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Multiple Reaction Monitoring-Mass Spectrometric Assays Can Accurately Measure Many Protein Concentrations in Complex Mixtures

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Abstract

Background—Mass spectrometric assays have the potential to replace protein immunoassays in basic science, clinical research, and clinical care. Previous studies have demonstrated the utility of assays using multiple-reaction monitoring mass spectrometry (MRM-MS) for the quantification of proteins in biological samples and many examples of the accuracy of these approaches to quantify *spiked* analytes have been reported. However, a direct comparison of multiplexed assays using liquid chromatography-tandem mass spectrometry with established immunoassays to measure *endogenous* proteins has not been reported.

Methods—We purified the HDL from the plasma of 30 human subjects enrolled in a clinical nutrition research study and used label-free shotgun proteomics approaches to analyze each sample. We then developed two different 6-plex assays that used isotope dilution MRM-MS: one assay used stable isotope labeled peptides and the other used stable isotope labeled apolipoprotein A-I (apoA-I), the most abundant protein in HDL, as internal standards to control for matrix effects and mass spectrometer performance. The shotgun and MRM-MS assays were then compared with commercially available immunoassays for each of the six analytes.

Results—Quantification by shotgun proteomics approaches correlated poorly with the six protein immunoassays. However, the MRM-MS approaches that used internal standard peptide or a single internal standard protein correlated well. In addition, MRM-MS approaches had good repeatability (<10% CV) and linearity.

Conclusions—Multiplexed MRM-MS assays correlate well with immunochemical measurements and have acceptable operating characteristics in complex samples. Our results support the proposal that MRM-MS could be used to replace immunoassays in a variety of settings.

Keywords

Mass spectrometry; multiple reaction monitoring; endogenous; proteins; high density lipoprotein; targeted proteomics

Multiplexed accurate quantification of proteins is becoming increasingly important in both basic biology and in clinical biomarker studies. Multiplexing immunoassays can be

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problematic (1) and migration of clinical protein assays from immunoassay platforms to mass spectrometric platforms could result in great benefits to patients and laboratories.(2) It is currently unknown how well multiplexed quantification of endogenous proteins by mass spectrometry agrees with traditional immunoassays, the current standard of care, but simultaneous measurement of two apolipoproteins in plasma using multiple reaction monitoring-mass spectrometry (**MRM-MS**) has shown great promise.(3)

Until recently, proteomic studies have focused primarily on the global identification of as many proteins as possible. This paradigm has shifted and the precise, simultaneous quantification of relative protein abundance of many proteins has become an important goal for many applications. Targeted proteomics experiments based on MRM-MS, a method that has been used clinically for many years for small molecule quantification, may provide precise relative quantification of known proteins in complex mixtures. In targeted proteomics peptides produced by protease digestion—typically with trypsin—serve as surrogate markers of protein abundance. MRM-MS assays offer many advantages over traditional immunoassays used for protein quantification in biological samples; in particular MRM-based methods can be rapidly developed and validated. Furthermore, such assays are readily multiplexed for quantification of many proteins (>50) in a single analysis over wide range of relative concentrations without cross-reacting interferences often found in multiplexed immunoassays.(4) Combined with isotope dilution using stable isotope-labeled peptides as internal standards (**IS_{pep}**), MRM has been established as the most promising approach to precise relative protein quantification.(5-8)

A recent multi-laboratory study has demonstrated good reproducibility of MRM-based assays across several laboratories.(9) However, this study also underscored the importance of the reproducibility of the enzymatic digestion of proteins, a critical step in MRM-based assays. More specifically, the CV of measurements that included the digestion step were more than 2-fold higher than the CV of measurements using pre-digested samples.(10) Importantly, digestion reproducibility cannot be corrected for by using IS_{pep}. For quantification of a specific endogenous protein, this limitation can be largely overcome when a stable-isotope labeled protein analog (**IS_{prot}**) is included as the internal standard.(11 up to 25-fold differences between the two approaches.(12) We therefore aimed to compare MRM-MS with more clinically relevant immunoassays.

As an example of a complex mixture of proteins, we chose HDL because it is an important plasma protein-lipid complex directly involved in cardiovascular disease. Elevated plasma HDL cholesterol is one of the only known negative risk factors for cardiovascular disease. HDL may mitigate atherosclerosis by multiple mechanisms including cholesterol efflux and antioxidant and anti-inflammatory properties, and its in vitro activity correlates with atherosclerosis.(13) Although a much simpler proteome compared to plasma, over 80 proteins have been identified in HDL with concentrations spanning four orders of magnitude,(14) HDL poses a major analytical challenge due to the very high content of lipids (50% by weight), especially phospholipids (30%), which are well-recognized ionization suppressants during atmospheric pressure ionization.(15) Importantly, we have recently shown that HDL from subjects with cardiovascular disease carries a unique ensemble of proteins which is modulated by lipid-lowering therapy.(16, 17) Precise quantification of the concentration of proteins in HDL could potentially serve as a clinical diagnostic tool and as a test of therapeutic efficacy.

