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"Product Ion Monitoring" Assay for Prostate-Specific Antigen in Serum Using a Linear Ion-Trap

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While numerous strategies exist for biomarker discovery, the bottleneck to product development and routine use at the clinic is in the verification phase of candidate biomarkers. The aim of this study was to establish a robust and high-throughput product ion monitoring (PIM) assay that is potentially capable of rapidly verifying candidates from discovery phase experiments. Using prostate-specific antigen (PSA), a model biomarker, and a routinely used mass spectrometer for discovery platforms, an ion trap (LTQ, Thermo), the utility of this instrument to perform PIM was explored. The proteotypic doubly charged intact peptide LSEPAELTDAVK (*m*/*z* 637) fragmenting to *m*/*z* 943 (PAELTDAVK) was monitored. A limit of detection of 10 attomoles with a coefficient of variation (CV) of <20% was obtained for a purified recombinant PSA digest. Immunoextraction of endogenous PSA from serum using a monoclonal antibody on a 96-well microtiter plate, followed by PIM on the LTQ, enabled quantification of PSA down to less than 1 ng/mL with a limit of detection of 0.1 ng/mL and CVs < 20%. Mascot searching and ion ratio confirmation further supported the conclusion that the quantified moiety in serum was the PSA peptide. We conclude that this methodology could be adapted quickly and easily to other candidates, thus providing a much needed technology to bridge the gap between discovery and validation platforms.

Keywords: biomarkers • multiple reaction monitoring • prostate-specific antigen • quantification • targeted proteomics • verification phase

Introduction

Rapid advances in proteomic and genomic technologies have created optimistic views that many more biomarkers will be discovered through various high-throughput techniques. However, these predictions have yet to come true.[1] To date, very few serum biomarkers have been introduced at a clinic over the last 15 years. While numerous strategies exist for biomarker discovery, the bottleneck to product development and routine clinical use is in the verification phase of candidate biomarkers. A verification and validation platform is more costly and labor-intensive and requires more time than a discovery program.[1,2] There is no doubt that meticulous verification steps are essential, and the impact of the end product on the future of diagnostics would be enormous.

Mass spectrometry (MS) is emerging as one of the most promising analytical techniques to simultaneously examine thousands of samples quickly, precisely, and accurately, using minute sample volumes. While MS has been used widely as a biomarker discovery platform, there are not as many studies applying MS as a tool for absolute protein quantification and thus validation of the candidates, although it has been used extensively for small organic compound quantification.[3] Given that different compounds with equal concentrations do not necessarily ionize with the same efficiency, techniques have been implemented to enable differential quantification of proteins with MS. Metabolic stable-isotope labeling, isotope tagging by a chemical reaction, and stable-isotope incorporation via enzymatic reaction are all ways by which identification of the relative abundance of molecular species can be achieved.[4,5] For absolute quantification of proteins, an internal standard peptide is spiked into the sample, and the ratio between the synthetic and endogenous peptide is determined by MS.[6–8] The premise for such an approach is that a peptide that is cleaved from a protein can serve as a representation of the concentration of that intact protein when cleavage is complete.[9] Given the above, it is important to optimize for a unique peptide to represent a given protein because not all tryptic peptide fragments are observed with equal likelihood. The peptides that are repeatedly identified for any given protein

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in a complex mixture are referred to as proteotypic.[10] Often, these experiments are performed on a triple quadrupole mass spectrometer in multiple reaction monitoring mode (MRM). Measurement of precursor ion/product ion transitions is referred to as selective reaction monitoring (SRM).[11] In this approach, the discriminating power of mass analyzers to select a specific analyte is utilized to transmit only ions of a specific mass-to-charge ratio (m/z) which is fragmented, and the resulting product ion(s) are monitored in a two-stage mass selection process. In an ion-trap mass spectrometer, precursor mass selection, collision-induced dissociation (CID), and detection of the product ion of interest occurs in the same region. In addition, given that the scan rate does not change in an ion-trap instrument, it is possible to perform product ion monitoring (PIM) or product ion MS/MS, that is, the monitoring of all product ions (instead of only one product) from the fragmentation of the precursor ion. In this manner, additional structural information can be gathered while not increasing the duty cycle of an ion trap relative to MRM.

In this respect, peptide MRM/SRM, on a triple quadrupole or PIM on an ion trap, measurements in serum digests are appealing since they provide a rapid and specific assay platform for biomarker verification. Nonetheless, the sensitivity of the assay is limited by the dynamic range of mass spectrometers.[12] Therefore, an enrichment strategy involving immunodepletion and gel-based or chromatography techniques are often coupled to MRM assays to increase the sensitivity of detection by mass spectrometers. Proteomic and biomarker studies that have utilized MRM analysis recognize the significant method development and optimization time required for each peptide studied.[13–17] Anderson et al. successfully designed MRM assays for 47 high-to-medium abundance proteins of human plasma. Using this assay and immunodepletion of high abundance proteins, the authors were able to measure L-selectin in plasma at 670 ng/mL concentration using a hybrid triple quadrupole ion-trap instrument (Q-Trap 4000).[12] In another study, the accuracy and reproducibility of absolute quantification of a protein found in serum without purification using a standard LC-MS/MS platform was evaluated on a triple quadrupole mass spectrometer.[9] Using prostate-specific antigen (PSA), Barnidge et al. spiked female serum with recombinant PSA and a stable isotope-labeled internal peptide standard of PSA. They were able to reproducibly and accurately quantify 2000 ng/mL of PSA.

