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Analysis of Serum Total and Free PSA Using Immunoaffinity Depletion Coupled to SRM: Correlation with Clinical Immunoassay Tests

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Abstract

Recently, selected reaction monitoring mass spectrometry (SRM-MS) has been more frequently applied to measure low abundance biomarker candidates in tissues and biofluids, owing to its high sensitivity and specificity, simplicity of assay configuration, and exceptional multiplexing capability. In this study, we report for the first time the development of immunoaffinity depletion-based workflows and SRM-MS assays that enable sensitive and accurate quantification of total and free prostate-specific antigen (PSA) in serum without the requirement for specific PSA antibodies. Low ng/mL level detection of both total and free PSA was consistently achieved in both PSA-spiked female serum samples and actual patient serum samples. Moreover, comparison of the results obtained when SRM PSA assays and conventional immunoassays were applied to the same samples showed good correlation in several independent clinical serum sample sets. These results demonstrate that the workflows and SRM assays developed here provide an attractive alternative for reliably measuring candidate biomarkers in human blood, without the need to develop affinity reagents. Furthermore, the simultaneous measurement of multiple biomarkers, including the free and bound forms of PSA, can be performed in a single multiplexed analysis using high-resolution liquid chromatographic separation coupled with SRM-MS.

Keywords

Selected reaction monitoring; immunoaffinity depletion; total PSA; free PSA; serum; immunoassay

Introduction

Cancer-specific protein biomarkers have great potential for informing early detection, diagnosis, and prognosis, as well as monitoring disease progression, response to treatment and therapy, and detection of early recurrence^{1–4}. One of the most familiar examples is the

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development and subsequent routine application of measuring prostate-specific antigen (PSA, also known as human kallikrein 3, hKLLK3), a protein biomarker that has revolutionized the management of prostate cancer⁵. The American Cancer Society has recommended routine use of the total PSA test for early detection of prostate cancer, in combination with digital rectal exam, for males age 50 and older^{6, 7}. However, the increase in early detection of prostate cancer achieved via application of routine PSA testing has also introduced clinical challenges, particularly in over-diagnosis and in the stratification of risk for subsequent development of highly invasive prostate cancer^{8, 9}. The inability of current PSA tests to distinguish between indolent and aggressive disease has prompted a recent draft guideline from the US Preventive Service Task Force recommending against PSA screening for anyone without obvious symptoms of prostate cancer⁷. Thus, there have been numerous efforts to refine PSA assays to improve the discriminatory power of the test.

One such approach to increase the specificity of PSA screening exploits the biochemical characteristics of PSA. PSA is a 237-amino acid, single chain, serine protease, synthesized in the ductal epithelium and prostatic acini and secreted as a glycoprotein into the lumina of the prostatic ducts. In blood there are two mature forms of PSA, free and bound. The bound form is primarily complexed with protease inhibitors, such as alpha-1- antichymotrypsin (ACT) or alpha-2-macroglobulin (A2M). Even though the molar concentration of ACT and A2M are 1,000-fold higher than that of PSA, up to 45% of PSA in blood is still in an unbound or free form. Clinical assays for 'total PSA' do not discriminate between the complexed and the free forms of PSA. However, since A2M engulfs the PSA molecule and blocks the access of anti-PSA antibodies, the 'total PSA' assay measures essentially free PSA and the PSA-ACT complex.

Although a total serum PSA concentration of 4.0 ng/mL is typically used as an indication for prostate biopsy¹⁰, results from the Prostate Cancer Prevention Trial indicated that up to 27% of men with total PSA concentrations between 3.1–4.0 ng/mL have prostate cancer and that there is a significant risk of detection for prostate cancer over all PSA concentrations^{11, 12}. At a total PSA concentrations exceeding 10 ng/mL, approximately 50% of the patients had cancer^{1, 5}. At the intermediate range of total PSA concentrations, 4.0–10.0 ng/mL, 25 – 35% of patients had cancer based on biopsy¹. In an attempt to improve the predictive power of PSA assays, assays have been developed that discriminate between 'free' (uncomplexed) and 'total' (free + ACT-bound) PSA. Determination of the percent free PSA (%fPSA = free PSA/total PSA × 100) is recommended for risk assessment of patients with total PSA concentrations between 4–10 ng/mL. A %fPSA of >25% indicates a low risk of cancer (e.g., probability = 8%) whereas a %fPSA of <10% suggests a high risk (e.g., probability = 56%)^{13–15}. A cut-off value of 25% for %fPSA detected 95% of cancers and reduced the biopsy rate by 20% when total PSA levels were between 4–10 ng/mL¹³.

