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APPLICATION NOTE 46

Stable Isotope-Labeled ApoA-1 as a Global Standard for Quantitative Proteomic Studies

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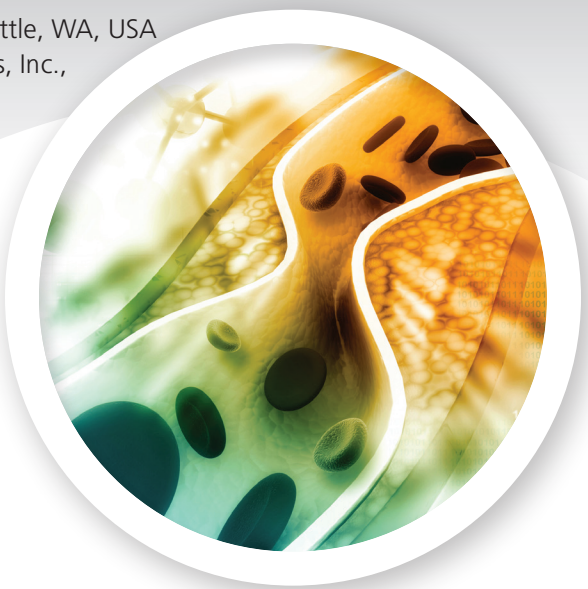

Overview

- Bottom-up LC-SRM/MS with ^{15}N ApoA-1 as internal standard.
- Protein quantification of HDL and plasma samples.
- ^{15}N ApoA-1 standard enables:
  - global protein quantification
  - accurate and precise measurement
  - enhanced peptide multiplexing
  - reduced assay cost

Cardiovascular disease (CVD) is the number one cause of morbidity and mortality worldwide.[1] However, reliable diagnostic tests of CVD risk are lacking, and in nearly 1/3 of patients the first indication of CVD is an acute, often fatal, cardiovascular event (i.e., myocardial infarction). Epidemiological studies have demonstrated an inverse association of CVD risk with plasma concentration of high-density lipoprotein (HDL), a complex comprising protein and lipids.[2] HDL is thought to mitigate atherosclerosis through a number of mechanisms (e.g., cholesterol efflux, anti-thrombosis, and anti-inflammation).[3,4] However, whether HDL proteins (>80 identified)[4,6] are associated with the cardiovascular protection remains unknown. Because HDL is in the causal pathway of atherosclerosis and CVD, and is a less complex mixture than plasma to analyze (e.g., ca. 100 vs. thousands of proteins and 4 vs. >10 order of magnitude concentration range),[7,8] HDL is an attractive target for quantifying the potentially cardioprotective proteins. The major analytical challenge stems from the phospholipids present in HDL (phospholipids represent about 30% of HDL by weight and are present at ca. 100× molar excess over an average protein),[7] which are recognized electrospray ionization (ESI) suppressants. If the HDL proteins can be quantified in a precise and accurate manner, they can potentially serve as a diagnostic tool of CVD and be used to help address the deficiencies of current clinical practices.

Protein quantification by targeted MS techniques, such as selected (or multiple) reaction monitoring (SRM or MRM) and parallel reaction monitoring (PRM), has become an indispensable approach in biological research and in clinical translational studies.[9,10] The selectivity, specificity, and multiplexing capability of these MS technologies are critical merits for protein biomarker analysis. Experimentally, quantification is based on a well-established methodology that has been used for decades in the quantification of small molecules (e.g., drugs, metabolites, and hormones).[11] The peptides produced by a proteolytic digest (typically with trypsin) are fragmented and specific fragments are monitored by MS (e.g., in triple quadrupole or hybrid quadrupole/Orbitrap instruments), with their responses serving as a surrogate quantitative measure of the intact protein concentration. To help correct for matrix and suppression effects,[9] stable isotope-labeled standard (SIS) have been employed. Although SIS peptides (also referred to as AQUA peptides)[12] have primarily been used, this type of internal standard does not account for the analytical variability associated with proteolysis. To accurately and precisely quantify proteins, as required in a clinical setting, the variability of all steps of the analytical process must be controlled. Inserting an isotopically labeled protein(s) at the beginning of an analytical workflow provides such control. Moreover, absolute quantification of the target protein can then be achieved. Here we demonstrate the utility of quantifying proteins in HDL and plasma samples using a SRM-based approach with ^{15}N-labeled human ApoA-1 serving as the internal protein standard. This standard is used to quantify not only endogenous ApoA-1, but also other target proteins in the analyzed matrices.

*These authors contributed equally to this application note.

