Quantification of Prostaglandin D Synthase and Other Eicosinoid Enzymes in Human Cerebrospinal Fluid by Isotope Dilution Using a Linear Ion Trap Mass Spectrometer

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Overview

Purpose: Evaluate a linear ion trap mass spectrometer for quantification of prostaglandin D synthase and ten other eicosanoid pathway enzymes in human cerebrospinal fluid.

Methods: A dual-pressure linear ion trap full-scan MS/MS method was used for quantification of ten peptides in CSF with stable isotope internal standards.

Results: Ion trap sensitivity (0.5 fmol on column) and linear dynamic range (4 orders) are well suited to quantification of peptides in CSF.

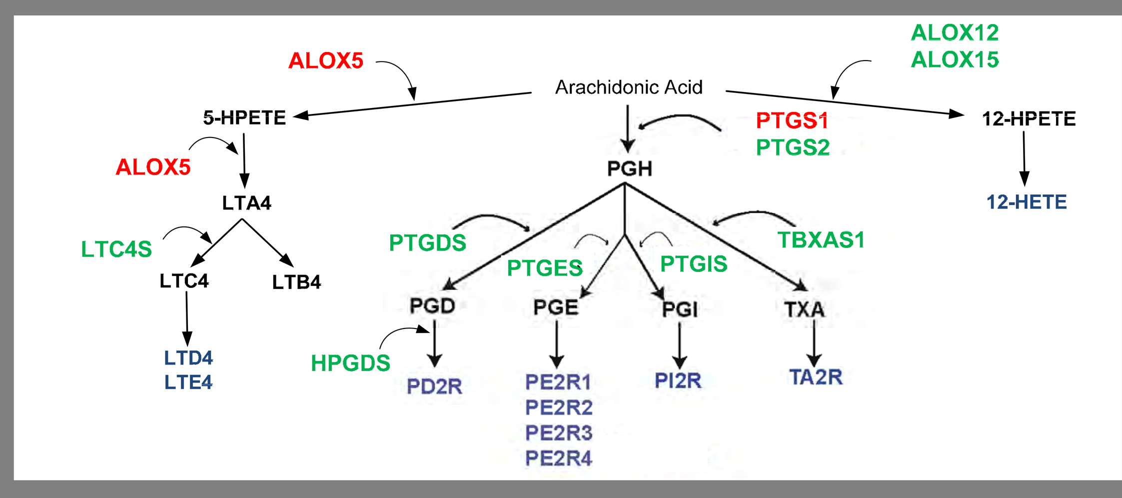
Introduction

The Prostanoid family represents a class of eicosanoids that are synthesized from arachidonic acid (FIGURE 1) and serve as signaling molecules for numerous biological processes. They exert complex control, mainly in inflammation or immunity, and as messengers in the central nervous system. Our interest centers on their involvement in migraine for which effective rescue medications inhibit the PGHS-1 & 2, but the downstream effects are not known. We are interested in identifying the changes in the eicosanoid pathway, from free arachidonic acid to the specific receptors, by identifying the pathway enzymes and quantifying their changes. This study examines the feasibility of quantifying the eicosanoid pathway enzymes using a targeted ion-trap based approach.

We began this study with the high abundance PTGDS. To quantify PTGDS and other eicosanoid pathway enzymes in CSF, peptides containing [^{12}C] or stable isotopes ([^{13}C]/[^{15}N]) of amino acids were synthesized (FIGURE1). CSF was spiked with 40 pmol of each of the stable isotopes before digestion with a proteolytic enzyme. Standard curves for quantification and determination of limit of detection (LOD) were obtained using serial dilutions of synthesized [^{13}C]/[^{15}N] peptides in the presence and absence of CSF. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) with ion trap full scan MS/MS of each precursor was used to quantify prostanoid pathway enzymes in CSF. Comparison of the chromatographic peak area for the heavy labeled peptide of known concentration to the corresponding light peptide present in the digested CSF, allows for calculation of the absolute amount of this peptide, and by extrapolation, to the corresponding protein.