The studies published thus far utilizing mass spectrometry as a quantification tool have successfully achieved levels of 300–500 ng/mL for proteins in serum with minimal or no purification steps. However, for an assay to be clinically relevant, the detection limits should be in the low nanogram/milliliter range. PSA, one of the best cancer biomarkers, has a reference range of 1–4 ng/mL. To achieve this level of sensitivity with MS, a purification step is essential. But it is also equally important for these MRM assays to be relatively simple to perform, robust, and high-throughput. The gold standard for determining the concentration of a biomarker in serum is the immunoassay. Establishment of an ELISA (enzyme-linked immunosorbent assay) is labor-intensive and not feasible for large candidate biomarker verification phases where the aim is to screen 100 or so candidates for clinical relevance in biological fluids.

Notably, antibody capture of the analyte is one of the most sensitive purification methodologies.[1] Combining antibody immunoextraction with MRM assays may achieve low nanogram/milliliter sensitivity. In the current study, we established a robust and high-throughput product ion monitoring (PIM) assay that is potentially capable of rapidly verifying candidates from discovery phase experiments using a linear ion-trap instrument. Specifically, we performed an immunoextraction of PSA from serum using a monoclonal antibody on a 96-well microtiter plate, followed by PIM on the LTQ, a linear ion trap. To our knowledge, this is the first study to use the LTQ for PIM assays and quantification on a 96-well microtiter plate. A limited number of studies have used the LCQ (an earlier version of LTQ) in SRM mode to determine the levels of certain drugs in plasma,[18–21] and some have attempted to examine the ability of a linear ion trap (LTQ) to quantitate peptides in serum in SRM mode.[11,16,17,22] PSA was chosen as the model protein since it can be obtained in a purified form, and an excellent immunoassay is available to validate the mass spectrometry data. Using an immuno-MS (immunoassay in combination with mass spectrometry) approach, with a minimal prefractionation step, a fast LC-MS gradient, and potentially preparing 96 samples simultaneously on a microtiter plate, we were able to demonstrate that PIM can be performed on the LTQ using a purified recombinant PSA digest to achieve a limit of detection of 10 attomoles with a coefficient variation of <20%. Furthermore, we successfully quantified PSA in serum of prostate cancer patients down to less than 1 ng/mL, with a detection limit of 0.1 ng/mL, using the monoclonal antibody immunoextraction approach followed by PIM, as validated by ELISA.

Materials and Methods

Reagents. The following chemicals were used: sequencing-grade modified trypsin (Promega; Madison, WI, USA), iodoacetamide, and dithiothreitol (DTT) (Sigma-Aldrich; St. Louis, MO, USA), and monoclonal PSA-antibody (Medix Biochemica; Finland).

PSA Protein Standard. Purified human recombinant pro-PSA purchased from Spectral Diagnostics (Toronto, Ontario, Canada) and Beckman Coulter (San Diego, CA) was used for generating the calibration curve and for determining the lowest limit of detection on the LTQ via product ion MS/MS or PIM. The concentration of stock rPSA (Spectral Diagnostics) was 250 μg/mL. The concentration of stock rPSA (Beckman Coulter) was 668 μg/mL. Ten micrograms of rPSA was digested at 37 °C with trypsin (1:10 ratio of trypsin:rPSA) in 50 mM ammonium bicarbonate and methanol (20%) aqueous solution. The following amounts of rPSA digests were prepared by dilution: 100, 10, 1, 0.1, 0.01 femtomoles (fmol). These values translate to 3000, 300, 30, 3, and 0.3 picograms (pg) of rPSA digest. In terms of concentrations, these values translate to 75, 7.5, 0.75, 0.075, and 0.0075 ng/mL. The purity of rPSA was confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Coomassie staining, as well as by tandem mass spectrometry.

LC-MS/MS Conditions. Tryptic peptides were separated on a 2 cm C_{18} trap column (inner diameter 200 μm). The peptides were eluted from the trap column onto a resolving 5 cm analytical C_{18} column (inner diameter 75 μm) with a 15 μm tip (New Objective). The LC setup was coupled online to a 2-D linear ion trap (LTQ, Thermo Inc.) mass spectrometer using a nanoelectrospray ionization source (nano-ESI). Buffer A contained 0.1% formic acid, 5% ACN, and 0.02% TFA in an aqueous water solution, and buffer B contained 90% ACN, 0.1% formic acid, and 0.02% TFA in water. The eluted peptides were analyzed by tandem mass spectrometry (MS/MS) for identifica-

tion purposes and by PIM for quantification purposes in positive-ion mode. A linear gradient was used with an injection volume of 40 μL, which was loaded onto the column via an Agilent 1100 Cap-LC series autosampler. A 25 min method was developed with a 5 min gradient and used for all experiments.

