce clean samples for analysis. Because of this fact, robustness testing of a novel 150 µm iKey Separations Device was carried out under these conditions as they provided the most challenging of the samples preparation techniques of biological fluids. The ionKey/MS System, comprised of the Xevo TQ-S Mass Spectrometer, the ACQUITY UPLC M-Class, the ionKey Source, and the iKey Separation Device is shown in Figure 1. The iKey Separation Device consists of the ceramic-based separations device with an integrated emitter together in a single device that is placed directly into the source of the mass spectrometer.

Figure 1. ionKey/MS System: comprised of the Xevo TQ-S, the ACQUITY UPLC M-Class, the ionKey Source, and the iKey Separation Device.

Figure 2 illustrates the separation of dextromethorphan and propranolol, during a generic five minute LC gradient. Here we observe a peak width at 10% height for dextromethorphan and propranolol of one second. The calculated resolution at this peak height was 0.8. In Figure 2 we again show the injection of the QC standard after 1,000 injections (5 days) of continuous 1 µL injections of the protein precipitated human plasma. It should be noted that a 1 µL injection on the 150 µm I.D. iKey Separation Device is equivalent to 200 µL on a traditional 2.1 mm I.D. analytical column. The comparison of the data shown in Figure 2 indicates that excellent peak symmetry and chromatographic resolution of 0.9 were maintained over the course of the testing period.

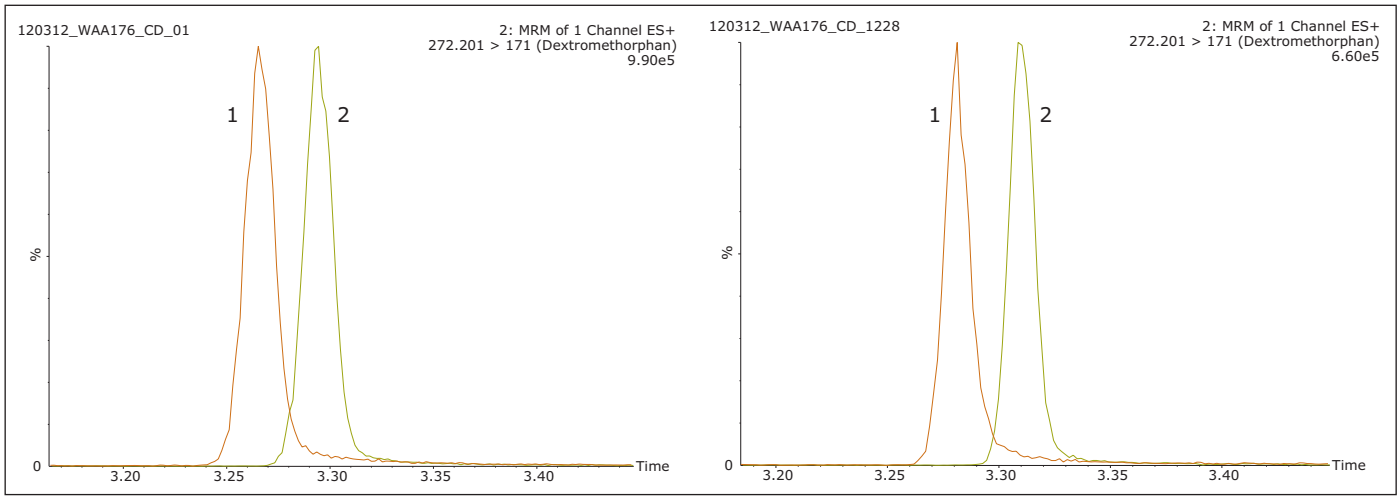


Figure 2. Resolution of propranolol (1) and dextromethorphan (2) at the start and finish of 1,000 continuous 1 µL injections of human plasma, prepared by protein precipitation.

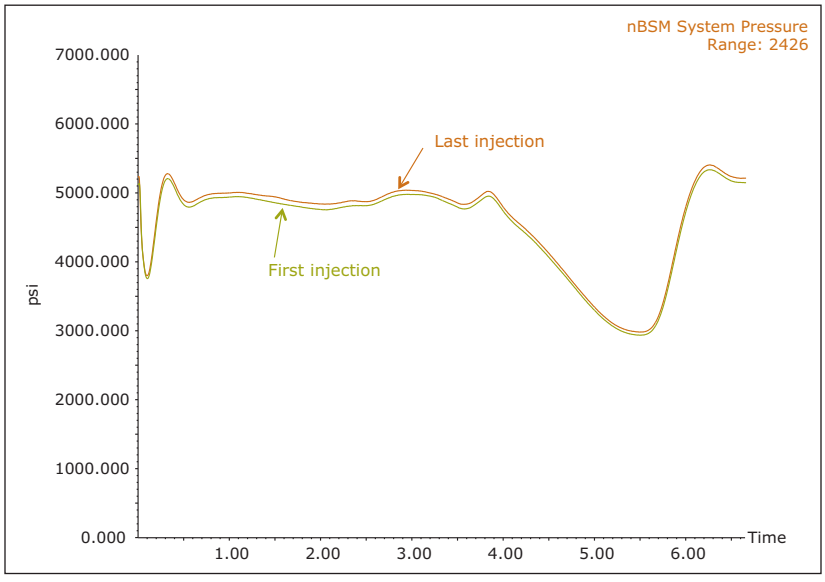


Figure 3. IonKey/MS System pressure traces at injection 1 and injection 1,000, after continuous injections of 1 µL of protein precipitated human plasma.

The system pressure traces from the study are shown in Figure 3. Here we observe no discernible increases in the system pressure, indicating that none of the frits, tubing or connective fittings have been blocked over the course of the study. It should again be noted that this injection volume of 1 µL is analogous to injecting roughly 200 times this volume or roughly 200 µL onto a standard 2.1 mm I.D. column. Cleanliness of the MS source is often a key parameter in the continued acquisition of quality data over the course of a study. The inherent ability in the use of smaller volumes to achieve similar sensitivity results as with standard 2.1 mm I.D. scale LC-MS equates to less contamination of the MS source from the sample.

Figure 4 illustrates the stability of the peak area from the QC sample over a course of 1000 injections of the protein precipitated human plasma sample. This data further illustrates the robustness, not only of the iKey Separations Device, integrated emitter, MS source but the entire system. The system was next challenged with human plasma samples prepared by LLE. This test utilized the same experimental conditions as with the previous example. Figure 5 shows the QC standard of propranolol and dextromethorphan at injection 1 and injection 1000 and illustrates that both the chromatographic peak shape and the resolution where maintained over the course of the study, much in the same manner as with the previous example. It should be further noted that again no discernible increase in system backpressure was observed.

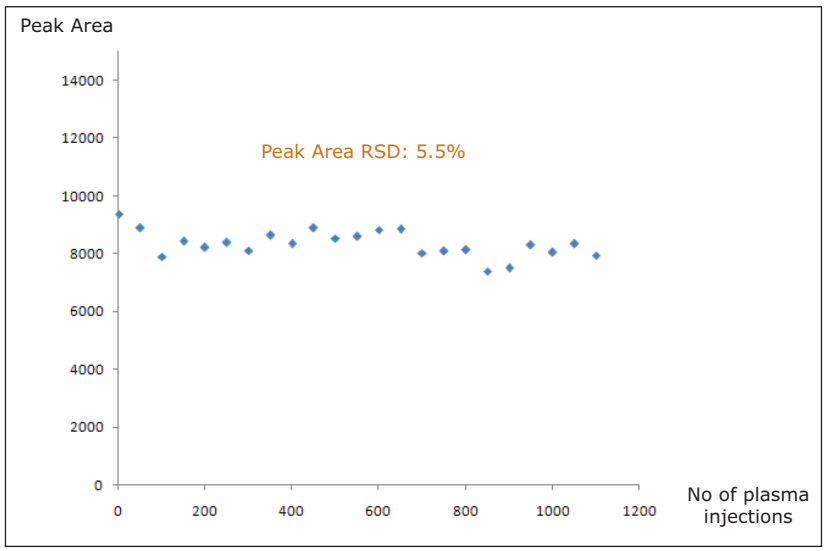


Figure 4. Dextromethorphan peak area counts from various test time points for the QC sample taken during the robustness testing with human plasma prepared by protein precipitation.

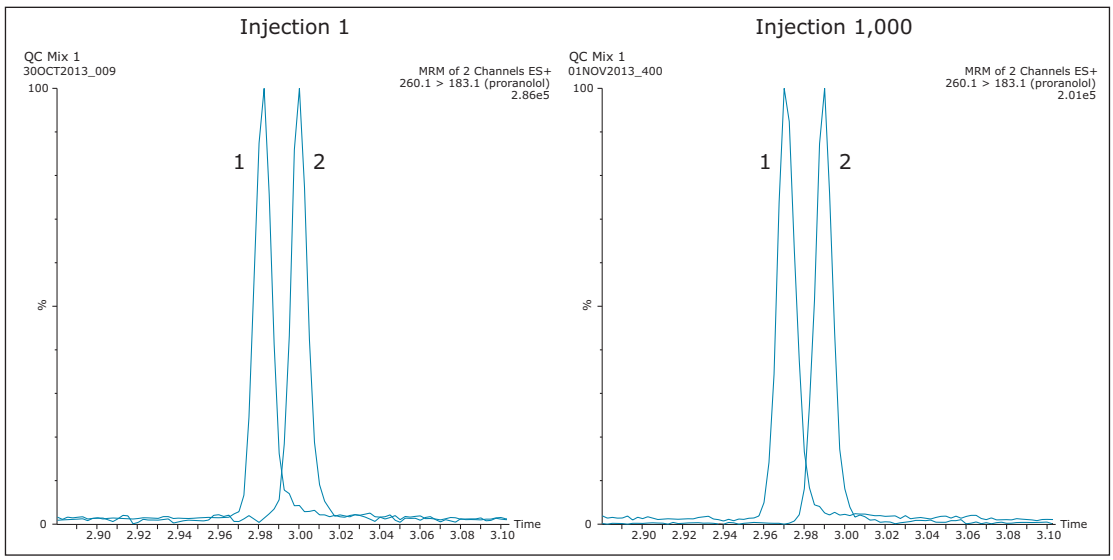


Figure 5. Resolution of propranolol (1) and dextromethorphan (2) at the start and finish of 1,000 continuous 1 µL injections of human plasma, prepared by liquid-liquid extraction.

Due to the increase in the development of biopharmaceuticals, such as antibody therapeutics, the robustness of the ionKey/MS System was tested with a common sample preparation scheme of affinity isolation followed by digestion and subsequent analysis of peptides generated from the sample preparation. As with the previous studies chromatographic performance and system pressure where monitored. In this example, specific peptides produced by the trypsin digestion of the antibody where monitored over the course of the study. Figure 6 illustrates the peak shape at injection 1 and injection 1,000 for two of the signature peptides monitored during the study. The typical peptide peak widths (at 10% peak height) observed under the experimental conditions employed were 3–4 seconds.

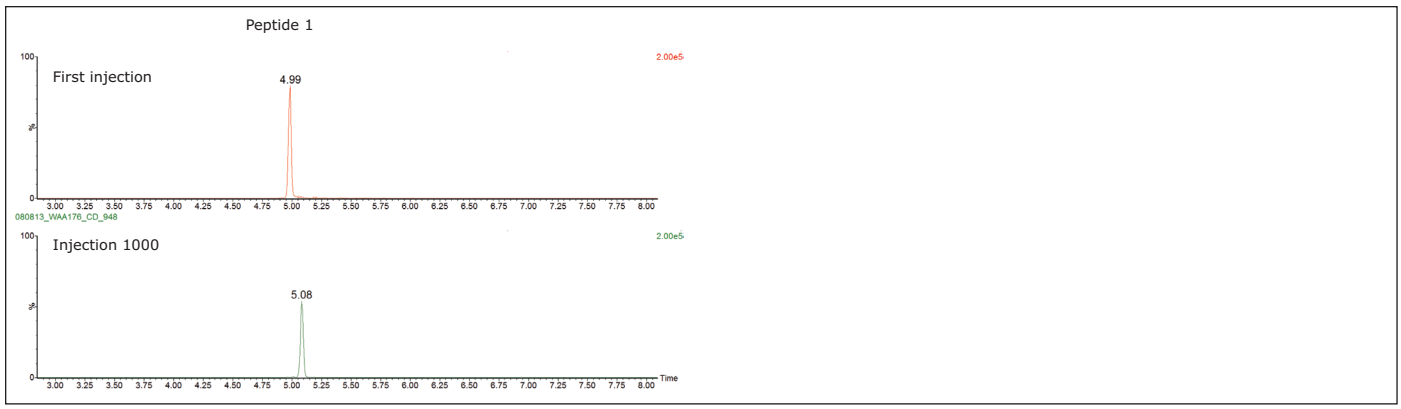


Figure 6. MRM chromatograms of two signature peptides from a therapeutic mAb digested with trypsin following immunoaffinity isolation from mouse serum.

As in the previous experiments, pressure traces were recorded throughout the entire study and the first and the last pressure traces are displayed in Figure 7.

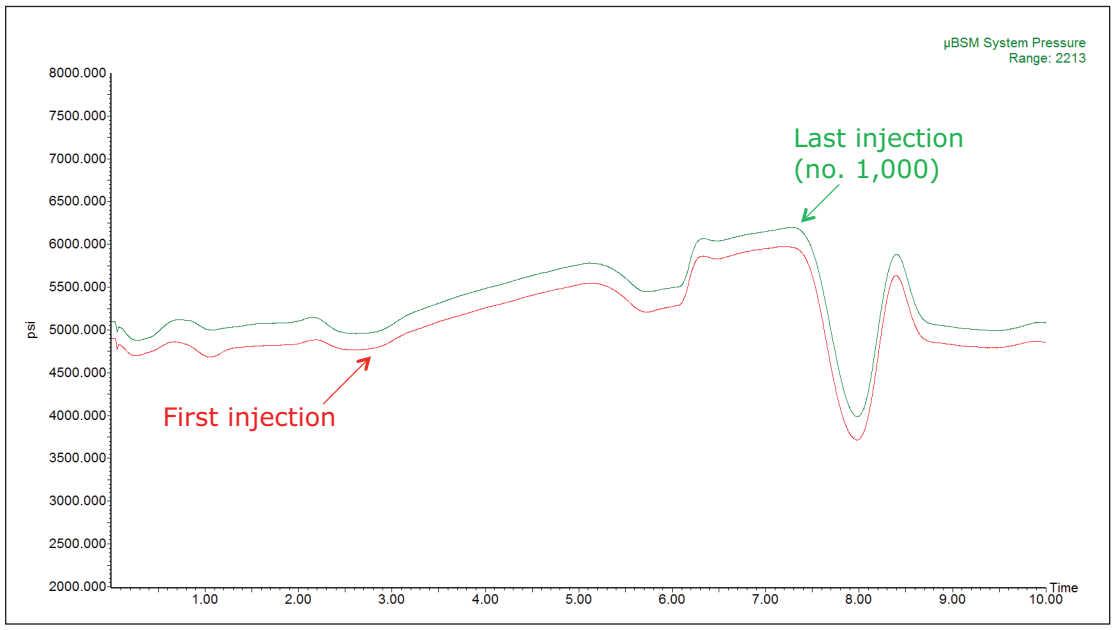


Figure 7. ionKey/MS System pressure traces recorded for the first and the last (1,000th) injection of a mAb isolated by immunoaffinity from mouse serum and digested with trypsin.

CONCLUSIONS

The performance of the ionKey/MS System, using a novel 150 µm I.D. iKey Separation Device packed with 1.7 µm chromatographic particles, in the analysis of biological fluids was shown to be:

- Robust for over 1,000 injections of human plasma prepared by protein precipitation, liquid-liquid extraction, and by immunoaffinity isolation with trypsin digestion, using injection volumes of 1 µL. This volume is roughly equivalent to a 200 µL injection on a standard 2.1 mm I.D. column.
- Capable of maintaining excellent peak symmetry and resolution for a critical pair of small molecules over a continuous testing period of 5 days. The resolution and peak width measured at 10% peak height was 0.8 and 1 second, at injection 1 and injection 1,000 respectively.
- Capable of maintaining very good chromatographic performance in terms of peak shape and peak width over 1,000 injections of a complex mAb digest sample.
- Maintaining consistent system pressure over the course of the study, indicating no accumulation of plasma components in the narrow I.D. tubing, or frits that could lead to poor chromatographic performance and system over-pressure.

References

1. Fraser, I., et al., The use of capillary high performance liquid chromatography with electrospray mass spectrometry for the analysis of small volume blood samples from serially bled mice to determine the pharmacokinetics of early discovery compounds, *Rapid Communications in Mass Spectrometry*, 1999, 13, 2366–2375.
2. Dear, G., et al., Applications of capillary LC(-MS) to drug metabolism studies, *LCGC Europe*, 2001, 2–8.
3. Wells, D.A., High throughput bioanalytical sample preparation, 2003, Elsevier, St. Paul, Minnesota, USA.

Waters would like to thank Eugene Ciccimaro, Bogdan Sleczka, Celia D'Arienzo, and Timothy Olah from BMS for the production and use of the digested antibody samples utilized in this study.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

SYSTEMATIC METHOD DEVELOPMENT FOR QUANTIFICATION OF PROTEINS VIA THE SURROGATE PEPTIDE APPROACH USING LC-MS/MS, MASSLYNX™ AND SKYLINE

Nikunj Tanna, Kerri Smith, Kelly Doering, Anthony Marcello and Mark Wrona
Waters Corporation, Milford, MA

INTRODUCTION

Biotherapeutics are increasingly becoming a significant part of the pharmaceutical arsenal as more and more companies work towards using them individually or in combination with other large or small molecules as drugs of choice. This trend manifests itself in the growing number of bioanalytical laboratories across the globe incorporating technological and scientific expertise required to deal with the complexities such analyses bring. The diversity within biotherapeutics ranges from small linear or cyclic peptides, all the way to complex monoclonal antibodies and antibody drug conjugates. The instruments of choice for performing these types of analysis are largely tandem quadrupole mass spectrometers, like the Waters Xevo TQ-S.

Bioanalysis of large proteins brings unique challenges for scientists. Sample preparation for large molecules often involves denaturation, reduction, alkylation followed by digestion. Depending upon the analyte of interest and the matrix in which the analyte is being quantified, the need for reduction and alkylation also needs to be evaluated. Each one of these steps needs optimization. Determining the best MRM transitions for peptides generated after digestion is also complicated, as each peptide can have multiply charged precursors and products. MRM methods are optimized through LC injections, creating multiple acquisition methods, modifying one parameter at a time, effectively generating upwards to 15-20 acquisition methods. Once the data is acquired, manually mining through the data is very time consuming, and could take hours, or even days to distill down to the best acquisition method to be used.

Skyline is a freely-available, open-source Windows client application for building Selected Reaction Monitoring (SRM) / Multiple Reaction Monitoring (MRM) quantitative methods and analyzing the resulting mass spectrometer data. It aims to employ cutting-edge technologies for creating and iteratively refining targeted methods for large-scale quantitative mass spectrometry studies in life sciences. This software has been developed and is being constantly maintained and improved by the MacCoss Lab at University of Washington.

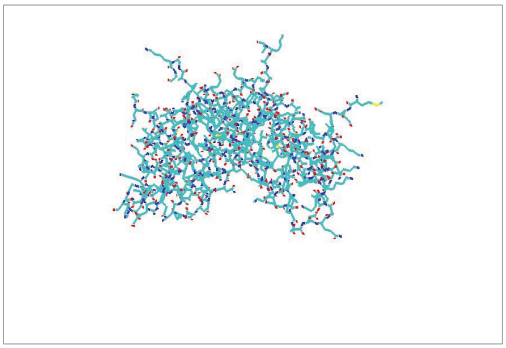


Figure 1. Interferon γ crystal structure[1] and amino acid sequence[2]

In this poster, we present a detailed workflow covering sample preparation, generating in-silico peptides, determining precursor and fragment ions, generating multiple acquisition methods optimizing MRM parameters, and eventually analyzing the data generated in an easy to interpret visual format, using Skyline and Waters MassLynx™ software. Interferon gamma will be used as a candidate molecule for this poster. However, a similar approach can be applied for the quantification of most proteins via the surrogate peptide approach.

METHODS

The protein sequence for Interferon γ was inserted in Skyline. In the settings menu, peptide settings were chosen to allow for digestion using trypsin, allowing 1 miscleavage. No background proteome was chosen. The minimum and maximum length of the peptides was chosen as 5 and 25 amino acids long respectively, and no modifications were excluded. Skyline instantly performed an in-silico digest of the protein sequence based on the parameters selected in the peptide settings.

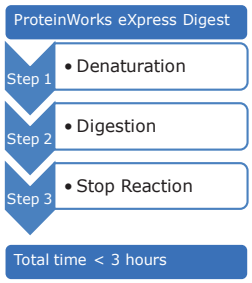
In the transition settings, Waters Xevo instrument was selected for the collision energy calculation. Precursor and product ions(b and y only) with 1, 2 and 3 charges were selected. The minimum m/z was set at 300 and the maximum was set at 2048.

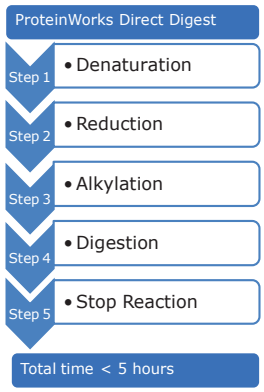
MS acquisition method were then created for MassLynx™ using Skyline. In the File option on the menu bar, export method was selected. Waters Xevo was chosen as the instrument type. Minimum transitions per sample was 150. Multiple methods optimizing collision energy was selected. Skyline generated 9 acquisition methods varying the CE for each transition of each peptide, in a MassLynx™ compatible format.

Sample Preparation:

Quantification of proteins via the bottom up approach usually involves multiple steps of denaturation, reduction, alkylation and digestion. At each one of these steps, multiple variables, like grade/vendor of reagent, time, temperature have to be evaluated and optimized. A standardized, kit based approach to sample preparation in the form of ProteinWorks™ kits was evaluated for this assay.

Different digestion protocols described below were evaluated during method development.





Chromatographic conditions:

Mobile Phase A: 0.1% Formic Acid in Water

Mobile Phase B: 0.1% Formic Acid in Acetonitrile

Column: ACQUITY UPLC HSS T3 1.8 µm, 2.1 x 150 mm

Column Temperature: 60 °C

Injection Volume: 10 µL

Xevo TQ-S source conditions:

Cone Voltage (kV): 2.50

Capillary Voltage (V): 40

Desolvation Temperature (°C): 500

Desolvation Gas (L/Hr) 1000

Cone Gas (L/Hr): 150

Time (mins)	Flow (mL/min)	%A	%B	Curve
Initial	0.400	95	5	Initial
2.00	0.400	95	5	6
2.10	0.400	95	5	6
6.00	0.400	50	50	6
6.10	0.400	5	95	6
8.00	0.400	5	95	6
8.10	0.400	95	5	6
10.00	0.400	95	5	6

Systematic method development for quantification of proteins via the surrogate peptide approach using LC-MS/MS, MassLynx™ and Skyline

RESULTS

A high concentration sample of INF γ spiked in buffer was digested using the 5 step ProteinWorks™ protocol. A total of 9 injections were made, 1 using each of the 9 methods created by Skyline. The results were imported back into Skyline through the File -> Import -> Results -> Add New replicate -> Optimizing Collision Energy. Once the import is complete, Skyline detects the best peak for each transition of each peptide and plots the corresponding peak area for each of the collision energy tested in a bar graph format as shown below (Figure 2). This easy to interpret visual format allows the scientist to quickly review the data and pick the optimal peptide transitions for a given peptide. The transitions which do not yield a good signal can simply be deleted using the delete/backspace key. Once the initial review is complete and the weak transitions are deleted, the scientist can either choose to make a final MRM method directly, or further fine-tune the method. To further fine-tune, one can undergo another round of method generation using the export function, changing the collision energy step size and step count. This can be done in the transition settings section, in the Collision Energy drop-down by choosing to edit the current instrument settings. If the scientist is content with the results form the first set of data, they can choose to create a final MRM method by simply exporting a single method in the File -> Export -> Method dialogue box. On doing so, Skyline will automatically pick the optimal collision energy for all the transitions left in the transition list.

INF γ spiked in buffer was digested using the 5 step protocol, and used to generate the final acquisition method as listed below (Figure 3). Blank human plasma and human plasma spiked with different concentrations of INF γ were then digested using the 5 step digestion protocol. Results showed background interfering peaks from the matrix present at the retention time of the peptide of interest, having very close MRM transitions (Figure 4). To resolve the matrix issue, INF γ spiked in buffer was digested using the 3 step protocol. Data showed that a subset of INF γ peptides were generated without the need for reduction and alkylation (Figure 3). A calibration curve of INF γ spiked in buffer and digested using the 3 step protocol showed good linearity and precision (figure 5).

Thus, for this molecule, the reduction and alkylation steps can be skipped, simplifying the sample preparation protocol. This also reduces the total number of peptides being generated from the sample matrix, therefore reducing the possibility of ion suppression due to fewer ionisable species. This may result in higher sensitivity from more area counts for analyte of interest and better signal to noise due to lower matrix background.

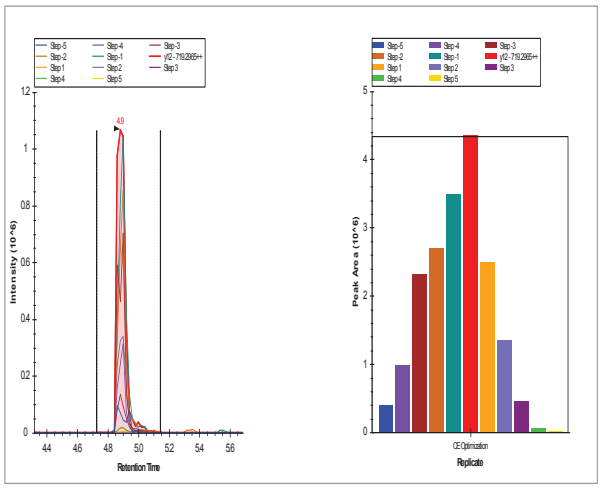


Figure 2. Data review pane for Collision Energy optimization within Skyline. The different bars indicate the different CE's for a single transition.