Discovery experiments using Thermo Scientific Proteome Discoverer 1.1 data analysis software identify marker peptides from which precursor *m/z*'s are extracted. Using specialized software, a precursor-ion/retention-time-pair table is constructed to carry out subsequent targeted MS/MS quantification experiments on a dual-pressure linear ion trap. Peptide quantification experiments have traditionally been performed on triple quadrupole instrumentation. With the increased speed and sensitivity of today's ion trap technology, and the increased number of transitions that can be monitored per peptide, it was hypothesized that an ion-trap based method using scheduled full-scan MS/MS scan events could deliver comparable performance without the need to determine parent-product ion transitions in advance. The resulting standard curves are linear for GPGEDFR (4-4800 fmol) with on-column LODs for nine labeled peptides in CSF of 0.5 fmol.

FIGURE 1. Eicosanoid pathway. All of the enzymes in the figure have been previously identified in our shotgun sequencing of CSF. Red highlighted enzymes were present at too low of a concentration to be quantified. Green highlighted enzymes have been quantified in human CSF in this study.



Methods

Sample Preparation

The local Institutional Review Board for Human Research approved the study and all participants gave signed informed consent. Diagnosis was migraine-without-aura.[1] CSF was obtained by routine lumbar puncture collection (all between 1:00 and 6:00 pm), centrifuged (3000 g for 3 min) for cell count, and the supernatant stored at -80 °C. Total protein was determined using the Quant-iT™ protein assay kit (Invitrogen/Molecular Probes, Eugene, OR), 40 pmol of stable isotope peptides were added, followed by routine dithiothreitol reduction, alkylation with iodoacetamide, and overnight digestion with proteolytic enzyme. Membrane associated proteins were isolated as described by Harrington et al.[2] and as depicted in the inset in Figure 5.

LC/MS and Data Processing

A sample containing 20 pmol unlabeled and 40 pmol enzymatically-digested [^{13}C],[^{15}N] labeled peptides was analyzed by LC-data dependent MS^2 using a Thermo Scientific LTQ Velos dual-pressure linear ion trap mass spectrometer to obtain parent ion/retention time pairs. The [^{13}C]/[^{15}N]- labeled peptides were then serially diluted (0-100 pmol) to determine limits of quantification.

CSF samples were spiked with 40 pmol [^{13}C],[^{15}N]-labeled peptide standards, denatured, digested and subjected to targeted LC-MS/MS. Extracted ion chromatographic peak area ratios of endogenous light to spiked heavy peptides were used to determine light peptide concentration, equivalent to the endogenous protein concentration. Quantities of eicosanoid pathway proteins in CSF were determined from standard curves. All LTQ Velos™ dual-pressure linear ion trap mass spectrometer quantification data were analyzed using Thermo Scientific Pinpoint 1.1 software

TABLE 1. Synthetic peptides containing stable heavy isotopes used in this study. The bold portion of the sequence represents the reporter peptide formed after digestion. The underlined letter indicates the heavy residue.

