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Rapid development of sensitive, high-throughput, quantitative and highly selective mass spectrometric targeted immunoassays for clinically important proteins in human plasma and serum

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Abstract

Objectives—The aim of this study was to develop high-throughput, quantitative and highly selective mass spectrometric, targeted immunoassays for clinically important proteins in human plasma or serum.

Design and methods—The described method coupled mass spectrometric immunoassay (MSIA), a previously developed technique for immunoenrichment on a monolithic microcolumn activated with an anti-protein antibody and fixed in a pipette tip, to selected reaction monitoring (SRM) detection and accurate quantification of targeted peptides, including clinically relevant sequence or truncated variants.

Results—In this report, we demonstrate the rapid development of MSIA-SRM assays for sixteen different target proteins spanning seven different clinically important areas (including neurological, Alzheimer's, cardiovascular, endocrine function, cancer and other diseases) and ranging in concentration from pg/mL to mg/mL. The reported MSIA-SRM assays demonstrated high sensitivity (within published clinical ranges), precision, robustness and high-throughput as well as specific detection of clinically relevant isoforms for many of the target proteins. Most of the assays were tested with *bona-fide* clinical samples.

In addition, positive correlations, (R^2 0.67–0.87, depending on the target peptide), were demonstrated for MSIA-SRM assay data with clinical analyzer measurements of parathyroid hormone (PTH) and insulin growth factor 1 (IGF1) in clinical sample cohorts.

Conclusions—We have presented a practical and scalable method for rapid development and deployment of MS-based SRM assays for clinically relevant proteins and measured levels of the target analytes in *bona fide* clinical samples. The method permits the specific quantification of individual protein isoforms and addresses the difficult problem of protein heterogeneity in clinical proteomics applications.

Keywords

Biomarker; SRM assay; Mass spectrometry; Proteomics; Immunoassay; Clinical proteomics

Introduction

Immunoassays are currently the gold standard for clinical protein measurement, although they may have limitations, primarily with respect to antibody specificity [1]. Quantitative measurements using traditional immunoassay methods, such as enzyme-linked immunosorbent assays (ELISA), are often affected by components in the sample matrix and other confounding factors [2–4]. Furthermore, they are often unable to distinguish among sequence variants and PTM heterogeneity of the target proteins [5]. Mass spectrometry (MS) has been used for quantification of small molecules in clinical laboratories for years, and, more recently, has been applied to protein quantification and analysis [6,7]. Because MS can resolve proteins at the sequence level, it provides the selectivity to address the issues outlined above. Nevertheless, the routine measurement of proteins by MS in biofluids, especially blood plasma or serum, have not been widely adopted due to a perceived lack of sensitivity, robustness, speed of analysis and therefore throughput.

Recent technological advances in instrumentation and sample preparation and enrichment have begun to make MS-based targeted protein assays an attractive alternative to classical ELISAs and other automated immunoassay-based methods using clinical analyzers [8–10]. Several studies have demonstrated that MS technology is sufficiently robust to allow the inter-laboratory reproducibility and throughput necessary for clinical applications [11–14]. In spite of these advances, it has still been difficult to couple high-sensitivity, especially for detection of low-abundance analytes in plasma and serum, with the robustness and throughput requirements of routine clinical assays. The large dynamic range of proteins in serum/plasma samples compromises the ability of MS to achieve sufficient sensitivity (signal-to-noise ratio) for accurate quantification of many clinically important markers and therefore requires prior enrichment of the less abundant species [15,16]. Numerous approaches for complexity reduction of plasma and serum before MS detection have been reported, including fractionation using multiple LC columns, prior depletion of abundant proteins, enrichment using solid phase extraction (SPE), nanoparticles or immunoaffinity enrichment by various techniques including magnetic beads [17–22].

Most of the previously published methods have not demonstrated the necessary combination of sensitivity, *i.e.* unambiguous analyte detection across the clinical range *in actual clinical samples*, with high precision (CVs < 20%) and speed/throughput (potential for STAT assays or at most 24 h sample turnaround times). For example, albumin depletion of serum or plasma samples results in depletion of potentially important biomarkers that may be bound to albumin [23]. SPE may be useful for analytes in a limited mass range but when applied to complex mixtures may result in significant loss of high molecular weight proteins and therefore limits sensitivity [22]. Magnetic beads are often used for immunoenrichment in gel

and western-blotting applications but when coupled with MS detection significant non-specific binding becomes problematic (Fig. S1, [24]). Beads also have limited capacity to handle a broad range of sample volumes. This is especially true when low abundance proteins are enriched from plasma or serum, where relatively large volumes (*ca.* 1000 μ L for an analyte present in pg/mL) may be needed in order to meet the detection limit of the MS. Large bead volumes are expensive, awkward to automate and slow since analyte binding to the antibody on the bead surface is dependent on batch adsorption. In addition, none of the enrichment methods described above have addressed one of the most profound challenges presented by the measurement of clinically relevant proteins, *i.e.* protein heterogeneity and isoforms [25].