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Methods

HDL Isolation and Proteolytic Digestion. HDL was isolated from EDTA-anticoagulated human plasma (from healthy subjects, n=33, and subjects with established CVD, n=32) as described previously.[3,13,14] Briefly, the isolation was performed by sequential ultracentrifugation (density: 1.063-1.21 g/mL using KBr) on an Optima XL ultracentrifuge (TL100.1 rotor; Beckman Instruments; Fullerton, CA, USA), with the collected HDL fractions extensively dialyzed against a buffer of 20 mM K_2HPO_4 and 100 µM DTPA (pH 7.4). Aliquots of HDL were kept frozen at -80°C and thawed only prior to analysis. An HDL sample (10 µg protein, as determined by Bradford assay) was diluted with 0.2% RapiGest (final concentration, C_f, 0.1%) in 100 mM ammonium bicarbonate to a final protein concentration of 0.1 µg/mL then spiked with 0.5 µg of ^{15}N ApoA-1 (catalog no. NLM-9539). Note: the sequence of the labeled ApoA-1 has a polyhistidine tag that is different from endogenous ApoA-1. Also note that this ^{15}N ApoA-1 is contained within ProteusQC™ (10 µL, 12.4 µM; catalog no. CNLM-9919). The protein mixture was reduced with dithiothreitol (DTT, C_f 5 mM), alkylated with iodoacetamide (IAA, C_f 15 mM), and digested at 37°C with two sequential additions of trypsin (1:20 w/w enzyme:protein ratio, for 3 and 16 h, respectively). Following digestion, the peptide mixture was quenched and RapiGest precipitated by addition of trifluoroacetic acid to a final concentration of 0.5% (pH < 2) with incubation at 37°C for 45 min. The resultant supernatant was collected, dried down, and stored at -20°C until LC-SRM/MS. Prior to analysis, the samples were reconstituted in 5% acetonitrile (ACN)/0.1% formic acid (FA).

Plasma Proteolytic Digestion. Plasma samples from three healthy human subjects and two transgenic mice (C57BL/6) expressing human ApoA-1 were spiked with 0.1 µg of ^{15}N ApoA-1 per µL of plasma. The protein mixture was denatured with 0.5% sodium deoxycholate (SDC), reduced with DTT (C_f 5 mM), alkylated with IAA (C_f 15 mM), and digested with a single trypsin addition (Worthington Biochemical; Lakewood, NJ, USA) for 2 h at 2 µg per 0.5 µl of plasma. Following digestion, the SDC was precipitated with trifluoroacetic acid (C_f 0.5%) and the supernatant frozen until LC-SRM/MS analysis. A four-point standard curve was prepared in triplicate in wild-type mouse plasma using a constant amount of ^{15}N ApoA-1 (0.1 µg/µL plasma) and a variable amount of human isolated ApoA-1 (0.1 – 10 µg/µL; Academy Biomedical; Houston, TX, USA). Sample preparation proceeded as described above.

Target Peptide Selection. A minimum of two peptides per protein were chosen for SRM analysis. Selection was stepwise based first on preliminary shotgun experiments and observed frequency in the PeptideAtlas mass spectral database, and then on clinical screening experiments where peptides with correlation exceeding r = 0.9 were desired. Peptides containing methionine residues or known glycosylation sites were selected. For standardization and quantification, peptides DYVSQFEGSALGK and VQPYLDDFQK from human ^{15}N ApoA-1 (UniProt accession number P02647) were selected as internal standards based on their chromatographic behavior and signal intensity.

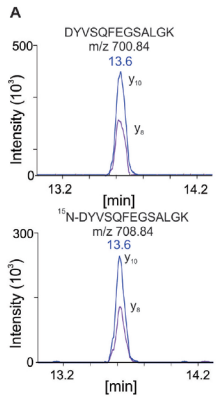
LC-SRM/MS Analysis. Sample analysis was performed by LC-SRM/MS on a nanoACQUITY UPLC system (Waters; Milford, MA, USA) connected to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific; San Jose, CA, USA), as described previously.[7,14] Briefly, the tryptic digests were first desalted on an XBridge BEH C18 capillary trapping column (40 × 0.1 mm, 5 µm, 100 Å; Waters) at a flow rate of 4 µL/min for 5 min then separated on a capillary XBridge BEH C18 analytical column (100 × 0.075 mm; 3.5 µm, 100 Å; Waters) at 0.6 µL/min over a 25 min linear gradient (1-35% eluent B). The mobile phase compositions were 0.1% FA in water for A and 0.1% FA in ACN for B. A column wash at 80% B and a 7 min re-equilibration at 1% B followed the gradient. A home-built nanoESI source was operated in the positive ion mode and the MS in the SRM mode with a 10 ms dwell/transition, unit mass resolution (0.7 Da) on both quadrupoles, and a collision gas pressure of 1.5 mTorr. The collision energy for selected transitions was optimized empirically based on signal intensity from multiple "scout" injections of a single pooled HDL sample.

