APPLICATION NOTE 22059

Simple and sensitive quantitation of large therapeutic proteins in plasma in 90 minutes

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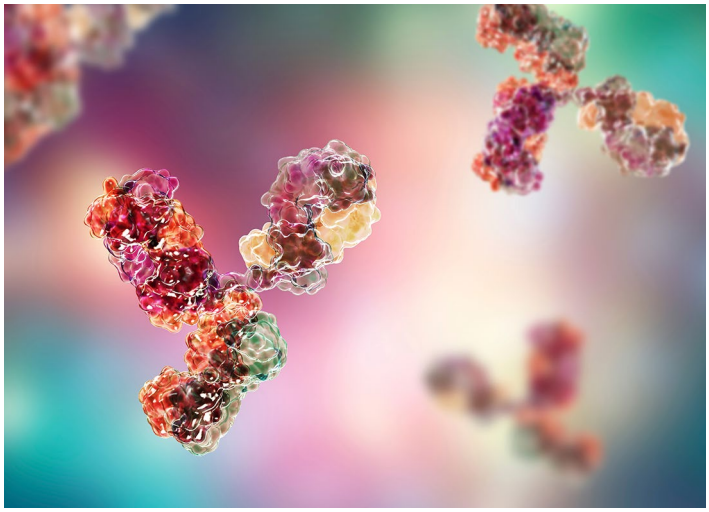

Goal

To simplify the workflow of liquid chromatography tandem mass spectrometry (LC-MS/MS) quantification of large therapeutic proteins in plasma while generating superior results to existing methods.

Introduction

Thermo Scientific™ SMART Digest™ soluble digestion kits are designed to remove issues often associated with sample preparation of proteins by delivering a process that is fast, simple, highly reproducible and sensitive.

The SMART Digest kits achieve this due to their heat-activated, thermally-stable enzyme design. this enables processing of the samples in 90 minutes compared to 24-hours with standard protocols.

Mass spectrometry-based methods are unique in their ability to directly measure peptide mass and, following fragmentation, determine peptide sequence. Recent advances in instrumentation and reagents have enabled routine quantification of large proteins by mass spectrometric methods in their intact form or by quantifying a set of their specific peptides.

Most large therapeutic proteins have relatively long half-lives and, as such, are present in plasma at concentrations exceeding 500 ng/mL throughout the duration of action while in circulation. At these concentrations, biotherapeutics require no further enrichment prior to enzymatic cleavage with trypsin and measurement by mass spectrometry. However, tryptic digestion of complex specimens is variable and typically does not result in complete digestion of all proteins in the sample.[1]

Trypsin digestion is a multifaceted process. Complete digestion is kinetically unfavorable. Autolysis cuts activity, changes enzyme specificity, and mitigates the use of high concentrations of enzyme. Furthermore, folded tertiary protein structure restricts access to cleavage sites. A typical digestion protocol involves the addition of chaotropes or strong surfactants to the sample, to promote denaturation and enhance solubility, followed by reduction and alkylation. Dilution of the sample following reduction and alkylation is required as chaotropes and strong surfactants also denature and deactivate trypsin. Trypsin is typically added in concentrations ranging from 1:50 to 1:10 (enzyme to substrate ratio). Once trypsin is added, incubation times can range from 3 to 24 hours at temperatures ranging from 37 to 50 °C. Attempts to accelerate this reaction by increasing enzyme concentration are typically limited by cost and enzyme kinetics. The use of sequencing grade trypsin can be cost prohibitive at a 1:10 enzyme to substrate ratio as 5 µL of plasma contains roughly 350 µg of protein. Methods that utilize pancreatic trypsin sourced from either pigs or cows can be performed at a fraction of the cost. However, pancreatic trypsin is prone to autolysis, which changes the specificity of trypsin and results in peptide degradation.[2] It is possible to mitigate the impact of peptide decay on quantitation by adding standards prior to digestion.[3] However, this method relies on the identification of rapidly forming peptides and does not prevent the loss of sensitivity inherent to peptide over-digestion. As an alternative to historical approaches, we present here a thermally enhanced digestion using thermophilic trypsin.