Many disease-related proteins are either truncated, modified by post translational modifications (PTMs) or single nucleotide polymorphisms (SNPs) [26] and are present in multiple active and inactive isoforms. The elucidation of the biological effects of protein heterogeneity has become one of the next big problems in clinical proteomics research. It is imperative in many cases to measure not just total protein concentration but also relative concentrations of clinically relevant isoforms in order to gain the necessary specificity in protein assays [27–33].

It is the protein heterogeneity problem that has frustrated efforts to develop, for instance, a prostate specific antigen (PSA) assay that delivers clinical results that are more easily interpretable and consistent [34,35]. Since PSA is typically present in numerous truncated and modified isoforms, the collective quantification of all forms (together) may not provide enough specificity for an accurate disease-related prognosis and has likely resulted in a high false-positive rate (FPR) for the assay, limiting its application [36].

Fortunately, the relative lack of specificity of most antibodies (depending on the epitope) to minor changes in protein sequence or the presence of PTMs also presents an opportunity for collectively enriching an entire range of isoforms of a particular disease-related protein of interest. This enrichment strategy coupled with specific detection at the level of amino acid (aa) sequence provided by the MS can provide a next-generation, highly specific assay for clinically relevant protein isoforms [37]. The process for development of an assay of this type requires initial mapping of the protein isoforms (using high-resolution LC-MS/MS) present in a collection of clinical samples likely to contain most or all disease-related variants. Once the sequence variants are identified, targeted assays can be constructed based on variant specific peptides for a multiplexed selected reaction monitoring (SRM) MS assay, or intact species if the protein is of low enough molecular weight to be clearly and unambiguously resolved in the MS [27–33,37].

Recently, we reported the development of SRM-based MS assays for parathyroid hormone (PTH) and PSA using this approach [13,37]. The described methods coupled mass spectrometric immunoassay (MSIA), (a previously developed technique for immunoenrichment on a monolithic microcolumn activated with an anti-protein antibody and fixed in a pipette tip [38]), to SRM detection and accurate quantification of targeted surrogate peptides, including clinically relevant truncated variants. Immunoenrichment using MSIA provides several advantages (i) the monolithic matrix demonstrates less non-specific binding than most bead methods ([24], Fig. S1), (ii) forced contact of the analyte with the antibody in repeated binding cycles results in reduced binding times (iii), A wide range of sample volumes can be accommodated, thus allowing rapid and quantitative enrichment of very low abundance (pg/mL) analytes. The reported MSIA-SRM assays demonstrated higher sensitivity for the detection of multiple forms of PTH with *bona-fide* clinical samples than other published methods [37].

In this report, we extend the initial panel of two proteins to sixteen, spanning seven different diseases/clinically important areas [38–54]. The 16 proteins include members of the Apolipoprotein family (ApoE, ApoA1, ApoC1, ApoCIII and ApoJ [clusterin]), some medium-to-high abundance proteins (ceruloplasmin, vitamin D binding protein, beta-2 microglobulin and C-reactive protein), and many clinically important low abundance proteins (procalcitonin, parathyroid hormone, insulin-like growth factor 1, prostate-specific antigen, erythropoietin, proprotein convertase subtilisin/kexin type 9 and amyloid beta). In addition, we have added multiple peptides to our initial PTH and PSA assays and included numerous variant and isoform specific peptides for other proteins not previously detected with SRM assays. Most of the assays in this report were demonstrated on clinical samples and we also demonstrated the correlation of two MSIA-SRM assays with clinical analyzer results.

Materials and methods

Clinical samples

Table 1 summarizes the clinical samples used in assay development. All samples were procured in accordance with the approval of institutional IRB protocols.

Antibodies and recombinant proteins

Supplementary Table S1 lists the sources for antibodies and recombinant proteins used in the development of all assays. Epitopes for the PTH, insulin-like growth factor (IGF1) and procalcitonin (PCT) antibodies were mapped using phage display technology (Differential Proteomics, Research Triangle, NC) and were determined to be aa 72–79, 87–92, and 99–106, respectively.