Peptide Selection and PIM Design. LC-MS/MS analysis of rPSA digest was performed, in which the major ions observed were subjected to MS/MS using the LTQ. The identified peptides showing the best signal intensity and chromatographic peak shape for rPSA were selected. To assist in the determination of SRM transitions, the Global Proteome Machine database[23] was employed to select peptides from PSA that were frequently detected. Using SRM transitions available from LC-MS/MS proteomic survey data and comprehensive testing of all candidate peptides of PSA, PIM assays were developed for the precursor−fragment ion transition of the doubly charged intact proteotypic peptide with m/z 637 (LSEPAELTDAVK) to m/z 943 (PAELTDAVK) and used for all further experiments. The other precursor−fragment ion transitions such as HSQPWQVLVASR (m/z 705), FLRPGDDSSHDLMLLR (m/z 936), and HSLFHPEDTGQVFQVSHSFPHPLYDMSLLK (m/z 1164) did not ionize as efficiently as m/z 637. Given that the scan rate in an ion trap is the same whether we are interested only in the product ion of m/z 943 or all products generated from m/z 637, we developed two different methodologies. The first method (referred to as SRM) consisted of scan type SRM with a parent mass of 637, isolation width (m/z) of 2.0, collision energy of 21%, and scan ranges of m/z 943–944. The second method (referred to as PIM or product ion MS/MS) consisted of the same parameters as above but with m/z scan ranges of 600–1100 and with a full scan type. This method is called "product ion monitoring (PIM)" because we isolated the m/z 637 peptide and monitored the entire product of its fragmentation. By monitoring multiple product ions generated from m/z 637 such as 646.38, 775.42, 943.51, and 1072.55, the results were searched with Mascot (Matrix Science) to further confirm the identity of the observed moiety. An additional supplemental confirmation was obtained by using the relative ion ratio distributions among the four fragments to identify the PSA peptide. Conditions were optimized for PIM experiments using the precursor−fragment ion transition of the doubly charged intact peptide m/z 637 (LSEPAELTDAVK) to m/z 943 (PAELTDAVK) and three other fragments.

rPSA Standard Curve Generation on LTQ. The following amounts of rPSA digests were injected into the LTQ in PIM mode: 100, 10, 1, 0.1, and 0.01 femtomoles (fmol). The samples were injected 10 times each.

Serum Digestion for Direct Analysis. All digests were performed using 5 μL of serum, without prior purification or removal of high-abundance proteins. Serum containing 5000 or 300 ng/mL of PSA and female serum (0 ng/mL of PSA) (as determined by ELISA) were denatured with 8 M urea, and the disulfide bonds were reduced with 200 mM dithiothreitol (DTT, Sigma). Following reduction, the samples were alkylated with 500 mM iodoacetamide (Sigma) and desalted using a NAP5 column (GE Healthcare). They were then lyophilized and trypsin-digested overnight in a 37 °C water bath. The samples were C_{18}-extracted and injected into the LTQ in SRM mode. The female serum containing no PSA was also used to perform spiking experiments whereby 0.1 fmol (3 pg) of rPSA digest was added to the mixture prior to injection. Three replicates were analyzed per sample.

Immunoextraction and In-Well Digestion for Immuno-MS. 96-well polystyrene plates were coated with 500 ng/well of PSA monoclonal antibody, as previously described.[24] After overnight incubation, the plates were washed 5 times with 0.9% NaCl solution, and samples were loaded in triplicate, 50 μL of serum (ranging in PSA concentration from 0 to 700 ng/mL) and 50 μL of an aqueous buffer for 1 h. After thoroughly washing the plate with a wash solution, the samples were resuspended in ammonium bicarbonate, methanol, and water and reduced with 2 μL of 20 mM DTT for 30 min at room temperature, yielding a final volume of 100 μL. Alkylation was performed in the dark for 1 h at RT by adding 2 μL of 100 mM iodoacetamide. Finally, in-well trypsin digestion was carried out at 37 °C overnight by adding 0.25 μg of trypsin, such that the final volume in the well was 104 μL. The samples were transferred to microcentrifuge tubes and zip-tipped using the $ZipTip_{C18}$ pipet tip (Millipore) and eluted in 2–5 μL of a buffer consisting of 70% ACN, 0.1% formic acid, 10% water, and 0.02% TFA. Buffer A (40μL) was added, and the entire sample was injected into the LTQ in "PIM" mode.

Data Analysis. The peak area responses recorded for each sample digest were analyzed using QuanBrowser, on Xcalibur software (version 2.0 SR2). Using the PIM methodology, the peak areas for m/z 646.38, 775.42, 943.51, and 1072.55 product ions of m/z 637 were examined manually for verification purposes, and only the peak area for m/z 943 was used for quantification. In this work, we define the limit of detection (LOD) as the lowest concentration of PSA that can be discriminated from zero with 95% confidence (analyte concentration at a signal of background plus 2 standard deviations) and the limit of quantification (LOQ) as the concentration of PSA that can be measured with <20% CV.

