Application Note: 509

Absolute Quantification of Eicosanoid Pathway Proteins Using a Linear Ion Trap Mass Spectrometer

Roger G. Biringer[1], Julie Horner[1], Alfred N. Fonteh[2], Stephen Kauffman[2], Andreas F. R. Hühmer[1], Michael G. Harrington[2]
[1]Thermo Fisher Scientific, San Jose, CA; [2]Molecular Neurology Program, Huntington Medical Research Institutes, Pasadena, CA

Key Words

- Velos Pro
- Prostanoids
- Cerebrospinal fluid (CSF)
- Peptide quantification

Introduction

Absolute quantification of proteins utilizing heavy-labeled peptide analogs is the most sensitive and accurate methodology for targeted, quantitative proteomics. This study outlines the absolute quantification of a subset of eicosanoid pathway proteins using stable isotope labeled (SI) peptide surrogates and a targeted linear ion trap-based approach. Typically, SI-based quantification studies use a triple quadrupole mass spectrometer because selected reaction monitoring (SRM) experiments on triple quadrupole systems allow large numbers of proteins to be quantified quickly and with high sensitivity in a single experiment.[1,2] However, the fast scanning capability and improved sensitivity of the Thermo Scientific Velos Pro dual-pressure, linear ion trap mass spectrometer provides an alternative to SRM-based analyses with comparable analytical performance. When combined with Thermo Scientific Pinpoint software and Thermo Scientific Proteome Discoverer software for method development, peptide verification, and quantification, the net result is a robust platform for pathway analysis.

The prostanoid family represents a class of eicosanoids that are synthesized from arachidonic acid (Figure 1). Prostanoids serve as signaling molecules for numerous biological processes. They exert complex control in inflammation or immunity and as messengers in the central nervous system. They also play a role in migraines. Effective migraine rescue medications inhibit prostaglandin-endoperoxide synthase (PTGS-1 & -2), but the downstream effects of these drugs are not known. A long-term goal of this study is to identify the changes in the eicosanoid pathway from free arachidonic acid down to the specific receptors, by identifying pathway enzymes and quantifying their changes.

Velos Pro as a Qual/Quan Platform

The Velos Pro™ linear ion trap provides a simplified discovery and quantification platform that expedites method development for targeted analysis without compromising sensitivity, accuracy, or confidence in the results. As well as eliminating the need for an additional instrument, as is typically required for high-throughput quantitative workflows, utilization of the same LC/MS platform for both qualitative (qual) and quantitative (quan) applications streamlines method development in a number of ways. Employing the same chromatographic system eliminates the need for verification and harmonization of retention times on two instruments. Using the same nanoelectrospray source eliminates the need to determine the optimal charge state for quantification. The time and effort spent selecting and optimizing triple quadrupole collision conditions and transitions is completely eliminated. Only the precursor ion *m/z* need be known (all fragment ions are observed) and ion trap collision conditions are normalized automatically without the need for user intervention.