MSIA tip protocols

Custom MSIA tips activated with relevant antibodies (Table S1) were obtained from Thermo Fisher Scientific (Tempe, AZ). Samples were processed and subjected to binding and elution using a Versette or Platemate Robotic workstation (Thermo Fisher Scientific) as previously described [13,37].

Proteolytic enzyme digestion, reduction/alkylation and desalting

Enriched serum or plasma extracts eluted from MSIA tips were processed as previously described [13,37]. In some cases, other proteolytic enzymes such as SV8 (Thermo Fisher Scientific) were used to generate peptides with the desired target sequences. Previous experiments using a concatenated, heavy isotope-labeled synthetic concatenated peptide standard demonstrated consistent tryptic digestion with an average digestion efficiency of 64% [55].

High resolution LC–MS/MS, data analysis and protein identification

High-resolution LC–MS/MS analysis was carried out on an LTQ-Orbitrap XL MS instrument (Thermo Fisher Scientific) as previously described [55]. All samples were analyzed using Proteome Discoverer software, version 1.3 (Thermo Fisher Scientific) for peptide and protein identification before SRM assay development.

SRM assays

SRM assays were developed on a TSQ Vantage triple quadrupole MS instrument, (Thermo Fisher Scientific) as previously described [13,37] with minor modifications. LC for all assays was carried out using Accucore aQ 2.1×50 columns (Thermo Fisher Scientific)

except for the amyloid beta (A beta) assay, where a Proswift C18 1.0×250 column (Thermo Fisher Scientific) was used. There were three full replicates per sample.

Calibration curve generation

We added recombinant proteins to control human blood plasma or serum to create calibration standards (typically N=9) using serial dilutions for all targeted proteins. During sample preparation, the spiked calibration curve point samples were processed with MSIA tips, eluted and subsequently reduced, alkylated and digested with trypsin (or other proteolytic enzyme) before measurement on the MS. Therefore, every point on the calibration curve reflected the complete MSIA workflow. Each point in the calibration curves (and every sample analyzed) also included 100 fmol of heavy isotope-labeled peptides, added after digestion of the recombinant protein and just before LC–MS analysis. The heavy isotope-labeled peptides were used as internal standards for QC of the LC and MS detection. In addition, all samples were dissolved in a solution of 15 µg/mL of glucagon in 100% Water 3% ACN and 0.2% FA to minimize binding to plastic surfaces [12,13]. Subtraction of endogenous target peptide signal in the background serum or plasma matrix was performed with the Pinpoint algorithm. Finally, the calibration curve replicate points were run adjacently. Since each calibration point contained the same amount of endogenous peptide, the heavy peptide concentration was used to calculate the run-to-run variance. Heavy peptides were also spiked into every clinical sample and the low run-to-run variance added confidence to the analysis.

Choice of proteins, peptides and transitions

Pinpoint software, version 1.2 or 1.3 (Thermo Fisher Scientific) was used for assay method development and targeted protein quantification [12,13,37]. Peptides were chosen by combining the four approaches outlined below:

1. Spectral libraries created by high-resolution LC–MS/MS discovery analysis of recombinant protein standards were imported into Pinpoint for assay development and optimization.
2. Algorithmic predictions of optimal peptides and transitions were carried out *in silico* using Pinpoint and these data were added to the list of peptides and transitions above.
3. Manual sequence analysis identified peptides that would theoretically result from tryptic (or other proteolytic enzymes such as SV8) digestion of natural variants such as apolipoprotein AI (Apo AI) peptides YTKKLNTQ and YTKKLNT) and these sequences were added to the methods.
4. Intact sequences of naturally occurring variants (such as A beta peptides aa 1–38, 1–40 and 1–42) were added to the desired methods.

Peptide identities were confirmed by chromatographic co-elution of light (endogenous) and heavy isotope-labeled transitions in the chromatographic separation. For additional verification and elimination of interferences, the SRM transition ratios were confirmed using discovery spectra. Time alignment and relative quantification of the transitions were performed with Pinpoint. Peptide sequences, transitions, collision energies and all other relevant parameters are given in Supplementary Table S2. All clinical samples were assayed in full triplicate (*i.e.* complete workflow including individual MSIA extractions).

Heavy isotope-labeled peptides

Heavy isotope-labeled versions (purity 97%) of each target peptide were synthesized (Thermo Fisher Scientific). Heavy isotope-labeled peptides had identical sequences to their

respective endogenous peptides, but the C-terminal Lysine or Arginine residues were fully-labeled (98.5%) with ^{13}C or ^{15}N [12,13,37], see Table S2 for peptide sequences.