Quantification of PSA in serum by ELISA. As a validation and verification step, the concentration of PSA was quantified with a PSA-specific noncompetitive immunoassay developed and validated in our laboratory.[24] Briefly, 96-well polystyrene plates were first coated with 500 ng/well of a PSA monoclonal antibody. After overnight incubation, the plates were washed and loaded with 50 μL of serum or standards and 50 μL of an assay buffer for 1 h. After washing the plate, 100 μL of another biotinylated PSA monoclonal antibody was added, creating a sandwich-type assay, and the plates were incubated for an additional 1 h with gentle shaking. After washing, alkaline phosphatase-conjugated streptavidin was added and incubated for 15 min and washed. Finally, diflunisal phosphate (DFP) and terbium-based detection was performed, essentially as described elsewhere.[25] Fluorescence was measured with a time-resolved fluorometer, the Cyberfluor 615 Immunoanalyzer (MDS Nordion, Kanata, ON, Canada). The calibration and data reduction were performed automatically.

Results

rPSA Peptide Selection and PIM Assay Design on the LTQ. Recombinant prostate-specific antigen (PSA) was digested and analyzed by LC-MS/MS (data-dependent full scan MS on the LTQ while scanning from m/z 450 to 1400). The RAW file was searched with Mascot (Matrix Science) (Figure 1). The MS/MS data provided information for peptide selection and the most abundant y-ion m/z value, allowing for improved design of PIM assays. Four product ions were selected to generate PIM assays (HSQPWQVLVASR (m/z 705), FLRPGDDSSHDLMLLR

MWVPVVFLTL SVTWIGAAPL ILSRIVGGWE CEK**HSQPWQV LVASR**GRAVC GGVLVHPQWV LTAAHCIRNK SVILLGR**HSL**
FHPEDTGQVF QVSHSFPHPL YDMSLLKNRF **LRPGDDSSHD LMLLRLSEPA ELTDAVK**VMD LPTQEPALGT TCYASGWGSI
EPEEFLTPKK LQCVDLHVIS NDVCAQVHPQ KVTKFMLCAG RWTGGKSTCS GDSGGPLVCN GVLQGITSWG SEPCALPERP
SLYTKVVHYR KWIKDTIVAN P

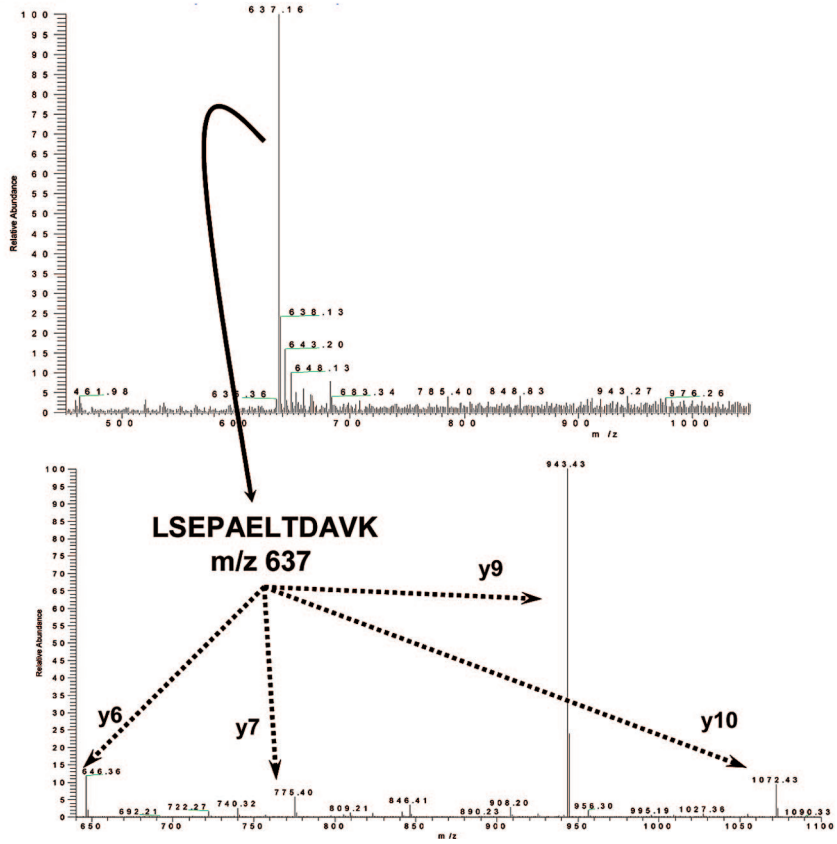
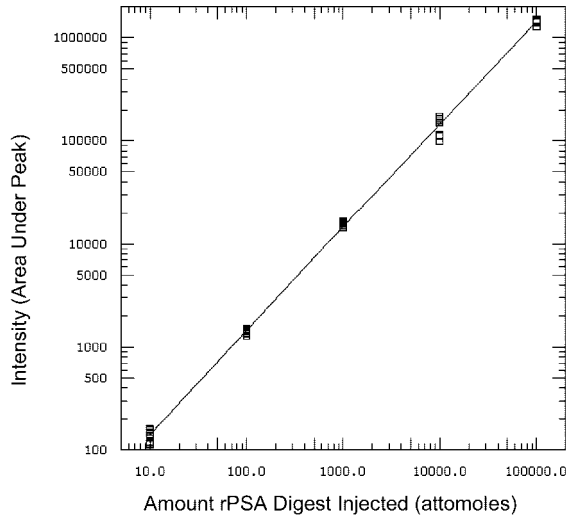