Experimental Design. The study and 10 replicates of control HDL were randomized prior to digestion. Repeatability assessment was based on the 10 replicates of the control HDL measured over the course of the three-day analysis. The plasma quantification samples were randomized and measured in analytical triplicate (refers to replicate preparations of the plasma samples). Samples included calibration curves constructed using human ApoA-1 and ^{15}N ApoA-1 in mouse plasma, plasma from wild type (WT) C57Bl/6 mice as a blank, and both homozygous (ApoA-1 $tg^{+/+}$) and heterozygous (ApoA-1 $tg^{+/-}$) human ApoA-1 transgenic mice. To determine the accuracy of the assay, the three human plasma samples were independently quantified at a certified laboratory (Northwest Lipid Metabolism and Diabetes Research Laboratories; Seattle, WA, USA) using a clinically validated nephelometric assay.

Data Analysis. All SRM data were processed with Skyline[15] and the data further analyzed with R software (version 3.1). The endogenous peptide response (i.e., peak area) was normalized to the response of a ^{15}N ApoA-1 peptide (DYVSQFEGSALGK or VQPYLDDFQK). Coefficients of variation (CVs) were calculated from the replicate digests (both HDL and plasma) to determine the analytical imprecision. The standard curve was used in the human/mouse plasma sample analysis to determine the concentration of ApoA-1.

Results

Global Normalization with ^{15}N ApoA-1. A major source of analytical variability in bottom-up proteomic methodologies is the proteolytic digestion. To account for the variability in proteolytic digestion and run-to-run variability in LC-SRM/MS analyses, we evaluated the use of ^{15}N ApoA-1 as an internal standard for ApoA-1 (major HDL constituent representing ca. 70% of its total protein content)[16] as well as for other HDL-associated proteins. Using our LC-SRM/MS method, the ApoA-1 peptides provided interference-free XICs with stable chromatographic characteristics (Figure 1A). To assess the repeatability of the assay, we used 10 replicate digests interspersed among a set of clinical CVD samples analyzed over the course of three days. The raw peptide peak areas were found to be highly variable, with average CVs exceeding 30% (Figure 1B). Normalizing to a peptide derived from ^{15}N ApoA-1 (either DYVSQFEGSALGK or VQPYLDDFQK; similar results obtained),



B

Protein	Peptide	Raw Response (%CV)	^{15}N ApoA-1 Normalized Response (%CV)
ApoA-1	DYVSQFEGSALGK	33.3	3.3
	VQPYLDDFQK	32.8	1.5
ApoB-100	IEIPLPFGGK	31.0	5.3
	SVSLPSLDPASAK	32.0	4.4
ApoE	AATVGSLAGQPLQER	28.1	4.1
	SELEEQLTPVAEETR	27.5	4.9
ApoC-III	DALSSVQESQVAQQAR	30.1	4.1
	GWVTDGFSSLK	27.0	6.0
Clusterin	ASSIIDELFQDR	32.7	5.2
	ELDESLQVAER	32.7	4.1

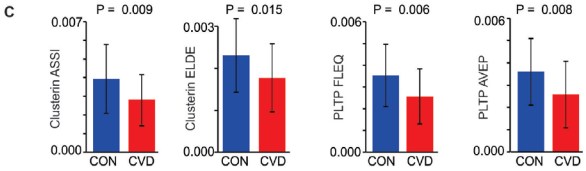


Figure 1. Use of ^{15}N ApoA-1 as a global internal standard for relative quantification of HDL proteins.
A. Representative XICs of an ApoA-1 peptides transitions in its endogenous and ^{15}N-labeled form.
B. Table showing the improvement in measurement precision with DYVSQFEGSALGK (from ^{15}N ApoA-1) used as internal standard for a panel of HDL-derived peptides.
C. Relative response comparison for CLU and PLTP measured in HDL samples (control and CVD) by bottom-up LC-SRM/MS with ^{15}N ApoA-1.

however, dramatically reduced the imprecision of the assay, not only for ApoA-1 (CV <3%), but also for other proteins (average CV 4.8%; Figure 1B). Although the precision of the global standard normalization approach is on par with the use of SIS peptides normalized against their endogenous peptide counterpart,[7] the use of a single protein as a global internal standard has a number of practical (e.g., increases multiplexing through decreased transition number) and economic (e.g., decreases assay cost) advantages. The utility of this normalization approach for HDL analysis was further demonstrated by the detection of a statistically significant reduction in the relative abundance of two HDL proteins – clusterin (CLU) and phospholipid transfer protein (PLTP) – in HDL of subjects with CVD, which suggests their potential as CVD biomarkers (Figure 1C).