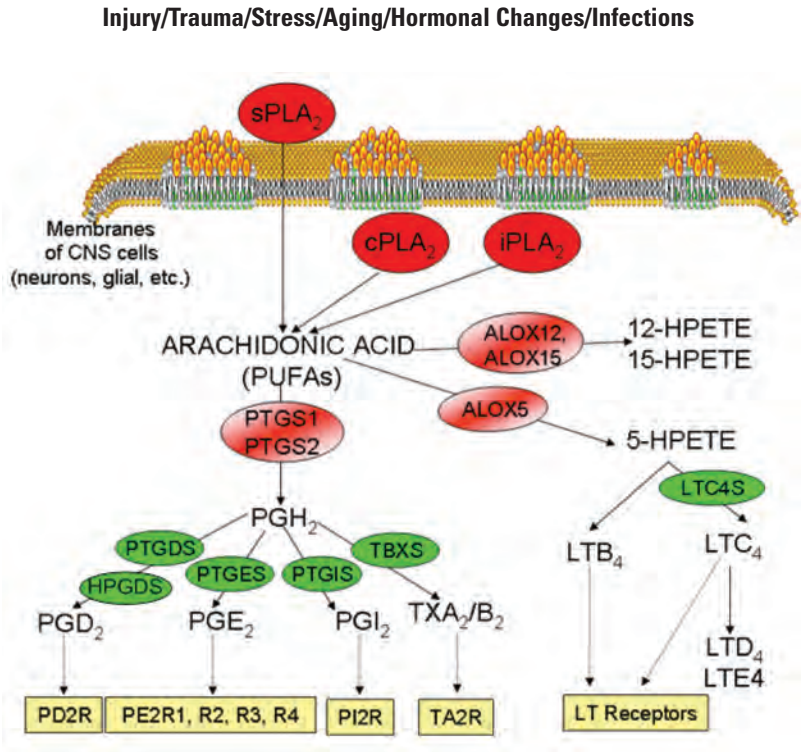

Figure 1. Eicosanoid pathway. Enzymes are indicated with ovals, metabolites with their text abbreviation, and receptors with rectangles. All of the enzymes in the figure have been previously identified in our shotgun sequencing of human CSF.

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Verification of peptides in the quantification step is an absolute requirement for a qual/quan workflow. Peptides with a similar sequence to the target can be mistakenly selected for quantification, especially for low abundance proteins in a complex matrix. Each full MS/MS scan contains all of the necessary information to identify the peptide, providing verification with each quantification measurement without the need for additional SRM-triggered MS/MS events.

The fast scanning (up to 10 Hz) of the Velos Pro instrument and its ability to schedule scan events is sufficient to simultaneously quantify proteins in a single LC/MS run in numbers required for most targeted studies.[3] The high dynamic range (5 orders) and low LOQs are more than sufficient for quantifying proteins at physiologically relevant concentrations in complex matrices.

Validation of the Quan/Qual Workflow

Validation of the ion trap quantification workflow is relatively straightforward.[2] Essential to validating any quantification workflow is selectivity so, first and foremost, all peptides selected for this study are proteotypic. Further, SI and endogenous peptides were verified with Pinpoint™ software version 1.1 by: 1) a statistically ranked comparison of each MS^2 spectrum to the corresponding library spectrum and 2) co-elution of the SI peptide/endogenous peptide pairs.[4]

All data were normalized to account for variation between samples (total protein) and technical replicates (SI peptide area). Each SI peptide was synthesized to include additional amino acid residues to provide at least one trypsin cleavage site on each peptide to normalize the trypsin efficiency across all samples.

In typical SRM-based quantification experiments, two or three peptides are monitored for each protein for verification of measured protein levels and to generate appropriate quantification statistics. In this pilot study we used one peptide per protein which is adequate for proof-in-principle of the methodology. Future work will involve multiple peptides per protein.

Goal

The ultimate goal of this work was to develop and test an absolute quantification workflow that employs a Velos Pro linear ion trap mass spectrometer and apply the workflow to a biologically relevant problem. To achieve this goal, the overall dynamic range, linearity of quantification, and LOD and LOQ values were first established for ten eicosanoid peptides. After these parameters were known, absolute quantification of proteins in migraine study participants was performed.

Experimental

Overview of the Ion Trap Full MS^2 Quantification Workflow

An overview of the quantification workflow is given in Figure 2. Each step is described in detail below.

Step 1: Design, synthesis and preparation of stock solutions of SI peptides

A combination of pre-existing experimental data and prediction with Pinpoint software version 1.1 was used to design proteotypic SI peptides.[2,4] To ensure at least one trypsin cleavage site on each peptide to serve as a digestion control, four of the ten peptides were synthesized with an additional residue on the N terminus and six of the ten peptides were synthesized with additional residues on both the C and N termini (Table 1)†. In all cases, the residues added matched those in the naturally occurring protein sequences.

Equal amounts of each SI peptide were spiked into tryptically digested cerebrospinal fluid (CSF) at various concentrations to establish optimal chromatographic conditions and enable creation of a standard curve for each peptide.