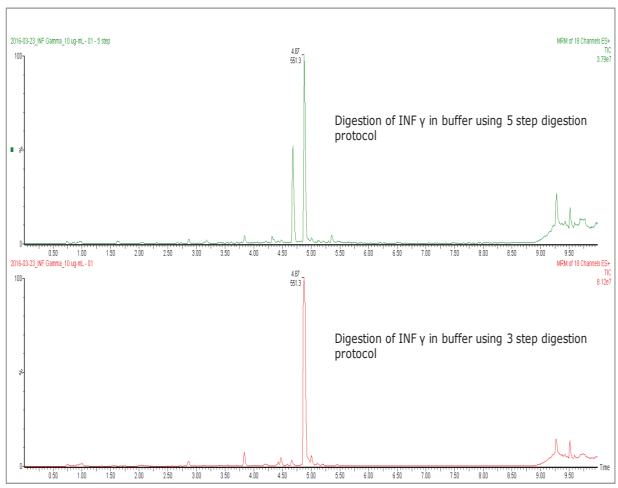


Figure 3. TIC of INF γ spiked in buffer and digested using the 5 step and 3 step ProteinWorks digestion protocol

Systematic method development for quantification of proteins via the surrogate peptide approach using LC-MS/MS, MassLynx™ and Skyline

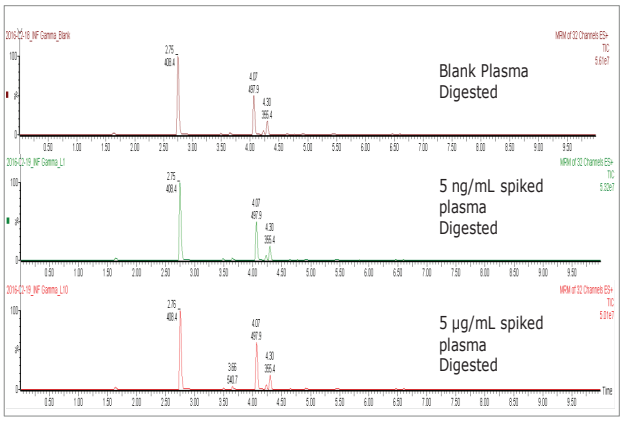


Figure 4. Blank human plasma digested using the 5 step protocol. Interference observed at the retention time of interest

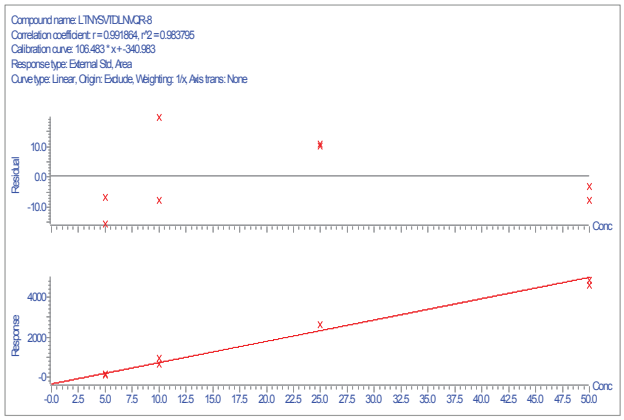
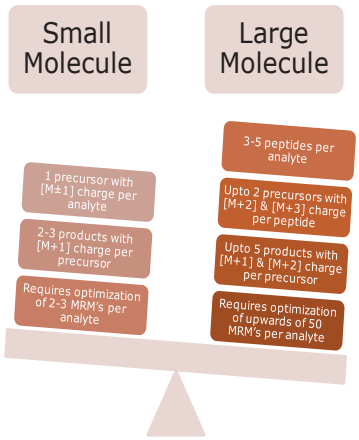


Figure 5. Calibration curve for INF γ spiked in buffer and digested using the 3 step protocol.

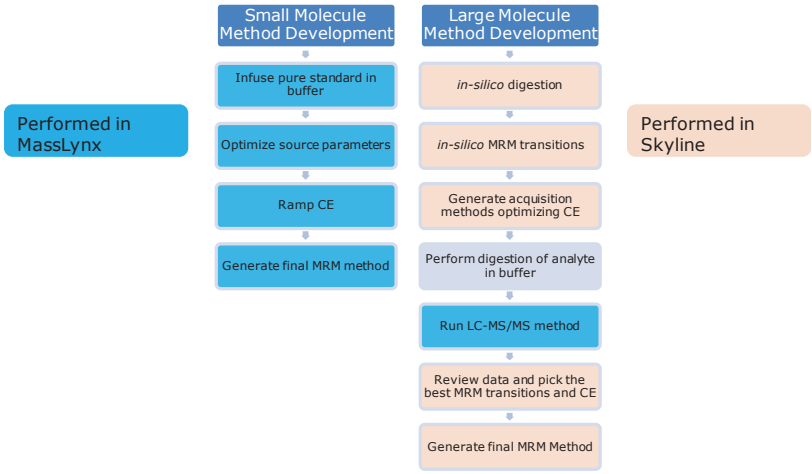
DISCUSSION

Quantitation of proteins using the surrogate peptide approach is rapidly becoming a key part of most bioanalytical laboratories have traditionally focused on performing small molecule quantification. Protein/peptide quantification brings with it unique challenges which are different from those typically experienced with a small molecule assay. Scientists are having to learn to navigate these challenges quickly. It has been shown multiple times that simple sample preparation techniques like protein precipitation, or liquid-liquid extraction rarely work for large molecules. The limited mass range of tandem quadrupole instruments, which are the instrument of choice for bioanalytical quantification, pose an additional challenge by requiring the proteins to be digested, to form small peptides which can then be detected within the mass range of these instruments.



Building an MRM method for a protein/peptide molecule is also very different compared to a small molecule. With small molecule method development, an infusion of the standard can be used to optimize all of the MS parameters and the spectrum is fairly clean. With peptides however, the spectrum gets extremely complicated. An infusion of even a pure synthetic peptide can generate a complex spectrum, making it impossible to fine-tune any parameters. The preferred option is to optimize MS parameters based on LC injections, varying one parameter at a time. For a large protein like a monoclonal antibody, there are anywhere between 3-5 possible peptides. Each peptide can have 8-10 MRM pairs which show some amount of signal. Optimizing the collision energy for each one of those MRM pairs would mean generating multiple acquisition methods, one by one. This process can get confusing and tiresome pretty quickly.

Using an open source software like Skyline removes many barriers associated with MRM method development. Its ability to not only generate in-silico peptides, but also to generate in-silico MRM transitions, and then generate acquisition methods optimizing collision energies for each one of those MRM transitions in minutes saves a lot of time and energy for the scientist. The ability to import the data into Skyline, review it in minutes, and then choose to either make a final MRM method with a few clicks can be extremely powerful. The software also affords ample flexibility to choose the type of in-silico digestion and MRM transitions based upon information about the specific analyte of interest.



CONCLUSION

- **Skyline works seamlessly with MassLynx™ software to make method development for the analytical scientist simple, easy and quick**
- **ProteinWorks™ kits provide a simple, standardized sample preparation kit for large molecule bioanalysis reducing the time required for method development and ensuring reproducibility across multiple laboratories**
- **Reduction and alkylation may not always be needed prior to digestion. Eliminating these steps may lead to increased area counts for analytes and/or reduced matrix background resulting in better sensitivity**
- **Elimination of these steps also result in a simpler sample preparation protocol, thus reducing the total error within an analysis, reagent cost and total assay time**

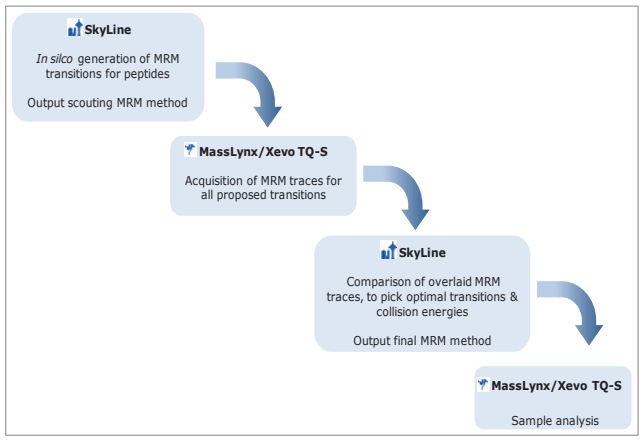


Figure 6. Workflow for MRM method generation using Skyline and MassLynx™ softwares

References:

1. Thiel DJ, le Du MH, Walter RL, D'Arcy A, Chène C, Fountoulakis M, Garotta G, Winkler FK, Ealick SE (September 2000). "Observation of an unexpected third receptor molecule in the crystal structure of human interferon-gamma receptor complex". Structure8 (9): 927–36. doi:10.1016/S0969-2126(00)00184-2.PMID 10986460
2. CAS Registry Number: 82115-62-6: CAS, A Division of the American Chemical Society

ACHIEVING GREATER SENSITIVITY, THROUGHPUT, AND ROBUSTNESS WITH IONKEY/MS AND TRAPPING

Gregory T. Roman, Jay S. Johnson, Yun W. Alelyunas, Mark Wrona, James P. Murphy

A number of advantages exist when configuring an ionKey/MS™ System with a trapping column. These include: 1) improved focusing and retention of hydrophilic compounds, 2) increased volumetric sample loading and corresponding larger total mass load, 3) reduced duty cycle times compared with direct injection (no-trap) methodologies, and 4) automated sample cleanup and downstream protection of the iKey™ Separation Device. These advantages improve the detection limits, sample throughput, and robustness capabilities of ionKey/MS.

SYSTEM CONFIGURATION

The additional chromatographic dimension prior to the analytical iKey is referred to as a trapping column. This column is designed to capture or trap analytes, remove matrix and non-binding components, and then elute these onto the iKey for further separation and analysis. Figure 1 outlines the fluidic configuration coupling a trapping column with the ionKey/MS System. The embedded animation provides an overview of the workflow spanning from sample injection, sample cleanup, trap refocusing, ionKey refocusing and ionKey ESI.

Figure 1. Schematic of the ionKey/MS System configured for single pump trapping.

The trapping column serves to purify the analyte by separating it from both the diluent and matrix components (i.e. salts and buffer ions), illustrated in Figure 2A, that has implications on peak shape and sensitivity. Ion suppression and ion adduction can also be dramatically influenced by the presence of a trap column, which helps to further desalt analyte, improve spectral quality, and reduce spectral complexity. Once the analyte has been trapped it can further undergo refocusing on the trap as the LC gradient is pumped through the trapping column, illustrated in Figure 2B.

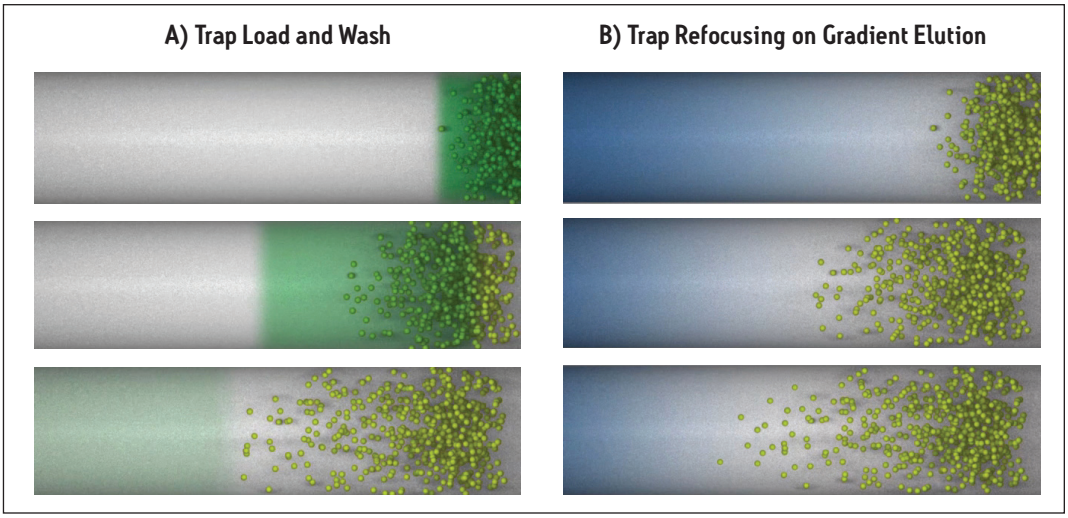


Figure 2. A) (left) Trap load and wash steps with diluent and non-binding matrix components (green) are washed from the trap. B) (right) Trap refocusing with gradient elution through the trap will serve to narrow the analyte peak as it is eluted from the trap.

The focusing is accomplished by differentially eluting the analytes contained within the peak. This differential elution occurs when the gradient flows over a peak of analytes that are spread over a given length. The larger the length that these analytes are spread, the larger extent of refocusing that will be required. Optimizing trap length for this process is an important consideration in method development. Also, the ID of the trap should be as close to the analytical column as possible.

Once the analytes are eluted from the trap they pass through transfer tubing and are refocused on the ionKey microfluidic (iKey). The refocusing that occurs on the iKey is critical to maintaining high peak capacity for analytes ranging from small molecules to intact proteins. Figure 3A illustrates the transfer from trap to iKey and the refocusing that occurs in this process to negate pre-column band broadening. Figure 3B shows data collected by analyzing the chromatography from the trap alone and comparing this to the refocused peak that occurs when a trap is coupled to an iKey.

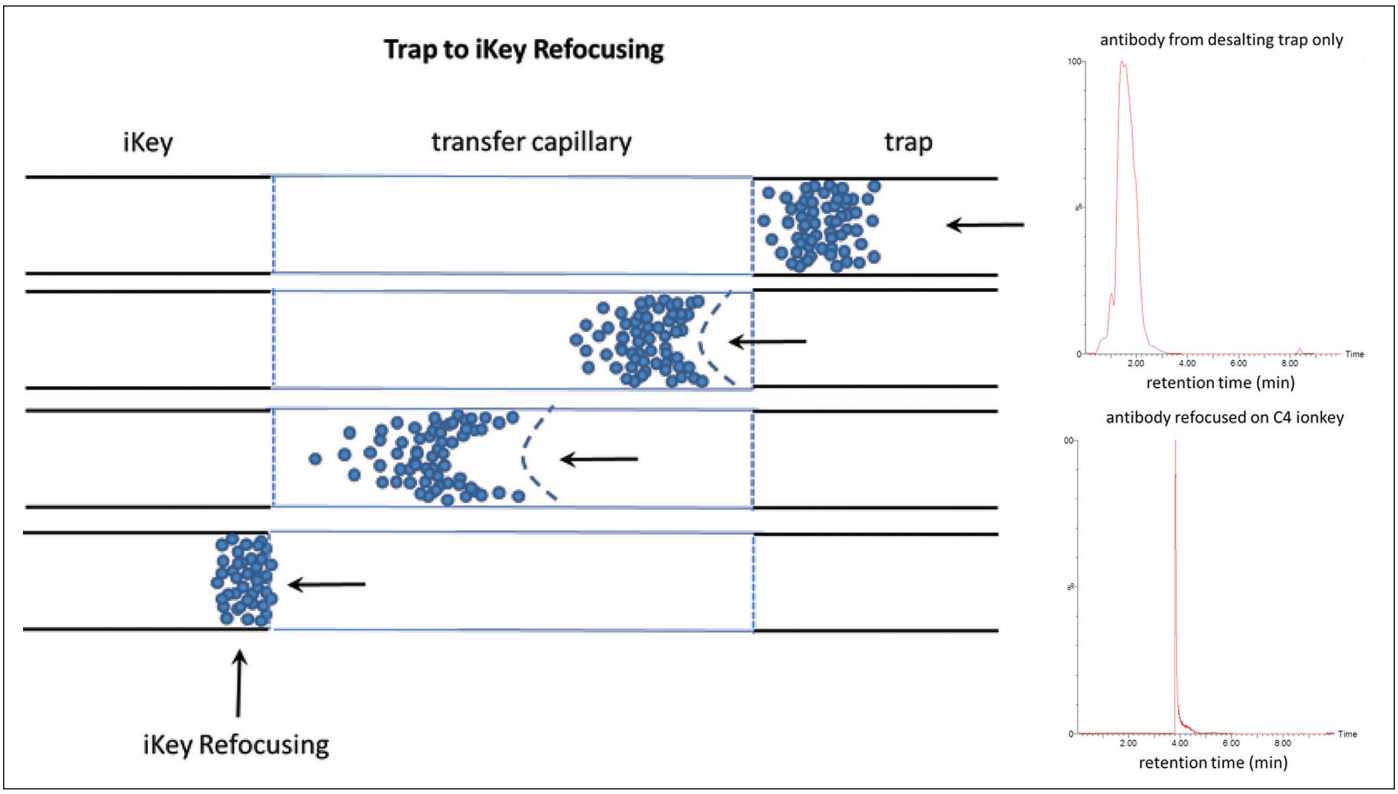


Figure 3. A) Trap to iKey sample transfer and analyte refocusing. B) Comparison between intact antibody eluted from trap only (top) and trap in combination with ionKey (bottom).

Peak shape improvement with trapping

The loading of the analyte onto a microscale column can vary depending upon both the hydrophobicity of the analyte and the organic strength of the diluent. Figure 2A illustrates the loading of a hydrophilic analyte onto a trap column. Upon loading the diluent is exchanged and the analyte peak will broaden slightly. Once the diluent has been exchanged and removed from the hydrophilic analyte it will no longer migrate laterally and one will be able to wash and remove contaminates without significant loss of the analyte.

It is possible to recover some of the losses associated with hydrophilic band broadening on loading by reverse elution using a gradient. This process of trap refocusing is illustrated in Figure 2B. As the gradient traverses the trap column the analytes will refocus. The refocusing occurs by moving the analytes on the edge of the peak into the center via differential elution. This process of differential elution is illustrated in Figures 1 and 2. The result is a focusing event that results in a narrower peak. Following trap refocusing the analyte is pumped through the transfer capillary, where there will be parabolic band broadening, and then refocusing on the iKey, illustrated in Figure 3.

It is critical that the trap be configured in the correct orientation to preserve the gains made in the refocusing of the analyte on the trap. There are two ways to plumb a trap (illustrated in Figure 4): 1) reverse load-forward flush, and 2) forward load-forward flush. The reverse load-forward flush is performed such that the analyte is loaded onto one side of the trap, and then eluted off that same side and into the transfer tubing. This is performed by switching the direction of the flow between trap load and trap elution. The forward load-forward flush is performed by loading the sample onto the head of the trap, and then eluting the sample through the entire length of the trap and then into the transfer tubing.

Figure 4 demonstrates the deleterious effects of eluting the peak through the entire length of the trap. When the system is configured for reverse load and forward flush the peak undergoes initial refocusing and is then pumped into the transfer tubing, however when the system is configured for forward load and forward flush the peak undergoes initial refocusing, but then must chromatograph through the remainder length of the trap column at a non-optimal linear velocity. This non optimal linear velocity causes the peak to broaden via longitudinal diffusion.

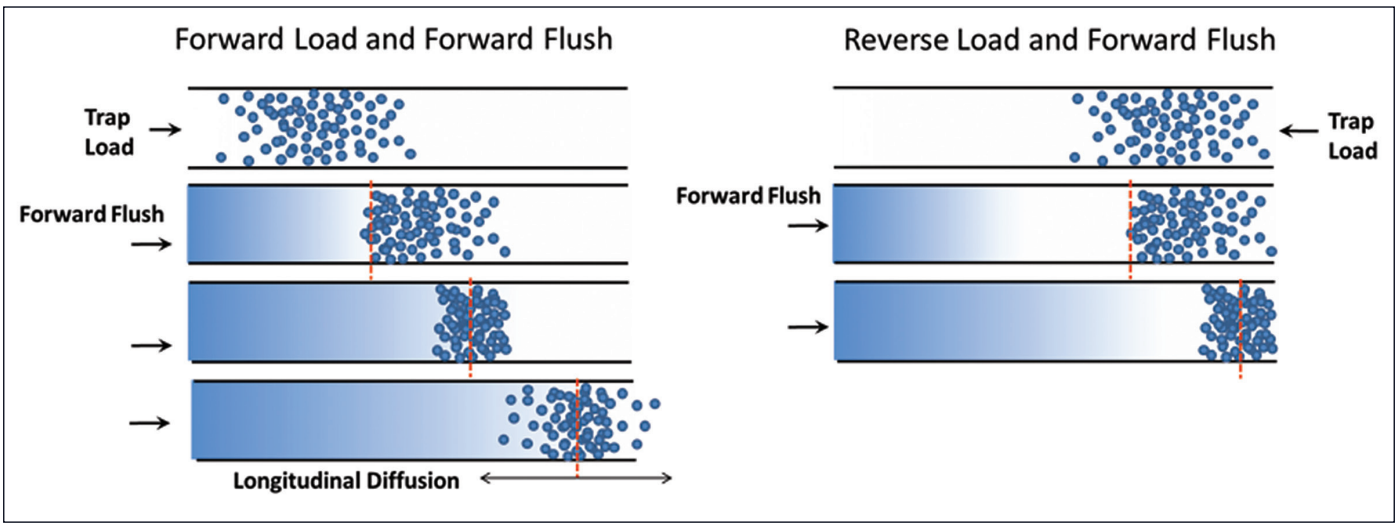


Figure 4. Illustrates the forward load of the analyte onto the trap and then forward flush of the trap column.

The refocusing on the iKey will be dependent on the differential retentivity between the trap and the iKey. This differential retentivity between trap and iKey provides significance in overcoming parabolic band broadening in the transfer tubing (Figure 3). In general, refocusing is accomplished by tuning the system so that the retentivity of the analyte is greater on the iKey, as compared to the trap column for the analyte of interest. Without a differential retentivity between the trap and iKey there will be no refocusing. Suggested trapping chemistry pairings and temperature combinations are tabulated in Table 1, which should serve as a guideline for where to start in terms of method development. More specific nanoscale trap and elute experiments have been published recently describing the chemistry and temperature relationships for peptide separations. In general, the guidelines in Table 1, follow similar trends as specified in this work.[1]

Analyte	Trap chemistry	iKey chemistry	Trap temp.	iKey temp.	Maximum injection volume
Intact protein	BEH C_4, 300Å, 5 µm	BEH C_4, 300Å, 1.7 µm	85 °C	80 °C	20 µL
Peptide	Symmetry C_{18}, 130Å, 5 µm	BEH C_{18}, 130Å, 1.7 µm	65 °C	65 °C	20 µL
Polar Small Molecule	HSS T3 C_{18}, 100Å, 5 µm	HSS T3 C_{18}, 100 Å, 1.7 µm	35 °C	30 °C	

Table 1. Chemistry and temperature pairing for intact protein, peptide bioanalysis, and polar small molecules. Also, maximum injection volumes for each configuration.

Improved peak shape for opioids using trapping over direct injection

Analyzing hydrophilic analytes in mixtures that require moderate organic content in the diluent can be challenging at the microscale. These challenges arise because of the relatively large injection volumes compared to column volumes, where both of these can be similar in magnitude (i.e. ~1 µL). A common way of working around this challenge involves utilizing a larger ID trap column to exchange the diluent and refocus analytes on the trap. Without efficient exchange of the diluent and trap refocusing hydrophilic small molecules will often coelute in the void, or simply break through the column with no noticeable peak. In the example of opioid drugs in oral fluid, this becomes a critical point. We found the best performance for these drugs by pairing both trap chemistry and iKey chemistry with HSS-T3 and operating both columns at low temperature.[2,3]

It is important to maximize retention across both trap and analytical tile for hydrophilic small molecules. Figure 5 illustrates the difference for these analytes between a direct injection onto an HSS-T3 iKey and a trapping configuration. The glucuronide opioids were significantly broader, or undetectable on the direct injection (bottom), as compared to the trapping configuration which enabled quantitative analysis of all hydrophilic glucuronides. The reduction or absence of early eluting glucuronides in the direct injection is due to column breakthrough, an event where the retention factor for the analyte is not great enough to cause significant focusing to form a sharp peak.

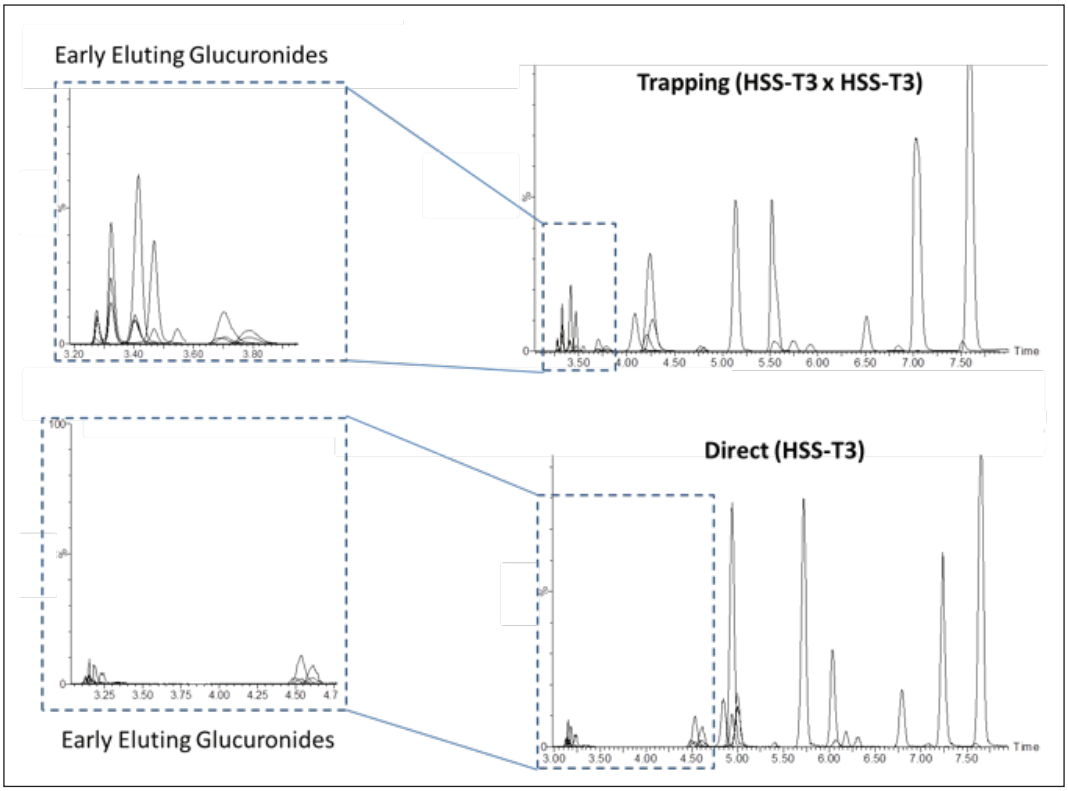


Figure 5. Chromatogram of hydrophilic glucuronides and opioids utilizing a HSS-T3 Trap coupled to an HSS-T3 iKey.

Improved robustness and on-column mass load

The trapping configuration also offers to improve the robustness and mass loading capabilities of the iKey system over direct injection. This configuration also enables us to remove matrix components by loading the sample to the trap and then "washing" the analyte on the trap, illustrated in Figure 2. Although many of the coeluting matrix components will not be removed in the trap wash step, many other non-coeluting small molecules, peptides and proteins will be removed, thus reducing the total protein load on the downstream ionKey consumable. Second, the large particle dimensions of the trap enable the larger porosity to withstand protein precipitation on the surface, as compared to the lower particle dimensions of the iKey Column itself which is less tolerant of protein precipitation. Following trapping the analyte is then eluted onto the iKey with an effectively lower total protein load that is within the operating conditions of the iKey System. Practical examples have successfully been demonstrated for protein bioanalysis from plasma.[4,5]

It is also important to note that with peptide and protein bioanalysis the total mass load depends on the sample prep methodology that is employed. Figure 6 compares the total peptide and protein load on-column for these different methodologies. It is recommended that the total protein load per injection for the ionKey/MS System configured for direct injection (no trap) iKey be <80 ng (on-column), when the system is configured for trapping the total protein load per injection is up to 1000 ng (on-column), which opens the available sample prep methodologies significantly.