Figure 1. LC-MS/MS analysis of rPSA for peptide selection and fragmentation of *m*/*z* 637. The PSA sequence is shown with single letter amino acid designations. The sequences highlighted in bold represent the product ions for which PIM assays were developed, and the sequences underlined correspond to the chosen proteotypic peptide. An example of a zoomed rPSA digest region of an MS/MS spectrum showing the LSEPAELTDAVK (*m*/*z* 637) peptide is shown. This unique tryptic peptide produces fragment ions shown in the lower panel of the spectrum view with their corresponding y-ion designation. Four fragments were monitored (highlighted with arrows).

(*m*/*z* 936), HSLFHPEDTGQVFQVSHSFPHPLYDMSLLK (*m*/*z* 1164), and LSEPAELTDAVK (*m*/*z* 637)). However, the precursor−fragment ion transition of the doubly charged intact peptide *m*/*z* 637 (LSEPAELTDAVK) to the y-9 ion *m*/*z* 943 was ultimately selected as the proteotypic peptide on the LTQ. This was confirmed with experimental proteomic survey data on the Global Proteome Machine database. Conditions were optimized for LC-PIM on *m*/*z* 637 as described in the Materials and Methods section.

PIM Limit of Detection Using rPSA Digest on the LTQ. A calibration curve was generated using digested recombinant PSA. Very similar data from two different sources of PSA were obtained. The purity of rPSA was verified by Coomassie staining on an SDS−PAGE gel (data not shown). The linear concentration ranges and limits of detection were determined for the pure sample digest. Various amounts of rPSA digests were injected directly into the LTQ in LC-PIM mode (100, 10, 1, 0.1, and 0.01 fmol of PSA). Multiple replicates were performed with good reproducibility and retention times. Figure 2 illustrates the PIM analysis of digested recombinant PSA where five different amounts were injected into the LTQ in multiple injections (10 times) performed on the same day. The integrated peak areas for each dilution of the peptide using QuanBrowser software were used to generate a calibration curve. The CV for the multiple injections was less than 20% with a linear range over 4 orders of magnitude. The limit of detection (LOD), as defined under Materials and Methods, was 10 attomoles. Using the PIM methodology, we were able to confirm the identity of the peaks by searching with Mascot. Furthermore, this approach enabled us to supplement peak identification by utilizing the ion ratios, which are routinely performed in GC-MS experiments as a validation of the target. The premise of this approach is that the fragmentation pattern of a peptide is more or less constant on the LTQ, and the ratios of the various b/y ions can be used to assist in validating a peptide. It is also known that all b/y ions derived from the *m*/*z* 637 ion must coelute. In the PIM spectrum of the *m*/*z* 637 peptide ion, the *m*/*z* 943 fragment is the largest peak (designated as 100%). The remaining three fragments have lower peak heights relative to the y-9 ion, *m*/*z* 646 (8%), 775 (4%), and 1072 (11%). Using QualBrowser, the individual ion chromatogram was extracted, and the relative ratios were determined by dividing the area of the *m*/*z* 943 peak by the area of the other ions (data not shown).

SRM Analysis of Whole Serum. With the optimized "SRM/PIM" assay for LSEPAELTDAVK, we analyzed serum without purification. Five micrograms of protein from unfractionated serum containing 5000 ng/mL of PSA, as measured by ELISA,



PSA, amol	10	100	1,000	10,000	100,000
PSA, ng/mL	0.0075	0.075	0.75	7.5	75
Mean Intensity	142	1408	15653	139776	1423788
Standard Deviation	19	86	642	27558	57424
CV (%)	13.2	6.1	4.1	19.7	4.0

Figure 2. rPSA calibration curve using PIM on the LTQ. Various amounts of rPSA digest were injected (10 times) directly into the LTQ in LC-PIM mode (10, 100, 1000, 10 000, and 100 000 attomoles [amol]). A linear range over 4 orders of magnitude was observed with a limit of detection of 10 attomoles. The mean intensity (arbitrary units), standard deviation (arbitrary units), and % CV are shown in the table below.