Step 2: Optimization of chromatography and creation of an inclusion list for a scheduled full-scan MS/MS method

The chromatographic standard prepared in Step 1 was analyzed by LC and Top 5 data-dependent MS^2 using a Velos Pro dual-pressure linear ion trap mass spectrometer equipped with a nanoelectrospray ion source. Chromatography was optimized to maximize the separation of the SI peptides. Identities were verified and retention times determined using Proteome Discoverer™ software version 1.2. The results were used with Pinpoint software version 1.1 to create a spectral library for verification and the retention times required for a scheduled full-scan MS/MS method.

Step 3: Run scheduled full-scan MS/MS to create standard curves and evaluate unknown samples

The Thermo Scientific Xcalibur software method for the scheduled full-scan MS/MS was created from the data acquired in Step 2. Both standards and samples from migraine study participant were evaluated using this method.

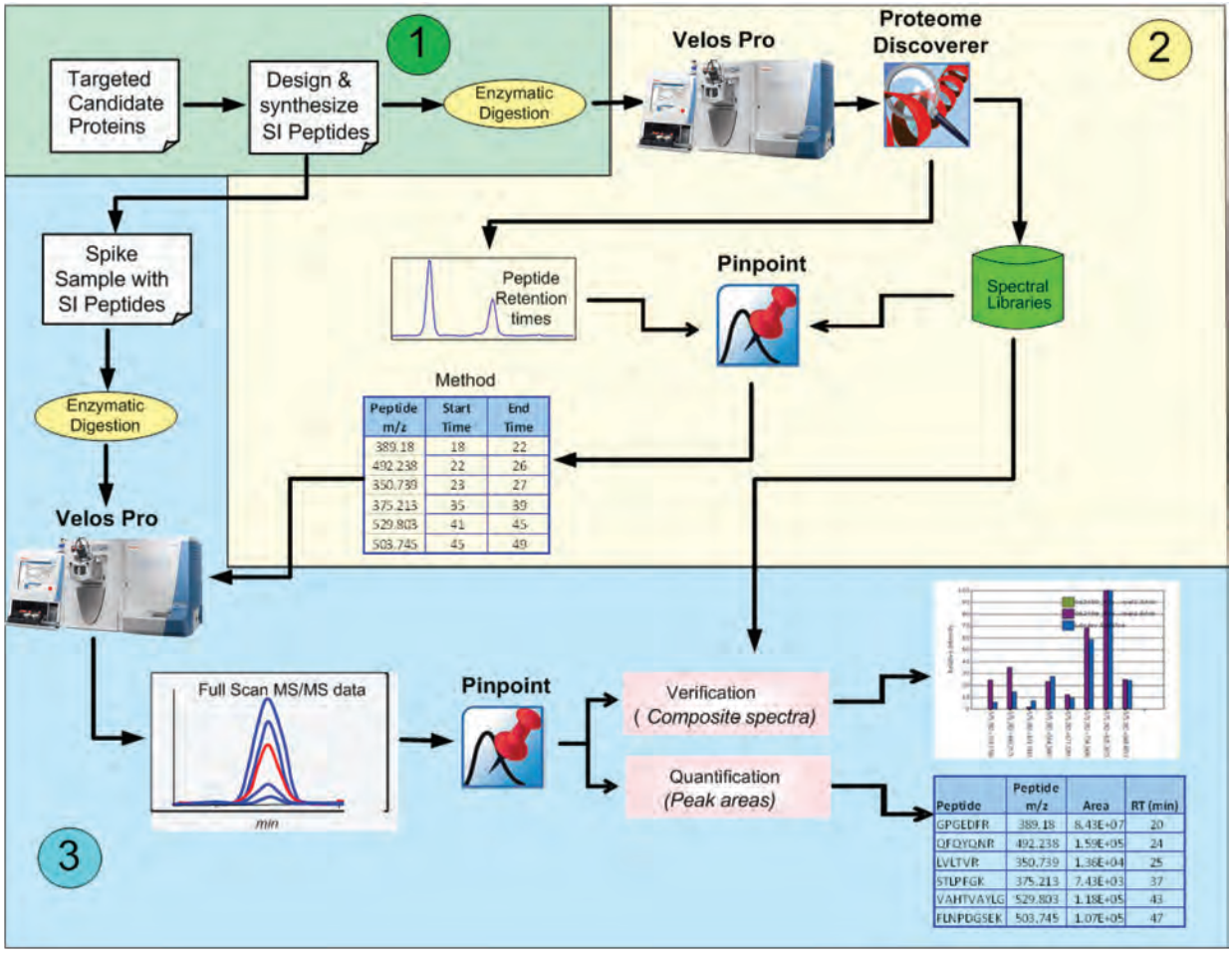

Figure 2. Optimized workflow: 1) Design, synthesis and preparation of stock solutions of SI peptides; 2) Optimization of chromatography and creation of an inclusion list for a scheduled full-scan MS/MS method; 3) Run scheduled full-scan MS/MS to create standard curves and evaluate unknown samples.

Table 1. Standard peptide results. Quality of standard curves are indicated by R^2. LOD represents the lowest injected amount that produced an extracted ion chromatographic peak with S/N > 3. LOQ was determined by: LOQ = 3.3*LOD. Average observed (Avg. Obs.) represents the average measured amount of the endogenous peptide measured in all migraine participant CSF. The red highlighted character in the synthetic peptide column indicated which amino acid residue was heavy labeled.