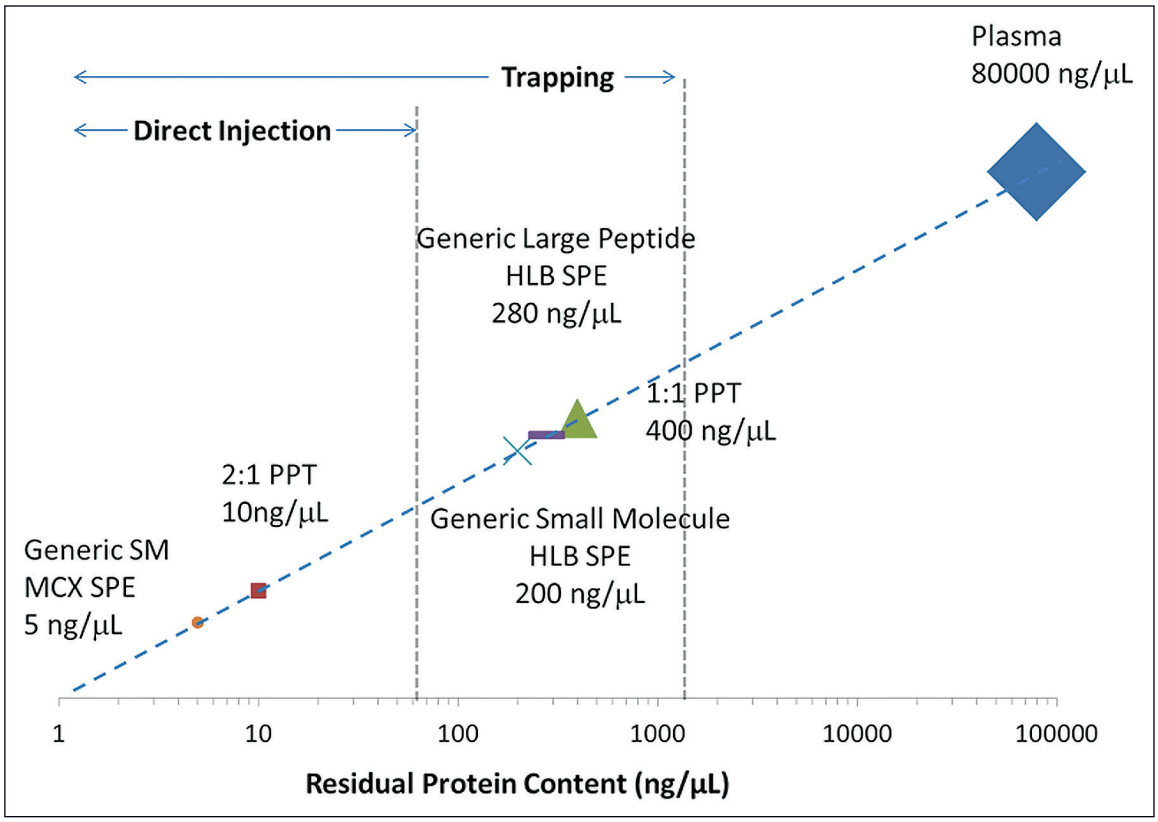


Figure 6. Correlation between sample prep and on-column load capabilities of ionKey in direct and trapping mode.

Column I.D. is an important variable with respect to the mass load capacity of a column. Although mass load limits will increase with column I.D., there are many molecular characteristics that can change the loadability across column I.D. Selection of the 300 µm I.D. x 5 cm column geometry was done so to optimize both loading and unloading of the trap column with respect to peak width. Larger I.D. trap columns demonstrated larger band broadening associated with the non-optimal linear velocities on gradient elution from the trap to the iKey. Lower I.D. trap columns were associated with limited mass load capacities. In sum, 300 µm I.D. x 5 cm provided the best peak width for gradient elution and greatest limit for mass loading.

The process of removing matrix components that do not retain to the trap column can help with respect to downstream ion suppression and sensitivity of the analytical assay. It is commonly known that ion pairs or ion adducts, whether these be inorganic or organic in nature, can cause significant ion suppression in an ESI source. It has been demonstrated that nanoscale ESI assists in reducing ion suppression as compared to high flow ESI,[6] however coupling this technique to a trap, wash, and elute method will assist in further reducing ion pairs and adducts. Figure 2B demonstrates the process of ion cleanup and washing, which helps prepare it for ionization. An example of both efficient and inefficient on-line desalting of an intact monoclonal antibody (mAb) is illustrated in Figure 7.[7]

An antibody will easily form non-covalent adducts with ions that will not dissociate through the MS analysis. The result is an extremely complex spectra that is difficult or impossible to interprete. Compare this to the antibody that has been desalted with a trap wash and elute method, which provides a much less complex spectra with better resolution. Trapping coupled to ionKey offers the most efficient method for desalting and antibody cleanup. Ion optics are also commonly fouled with salts, peptides and proteins, and need regular cleaning. Removing these ions so that they do not enter the ESI source and MS can further improve the cleanliness of the ion optics and reduce the preventative maintenance duty cycle.

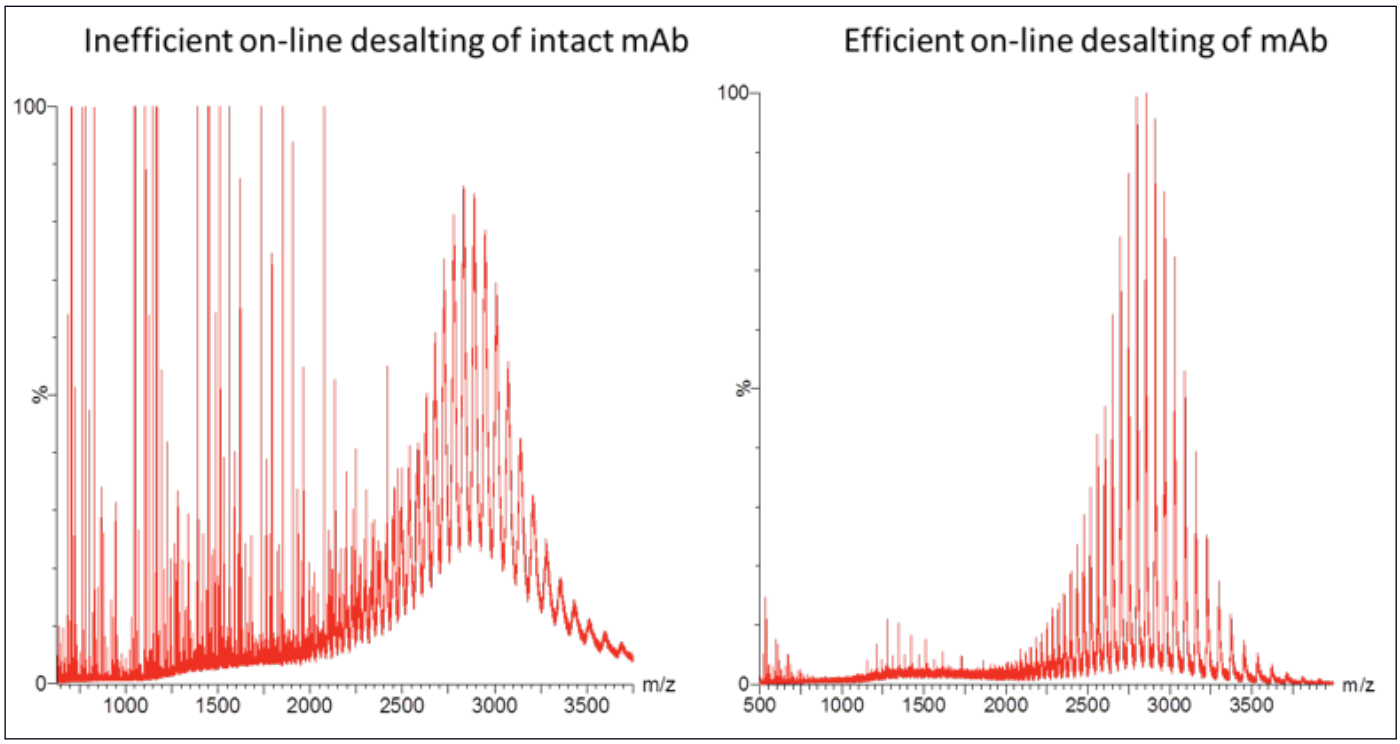


Figure 7. Left) Inefficient desalting of an antibody standard illustrates a complex spectra with an elevated charge state distribution envelope. Right) properly desalted antibody with lower background and improved charge state distribution resolution.

Improved volume load capabilities

The increased volumetric loading capacity of an ionKey/MS System configured for trapping is due to several reasons. First, larger volumes can be loaded faster onto a larger I.D. column with 5 micron particle diameter due to the flow rate capabilities of the trap. Second, the volumetric component of the trapping column increases the total chromatographic column volume which increases the capacity. Finally, the volumetric component of the trap enables more effective diluent exchange which assists in hydrophilic compound analysis.

The capability to inject larger volumes onto a microscale system enables further extending limits of detection. For example, it is recommended to inject 1–5 µL volume loops onto an ionKey in direct inject mode. However, integrating a trapping column enables the injection of 20 µL volume loops, with linearity that ranges from 1–20 µL. An example is shown below in Figure 8, illustrates utilizing injection volumes from 1–20 µL using a trapping configuration that are linear for small molecules propranolol, buspirone, and verapamil.[8]

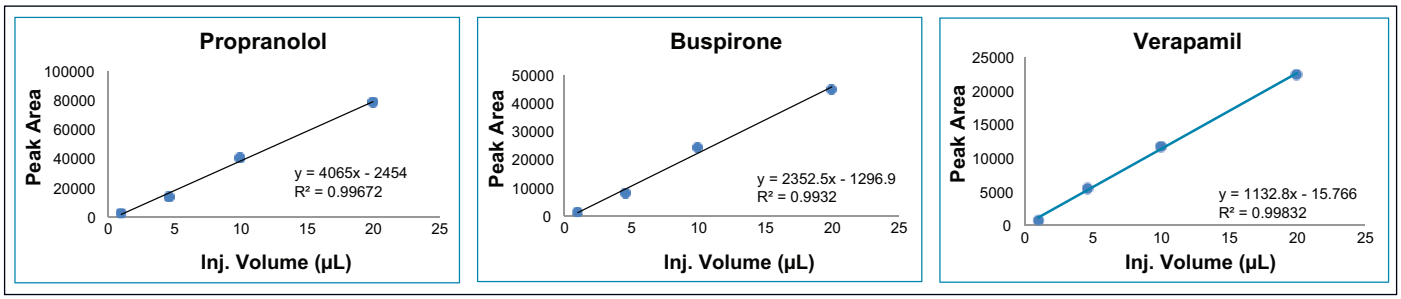


Figure 8. ionKey configured with dual pump trapping mode utilizing a HSS-T3 Trap coupled to an HSS-T3 iKey for small molecules propranolol, buspirone, and verapamil.[8]

CONCLUSION

The trap in conjunction with ionKey/MS provides improved separation capabilities, sensitivity and robustness for a wide range of analytes spanning small molecules, peptides and intact proteins. These improvements enable the microscale system to inject similar volumes as a traditional high flow analytical UPLC® instrument, and continue to hold sensitivity advantages with equivalent injection volumes. It also expands the realm of samples and sample prep methodologies that are amenable with such a technology. The hardware integration and ease of use demonstrated here will ease the path to utilization by research areas in biotechnology, health, food, and environment related research areas.

References

1. Matthew A. Lauber, Stephan M. Koza, and Kenneth J. Fountain. Optimizing Peak Capacity in Nanoscale Trap-Elute Peptide Separations with Differential Column Heating. Waters Application Note 720005047EN.
2. M. Gilar, J.T.S. McDonald, G. Roman, J.S. Johnson, J.P. Murphy, J.W. Jorgenson, *Journal of Chromatography A*, 2015, 1381, 110–117.
3. Gregory Roman, Robert Lee, James Murphy, and Michelle Wood. High Sensitivity Analysis of Opioids in Oral Fluid Using ionKey/MS. Waters Application Note 720005296EN.
4. Jay S. Johnson, Morteza Razavi, James Murphy, Selena Larkin, Paul Rainville. Improving the Detection of Thyroglobulin in Human Plasma for Clinical Research by Combining SISCAPA Enrichment and Microflow LC-MS. Waters Application Note 720005509EN.
5. Mary E. Lame, Erin E. Chambers, and Kenneth J. Fountain. Increasing Sensitivity and Minimizing Sample Volume for the Quantification of Therapeutic and Endogenous Cyclic Peptides in Plasma Using ionKey/MS. Waters Application Note 720005128EN.
6. Jay S. Johnson, Paul D. Rainville, James P. Murphy: Reductions in ion suppression using ionKey/MS compared to standard flow LC-MS. Waters White Paper 720004966EN.
7. Gregory Roman, Henry Shion, James Murphy, Weibin Chen. High Sensitivity Analysis of Antibodies (IgG1) using ionKey/MS. Waters Application Note 720005378EN.
8. Yun Wang Alelyunas, Mark D. Wrona, Jim Murphy, Angela Doneanu, Gregory Roman, and Paul Rainville. Exploring Extra Sensitivity Using ionKey/MS with the Xevo G2-XS QTof HRMS for Small Molecule Pharmaceutical Analysis in Human Plasma. Waters Application Note 720005371EN.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Sensitive and Reproducible LC-MS Quantification of C-Reactive Protein in Plasma: A Potential Biomarker of Inflammation

Paula Orens, Mary Lame, Steven Calciano, and Erin Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

High sensitivity quantification of a protein biomarker, demonstrated speed and reproducibility of a generic kit-based approach for protein quantification, high sensitivity of the Xevo® TQ-XS mass spectrometer, mixed-mode SPE specificity, no affinity purification required.

WATERS SOLUTIONS

ProteinWorks™ eXpress Direct Digest Kit

ProteinWorks µElution SPE Clean-Up Kit

ACQUITY UPLC® HSS T3 Column

ACQUITY UPLC System

Xevo TQ-XS Mass Spectrometer

MassLynx® Software

TargetLynx™ Application Manager

KEYWORDS

C-reactive protein, CRP, biomarker, protein digestion, tryptic peptides, protein quantification, ProteinWorks, eXpress Direct Digest, µElution SPE, Oasis® MCX, Xevo TQ-XS, Skyline

INTRODUCTION

C-Reactive Protein (CRP)[1] is naturally synthesized in the liver and released into the bloodstream in response to inflammation. There is, therefore, interest in measuring CRP both in cases of chronic inflammation such as Rheumatoid Arthritis (RA),[2,4] as well as to evaluate cardiovascular risk[3] and inflammatory cancerous processes.[4] Tissue injury or inflammation causes a > 100-fold[2] increase in plasma CRP levels. While endogenous plasma levels in healthy individuals are relatively low, generally between 0.1 and 3 µg/mL (4–120 nM),[2] levels present in diseased patients are so highly elevated, multiple ELISA or immunoturbidimetric[3] tests are required to achieve accurate CRP quantification. The need for multiple tests arises out of the limited linear dynamic range of ligand binding assays (LBA). This inherent short coming, as well as lack of standardization, possible cross-reactivity, and expensive, difficult to reproduce reagents are just a few of the reasons that the industry is moving towards LC-MS. Mass Spectrometry detection offers many benefits for protein quantification such as sensitivity, specificity, broad linear dynamic range, fast method development times, and the ability to multiplex. However, LC-MS protein quantification still presents challenges. There is no single standardized workflow and the various workflow options can be complex and laborious, making it difficult for a scientist to achieve success. In this application note, we describe a generic, kitted approach which requires only 2 hours for digestion vs. a standard 24 hour method described in the literature.[5] Digestion and subsequent peptide level purification are performed using the ProteinWorks eXpress Direct Digest (p/n: 176003688) and ProteinWorks µElution SPE Clean-Up Kit (p/n: 186008304) for the accurate and reliable quantification of endogenous CRP in human plasma. The kits and their included protocols achieve LLOQs between 0.025-0.1 µg/mL (1-4 nM) from only 35 µL of plasma.

EXPERIMENTAL

To prepare calibration curve standards and quality control (QC) samples, CRP (human sequence) was spiked into rat or human plasma at various concentrations over the range of 0.025–100 µg/mL (1–3987 nM). CRP QC samples were prepared in one lot of rat plasma and four lots of human plasma. Calibration curve standards were prepared in duplicate, each QC level prepared in triplicate, and blank (non-spiked) plasma samples were prepared in quadruplicate. Plasma samples (35 µL) were digested for 2 hours using the ProteinWorks eXpress Direct Digestion Kit, specifically, the 3-Step (no reduction/alkylation) method included in the kit with an abbreviated denaturation time of 5 minutes (p/n: 176003688). Post digestion purification of signature peptides was done using the ProteinWorks µElution SPE Clean-Up Kit (p/n: 186008304) and included protocol.

Method conditions

LC system: ACQUITY UPLC

Column: ACQUITY UPLC HSS T3, 1.8 µm, 2.1 x 50 mm (p/n: 186003538)

Temp.: 55 °C

Sample temp.: 10 °C

Injection volume: 5 µL

Mobile phases: A: 0.1% Formic acid in H_2O
B: 0.1% Formic acid in ACN

Gradient:

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.300	100.0	0.0	6
1.0	0.300	100.0	0.0	6
5.0	0.300	70.0	30.0	6
5.5	0.300	10.0	90.0	6
6.3	0.300	10.0	90.0	6
6.4	0.300	100.0	0.0	6
7.5	0.300	100.0	0.0	6

MS conditions

MS system: Xevo TQ-XS

Ionization mode: ESI+

Capillary: 3.5 kV

Source offset (V): 60 V

Source temp.: 150 °C

Desolvation temp.: 600 °C

Cone gas flow: 150 L/Hr

Desolvation gas flow: 1000 L/Hr

Collision gas flow: 0.15 mL/Min

Nebulizer gas flow: 7 Bar

Data management: MassLynx (v4.1)

Quantification software: TargetLynx

RESULTS AND DISCUSSION

Early detection of elevated CRP levels could be imperative for proper treatment or prevention of RA or heart disease. Availability of a sensitive analytical method that can detect low levels and differentiate between small concentrations can facilitate early detection. Mass spectrometric detection, though not traditionally used for CRP quantification, has the sensitivity and reproducibility required for measuring both the low endogenous CRP levels in healthy individuals as well as elevated levels in diseased populations.

MASS SPECTROMETRY

Protein quantification by LC-MS is typically performed via the bottom up approach which uses enzymatic digestion (usually trypsin) and relies on analysis of resulting (tryptic) peptides. To identify an appropriate representative or surrogate peptide for human CRP (Figure 1), an in-silico digestion was performed using Skyline Software (MacCoss Labs, University of Washington).[6] In addition to identifying potential tryptic peptides, Skyline facilitated MS method development by selecting and optimizing precursors and collision induced fragments for MRM (multiple reaction monitoring) analysis. The full amino acid sequence of CRP[7] and the unique signature peptides: AFVPFK, ESDTSYVSLK, and GYSIFSYATK (highlighted in blue), are illustrated in Figure 2. Optimized MS conditions and MRM transitions for the CRP tryptic peptides are listed in Table 1. Both primary and confirmatory (secondary) transitions are included for each peptide.

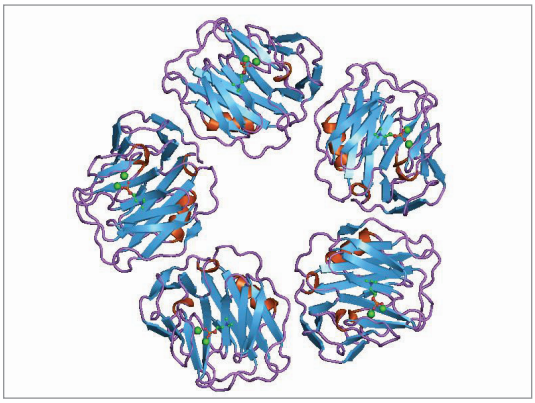


Figure 1. C-Reactive Protein (CRP) structure.[1]

MEKLLCFLVLTSLSHAFGQTDMSRKAFVFPKESDTSYVSLKAPLTKPLKAFTVCLHFYTELS
STRGYSIFSYATKRQDNEILIFWSKDIGYSFTVGGSEILFEVPEVTVAPVHICTSWESASGI
VEFWVDGKPRVRKSLKKGYTVGAEASIILGQEQDSFGGNFEGSQSLVGDIGNVNMWDF
VLSPDEINTIYLGGPFSPNVLNWRALKYEVQGEVFTKPQLWP

Figure 2. Amino acid sequence of human CRP; tryptic peptides used for quantification are highlighted in blue.

Peptide	Precursor charge state	MRM transition	Cone voltage (V)	Collision energy (eV)	Product ion identification
AFVFPK	$[M+2H]^{2+}$	354.71>244.17 (Primary)	35	9	[1H+]1/y2
	$[M+2H]^{2+}$	354.71>219.11 (Confirmatory)	35	3	[1H+]1/b2
ESDTSYVSLK	$[M+2H]^{2+}$	564.77>347.23 (Primary)	35	17	[1H+]1/y3
	$[M+2H]^{2+}$	564.77>696.39 (Confirmatory)	35	17	[1H+]1/y6
GYSIFSYATK	$[M+2H]^{2+}$	568.78>221.09 (Primary)	35	11	[1H+]1/b2
	$[M+2H]^{2+}$	568.78>716.36 (Confirmatory)	35	11	[1H+]1/y6

Table 1. Final MS conditions for CRP tryptic peptides, including precursor and fragment ions.

In an earlier study, CRP quantification was performed on a Xevo TQ-S MS. Quantification was subsequently transferred to a newer generation platform, a Xevo TQ-XS triple quadrupole mass spectrometer, which provided the benefit of improved sensitivity. Signal-to-noise (S/N) for the three CRP peptides used for quantification increased 2.5 X (AFV), 3.5 X (ESD) and 1.4 X (GYS) using the Xevo TQ-XS vs the older platform. This improvement in sensitivity for the AFV peptide is demonstrated in Figure 3. For the ESD, peptide (RT 3.63 minutes), the intensity for an interfering matrix peak (RT 3.72 minutes), present as a shoulder on the main peak, was decreased 7 X. This is shown in Figure 4. The reduced matrix interference facilitated easier peak integration and resulted in improved limits of detection.

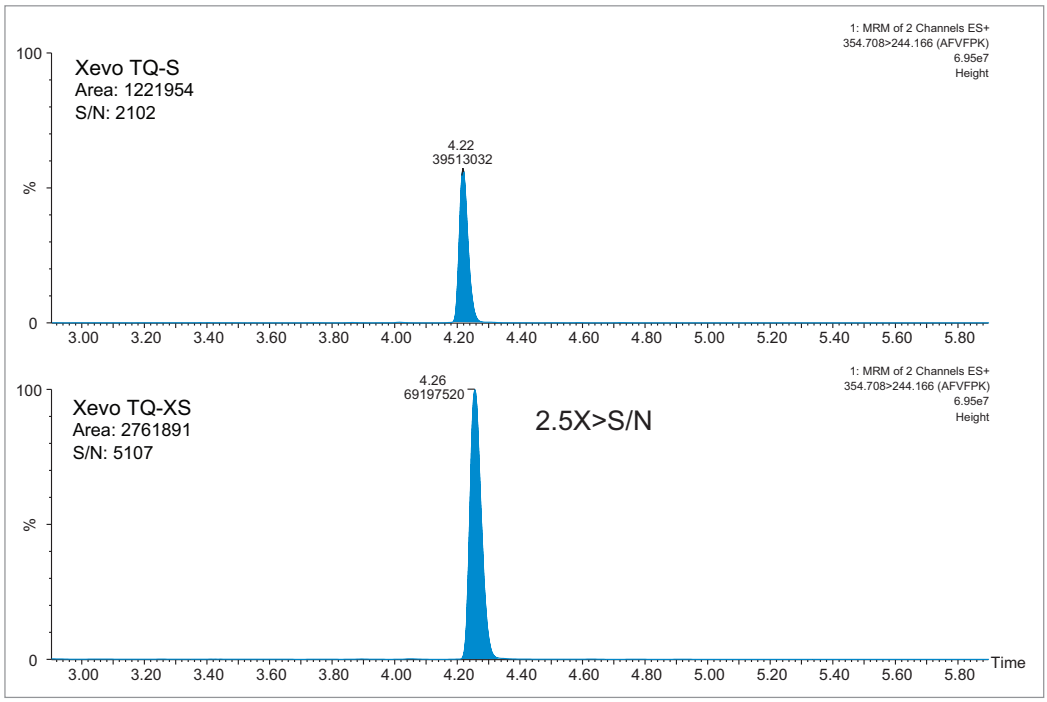


Figure 3. Representative chromatogram demonstrating improved sensitivity and S/N for the AFVFPK CRP peptide digested in rat plasma (100 µg/mL) and purified by SPE: Xevo TQ-S vs. Xevo TQ-XS.

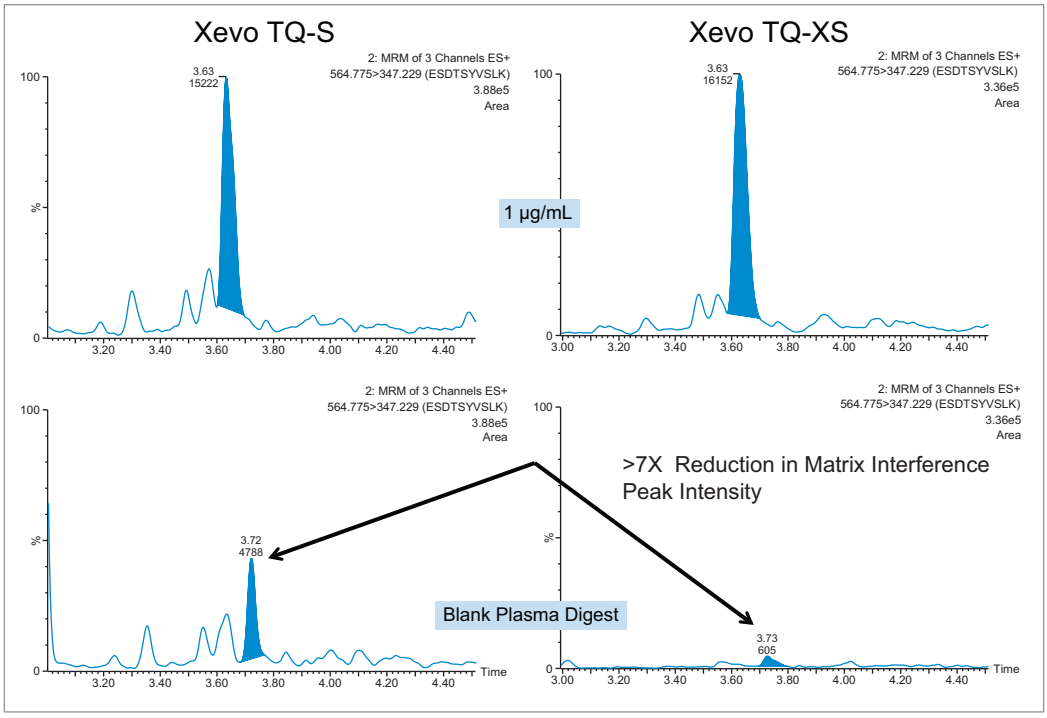


Figure 4. Representative chromatogram demonstrating reduced matrix interferences for the ESDTSYVSLK CRP peptide digested in rat plasma (blank and 1 µg/mL) and purified by SPE: Xevo TQ-S vs. Xevo TQ-XS.