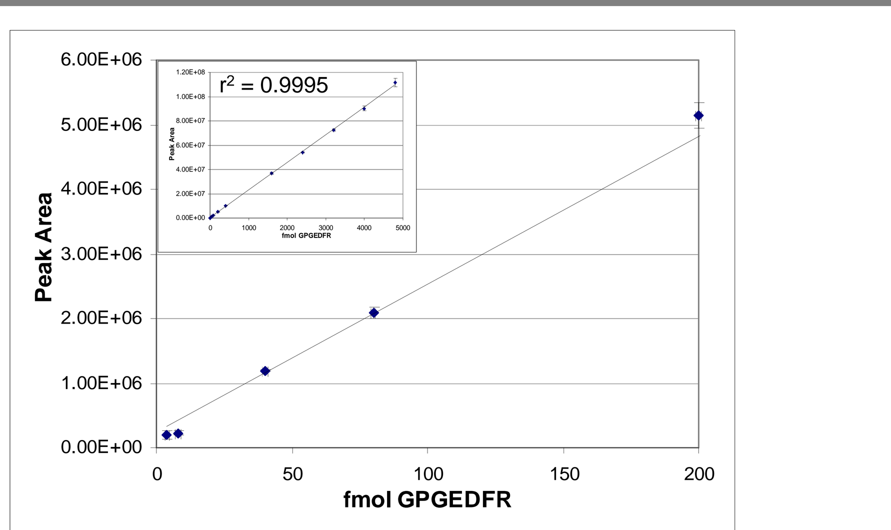
Abbrev	NAME	Peptide sequence
PTGDS	Prostaglandin-D-synthase	*GSK***GPGEDFR**
PTGS2	Prostaglandin G/H synthase 2	*FNK***QFQYQNR**
PTGS1	Prostaglandin G/H synthase 1	*R***LVLTVR***SNL*
HPGDS	Hematopoietic prostaglandin D synthase	*K***STLPFGK**
PTGES	Prostaglandin E synthase	*VGR***VAHTVAYLGK***LR*
PTGIS	Prostacyclin synthase	*YNR***FLNPDGSEK***KD*
TBXAS1	Thromboxane-A synthase	*EFK***SVADSVLFLR***DK*
ALOX15	Arachidonate 15-lipoxygenase	*HLR***YTLEINVR**
ALOX5	Arachidonate 5-lipoxygenase	*RK***ELETR***QKQ*
ALOX12	Arachidonate 12-lipoxygenase, 12S-type	*ALR***LWEIIAR***Y*
LTC4S	Leukotriene C4 synthase	*LR***YFQGYAR***SA*

Results

Quantification of [$^{13}C_9$],[^{15}N]-Phe GPGEDFR

A standard curve (Figure 2) was constructed from quantification results for a range of concentrations of [$^{13}C_9$],[^{15}N]-Phe GPGEDFR using the LTQ Velos dual-pressure linear ion trap operated in a Top 10 data-dependent full-scan MS/MS mode where all and only precursors to be analyzed populate a parent list. Average peak areas were calculated from the average of three extracted product ion chromatograms for each concentration. The dynamic range of the standard curve is three orders of magnitude (4-4800 fmol on column) with a limit of quantification (LOQ) for [$^{13}C_9$],[^{15}N]-Phe GPGEDFR of 4 fmol at maximum (on column injections of less than 4 fmol were not examined).

FIGURE 2. Limits of quantification of GPGEDFR and linearity of response using a LTQ Velos dual-pressure linear ion trap operated in Top 10 data dependent full-scan MS/MS mode with parent ions selected from a list.



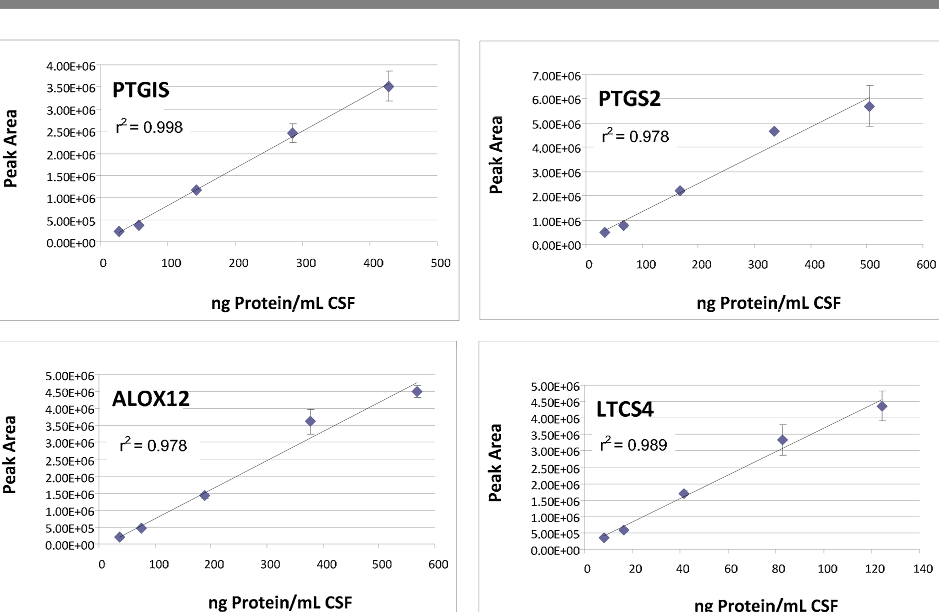
Quantification of Eight ([^{13}C],[^{15}N]) Labeled Peptides

Stable-isotope-labeled peptides were added to human cerebrospinal fluid over the concentration range spanning 500-7500 amol/µL (equivalent to approximately 8-500 ng/mL of the corresponding protein) in order to determine limits of quantification for each.