Protein	Peptide	Retention Time Average	Retention Time SD	LOD (amol)	R^2	LOQ (amol)	Avg. Obs (amol)	Synthetic Peptide
PTGDS	GPGEDFR	29.8	0.6	10	0.98	33	3.3E+06	GSKGPGEDFR
PTGS2	QFQYQNR	33.3	0.5	250	0.857	825	556	FNKQFQYQNR
PTGS1	LVLTVR	42.9	0.4	10	0.95	33	716	RLVLTVRSNL
HPGDS	STLPFGK	41.5	0.5	250	0.985	825	1915	KSTLPFGK
PTGES	VAHTVAYLGK	37.2	0.5	500	0.985	1650	3612	VGRVAHTVAYLGKLR
PTGIS	FLNPDGSEK	34.8	0.5	25	0.99	82.5	986	YNRFLNPDGSEKKD
TBXA1	SVADSVLFLR	60.9	0.4	500	0.969	1650	4738	EFKSVADSVLFLRDK
ALOX15	YTLEINVR	51.6	0.4	250	0.872	825	983	HLRYTLEINVR
ALOX12	LWEIIAR	57.0	0.4	50	0.99	165	1090	ALRLWEIIARY
LTCS4	YFQGYAR	38.7	0.4	50	0.982	165	1549	LRYFQGYARSA

Sample Preparation

CSF Samples

Cerebrospinal fluid samples were collected and total protein quantified as described by Harrington.[5] CSF was then deglycosylated with n-glyconase. Lumbar samples were obtained from migraine study participants, matched for well and sick states. SI peptides were added to CSF (260-270 fmol/µL each) from migraine study participants, followed by routine dithiothreitol reduction, alkylation with iodoacetamide, and overnight tryptic digestion.

Standards

To establish optimal chromatographic conditions and create a standard curve for each peptide, equal amounts of each SI peptide were spiked into CSF at various concentrations (5 amol/µL to 7.5 fmol/µL). Both the SI peptides and CSF were reductively alkylated and trypsin digested prior to mixing. To establish the overall linear dynamic range for this method, a dilution series of [^{13}C]/[^{15}N]-Phe GPGEDFR (SI-GPGEDFR) was prepared neat in 0.1% formic acid (1 fmol/µL to 2 pmol/µL).

