Advances in multiplexed MRM-based protein biomarker quantitation toward clinical utility ☆

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ABSTRACT

Accurate and rapid protein quantitation is essential for screening biomarkers for disease stratification and monitoring, and to validate the hundreds of putative markers in human biofluids, including blood plasma. An analytical method that utilizes stable isotope-labeled standard (SIS) peptides and selected/multiple reaction monitoring-mass spectrometry (SRM/MRM-MS) has emerged as a promising technique for determining protein concentrations. This targeted approach has analytical merit, but its true potential (in terms of sensitivity and multiplexing) has yet to be realized. Described herein is a method that extends the multiplexing ability of the MRM method to enable the quantitation 142 high-to-moderate abundance proteins (from 31 mg/mL to 44 ng/mL) in undepleted and non-enriched human plasma in a single run. The proteins have been reported to be associated to a wide variety of non-communicable diseases (NCDs), from cardiovascular disease (CVD) to diabetes. The concentrations of these proteins in human plasma are inferred from interference-free peptides functioning as molecular surrogates (2 peptides per protein, on average). A revised data analysis strategy, involving the linear regression equation of normal control plasma, has been instituted to enable the facile application to patient samples, as demonstrated in separate nutrigenomics and CVD studies. The exceptional robustness of the LC/MS platform and the quantitative method, as well as its high throughput, makes the assay suitable for application to patient samples for the verification of a condensed or complete protein panel. This article is part of a Special Issue entitled: Biomarkers: A Proteomic Challenge.

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1. Introduction

NCDs are identified by the World Health Organization as "Group II Diseases" and encompass various disorders (e.g., endocrine), diseases (e.g., cardiovascular, respiratory), and congenital anomalies (e.g., Down syndrome) [1]. Collectively, NCDs are a global epidemic that accounts for approximately 66% of all fatalities [2], and their incidence is estimated to increase substantially across all demographics in the coming years [1,3]. While exhaustive efforts are being made toward increasing public awareness of the preventable risk factors (e.g., poor diet, physical inactivity), efforts are also being devoted to establishing techniques for improved disease diagnosis, prognosis, and stratification. The latter is being accomplished through the discovery and analysis of biomarkers.

A biomarker is defined as a biological indicator of an individual's physiological status. It can be measured and assessed through a recording (e.g., electrocardiogram), an imaging test (e.g., computed tomography scan), or a biospecimen test (e.g., urine). Through screening of a body fluid, evidence of toxic metabolites, aberrant signaling pathways, abnormal cellular secretions, and/or tissue leakage proteins can be obtained, which can help guide personalized medicine. Blood represents an ideal fluid to sample since its collection is relatively non-invasive, it is inexpensive to collect, and it contains the largest repository of circulating analytes, with proteins being the subject of this manuscript. Due to the paucity of FDA-approved protein biomarkers [4] – and the need for additional ones of high specificity for increased confidence [5] – considerable research is currently being focused on verifying the multitude of candidate markers that have been discovered through genomic (e.g., transcriptome profiling [6]) or proteomic (e.g., shotgun or multidimensional separations with tandem MS [7,8]) technologies.

Biomarker verification is the bottleneck of the biomarker pipeline [9,10]. It is at this stage that hundreds of candidate markers need to be screened against hundreds to thousands of patient cohorts for evaluation of their true clinical utility [11]. Verification is traditionally performed with enzyme-linked immunosorbent assays (ELISAs), since this is the "gold standard" method used in clinical laboratories. Its use is understandable, since a developed and reliable ELISA is capable of delivering exceptional sensitivity, extraordinary accuracy, and high throughput.

However, the problems associated with this technique are significant (e.g., it is costly and has extended development time with a high failure rate) and it cannot be used for highly multiplexed biomarker verification [11], or where rapid and inexpensive analyses are desired for prioritizing the markers that proceed to clinical validation.

An alternative verification approach is MS-based, which utilizes targeted MRM technology in conjunction with isotopically labeled standards [12,13]. This approach capitalizes on the high specificity of MRM (also referred to as SRM) detection (performed on a triple quadrupole mass spectrometer), and is capable of multiplexed protein quantitation in a rapid and inexpensive manner. To reduce sample complexity with the goal of enhancing sensitivity, depletion [14–18], enrichment [19–24], and multidimensional fractionation [25] have independently been applied to the bottom-up proteomic workflow. While great strides have been made, the true multiplexing ability of the method has yet to be fully realized. The quantitation of 67 CVD-related proteins (inferred from the analysis of 135 peptides) represents one of the largest panels quantified to date, using a MRM–MS with internal standard approach [26].

Described here is a rapid and robust approach for the targeted, interference-free, MRM quantitation of an expanded panel of 142 NCD-related proteins in human plasma that remains unprecedented by an MRM with SIS peptide approach. The method is antibody-free, which would cause putative markers such as transthyretin (a potential marker for Alzheimer's disease [27]) to be removed, and does not require protein- or peptide-based fractionation. The absence of these sample processing techniques reduces both the cost and the variability of the assay, and increases the throughput. The analytical platform consists of a latest generation ultra-high performance liquid chromatography (UHPLC) system and a state-of-the-art triple quadrupole mass spectrometer. Together, this platform delivers the sensitivity and reproducibility required for the highly multiplexed quantitation of these 142 high-to-moderate abundance plasma proteins. The concentrations of these proteins span a 6 order-of-magnitude range (albumin, 31 mg/mL; myeloblastin, 44 ng/mL), as measured by the linear regression analysis of their peptide standard curves (ca. 2 peptides per protein on average, 312 peptides in total). It is the use of a single transition/peptide and multiple peptides per protein, together with linear regression analysis of standard curves obtained from peptide standards containing constant endogenous (unlabeled) peptide amounts and variable exogenous (labeled) peptide amounts, that makes our final method different from previous methods recently developed in our laboratory [26,28] and other research laboratories [21,29–31]. The use of a single transition/peptide is justifiable since we have 4 dimensions of analyte specificity (i.e., precursor ion m/z, product ion m/z, peptide retention time, relative response) and the transitions are rigorously screened for interferences in the control and unknown plasma, with only those transitions that qualify being used for quantitation. The use of a single peptide helps to increase peptide multiplexing and improves the MS duty cycle by removing the time spent collecting data on the qualifier transitions. Linear regression analysis enables the facile application to patient samples, as demonstrated in separate nutrigenomic and CVD studies. Collectively, this modified protocol should prove useful to the proteomics community for expediting the verification of panels of candidate disease markers in human plasma.