While commercially available immunoassays for both total and free PSA work well and have FDA approval, the development of these assays required the identification and characterization of immunoassay-qualified antibodies that could accurately and reproducibly discriminate between the two biochemical forms of PSA. The investment in time and resources required to generate such immunoassays is considerable, and this requirement often impedes the development of clinically useful protein-based assays in the absence of compelling pre-clinical data. A case in point is the fusion transcript TMPRSS2-ERG, which is present in approximately 50% of prostate cancers^{16–18}. The fusion transcript is expressed as a truncated protein^{19, 20}. However, no commercial immunoassays to detect the truncated ERG as a cancer biomarker have been developed.

The focus of this report is the development and evaluation of an alternative approach that circumvents the necessity to develop affinity reagents, known as selected reaction

monitoring (SRM) or multiple reaction monitoring²¹. SRM exploits the unique features of the triple quadrupole mass spectrometer that enable two levels of mass selection (precursor and fragments) and a relatively long dwell time over a narrow m/z range of interest, resulting in high selectivity and sensitivity. A further analytical requirement, identical liquid chromatography (LC) elution times for multiple transitions of the same target analyte, filters out the co-eluting background ions with great effectiveness, even from an extremely complex biological matrix, e.g., tryptic digest of plasma. The ion currents of fragment ions can provide accurate quantification of analyte concentration with stable isotope-labeled internal standards. Applying modern triple quadrupole mass spectrometers with high-duty cycles and “smart” SRM assay configurations (e.g., utilizing the peptide LC elution time to “schedule” SRM events), a large number of protein targets can be monitored during a single LC-SRM-MS analysis. These features, combined with several front-end enrichment methods that have been recently developed, e.g., major serum/plasma protein depletion alone, or in conjunction with chemical²², chromatographic²³, and antibody-based²⁴ enrichment, have led to reliable detection of targeted proteins at the low ng/mL level or better in serum/plasma²⁵.

Published detection limits for total PSA in plasma/serum using SRM-MS are in the 1–10 ng/mL range, and are highly dependent on the sample preparation and the MS detection methods used^{23, 26–28}. In this study, we describe simple, yet effective, immunoaffinity depletion-based workflows and demonstrate for the first time the detection of both the total and free PSA at the low ng/mL concentration in human clinical serum samples, using LC-SRM-MS without the requirement for specific PSA antibodies. Furthermore, the correlation observed between clinically approved immunoassay tests and SRM-based assays for both the total and free PSA measurements exceeded 0.90, even in a set of blinded samples. This LC-SRM-MS approach can obviously be extended to the quantitative analysis of many other biomarkers that have similar bound and free interactions. More generally, the strong correlations obtained between the LC-SRM-MS analyses and clinical immunoassays suggest that SRM can be used as a reference method for preliminary determination of assay validity, prior to the development of more conventional immunoaffinity-based assays that would be used in clinical laboratories.

Material and Methods

Materials and chemicals

PSA protein purified from human seminal fluid was procured from Calbiochem (San Diego, CA). Ammonium bicarbonate, dithiothreitol (DTT), and iodoacetamide were from Sigma-Aldrich (St. Louis, MO). Sequencing grade modified porcine trypsin was from Promega (Madison, WI).

Serum samples and standard PSA protein for spiking

The human female serum sample was purchased from the Biochemed Services (Winchester, VA). It was a pooled sample from multiple healthy female volunteers. The initial protein concentration was 80.6 mg/mL as determined by BCA protein assay (Pierce, Rockford, IL). All protein sample processing was performed at 4 °C. Purified human PSA was spiked into the female serum sample at different concentrations (0.5, 1, 1.5, 2.5, 5, 7.5, 10, 25, and 50 ng/mL of each protein in serum) to construct a calibration curve. Control serum without any spiked proteins was also prepared and went through the same sample preparation process.