Clinical analyzer measurements

Intact PTH was measured with the Advia Centaur System (Siemens) according to the manufacturer's instructions. The assay is a sandwich immunoassay where the primary antibody is a polyclonal goat anti human to PTH aa 1–34, and the secondary antibody is a biotinylated goat anti-human polyclonal to the aa 39–84 region.

Intact IGF1 was measured using the Immulite 2000 Platform (Siemens) according to the manufacturer's protocols. This assay is a solid phase enzyme-labeled chemiluminescent immunometric assay. The capture antibody is a murine monoclonal (epitope not disclosed by the manufacturer) and the detection antibody is a rabbit polyclonal.

Results

SRM method development and optimization

Fig. 1 shows the automated workflow for SRM method development. The strategy combines algorithmic and spectral library prediction with empirically collected high-resolution LC–MS/MS data for determination of the optimal peptides and transitions. This approach initially creates an SRM method containing an exhaustive list of target peptides and transitions. Once the initial method is built and if recombinant protein standards (digested or intact) are available for testing, the list can be rapidly shortened by iterative optimization using an automated process. If recombinant proteins are not available, synthetic peptides can be used. Parameters such as collision energy, LC gradient and SRM scheduling windows are varied within each iteration (approximately 20–30 min each) and the highest intensity transitions are retained for the next iteration. After approximately 3–4 iterations, the method is optimized and ready for sample analysis. The entire MS method development process typically can be completed in approximately 2–3 h.

MSIA-SRM workflow

Fig. 2 illustrates the workflow developed for high-throughput and rapid sample turnaround. All steps were carried out using an automated pipetting workstation in 96 well microtiter plates. Plasma or serum sample volumes ranged from 1 to 1000 μL , depending on the abundance of target proteins. Capture antibodies (monoclonal or polyclonal) were covalently bound to the MSIA microcolumns ([37,38], Table S1). The best performing antibodies were selected after testing several commercially available sources with standard commercially available recombinant proteins (Table S1). Sample extraction and elution were typically completed in 2 h or less, depending on the number of binding cycles. The optimal number of repetitive binding cycles (200–1500) was determined empirically in initial experiments and depended on (i) the affinity of the antibody for the analyte and (ii) the analyte abundance in the samples. Once the captured analyte was eluted into the 96 well microtiter plate, the extracts were pH neutralized, reduced, alkylated and digested with trypsin at 50 °C. The reduction/alkylation/digestion process was completed in 3 h and, at completion, the sample was ready for injection onto the LC–MS/MS system. Typical chromatographic separation times using fast-flow (240–800 $\mu\text{L}/\text{min}$) LC on a triple quadrupole MS instrument ranged from 1.25 to 7 min per sample, depending on the level of LC multiplexing. Some low molecular weight proteins or protein fragments such as A beta aa 1–38, 1–40, 1–42 were measured intact, *i.e.* without trypsin (or other enzyme) digestion. In that case, sample introduction to the MS was carried out immediately following elution from the MSIA tips. Intact protein assays will typically require slower LC flow rates or potentially nanoflow LC in order to achieve the requisite resolution for separation of isotopes in highly charged

species. In addition, large intact proteins (>10 kDa) are best resolved in an ion-trap or hybrid instrument. Throughput for the entire workflow (fast-flow) was approximately 4 h per 96 analyses for the MSIA preparation (including digestion) with an added 5–7 min MS separation time per sample in single-plex mode. Combining multiple LC channels with a single MS instrument (multiplexing) increased the throughput by 2–4 times.

Assay sensitivity and precision

Fig. 3 shows representative extracted SRM chromatograms of surrogate peptides from digested human plasma or serum samples or recombinant proteins. As evident in the figure, peak shapes were typically symmetrical and relatively free of significant interferences. SRM transition ion ratios were within $\pm 15\%$ of internal standard reference ratios. Several SRM transitions [2–5] were used for the quantification of each peptide. This is important for SRM quantification of peptides to ensure selectivity, even in enriched samples. Representative calibration curves for each assay are shown in Fig. 4. Lower limit of quantification (LLOQ) values are given in Table 2. Detection limits and dynamic ranges for all targeted peptides were well within the useful clinical range for the selected analytes and the LLOQ values were close to or at the lower end of the range (Table 2). Calibration curves for all peptides demonstrated linear behavior (correlation coefficients ranged from 0.89 to 0.99 (triplicate analysis) and the precision (% CV of triplicates within one day) for all peptides was 20%.