2. Experimental

2.1. Chemicals and reagents

Chemicals (e.g., ammonium bicarbonate, iodoacetamide) and reagents (e.g., formic acid) were obtained from commercial sources at the highest purities available. All solvents (including water, acetonitrile, and methanol) were LC–MS grade and acquired from Sigma Aldrich (St. Louis, MO, USA).

2.2. Human blood plasma

Normal plasma (with K_2EDTA as an anticoagulant) for method development was purchased from Bioreclamation (Westbury, NY, USA; catalogue no. HMPLEDTA2, lot no. BRH43226), and was stored at -20 °C until use. Briefly, this plasma was harvested from whole blood, which was collected aseptically at FDA-inspected facilities from the antecubital fossa of 30 race- and gender-matched consenting donors (aged 18–50). The donors adhered to strict inclusion criteria (related to sexual activity and disease history), were negative for common viral markers, and passed a series of vital and physical checkpoints.

In a large-scale application, EDTA-treated plasma samples were collected by LifeLabs Medical Laboratory Services (Toronto, ON, Canada), stored at the University of Toronto (Toronto, ON, Canada), and processed at the University of Victoria - Genome BC Proteomics Centre. The investigated cohort was part of the Toronto Nutrigenomics and Health Study, and consisted of 438 consenting, ethnically diverse young adults (ages 20–29). The procedure for collecting subject characteristics and blood plasma followed that described previously [32–35].

For a second application, K_2EDTA-treated plasma from an ethnically diverse, CVD-afflicted cohort (n = 10 males, n = 8 females; ages 44–79) was supplied by Bioreclamation and stored at −20 °C until use. The donors were consenting, free of viruses, and not diagnosed with other diseases. Note that the use of unidentified human plasma samples was approved by the Human Research Ethics committee (protocol no. 13-113) at the University of Victoria (Victoria, BC, Canada).

2.3. Stable isotope-labeled peptide standards

The initial target panel was composed of 654 proteotypic peptides (corresponding to 291 NCD-linked plasma proteins). These peptides were selected through bioinformatics according to the rules outlined by Kuzyk et al. (e.g., absence of missed tryptic cleavages [36]); although, some have been empirically measured in our previous projects [26,37–39]. Briefly, C-terminal [^{13}C]/[^{15}N] labeled tryptic peptides were synthesized in-house using a standard Fmoc (N-(9-fluorenyl) methoxycarbonyl) procedure on an Overture or a Prelude peptide synthesizer (Protein Technologies; Woburn, MA, USA). As described previously in detail [38], purification was performed by HPLC and confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis, while characterization was conducted by capillary zone electrophoresis (CZE), performed at the University of British Columbia (Vancouver, BC, Canada), and amino acid analysis (AAA), performed at the Hospital for Sick Children (Toronto, ON, Canada). The post-synthetic purity of the 654 peptides was 94%. The AAA and CZE analyses enabled accurate SIS peptide concentrations to be determined for accurate protein quantitation.

2.4. Sample preparation

Samples were prepared following our standard bottom-up proteomic protocol [12,37,38,40]. Briefly, this involved denaturing and reducing a plasma sample (60 μL of 10-fold diluted undepleted plasma) with 5 mM Tris-(2-carboxyethyl)phosphine and 1% sodium deoxycholate for 30 min at 60 °C. Alkylation was performed with a 30 min incubation of 10 mM iodoacetamide at 37 °C. Residual alkylating agent was quenched with 10 mM dithiothreitol for 30 min at 37 °C. The buffer used throughout was 25 mM ammonium bicarbonate, which provided a pre- and post-digestion pH of approximately 8. Proteolytic digestion was accomplished by incubating overnight (16 h) with a 50:1 ratio of sample to sequencing-grade modified trypsin (Promega; Madison, WI, USA). Digestion was stopped by the addition of a chilled, acidified SIS peptide mixture, which contains either an equimolar mixture of SIS peptides at 100 fmol/μL for optimization or a concentration-balanced SIS peptide mixture for the reproducibility and quantitative analyses

(i.e., 174 μL of SIS mix per standard or unknown sample), followed by a chilled 1.2% formic acid solution (426 μL per standard or unknown sample). Note that the SIS peptide concentrations in the 7 samples used for calibration covered a 10,000-fold range (serially diluted from 250 fmol/μL at a 1:2:5:2.5:10:10 ratio), while the natural (NAT) peptide concentrations remained constant, but were at a constant concentration of 25 fmol/μL in the unknown samples. Also note that the SIS peptide mixture was "concentration balanced" by adjusting the SIS peptide concentrations in the mixture to yield nearly equal SIS/NAT peak areas when spiked into the plasma protein digest (estimated concentrations are based on the peak area measurements obtained from the analysis of the equimolar SIS-peptide mixture in plasma). The reduction of the pH to below 3 post-digestion caused precipitation of the deoxycholate, which was subsequently pelleted by centrifugation. Each supernatant was then desalted with an Oasis 10 mg hydrophilic/lipophilic balanced sorbent in a syringe-barrel cartridge (Waters; Milford, MA, USA). After elution with 50% acetonitrile (ACN, CH$_3$CN) and 0.1% formic acid (FA, HCO$_2$H), the samples were lyophilized to dryness and resolubilized in 0.1% FA. The final plasma protein concentration was 1.8 μg/μL, based on an initial protein concentration of 61.5 mg/mL (as measured by a Bradford assay kit). Apart from the standard curve, which had a SIS peptide concentration range of 0.03–300 fmol/μL for levels 1–7, the final SIS peptide concentration was 30 fmol/μL in all samples (buffer and plasma).

2.5. LC/MRM-MS analysis

Peptide separations (using 15-μL injections) were performed by RP-UHPLC on a Zorbax Eclipse Plus C$_{18}$ Rapid Resolution HD column (150 × 2.1 mm, 1.8 μM particles; Agilent Technologies; Palo Alto, CA, USA). The column and autosampler were maintained at 50 °C and 4 °C, respectively. The peptide mixture was separated using a 43 min ACN gradient from 3 to 90% mobile phase B (composition: 0.1% FA in 90% ACN) at a flow rate of 0.4 mL/min. The specific gradient employed was as follows (time, % B): 0, 3; 1.5, 7; 16, 15; 18, 15.3; 33, 25; 38, 45; 39, 90; 42.9, 90; and 43, 3. Each analysis was followed by a 4 min column equilibration. To reduce carryover, a blank injection of mobile phase A (composition: 0.1% FA) was run between each type of sample (i.e., buffer vs. plasma in interference screening), and between different concentration levels of the standard when determining the calibration curve.

