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Brief Communication

Multiple-Reaction Monitoring–Mass Spectrometric Assays Can Accurately Measure the Relative Protein Abundance in Complex Mixtures

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BACKGROUND: Mass spectrometric assays could potentially replace protein immunoassays in many applications. Previous studies have demonstrated the utility of liquid chromatography–multiple-reaction monitoring–mass spectrometry (LC-MRM/MS) for the quantification of proteins in biological samples, and many examples of the accuracy of these approaches to quantify supplemented analytes have been reported. However, a direct comparison of multiplexed assays that use LC-MRM/MS with established immunoassays to measure endogenous proteins has not been reported.

METHODS: We purified HDL from the plasma of 30 human donors and used label-free shotgun proteomics approaches to analyze each sample. We then developed 2 different isotope-dilution LC-MRM/MS 6-plex assays (for apoliporoteins A-I, C-II, C-III, E, B, and J): 1 assay used stable isotope-labeled peptides and the other used stable isotope-labeled apolipoprotein A-I (an abundant HDL protein) as an internal standard to control for matrix effects and mass spectrometer performance. The shotgun and LC-MRM/MS assays were then compared with commercially available immunoassays for each of the 6 analytes.

RESULTS: Relative quantification by shotgun proteomics approaches correlated poorly with the 6 protein immunoassays. In contrast, the isotope dilution LC-MRM/MS approaches showed correlations with immunoassays of $r = 0.61–0.96$. The LC-MRM/MS approaches had acceptable reproducibility (<13% CV) and linearity ($r \geq 0.99$). Strikingly, a single protein internal standard applied to all proteins performed as well as multiple protein-specific peptide internal standards.

CONCLUSIONS: Because peak area ratios measured in multiplexed LC-MRM/MS assays correlate well with immunochemical measurements and have acceptable operating characteristics, we propose that LC-MRM/MS could be used to replace immunoassays in a variety of settings.

Multiplexed precise quantification of proteins is becoming increasingly important in both basic biology and in clinical biomarker studies. Multiplexing of immunoassays can be 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 2 apolipoproteins in plasma by use of multiple-reaction monitoring–mass spectrometry (LC-MRM/MS) has shown 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 the relative protein abundance of many proteins has become an important goal for many applications. Targeted proteomics experiments based on LC-MRM/MS, a method 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. LC-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 in a single analysis over a 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 multilaboratory 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 CVs of measurements that included the digestion step were more than 2-fold higher than the CVs of measurements performed with predigested samples *(10)*. Importantly, digestion reproducibility cannot be corrected for by using IS_{pep}. For relative quantification of a specific en-

dogenous protein, this limitation can be largely overcome when a stable-isotope–labeled protein analog (IS_{prot}) is included as the internal standard *(11)*. A recent study in yeast cell lysates showed limited agreement between LC-MRM/MS and quantification by immunoblotting with up to 25-fold differences between the 2 approaches *(12)*. We therefore aimed to compare LC-MRM/MS with quantification by more accurate 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 that may mitigate atherosclerosis by multiple mechanisms *(13)*. Although HDL is a much simpler proteome compared to plasma, more than 80 proteins have been identified in HDL with concentrations spanning 4 orders of magnitude *(14)*, and HDL poses a major analytical challenge owing to the very high content of lipids (50% by weight), especially phospholipids (30%), well-recognized ionization suppressants *(15)*. We have recently shown that HDL from patients with cardiovascular disease carries a unique ensemble of proteins that 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.