CHROMATOGRAPHY

Reversed-phase chromatographic retention can be challenging due to the relatively small size and polar nature of tryptic peptides. Use of an ACQUITY HSS T3 Column, 1.8 µm, 2.1 x 50 mm (p/n: 186003538) for the CRP peptides afforded improved retention as compared to a BEH C18 column and facilitated resolution from endogenous matrix interferences. Representative chromatograms of the three peptides: AFVPFK, ESDTSYVSLK, and GYSIFSYATK are illustrated in Figure 5. Peak widths were <4.5 seconds wide.

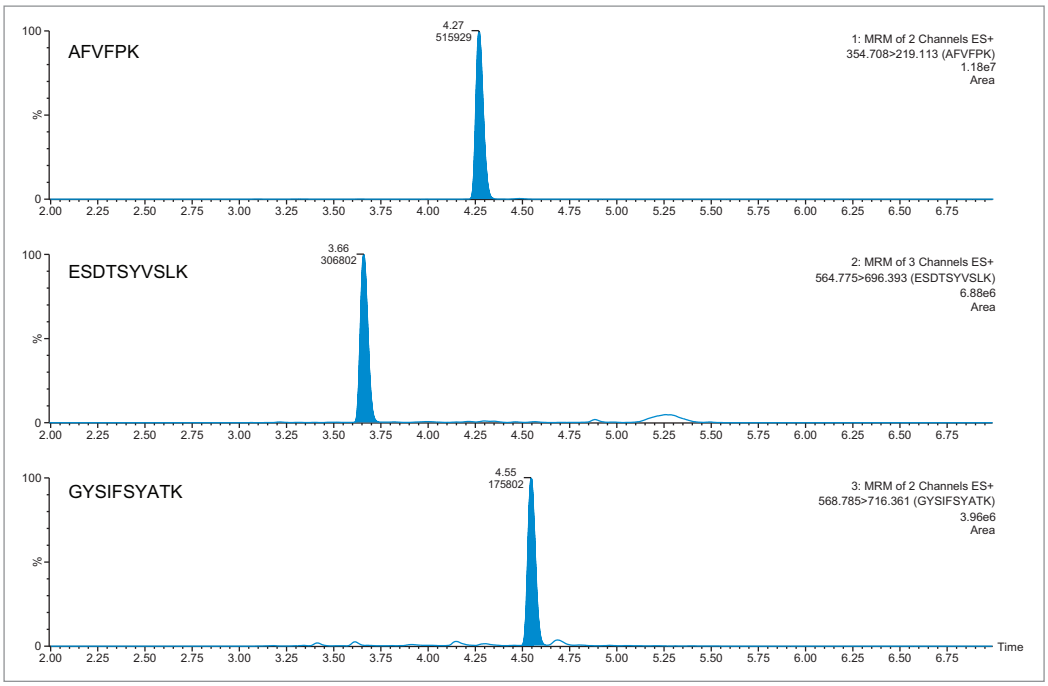


Figure 5. UPLC chromatographic separation of CRP tryptic peptides: AFVPFK, ESDTSYVSLK, and GYSIFSYATK, digested in rat plasma (50 µg/mL) using an ACQUITY HSS T3, 1.8 µm, 2.1 x 50 mm Column.

SAMPLE PREPARATION

Typical sample preparation workflows for protein quantification are often complex and laborious. In this application, we used the ProteinWorks eXpress Direct Digest Kit (p/n: 176003688) to directly digest plasma samples in only 2 hours. Subsequent purification of the peptides with the ProteinWorks µElution SPE Clean-Up Kit (p/n: 186008304) and protocol removed buffer salts, phospholipids, and excess digestion reagents post digestion. The SPE kit relies on a mixed-mode sorbent (Oasis® MCX, reversed-phase, and ion-exchange) to provide enhanced specificity. Tryptic peptides are bound to the ion-exchange moiety of the SPE sorbent, thereby imparting orthogonality, and thus greater specificity, into the method as a whole. In addition, very polar peptides are more efficiently trapped by ion exchange than by traditional reversed-phased only sorbent. This was particularly important for the most polar peptide, ESD. Recoveries for all three CRP peptides were excellent, with greater than ≥90% recovery using the generic protocol provided.

LINEARITY, PRECISION, AND ACCURACY

Using only 35 µL of sample and the aforementioned ProteinWorks kits, quantification limits of 0.025 and 0.05 µg/mL (1–2 nM) were achieved in rat and human plasma, respectively. Calibration curves from the tryptic peptides, in both rat and human plasma, were linear with R^2 values >0.99 using 1/x or $1/x^2$ weighted regressions. A summary of standard curve performance in rat and human plasma is shown in Tables 2 and 3. Linearity, accuracy and precision data met typical method validation requirements. For both rat and human, standard curves were linear over 4 orders of magnitude with mean accuracies ranging from 94–105%. The precision and accuracy for the QC samples was excellent with mean % RSDs all <5% and % QC accuracy ranges of 94.3–106.2 (rat) and 89.4–103.5 (human). Rat QC performance is highlighted in Table 4. Human QC performance for the AFV and ESD peptides is shown in Table 5, Panels A and B, respectively. Demonstration of this performance is illustrated in Figure 6, Panels A and B.

Peptide	Curve (µg/mL)	Curve (nM)	Weighting	Linear Fit (R^2)	% Accuracy Range
AFVFPK	0.025–100	1.00–3987	$1/x^2$	0.999	95.4–103.2
ESDTSYVSLK	0.100–100	3.99–3987	1/x	0.997	92.9–105.1
GYSIFSYATK	0.050–100	1.99–3987	1/x	0.998	95.2–104.0

Table 2. Rat plasma: linear dynamic range and standard curve statistics for the CRP tryptic peptides used for quantification. Plasma samples were digested and extracted using ProteinWorks eXpress Direct Digest and µElution SPE Clean-Up Kits

Peptide	Curve (µg/mL)	Curve (nM)	Weighting	Linear fit (R^2)	% Accuracy range
AFVFPK	0.050–100	1.99–3987	$1/x^2$	0.998	93.6–104.4
ESDTSYVSLK	0.050–100	1.99–3987	1/x	0.999	96.8–102.4

Table 3. Human plasma: linear dynamic range and standard curve statistics for the CRP tryptic peptides used for quantification. Plasma samples were digested and extracted using ProteinWorks eXpress Direct Digest and µElution SPE Clean-Up Kits.

Peptide	CRP QC concentration (µg/mL)	CRP QC concentration (nM)	Mean (N=3) calculated concentration (µg/mL)	Mean (N=3) calculated concentration (nM)	Mean (N=3) % accuracy	%RSD
AFVPFK	0.075	2.990	0.071	2.818	94.3	2.16
	0.750	29.904	0.763	30.422	101.7	3.18
	7.500	299.035	7.691	306.664	102.5	1.23
	75.000	2990.353	74.946	2988.213	99.9	3.49
ESDTSYVSLK	0.250	9.968	0.265	10.579	106.2	2.08
	0.750	29.904	0.738	29.425	98.4	0.72
	7.500	299.035	7.210	287.459	96.1	0.97
	75.000	2990.353	75.399	3006.262	100.6	3.77
GYSIFSYATK	0.075	2.990	0.078	3.097	104.0	2.68
	0.750	29.904	0.735	29.292	98.0	6.15
	7.500	299.035	7.394	294.822	98.6	1.98
	75.000	2990.353	74.918	2987.070	99.9	5.63

Table 4. Rat plasma: QC sample statistics for tryptic peptides used to quantify CRP.

Table 5A.

Peptide	CRP overspike concentration (µg/mL)	CRP overspike concentration (nM)	CRP QC concentration (µg/mL)	CRP QC concentration (nM)	Mean (N=3) calculated concentration (µg/mL)	Mean (N=3) calculated concentration (nM)	Mean (N=3) % accuracy	%RSD
AFVPFK Lot #1	0.000	0.000	0.387	15.420	0.387	15.420	100.0	3.66
	0.075	2.990	0.462	18.421	0.481	19.191	104.3	0.60
	0.750	29.904	1.137	45.334	1.176	46.889	103.5	2.51
	7.500	299.035	7.887	314.466	7.941	316.605	100.7	2.23
	75.000	2990.353	75.387	3005.783	74.678	2977.515	99.1	1.25
AFVPFK Lot #2	0.000	0.000	1.162	46.331	1.162	46.331	100.0	4.55
	0.075	9.968	1.237	49.321	1.167	46.543	94.4	4.52
	0.750	29.904	1.912	76.234	1.750	69.775	91.5	1.15
	7.500	299.035	8.662	345.366	8.398	334.853	96.9	2.29
	75.000	2990.353	76.162	3036.684	72.141	2876.348	94.7	1.74
AFVPFK Lot #3	0.000	0.000	1.867	74.440	1.867	74.440	100.0	1.20
	0.075	9.968	1.942	77.430	1.787	71.250	92.0	3.92
	0.750	29.904	2.617	104.343	2.290	91.319	87.5	3.17
	7.500	299.035	9.367	373.475	8.527	339.983	91.0	1.29
	75.000	2990.353	76.867	3064.793	72.444	2888.429	94.2	13.31
AFVPFK Lot #4	0.000	0.000	18.128	722.778	18.128	722.778	100.0	2.26
	0.075	9.968	18.203	725.779	17.612	702.201	96.8	6.47
	0.750	29.904	18.878	752.692	17.586	701.178	93.2	1.72
	7.500	299.035	25.628	1021.824	23.988	956.421	93.6	5.59
	75.000	2990.353	93.128	3713.141	83.284	3320.634	89.4	1.37

Table 5B.

Peptide	CRP overspike concentration (µg/mL)	CRP overspike concentration (nM)	CRP QC concentration (µg/mL)	CRP QC concentration (nM)	Mean (N=3) calculated concentration (µg/mL)	Mean (N=3) calculated concentration (nM)	Mean (N=3) % accuracy	%RSD
ESDTSYVSLK Lot #1	0.000	0.000	0.439	17.514	0.439	17.514	100.0	5.21
	0.075	2.990	0.514	20.494	0.507	20.215	98.7	1.61
	0.750	29.904	1.189	47.407	1.196	47.686	100.5	5.37
	7.500	299.035	7.939	316.539	7.781	310.252	98.0	0.73
	75.000	2990.353	75.439	3007.857	73.159	2916.937	97.0	1.19
ESDTSYVSLK Lot #2	0.000	0.000	1.188	47.347	1.188	47.347	100.0	2.36
	0.075	9.968	1.263	50.358	1.269	50.597	100.5	2.99
	0.750	29.904	1.938	77.271	1.894	75.503	97.7	1.26
	7.500	299.035	8.688	346.403	8.295	330.746	95.5	1.40
	75.000	2990.353	76.188	3037.720	74.171	2957.300	97.3	1.36
ESDTSYVSLK Lot #3	0.000	0.000	1.867	74.440	1.867	74.440	100.0	1.20
	0.075	9.968	1.942	77.430	1.787	71.250	92.0	3.92
	0.750	29.904	2.617	104.343	2.290	91.319	87.5	3.17
	7.500	299.035	9.367	373.475	8.527	339.983	91.0	1.29
	75.000	2990.353	76.867	3064.793	72.444	2888.429	94.2	13.31
ESDTSYVSLK Lot #4	0.000	0.000	18.128	722.778	18.128	722.778	100.0	2.26
	0.075	9.968	18.203	725.779	17.612	702.201	96.8	6.47
	0.750	29.904	18.878	752.692	17.586	701.178	93.2	1.72
	7.500	299.035	25.628	1021.824	23.988	956.421	93.6	5.59
	75.000	2990.353	93.128	3713.141	83.284	3320.634	89.4	1.37

Table 5. Human plasma: QC sample statistics for tryptic peptides, AFVPFK (A) and ESDTSYVSLK (B), used to quantify CRP in four lots of human plasma.

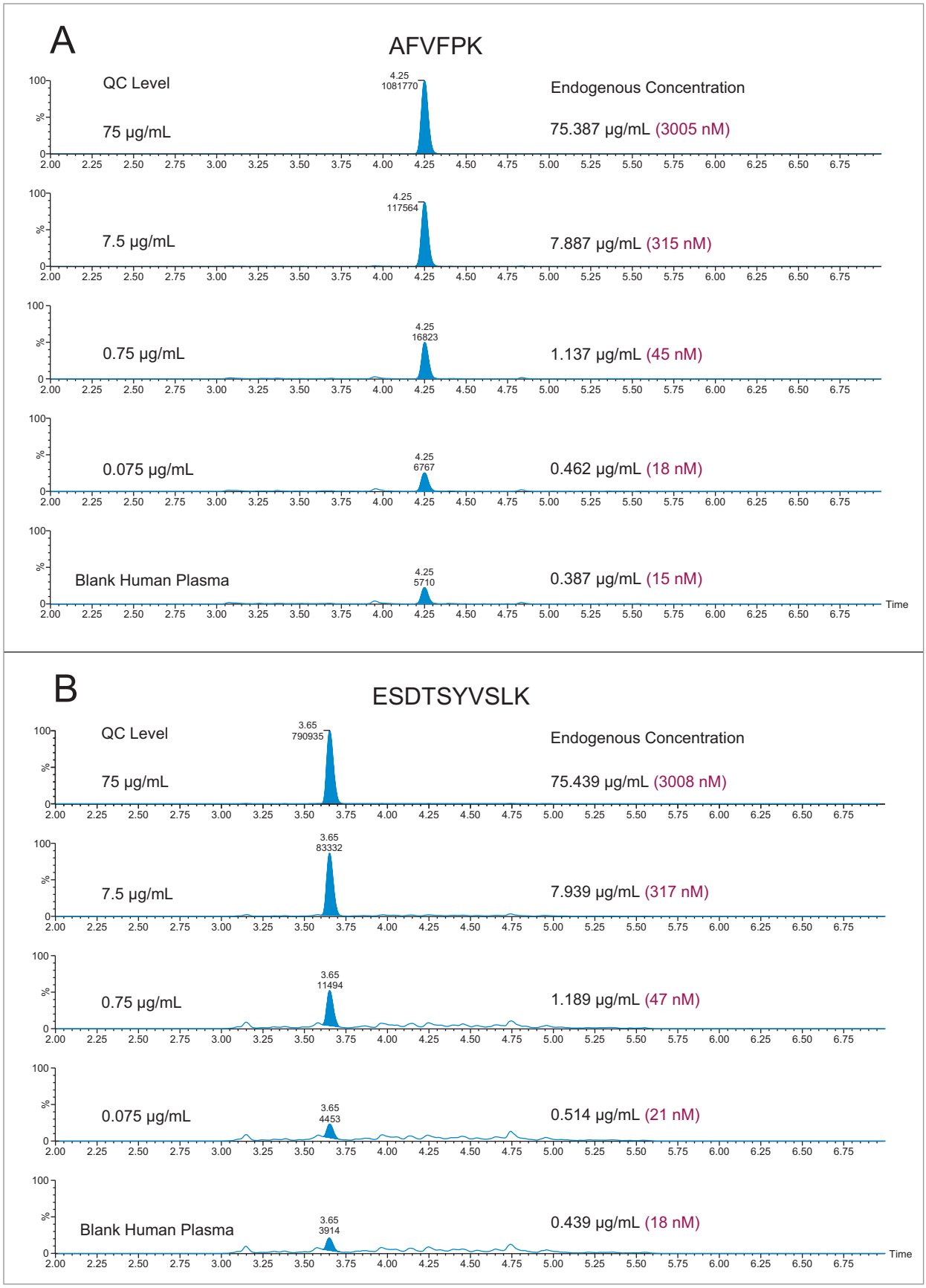


Figure 6. QC chromatograms for AFVPFK (A) and ESDTSYVSLK (B) in human plasma (Lot #1). QC concentrations (overspike concentration+endogenous concentration) can be seen to the right of the QC level.

Endogenous human CRP concentrations were accurately quantified in a total of four lots of human plasma. The lowest level of CRP that could be accurately and precisely quantified above the endogenous level in human plasma was 0.05 µg/mL (2 nM). For the blank human plasma digest (Figure 6), there is a strong signal from the human CRP peptide, due to endogenous CRP concentrations in the range of 0.4–0.666 µg/mL. In rat plasma blank digest (Figure 4), human CRP peptide is not detected. This is expected as the rat CRP amino acid sequence is different than human CRP sequence. Calculated endogenous CRP levels for the four lots of human plasma are summarized Table 6 and illustrated in Figure 7, panels A (AFV peptide) and B (ESD peptide). Within each plasma lot, the calculated endogenous CRP concentrations derived from either the AFV or ESD tryptic peptides were within 10% agreement. Confirmatory transitions for each peptide were used to verify endogenous concentrations of each plasma lot. With the exception of Lot 1, the concentrations calculated from the primary and confirmatory transitions were within 10% of each other. Endogenous CRP concentrations calculated from the GYS peptide did not correlate with those calculated by the AFV and ESD peptides. It is speculated that an underlying, co-eluting interference impeded accurate quantification using the GYS peptide.

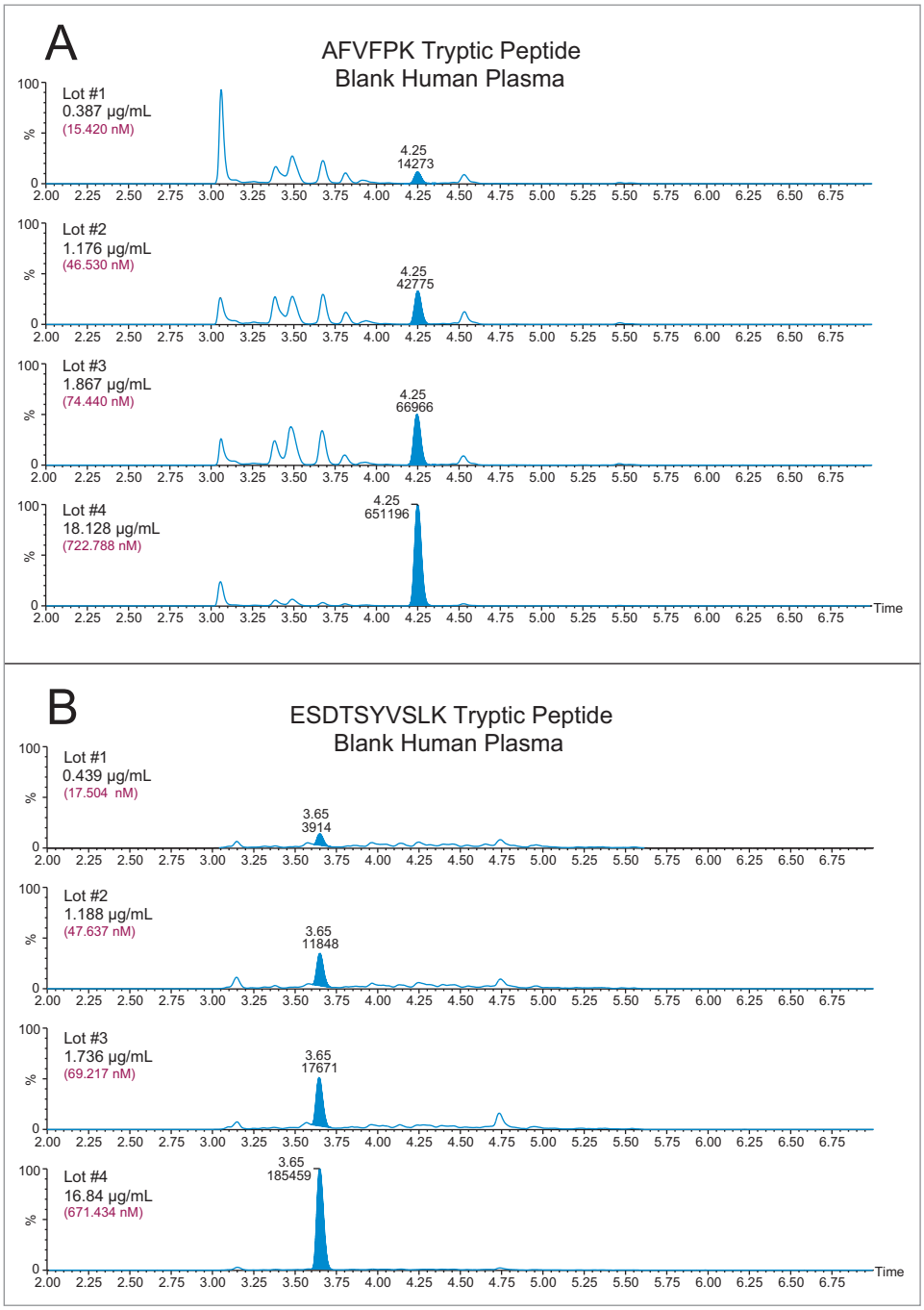


Figure 7. Representative chromatograms highlighting endogenous CRP concentrations in four lots of human plasma represented by the AFVPFK (A) and ESDTSYVSLK (B) tryptic peptides of CRP.

Peptide	Plasma	Mean (N=3) Calculated Endogenous Concentration (µg/mL) Primary MRM	Mean (N=3) Calculated Endogenous Concentration (µg/mL) Confirmatory MRM	Mean (N=3) Calculated Endogenous Concentration (nM) Primary MRM	Mean (N=3) Calculated Endogenous Concentration (nM) Confirmatory MRM
		354>244	**354>219**	**354>244**	**354>219**
AFVPFK	Lot #1	0.387	0.381	15.420	15.191
	Lot #2	1.167	1.145	46.331	45.653
	Lot #3	1.867	1.89	74.440	75.357
	Lot #4	18.128	18.273	722.778	728.570
		364>347	**364>696**	**364>347**	**364>696**
ESDTSYVSLK	Lot #1	0.439	0.666	17.514	26.554
	Lot #2	1.188	1.145	47.347	45.653
	Lot #3	1.736	1.952	69.197	77.829
	Lot #4	16.84	17.015	671.414	678.411

Table 6. Calculated endogenous CRP concentrations in four lots of human plasma using the AFVPFK (A) and ESDTSYVSLK (B) tryptic peptides of CRP.

CONCLUSION

Endogenous CRP was reliably quantified down to 0.025 µg/mL (1 nM) using commercially available digestion and purification kits. Plasma (35 µL) was directly digested (no affinity purification necessary) and subsequent tryptic peptides purified using the generic protocols provided in the kits. The combination of mixed-mode SPE and protocol resulted in >90% recovery for the three peptides. Total sample preparation time, including SPE, was <3 hours. Updating the MS platform, Xevo TQ-S to Xevo TQ-XS, improved sensitivity and S/N, consequently improved the LOQ. This method demonstrates the accurate, reproducible quantification of endogenous CRP levels in plasma using a generic kit-based approach. The broad dynamic range and specificity of LC-MS reliably measures low endogenous levels and the elevated levels expected in diseased patients.

References

1. C-reactive protein, pentameric structure In Wikipedia. Retreived 19Sep2016 from https://commons.wikimedia.org/w/index.php?curid=6003861
2. Kun, E., Wu, J., Karl, J., Liao, H., Zolg, W., Guild, B. (2004) Quantification of c-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and ^{13}C-labeled peptide standards. *Proteomics* (Issue 4), pages 1175–1186.
3. C-reactive protein test (2014, 12 Dec.) Retreived from http://www.mayoclinic.org/tests-procedures/c-reactive-protein/basics/definition/PRC-20014480
4. Allin. K.H., Nordestgaard, B.G., (2011) Elevated c-reactive protein in the diagnosis, prognosis, and cause of cancer. *Critical reviews in clinical laboratory sciences*. Volume 48 (Issue 4), pages 155–170.
5. Rezelia, M., Végvária, A., Silajdžićb E., et.al. (2014) Inflammatory markers in Huntington's disease plasma-A robust nanoLC-MRM-MS assay development. *EuPA Open Proteomics* (3) pages 68–75.
6. Skyline targeted mass spec environment (2016). Downloaded March 2016 from https://skyline.gs.washington.edu/labkey/project/home/software/Skyline/begin.view
7. UniPro, (16May2014). UniProtKB – P02741 (CRP_Human). Primary accession number: P02741. Retreived from http://www.uniprot.org uniprot/P02741

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Antibody Drug Conjugate (ADC) Bioanalysis

Consisting of an antibody coupled with a potent cytotoxic agent, antibody drug conjugates (ADCs) are a rapidly growing class of biotherapeutics. The antibody enables targeted delivery of cytotoxic drugs, while minimizing damage to healthy cells, which has proven to be an enormous advance in cancer treatment in recent years. With the progress of antibody development over the past decades, interest from pharmaceutical companies to develop ADCs is intensifying.

The inherent complexity of ADCs presents unique challenges to bioanalysts due to their large size and heterogeneous nature. In addition to typical challenges associated with quantifying antibodies (multiply charged species, fragmentation, complex sample prep and quantifying by the surrogate peptide method), the payload on ADCs adds another layer of complexity with the need to characterize the drug antibody ratio (DAR) and the drug conjugation sites. Waters' experience and leadership in ADC characterization and quantification by LC-MS is illustrated in the selection of application notes and poster notes presented here.

For a more in-depth view of the LC-MS characterization of ADCs, please visit **www.waters.com/adc**.