The 1290 Infinity LC system was interfaced to a 6490 triple quadrupole mass spectrometer (both Agilent Technologies) via a standard-flow ESI source that was operated in the positive ion mode. The general MS parameters were identical to those used previously in our quantitative proteomics projects [12,26,37,39,40]. Specific parameters, such as collision energy and retention time, were optimized as described below. Four MRM methods were employed for interference screening (1050 transitions/method), with 66 being the maximum number of transitions concurrently monitored in each 800-ms cycle at a minimum dwell time of 10 ms. The final method for quantitation had dwell times between 13 and 256 ms, at an overall cycle time of 515 ms. This provided a maximum of 36 concurrent transitions during each 1-min detection window.

2.6. Peptide- and transition-specific optimizations

MRM transitions (for charge state and collision energy) were tuned empirically during direct infusion experiments, as described previously [26,37,38]. Briefly, mixtures of synthetic peptides (at 1 μM in 0.1% FA/30% ACN) were infused for unscheduled MRM analyses over the course of several weeks, where each precursor/product ion pair was screened for 20 ms while collision energies were ramped from 5 to 53 V. The transitions were chosen without regard to their precursor charge (double or triple), product ion series (b or y), or product ion-to-precursor ion m/z relationship. The only caveat was that the m/z

values lie within m/z 300 and 1400. From these optimizations, a list of the 3 most abundant ion pairs for each peptide was compiled, along with their corresponding collision energies. Identical collision energies were used for the NAT peptides because their physicochemical properties are the same.

Peptide retention times were scheduled according to the LC/MRM-MS analysis of the equimolar SIS mix (in 0.1% FA). This involved the unscheduled monitoring of groups of approximately 120 transitions (3 transitions/peptide), with a 20-ms dwell time per transition. Since the ^{13}C/^{15}N-labeled peptides and native peptides co-chromatograph (unlike deuterated analytes), identical retention times can be used for monitoring the NAT peptides. Furthermore, since nearly equivalent retention times are observed in both plasma and buffer, the scheduled methods can be applied to plasma for interference screening.

2.7. Interference screening

Interference in the SIS and NAT MRM ion channels of the development runs was determined from the LC/MRM-MS analysis of each of the peptide's 3 most abundant transitions in buffer and in normal plasma (n = 2 for both), as we have described previously [26,37,38]. To obtain the relative ratios, the response (i.e., peak area) of each SIS transition in buffer, SIS transition in plasma, and NAT transition in plasma was then referenced against the response for the most intense transition for a given peptide. The average relative ratios between the SIS peptide in buffer, the SIS peptide in plasma, and the NAT peptide in plasma were then calculated. For a peptide to be qualified as interference-free, at least 2 of its 3 transitions must have coefficient of variations (CVs) below 25% for the average relative ratios of the 3 groups (SIS in buffer, SIS in plasma, NAT in plasma). In addition, the extracted ion chromatogram (XIC) traces of the SIS and NAT peptides must chromatographically co-elute and exhibit identical peak symmetry, shape, and width. The symmetry and shape assessments were performed visually, while the retention time and peak width comparisons were performed in Excel using the values obtained in MassHunter Quantitative Analysis software (these are described further in the MRM Data Analysis section).

2.8. MRM data analysis

MRM data was processed and visualized with MassHunter Quantitative and Qualitative Analysis software (version B.05.00; Agilent Technologies), as described previously [26,37,38]. Peaks were first manually inspected to determine the accuracy of peak selection and integration, before peak (i.e., retention time, peak width, response) and quantitative information (including limits of quantitation, and dynamic range) were extracted. For protein quantitation, 7-point standard curves were prepared with a $1/x^2$ weighting. The qualification criteria used for each concentration level adhered to the FDA guidelines [41,42], where an average precision below 20% CV and an average accuracy within 80 and 120% were required for the 5 replicates. The LLOQ reflected the SIS concentration at the lowest qualified concentration level and the dynamic range referred to the difference between the SIS concentrations at the lowest and highest qualified levels, while the precision of quantitation was determined from the NAT/SIS response at level 5 (level at which balanced NAT/SIS concentrations are present). For applicability to patient samples, a new data analysis strategy for determining protein concentrations was employed. This required the concentration of NAT to first be estimated (product of average NAT/SIS relative response and SIS peptide concentration at level 5) before a plot of relative response as a function of relative concentration (both SIS/NAT) for the qualified levels could be used to produce a linear regression equation. By substituting the measured relative response (RR) into the peptide-specific equation and solving

for NAT_{conc}, as shown below, the concentration of an endogenous plasma protein (in fmol/μL) can readily be calculated.

$$NAT_{conc} = \frac{(SIS_{conc} \times m)}{(RR - b)}:$$

The known variables in the equation are the slope (denoted as m), the y-intercept (denoted as b), and the SIS peptide concentration (SIS_{conc}). All SIS peptide concentrations were corrected with the composition and purity information obtained from the AAA and CZE analyses. Concentrations were converted to ng/mL by using the protein molecular weights, as determined from ExPASy's "Compute pI/Mw tool" [43].

3. Results and discussion

Targeted MS approaches are emerging as a viable alternative to immunoassays for candidate biomarker verification. A method with increasing appeal centers on the use of MRM-MS with internal standards. Although the method has demonstrated its ability to satisfy several of the required elements of a verification, and perhaps validation, assay (e.g., specificity, reproducibility), it has yet to reach its full potential. To that end, we have developed a highly multiplexed MRM assay for the simultaneous quantitation of 142 disease-associated proteins in human plasma. Presented and discussed below are the developed method, its analytical merits, and its application to patient samples.