LC method

The liquid chromatograph was operated as follows:

LC system:	Thermo Scientific Surveyor M-plus pump and MicroAS autosampler utilizing a 150:1 split
Column:	C18 Picofrit packed-tip column, 75 µm x 100 mm
Mobile phase A:	0.1% Formic acid
Mobile phase B:	0.1% Formic acid in acetonitrile
Flow rate:	400 nL/min
Gradient:	2% B to 40% B in 60 minutes
Sample loading:	Direct injection

MS Method for Establishing Retention Times and Spectral Libraries

The Velos Pro dual-pressure linear ion trap with nanospray ion source was operated as follows:

Top 5 MS^2, 1 microscan	
Repeat count:	2
Full AGC target:	3e4
MSn AGC target:	1e4
Dynamic exclusion enabled	
Scan range:	*m/z* 300-2000
Global parent and reject mass lists enabled	
Parent mass list:	380.23, 394.19, 454.28, 457.73, 497.25, 506.75, 533.32, 557.32
Start time:	0 min
End Time:	85 min

MS Method for Targeted Absolute Quantification of Proteins

The Velos Pro dual-pressure ion trap with nanospray ion source was operated as follows:

MS/MS Mass list enabled, 1 microscan
Full AGC target: 3e4
MS^n AGC target: 1e4
Global parent and reject mass lists enabled
MS mass: See Table 1
Last mass: 2X MS mass
End time and start time: Peptide retention time ± 5 min respectively

Database Search, Method Parameters and Quantification

Proteome Discoverer software version 1.2 with SEQUEST® search engine was used to identify peptides, provide a spectral database, and determine retention times. The data was searched using the following parameters:

Fixed modifications: Carboxyamidomethyl (C)	
Dynamic modifications:	13C(5)15N(1)/+6.014 Da (V,P) 13C(6)15N(1)/+7.017 Da (I,L) 13C(9)15N(1)/+10.027 Da (F)
Precursor mass tolerance: 1.0 Da	
Fragment mass tolerance: 1.0 Da	
Enzyme: Trypsin (full cleavage)	
Maximum missed cleavages: 2	
Database: Custom database containing 10 proteins	

Pinpoint software version 1.1 was used for quantification and validation of peptides. A custom FASTA database containing 10 proteins was imported to establish expected precursor and product ion *m/z* values for both heavy and endogenous peptides. Proteome Discoverer results files were imported to establish retention times and spectral libraries for validation. Precursor mass lists and retention times were then exported in .csv format for subsequent importation into the mass time window list within the Xcalibur software targeted quantification method. Following the acquisition of data, SI and endogenous peptides were verified by: 1) comparison of each MS^2 spectrum to the corresponding library spectrum and 2) co-elution of the SI/endogenous peptide pairs (Figure 3). A Costa-Soares correlation was computed for each spectral comparison and values smaller than 0.07 were considered confident identifications.[4]