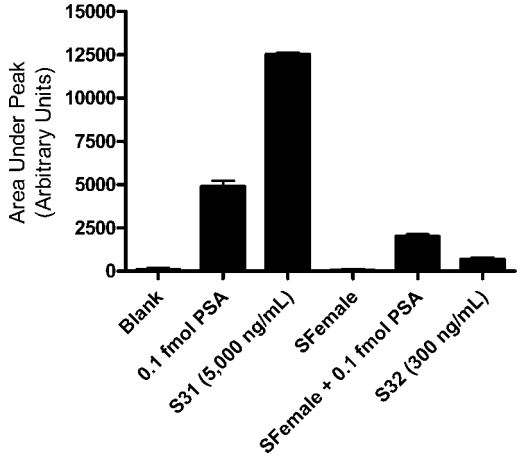


Figure 3. SRM analysis of whole serum without prior purification. Five micrograms of trypsin-digested total protein from serum containing 5,000 ng/mL of PSA (S31), female serum (SFemale) (containing no PSA), and serum containing 300 ng/mL of PSA (S32) was injected into the LTQ in SRM mode. Blank injection refers to buffer A (95% water, 0.1% formic acid, 5% ACN, and 0.02% TFA) only. Spiking experiments were performed whereby 0.1 fmol of digested rPSA was added to digested female serum and injected into the mass spectrometer. The samples were injected in triplicate. Other details of the method are described under the Materials and Methods section. Lower recoveries of spiked samples are due to ion suppression from high-abundance peptides.

was digested with trypsin and loaded onto the LTQ in LC-SRM mode. An intense peak was generated for this sample, as shown in Figure 3. Female serum, containing no detectable PSA by ELISA, was digested and subjected to direct analysis as well. The intensity and peak area for female serum was similar to a blank injection of buffer A only. To further confirm that the detectable moiety was indeed the PSA peptide, spiking experiments were performed whereby 0.1 fmol of digested rPSA was added to digested female serum and injected into the mass spectrometer. An intensity greater than female serum alone was seen in the spiked sample with the correct retention time (Figure 3); however, the area was lower than 0.1 fmol of digested PSA alone. Finally, serum containing 300 ng/mL of PSA was also subjected to the same analysis as above. Using LC-SRM methodology for this sample, PSA was identified; however, the signal was very close to background. We have thus considered that this PSA concentration (300 ng/mL) was the detection limit of this method.

Immuno-MS Assay to Quantitate PSA in Serum. A number of different experimental approaches were examined to quantitate PSA in serum using PIM. These included the use of magnetic beads to immunoextract PSA from serum, in-gel digestion after Coomassie staining, and using a modified-ELISA approach on a microtiter plate. The use of magnetic beads was labor-intensive and not suitable for high-throughput analysis when compared to the alternative approach (modified-ELISA). However, a recent study has shown the utility of using magnetic beads for peptide capture and subsequent LC-MRM on the LTQ.[22] The Coomassie in-gel digest method performed well for rPSA but failed to detect low levels of PSA in a complex mixture such as serum (data not shown). The immuno-MS (modified-ELISA) methodology, as developed here, appeared to be the most promising approach for low level quantification of PSA in serum (Figure 4). The approach consisted of coating a 96-well microtiter plate with a monoclonal PSA antibody that captures both free and antichymotrypsin-bound PSA. The PSA antibody and the captured antigen (PSA, along with any other proteins that were complexed to PSA, and proteins that

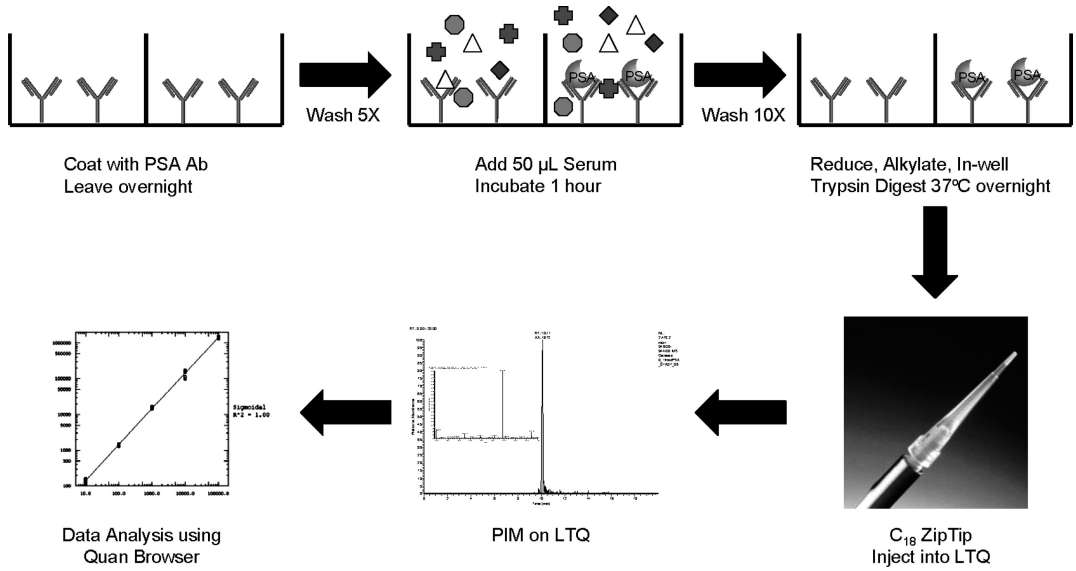


Figure 4. Immuno-MS assay. An illustration of the 96-well microtiter plate methodology used to immunoextract PSA from serum and detect one proteotypic peptide by LC-PIM on the LTQ. More details are given in the text.