Correlation with commercial clinical analyzer immunoassays

We compared MSIA-SRM assay quantitative results for IGF1 and PTH with clinical analyzer measurements for the same sample sets (Table 1, IGF1 n=289; PTH, n=357). The correlation plots (Fig. 5) demonstrated that surrogate peptide measurements by MSIA-SRM were positively correlated with protein measurements from the clinical analyzer data. The correlation of the clinical analyzer intact PTH and the PTH MSIA-SRM N-terminal peptide (aa 1–13) was 0.67 and resulted in a slope of 0.67 with a negative Y intercept, (Fig. 5A). When the MSIA-SRM concentrations for all the tryptic peptides from PTH (aa 1–13, 14–20, 28–44, 73–80) were averaged, the correlation with the clinical analyzer data increased to 0.87 (Fig. 5B). The difference in these values may reflect the capture and quantification of other N-terminally truncated PTH fragments as well as intact PTH in the clinical analyzer assay (see Discussion).

In addition, averaging the MSIA-SRM concentrations for all the tryptic peptides from PTH (aa 1–13, 14–20, 28–44, 73–80) resulted in a higher PTH concentration than that measured by the clinical analyzer (slope 1.79 and positive y-intercept). This may be due to inclusion of C-terminal fragments that would not be detected in the clinical assay.

The correlation of the MSIA-SRM IGF1 peptide and the clinical analyzer data for intact IGF1 was 0.71 (Fig. 5C). The epitope for the IGF1 antibody used in the clinical analyzer assay was not disclosed by the manufacturer (see Materials and methods).

Discussion

We have developed high-throughput, sensitive and highly selective MS-based assays using MSIA-SRM for 16 different, clinically important analytes. The 16 proteins included members of the Apolipoprotein family (ApoE, ApoA1, ApoC1, ApoCIII and ApoJ [clusterin]), some medium-high abundance proteins (ceruloplasmin, vitamin D binding protein, beta-2 microglobulin and C-reactive protein), and many clinically important low abundance proteins (procalcitonin, parathyroid hormone, insulin-like growth factor 1, prostate-specific antigen, erythropoietin, Proprotein convertase subtilisin/kexin type 9 and amyloid beta). The MS assay development was rapid (on the order of hours), semi-

automated and resulted in a method that provided precise quantification within the established and required clinical range for each analyte. The MSIA-SRM assays are highly specific since detection using MS provides sequence information.

Correlations with clinical analyzer immunoassays were tested for two analytes, PTH and IGF1 and demonstrated R^2 values ranging from 0.67 to 0.87, depending on the surrogate target peptide. Specifically for PTH, the correlation of the clinical analyzer data with MSIA-SRM of the single N-terminal peptide ($R^2=0.67$, Fig. 5A) *versus* the average values of all peptides ($R^2=0.87$, Fig. 5B) suggests that the traditional immunoassay may include intact and truncated protein species in the calculated amounts due to a lack of specificity in the capture or detection antibodies (or both). Also, averaging the MSIA-SRM concentrations for all the tryptic peptides from PTH (aa 1–13, 14–20, 28–44, 73–80) resulted in a higher PTH concentration than that measured by the clinical analyzer, (Fig. 5B), possibly indicating detection of C-terminal fragments that would not be seen in the clinical assay. This presents a problem for analytes where truncated species or other variants are clinically relevant (such as PTH) [56,57]. For example, the PTH active site is aa 1–10 and the intact protein is rapidly cleaved *in vivo*. Therefore, measurement of the N-terminal aa 1–13 tryptic peptide using SRM can provide a more accurate estimation of *active* protein. Although the primary antibody for the clinical analyzer assay is directed at aa 1–34, it may not be absolutely specific since it is polyclonal. Therefore, it may capture some fragments that are missing the active site at the N-terminus, resulting in an overestimation of *active* protein. Detection with MS allows the specific quantification of these different isoforms and therefore excludes any fragments not containing the active site at the N-terminal. Any analyte that exhibits fragmentation or cleavage *in vivo* would be subject to similar *caveats* when measured with traditional immunoassays.

A discussion of immunoenrichment techniques coupled to MS detection should also include the choice of anti-peptide *versus* anti-protein antibodies [24,58,59]. Although there are instances where anti-peptide antibodies may have applications, (for example in the case of proteins where good antibodies are not available), for most practical purposes capture at the protein level provides significant advantages. Anti-protein antibodies provide global enrichment of multiple protein isoforms, simplifying the process of distinguishing active from inactive forms. In addition, it is not necessary (nor even desirable) to develop highly specific antibodies for this application since capturing a collection of protein isoforms is preferred. Using an anti-protein antibody raised toward an epitope in common to all isoforms/variants is more economical since only one is needed to measure multiple forms. If anti-peptide antibodies are used, they must be highly specific and a separate one is needed for each target peptide. For example, the PTH assay described in this report would require 7 different anti-peptide antibodies in order to provide the same information. Not only is this approach much more expensive, but it does not allow for the addition of new isoforms to an assay without long delays (months) while new antibodies are being developed. Adding new isoforms to an assay that is based on an anti-protein antibody can be accomplished in a matter of hours (Fig. 1).