IgY14 immunoaffinity depletion

The commercially available Seppro IgY14 LC10 column from Sigma-Aldrich was used to remove fourteen high abundance proteins (albumin, α 1-antitrypsin, IgM, haptoglobin, fibrinogen, α 1-acid glycoprotein, HDL, LDL, IgG, IgA, transferrin, α 2-macroglobulin, and

complement C3) from human serum samples. This column is based on avian antibody (IgY)-antigen interactions and optimized with buffers for sample loading, washing, eluting, and column regeneration. Specific removal of the 14 high abundance proteins depletes approximately 95% of the total protein mass from human plasma²⁹. Serum samples were diluted 6-fold with the dilution buffer, passed through a 0.45 μm spin filter, and centrifuged for 1 min at $9,200 \times g$ to remove any particulate materials present in the sample. Fifty microliters of the serum samples with and without spiked PSA proteins were passed through the IgY14 column using an Agilent 1200 series HPLC system (Santa Clara, CA) per the manufacturer's recommendation. The three buffers (dilution: 100 mM Tris-HCl with 0.15 M NaCl, pH 7.4; stripping: glycine-HCl, 0.1 M, pH 2.5; and neutralization: Tris-HCl, 0.1 M, pH 8.0) were used in this depletion process that consisted of sample loading, washing, eluting, and neutralization followed by a re-equilibration with a total cycle time of about 70 min. The flow-through fractions were collected and concentrated in Amicon Ultra-15 (3-kDa nominal molecular mass limit; Millipore, Bellerica, MA) concentrators followed by buffer exchange to 50 mM NH_4CO_3 , pH 8.0. Protein concentration was then determined by BCA protein assay.

Tandem IgY14/SuperMix immunoaffinity depletion

In the development of the SuperMix column, chickens were immunized with human plasma proteins after removal of 14 high abundance proteins. The majority of the resultant antibodies incorporated into the SuperMix resin recognize moderate abundance proteins and/or immunoreactive proteins³⁰. The SuperMix LC5 column (Sigma-Aldrich) is designed for use in conjunction with the IgY14 LC10 column to enable the detection of low abundance proteins. In an in-line tandem column setting and fully automated fashion, 150 μL of the sample was continuously passed through first the IgY14 LC10 column then the SuperMix LC5 column. The separation conditions used in the tandem depletion were similar to those described above for the IgY14 depletion. The flow-through fractions were collected, concentrated and buffer exchanged as described above. Protein concentration was measured using the BCA protein assay. The flow-through fraction contained approximately 1% of the original total protein mass³⁰.

Protein digestion

One hundred micrograms of proteins from the IgY14 and IgY14/SuperMix flow-through fractions were denatured and reduced in 50 mM NH_4CO_3 , pH 8.0 with 8 M urea and 10 mM DTT for 1 h at 37 °C. Protein cysteinyl residues were alkylated with 40 mM iodoacetamide for 30 min at room temperature in the dark. The resulting protein mixtures were diluted 10-fold with 50 mM NH_4CO_3 , pH 8.0, before sequencing grade modified porcine trypsin (Promega) was added at a trypsin:protein ratio of 1:50 (w/w). The samples were incubated at 37 °C for 3 h. The tryptically digested samples were then loaded onto 1-mL SPE C18 columns (Supelco, Bellefonte, PA) and washed with 4 mL of 0.1% TFA, 5% acetonitrile. Peptides were eluted from the SPE column with 1 mL of 0.1% TFA, 80% acetonitrile and lyophilized. Final peptide concentration was determined by BCA protein assay. Peptide samples were stored at -80 °C until time for further analysis.

LC-SRM-MS analysis

Immediately prior to LC-SRM-MS analysis, ^{13}C - and ^{15}N -labeled (at C-terminal Lysine) synthetic peptides (AQUA-Basic grade with >95% purity and >99% isotopic enrichment, Thermo Scientific, Waltham, MA) were added to the digested serum samples at a final concentration of 0.25 fmol/ μL . Peptide samples were analyzed using a nanoACQUITY UPLC (Waters, Milford, MA) coupled on-line to a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific). The LC system was equipped with a Waters nanoACQUITY UPLC BEH C18 analytical column (particle size 1.7 μm , 75 μm i.d., 250

mm in length, pore size 130 Å) and a Waters nanoACQUITY UPLC Symmetry C18 trap column (particle size 5 µm, 180 µm i.d., 20 mm in length, pore size 100 Å). Peptides were loaded into the trap column for 1 min with 99% mobile phase A (0.1% formic acid in water) using a flow rate of 10 µL/min and were eluted using a flow rate of 350 nL/min. This capillary column was connected via a Valco 100-µm i.d. stainless steel union to a chemically etched fused 20 µm-i.d. silica emitter produced in-house³¹. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). After loading 2 µL of the peptide solution onto the column (1.0 µg nominally), a linear gradient elution was performed by increasing the mobile phase composition from 0 to 70% B over 45 min. The inlet capillary of the mass spectrometer was maintained at 200 °C with an electrospray ionization voltage of 2.5 kV that was applied to the Valco union.