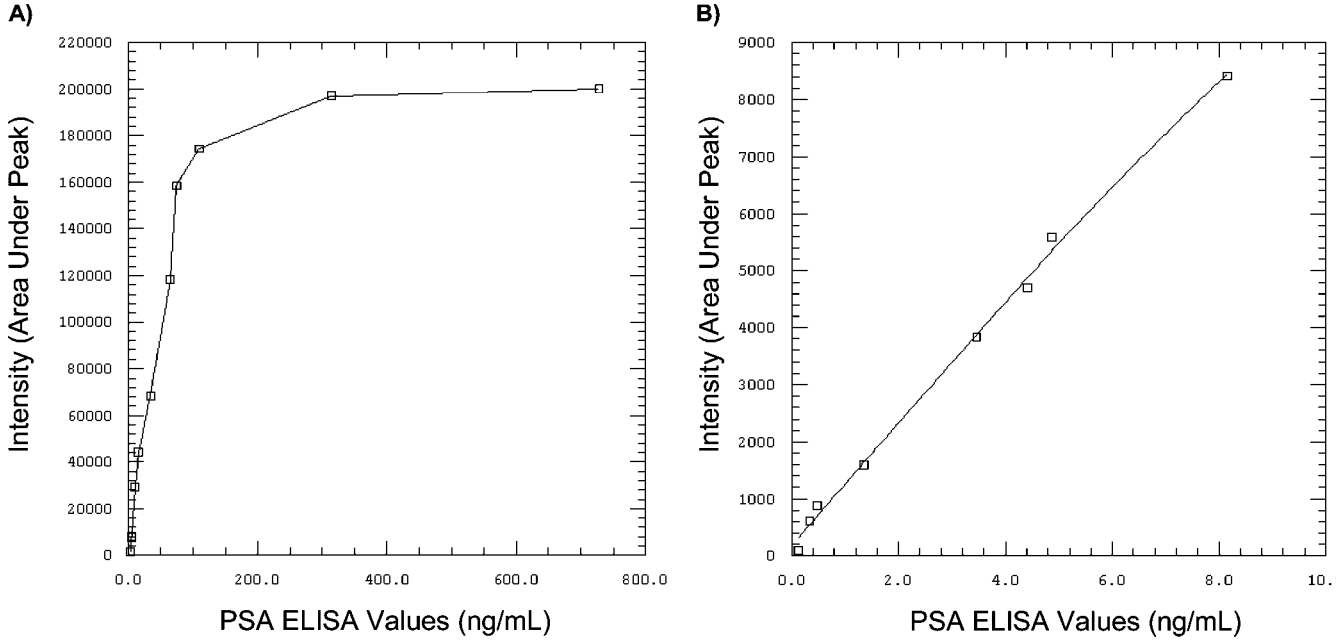


Figure 5. Detection of clinically relevant levels of PSA in serum using a monoclonal antibody. Using the monoclonal immuno-MS methodology, various serum samples, in triplicate, were analyzed. (A) 12 serum samples containing 0–700 ng/mL of PSA. (B) 9 serum samples containing 0–8 ng/mL of PSA. The LOD and LOQ were 0.1 and 1 ng/mL, respectively.

remained after washing) were reduced, alkylated, and trypsin-digested in-well. A number of different conditions were examined to optimize the immunoextraction procedure including type of buffers, amount of coating antibody used, washing conditions, in-well digestion versus glycine elution, followed by trypsin digestion, and so forth. During optimization, the in-well digested samples were run in identification mode (LC-MS/MS). Approximately 10 proteins were identified in each well. These were mainly made up of albumin, apolipoproteins, a few other high-abundance serum proteins, and PSA (data not shown). Serum samples, in triplicate, were analyzed with this method, and in-well digestion was performed, followed by C_{18} extraction of the peptides and injection into the LTQ in LC-"PIM" mode. Serum PSA values ranged from 0 to 700 ng/mL (Figure 5A). No detectable peak area was observed for the serum sample containing 0 ng/mL of PSA, as measured by ELISA. At levels above 100 ng/mL of PSA in serum, the signal reached a plateau. Lower concentrations of PSA in serum (0–8 ng/mL) were examined to determine the LOD and LOQ (Figure 5B). The LOD was <0.1 ng/mL, and the LOQ was 1 ng/mL. The % CV of serum samples with 1, 3, and 8 ng/mL was 20, 10, and 14%, respectively. To examine the background signals, wells with no sample added (only the coating antibody) were processed in the same manner, yielding areas similar to serum with no PSA (data not shown).

Polyclonal Immuno-MS Assay to Quantitate PSA. Since there are no commercially available monoclonal antibodies for many candidate biomarkers identified from discovery phase experiments, the utility of expanding this method to a polyclonal antibody was explored. The samples were processed in

a manner similar to that outlined in Figure 4using an anti-PSA polyclonal antibody. The LOD was 0.5 ng/mL, while the LOQ was 1 ng/mL (data not shown).