We isolated HDL from the plasma of 30 normal subjects that were collected during an IRB-approved clinical research study(18) and performed shotgun proteomics on a high resolution mass spectrometer [see Supplemental Material for an overview (Supplemental Fig. 1A) and complete description of methods] to identify and quantify proteins associated with HDL.

Based on these results, we selected six proteins for which commercial immunoassays were readily available and whose range of relative concentrations across the study population (as assessed by shotgun proteomics and spectral counting) was at least 50% of the mean concentration (Supplemental Fig. 1B). The selected set of proteins also had wide range of molecular weights (11-550 kDa; Supplemental Fig. 1C). For each protein, two peptides were then selected for the targeted analysis based on the mean spectral count of each peptide across the study population and the number of observations in the PeptideAtlas database.(19) Two peptide precursor ion m/z-to-fragment ion m/z pairs (known as transitions) were then chosen for each peptide based on fragment intensity in the tandem mass spectra from the shotgun experiment (Supplemental Table 1). Stable-isotopically labeled peptides with a C-terminal labeled arginine or lysine were synthesized for each of the six proteins (IS_{pep}). In parallel we also generated recombinant ^{15}N-labeled apoA-I (IS_{prot}).

Much like albumin in serum, the major constitutive protein in HDL, apolipoprotein A-I (**apoA-I**), represents ~70% of the total protein content. We first evaluated the accuracy of different mass spectrometric approaches for quantification of apoA-I using as the comparative method a clinically-used nephelometric immunoassay that is calibrated against the WHO standard reference material (Siemens BN-II). We spiked two separate HDL digests with IS_{pep} after trypsin digestion and/or IS_{prot} before trypsin digestion and performed a targeted MRM analysis with IS_{prot} (*MRM-protein*) and IS_{pep} (*MRM-peptide*), respectively. In parallel we estimated the abundance of apoA-I from the shotgun analysis using spectral counting and extracted ion chromatogram peak areas (**XIC**) for two peptides.(20) To quantify relative concentrations of apoA-I across the study population we used following measures: *MRM peak area* (LC-MRM/MS assay with no internal standard), *MRM-peptide ratio* (LC-MRM/MS assay with IS_{pep}), *MRM-protein ratio* (LC-MRM/MS assay with IS_{prot}), *normalized MRM-protein ratio* (average of the MRM-protein ratio for two peptides in each protein), *spectral counting*, and *XIC peak areas*.

As expected, the *MRM peak areas* of peptides from the same protein correlated well with one another, as did *XIC peak areas* (Supplemental Fig. 2). Our results clearly demonstrate that normalization to IS_{pep} internal standard peptides, *MRM-peptide ratio*, (Supplemental Fig. 2E-H) improves the accuracy of the targeted MRM method over the peak area alone (Supplemental Fig. 2A-D), which is likely due to correction of variable matrix effects across the population, injection volume variability, and deterioration of mass spectrometer performance over the time of analysis. The use of IS_{prot} further improved the accuracy of the MRM-based method (Supplemental Fig. 2I-L), providing excellent correlation with the clinical immunoassay ($r = 0.96$, Fig. 1). Strikingly, *spectral counting* and *XIC*, which are commonly used as quantitative measures in shotgun proteomics experiments in basic science research, correlated poorly with the immunoassay (Supplemental Fig. 2M-P), demonstrating that these two label-free shotgun proteomics approaches yield only a rough semi-quantitative measure of relative protein abundance.(17) These initial results confirmed that the *normalized MRM-protein ratio* approach was the most accurate for the quantification of a single protein by MRM-based methods.(11)

To further test the analytical characteristics of the *normalized MRM-protein ratio* approach, we evaluated its repeatability and linearity as outlined in Supplemental Figure 1A. To test the repeatability of the LC-MRM/MS step alone we analyzed a single digestion of the same HDL 12 times over the course of 12 days interspersed between injections of other HDL samples. We also tested the overall repeatability of the assay by analyzing 5 replicate digestions of a single pooled HDL sample twice (day 1 and day 12). The *normalized MRM-protein ratio* approach demonstrated excellent repeatability (<2%CV) in both experiments (Table 1). To assess the linearity of the IS_{prot} *(normalized MRM-protein ratio)* and IS_{pep} *(normalized MRM-peptide ratio)* methods, we analyzed single digestions of a 9-point serial

dilution of human HDL into mouse HDL. Both approaches demonstrated acceptable linearity over more than two-orders of magnitude with IS_{prot} ($r^2 = 0.9996$) and IS_{pep} ($r^2 = 0.9936$) (Supplemental Fig. 3).