Thermally enhanced digestion overcomes the fundamental limitation of historical approaches, mainly the need to denature proteins by means of chemicals that deactivate trypsin. The ability to digest and denature the proteins simultaneously using heat simplifies the workflow while dramatically improving the efficiency of the digestion. In cases where the sample of interest is difficult to digest, the improved efficiency can improve sensitivity more than 10-fold. In the case of native IgG, the transition from 50 to 70 °C results in an 800-fold increase in signal. Furthermore, the increased efficiency translates into significant cost savings as relatively small amounts of enzyme can digest large quantities of protein. Lastly, with reaction times under 2 hours, this approach enables rapid method development.

Table 1. Digestion methods

	Recombinant trypsin	Pancreatic trypsin	Soluble SMART Digest
Digestion steps	3	3	1
Digestion time	3-24 hours	3-24 hours	1-2 hours
Over digestion	No	Yes	No
Method development time	Weeks	Weeks	Days

Experimental

Consumables

Digestion

- Thermo Scientific™ SMART Digest™ Soluble Trypsin Kit (P/N 60113-101)

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Optima™ acetonitrile (ACN) (P/N A955-4)
- Fisher Scientific™ formic acid (FA) (P/N F/1900/PB08)
- Mouse plasma from a reputable supplier
- Human IgG from a reputable supplier

Sample handling

- Thermo Scientific™ Matrix™ SepraSeal (P/N 4463)
- 96-deepwell low-adsorption plates
- Thermomixer from a reputable supplier

Preparation of calibration and quality control (QC) samples

Human IgG was spiked into mouse plasma at concentrations between 0.5 ug/mL and 500 µg/mL. Quality control samples were prepared at 0.5, 5, 50, and 500 µg/mL in mouse plasma.

Quantification of human IgG in animal plasma using SMART Digest kit

To prepare samples of analysis, 5 µL of plasma, 45 µL ultrapure water, 150 µL of SMART Digest Buffer, and 5 µL of Soluble SMART Digest were added to wells of a 96 Deepwell plate. Once plated, the plate was sealed using SepraSeals and incubated for 90 min at 70 °C. Following digestion, the samples were allowed to cool for at least 5 min. Then, 150 µL of the digested sample was decanted into a new plate containing 150 µL of 1% TFA in each well (alternatively, the sample plate can be acidified directly). 4 µL of the final solution was injected for analysis with the LC-MS system.

Separation conditions

Instrumentation

- Thermo Scientific™ UltiMate™ 3000 Rapid Separation Dual system equipped with:
  - SRD-3600 Solvent Racks with Degasser (P/N 5035.9230)
  - DGP-3600RS Rapid Separation Pump (P/N 5040.0066)
  - WPS-3000TRS Rapid Separation Thermostatted Well Plate Autosampler (P/N 5841.0020)
  - TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)

Column

- Thermo Scientific™ Accucore™ C18 column, 2.1 mm × 50 mm, 2.6 µm (P/N 17126-052130)

LC settings

Parameter	Value
Mobile phase A	0.1% formic acid in 98% water and 2% acetonitrile
Mobile phase B	0.1% formic acid in 10% water and 90% acetonitrile
Flow rate	0.5 mL/min
Column temperature	50 °C
Injection details	4 µL
Gradient	Table 2

Table 2. LC gradient conditions

Time (min)	%A	%B
0	90	10
1	90	10
5	30	70
5.1	10	90
6.5	10	90
6.6	90	10
8	90	10

Table 3. MS fragment information

Q1 mass	Q3 mass	Act Q	Act time (ms)	CE	Peptide sequence
603.4	805.4	0.25	10	35	VVSVLTVLHQDWLNGK
937.7	836.5	0.25	10	35	TTPPVLDSDGSFFLYSK