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Automating the Determination of Drug-to-Antibody Ratio (DAR) of Antibody Drug Conjugates (ADCs) Based on Separation by Hydrophobic Interaction Chromatography (HIC)

Robert Birdsall, Eoin Cosgrave, Henry Shion, and Weibin Chen
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Increase productivity through the automation of DAR calculations
- Streamline data processing with custom calculations for efficient data review
- Integrate functionality to improve reporting of results

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY UPLC Autosampler with FTN

ACQUITY UPLC TUV Detector

Protein-Pak™ Hi Res HIC Column

Empower® 3 Software

KEY WORDS

Antibody drug conjugate, ADC, cytotoxic agent, drug payload, linker, cysteine conjugate, drug antibody ratio, DAR, hydrophobic, HIC, therapeutic antibody, protein

INTRODUCTION

Antibody drug conjugates (ADCs) represent a rapidly growing class of biotherapeutic drugs for the treatment of cancer.[1] ADCs offer the selectivity of an antibody with the potency of a cytotoxic agent such as a synthetic drug.[2] ADC design, in part, relies on predictable conjugation chemistry that preserves antibody binding activity (Figure 1A) while facilitating reproducible characteristics that can be used as metrics for assessing critical quality attributes (CQAs) to ensure a safe and effective ADC product.[3]

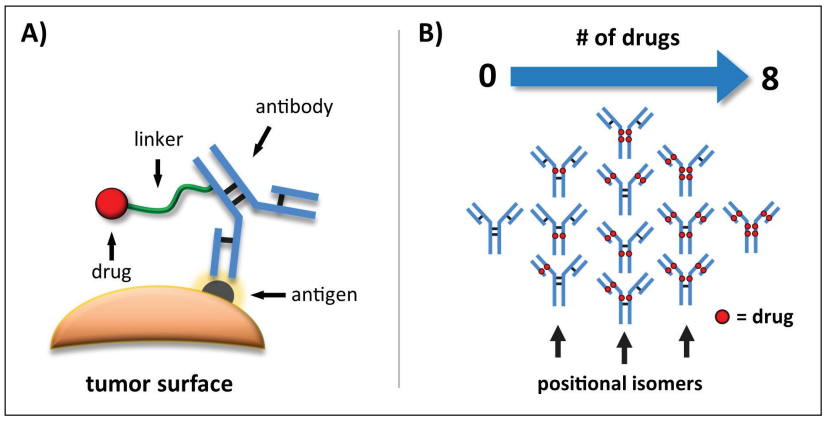


Figure 1. Biotherapeutic ADC Modality from Effective Drug Design. A) An ADC facilitates a targeted treatment of cancerous tumors, in which a drug payload is brought within close proximity of a tumor through the specificity of a therapeutic antibody. B) Example of cysteine-conjugated ADCs, illustrating the conjugation of drugs to the antibody via inter-chain disulfide bonds. Expected drug loads occur in intervals of 2, 4, 6, and 8.

Cysteine-conjugated ADCs are a sub-class of biotherapeutic drugs that are manufactured with well-known conjugation chemistry. Drug payloads are attached through a linker to monoclonal antibodies (mAbs) via the thiol groups. Thiols are generated by the reduction of inter-chain disulfide bonds, and expected drug load occurs in intervals of 2, 4, 6, and 8 as shown in Figure 1B due to the formation of two thiols from each disulfide.

EXPERIMENTAL

The Protein-Pak Hi Res HIC column (4.6 x 100 mm, 2.5 µm, p/n 186007583) was conditioned prior to use according to its care and use manual. Chemical reagents were purchased from Sigma-Aldrich and used as received. A cysteine-conjugated ADC was prepared by a collaborator at a concentration of 10 mg/mL in formulation buffer. Samples were prepared for analysis at a concentration of 2 mg/mL in 1M ammonium sulfate ($(NH_4)_2SO_4$).

Conditions

LC system:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC TUV
Absorption Wavelength:	280 nm
Vials:	Total Recovery vial: 12x32 mm glass, screw neck, cap, nonslit (p/n 6000000750cv)
Column:	Protein-Pak Hi Res HIC, 4.6 x 100 mm, 2.5 µm
Column temp.:	25 °C
Sample temp.:	4 °C
Injection vol.:	10 µL
Mobile phase A:	125 mM Phosphate buffer, pH 6.7 with 2.5 M $(NH_4)_2SO_4$
Mobile phase B:	125 mM phosphate buffer, pH 6.7
Mobile phase C:	Isopropyl alcohol
Mobile phase D:	Water

Gradient

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.700	50	0	5	45
10.00	0.700	0	50	5	45
15.00	0.700	0	50	5	45
15.01	0.700	50	0	5	45
30.00	0.700	50	0	5	45

Informatics for data collection & processing

Empower 3 Software, SR1, FR2

The conjugation process, which is dependent on the reactant concentrations, can result in variable drug-to-antibody-ratio (DAR, Figure 2) and subsequently impact the efficacy and safety of the ADC. Therefore monitoring the drug distribution and drug load of ADCs during the manufacturing process is of critical importance for pharmaceutical companies.

In the absence of a dedicated workflow, analysts will often perform manual calculations based on hydrophobic interaction chromatography (HIC) peak area for the determination of DAR based on drug load distribution. Waters' chromatographic data system (CDS), Empower 3 Software, can be used to streamline the analysis of ADCs through an integrated approach to data acquisition, processing, and reporting of experimental results. This process, which can be fully automated, makes the Empower CDS ideal for increasing productivity through efficient method deployment in the characterization of ADCs.

The objective of this application note is to demonstrate the ability to automatically determine DAR values corresponding to the drug load distribution of cysteine-conjugated ADCs using Empower 3 Chromatography Data Software. A cysteine-conjugated ADC was used as a model conjugate to test the application.

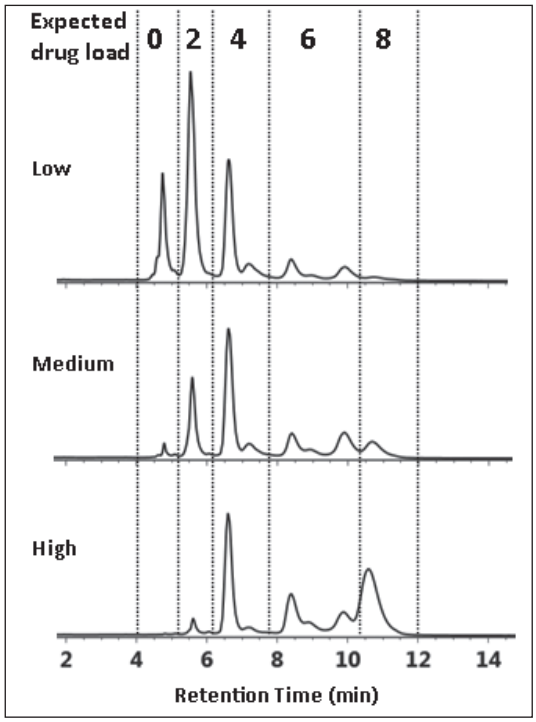


Figure 2. Monitoring drug load variability. Three batches of cysteine-linked ADCs were synthesized, each with a different level of drug conjugation (low, medium, high) and separated using hydrophobic interaction chromatography (see experimental). The drug load distribution shifted from low to high corresponding to an increase in the load of the hydrophobic drug.

RESULTS AND DISCUSSION

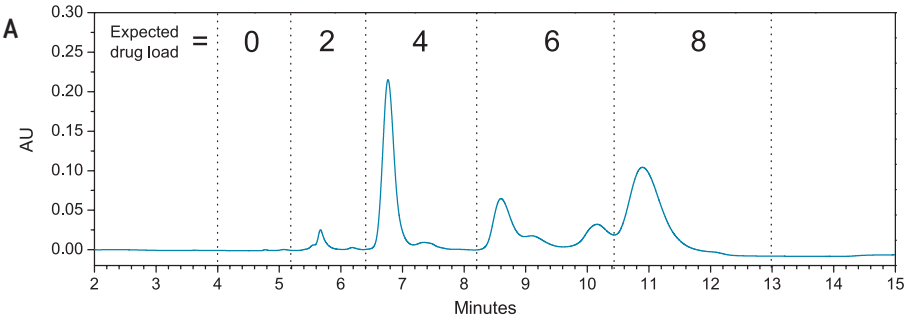
Efficient component management for challenging biotherapeutic samples

Chromatograms of separations of cysteine-conjugated ADCs using HIC are often comprised of multiple peaks where groups of peaks represent positional isomers of a conjugated antibody (Figure 3A).[3]

With its high-fidelity separations, the ACQUITY UPLC H-Class System delivers reproducible analyses; the researcher can then take advantage of informatics tools in Empower 3 Software to accurately group peaks by retention time for calculation of the DAR value based on drug distribution. This results in a highly efficient approach to method development during characterization.

As shown in Figure 3A, the multiple components associated with individual drug loads (DAR 0 through DAR 8) are entered into the Timed Groups tab (Figure 3B) of the processing method based on expected retention times. The ability to manage identification of components in separations like this makes Empower 3 Software ideal for characterizing challenging biotherapeutics such as ADCs.

A



B

Integration | Smoothing/Offset | Components | Impurity | Peak Ratios (MS Ion Ratios) | Default Amounts/Purity | Named Groups | **Timed Groups** | Suitability | Limits | Noise

	Name	Start (min)	Stop (min)	Source of Calibration X Value
1	DAR 0	4.000	5.200	Not Calibrated, Sum Peaks for Quantitation
2	DAR 2	5.200	6.400	Not Calibrated, Sum Peaks for Quantitation
3	DAR 4	6.400	8.200	Not Calibrated, Sum Peaks for Quantitation
4	DAR 6	8.200	10.400	Not Calibrated, Sum Peaks for Quantitation
5	DAR 8	10.400	13.000	Not Calibrated, Sum Peaks for Quantitation

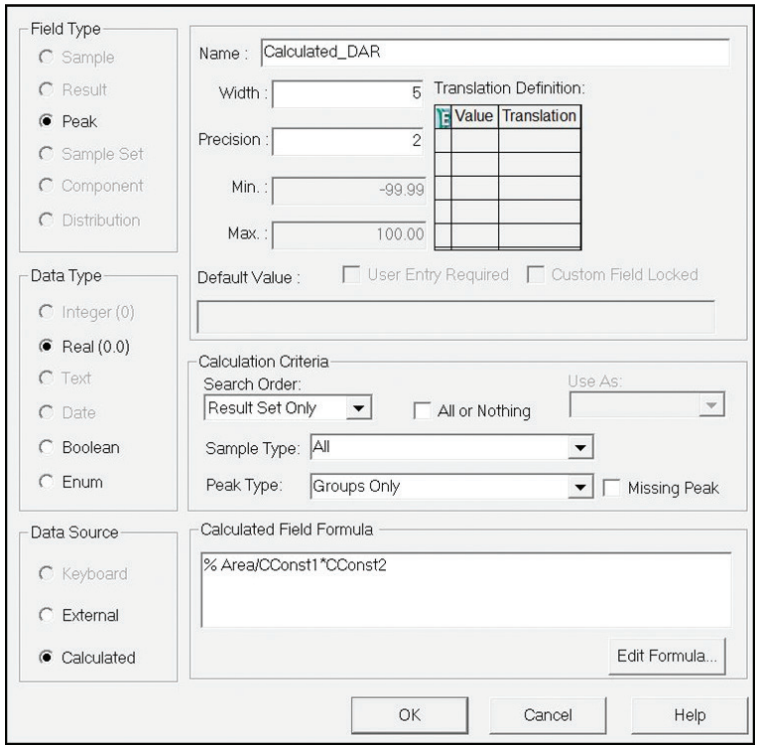
Figure 3. Managing components through method design. A) Empower offers the ability to manage groups of separation components (peaks) associated with individual drug loads. B) Using the Timed Groups feature of the processing method, retention time windows can be used for the grouping of multiple components for efficient peak identification and management in analysis results.

Improving productivity through automated calculation of DAR values

Empower 3 Software provides a myriad of informatics tools – which are designed to increase productivity in data analysis – that can be deployed for the automation of calculations associated with CQAs of cysteine-conjugated ADCs such as DAR values. This is achieved through the use of the Custom Fields, which are generated in the project properties from the system configuration manager as shown in Figure 4A.

Efficient processing of results using Empower is, in part, made possible through the ability to set criteria for custom field calculations. As shown in Figure 4A, by selecting Groups Only in the Peak Type window, the custom calculation shown in the formula window will only be applied to the grouped data as defined in Figure 3B. Furthermore, Empower's flexibility to use numerical constants from the Component Manager (Figure 4B) facilitates the use of custom calculations such as DAR values for cysteine-conjugated ADCs.

A



B

Integration | Smoothing/Offset | Components | Impurity | Peak Ratios (MS Ion Ratios) | Default Amounts/Purity | Named Groups | Timed Groups | Suitability | Limits

Average By: None | Update RT: Never | RT Window (%): 5.00 | CCalRef1 | Include Internal Std Amounts in % Amount Calculation | Sample Value Type: Amount | Auto Peak Label | RT Reference Used to Name Unnamed Peaks by RRT:

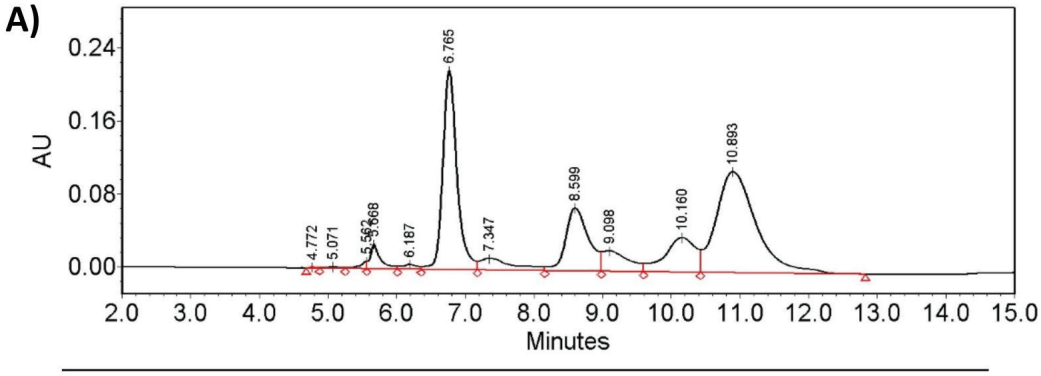
	Name	Component Type	Retention Time (min)	CConst1	CConst2
1	DAR 0	Main Component	4.000	100.00	0.00
2	DAR 2	Main Component	5.200	100.00	2.00
3	DAR 4	Main Component	6.400	100.00	4.00
4	DAR 6	Main Component	8.200	100.00	6.00
5	DAR 8	Main Component	10.400	100.00	8.00

Figure 4. Automating calculations using flexible custom fields. A) Empower 3 Software's Custom Fields feature is flexible and can accommodate custom calculations that use B) numerical constants in the calculation formula. In this example, CConst2 (Expected DAR) associated with the HIC separation of the cysteine-conjugated ADCs is used in the calculated field formula (DAR = % Area / 100 x Expected DAR).

The custom fields, upon initial set-up, can be incorporated into the analysis workflow. To demonstrate this, 20 µg of a cysteine-conjugated ADC sample at a concentration of 2 mg/mL in 1M $(NH_4)_2SO_4$ was injected on a Protein-Pak Hi Res HIC column (4.6 x 100 mm, 2.5 µm) and separated using a 10-minute gradient as shown in Figure 5A. As shown in Figure 5B, Empower automatically reports the retention time and associated peak area for the individual peaks as well as the grouped peaks as defined in Figure 3B.

As part of the processing method, Empower automatically calculates and displays the individual DAR values based on drug distribution (DAR 0 = 0.00, DAR 2 = 0.08, DAR 4 = 1.32, DAR 6 = 1.70, DAR 8 = 2.75) for the cysteine-conjugated ADC sample using the custom field calculation defined in Figure 4A. This process, which automates the calculation of CQAs, demonstrates that the Empower 3 Software is well-suited for increasing productivity in the characterization of antibody drug conjugates.

A)



B)

	Name	Retention Time (min)	Area (µV*sec)	% Area	Height (µV)	Int Type	Calculated_DAR
8		6.765	3089401	27.42	217325	VV	
9		7.347	321935	2.86	11808	VV	
10		7.931	39516	0.35	3207	VV	
11		8.598	1523740	13.53	67630	VV	
12		9.094	523160	4.64	20682	VV	
13		10.160	1060635	9.42	35666	VV	
14		10.897	4279225	37.99	108315	VB	
15	DAR 0	4.051	40995	0.36	4049	Group	0.00
16	DAR 2	5.562	386488	3.43	39551	Group	0.07
17	DAR 4	6.765	3450852	30.63	232340	Group	1.23
18	DAR 6	8.598	3107534	27.59	123978	Group	1.66
19	DAR 8	10.897	4279225	37.99	108315	Group	3.04

Individual peaks

Grouped peaks

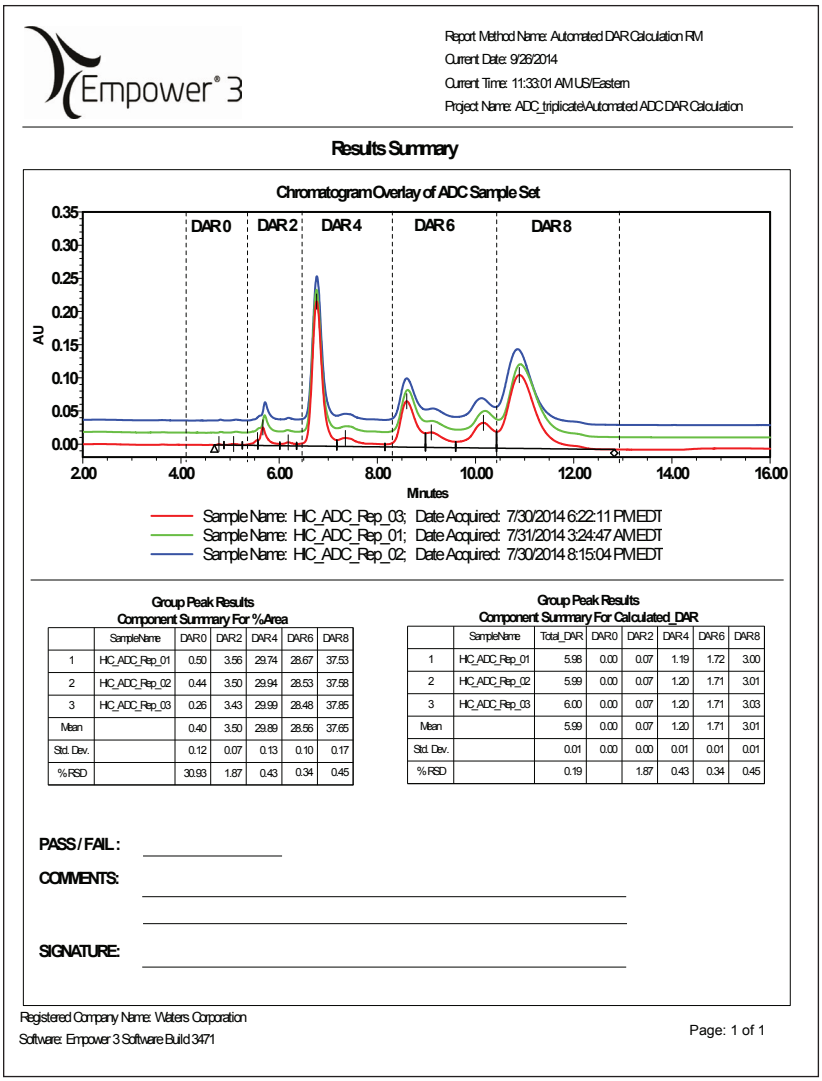
Figure 5A. Incorporation of Custom Fields in data analysis. A) Integration of a cysteine-conjugated ADC separation using a Protein-Pak Hi Res HIC Column with custom calculations. B) As a part of the processing method, Empower 3 Software seamlessly incorporates identification of individual and groups of chromatographic peaks, while custom field calculations for the individual DAR values are displayed in the right column for the grouped peaks.

Informatics tools that automate reporting of CQAs

Empower 3 Software additionally features powerful reporting functionality that is designed to provide researchers with meaningful analytical data and summaries for management review. Report templates can be readily constructed and customized for assessment of results.

An example of a report template designed for reporting the DAR and drug distribution for cysteine-conjugated ADC characterization is shown in Figure 6. Using the results from Figure 5B, a summary report of the relative area and corresponding individual and total DAR values based on drug distribution along with the corresponding statistical results (*e.g.*, mean and % RSD) is generated after data acquisition and processing.

Offering the flexibility of custom reporting templates that can be designed to meet a laboratory's specific analytical and communication needs, Empower 3 Software is a powerful partner to an ACQUITY UPLC H-Class System. Together they provide an integrated method development approach for the acquisition, processing, and reporting of results in the characterization of cysteine-conjugated ADCs.



	SampleName	DAR0	DAR2	DAR4	DAR6	DAR8
1	HIC_ADC_Rep_01	0.50	3.56	29.74	28.67	37.53
2	HIC_ADC_Rep_02	0.44	3.50	29.94	28.53	37.58
3	HIC_ADC_Rep_03	0.26	3.43	29.99	28.48	37.85
Mean		0.40	3.50	29.89	28.56	37.65
Std. Dev.		0.12	0.07	0.13	0.10	0.17
%RSD		30.93	1.87	0.43	0.34	0.45

	SampleName	Total_DAR	DAR0	DAR2	DAR4	DAR6	DAR8
1	HIC_ADC_Rep_01	5.98	0.00	0.07	1.19	1.72	3.00
2	HIC_ADC_Rep_02	5.99	0.00	0.07	1.20	1.71	3.01
3	HIC_ADC_Rep_03	6.00	0.00	0.07	1.20	1.71	3.03
Mean		5.99	0.00	0.07	1.20	1.71	3.01
Std. Dev.		0.01	0.00	0.00	0.01	0.01	0.01
%RSD		0.19		1.87	0.43	0.34	0.45

Figure 6. Report Template example. Custom calculations for the individual and total DAR values based on drug distribution are shown in the report for the cysteine-conjugated ADC sample.

CONCLUSIONS

With a modality that has the potential to redefine cancer treatment, interest from pharmaceutical companies to deliver ADC biotherapeutics to market is intensifying. To support this, characterization methods that are efficient and adaptable to evaluate novel CQAs associated with this new class of biotherapeutic drug are needed.

Empower 3 Software represents one of Waters' solutions to these challenging problems. This chromatographic data system can be used to streamline the analytical workflow through an integrated approach to data acquisition, processing, and reporting of experimental results. As one part of its integrated informatics toolset, custom field calculations are seamlessly incorporated into the ADC data analysis workflow to address processing challenges facing today's analysts.

This end-to-end automatable workflow illustrates that Waters' integrated analytical technology solutions are ideal for increasing productivity through efficient method deployment in the characterization of antibody drug conjugates.

References

1. Alley SC, Okeley NM, Senter PD. Antibody-drug conjugates: targeted drug delivery for cancer. *Curr Opin Chem Biol*. 2010 Aug;14(4):529–37. doi: 10.1016/j.cbpa.2010.06.170.
2. Wu AM, Senter PD. Arming antibodies: prospects and challenges for immunoconjugates. *Nat Biotechnol.* 2005 Sep;23(9):1137–46.
3. Wakankar A, *et al*. Analytical methods for physicochemical characterization of antibody drug conjugates. MAbs. 2011 Mar-Apr;3(2):161–72.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Automated Quantitative Analysis of Antibody Drug Conjugates Using an Accurate Mass Screening Workflow in the UNIFI Scientific Information System

Liuxi Chen, Steven Cubbedge, Ying Qing Yu, and Rose Lawler
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The Waters® UNIFI® Scientific Information System provides the Accurate Mass Screening workflow to streamline and facilitate the challenging quantitative analysis of antibody drug conjugate (ADC) peptides. This workflow utilizes the UNIFI scientific library as the means to share the peptide and peptide-drug conjugate target component information characterized by the Peptide Mapping workflow within the Biopharmaceutical Platform Solution. This component information is then used to create the target lists and Custom Field to automatically quantify these key attributes of ADCs.

WATERS SOLUTIONS

Accurate Mass Screening workflow with UNIFI

Peptide Mapping (MS^E) Workflow within the Biopharmaceutical Platform Solution

ACQUITY UPLC® H-Class Bio System

Xevo® G2-S QTof Mass Spectrometer

Xevo G2-XS QTof Mass Spectrometer

KEY WORDS

Monoclonal antibodies (mAbs), biotherapeutic characterization, antibody drug conjugates, ADC, quantitative analysis, peptide mapping, MS^E

INTRODUCTION

Antibody drug conjugates (ADCs) are a sub-class of biotherapeutics that consist of monoclonal antibodies (mAbs) and cytotoxic drugs linked to mAbs by chemical linkers. ADCs are a complex mixture of conjugates that differ in the number of drugs attached as well as the location of the drug linkage. Previous studies demonstrated that the location, distribution, and site occupancy of the drug molecules play a critical role in the Complementarity Determining Region (CDR) binding efficiency, efficacy, toxicity, stability, and pharmacokinetic profile of ADCs. It is crucial to establish efficient and robust analytical capabilities to characterize the heterogeneity of drug-linker content and modification sites as well as to demonstrate lot-to-lot consistency, stability, and process comparability. Structural characterization, along with qualitative and quantitative analysis of ADCs, is challenging due to the heterogeneous nature of the conjugates and the low abundancies of individually modified species. The quantitation of conjugated peptides and determination of site occupancy ratio has traditionally been done by LC-UV methods, which is limited by the spectroscopic nature of the drug, low sensitivity, and insufficient selectivity. Compared to UV-based methods, MS-based methods can provide improved sensitivity and selectivity. However, MS-based quantitation has its own challenges that often involve monitoring a large number of MS peaks per sample for complex ADC peptides. Currently, efficient quantitation of ADC peptides and direct generation of the key attributes used for ADC comparability studies are impeded by a lack of sophisticated software tools. Waters Accurate Mass Screening (MS^E) workflow within the Screening Platform Solution with UNIFI addresses these challenges and provides targeted component quantitation across samples, Custom Field for key ADC attributes (such as relative conjugation site occupancy ratio), and generation of reports in a streamlined and automated fashion.

EXPERIMENTAL

Liquid chromatography

System:	ACQUITY UPLC H-Class Bio System
Detector:	ACQUITY UPLC Tunable UV (TUV) Detector
Column:	ACQUITY UPLC BEH300 C_{18} Column, 300 Å, 1.7 µm, 2.1 mm x 100 mm (P/N 186003686)

Mass spectrometer

Mass spectrometer:	Xevo G2-XS QTof
Acquisition mode:	MS^E

Informatics

UNIFI 1.8 Scientific Information System

Analysis type:	Accurate Mass Screening (MS^E) workflow within the Screening Platform Solution with UNIFI, Peptide Mapping (MS^E) workflow within the Biopharmaceutical Platform Solution.

Sample information

Antibody drug-conjugated samples were denatured, alkylated, and digested by Asp-N endoproteinase. Leucine enkephalin (LeuEnk) was added to each sample at a final concentration of 50 fmol/ul as an internal standard.

