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Method for the Simultaneous Quantitation of Apolipoprotein E Isoforms using Tandem Mass Spectrometry

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Abstract

Using Apolipoprotein E (ApoE) as a model protein, we developed a protein isoform analysis method utilizing Stable Isotope Labeling Tandem Mass Spectrometry (SILT MS). ApoE isoforms are quantitated using the intensities of the *b* and *y* ions of the ¹³C-labeled tryptic isoform-specific peptides versus unlabeled tryptic isoform-specific peptides. The ApoE protein isoform analysis using SILT allows for the simultaneous detection and relative quantitation of different ApoE isoforms from the same sample. This method provides a less biased assessment of ApoE isoforms compared to antibody-dependent methods, and may lead to a better understanding of the biological differences between isoforms.

Keywords

quantitative mass spectrometry; stable isotopes; cerebrospinal fluid; Alzheimer's disease; apolipoprotein E; isoforms; protein kinetics

Currently, many protein isoform quantitation methods depend upon the availability of specific antibodies to each protein isoform. Development of antibodies includes many steps, which can take months to years to complete, and often fail to produce an antibody with the desired specificity. Due to the difficulty, time, and resources required for antibody development, an alternative and more generalized quantitation method is highly desirable. We present an antibody-independent LC-MS method which enables the quantitation of multiple protein isoforms within a single sample.

ApoE is the greatest genetic risk factor for Alzheimer's disease (AD) due to risk associated with the three common isoforms. These ApoE isoforms vary by a single amino acid change: ApoE2 (cys112, cys158), ApoE3 (cys112, arg158), and ApoE4 (arg112, arg158). ApoE2 decreases the risk for developing AD [1] while ApoE4 increases the risk of developing AD. [2] The only commercially available isoform-specific ApoE antibody is for the ApoE4 isoform. [3;4] Current assays are limited by antibody specificity and are unable to detect the protein isoforms simultaneously. A method that can measure the ApoE isoforms within the same

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sample, independent of isoform-specific antibodies, will be useful in addressing important biological questions.

In this report, we present an antibody-independent method of detecting and quantitating ApoE isoform-specific proteins by combining general lipoprotein purification, stable-isotope-labeled internal standard, and directed tandem mass spectrometry quantitation. We utilize PHM-Liposorb™ (Calbiochem, San Diego, CA), an absorbent typically used to remove lipids and lipoproteins from serum or plasma, to capture ApoE from biological fluids (Supplemental Figure 1. After tryptic digestion, ApoE isoform-specific peptides are quantitated using the Stable Isotope Labeling Tandem (SILT) MS approach. SILT MS maximizes sensitivity and selectivity by using the intensities of the MS/MS *b* and *y* ions for quantitation in lower resolution mass spectrometers.[5] The proteomic testing of SILT MS has been verified *in vitro* [6], and SILT has been applied to *in vivo* human protein kinetics studies.[7; 8]

ApoE2 and ApoE4 isoforms were obtained from the media of immortalized astrocytes derived from knock-in mice expressing human ApoE2 or ApoE4.[9] When pooled together and digested with trypsin, ApoE2 and ApoE4 media yield all four potential ApoE2, ApoE3 and ApoE4's isoform-specific peptides: LGADMEDVC₁₁₂GR, LGADMEDVR₁₁₂, LAVYQAGAR, and C₁₅₈LAVYQAGAR (Supplemental Table 1). Pooled ApoE2 and ApoE4 media were incubated with PHM-Liposorb™ (10:1 media:liposorb, 30 min 4°C). The PHM-Liposorb™ was prepared according to the manufacturer's instructions (1 g/50 mL of PBS). Adsorbed ApoE was denatured in 40% trifluoroethanol (TFE)/100mM triethylammonium bicarbonate (TEABC) (1h, 37°C). BSA(0.1 µg) was added during the TFE denaturation step to serve as an internal control as well as a means to normalize the matrix. Samples were reduced with 5 mM dithiothreitol (DTT) (30 min, RT), alkylated with 20 mM iodoacetamide (IAM) (30 min RT in the dark), and quenched with an additional 5 mM DTT (15 min, RT). Samples were diluted to 10% TFE with 100 mM TEABC, and then digested with trypsin (0.5 µg, 18h, 37°C). Before analysis, samples were desalted using Carbon Nu-tips following the manufacturer's instructions (Glygen, Columbia, MD). After centrifugal evaporation (30 min at 25°C), samples were resuspended in 15 µL 1% acetonitrile/1% formic acid. Separation of peptides was carried out using an Eksigent nanoLC flowing at 200 nL/min. A NewObjective picofrit column (75 µm) was packed with Michrom Magic C18aq (5µm, 10cm). The gradient was held at 2%B increasing to 10%B over 20 min to 15%B at 30 min, and then to 95%B at 40 min for 5 min (A= 0.1% formic acid in water, B= 0.1 formic acid in acetonitrile). Peptides were detected by a Thermo LTQ operated in positive ion mode using a spray voltage of 1.8kV, 200°C capillary temperature, and 25% collision energy for MS². The MS method monitored the doubly-charged species of each isoform-specific peptide. All four isoform-specific peptides were detected with abundant signal in a single LC-MS run (Figure 1). The matching *b* and *y* ions for each peptide were identified in their respective mass spectra. Isoform-specific peptides were also identified in 0.1 mL human CSF from heterozygous young normal control individuals (E3/2 and E3/4). The identified isoform-specific peptides were consistent with the respective genotype of each individual (Supplemental Figure 2).