Optimization of SRM assay

Initially, 5 transitions were used for each of the peptides according to their strong signal intensity in the GPM spectral library and/or Skyline selection³². The three best transitions were selected for further analysis and the most intensive transitions from each peptide were used for final quantification. For collision energy (CE) optimization, five CE values were used for individual transitions with a range of 8 voltages where manufacturer-provided CE equation values were maintained in the middle. The raw data were imported to Skyline to evaluate the optimal CE values.

Data analysis

All raw data acquired from the triple quadrupole MS were imported into 'Skyline' data analysis software³² for peptide quantification. Peak detection and integration were determined based on two criteria: 1) the same retention time and 2) approximately the same relative SRM peak intensity ratios across multiple transitions between light peptides and heavy peptide standards. All data were manually inspected to ensure correct peak detection and accurate peak integration. Salvitzky-Golay smoothing was used. The Skyline results were then exported to Microsoft Excel for further analysis (e.g., linear regression and Pearson correlation) and plotting.

Immunoassay measurements of clinical specimens

Clinical serum samples from patients undergoing PSA testing for prostate cancer were provided by UT Health Science Center at San Antonio (I. Thompson and R. Leach) and the Johns Hopkins Medical Institutions (D. Chan and L. Sokoll) and were analyzed in a CLIA certified laboratory at Johns Hopkins Medical Institutions. All experimental procedures were approved by the Institutional Review Boards of the Johns Hopkins Medical Institutions (Baltimore, MD), UT Health Sciences Center (San Antonio, TX) and Pacific Northwest National Laboratory (Richland, WA) in accordance with federal regulations. Analysis of the 33 blinded serum specimens for total PSA was performed using the Tosoh AIA-2000 ST PSA assay (Tosoh Bioscience, South San Francisco, CA). All other samples, including the PSA spike-in female serum samples, were analyzed for total and free PSA using the Beckman Coulter Hybritech assays on the Access Immunoassay System (Beckman Coulter, Inc., Brea, CA).

Results and Discussions

A number of recent studies have reported the detection of total PSA at low ng/mL levels in blood plasma/serum using different sample preparation strategies and SRM methods^{23, 26, 27}. The aim of the present work was to develop robust analytical workflows and SRM assays that enable highly sensitive detection of both total and free PSA using LC-SRM-MS without the requirement for specific PSA antibodies. The optimized sample

processing workflows, illustrated in Figure 1, allow for accurate quantification of both total and free PSA in blood serum using single-stage IgY14 and tandem IgY14/SuperMix immunoaffinity depletion, respectively, through external calibration. Independent sets of clinical serum samples were then analyzed using both clinical immunoassay tests and the SRM-based assays.

Principle of using IgY14 and tandem IgY14/SuperMix immunoaffinity depletion approaches for measurements of total and free PSA in blood plasma/serum

Immunoaffinity depletion of multiple high abundance proteins from blood serum/plasma represents an effective and reproducible strategy for highly sensitive, quantitative SRM measurements of blood proteins, without the need for specific antibodies (thus avoiding the high cost and long lead time for antibody development against newly discovered protein targets)²³. Much of the PSA in blood serum is complexed to carrier proteins, predominantly ACT, which can bind up to 85% of all PSA molecules in blood³³. In the tandem immunoaffinity depletion (IgY14/SuperMix) method, ACT is not removed in the first stage IgY14 depletion, as ACT is not one of the top 14 high abundance proteins targeted by IgY14; however, ACT is targeted in the second stage SuperMix depletion which removes the next level of moderate abundance proteins (including ACT). In addition to ACT, a second major blood serum/plasma protein, A2M, is targeted by IgY14 immunoaffinity depletion; A2M can form a complex with PSA³⁴ that is not detected by conventional immunoassays for total PSA. Therefore, immunoaffinity depletion with only the IgY14 column should produce samples representing 'total PSA', while samples produced with tandem IgY14/SuperMix depletion should contain only 'free PSA' in the flow-through fraction. The guiding principles established here for measuring both free and total PSA represent a new experimental strategy that may be extended to other biomarkers present in blood plasma/serum that display similar complexation/binding characteristics and that can be analyzed in a single multiplexed analysis using high-resolution liquid chromatographic separation coupled with SRM-MS.