In this application note, lysine-conjugated ADCs were used to demonstrate the utilization of the Accurate Mass Screening workflow to monitor the relative abundance of ADC conjugated peptides across samples and to complete relative conjugation site occupancy ratio calculations automatically. The reported method is universal and can be applied to ADC therapeutics generated by different conjugation chemistries. Multiple features in UNIFI facilitate the quantitation analysis. First, the library of target components, including accurate mass values and optional retention times, is created. This was achieved by processing representative samples using the Peptide Mapping (MS^E) analysis that identifies and labels the various peptide and peptide-drug conjugates. These are then selected and the relevant information entered into the UNIFI scientific library. The target components are therefore stored and easily retrievable to construct analysis methods. Custom Field enables the automated calculation of important product attributes of the ADCs. This is completed by using a Custom Field when setting up the processing method. In addition, UNIFI features a review panel that provides the ability to easily organize and save data review workflow steps, including user-configured plots and tables for consistent data display and straightforward communication. All of the relevant information including the use of result filters is available to the user and can be copied into reporting templates to visualize the results and generate analysis-specific reports.

RESULTS AND DISCUSSION

Step 1: Data acquisition and processing to create the library of target components

LC-MS^E peptide mapping data were acquired using the ACQUITY UPLC H-Class Bio System (with both UV and MS detection) and Xevo G2-XS QTof MS. MS^E data acquisition enables sufficient points across the chromatographic elution peaks and provides MS (Low CE) and MS-MS (High CE) spectra in alternate scans. The data was processed using the Peptide Mapping MS^E workflow. This software identifies the potential peptide and peptide-drug conjugate components through de-novo digestion of a target protein and searches the accurate mass MS and MS-MS data considering adducts and modifications, including the linker and drug. The identified components can then be saved into a user-defined UNIFI scientific library. The Peptide Mapping workflow presents identified components and quality attributes that indicate the relative confidence of the identification. User-defined filters can be created and saved such that these quality attributes can be applied to the components that will be stored within the library and used as the basis for the creation of the target list in the Accurate Mass Screening method.

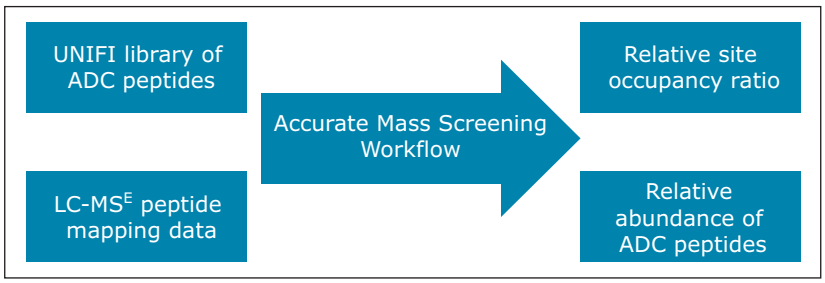


Figure 1. Quantitative analysis workflow of antibody drug conjugates with UNIFI Accurate Mass Screening.

Step 2: Contents and use of the UNIFI scientific library

The UNIFI scientific library is a central library function within the software that contains commercial and user-created libraries. These libraries can be used to store component-centric chemical and physical information as well as detection results. This information can be used to facilitate the creation of analysis methods. Figure 2 shows an example of an ADC peptide library and detection results, including information such as the expected mass-to-charge ratio (*m/z*), expected retention time, and expected fragment ion *m/z*. Information can be imported through an excel spreadsheet, input into the library manually, or sent from the results of certain UNIFI analysis types.

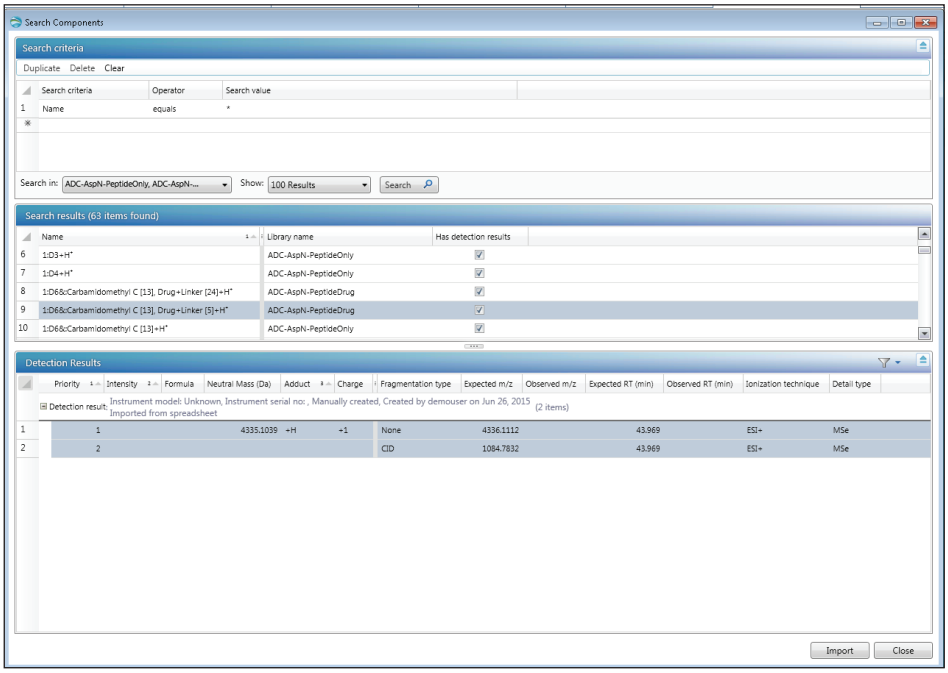
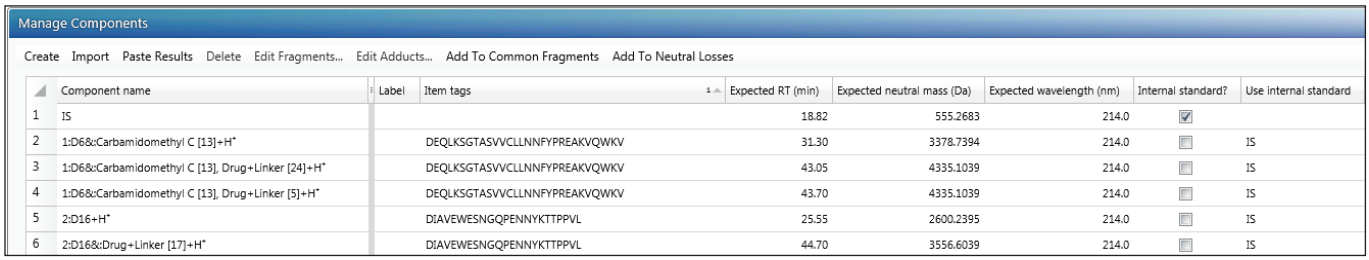


Figure 2. An example of an ADC peptide library entry.

Step 3: Accurate Mass Screening workflow method settings

a. Manage Components table

First, the Manage Components table is created. Screening components can be imported directly from the scientific library. Figure 3 shows an example Manage Components table. Please note that in order to recognize the conjugated peptide with its unconjugated peptide counterpart, the peptide sequence must be included as an "item tag" for each component. This item tag is stored in the library along with the peptide sequence from the result fields in the Peptide Mapping MS^E analysis. The internal standard can be added to the component table as shown in Figure 3. The expected retention time (RT) field is not required; however it can increase screening accuracy if the value is known from a previous analysis. The screening time window can be defined by the "target by retention time" setting in the analysis method. The expected neutral mass (Da) value for each component is required for the screening workflow. Mass-to-charge (*m/z*) values of different charge states or peptide adduct ions (*m/z*) are automatically calculated from the neutral mass during data processing.



	Component name	Label	Item tags	Expected RT (min)	Expected neutral mass (Da)	Expected wavelength (nm)	Internal standard?	Use internal standard
1	IS			18.82	555.2683	214.0	☑	
2	1:D6&:Carbamidomethyl C [13]+H+		DEQLKSGTASVVCLLNNFYPREAKVQWKV	31.30	3378.7394	214.0	☐	IS
3	1:D6&:Carbamidomethyl C [13], Drug+Linker [24]+H+		DEQLKSGTASVVCLLNNFYPREAKVQWKV	43.05	4335.1039	214.0	☐	IS
4	1:D6&:Carbamidomethyl C [13], Drug+Linker [5]+H+		DEQLKSGTASVVCLLNNFYPREAKVQWKV	43.70	4335.1039	214.0	☐	IS
5	2:D16+H+		DIAVEWESNGQPENNYKTTPPVL	25.55	2600.2395	214.0	☐	IS
6	2:D16&:Drug+Linker [17]+H+		DIAVEWESNGQPENNYKTTPPVL	44.70	3556.6039	214.0	☐	IS

Figure 3. Manage Components table in the Accurate Mass Screening workflow.

b. Processing parameters

Figure 4 shows the "processing sections" of the accurate mass screening workflow. The quantitative analysis of ADC peptides is facilitated through multiple features of UNIFI, some of which are highlighted and described in detail in Figure 4. The users have the flexibility to define the channel (UV or MS) or ions used for quantitation (most intense monoisotopic or sum of all adducts and isotopes). MS components can be quantified using the 2D peak (2D extracted ion chromatograms (XIC) area or height) or 3D peak volume. In addition, all the quantitation settings can be set on a per-component basis to allow flexibility in the application of different settings for separate target groups.

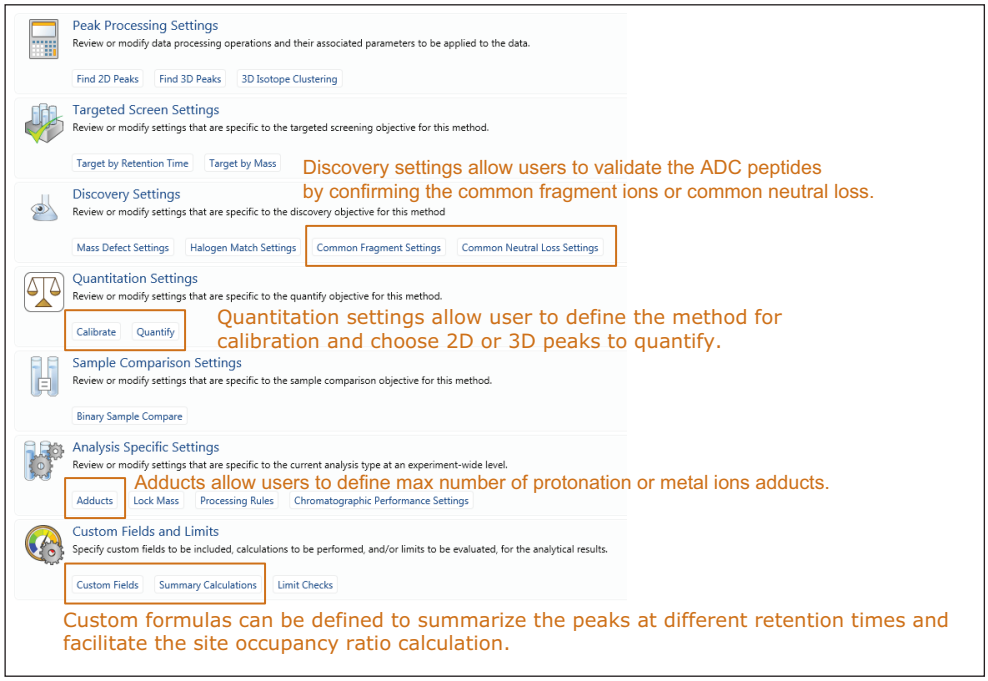


Figure 4. Processing parameters overview of the Accurate Mass Screening workflow.

Step 4: Setting up Custom Fields

Custom Field is designed to perform the specific calculations for target analysis. The Custom Field can be stored in the analysis method with the results directly available for viewing, plotting, and reporting. Automated calculations within the analysis method eliminate the tedious extra steps for manual data manipulation and re-processing. Custom Field and analytical methods are protected by a secure framework via configurable user access controls based upon roles that facilitate their use in routine analysis, particularly in regulated laboratories.

Custom Field and the formulae to support the calculation of ADC attributes in a quantitative analysis are shown in Table 1. The Custom Field is formulated generically so that it can be applied to other types of ADC peptide analyses. The text marker "drug" is used to identify the conjugated peptide components in the screening results. As long as the component name of the conjugated peptides contains "drug" in the Manage Components table, the Custom Field will find these components and apply the determined values. "Calculate amount" is a standard field generated by UNIFI processing that is obtained by normalizing the intensity of each identified quantitative response against the spiked-in internal standard response. The peptide sequence values are included in the "item tag" field, so that the peptide components with the same sequence can be summed using the "Peptide Tot AMT" Custom Field. Using these four Custom Fields, users can effectively monitor the amount of conjugated peptides and relative site occupancy ratios across sample sets.

Custom field name	Description	Data type	Formulas
DRUG	Identify the conjugated peptides components	Component	IF(FIND(Component name, "Drug"),1,0)
Peptide-Drug AMT	Sum of conjugated peptide amount	Component	SUM(Calculated amount ,Item tags=VALUE(Item tags,,CURRENT)&DRUG=1)
Peptide Tot AMT	Sum of amount of conjugated peptide and unconjugated peptide	Component	SUM (Calculated amount, Item tags=VALUE (Item tags,,CURRENT))
Site occupancy ratio	% amount of the conjugated peptide in the total amount for the peptide based on the same sequence	Component	Peptide-Drug AMT/Peptide Tot AMT

Table 1. Custom Field settings for the conjugated peptide amount and site occupancy ratio calculations. Blue indicates "functions." Red indicates "fields." Green indicates a vector of a function. Site occupancy ratio = area (conjugated pep. peak)/[area(unconjugated pep. peak) + area(unconjugated pep. peak)].

Step 5: Results review

The detection and calculation results are displayed in the UNIFI review tab as shown in Figure 5. It consists of three areas:

a. The Component Summary table which includes the user-specified result fields in this example for all of the identified components which were selected through the use of a filter.

b. The user-configured Chromatogram panel that displays the XICs of the selected component(s). In this example it contains the three XICs for the two diastereomers of the conjugated peptide 2:D13 and the unconjugated peptide 2:D13 (Heavy chain, the thirteenth Asp-N peptides from N-terminus).

c. The summary plot can be used to plot the value of any component field across the samples in a batch. This includes Custom Field, which is illustrated by the plotting of the relative site occupancy ratio plotted as a bar chart from duplicate injection of three ADC samples.

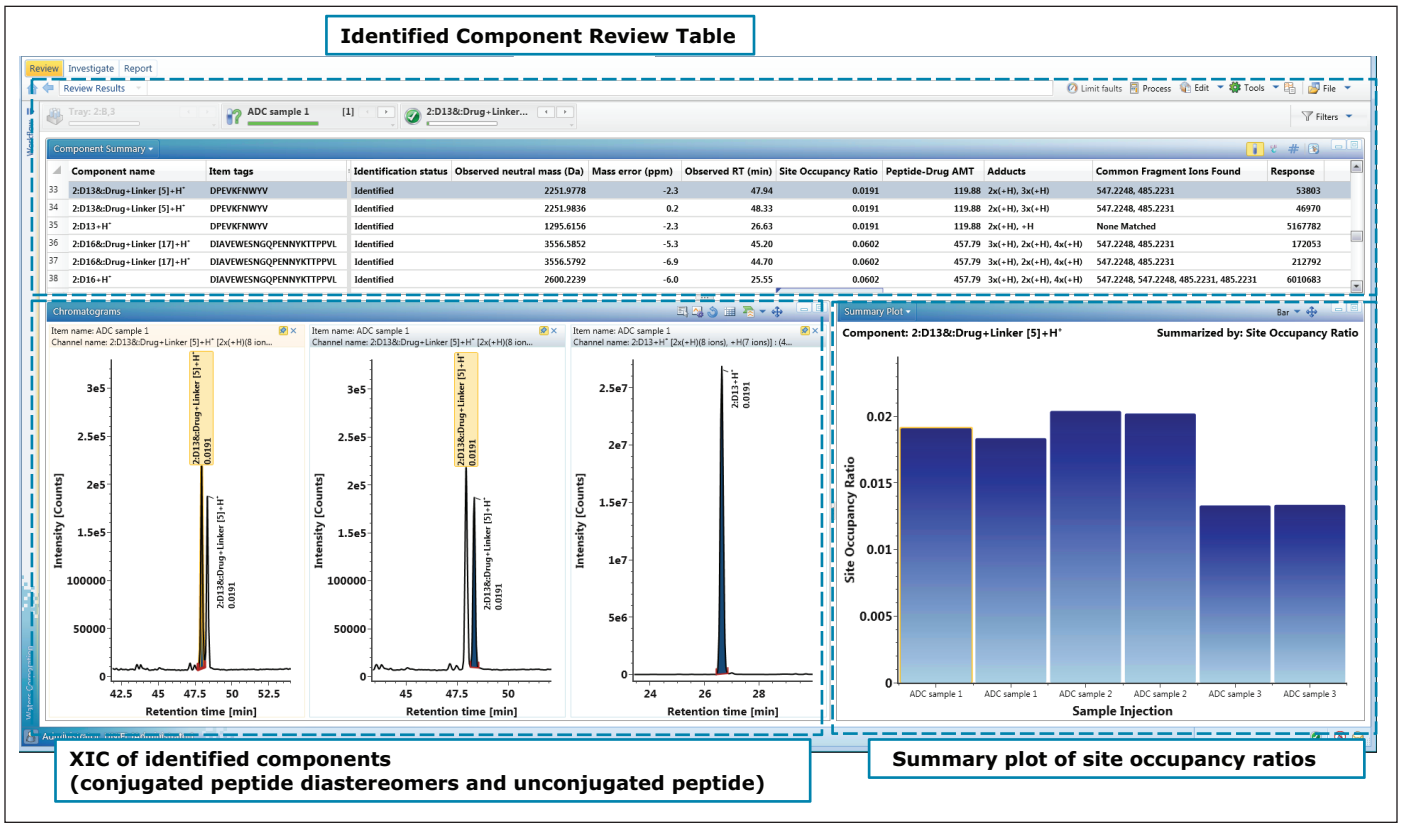


Figure 5. Identified Component Review table of the three ADC samples.

CONCLUSIONS

This application note describes how to perform ADC peptide quantitation using the Accurate Mass Screening workflow in UNIFI and further demonstrates the capability of comparing the key product attributes of ADCs across samples through this automated workflow. This eliminates tedious and labor-intensive manual data processing, which reduces associated human errors. Furthermore, the defined workflow in UNIFI can be saved and transferred to promote high quality data generation, consistent processing, and reporting for complex ADC samples. Combined with the UNIFI Peptide Mapping MS^E workflow, this will enable scientists to overcome the analytical challenges for both structural characterization as well as qualitative and quantitative analysis of complex ADCs. Collectively the Accurate Mass Screening workflow (quantitative tool) combined with UNIFI scientific library and Custom Field is a great addition to the traditional Peptide Mapping MS^E workflow (qualitative tool) within the same UNIFI architecture to facilitate the challenges of ADC biotherapeutics research and development.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Quantification of the Antibody Drug Conjugate, Trastuzumab Emtansine, and the Monocolonal Antibody, Trastuzumab, in Plasma Using a Generic Kit-Based Approach

Hua Yang, Mary Lame, Sherri Naughton, and Erin Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

Simple, standardized approach for accurate and reproducible quantification of ADC and mAb therapeutics; broadly applicable optimized digest kit eliminates method development.

WATERS SOLUTIONS

ProteinWorks™ eXpress Direct Digest Kit (p/n 176003688)

Intact mAb Check Standard (p/n 186006552)

ACQUITY UPLC® Peptide, BEH C_{18}, 1.7 µm, 2.1 x 150 mm Column (p/n 186003687)

ACQUITY UPLC System

Xevo® TQ-S Mass Spectrometer

KEY WORDS

antibody drug conjugate, monoclonal antibody, ado-trastuzumab emtansine, T-DM1, trastuzumab, protein quantification, eXpress Direct Digest, ProteinWorks

INTRODUCTION

Monoclonal antibodies (mAbs), as well as antibody-drug conjugates (ADCs) represent a growing class of therapeutics due to their target specificity, lower toxicity and higher potency. With the increasing interest in mAb and ADC therapeutics, the desire for LC-MS bioanalytical quantification in support of drug development is also increasing. Historically, mAbs and ADCs have been quantified using ligand binding assays (LBAs), such as the gold-standard ELISA. While these immuno-based methods are sensitive and simple to execute, poor reagent reproducibility, lack of standardization, cross-reactivity, limited linear dynamic range, and other short-comings have led the drive to convert to LC-MS. In contrast, MS based methodologies offer many advantages over traditional LBAs, such as: multiplexing, broad dynamic range, superior selectivity, and shorter method development times. However, for LC-MS protein quantification challenges still exist. There is no single standardized workflow and the various workflow options can be complex and laborious, making it difficult for the novice bioanalytical scientist to achieve success. Additionally, due to their complex and heterogeneous nature, ADCs often require multiple bioanalytical assays to determine efficacy, toxicity, and PK/PD response during drug development stages. The bottom up approach, using enzymatic digestion of the ADC/mAb, followed by LC-MS/MS analysis is becoming routine for ADC and mAb quantification. Of the many experiments required to characterize and quantify ADC's, total antibody measurements are important. This application note describes the total mAb quantification of the ADC, ado-trastuzumab emtansine, and the mAb, trastuzumab, from rat plasma using the ProteinWorks eXpress Direct Digest Kit and Protocol.

EXPERIMENTAL

Sample description

To prepare standards and quality control samples (QC), trastuzumab or T-DM1 was spiked into rat plasma at various concentrations (0.1–500 µg/mL). An intact murine monoclonal antibody standard (p/n 186006552) was used as a generic internal standard. Plasma samples (35 µL) were then prepared for LC-MS analysis using the ProteinWorks eXpress Direct Digest Kit and a 5-step digestion protocol which included reduction and alkylation.

Method conditions

LC system:	ACQUITY UPLC
Detection:	Waters Xevo TQ-S Mass Spectrometer, ESI+
Column:	ACQUITY UPLC Peptide BEH C_{18}, 300Å 1.7 µm, 2.1 x 150 mm Column
Temp.:	55 °C
Sample temp.:	10 °C
Injection vol.:	10 µL
Mobile phases:	A: 0.1% formic acid in water
	B: 0.1% formic acid in acetonitrile

Gradient:

Flow rate (mL/min)	Time (min)	Profile %A	%B	Curve
0.3	0.0	100	0	6
0.3	1.0	100	0	6
0.3	16.0	50	50	6
0.3	16.5	10	90	6
0.3	17.5	10	90	6
0.3	18.0	100	0	6
0.3	20.0	100	0	6

Data management: MassLynx (v4.1)

MS conditions

Capillary (kV):	3
Cone (V):	30
Source offset (V):	50
Source temp. (°C):	150
Desolvation temp. (°C):	600
Cone gas flow: (L/Hr):	150
Desolvation gas flow: (L/Hr):	1000
Collision gas flow (mL/Min):	0.15
Nebuliser gas flow (Bar):	7

RESULTS AND DISCUSSION

Trastuzumab is a humanized anti-HER2 monoclonal antibody that was approved by the FDA in 1998. With EU patent expiry in July 2014, and impending US patent expiry in 2019, the focus on this drug, as well as next and new generation drugs, such as ADCs, has steadily increased. Ado-trastuzumab emtansine (T-DM1) is an FDA approved ADC, marketed under the brand name Kadcyla, and used as treatment for patients with advanced breast cancer.[1-3] ADCs, like T-DM1, are composed of cytotoxic small molecule drug (payload) covalently bound to an antibody by a linker. Due to their complex and heterogeneous nature, ADCs require multiple bioanalytical assays to quantify both conjugated and unconjugated forms of the ADC, total mAb, cytotoxic payload, and various other catabolites/metabolites. LC-MS quantification of ADCs and mAbs typically employs enzymatic digestion (most commonly trypsin), followed by quantification of one or multiple representative tryptic peptides using multiple reaction monitoring (MRM).

Using the ProteinWorks eXpress Direct Digest Kit and protocol, a direct digest of plasma (35 µL) containing either T-DM1 or trastuzumab was performed. LC-MS/MS quantification of signature peptides was performed using a Xevo TQ-S triple quadrupole MS. Chromatographic separation was achieved using an ACQUITY UPLC System with an ACQUITY UPLC Peptide BEH C_{18}, 300A, 1.7 µm, 2.1 x 150 mm Column. Three signature tryptic peptides were used for quantification: IYPTNGYTR, FTISADTSK, and GPSVFPLAPSSK. MS conditions are summarized in Table 1.

Protein	Peptide	MRM transition	Cone voltage (V)	Collision Energy (eV)
T-DM1/Trastuzumab	IYPTNGYTR	542.77>249.16	36	16
		542.77>808.40	12	16
Trastuzumab	FTISADTSK	485.20>721.40	28	22
		485.20>608.30	28	22
Trastuzumab	DTYIHWVR	543.30>597.30	28	24
		545.30>710.40	28	28
Trastuzumab	GPSVFPLAPSSK*	593.83>699.40	31	21
T-DM1 miscleavage with small molecule drug attached	FTISADTSKNTAYLQMNSLR	1073.17>547.20	35	38
		1073.17>485.22	35	38
	GPSVFPLAPSSKSTSGGTAALGCLVK*	1149.23>547.20	35	38
Murine mAb (IS)	SVSELPIMHQDWLNGK*	618.64>834.41	16	12
	VNSAAFPAPIEK	622.30>654.44	28	16

Table 1. MRM conditions for trastuzumab and trastuzumab emtansine (T-DM1).
**Generic IgG peptide*

From an analytical perspective, tryptic digestion and choice of signature peptide poses a challenge for quantification of T-DM1, since it is a lysine-conjugated ADC. Trypsin cleaves peptides on the C-terminal side of lysine amino acid residues and if a lysine residue is occupied with the cytotoxic drug, cleavage will not occur ("miscleavage"). Thus, if one were to choose a lysine containing peptide to quantify T-DM1, there is potential for miscleavage on the lysine residue when it is conjugated with the small molecule drug. Because the signature peptide IYPTNGYTR lacks a lysine residue, one can confidently and accurately use it to quantify both T-DM1 and trastuzumab. For this same reason one would need to be cautious of using the two lysine containing peptides, FTISADTSK and GPSVFPLAPSSK for accurate quantification of T-DM1. Both of these peptides have some degree of small molecule drug occupancy and thus, due to potential miscleavage of the lysine residue may result in lower calculated concentrations than a non-lysine containing peptide.

For this application, sensitivity, linearity, accuracy and precision data met typical method validation requirements.[4] Standard curves were linear over 3.5 orders of magnitude with the average accuracies of 100% for the standard curve points. For the IYPTNGYTR, FTISADTSK, and GPSVFPLAPSSK tryptic peptides, quantification limits between 0.5–1.0 µg/mL were achieved. Summary statistics from standard curves for trastuzumab are shown in Table 2. In addition, the accuracy and precision for trastuzumab and T-DM1 QC samples, quantified using the trastuzumab standard curve, were excellent with % CVs <8. This is summarized in Table 3.