Using sequential ultracentrifugation we isolated HDL (density fraction = 1.063–1.21 g/mL) from the plasma of 30 healthy individuals who participated in an institutional research board–approved clinical research study *(18)*, and we performed shotgun proteomics analysis on a high-resolution mass spectrometer to identify and quantify proteins associated with HDL (for an overview and description of methods see the Supplemental Material file and Supplemental Fig. 1, A and B, in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol58/issue4). On the basis of these results, we selected 6 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 (see online Supplemental Fig. 1C). The selected set of proteins also had a wide range of molecular weights (11–550 kDa) and their concentrations spanned more than 2 orders of magnitude (see online Supplemental Fig. 1, D and E). For each protein, 2 peptides were then selected for the targeted analysis on the basis of the mean spectral count of each peptide across the study population and the number of observations in the PeptideAtlas database *(19)*. Selected peptides were then verified by using proteotypic peptide scoring algorithms as described in the online Supplemental Material file. Peptides containing methionine residues or known glycosylation sites were excluded. Two peptide precursor ion *m/z*-to–fragment ion *m/z* pairs (known as transitions) were then chosen for each peptide on the basis of fragment intensity in the tandem mass spectra from the shotgun experiment (see online Supplemental Table 1). Stable-isotope–labeled peptides with a C-terminal–labeled arginine or lysine were synthesized for each of the 6 proteins (IS_{pep}). In parallel we also generated recombinant ^{15}N-labeled apolipoprotein A-I (apoA-I) (IS_{prot}).

Much like albumin in serum, the major constitutive protein in HDL, apoA-I, represents approximately 70% of the total protein content. We first evaluated the accuracy of different mass spectrometric approaches for relative 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 supplemented HDL digests with IS_{prot} before trypsin digestion and IS_{pep} after 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 (XIC) peak areas for 2 peptides *(20)*. To quantify relative concentrations of apoA-I across the study population we used the following measures: MRM peak area [liquid chromatography–MRM/mass spectrometry (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 (mean of the MRM–peptide ratio for 2 peptides in each protein), normalized MRM–protein ratio, spectral counting, and XIC peak areas.

As expected, the MRM peak areas of peptides from the same protein correlated well with one another ($r = 0.90$), as did XIC peak areas ($r = 0.94$; also see online Supplemental Fig. 2, A and Q). Our results demonstrated that normalization to IS_{pep} internal standard peptides, the MRM–peptide ratio (see online Supplemental Fig. 2, E–H), improved the relative accuracy of the targeted MRM method over the peak area alone ($r = 0.79$ vs 0.30; also see online Supplemental Fig. 2, A–D), 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 relative accuracy of the MRM-based method (see online Supplemental Fig. 2, I–L), and these peak area ratios provided good correlation with the clinical immunoassay ($r = 0.96$, Fig. 1). In contrast, spectral counting and XIC, which are commonly used as quantitative measures in shotgun proteomics exper-

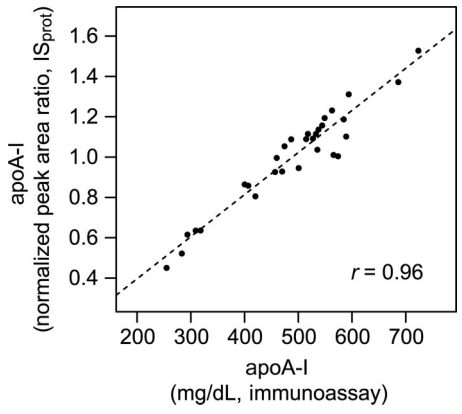

Fig. 1. IS_{prot} in LC-MRM/MS assay for apoA-I in HDL. The relative concentration of apoA-I in HDL, measured as the normalized peak area ratio by using IS_{prot}, was determined in 30 samples by LC-MRM/MS and was compared with the concentration measured by using a nephelometric immunoassay. The line of the equation of the Deming regression is denoted with a dashed line. The Pearson correlation coefficient (*r*) is shown.

iments in basic science research, correlated poorly with the immunoassay (r = 0.56 and 0.61; also see online Supplemental Fig. 2, P and T), demonstrating that these 2 label-free shotgun proteomics approaches yield only a rough semiquantitative measure of relative protein abundance *(17)*. These initial results confirmed that the normalized MRM–protein ratio approach is the most accurate for the relative 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 online Supplemental Fig. 1A. To test the repeatability of the LC-MRM/MS step alone we analyzed digested pooled HDL 59 times over the course of 9 days across 2 experiments. We also tested the repeatability of the digestion by analyzing 5 replicate digestions of a single pooled HDL sample 4 times (injected twice on each of days 1 and 9). The normalized MRM–protein ratio approach demonstrated excellent repeatability (<10%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 across the concentration range studied, we analyzed single digestions of a 4-point dilution series of human HDL into mouse HDL. Both approaches demonstrated acceptable linearity with IS_{prot} (r^2 = 0.9994) and IS_{pep} (r^2 = 0.9982) (see online Supplemental Fig. 3).