Discussion

A highly sensitive and high-throughput verification platform is desperately needed to bridge the gap between discovery and validation phases of candidate biomarkers. In addition to its important role as a discovery platform, the use of MS as a quantification tool is gaining popularity. It has been suggested that multiple reaction monitoring (MRM) assays will enable fast, high-throughput verification of candidate biomarkers in blood. The introduction of software tools to predict proteotypic peptides, along with improved resolution of MS instruments, will facilitate faster optimization of MRM assays.[10] The MRM Initiated Detection And Sequencing (MIDAS) strategy has enabled detection of low level phosphorylated peptides with increased confidence.[26,27] In this approach, the protein sequence is digested in-silico. Highly favorable fragment ions are predicted, and potential MRM transitions are generated for all the peptides. Recent studies suggest that at least 200 MRM assays can be performed in a single LC-MRM-MS run.[12] Proteins present at 1 μg/mL are measurable by MRM in whole plasma with minimal sample fractionation.[12] Liao et al. used 2D-LC-MS/MS to identify candidate biomarkers in synovial fluid proteome and used MRM for validation of 33 candidate proteins in serum on a triple quadrupole mass spectrometer.[28] Serum samples were depleted of abundant proteins, followed by size-fractionation prior to MRM. The lowest detectable concentration was 350 ng/mL. Another recent example is the quantification of the candidate biomarker Zn-α_2 glycoprotein (ZAG) for prostate cancer.[29] A LOD of 80 ng/mL and a LOQ of 320 ng/mL of ZAG in serum was achieved. However, to evaluate the potential of a candidate molecule as a cancer biomarker, serum levels in the low ng/mL range should be readily quantifiable to be able to differentiate cancer from normal.

Stable Isotope Standards with Capture by Anti-Peptide Antibodies (SISCAPA) is an emerging technology that is gaining popularity. In this approach, the sample is digested; an internal standard peptide labeled with a stable isotope is added; and enrichment of low-abundance peptides is achieved by capture with immobilized peptide antibodies, followed by MS.[30] The sensitivity of this method was demonstrated by detecting two moderate-abundance proteins (hemopexin and ACT), which were present at 0.3–1.15 mg/mL in serum. In the present study, we report a PIM assay that can be performed on the LTQ and using a purified recombinant PSA digest, a limit of detection of 10 attomoles with CV < 20%, and a linear range over 4 orders of magnitude was achieved. Furthermore, we successfully quantified PSA in serum of prostate cancer patients down to less than 1 ng/mL and obtained detection limits of 0.1 ng/mL using the monoclonal immuno-MS approach followed by PIM on the LTQ. The linear concentration range and limit of detection are affected by the binding capacity of the solid phase and instrument sensitivity. Therefore, the LOD for the pure analyte and a complex mixture such as serum will differ.

PSA is a glycoprotein that belongs to the serine protease family. Its catalytic triad involves residues His 57, Asp 102, and Ser 195.[31] The proteotypic peptide used in this study is not in the vicinity of the glycosylation site or near the catalytic triad.[32] In addition, most of the serum PSA is complexed with the protease inhibitor alpha-1-antichymotrypsin (ACT); however, a small fraction remains free in circulation.[33,34] The area of the theoretical ACT binding site on PSA is not near the proteotypic peptide.[35,36] Therefore, the presence of ACT or glycosylation should not interfere with the m/z 637 peptide of interest in the immuno-MS assay. The monoclonal antibody used in this study recognizes total PSA (both free and bound PSA). While our approach is antibody-dependent, we believe that at present achieving levels of sensitivity in the low nanogram/milliliter range using a mass spectrometer is highly unlikely without antibody purification, while maintaining a simple protocol, robustness, reproducibility, and high-throughputness. For candidate molecules without an available antibody, it is possible that our methodology could work with a peptide antibody.

In conclusion, our methodology clearly demonstrates numerous advantages, as also discussed by others.[22] Typical MRM assays are rather slow, making them time-consuming for examining multiple serum samples for biomarker verification of a candidate. Our approach, based on a 25-min gradient for each sample, combined with processing 96 serum samples (premass spectrometry) at once in a microtiter plate, enabled a much faster processing time, despite the manual C_{18} extraction step. Furthermore, similar in principle to a sandwich ELISA, our method works with a single antibody (monoclonal or polyclonal) on a 96-well microtiter plate with the mass spectrometer serving as the specific detector. As well, the advantages of an ion trap (a widely used instrument) for assay optimization of candidate biomarkers are evident, as presented in this study. Using the same instrument for discovery and validation phases facilitates choosing the proteotypic peptide to optimize MRM assays, with the aid of publicly available databases such as GPM and PeptideAtlas. Taken together, the use of emerging software tools such as MIDAS in combination with discovery phase instruments such as ion traps for early stage optimization shows the promise of peptide serum MRM may become a reality.

Nonstandard Abbreviations: ACN, acetonitrile; CID, collision-induced dissociation; CV, coefficient of variation; DFP, diflunisal phosphate; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; LC-MS/MS, liquid chromatography tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; MS, mass spectrometry; PIM, product ion monitoring; PSA, prostate-specific antigen; SRM, selected reaction monitoring; TFA, trifluoroacetic acid.

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