Development and evaluation of the LC-SRM-MS method for detection of PSA in serum

Selection of appropriate proteotypic peptides and their corresponding SRM transitions is critical and largely responsible for the ultimate sensitivity and specificity of an LC-SRM-MS assay. Previous studies reported that two peptides, LSEPAELTDAVK and IVGGWECEK (the cysteine is carboxyamidomethylated), provided the most intensive ESI signals for PSA^{23, 26}, which was confirmed in our analyses of a tryptic digest of pure PSA using both LC-MS/MS on an LTQ-Orbitrap hybrid instrument and direct infusion on the triple quadrupole instrument (data not shown). Synthetic peptides were obtained for these two peptides and collision energies for corresponding transitions (636.8/943.5, 636.8/533.3 and 636.8/472.3 for LSEPAELTDAVK; 539.3/964.4, 539.3/865.4 and 539.3/436.2 for IVGGWECEK) were optimized accordingly. The sensitivity of these two peptides in terms of PSA detection in blood serum was further evaluated by spiking a female serum sample with PSA protein followed by LC-SRM-MS analysis.

To determine the LOD and limit of quantification (LOQ) of our LC-SRM-MS workflows for detection of PSA in serum, intact PSA protein was spiked into a pooled control female serum sample at 10 different, clinically relevant concentrations (50, 25, 10, 7.5, 5, 2.5, 1.5, 1, 0.5, and 0 ng/mL); the endogenous total and free PSA concentrations in this control female serum sample were 0.14 ng/mL and 0.03 ng/mL, respectively, as determined by immunoassay. As the ratio of free to total PSA was stable over incubation times of 5 min to 5 h (data not shown), the spiked PSA was incubated in female serum samples for 30 min prior to processing separately by single-stage IgY14 and tandem IgY14/SuperMix depletion, each in three process replicates, and each replicate was analyzed three times by LC-SRM-

MS (i.e., for a total of 9 analyses for each data point for both the total PSA and free PSA calibration curves). Heavy-isotope-labeled synthetic peptides (LSEPAELTDAVK* and IVGGWECEK*; where * denotes the ^{13}C - and ^{15}N -labeled Lys residues) were added to each of the samples at a fixed concentration of 0.25 fmol/ μL , and the light-to-heavy peptide peak area ratio (L/H) and the PSA protein spike-in concentrations were used to generate the calibration curve equation. The same concentrations of heavy-isotope-labeled peptide standards were also spiked into the clinical samples, and the measured L/H values and established equation were then used to calculate the endogenous surrogate peptide concentrations in the clinical samples.

Three transitions for both light and heavy versions of the two PSA peptides (i.e., a total of 12 transitions) were monitored for the entire LC-SRM-MS analysis and acquired at unit resolution in Q1 and Q3 with a dwell time of 20 ms for each transition; approximately 30 data points per chromatographic peak were obtained and no scheduling was used given the simplicity of the SRM assay. Transitions of peptide LSEPAELTDAVK provided a much higher sensitivity (>10-fold; data not shown) than transitions tracking peptide IVGGWECEK (not detectable in many of the lower spike-in levels) in the serum background. Therefore, only peptide LSEPAELTDAVK was considered for the quantification of PSA in serum. All three transitions, as well as the corresponding heavy peptide transitions, were required to be detected within the same retention time in order to be considered as confident detection of the peptide. Ideally, multiple transitions should be used per peptide for accurate quantification by LC-SRM-MS. However, it was evident that two of the three transitions (636.8/533.3 and 636.8/472.3) had interference from the serum matrix (higher than normal and more variable intensities), as compared to the transition intensity ratios of the heavy peptide (data not shown), and therefore, only transition 636.8/943.5 was used for the final quantification of PSA (although the other two transitions were also detected at the same LC elution time). This is consistent with previous reports on SRM studies of PSA in plasma/serum²⁶. The noise in the analyses was evaluated in the local background areas at both the left and right sides of the chromatographic peak of peptide LSEPAELTDAVK in both the IgY14- and IgY14/SuperMix-depleted samples.