MS conditions for Thermo Scientific™ Velos Pro™ ion trap mass spectrometer

Parameter	Value
MS settings	HESI
Mode	Positive
Heater temp	350 °C
Sheath gas	60
Aux gas	20
Spray voltage	4 kV
Capillary temp	375 °C
S-Lens RF level	55%
MS fragment	(See Table 3) *m/z* 603.4, 937.7
Resolution	Normal

Note: Flow is diverted to waste using the divert valve until 1.5 min into the gradient. Flow is sent to the source from 1.5 min to 3 min into the gradient, and then sent to waste again at 3 min into and until the end of the gradient.

Results and discussion

Calibration and quantification for human IgG with SMART Digest Soluble kit

The SMART Digest Soluble kit provided excellent reproducibility and linearity across a wide dynamic range. Calibration and quality control samples were prepared as previously described in the experimental section. Calibration points ranged from 0.5 to 500 µg/mL. Quality control samples were prepared at low, medium, and high concentration levels. Seven-point calibration curves of IgG in plasma yielded linear fits for R^2 of 0.9997 and 0.9998, respectively, for the front and back calibration curves generated monitoring peptide sequence VVSVLTVLHQDWLNGK (*m/z* = 603.4, Figure 1 and Table 4). Furthermore, the seven-point calibration curves yielded linear fits of 0.9999 and 1, respectively, for the front and back calibration curves generated monitoring peptide sequence TTPPVLDSDGSFFLYSK (*m/z* = 937.7, Figure 2 and Table 5).

The results generated for quality control samples prepared at 0.5, 5, 50, and 500 µg/mL were accurate and precise (Table 5). Variability was determined and coefficient of variation (CV) values were obtained for IgG in mouse plasma ranging from 9.9% at the lower limit of quantitation (LLOQ) to 4.7% at the upper limit of quantitation (ULOQ) (n=6) monitoring peptide sequence VVSVLTVLHQDWLNGK (*m/z* = 603.4) and plasma ranging from 11.6% at the LLOQ to 3.8% at the ULOQ (n=6) monitoring peptide sequence TTPPVLDSDGSFFLYSK (*m/z* = 937.7) even without internal standard (Table 7).

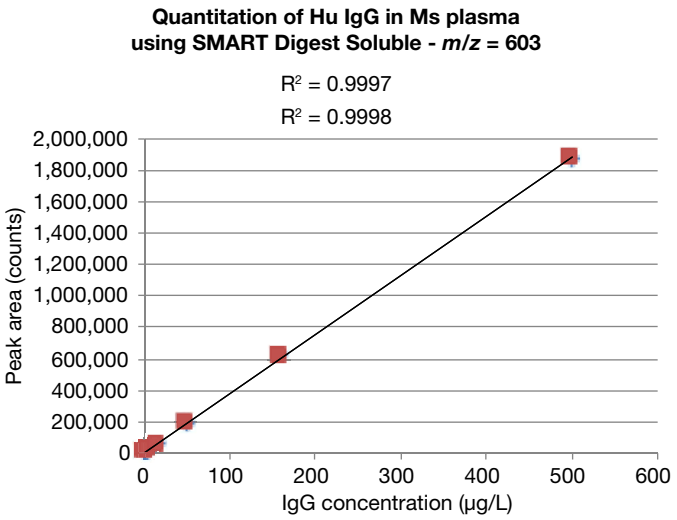

Figure 1. Analysis of human IgG in mouse plasma using SMART Digest Soluble kit, calibration curves for peptide VVSVLTVLHQDWLNGK

Table 4. Analysis of human IgG in mouse plasma using SMART Digest Soluble kit, calibration curves for peptide VVSVLTVLHQDWLNGK (n=6)

Analysis of peptide VVSVLTVLHQDWLNGK- MS^1/MS^2 = 603.56/805.69							
Concentration of IgG (in µg/mL)							
	0.00	1.58	5.00	15.8	50.0	158	500
Peak areas							
Front	2838	6197	18384	58795	188782	623676	1876744
Back	2540	6424	19060	55331	191955	619172	1878470