We then evaluated the performance of mass spectrometric methods for the measurement of 5 other HDL proteins. For each protein we used stable isotope-labeled analogs of each peptide selected for the LC-MRM/MS assay (i.e., IS_{pep}) (see online Supplemental Table 3) or used labeled apoA-I as a global internal standard for all measured proteins (i.e., IS_{prot}). Similar

Table 1. Performance characteristics of a multiplexed LC-MRM/MS assay.

	IS_{pep}[a]					IS_{prot}[b]				
		Imprecision, % CV					Imprecision, % CV			
	r[c]	Linearity[d]	LC-MS[e]	Digest[f]	Total[g]	r	Linearity	LC-MS	Digest	Total
A-I	0.79	0.9982	2.6	9.4	9.8	0.96	0.9994	5.6	2.3	6.1
B	0.67	0.9983	6.4	2.6	6.9	0.61	0.9975	8.9	3.5	9.6
C-II	0.90	0.9948	4.1	4.6	6.1	0.92	0.9937	6.1	4.1	7.4
C-III	0.89	0.9962	9.5	5.0	10.7	0.88	0.9992	11.8	4.8	12.8
E	0.96	0.9984	2.2	3.3	4.0	0.92	0.9984	2.2	2.1	3.1
J	0.81	0.9994	9.3	6.8	11.5	0.79	0.9991	12.4	3.3	12.8

[a] Isotope-labeled peptides were spiked after digestion and used as internal standards in a multiplexed LC-MRM/MS assay to quantify the relative concentration of 6 proteins in HDL (normalized MRM–peptide ratio).
[b] ^{15}N isotope-labeled apoA-I was spiked prior to digestion and used as an internal standard in the LC-MRM/MS assay for the same 6 proteins (normalized MRM–protein ratio).
[c] Pearson correlation coefficient (*r*) of the relative protein concentration determined by LC-MRM/MS compared with immunoassay (n = 30).
[d] Linearity determined across a 4-point dilution series of human HDL into mouse HDL.
[e] Imprecision of the LC-MS step was determined by injecting digested pooled HDL 35 times over 3 days and 4 days later 24 times over 2 days. The mean imprecision of the 2 experiments is presented.
[f] Five separate digestions of HDL were injected 4 times (twice on each of days 1 and 9). The mean of the 4 injections was used as the relative concentration. The imprecision of the 5 digestions is presented.
[g] Total imprecision was estimated from the variability of the digestion and LC-MS steps (see online Supplemental Material).

to apoA-I, the IS_{pep} and IS_{prot} approaches demonstrated good reproducibility (<13% CV) and linearity (r^2 >0.99) for the other 5 proteins (Table 1; also see online Supplemental Fig. 4). For each protein we also calculated the correlation of the peak area ratios measured by each method with the concentration determined by an immunoassay. Both approaches yielded very similar results for all 5 proteins (r = 0.67–0.96 vs 0.61–0.96, Table 1). As we observed for apoA-I, the label-free shotgun proteomics quantitative measures (spectral counting and XIC) correlated less strongly overall with the immunoassay data (r = 0.38–0.71 and 0.58–0.81, respectively; see online Supplemental Figs. 5–9). The performance of spectral counting and the targeted approaches for apoB were very similar, possibly owing to the high molecular weight of this protein relative to the other proteins.

The overall aim of the present study was to determine whether multiplexed protein quantification using mass spectrometry can provide relatively accurate, linear, and reproducible measurements of endogenous protein concentrations in complex human specimens. We found that the isotope dilution LC-MRM/MS methods using either internal standard peptides or a single internal standard protein have operating characteristics and accuracy comparable to biochemical approaches. Importantly, the IS_{prot} approach is capable of correcting for digestion variability but the IS_{pep} approach is not. There are of course drawbacks to LC-MRM/MS assays compared with immunoassays, most particularly the high skill level of the operator, the expense of the instrumentation, and the lower throughput. However, our data support the proposal that peak area ratios measured in LC-MRM/MS methods may provide accurate and reproducible quantitative data for basic and clinical studies and have the potential to be readily translated into clinical practice.

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