Peptide	Std. curve range (µg/mL)	Weighting	Linear fit (r^2)	Mean % accuracy
IYPTNGYTR	0.5–500	$1/x^2$	0.995	100.01
FTISADTSK	1.0–500	$1/x$	0.999	100.01
GPSVFPLAPSSK*	0.5–500	$1/x^2$	0.990	100.00

*Table 2. Linear dynamic range, weighting and average accuracy for standard curves for Trastuzumab plasma digested and extracted using the ProteinWorks eXpress Direct Digest Kit. *Generic IgG peptide*

mAb/ADC	Peptide	QC conc. (µg/mL)	Mean cal. conc. (µg/mL)	Std. dev.	%CV	Mean % accuracy
Trastuzumab	IYPTNGYTR	0.65	0.64	0.03	4.58	99.77
		3.5	3.25	0.19	5.96	92.90
		6.5	6.83	0.16	2.29	105.13
		35	36.41	0.42	1.16	104.03
		65	63.31	2.18	3.44	97.40
		350	345.64	18.66	5.40	98.73
T-DM1	IYPTNGYTR	0.65	0.65	0.05	6.94	100.50
		3.5	3.36	0.24	7.10	95.87
		6.5	7.1	0.05	0.66	109.20
		35	34.51	1.09	3.17	98.57
		65	59.74	3.72	6.22	91.90
		350	324.72	17.06	5.25	92.80
mAb/ADC	Peptide	QC conc. (µg/mL)	Mean cal. conc. (µg/mL)	Std. dev.	%CV	Mean % accuracy
Trastuzumab	FTISADTSK	3.5	3.47	0.17	4.89	99.20
		6.5	6.70	0.11	1.69	103.07
		35	38.30	0.28	0.73	109.47
		65	64.12	1.68	2.63	98.67
		350	357.47	9.65	2.70	102.13
T-DM1	FTISADTSK	3.5	3.03	0.06	1.87	86.60
		6.5	6.91	0.30	4.37	106.30
		35	33.35	0.65	1.94	95.27
		65	58.70	2.93	4.99	90.30
		350	322.02	7.51	2.33	92.00
mAb/ADC	Peptide	QC conc. (µg/mL)	Mean cal. conc. (µg/mL)	Std. dev.	%CV	Mean % accuracy
Trastuzumab	GPSVFPLAPSSK	0.65	0.66	0.05	7.97	100.87
		3.5	3.04	0.07	2.29	86.90
		6.5	6.27	0.09	1.41	96.50
		35	35.5	1.62	4.55	101.43
		65	71.38	3.04	4.26	109.83
		350	379.79	21.64	5.70	108.50
T-DM1	GPSVFPLAPSSK	0.65	0.67	0.02	2.84	103.30
		3.5	3.1	0.06	1.80	88.47
		6.5	6.19	0.31	4.98	95.15
		35	33.55	1.44	4.29	95.87
		65	63.08	4.04	6.40	97.03
		350	336.36	15.35	4.56	96.10

Table 3. Statistics for Trastuzumab and T-DM1 QC samples from the peptides, IYPTNGYTR, FTISADTSK, and GPSVFPLAPSSK, used for quantification.

QC chromatographic performance and demonstration of sensitive quantification for all three signature peptides is highlighted in Figures 1–3, Panels A (trastuzumab) and B (T-DM1), respectively.

Due to the hydrophobic nature of the cytotoxic drug molecule attached to the antibody and differences in stereo chemical configurations, conjugated TDM-1 peptides generally will elute later in a chromatographic run as diastereomeric pairs. Additionally, TDM-1 peptides, by collision induced disassociation (CID), produce a common fragment (547.2 *m/z*). This fragment corresponds to part of the drug molecule broken down by the CID process.

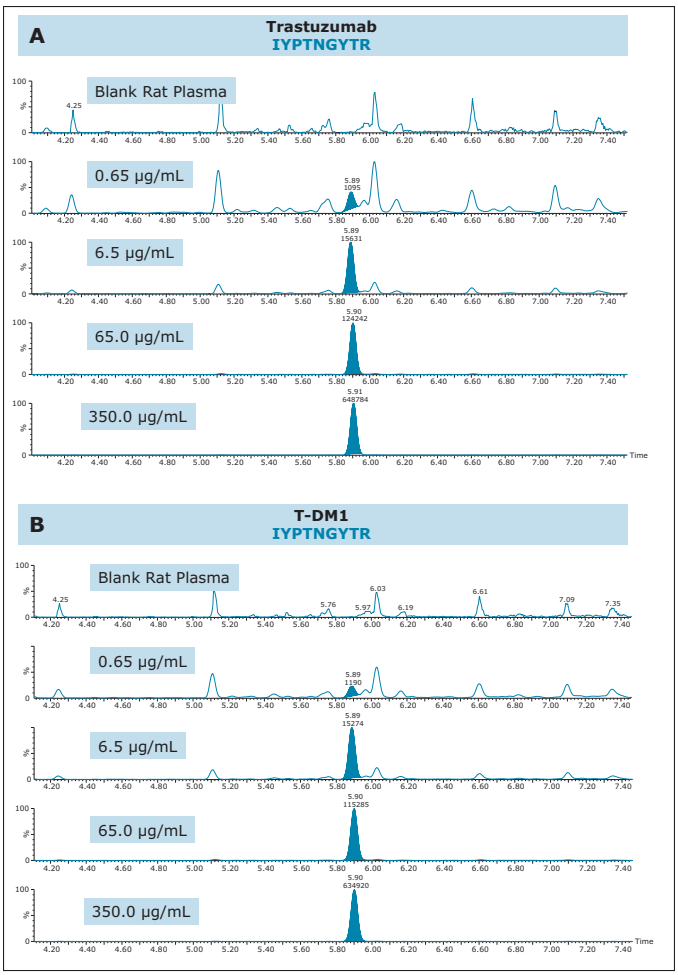


Figure 1. QC chromatograms of Trastuzumab (A) and T-DM1 (B) for the IYPTNGYTR unique signature peptide.

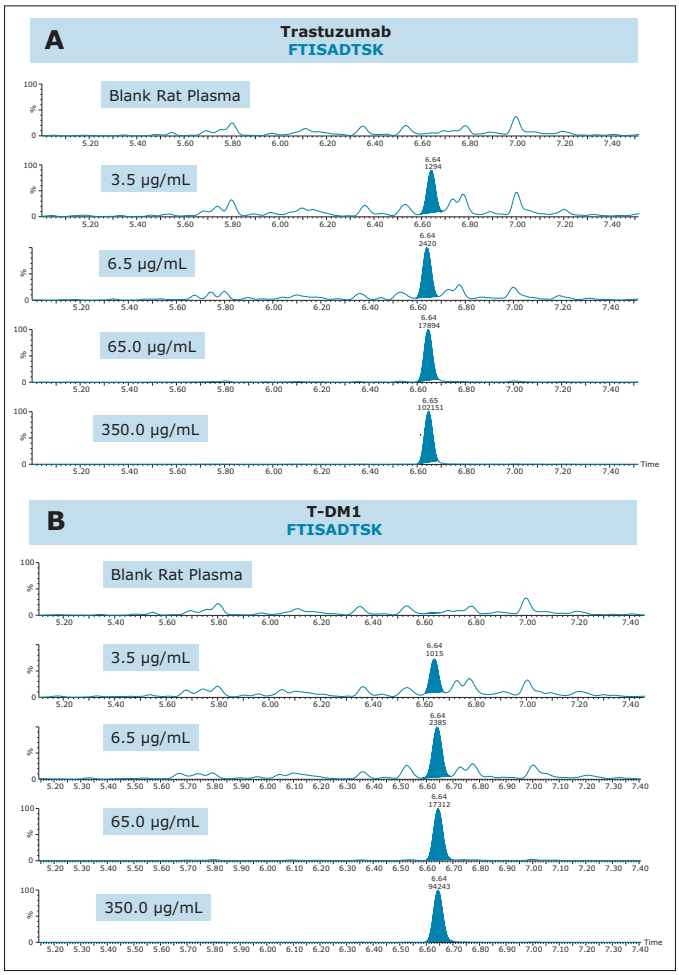


Figure 2. QC chromatograms of Trastuzumab (A) and T-DM1 (B) for the FTISADTSK unique signature peptide.

In this application, we were successfully able to detect two conjugated, "miscleavage" peptides of TDM-1 (FTISADTSKNTAYLQMNSLR and GPSVFPLAPSSKSTSGGTAALGCLVK). These conjugate peptides contained a common fragment 547.2 *m/z* from the conjugated payload and eluted later in the chromatographic run, as pairs (isomers from the conjugation). Figure 4, panels A and B illustrate the presence of these conjugated peptides in TDM-1 plasma samples (350 µg/mL), as compared to Trastuzumab (350 µg/mL), and blank rat plasma. Presence of the FTISADTSKNTAYLQMNSLR conjugated peptide was confirmed by multiple MRM transitions, and is shown in Figure 5. Additionally, both of these conjugated TDM-1 peptides increased with increasing concentration of T-DM1. This is highlighted in Figure 6, panels A and B.

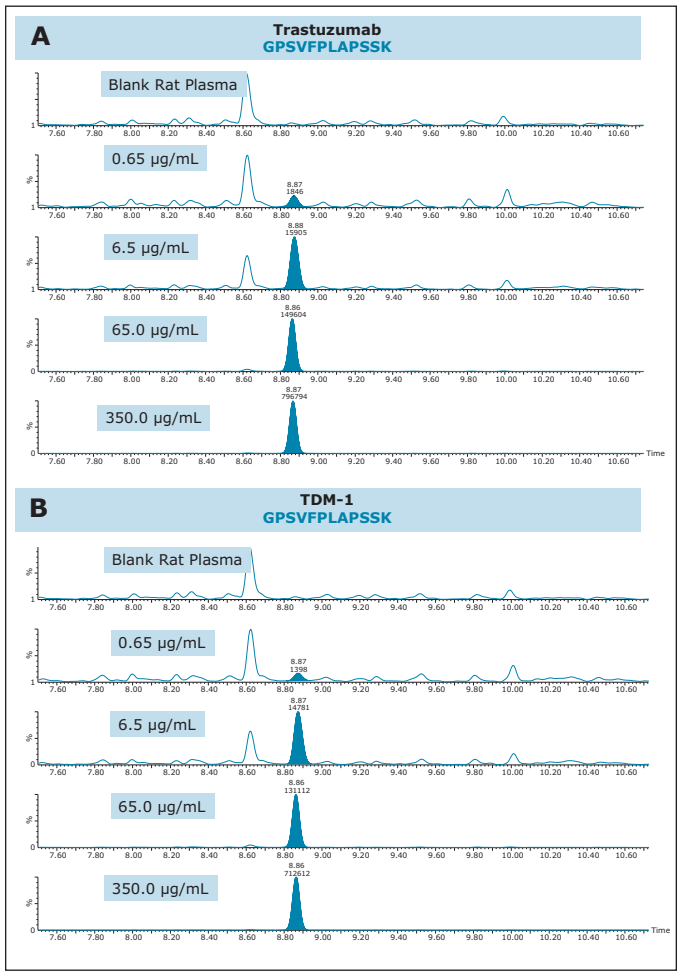


Figure 3. QC chromatograms of Trastuzumab (A) and T-DM1 (B) for the GPSVFPLAPSSK generic IgG signature peptide.

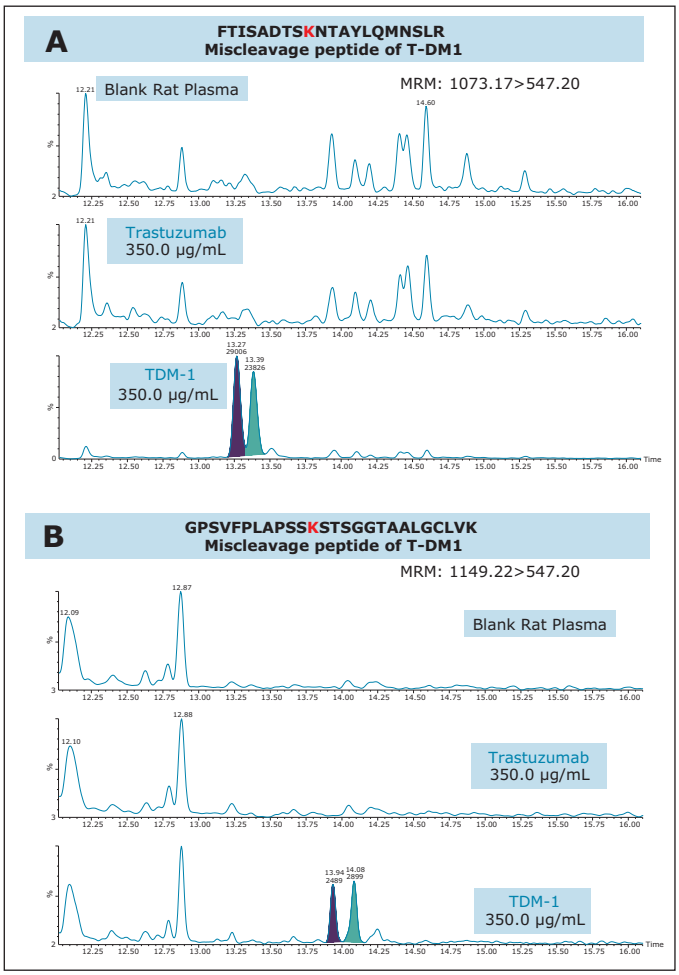


Figure 4. Chromatograms demonstrating the presence of the miscleavage peptides in T-DM1 (350 µg/mL), as compared to Trastuzumab (350 µg/mL), and blank rat plasma when digested and extracted using the ProteinWorks eXpress Direct Digest kit; Panel A: FTISADTSKNTAYLQMNSLR and Panel B: GPSVFPLAPSSKSTSGGTAALGCLVK.

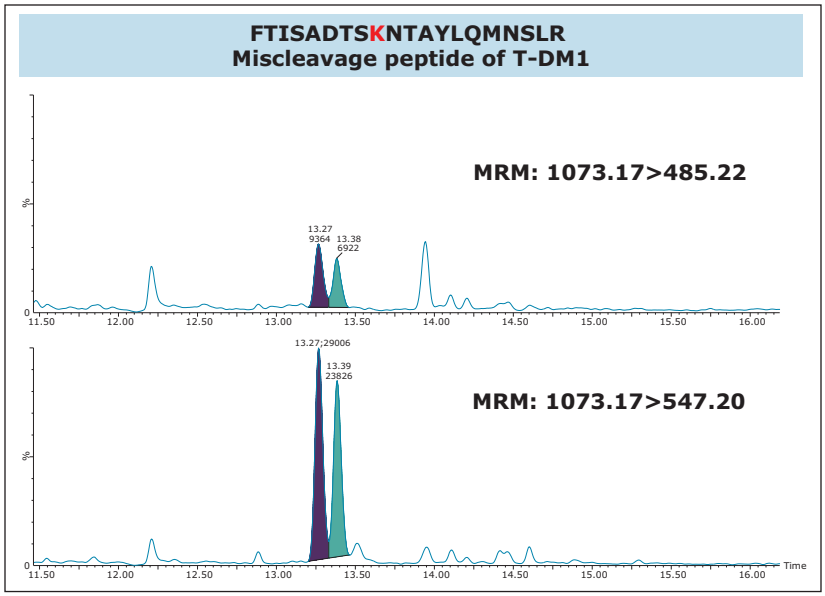


Figure 5. Chromatograms demonstrating the presence of the FTISADTSKNTAYLQMNSLR conjugated peptide confirmed by multiple MRM transitions (1073.17>547.20 m/z and 1073.17>485.22 m/z).

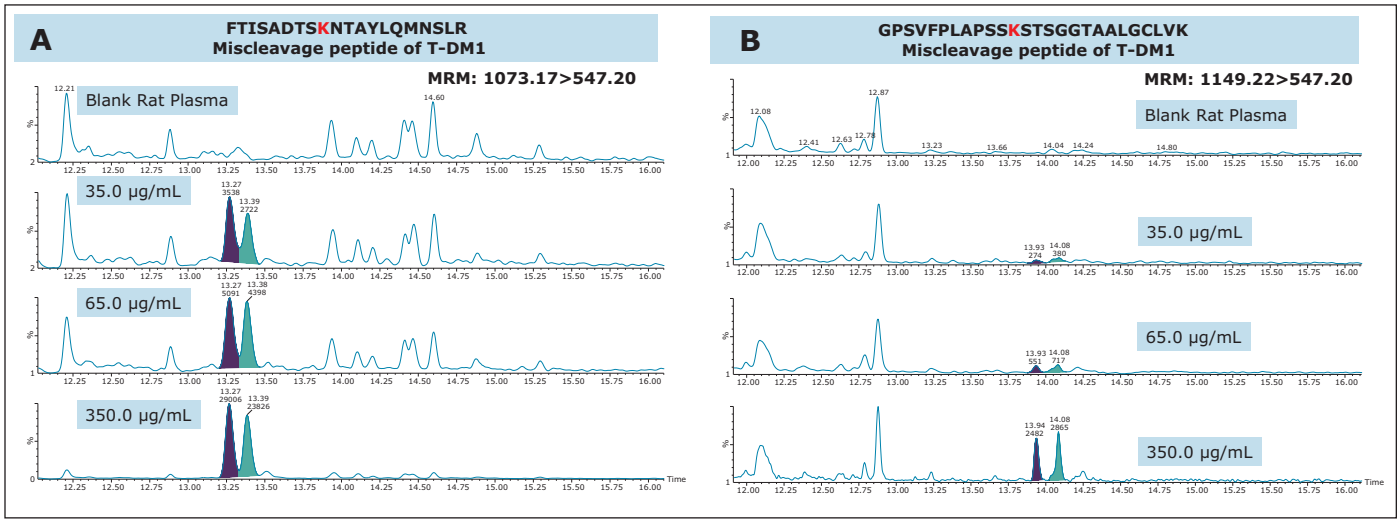


Figure 6. Chromatograms demonstrating increase of the conjugated peptides of T-DM1 with increasing T-DM1 concentration in plasma, when digested and extracted using the ProteinWorks eXpress Direct Digest Kit; Panel A: FTISADTSKNTAYLQMNSLR and Panel B: GPSVFPLAPSSKSTSGGTAALGCLVK.

CONCLUSIONS

The ProteinWorks eXpress Direct Digest Kit was successfully used to quantify trastuzumab and the ADC, T-DM1, from a typical set of standard curve and QC samples in plasma. Through direct digestion of 35 µL of plasma, quantification limits of 0.5–1.0 µg/mL were achieved, while maintaining excellent linearity, precision and accuracy. The universal, kit-based approach allows novice users to achieve high sensitivity with a simple step-wise protocol and standardized, pre-measured reagents, ensuring both the sensitivity and reproducibility required in discovery studies to make time sensitive and critical project decisions.

References

1. Peddi PF, Hurvitz SA. Trastuzumab emtansine: the first targeted chemotherapy for treatment of breast cancer. *Future oncology (London, England).* 2013;9(3): 10.2217/fon.13.7. doi:10.2217/fon.13.7.
2. Barok M, Joensuu H, IsolaJ. Trastuzumab emtansine: mechanisms of action and drug resistance. *Breast Cancer Res*. 2014 Mar5;16(2):209.doi: 10.1186/bcr3621.
3. http://www.drugbank.ca/drugs/DB05773
4. FDA Guidance for Industry for Bioanalytical Method Validation, CDER.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

[POSTER NOTE]

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

QUANTIFICATION OF INTACT PROTEIN BIOTHERAPEUTICS BY A MICROFLUIDIC LC/MS PLATFORM

Gregory T. Roman, James P. Murphy, Catalin E. Doneanu, Ying Qing Yu, Kelly Doering, Weibin Chen
Waters Corporation, 34 Maple Street, Milford, MA 01757

OVERVIEW

To characterize the analytical capabilities of the ionKey/Xevo G2-XS system for quantification of intact proteins.

INTRODUCTION

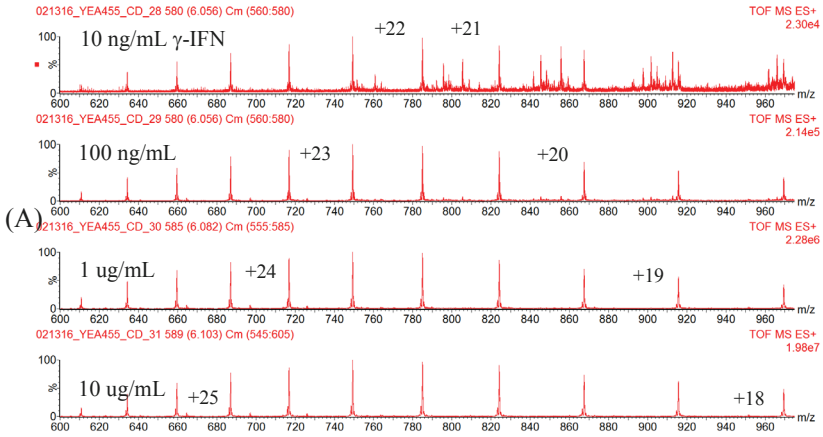
Structural characterization of biotherapeutic proteins is routinely performed by high-resolution mass spectrometry. In contrast, quantification of proteins in biological fluids is typically performed either by ligand binding assays (LBA) or by tandem mass spectrometry using the signature peptide approach (MRM method).

Here we evaluated the analytical capabilities of an integrated microfluidic platform coupled to a high-resolution QTOF instrument (the IonKey Xevo G2-XS) for quantification of three protein biotherapeutics: gamma interferon (g-IFN, a 17 kDa cytokine), an 150 kDa mAb (Waters mAb standard) and an antibody drug conjugate (trastuzumab emtansine ADC).

The IonKey microfluidic platform provides significant sensitivity enhancements for analysis of intact proteins compared to standard (analytical scale) LC/MS platforms because of (1) increased electrospray ionization efficiency; (2) higher sampling efficiency of multi-charged protein ions; and (3) decreased ion suppression effects caused by co-eluting matrix components.

Several figures of merit, including linear dynamic range, LLOD, assay reproducibility and mass measurement accuracy of deconvoluted mass, were compared across the 3 assays reported here.

Figure 1. (top) Series of ionKey devices illustrating integrated chromatography-ESI. (bottom) Plug and play ionKey source illustrating insertion of iKey devices.



(A)

Figure 2. (A) ESI-MS spectra of g-IFN at four different concentrations in the range of 10 ng/mL-10 µg/mL.

TO DOWNLOAD A COPY OF THIS POSTER NOTE, VISIT WWW.WATERS.COM/POSTERS

METHODS

Sample Preparation

A stock solution of gamma-interferon (g-IFN, 1 mg/mL European Pharmacopeia standard) was diluted with 0.1% FA (formic acid) to prepare 4 solutions with concentrations of 10, 100, 1000 and 10,000 ng/mL. Dilutions were prepared in plastic vials in the presence of 1 µM BSA (bovine serum albumin).

For the analysis of non-glycosylated samples, the Waters mAb standard (10 mg/mL stock) was diluted with 3% ACN, 0.1% FA to achieve the desired concentrations. Another sample set was prepared in the same solvent following overnight PNGase F deglycosylation.

The ADC sample (trastuzumab emtansine) was isolated from rat plasma following generic affinity capture.

Chromatographic Conditions

The g-IFN assay was performed on a 150 µm x 5 mm C4 iKey, kept at 60 deg C, using a 3 min gradient from 5 to 50% Solvent B. The flow rate was 2 µL/min and the mobile composition was 0.1% FA in DI water (Solvent A) and 0.1% FA in 100 % ACN. The injection volume was 1 µL and the assay was performed in direct inject mode.

The same mobile phases were used for the mAb and ADC assays, but the IonKey system was configured in trapping mode. Samples (5 µL) were loaded on a 300 µm x 100 mm trap column at 15 µL/min and separated on a 150 µm x 10 mm iKey, kept at 80 deg C, using a 3.5 min gradient from 3 to 95% Solvent B. The elution flow rate was 3 µL/min.

MS Conditions

MS System:	Xevo G2-XS QTOF
Ionization Mode:	ESI positive, Sensitivity Mode
Capillary Voltage:	3.5 kV
Sampling Cone:	30 V (g-IFN) or 150 V (mAbs/ADC)
Source Temp:	150 oC
Mass Range:	m/z 500 – 2,000 (g-IFN) or m/z 500 – 4,000 (mAbs/ADC)

RESULTS

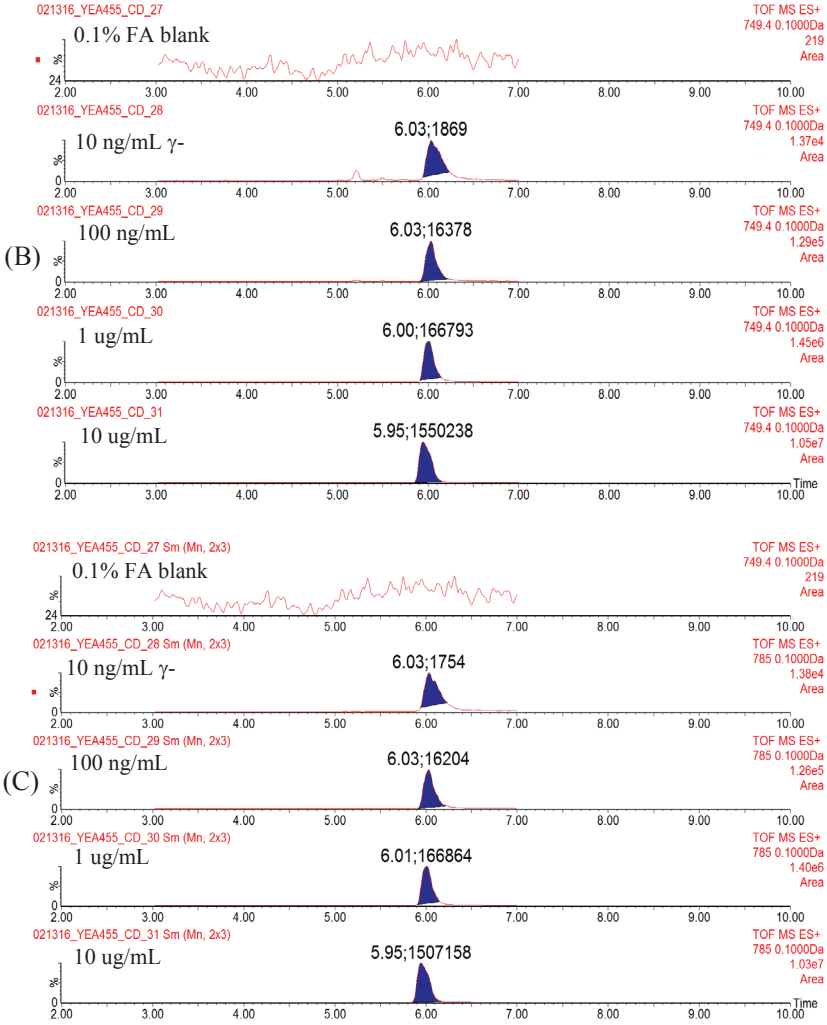


Figure 2. (B,C) Mass chromatograms recorded for the most intense charge states—749.4 (+21) and 785.0 (+21)- across the same concentration range; (D) Calibration curve of γ-IFN.