Trypsin digestion is another area where anti-protein antibody capture provides clear advantages. Digestion in solution is highly dependent on the sample protein-to-trypsin ratio and complex samples such as raw plasma or serum require relatively large amounts of trypsin and long digestion times (12–24 h) [18]. This requirement is further complicated by the samples themselves being different. Essentially, samples from different states of health and disease are expected to be different (thus the very reason to analyze them), the effects of which have never been studied to the degree necessary to convincingly demonstrate that digestion variability due to the diseases themselves does not exist. Due to these reasons, the use of anti-peptide antibodies, which requires digestion of the crude sample *prior* to capture

is and therefore more expensive and time consuming, as well as a potential source of errors stemming from uncontrolled, or unknown causes. Conversely, samples enriched at the protein level are digested *after* capture and are therefore far less complex. In this case, trypsin digestion is typically fast (2–4 h), more economical and highly reproducible ([13,37,55], this paper). Finally, there is the problem of quantification of non-unique peptides with anti-peptide antibodies. If the surrogate peptides are not carefully chosen and are not unique, digestion of a complex mixture with subsequent anti-peptide immunoenrichment and quantification will result in incorrect measurements since one peptide sequence may be present in multiple proteins. This problem is not encountered when enrichment is done at the protein level.

Of course, the ideal assay for protein measurement might exclude the need for trypsin digestion altogether. However, with the present technology, MS instruments have a practical upper limit for rapid, sensitive and fully quantitative intact protein measurement that lies at approximately 20 kDa, although qualitative analysis can be done on much larger molecules [60].

A question that will certainly be raised is whether or why immunoenrichment is necessary for medium or high abundance analytes. In fact, enrichment of even high abundance analytes serves several important functions. As described above, trypsin digestion of complex mixtures is expensive and time consuming. Also, although protein targets may be present in high abundance, clinically important variants are likely to be less so. An example is Apo AI, one of the most abundant proteins in plasma and the major component of high density lipoprotein (HDL). The ability of Apo AI to bind lipid effectively is highly dependent on the C terminal [50]. Truncated Apo AI differs from full length Apo AI by a single amino acid at the C-terminus (<http://www.uniprot.org/uniprot/P02647>). Immunoenrichment and detection of Apo AI with MSIA-SRM permits the quantification of both isoforms (this paper) as well as other C and N-terminally truncated variants that may be present in much lower amounts. An additional, more practical advantage is that injecting simple mixtures (*versus* raw plasma or serum digests) onto the MS instrument prevents fouling of the LC columns and MS source, keeping cleaning and instrument maintenance to a minimum and adding robustness and consistency to routine measurements.

One of the most compelling reasons for the implementation of MS in the clinical lab is the potential for measuring panels of several analytes at once. Current multiplexed ELISA or other immunoassay technologies present serious limits since as the number of measured analytes is increased in one assay, the sensitivity is typically compromised. The technology described in this report allows multiplexing of analytes by serial extraction of the same sample with different MSIA tips. This approach can be fast, efficient, conservative with sample volume and avoids the problem of accommodating several antibodies with different optima for binding and elution conditions on a single substrate. The serially extracted fractions are then measured at the same time in the SRM assay (data not shown).

In conclusion, we have presented a practical and scalable method for rapid development and deployment of MS-based SRM assays for clinically relevant proteins and demonstrated results with 16 different proteins previously correlated with 7 different disease groups, ranging in concentration from pg to mg/mL. The described method couples MSIA, a high-throughput method for immunoenrichment, with fully quantitative SRM assays that permit detection of target proteins within the established clinical ranges in plasma or serum. Finally, the method allows the specific quantification of individual protein isoforms and addresses the difficult problem of protein heterogeneity in clinical proteomics applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

MS	mass spectrometry
LC	liquid chromatography
MS/MS	tandem mass spectrometry
SPE	solid phase extraction
ESI	electrospray ionization
ACN	acetonitrile
m/z	mass to charge ratio
SRM	selected reaction monitoring
Apo	apolipoprotein
LLOQ	lower limit of quantification
FPR	false positive rate
PTH	parathyroid hormone
DBP	vitamin D binding protein
B2M	beta 2 microglobulin
CRP	C-reactive protein
PCT	procalcitonin
IGF1	insulin-like growth factor 1
PSA	prostate-specific antigen
EPO	erythropoietin
PCSK9	proprotein convertase subtilisin/kexin type 9
A beta	amyloid beta
PTM	post translational modification
MSIA	mass spectrometric immunoassay
ELISA	enzyme-linked immunosorbent assay
SNP	single nucleotide polymorphism
aa	amino acid
FA	formic acid

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Fig. 1.
Workflow for automated development and optimization of SRM assays using Pinpoint software.