3.1. Overview of developed method

The quantitative plasma proteomic approach shown here utilizes bottom-up LC-MRM-MS with quantitation based on SIS peptides (labeled C-terminally with $^{13}C/^{15}N$ isotopes). Each endogenous target peptide is represented by its corresponding isotopically coded analog (synthesized in-house), with 654 peptides constituting the initial panel of analysis. The 291 proteins corresponding to these peptides have been reportedly linked to various NCDs [44,45], with CVD having the highest correlation. At this time, most of these associations are still putative; although, some, such as C-reactive protein [46] and thyroglobulin [47], have already been validated as clinically-relevant markers of disease [4]. Collectively, the 291 plasma proteins cover a 10 order-of-magnitude concentration range, from albumin at 43 mg/mL to interleukin 1-beta at 1.2 pg/mL. While we certainly did not expect to be able to quantify this entire range in undepleted human plasma, the panel is intended to serve a dual role as we will also be evaluating its use on a fractionated plasma sample, which will be the subject of a separate study. Nonetheless, the selected target panel can be used for assessing the sensitivity and multiplexing capability of an MRM-based workflow for the ultimate verification of plasma protein biomarkers.

To improve quantitative accuracy and to maximize sensitivity the peptide retention times and transition-specific collision energies were optimized. Since SIS peptides are chemically identical to their endogenous counterpart, the optimizations were achieved with simple SIS mixes, with the results subsequently applied to the corresponding NAT peptides. Chromatographic separations were performed in 43 min at standard flow rates, since we had previously found that the standard-flow system utilized was analytically superior (in terms of retention time reproducibility and detection sensitivity) to nano-flow, when interfaced to the same mass spectrometer and loaded with 10 times as much sample [37]. Based on the optimal loading capacity range (10–50 μg) for the standard-flow UHPLC column [26,37], a plasma digest loading amount of 27 μg (corresponding to 0.4 μL of undepleted plasma) was used.

To ensure that the peptides monitored during quantitation are detectable and interference-free, duplicate LC/MRM-MS analyses of SIS peptides in buffer and SIS peptides in normal plasma were conducted,

with 3 transitions being monitored per peptide. Stringent criteria (related to peak profiles and relative ratios) were used for peptide qualification. This screening exercise shortened the panel of target peptides/proteins from 291 proteins (654 peptides) to 149 proteins (348 peptides; see Fig. 1 for an overview of the peptide filtering process). Peptides derived from low-abundance proteins (e.g., atrial natriuretic peptide, macrophage colony-stimulating factor) were eliminated due to a lack of NAT peptide detectability, while some peptides from higher-abundance proteins were rejected due to either chemical interference (e.g., VQQNVPSGTDTGDPQSK, apolipoprotein L1; AAQAAVASYNMGSNSIYYFR, cystatin-M) or poor digestion efficiency (e.g., IDGSGNFQVLLSDR, von Willebrand factor; ELHHLQEQNVSNAFLDK, ceruloplasmin). The final method for protein quantitation in normal plasma consisted of only the quantifier transitions (696 transitions in total for 348 interference-free peptides; see Supplemental Table 1 for the final MRM panel and optimized parameters). These transitions represent the highest-responding MRM transitions for each peptide and enable enhanced peptide multiplexing. Note that when implementing this method, 3 transitions per peptide should first be monitored in buffer, followed by monitoring in normal plasma, in order to schedule peptide retention times and for interference screening, before reducing the number of transitions to one per peptide for protein quantitation. Although we advocate scheduling retention times empirically, this exercise can also be performed with reference peptide standards [48].

Our method also utilized a concentration-balanced SIS peptide mixture (average NAT/SIS ratio of 2), since ratio balancing had been previously demonstrated to reduce the analytical variation between analyses [49]. The elution distribution was fairly even (see Fig. 2 for a representative chromatogram), with 36 being the maximum number of ion transitions monitored simultaneously during the 1-min detection windows. Interestingly, the quantifier transitions were predominantly those forming y ions (see Supplemental Table 1), since these are the dominant fragment ions generated during low-energy collision-induced dissociation of tryptic peptides [50].

Proteins were quantified based on the peptide response curves. These curves were generated from the LC/MRM-MS analysis of standard peptide samples, and required that a given concentration level
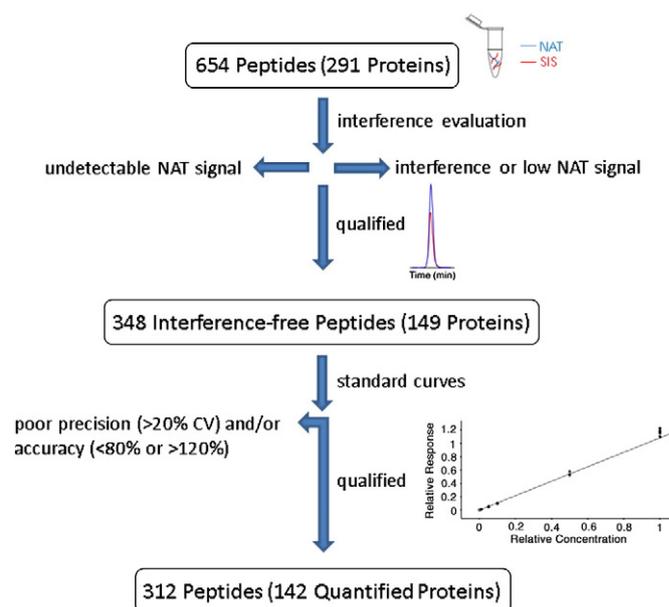
Fig. 1. Overview of peptide filtering steps for precise and accurate protein quantitation in normal human plasma. The two checkpoints are at interference screening and standard curve assessment, where only those that qualify proceed. Qualification criteria are based on NAT/SIS peak symmetry and precision (<25% CV in average relative ratio) in the first step, and both precision (<20% CV, on average) and accuracy (80–120%, on average) for each concentration level (n = 5/level) in the second step.

exhibit high precision and accuracy in order for it to qualify. Seven of the 149 proteins were unable to be quantified (see Fig. 1) because they did not meet our qualification criteria (i.e., an average precision of <20% CV per concentration level, an average accuracy of 80–120% per concentration level) for a minimum of 3 consecutive concentration levels, as well as a variety of additional factors (e.g., co-eluting noise in the SIS MRM ion channels, poor NAT signal intensity). The remaining 142 proteins were quantified based on 312 qualified peptides with their curves demonstrating strong linear correlation (average R^2 of 0.98) and average dynamic ranges between 10^2 and 10^3 (see Supplemental Fig. 1 for a pie chart showing the number of peptides per protein, Fig. 3 for two representative curves, and Supplemental Table 2 for performance details of each peptide). Where multiple peptides were present, the protein concentration was based on the peptide that yielded the highest concentration. Overall, the calculated concentrations were found to span 6 orders of magnitude, from 31 mg/mL (albumin) to 44 ng/mL (myeloblastin), as illustrated in Fig. 4. Based on recent literature reports, ca. 72% of the 142 quantified proteins have been linked to at least one type of CVD or one type of cancer; 40 of the 142 proteins have been associated with 40 of the 142 proteins have been associated with multiple diseases (see Supplemental Table 2 for reported associations and Supplemental Fig. 2 for the breakdown by NCD category).