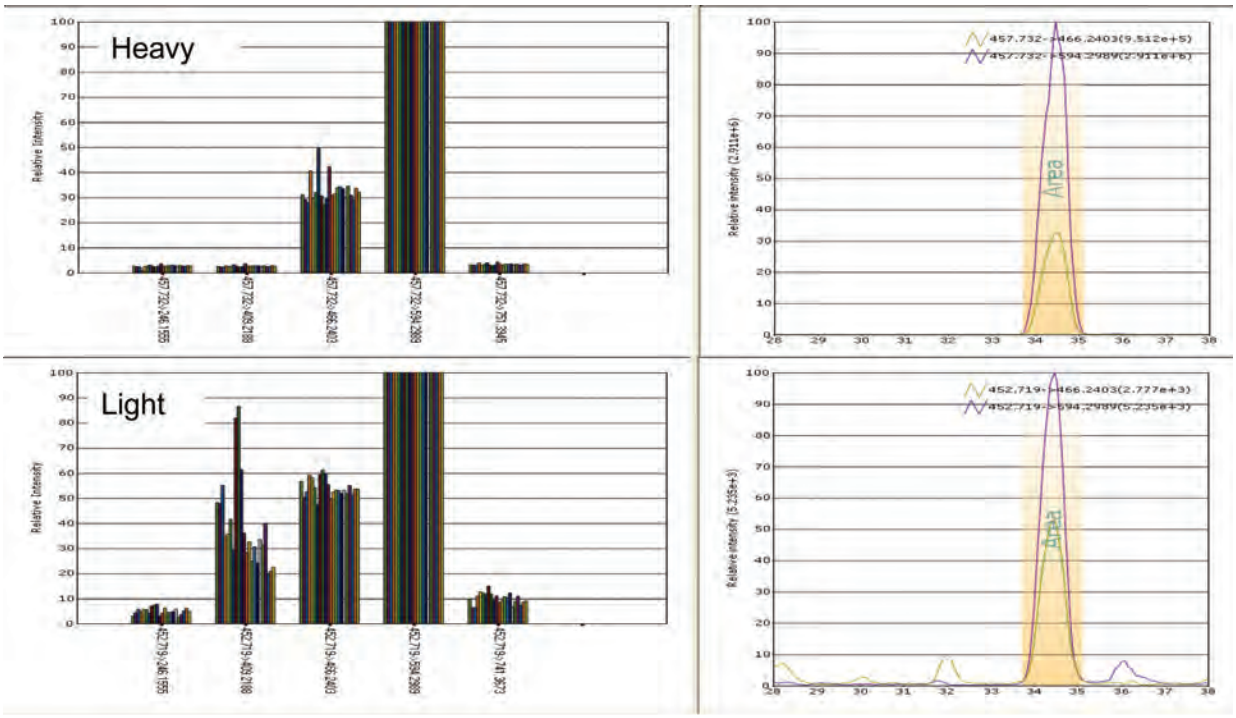

Figure 3. Pinpoint software quantification results for the LTCS4 peptide YFQGYAR in migraine study participant CSF. Composite verification spectra (left) for multiple samples and replicates and extracted ion chromatograms for a single sample (right) are given for both the spiked heavy peptide (top) and the endogenous light peptide.

Quantification of peptides was automatically performed with Pinpoint software as the sum of the extracted ion chromatogram peak areas for the two most intense fragment ions (Figure 3). Standard curves were automatically generated for each SI peptide (Figure 4). Absolute quantification of endogenous peptides was accomplished by multiplying the ratio of the summed peak areas for the endogenous peptide to its respective SI peptide by the known amount of SI peptide spiked into the sample. Quantification results were normalized to the original volume of CSF sampled and by protein concentration‡ to the average measured protein concentration, and then converted to ng/mL protein using Microsoft® Excel®. Data from three technical replicates were evaluated for each sample.

Results and Discussion

Establishing Limits of Detection and Quantification for SI-Peptides

Standard curves (Figure 4) for all ten SI peptides (n = 3 for each amount injected) were obtained and LOD and LOQ values calculated from the results (Table 1, on page 3). Injections for all peptides ranged from 10 amol to 15 fmol. The injection range for SI-GPGEDFR was extended to 8 pmol with neat peptide solutions. The median Pearson coefficient for these standard curves was 0.98 and the median %CV was 3%, affirming the high quality of the results. LOD represents the lowest injected amount of the given peptide that produced an extracted ion chromatographic peak with S/N > 3. LOQ was determined using the standard chromatographer's rule: LOQ = 3.3*LOD.[6]

Quantification of Eicosanoid Proteins in CSF Obtained from Migraine Study Participants

CSF samples obtained from migraine study participants and matched for well and sick states were examined and all ten peptides simultaneously quantified. The average measured amounts for all proteins across all six migraine study participant samples are given in Table 1, and the range measured for all six migraine study participants are depicted in Figure 4 as the green colored region of the standard curve. The %CV across all participant samples are both peptide and sample dependent. The median %CV across all samples and peptides is 15% and the %CV of nearly half the samples are below 10%. Quantification results translated to ng protein/mL CSF are depicted in Figure 5. The dashed red line in Figure 5 indicates the LOQ obtained from standard curves translated to the units and conditions for the study participant samples. Eight of the ten proteins can be confidently quantified for all study participants. PTGS2 and ALOX15 are confidently quantified for one and two study participants, respectively. Increasing the amount of sample injected by two to three fold would allow confident quantifications for these proteins, but the limited amounts of study participant samples precluded additional experimentation.