We then evaluated the performance of mass spectrometric methods for the measurement of five other HDL proteins. For each protein we synthesized stable isotope-labeled analogs of each peptide selected for the LC-MRM/MS assay (*i.e.,* IS_{pep}) (Supplemental Table 3) or used labeled apoA-I as internal standards (*i.e.,* IS_{prot}). Similar to apoA-I, the IS_{pep} and IS_{prot} approaches demonstrated good repeatability (<10%CV) and linearity for the other 5 proteins (Table 1, Supplemental Fig. 4). For each protein we also calculated the correlation of the relative concentration measured by each method with the concentration determined by an immunoassay. Remarkably, both approaches yielded very similar results for all 5 proteins (Table 1), with IS_{prot} providing better overall correlations than IS_{pep}. As we observed for apoA-I, the label-free shotgun proteomics quantitative measures (*spectral counting* and *XIC*) correlated poorly with the immunoassay data (Supplemental Figs. 5-9).

The overall aim of the present study was to determine whether multiplexed protein quantification using mass spectrometry can provide accurate, linear, and reproducible measurements of endogenous protein concentrations in complex human specimens. We found that the isotope dilution MRM-MS methods using either internal standard peptides or a single internal standard protein have operating characteristics and accuracy comparable to biochemical approaches. Our data strongly support the proposal that MRM-MS methods may provide accurate and reproducible quantitative data for basic and clinical studies and have great potential to be readily translated into clinical practice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MRM-MS	multiple-reaction monitoring mass spectrometry
IS_{pep}	stable isotope labeled peptide internal standard
IS	stable isotope labeled protein internal standard
apoA-I	apolipoprotein A-I
XIC	extracted ion chromatogram

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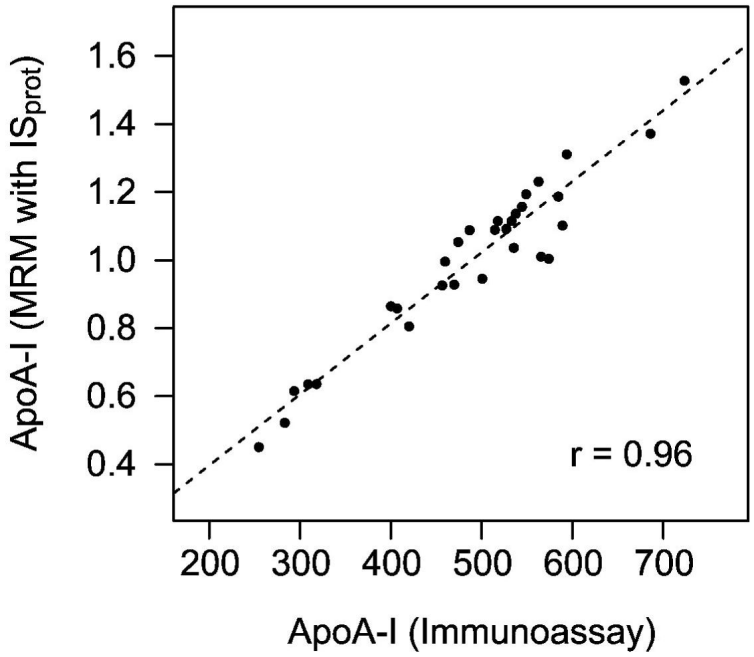

Figure 1. Internal standard protein in MRM-MS assay for apoA-I in HDL

The relative concentration of apoA-I in HDL measured in 30 samples by MRM-MS is compared with the concentration measured using a nephelometric immunoassay. The line of the equation of the Deming regression is denoted with a dashed line. The Pearson correlation coefficient (r) is shown.

Table 1
Performance characteristics of a multiplexed MRM-MS assay

Isotope labeled apoA-I was used as the internal standard for all six proteins in the assay and correlation with immunoassay was determined along with linearity and imprecision.

			Imprecision		
	Correlation with Immunoassay (N=30)	Linearity	LC-MS (%CV)	Digestion (%CV)	Overall (%CV)
A-I	0.95	0.9996	1.15%	0.25%	1.18%
B	0.61	0.9997	4.59%	4.43%	6.38%
C-II	0.92	0.9948	8.38%	7.99%	11.58%
C-III	0.88	0.9976	8.39%	4.41%	9.48%
E	0.92	0.9980	6.03%	4.93%	7.79%
J	0.79	0.9996	9.51%	7.43%	12.07%