Stable-isotope-labeled internal standards provide an ideal control to account for processing steps as both the labeled and unlabeled peptides respond similarly to biological and chemical processes. The ratios of labeled to unlabeled peptides can be used for absolute quantitation with exogenous labeled standards, or to measure the relative incorporation of amino acids into proteins during translation. Labeling with stable-isotope-labeled amino acids allows for calculations of protein production and clearance rates in cell culture, animal models, and human subjects.[7;10;11;12;13;14;15;16;17]

To demonstrate stable-isotope-labeling and relative quantitation of ApoE isoforms, immortalized ApoE4 and ApoE2 expressing astrocytes were grown to near confluency. The

media was changed from serum-containing to serum-replacement containing dialyzed and delipidated fetal bovine serum. After approximately 12 hours in serum-replacement media, $^{13}\text{C}_6$ -leucine was added at an equal concentration to the unlabeled leucine present in the media (maximum ^{13}C -leucine labeling = 50%, 1:1). Samples were collected at various intervals for up to 48 hours. The MS monitored the $^{13}\text{C}_6$ -labeled and unlabeled doubly-charged ions for all four isoform-specific peptides. Spectra were quantitated with the SILT method using the ratio of the sum of the *b* and *y* ion intensities of the labeled to unlabeled isoform-specific peptides.[5] (MS Excel spreadsheets customized for quantitation are available from the authors). To test the reproducibility of the method, standard error of the mean (SEM) of the tracer to tracee ratio (TTR) ($^{13}\text{C}_6$ -ApoE divided by $^{12}\text{C}_6$ -ApoE) was calculated for biological triplicates. SEM ranged from 0.5–6% for LGADMEDVcGR, 0.5–5% for LGADMEDVR, 0.1–3% for LAVYQAGR, and 0.7–8% for cLAVYQAGAR. The higher SEM values were observed at hours with higher labeling (24–48h). After 24 hours of labeling, the TTR approached steady-state (Figure 2). The fractional synthetic rates (FSR) for the ApoE2 and ApoE4 specific peptides were calculated from the slope of the initial incorporation of the ^{13}C -leucine divided by the TTR achieved at plateau.[16] The FSR for ApoE4 (averaged result for LGADMEDVR and LAVYQAGAR) and ApoE2 (averaged result for LGADMEDVcGR and cLAVYQAGAR) was found to be 8.6% per hour and 8.5% per hour, respectively (Figure 2). The similar FSR observed for ApoE4 and ApoE2 may be due to the fact that expression of both isoforms in these astrocytes was under control of the same endogenous ApoE mouse promoter; the cell lines were immortalized.[9] Though it is interesting that the FSRs were found to be similar for the E2 and E4 cells, this likely does not provide physiologic information on ApoE production and regulation *in vivo*. The cell culture system is an artificial, model system used to demonstrate an application of the SILT quantitation method. The results of the cell culture kinetics were used to determine at which point the ^{13}C -labeling reached steady-state. The 48 hour time point was chosen to collect the 0–20% ^{13}C -ApoE2 and ^{13}C -ApoE4 media used to generate Supplemental Figure 3, which demonstrates the reproducibility of the method. The residuals of the four peptides' standard curves demonstrate strong linear correlations ($R^2 > 0.99$) over the 0–20% ^{13}C -leucine labeling range (Supplemental Figure 3).

Using Apolipoprotein E as a model protein, we detail a specific protocol for simultaneous ApoE2, ApoE3, and ApoE4 isoform quantitation. This mass spectrometry based approach to detect and quantitate isoform-specific tryptic peptides is useful to study the less common ApoE isoforms (in humans, the prevalence of the apoE2 allele is 7%, apoE3 is 78%, and apoE4 is 15%).[18] Thus, ApoE isoforms can be analyzed in ApoE heterozygous subjects. The described tandem MS quantitation method can be applied to metabolism or kinetics studies for the calculation of protein isoform production and clearance rates, and can also be adapted for absolute quantitation experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. Extracted Ion Chromatogram **B. MS/MS** **Figure 1.**

The Separation and Detection of ApoE Isoform-specific Tryptic Peptides. Human ApoE2 and ApoE4 from pooled astrocyte media was captured with PHM-Liposorb, denatured, reduced, alkylated, and digested. All four isoform-specific peptides are separated and detected in a single nanoLC-MS run with a high MS^2 signal-to-noise ratio. A. Extracted Ion Chromatographs for the doubly-charged ion of each isoform-specific peptide. B. Corresponding MS^2 spectra for each peptide with the *b* and *y* ions labeled. (Lower case *c* represents alkylated cysteine residue.)

A. ApoE4 Astrocytes

B. ApoE2 Astrocytes

Figure 2.

Application of ApoE Isoform SILT Quantitation *in vitro* Using Immortalized Human ApoE2 and ApoE4 Knock-in Murine Astrocytes. ApoE2 and ApoE4 expressing astrocytes were labeled to ratio of 1:1 ^{13}C -leucine: ^{12}C -leucine. Media was collected over 48 hours. The SILT method was used to determine the incorporation of ^{13}C -leucine into ApoE (biological triplicates, error bars SEM). (Lower case c represents alkylated cysteine residue.)