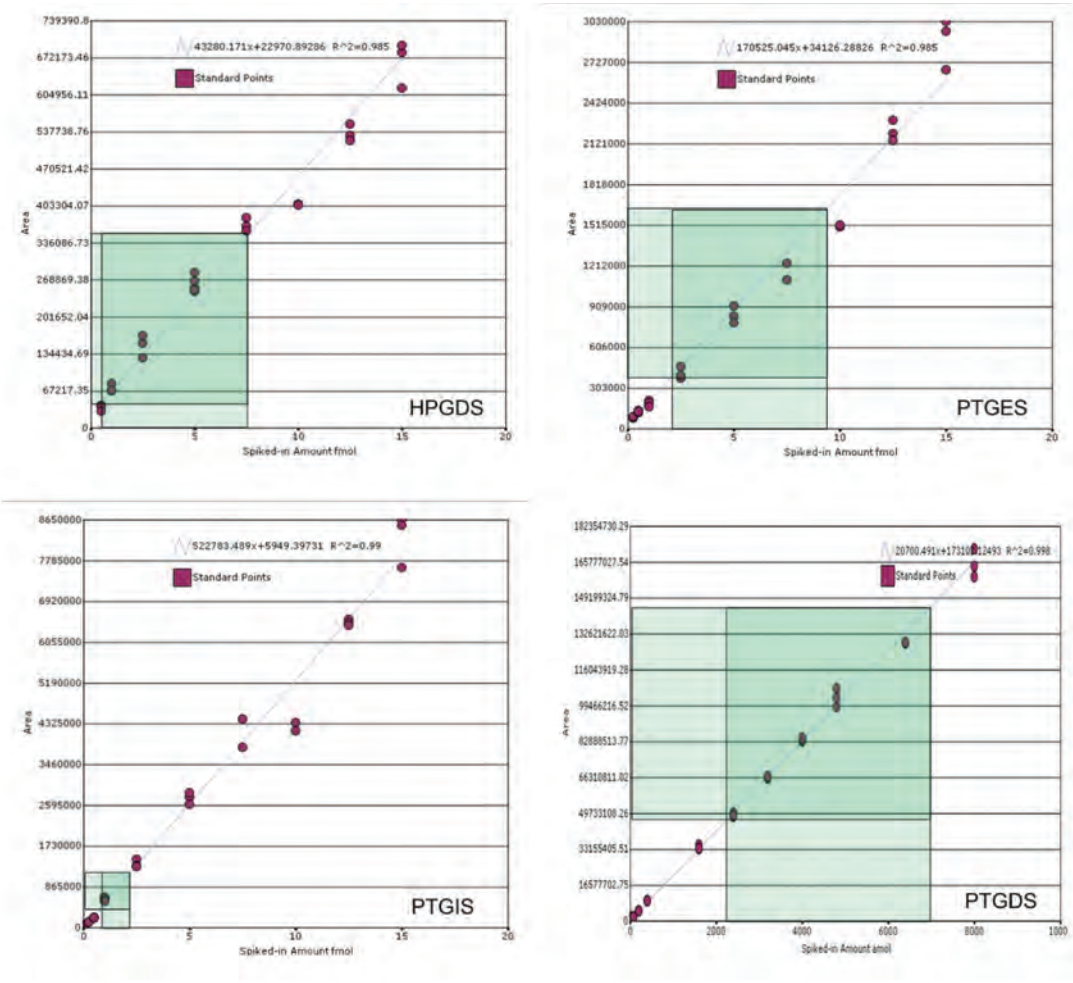

Figure 4. Example standard curves for quantification of low levels of eicosanoid enzymes utilizing stable isotope-labeled peptides spiked in human CSF as the surrogate. Data points were measured for each heavy peptide. The green shaded portion of each plot indicates the concentration range measured for endogenous peptides in CSF from migraine study participants.