3.2. Specificity of measurement

The targeted, quantitative approach provides 4 dimensions of analyte specificity. The first two are attributed to the MRM technique, wherein a specific precursor and product ion are mass selected and filtered from the background. Due to the isotopic labeling in SIS, the modification generates a +6 to +10 mass shift, which causes the m/z values of SIS and NAT to differ from one another in MS1 and/or MS2, depending on the ion type (i.e., b or y). As Sherman et al. accurately stated [51], these two dimensions are not specific enough to define a peptide uniquely. Not accounted for in their argument was that scheduling of peptide retention times and the use of relative ratios in labeling experiments can provide two additional dimensions of specificity to each MRM assay. Monitoring for a peptide only over the time in which it is expected to elute also enhances ion sensitivity,
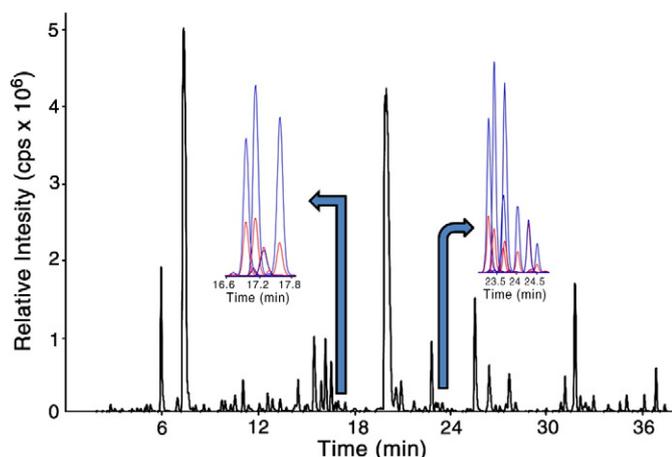
Fig. 2. Representative total ion chromatogram for 348 interference-free peptides (representing 149 NCD-associated proteins), monitored in the final scheduled MRM assay. The SIS peptide mixture spiked into the plasma tryptic digest was balanced to reflect their endogenous peptide concentrations. The insets illustrate extracted ion chromatograms (XICs) of 26 lower abundance peptides eluting within 1.2 and 1.5 min segments. Included are peptides from 6 proteins (carbonic anhydrase 1, creatine kinase M-type, hyaluronan-binding protein 2, insulin-like growth factor 1, myeloblastin, and protein S100-A9) at the lower end of the determined concentration range (44–160 ng/mL). The NAT peptides are displayed in blue, while the SIS peptides are shown in red.
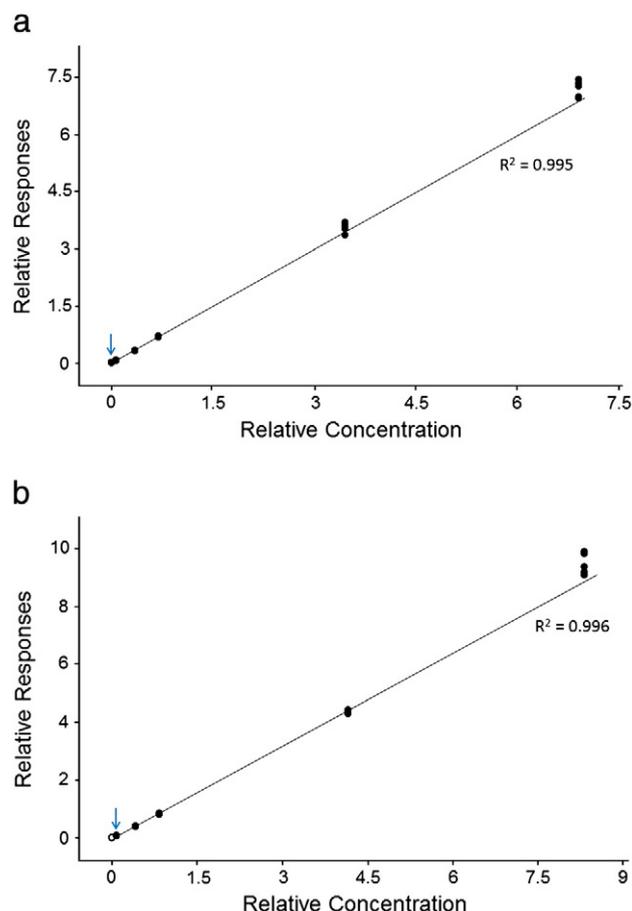
Fig. 3. Representative standard curves from the multiplexed LC/MRM-MS analysis of 348 peptides. Shown are the curves for two medium abundance proteins: a) AGALNSNDAFVLK (10^3 linear range, 69 μg/mL determined concentration) from gelsolin and b) LLPHANEVSQK (10^2 linear range, 40 μg/mL determined concentration) from apolipoprotein A-IV. The standard samples used to generate the curves contained a constant amount of plasma digest and a variable amount of the concentration-balanced SIS peptide mixture. The ordinate corresponds to the SIS/NAT relative response, while the abscissa refers to the SIS/NAT relative concentration. The five replicates for each concentration level are displayed as dots, with only those levels that qualified being filled. The blue arrow indicates the LLOQ.