Fig. 2.
Workflow and throughput for MSIA-SRM assay development.

* Tested on recombinant protein and clinical samples, ^Tested on recombinant protein only

* Tested on recombinant protein and clinical samples, ^Tested on recombinant protein only

Fig. 3. Extracted chromatograms of MSIA-SRM analyses of clinical samples or recombinant proteins. A. Apo E, B. ceruloplasmin, C. Apo AI, D. Apo CI, E. Apo CIII, F. clusterin, G. PTH, H. DBP, I. B2M, J. CRP, K. PCT, L. IGF1, M. PSA, N. EPO, O. PCSK9, P. A beta.

Fig. 4. Calibration curves for targeted peptides. Linear regression curves were created using recombinant proteins subjected to the complete MSIA workflow (Fig. 2). R^2 values ranged from 0.89 to 0.98 and %CVs of full triplicates ranged from 0 to 13%. A. Apo E, B. ceruloplasmin, C. Apo AI, D. Apo CI, E. Apo CIII, F. clusterin, G. PTH, H. DBP, I. B2M, J. CRP, K. PCT, L. IGF1, M. PSA, N. EPO, O. PCSK9, P. A beta.

Fig. 5. Linear regression correlation plots of clinical analyzer and MSIA-SRM data obtained from clinical samples. A. SRM-MSIA of PTH aa 1–13 (N-terminal) peptide and Advia Centaur intact PTH immunoassay (N=357). B. Average value of all tryptic PTH peptides (aa 1–13, 14–20, 28–44, 73–80) and Advia Centaur intact PTH immunoassay (N=357). C. SRM-MSIA of IGF1 aa 49–69 peptide and Immulite 2000 immunoassay for intact IGF1 (N=289).

Table 1

Clinical samples used in assay development and testing. All samples were procured in accordance with the approval of institutional IRB protocols.

Disease	Sample type	Number of samples
Alzheimer's/neurological	Plasma	40
Cardiovascular/cerebrovascular	Plasma	32
Renal failure/endocrine function/bone metabolism/low vit D	Plasma	357
Growth disorders	Plasma	289
Cancer	Serum	Pool

Table 2

Lower limits of quantification and clinical ranges for targeted proteins and peptides.

Disease	Protein	LLOQ ^a	Clinical range	aa	Peptide sequence	Peptide type
Alzheimer's/neurological	Amyloid beta	50 pg/mL	20–80 pg/mL	1–38	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG	Disease variant
				1–40	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGV	Disease variant
				1–42	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVIA	Disease variant
	Apolipoprotein E	10 ng/mL	10–100 µg/mL	81–89	ALMDETMK	Monitoring
				199–207	LGPLVEQGR	Monitoring
				210–224	AATVGSLAGQPLQER	Monitoring
				261–269	LEEQAQIR	Monitoring
				270–278	LQAEAFQAR	monitoring
				281–292	SWFEPLVEDMQR	Monitoring
				301–317	VQAAVGTSAAPVPSDNH	c-terminal
				177–185	LAVYQAGAR	Isoform specific
				176–185	C[Carboxymethyl]LAVYQAGAR (aa 176 R C)	Isoform specific
				20–33	VEQAVETEPEPELR	Isoform specific
Cardiovascular/cerebrovascular	Ceruloplasmin	62.5 µg/mL	14–450 µg/mL	20–29	VEQAVETEPK (aa 31 E K)	Isoform specific
				44–50	WELALGR	Isoform specific
				44–50	WEPALGR (aa 46 L P)	Isoform specific
				121–132	LGADMEDVC[Carboxymethyl]GR	Isoform specific
				121–130	LGADMEDVR (aa 130 C R)	Isoform specific
				70–81	ALYLYTYDTEFR	Monitoring
				427–439	EYTDASFTNR	Monitoring
				469–481	GAYPLSIEPIGVR	Monitoring
				548–558	DIFTGLIGPMK	Monitoring
				771–778	GEFYIGSK	Monitoring
	Apolipoprotein AI	0.31 mg/mL	1.06–2.20 mg/mL	153–160	GARQKLHE	Monitoring
				208–215	NGGARLAE	Monitoring
				216–222	YHAKATE	Monitoring
				230–236	KAKPALE	Monitoring
				237–247	DLRQGLLPVLE	Monitoring