A targeted, scheduled, full-scan MS/MS method was constructed for the precursor ion/retention time pairs obtained in the discovery experiment. At any time during the chromatographic run, a minimum of four and a maximum of ten concurrent scan events took place. Nine of the eleven peptides were found to be simultaneously quantifiable using this method; the remaining two eluted too early in the chromatogram and thus exhibited non-linear responses.

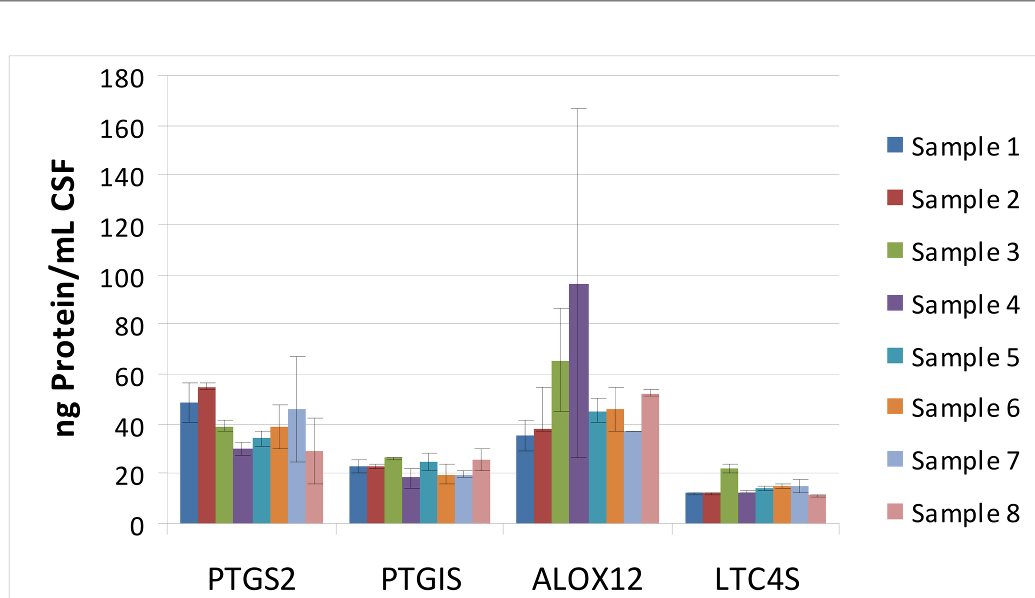
Figure 3 shows the standard curves for low-level injections of four of the eight quantifiable peptides translated to ng/mL of protein they represent.

FIGURE 3. Example standard curves for quantification of low levels of eicosanoid enzymes utilizing stable isotope labeled peptides spiked in human cerebrospinal fluid as the surrogate.



All quantifiable peptides are detected at the minimum injected amount of 500 amol using a targeted, scheduled full-scan MS/MS method. The method requires no a priori knowledge of precursor/product ion transitions, nor optimization of collision conditions.