while relative ratios help normalize for sample loss or variable instrument performance [52]. As an aside, even further specificity can be obtained by the analysis of secondary product ions through MRM^3 [53,54]. Although specificity can be enhanced with high resolution and accurate mass (HR/AM) measurements performed on the Q Exactive (quadrupole-Orbitrap) mass spectrometer [55–57], the Q Exactive is not a triple quadrupole and may result in differences in terms of sensitivity and linear dynamic range. Nonetheless, the 4 dimensions of specificity used here facilitate the creation of highly specific MRM protein assays which are suitable for the analysis of complex plasma matrices where the potential of transmitting non-target ions with isobaric, or near isobaric, m/z values is increased. In fact, this high specificity resulted in only 60 peptides (39 proteins) to be eliminated from screening of normal plasma due to chemical interference with the SIS or NAT transitions. Among those removed were peptides from several coagulation factors (V, VIII, IX, X, XI, and XIII A chain), granzyme (A, H, and K), and insulin-like growth factor-binding proteins 1 and 3. The remaining interference-free peptides did not exhibit m/z redundancy or sequence homology, and should provide highly precise and accurate quantitation. While not conducted here, the iSRM (intelligent SRM) instrument control software, which automatically assembles SRM/MRM transitions based on the product ion intensities collected from discovery experiments,
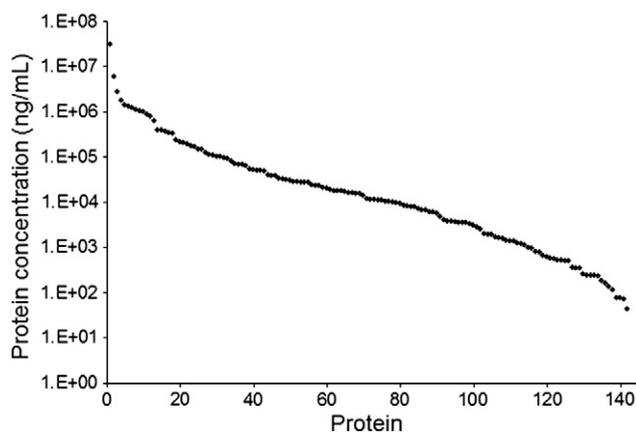
Fig. 4. Range of concentrations determined from the LC/MRM-MS analyses of undepleted and non-enriched human plasma. The concentrations are arranged from highest to lowest abundance, with the highest concentration obtained listed for each protein.

could be implemented for verification of the target peptide identities [58].

3.3. Multiplexing ability

The ability to multiplex MRM assays for a large number of peptides into a single analytical run for parallel processing is a significant advantage of the MRM technique and one that will certainly be of value in expediting biomarker verification. To maximize this feature, peptide retention times should be scheduled and monitored over narrow detection windows. The cycle time must be minimized to ensure that at least 10 data points are collected across the chromatographic profile of each peptide and to also ensure that the number of concurrent MRM transitions is limited in order to increase the MS duty cycle for those ions on which the quantitation is based. For this assay, the desired LC sampling rate was attained with 1-min integration windows and a 515-ms MS duty cycle. This high degree of multiplexing was aided by the high retention time reproducibility (0.05% average for 312 interference-free and quantifiable peptides) and narrow chromatographic peaks (5.7 s average at half height for 312 interference-free and quantifiable peptides) obtained from the UHPLC standard-flow separation. During quantitation, a total of 696 transitions were scheduled into a single, 43 min MRM method. Based on the elution-time distribution of the peptides, the low number of concurrent transitions (maximum of 36), and the low MS cycle time (515 ms), it is conceivable that an additional 300 transitions could be incorporated. Nonetheless, the set of 348 peptides (149 proteins) that form the final quantitative panel is already highly multiplexed and is one of the largest panels analyzed to date using calibration curves based on SIS peptides This peptide/protein list can serve as a useful guide for panel selection in disease-specific biomarker studies. It can also be used as a biomarker discovery tool to screen large numbers of proteins to find smaller biomarker panels, which can provide improved diagnostic accuracy compared to a single biomarker alone.

3.4. Assay throughput and cost

The developed MRM assay offers high throughput in preparation and in execution, with approximately 24 h required for preparing a maximum of 96 samples/day and 47 min for the LC/MRM-MS quantitative analysis of 312 peptides (142 proteins). The chromatographic run time for a panel of this magnitude cannot be compressed without adversely affecting the number and degree of interferences. The run

time could certainly be reduced for higher throughput, however, if a subset panel is to be interrogated in a disease-specific study, as long as the interference testing is verified for the new chromatographic conditions. Nonetheless, the developed method would consume approximately 3 days of instrument time for processing a 96-well plate of samples (7 controls and 89 unknowns).

We estimate the cost for developing and implementing a MRM assay to be ca. $1000 per peptide. This cost considers the reagents, personnel, and instrument wear for synthesizing the SIS peptides, the assessment of interference and detectability, the balancing of the SIS peptide mixture, and quantification of the proteins. Therefore, a MRM assay costs ca. $3000 per protein if 3 peptides are targeted, which is a significant improvement over the cost of obtaining a reliable ELISA [11,59]. The cost benefit analysis is aided by the omission of a depletion or enrichment step, which not only increases the cost but also negatively influences the variability and peptide recovery [60,61]. It should be noted, too, that the ability to multiplex MRM assays, further reduces the run cost and increases the throughput. Additionally, since SIS peptides are added post-digestion, smaller quantities are consumed per analysis.

3.5. Platform robustness

Robustness is a critical metric of any analytical method and is of heightened importance if a method is to be of use to the clinical proteomics community. In the method described in this paper, short-term method and platform robustness were measured through replicate inter-day analyses of analytical metrics such as retention time and peak width. For a panel of 348 interference-free peptides, high reproducibility in signal (6.9% CV in relative response, on average), retention time (0.05% CV, on average), and peak width (FWHM 5.4% CV, on average) was observed when 3 samples were independently prepared. These results are in-line with those obtained previously for this LC/MRM-MS platform and with our other projects involving alternate samples and targets [26,37–39], which helps to confirm the high reproducibility of this platform. Furthermore, since we have previously demonstrated the superior reproducibility of this standard-flow platform over a nano-flow [37], we recommend standard-flow incorporation in quantitative proteomic studies, provided that sufficient sample is available. In all of the studies using this platform, similar precision was achieved in the measurement of absolute SIS and NAT responses (data not shown). This is additional evidence for the robustness of the platform and demonstrates the reproducibility of the tryptic digestion. In the standard curves (where NAT functions as the normalizer), equally high precision was obtained for 312 interference-free NAT peptides across 7 concentration levels (see Fig. 5). Although the inter-laboratory variability of these assays was not investigated, we anticipate this to be low based on the results of the Addona multi-site study [62].