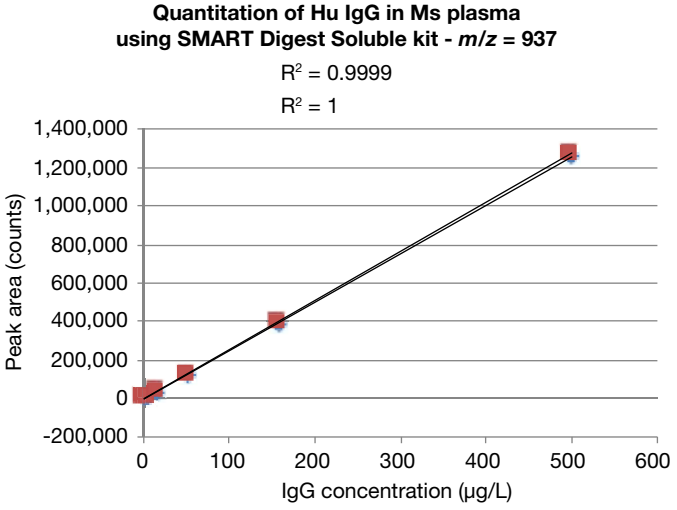

Figure 2. Analysis of human IgG in mouse plasma using SMART Digest Soluble kit, calibration curves for peptide TTPPVLDSDGSFFLYSK

Table 5. Analysis of human IgG in mouse plasma using SMART Digest Soluble kit, calibration curves for peptide TTPPVLDSDGSFFLYSK (n=6)

Analysis of peptide TTPPVLDSDGSFFLYSK – MS^1/MS^2 = 937.53/836.56							
Concentration of IgG (in µg/mL)							
	0.00	1.58	5.00	15.8	50.0	158	500
Peak areas							
Front	1414	3272	10977	34972	116501	391670	1255643
Back	1897	3964	11682	39009	123498	399954	1275570

Table 6. Analysis of human IgG in mouse plasma using SMART Digest Soluble kit, quality control results for peptide VVSVLTVLHQDWLNGK (n=6)

	Concentration of IgG (in µg/mL)			
	0.500	5.00	50.0	500
Sample 1	3347	21259	215475	2087737
Sample 2	3453	24421	222469	1947449
Sample 3	3005	22589	223450	2008473
Sample 4	3080	22023	221228	1895858
Sample 5	3389	22375	225447	1994173
Sample 6	2507	22055	218402	1896674
Sample 7	3279	21974	227053	1825963
Sample 8	2967	22880	215406	1828749
Average	3128	22447	221116	1935635
St Dev	310.2	931.8	4360.3	91463.8
CV (%)	9.9	4.2	2.0	4.7

Table 7. Analysis of human IgG in mouse plasma using SMART Digest Soluble kit, quality control results for peptide TTPPVLDSDGSFFLYSK (n=6)

	Concentration of IgG (in µg/mL)			
	0.500	5.00	50.0	500
Sample 1	1916	13971	136961	1397734
Sample 2	1939	16069	137909	1295532
Sample 3	1920	14300	140718	1382284
Sample 4	1658	13238	140623	1327008
Sample 5	1867	14338	149492	1379535
Sample 6	1827	14065	143456	1325248
Sample 7	1801	13267	142524	1250266
Sample 8	1322	13428	140901	1302293
Average	1781	14085	141573	1332488
St Dev	206.4	916.4	3853.4	50808.5
CV (%)	11.6	6.5	2.7	3.8

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Conclusion

The use of the SMART Digest Soluble kit enables a simple and efficient sample preparation method when compared to conventional denaturation and digestion.

This application shows a simple, rapid, and sensitive method for the quantification of human IgG in mouse plasma using the SMART Digest Soluble kit in a single step with a total time of 90 minutes when compared to a process that typically requires multiple steps and up to 24 hours.

References

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