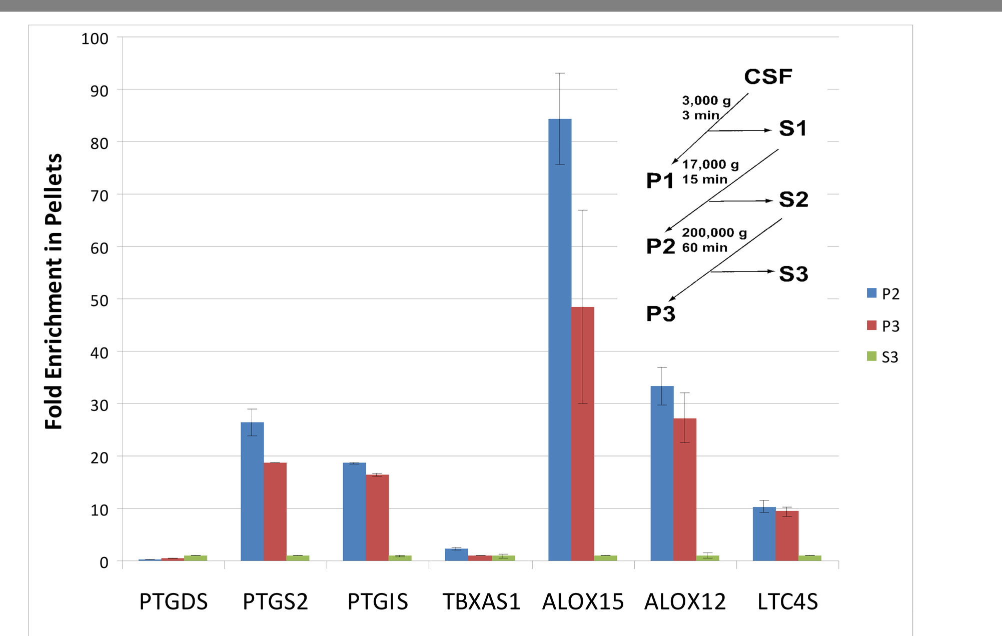
FIGURE 4. Four examples of the average amounts of eicosanoid pathway proteins quantified in human CSF. Replicates of eight different CSF samples are shown. Error bars represent ± 1 stdev.



Quantification of eicosanoid peptides in CSF

Human CSF samples spiked with 40 pmol of stable isotope labeled peptide standards, were analyzed using the targeted full-scan MS^2 ion trap method described earlier. These results indicate that endogenous peptides can be quantified to approximately 1 fmol on column (equivalent to 10-80 ng/mL of protein) with RSDs less than 20% (Figure 4). Differences in absolute protein levels across samples are attributed to biological variability.

FIGURE 5. Fold enrichment of eicosanoid pathway enzymes in pellets obtained by ultracentrifugation of CSF.[2]



Examination of eicosanoid enzyme levels in various fractions of CSF

Membrane associated eicosanoid pathway proteins were prepared as described in reference 2, and spiked with heavy peptides prior to digestion.

Following quantification, the amounts of peptides representing the indicated proteins were normalized as a fraction of total protein to indicate enrichment in the pellet preparations (Figure 5). The observed enrichments extend our previously reported results (reference 2) to the additional proteins.

Conclusions

- We have developed a targeted full-scan MS/MS linear ion trap-based quantitative assay for nine enzymes of the eicosanoid pathway (Figure 1).
- Quantification of the peptide GPGEDFR (PTGDS) on the LTQ Velos dual-pressure linear ion trap is linear over three orders of magnitude with LOD of 0.5 fmol with neat standard peptides (Figure 2).
- All heavy peptides examined, including the peptide GPGEDFR (PTGDS), can be reliably quantified using the LTQ Velos dual-pressure linear ion trap down to 0.5 fmol (equivalent to 8-40 ng/mL of corresponding protein) in the presence of CSF (Figure 3).
- Eight of the eleven targeted proteins were quantified in eight human samples, and the absolute amount of protein in CSF was determined (Figure 4). To our knowledge, with the exception of PTGDS, this is the first report of concentrations for these proteins in human CSF.
- The quantity of additional eicosanoid enzymes (LTC4S, PTGIS,TBXAS1, ALOX12, ALOX15) previously not quantified in human CSF were established for the first time in both the P2 and P3 pellets in this study.[2]

References

1. Olesen, J.; Steiner, T.J. The International classification of headache disorders, 2nd edn (ICDH-II). J Neurol Neurosurg Psychiatry. 2004;75:808-11.
2. Harrington, M.G.; Fonteh, A.N.; Oborina, E.; Liao. P.; Cowan, R.P.; McComb, G.; Chavez, J.N.; Rush, J.; Biringer, R.G.; Hühmer, A.F. The morphology and biochemistry of nanostructures provide evidence for synthesis and signaling functions in human cerebrospinal fluid. Cerebrospinal Fluid Res. 2009 Sep 7;6:10.

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