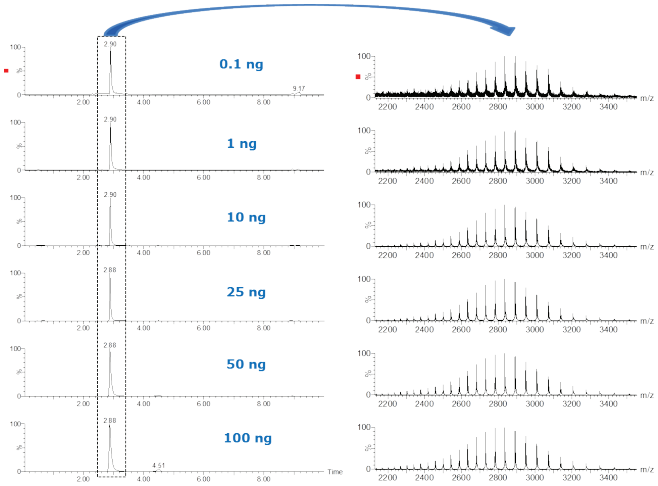


Figure 3. (Left panel) TIC chromatograms of the deglycosylated Waters mAb standard for several mass loads in the range of 0.1 to 100 ng covering 3 orders of magnitude;

(Right panel) The corresponding charge state distributions obtained for each mAb amount loaded on-column.

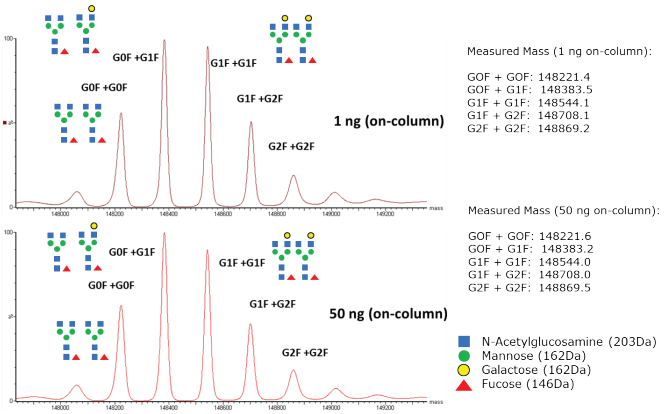


Figure 4. Deconvoluted ESI-MS spectra of non-deglycosylated Waters mAb standard for two mass loads on column (1 ng vs 50 ng) shows very similar mass accuracies for the five major glycoforms.

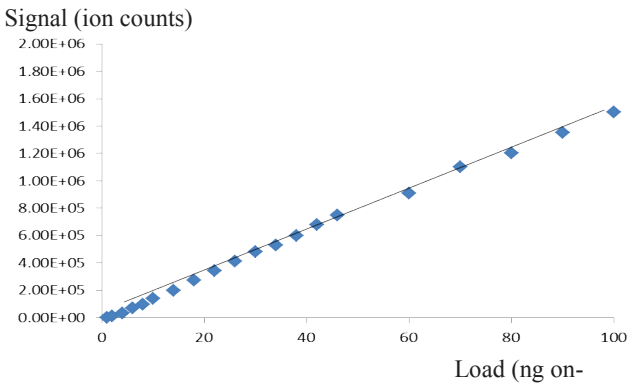


Figure 5. Calibration curve for multiple amounts of the

Waters mAb standard in the range of 0.1 to 100 ng loaded on-column.

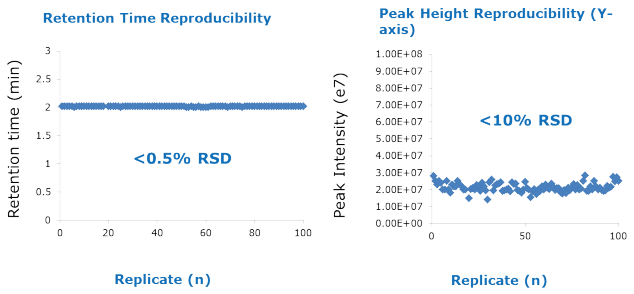


Figure 6. Reproducibility of retention time and peak height for intact antibody standard.

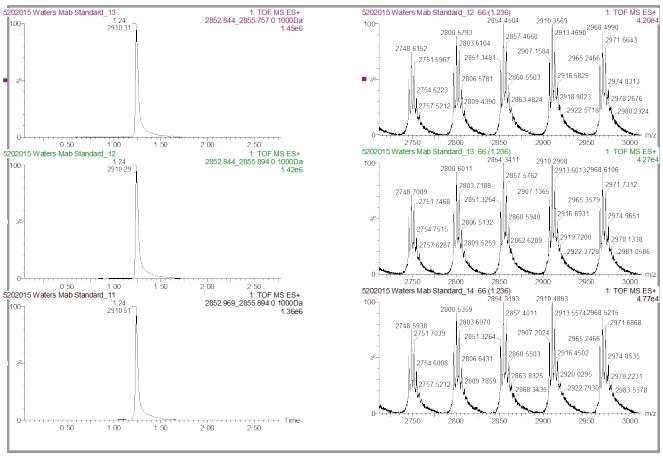


Figure 7. Example of high throughput mAb analysis (5 min runtime). Successive injections of the non-deglycosylated Waters mAb standard indicating very good retention time and peak height reproducibility.

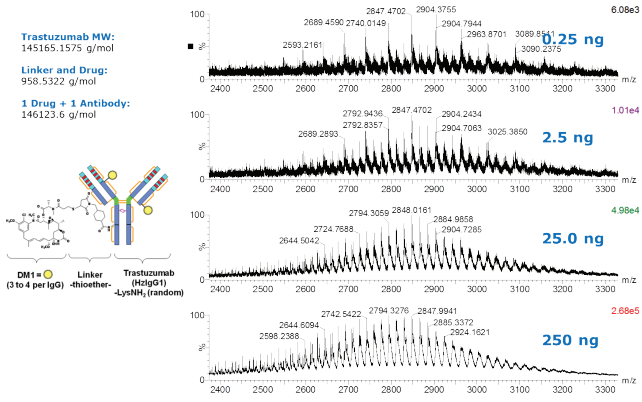


Figure 8. Deglycosylated Herceptin analyzed using the C4 ionKey.

Quantification of Intact Protein Biotherapeutics by a Microfluidic LC/MS Platform

CONCLUSION

- **The assay of gamma interferon (g-IFN) had a linear dynamic range of 3 orders of magnitude, from 10 ng/mL to 10 µg/mL.**
- **The LLOD of the intact Waters mAb ranged from 0.1 to 1 ng (on-column) and demonstrated a 10X improvement in sensitivity over standard (analytical) flow methods.**
- **Trapping capabilities of the IonKey system enabled improved mass load capabilities and mAb refocusing for improved peak shape.**
- **A high-throughput robust and reproducible LC/MS method, with limited sample preparation, is often required for sensitive analysis of mAbs and ADCs.**
- **The advantages of the ionKey/MS system for mAb and ADC analysis include: improved sensitivity, reduced sample consumption, ease of integration for high throughput analysis, ease of use, and reduced solvent consumption and operating costs.**

[POSTER NOTE]

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

IN-DEPTH CHARACTERIZATION OF LYSINE-CONJUGATED ANTIBODY -DRUG CONJUGATES (ADCs) BY LC/MS QUALITATIVE AND QUANTITATIVE ANALYSIS

Liuxi Chen, Henry Shion, Ying Qing Yu, Weibin Chen
Waters Corporation, Milford, MA 01757, USA

INTRODUCTION

Antibody drug conjugates (ADCs) is a sub-class of biotherapeutics which consists of monoclonal antibodies (mAbs) and cytotoxic drugs linked to mAbs by chemical linkers. The reactivity of primary amines and their compositional availability in monoclonal antibodies (mAbs) make them a popular target for chemical conjugation in antibody-drug conjugates (ADCs). However, conjugation reactions of lysine residues result in highly heterogeneous mixtures with drugs in many combinations at different lysine sites on the mAb. Hence, the structural complexity and intrinsic heterogeneity of lysine-conjugated ADCs impose a prominent analytical challenge to current characterization methods. Quantification of conjugated peptides and site occupancy ratio determination was traditionally done by UV methods. The drawbacks for UV quantification include low sensitivity, insufficient selectivity and relative long analysis time. MS based quantification can provide higher selectivity and sensitivity compared to UV based methods. The biopharmaceutical industry lacks a complete workflow that enables efficient identification and quantification of ADC peptides, therefore facing a great challenge to make direct comparison on the site occupancy of ADCs from multiple sources. In this study, we present an integrated approach that combines multiplexed MS/MS data acquisition strategy with multi enzyme digestion for the in-depth characterization of lysine-conjugated ADCs. Both data-independent acquisition and data-dependent acquisition (DDA) methods were used to identify the lysine-conjugated peptides, confirm the conjugation sites, determine the relative site occupancy ratio and compare conjugated peptide levels across samples.

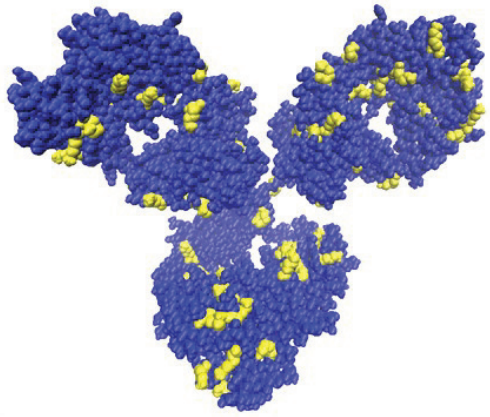


Figure 1. Surface exposed lysine residues on IgG1.

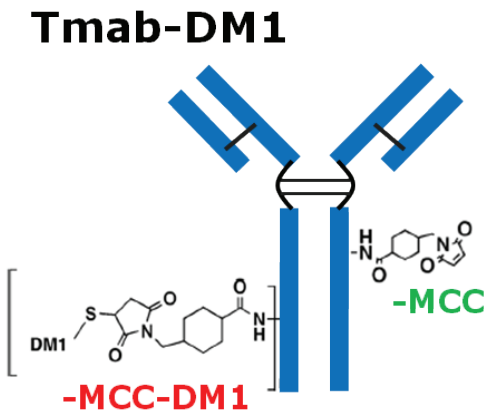


Figure 2: ADC Tmab-DM1 Structure Illustration

TO DOWNLOAD A COPY OF THIS POSTER NOTE, VISIT WWW.WATERS.COM/POSTERS

METHODS

Sample Preparation:

Peptide mapping analysis:

Trastuzumab (Tmab) and trastuzumab emtansine (Tmab-DM1) were denatured, alkylated and digested by trypsin and Asp-N endoproteinase, respectively. Leucine enkephalin (LeuEnk) was added to each sample at final concentration of 50 *fmol/ul* as an internal standard. Enzyme digests of Tmab and Tmab-DM1 were analyzed in triplicate injections.

Intact mass analysis:

All samples were treated with PNGase F overnight to remove the N-linked glycans in order to reduce the intact mass spectra complexity.

LC/MS:

LC:	*ACQUTIY UPLC H-Class System*
Column:	ACQUITY UPLC BEH C18, 300Å, 1.7 µm, 2.1 x 100 mm (p/n 186003686)
Column temperature:	65 °C
Mobile phase:	A. 0.1% Formic acid in water
	B. 0.1% Formic acid in acetonitrile
MS:	Xevo G2-XS QTof MS
Data Acquisition:	MS^E and FastDDA
Mode:	ESI positive mode
Capillary Voltage:	3.0 kV
Cone Voltage:	30 V
Source Temperature:	100 °C
Desolvation Temperature:	250 °C
Mass Range (*m/z*):	100-2000
Lock mass used:	100 fmol/ul of Glu-Fibrinopeptide B in ($[M+2H]^{2+}$, 785.8426)

MS^E settings:

Scan rate for alternating low/high Energy:	0.5 sec
Low energy:	6 V
High energy ramp:	20-45 V

FastDDA settings:

MS scan time:	0.2 sec
MSMS scat time:	0.2 sec
Peak detection:	Intensity threshold
Max. # MSMS scans/survey:	5

Dynamic peak exclusion: Acquire and then exclude for 8 sec (± 1.1 Da)

Collision Energy: m/z dependent ramp applied for low and high mass

Stop MS/MS criteria: TIC 5e8 or 0.4 sec

Informatics:

UNIFI 1.8

UNIFI scientific library

Intact Mass Analysis

The distribution of the drug load is determined by MS intact analysis. The deconvoluted mass spectra contain 8 major peaks with mass difference of 957 Da between adjacent peaks, which is in agreement to the mass of covalently linked DM1 drug with one MCC linker. In both the innovator and candidate biosimilar ADCs, 8 major peaks correspond to Tmab with 0-7 DM1 drug and linkers respectively (label as +0 drug, +1 drug, etc). The less abundant peaks right next to the major peaks with 219 Da, which attributes to the unreacted linkers that modified the antibody but do not react with DM1.

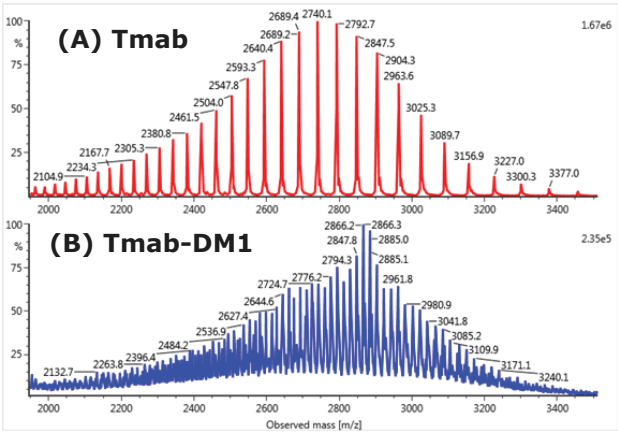


Figure 3. Combined raw spectra for Tmab (A) and Tmab-DM1 (B).

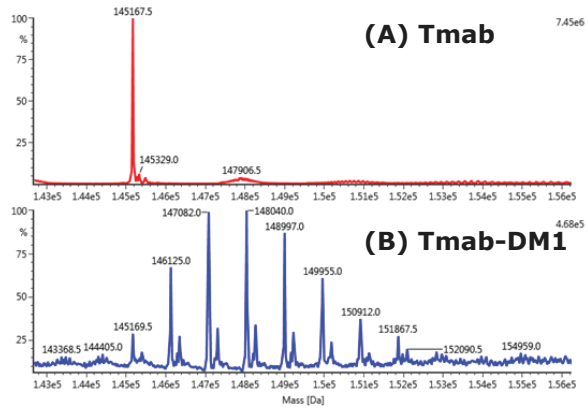


Figure 4. Deconvoluted spectra for Tmab (A) and Tmab-DM1 (B).

ADC Peptide Analysis Workflow

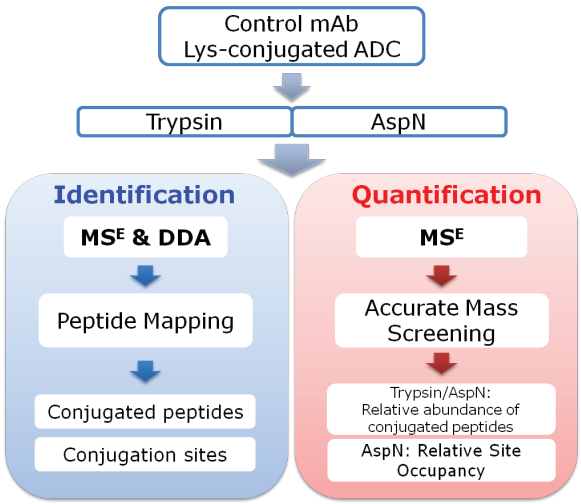


Figure 5. ADC Peptide level analysis identification and the quantification workflows. Lysine-conjugated ADC and unconjugated control mAb were digested by trypsin and Asp N respectively, followed by MS^E and DDA modes. UNIFI Peptide Mapping workflow was used to identify the conjugated peptides and pinpoint the conjugation sites. The same set of MS^E data were further analyzed using Accurate Mass Screening workflow in UNIFI to quantify the relative site occupancy and relative abundance of conjugated peptides across different samples.

	Trypsin	Asp N
Unconjugated peptide	K	K
Conjugated peptide	K miscleavage	K
Quantitation	Relative abundance of conjugated peptides	Relative site occupancy ratio

Table 1. The enzyme of choice for different quantification purposes. Trypsin digest was used to calculate relative abundance of conjugated peptides, while Asp-N digest was used to determine the relative site occupancy of individual site.

Peptide Mapping—Site Identification

Figure 6. LC/MS^E chromatogram (BPI) of tryptic peptide mapping analysis for Tmab vs Tmab-DM1 in comparison mode.

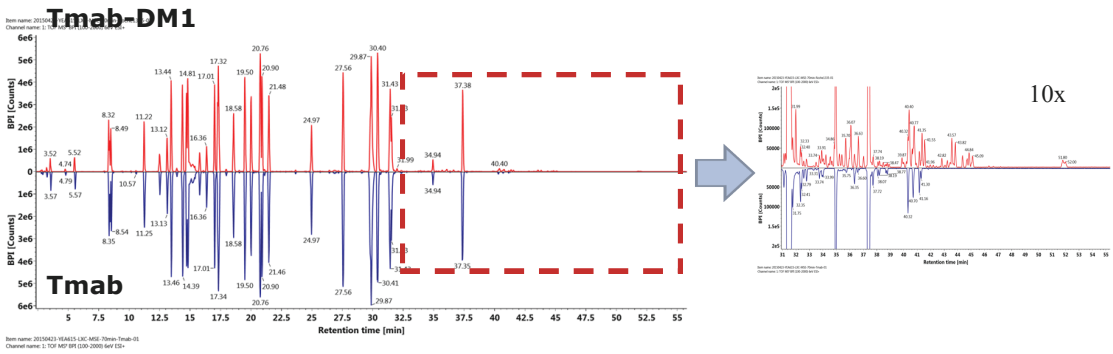


Figure 7. LC/MS^E chromatogram (BPI) of Asp-N peptide mapping analysis for Tmab vs Tmab-DM1 in comparison mode.

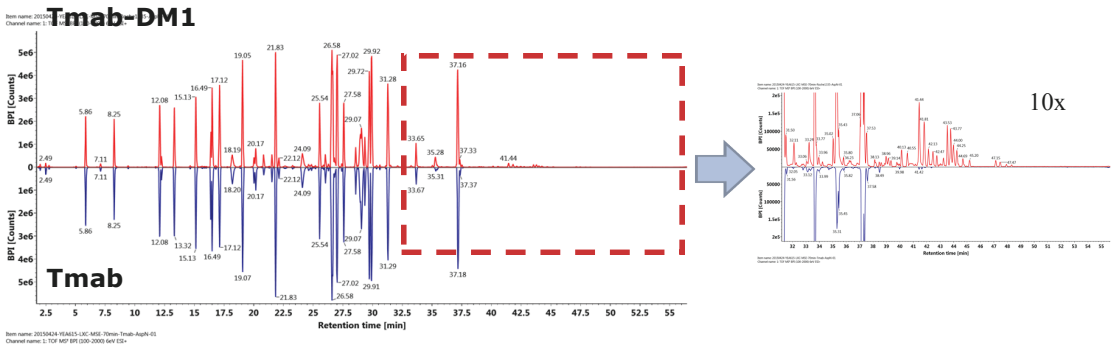
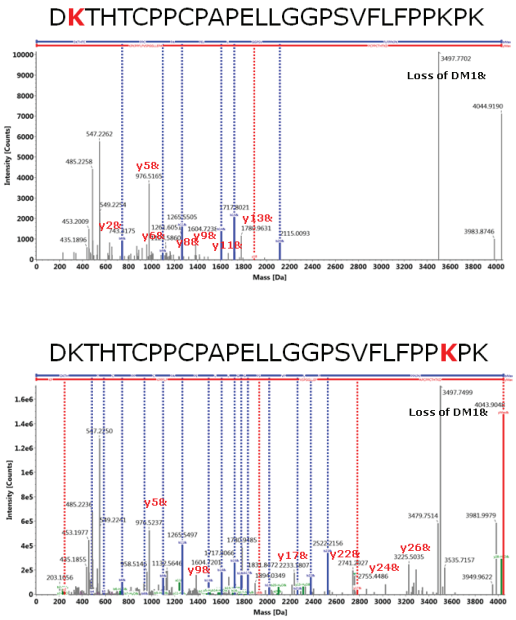


Table 2. Numbers of conjugation sites identified in different regions of Tmab using DDA and MS^E methods.

Region	Total # of Lys	Trypsin		Asp N	
		DDA	MSE	DDA	MSE
Variable Fab	12	9	10	6	8
Constant Fab	14	13	13	9	7
Fc	20	16	17	4	6
Total	46	38	40	19	21

Figure 8. MS/MS spectra to confirm conjugations sites for positional isomers for Asp-N peptide [224] DKTHTCPPCPAPELLGGPSVFLFPPKPK[251]



The CID fragmentation of the Tmab-DM1 generates a signature fragment ion (m/z 547.2, charge +1), commonly for all conjugated peptides. The signature fragment ion corresponds to a partial drug fragment.

Peptide Mapping—Site Quantification

Figure 9. Relative abundance of conjugated peptides (tryptic) across samples.

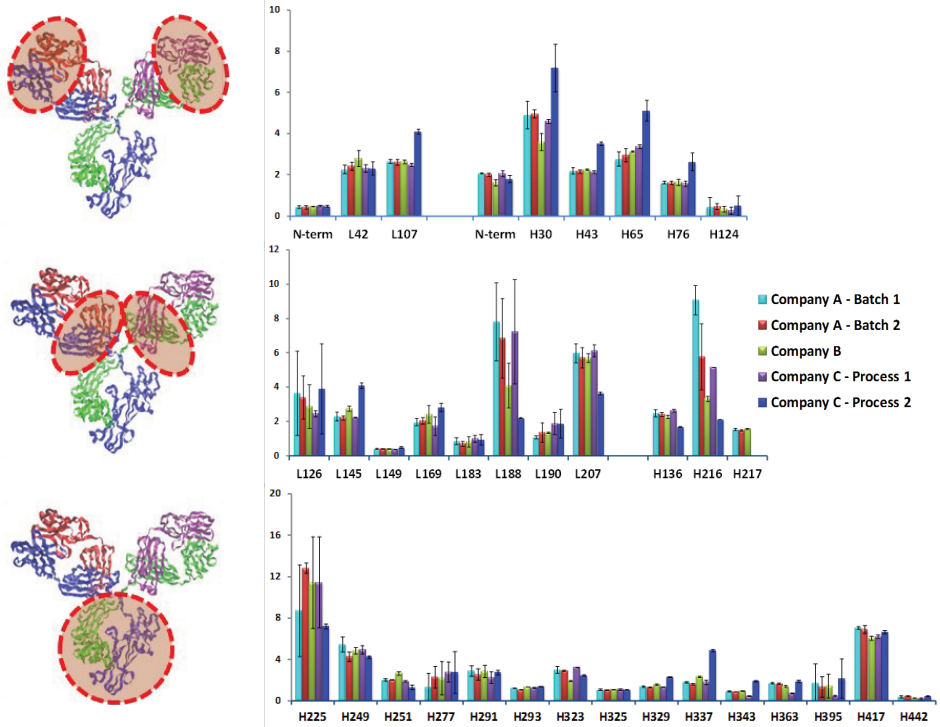
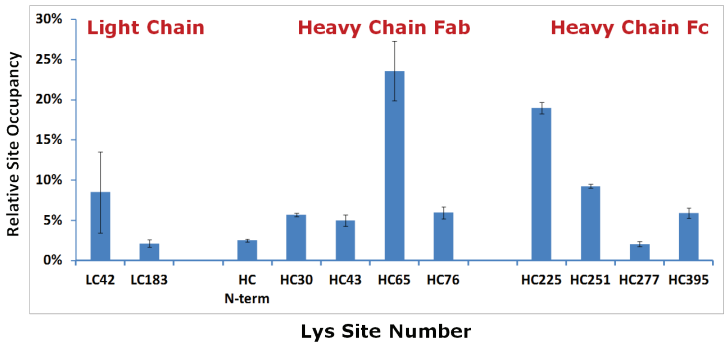


Figure 10. Relative site occupancy determined using Asp-N digestion.



Relative site occupancy ratio = Area (conjugated pep. peak)/

[Area(unconjugated pep.Peak) +Area(conjugated pep. Peak)]

CONCLUSION

- **For Tmab-DM1, 80 out of 92 conjugation sites were observed.**
- **UNIFI provided automated workflow for:**
  - **In-depth primary structure characterization of lysine-conjugated ADC.**
  - **Site specific localization of ADC conjugation (Peptide Mapping Workflow).**
  - **Quantification of relative site occupancy (Accurate Mass Screening Workflow).**
  - **While this presentation has focused on lysine-conjugated ADCs, these UNIFI workflows are directly applicable to other classes of ADC biotherapeutics.**

Reference:

1. Wang L, Amphlett G, Blattler WA, Lambert JM, Zhang W, Protein Sci. 2005 Sep;14(9):2436-46.
2. Waters Application Note (PN = 720005603). "Automated Quantitative Analysis of Antibody Drug Conjugates Using an Accurate Mass Screening Workflow in the UNIFI Scientific Information System".

Index of Analytes

Various proteolytic peptides not listed.

MW's are estimates and were obtained from published sources, package inserts and/or from experimentation.

Index of Analytes

[NOTES]

[notes]

[NOTES]

[NOTES]

Sales Offices:

Australia 61 2 9933 1777

Austria 43 1 877 18 07

Belgium and Luxembourg 32 2 726 1000

Brazil 55 11 4134 3788

Canada 1 800 252 4752

China 86 21 6156 2666

Czech Republic 420 2 617 11384

Denmark 45 46 59 8080

Finland 358 9 5659 6288

France 33 1 30 48 72 00

Germany 49 6196 400 600

Hong Kong 852 2964 1800

Hungary 36 1 350 5086

India 91 080 49292200 03

Ireland 353 1 448 1500

Israel 9723 3731391

Italy 39 02 265 0983

Japan 81 3 3471 7191

Korea 82 2 6300 9200

Malaysia 603 7841 1688

Mexico 52 55 52 00 1860

The Netherlands 31 76 508 7200

Norway 47 6 384 6050

Poland 48 22 101 5900

Portugal 351 21 893 61 77

Puerto Rico 1 787 747 8445

Singapore 65 6593 7100

Spain 34 93 600 9300

Sweden 46 8 555 115 00

Switzerland 41 56 676 7000

Taiwan 886 2 2508 5500

UAE 971 4 214 62 38

UK 44 208 238 6100

US 1 800 252 4752

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

October 2016 720005226EN LM-PDF