3.6. Detection sensitivity

The described method enabled the determination of plasma protein concentrations covering 6 orders of magnitude, from albumin at 31 mg/mL (470 μM) to myeloblastin at 44 ng/mL (1.8 nM) This corresponded to 10, 105, and 27 proteins in the mg/mL, μg/mL, and ng/mL concentration ranges, respectively. Included were 4 proteins with determined plasma concentration levels of less than 100 ng/mL (myeloblastin, 44 ng/mL; apolipoprotein C-IV, 72 ng/mL; carbonic anhydrase I, 76 ng/mL; protein S100-A9, 77 ng/mL), which brings the assay into the upper range where tissue leakage proteins, secreted during only necrosis or apoptosis, are detectable. Also quantified in the detectible range are proteins secreted or released from tissues (e.g., apolipoprotein B-100 from hepatic tissue [63], adiponectin from adipose tissue [64]) and from organs (e.g., serotransferrin and complement factor H from liver [65]), as well as intracellular proteins
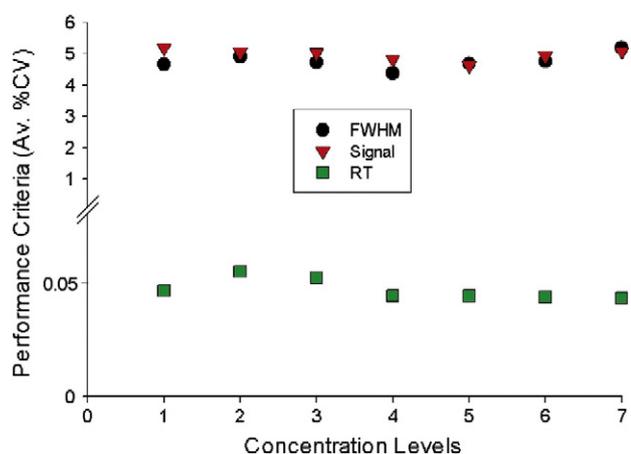
Fig. 5. Variability of performance metrics from the quantitation curves. The signal (as red triangles), FWHM (as black circles), and retention time (as green squares) for the 312 interference-free, quantifiable peptides are plotted as average %CV at each concentration level (n = 5 per level) for the NAT signals.

(e.g., hemoglobin and carbonic anhydrase from erythrocytes). While these proteins are collectively classed as high-to-moderate in abundance, they nonetheless represent potential markers of disease that must be verified for clinical relevance.

The true measure of the sensitivity of a MRM assay is the LLOQ, since it reflects the lowest concentration that can be accurately measured. The protein LLOQs that were derived from the highest-responding proteotypic surrogate peptides ranged from 6 ng/mL (for apolipoprotein E) to 261 μg/mL (for fibronectin; see Supplemental Table 2). There were instances in this dataset where poor linear dynamic ranges were observed despite high analytical precision and the absence of chromatography or interference issues. In these cases, quantitative accuracy was limited for unknown reasons. This suggests that alternative peptides should be selected. In cases where multiple peptides per protein were quantified, selecting a different peptide was found to extend the linear dynamic range (222-fold, on average, for the 5 proteins with LLOQs at the 100 μg/mL level), but reduced the final concentration determined for the protein. The variability in concentration derived from peptides is not surprising and is attributed to either incomplete tryptic digestion, insufficient denaturation, or resistance to digestion.

To be translatable to clinically relevant applications, the measured concentrations must be within the linear response range of the analyte and be above the LLOQ. These criteria were obeyed for 129 of the 142 NCD-linked proteins, with the average ratio of protein concentration to LLOQ being 2 orders of magnitude. By selecting different peptides, the concentrations derived for 4 proteins (corticosteroid-binding globulin, lipopolysaccharide-binding protein, serum paraoxonase/lactonase 3, and vitamin K-dependent protein C) reached acceptable levels on the standard curve. Also required for translation to clinical settings is that the linear dynamic range cover the concentrations expected from unknown samples, including those from patients with the targeted disease. Since the equation used to calculate the NAT concentration causes the ratio of SIS/NAT to be reversed in the application to relative responses from unknowns, a narrow dynamic range is acceptable.

3.7. Application to unknown plasma samples

The MRM method shown here has considerable analytical merit, as demonstrated above. It is able to specifically and robustly quantitate a large panel of high-to-moderate abundance candidate disease markers in untreated (i.e., no depletion, enrichment, or fractionation) human plasma, without sacrificing cost or throughput. Absent from the above discussion, however, is its extension to unknown patient plasma samples, which ultimately determines its clinical utility. This was evaluated here with two different sample cohorts that were designed to address two different applications.

In the first application, a smaller panel of 40 plasma proteins (104 peptides) with reported associations to CVD and multiple quantifiable peptides per protein was selected from our final MRM method. New 7-point standard curves were prepared from an independently prepared plasma tryptic digest, using the normal pooled plasma sample from development as the control, for precise and accurate quantitation of a small CVD cohort (n = 18). Compared to the quantitative results from the larger method development study, the determined concentrations in normal human plasma were nearly identical, as evidenced by a R^2 value of 0.96 and a regression line slope of 1.0 (see Supplemental Fig. 3). This high reproducibility in quantitation further highlights the robustness of this method and platform combination, which allows its extension to unknown samples. In this analysis, chemical interferences in the patient samples are expected to be different than those in healthy samples due to the presence of up- or down-regulated proteins, exogenous xenobiotics, and degradation or decomposition products that are related to the disease and its treatment. To assess for interferences in these sample types, we advocate the use of an approach with a single representative MRM ion pair per peptide but with multiple peptides representing each protein to be quantified, as developed here and as proposed previously [26,66]. In this approach, the peptide relative responses are first plotted against one another for each corresponding protein. The presence or absence of interference is determined by examining linearity of the resulting plot. A deviation suggests the presence of an interference that is usually validated by inspection of the peptide's XICs. This could possibly prevent quantitation of that particular protein from that specific sample if alternative peptides for this protein also contain interferences (see Fig. 6 for two contrasting examples). An added advantage of this approach (1 transition per peptide, multiple peptides per protein) is that it helps to maximize the ion statistics and facilitates improved multiplexing, which is necessary for the rapid verification of an expanding list of candidate disease biomarkers.