ApoA-1 Quantification in Plasma. Targeted MS assays have become frequently implemented in quantitative proteomics with the accuracy of the measurement being increasingly significant in clinical and translational studies. Using ^{15}N ApoA-1 as the internal standard, ApoA-1 was quantified in human plasma via a standard curve then quantitatively compared to a clinically validated method for accuracy assessment. The standard curve was generated in mouse plasma devoid of human ApoA-1. As expected, this yielded excellent linearity over the biologically relevant concentration range anticipated for the human and samples from mice expressing human apoA-1 (Figure 2A). The quantitative results of human plasma revealed excellent precision and agreement with the ApoA-1 concentration determined by an independent reference method (Figure 2B). Furthermore, the mice transgenic for human ApoA-1 were found to have a three- to six-fold higher concentration of human ApoA-1, with a two-fold higher value obtained with the homozygous mice (Figure 2B).

Discussion

Quantitative proteomics has become an indispensable tool for MS-based, biological and translational studies. While relative quantification is widely used, absolute quantification is required for translational and clinical studies. To achieve a high degree of precision, internal standards must be implemented and ideally added early in the sample preparation process to control for all aspects of the analytical workflow. In this respect, the widely used SIS peptides have significant limitation as they do not control for one of the critical steps – proteolytic digestion. To overcome this limitation, a quantitative approach based on peptide concatamers (QconCAT),[17] has been developed. A QconCAT construct is an artificial protein comprising of concatenated tryptic peptides containing stable isotope-labeled amino acids. This approach, however, is limited by the necessity for complete digestion to generate an equimolar peptide mixture of the intact QconCAT. An attractive alternative is to use a stable isotope-labeled protein, as developed initially by Brun V and colleagues,[18,19] which is a recombinantly expressed, full-length analogue of its endogenous protein. This approach, termed PSAQ (protein standard for absolute quantification), has been used to quantify CVD biomarkers in human sera.[20] A limitation of this approach is the time required to express and characterize individual SIS proteins for multiplex, targeted proteomic analyses. In an effort to overcome this shortcoming, we have evaluated the use of ^{15}N ApoA-1 as an internal standard for quantifying not only ApoA-1, but also other proteins.[7] Here we demonstrate that this normalization approach affords excellent analytical reproducibility and quantitative accuracy. In the CVD study, the analysis revealed that the HDL in subjects with established CVD are specifically depleted of CLU and PLTP, two proteins known to reside on a single particle.[21] Moreover, PLTP is one of the key proteins involved in remodeling HDL. Its decrease in CVD may be related to the impaired remodeling of HDL and contribute to HDL dysfunction as well as CVD development.[22]

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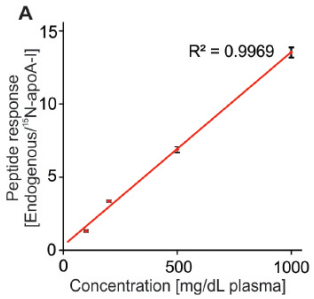
A



B

Sample	Nephelometry	LC-SRM/MS		Quantitative Accuracy
	Concentration (mg/dL)	Concentration (mg/dL)	Precision (%CV)	
Human Pls1	142	136.2	2.8	-4.1%
Human Pls2	160	156.2	4.4	-2.4%
Human Pls3	132	130.5	1.9	-1.2%
ApoA-1 tg$^{+/-}$		628.4	1.1	
ApoA-1 tg$^{+/+}$		305.5	2.9	

Figure 2. Use of ^{15}N ApoA-1 affords accurate and precise measurement of ApoA-1 in plasma.
A. Standard curve of human ApoA-1 constructed in mouse plasma using ^{15}N ApoA-1 as the internal standard.
B. Quantification of ApoA-1 in human plasma (Pls) and human apoA-1 transgenic mouse samples by LC-SRM/MS with ^{15}N ApoA-1 referenced against the values determined from a clinically validated biochemical method.

Beyond the presented studies, the SRM-based methodology and quantitative approach has been extended to the multiplexed analysis of 45 HDL proteins.[7,23] The use of ^{15}N ApoA-1 as a global normalization factor is not limited to LC-SRM/MS methodologies. We have recently demonstrated that PRM provides comparable quantitative results to SRM in the analysis of 26 HDL proteins from 44 human subjects.[23] For improved multiplexing, the application to datA-1ndependent acquisition (DIA) mass spectrometry is in progress.

Acknowledgements

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Additional Product of Interest

Catalog No.	Description
CNLM-9919	ProteusQC™ – Labeled ApoA-1 + 6 Labeled ApoA-1 Tryptic Peptides

For more information, please visit isotope.com → Applications → Proteomics → QC and Quantitation Kits → ProteusQC.

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