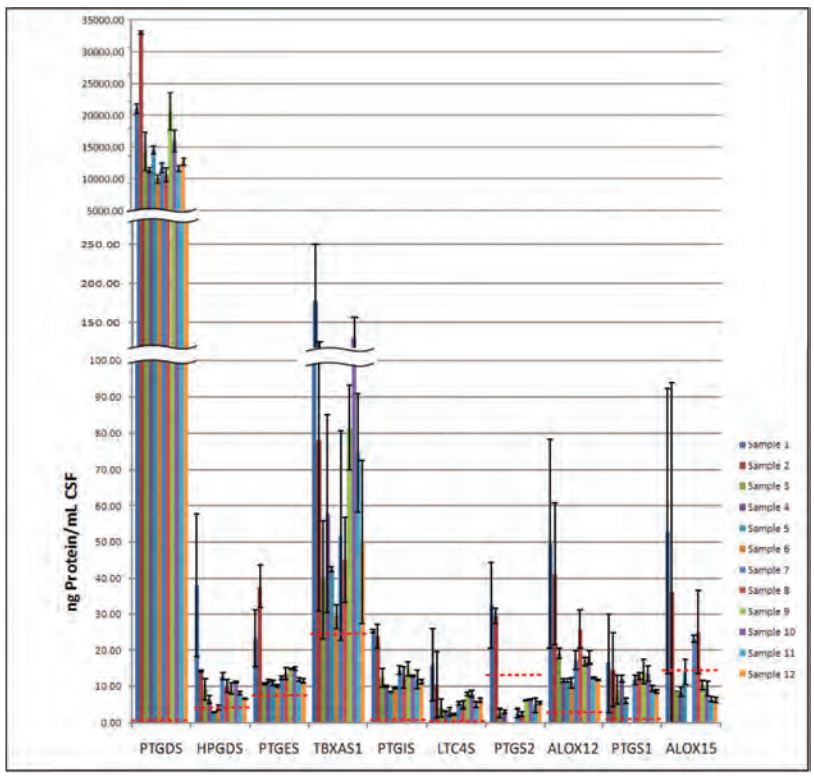

Figure 5. Pinpoint software quantification results for eicosanoid proteins in migraine study participant CSF translated to ng protein/mL CSF. Each bar represents the average of three LC/MS runs and the error bars represent ± 1 standard deviation. Red dashed lines represent the LOQ (Table 1) translated to ng protein/mL under these experimental conditions. Odd numbered samples represent study participants in the sick state and the even number following represents corresponding well state.

Detection of Disease-Relevant Proteins in the Eicosanoid Pathway at Physiological Levels

Concentrations of PTGDS measured in migraine study participant CSF ranged from 10,000 to 33,000 ng/mL, which compares well with the reported range of 16,000 to 40,000 ng/mL.[7] An equivalent comparison for the other proteins in this study is not possible at this time. To our knowledge, with the exception of PTGDS, this is the first report of concentrations for the nine other proteins in CSF. Differences in absolute protein levels across the samples are participant dependent, but there is no statistically confident difference between wellness states for the same study participant.

Conclusion

We have developed a dual-pressure linear ion trap-based workflow for the absolute protein quantification of a subset of enzymes of the eicosanoid pathway.

- Proteins were quantified in human CSF by employing stable isotope peptide standards and the identity of each peptide was verified with Proteome Discoverer software and by comparison with the internal standard using a targeted full-scan MS/MS spectrum.
- Selection of surrogate peptides and the Velos Pro mass spectrometer instrument method was easily achieved using Pinpoint software.
- Ten endogenous proteins of the eicosanoid pathway were confidently identified and eight confidently quantified at physiologically relevant concentrations in human CSF in a single LC/MS run spanning over three orders of magnitude in protein concentration.
- Using this qual/quan approach quantification accuracy was linear (median r^2= 0.98) with LOQs ranging from 33 amol to 1.6 fmol and median %CV = 3% for all proteins in CSF and allowed the observation of absolute protein levels in samples of migraineurs in sick and well states.

References

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Footnotes

† For those without local support for custom peptide synthesis we recommend the Thermo Scientific peptide synthesis service.
Website: http://www.thermohybaid.de/cgi-bin/start.app
Email: sales.biopolymers@thermofisher.com
Phone: +49 (0) 731395 79 290 (8:00-18:00 CET)

‡ Protein concentrations were measured using a BCA protein assay (Pierce)

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