Despite its merits, two notable limitations of this strategy must be considered. First, it increases data analysis time, particularly in cases where 4 or more peptides per protein are present and a minimum of 6 peptide-vs.-peptide relative response plots must be manually generated and evaluated. The number of outliers that require further inspection of their XICs then becomes considerable and this manual inspection needs to be performed prior to quantitating the interference-free peptides. Automation would be advantageous and would help diminish the subjective nature of this analysis. A second limitation also involves the number of peptides monitored per protein. When the correlation plots of the relative responses do not agree (e.g., a vs. b is linear, a vs. c is non-linear, b vs. c is linear) and evaluation of the XICs does not lead to a definitive result, an insufficient number of target peptides could cause "reliable" peptides to be disqualified from being used as a basis for quantitation.

In the second application, 120 of the 142 quantified proteins were selected by the El-Sohemy laboratory (University of Toronto) as targets for a large-scale nutrigenomics study. In this on-going study, the concentration of each protein is inferred from a single peptide and interferences in the unknowns are detected by monitoring 3 MRM transitions per peptide (1 quantifier and 2 qualifiers). It should be stressed that if interference is found in the quantifier transition of an unknown sample, a qualifier transition cannot instead be used for this sample since this will alter the SIS/NAT relative response and yield an erroneous protein concentration. The same set of peptide-specific MRM transitions must be monitored in both the control and unknown samples. Furthermore, if additional peptides are available, the same peptide should be used to quantify the protein since different peptides from the same can result in different plasma protein concentrations (see Supplemental Table 2, as well as [12,26,39]).
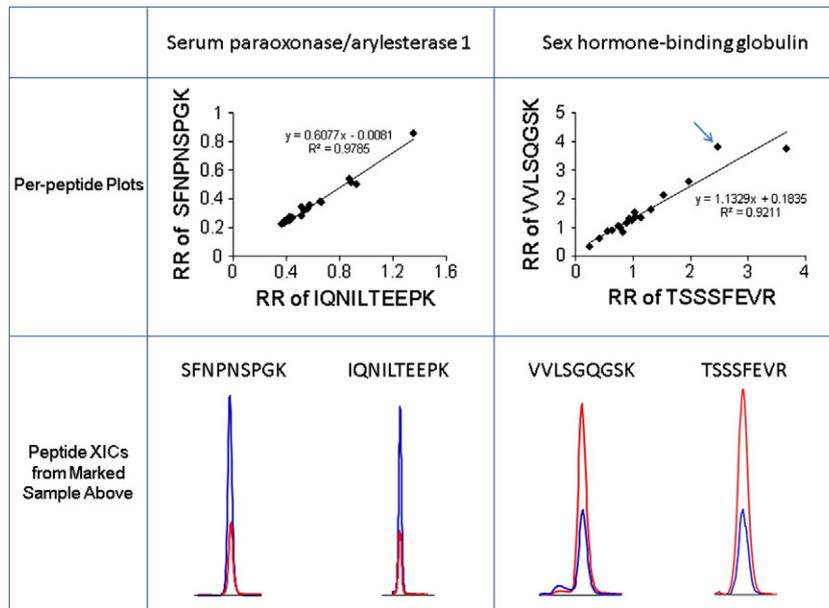
Fig. 6. Interference screening strategy with unknown samples. Interferences are identified through plots of the relative responses (RRs) for each peptide and from each protein, and are confirmed by inspection of the XICs for the different peptides. Due to its deviation from linearity, the sample indicated by the blue arrow contains an interference in the SIS and/or the NAT MRM transitions of peptide VVLSQGSK and/or TSSSFEVR. The XICs of the 2 interference-free peptides for serum paraoxonase/arylesterase 1 are shown for comparison.

Therefore, in the event of interference in the SIS or NAT quantifier transition, the interference-containing peptide must be disregarded and its protein not quantified for that particular sample. A total of 438 human plasma samples are currently being processed with the aim of identifying protein markers of dietary exposure and further investigating the effects of diet on human health. To simplify the data interpretation, the subjects are healthy young adults with no chronic disease prevalence. In a previous collaboration, we performed quantitative proteomic analyses on 54 plasma proteins to evaluate their relationship with circulating micronutrients (e.g., vitamins C and E [33,34]) and their role in regulating physiological processes. The biological mechanisms examined in this new study, however, will be presented in a future manuscript. It is worth noting that, despite the standard samples being prepared from a different plasma source, a different balanced SIS-peptide mixture, and by a different operator, similar quantitative results were obtained (R^2 of 0.91, regression line slope of 0.96; data not shown) as in the method development study described here. These results again confirm the high reproducibility of the described method and the analytical platform, which provides confidence in the results of this on-going study, as well as the extension of this method to other projects.

4. Conclusions and significance

In this paper, we have demonstrated a rapid and robust MRM method using concentration-balanced SIS peptides for the highly multiplexed quantitation of 142 candidate disease-related proteins in undepleted and non-enriched human plasma. The proteins quantitated cover a 6 order-of-magnitude concentration range, with determined values between 31 mg/mL (albumin) and 44 ng/mL (myeloblastin), which were inferred from 312 interference-free peptides. The corresponding response curves were linear, with an average R^2 of 0.98 and an average dynamic range between 10^2 and 10^3. Despite the high-to-moderate abundance levels of these proteins in human plasma, they still remain "putative" biomarkers that need to be verified and validated prior to clinical use. This verification and validation process is of critical importance since additional diagnostic or prognostic markers, or panels of biomarkers, could help to reduce the potential for false positive or false negative results, which in turn could diminish the economic burden on the healthcare system and/or lead to earlier treatment.

To reach this goal of large-scale biomarker verification, a novel analysis strategy has been developed and utilized for determining endogenous protein concentrations. The developed method and analysis strategy was then successfully applied to two separate applications — one related to CVD and one related to nutrigenomics. Apparent from these studies was the high degree of quantitative and analytical reproducibility, which provides a solid basis for the extension of these methods to alternative proteomic initiatives of biological importance. While we and others focus on improving the depth of quantitation and extending the multiplexing capabilities of the MRM technique, we must not overlook potential candidate markers of high-to-moderate abundance in plasma. The method described here fulfills the requirements for pre-clinical application and provides a convenient and reliable platform for evaluating these markers in a highly multiplexed and rapid manner.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbapap.2013.06.008.

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