Disease	Protein	LLO ^a	Clinical range	aa	Peptide sequence	Peptide type
Renal failure/endocrine function/bone metabolism	Apolipoprotein CI	10 ng/mL	60 µg/mL	260–267	YTKKLNTQQ	C-terminal
				260–266	YTKKLNT	Isoform specific
				27–36	TPDVSSALDK	N-terminal
				39–47	EFGNTLEDK	Monitoring
	Apolipoprotein CIII	25 µg/mL	100 µg/mL	66–74	EWFSETFQK	Monitoring
				21–37	SEAEDASLLSFMQGYMK	N-terminal
				45–60	DALSSVQESQVAQAR	Monitoring
				61–71	GWVTDGFSSLK	Monitoring
	Apolipoprotein J/clusterin	25 µg/mL	35–105 µg/mL	72–88	DYWSTVK	Monitoring
				82–89	EDALNETR	Monitoring
				168–182	QQTHMLDVMQDHFSR	Monitoring
				199–214	EPQDTHYLPFSLPHR	Monitoring
	PCSK9	37 ng/mL	30–3000 ng/mL	226–336	ELDESLQVAER	Monitoring
				47–66	SEEDGLAEAPHGTTATHR	Propeptide
				74–83	LPGTYYVVVLK	Propeptide
				153–160	SIPWNLER	Monitoring
	Parathyroid hormone	16 pg/mL	15–65 pg/mL	259–272	GTVSGTLGLEFIR	Monitoring
				422–434	DVINEAWFPEDQR	Monitoring
				511–525	AHNAFGGEGVYAIAR	Monitoring
				1–13	SVSEIQLMHNLOK	N-terminal
	Vitamin D binding protein	50 µg/mL	200–300 µg/mL	14–20	HLNSMER	Monitoring
				28–44	LQDVHNFVALGAPLAPR	Monitoring
				73–80	ADVNVLTk	Monitoring
				34–44	FVALGAPLAPR	Variant
				35–44	VALGAPLAPR	Variant
				7–13	LMHNLGK	Variant
				14–20	HLNS(phospho)MER	Variant
				38–50	EDFTSLSLVLYR	Monitoring
				208–218	HLSLLTTLNLR	Monitoring
				346–352	YTFELSR	Monitoring
				354–370	THLPEVFLSKVLEPTLK	Monitoring

Disease	Protein	LLOQ ^a	Clinical range	aa	Peptide sequence	Peptide type
Sepsis/infection	Beta 2 microglobulin	0.5 µg/mL	0.7–1.80 µg/mL	364–370	VLEPTLK	Monitoring
				395–402	ELSSFIDK	Monitoring
				69–78	VEHSDLFSK	Monitoring
	C reactive protein	5 µg/mL	8 µg/mL	101–111	VNHVTLSPK	Monitoring
				32–41	ESDTSYVSLK	Monitoring
				50–65	AFTVC(carboxymethyl)LFHYTELSSTR	Monitoring
Growth disorders	Procalcitonin	1 ng/mL	0.1 ng/mL	66–75	GYSIFSATK	Monitoring
				210–224	YEVQGEVFTKQLWP	C-terminal
				30–48	SALESSPADPATLSEDEAR	Monitoring
				91–102	C(carboxymethyl)GNLSTC(carboxymethyl)MLGTYTQDFNK	Monitoring
				103–118	FHTFPQTAIGVGAPGK	Monitoring
	IGF1	10 ng/mL	25–1000 ng/mL	121–128	DMSSDLER	Monitoring
				49–69	GPETLC(carboxymethyl)GAELVDALQFVC(carboxymethyl)GDR	Monitoring
				86–95	APQTGIVDEC(carboxymethyl)C(carboxymethyl)FR	Monitoring
					TLC(carboxymethyl)GAELVDALQFVC(carboxymethyl)GDR	Long standard
					SVILLGR	Isoform 3 specific
Cancer	PSA	1.56 ng/mL	2 ng/mL	71–77	DTIVANP	Isoform 4 specific, C terminal
				255–261		
Drug doping	Erythropoietin	100 pg/mL	10 pg/mL	70–77	KSVILLGR	Monitoring
				131–137	SLTTLR	Monitoring
				171–177	VYSNFLR	Monitoring
				73–79	VNFYAWK	Monitoring
				159–166	TITADTFR	Monitoring
				41–47	YLLEAK	Monitoring

^a Average of all peptides.