In the case of IgY14 depletion for detection of total PSA, owing to the effectively reduced dynamic range in protein concentration and an enrichment factor of approximately 20 for low abundance proteins after the depletion, and the optimized LC-MS/MS conditions (e.g., using sub-2- μm particles in the high-pressure LC separation, and a triple quadrupole instrument with more effective ion transmission), a LOD of 1.5 ng/mL and a LOQ of 5 ng/mL (both nominal PSA protein spike-in levels), as determined to be greater than 3 and 10 times the standard deviation of local noise, respectively, were achieved for detecting total PSA in serum without further fractionation²³, or chemical²⁸ or chromatographic enrichment²⁶. In the case of tandem IgY14/SuperMix depletion, although it removes the PSA-ACT complex and leaves a much smaller amount of 'free PSA' for the final LC-SRM-MS analysis, it further reduces the dynamic range in protein concentration and provides an enrichment factor of approximately 100 for the low abundance proteins in the final product. As a result, a LOD of 1.5 ng/mL and a LOQ of 5 ng/mL (both nominal "total" PSA protein spike-in levels) were achieved for detection of free PSA in serum. Example extracted ion chromatograms for the IgY14 and IgY14/SuperMix depletion SRM calibration curve experiments are available in Supplementary Figures 1 and 2, respectively. The calibration curves, established through linear regression function of the light-to-heavy peptide peak area ratio of LSEPAELTDAVK (transition 636.8/943.5) versus the nominal PSA spike-in concentration in both the IgY14 ($y = 0.0064x + 0.0255$) and IgY14/SuperMix ($y = 0.0263x + 0.0981$) depletion experiments, both showed good linearity in the measured concentration range with a determination (R^2) of 0.99 (Figure 2). The coefficient of variation (CV) for LC-SRM-MS analyses of the IgY14- and IgY14/SuperMix-depleted samples ranged from 5.2–

27.0% and 3.5–16%, respectively, for the process replicates of the calibrators. It is evident that in addition to further enrichment of the low abundance proteins, the IgY14/SuperMix depletion often provides a cleaner background and hence, less interference in the LC-SRM-MS analysis (unpublished results) due to the effective removal of the moderate abundance proteins. Therefore, for the same nominal PSA spike-in level, the calibration curve data from IgY14/SuperMix depletion showed better sensitivity than that from IgY14 depletion (comparing Figure 2B to 2A), even though a large portion of the PSA molecules (up to 85%³³) were co-depleted as the PSA-ACT complex in IgY14/SuperMix depletion.

PSA recovery information for the entire sample preparation process (including depletion, trypsin digestion, and sample clean-up), with and without co-depletion of PSA with ACT, was calculated for the IgY14/SuperMix and IgY14 calibration curve experiments (detected nominal PSA spike-in levels ranging from 1.5 to 50 ng/mL), respectively. Corresponding PSA recovery was calculated by comparing the SRM signal of the light peptides to that of the heavy peptides that were spiked into the samples (0.25 fmol/μL) right before the final SRM analysis: 18.8–25.5% recovery for the IgY14 depletion workflow (CV was on average 18.1% for process replicates) and 10.8–14.7% recovery for the IgY14/SuperMix depletion workflow (CV was on average 20.3% for process replicates). The calculated recovery was the highest for the 1.5 ng/mL spike-in levels in both the IgY14 and IgY14/SuperMix experiments, presumably due to the influence from the endogenous PSA. The data showed that: 1) both immunoaffinity depletion-based workflows are very reproducible; and 2) the measured PSA recovery was consistent with the postulate that IgY14 depletion provided measurement of “total PSA” (18.8–25.5% recovery) and that “free PSA” was generated in the tandem IgY14/SuperMix depletion method (10.8–14.7% recovery).

Because IgY14 depletion removes approximately 95% of protein mass whereas IgY14/SuperMix depletion removes approximately 99% of protein mass from blood serum/plasma, 3-fold larger serum sample volumes were used in the IgY14/SuperMix depletion to make certain that sufficient protein was available for downstream LC-SRM-MS analysis following depletion. After depletion, 35% and 15% of the depletion product (flow-through fractions) were used in the IgY14/SuperMix and IgY14 calibration curve experiments, respectively. Therefore, considering these factors, the PSA signal in IgY14/SuperMix depletion should be 7-fold stronger than that in IgY14 depletion, if there was no additional loss of PSA in second stage SuperMix depletion. The calibration curve experiments, however, showed only an approximately 4-fold increase in the IgY14/SuperMix experiments (comparing Figure 2B to 2A), again this is consistent with detection of total PSA and free PSA respectively in serum samples applying IgY14 only and tandem IgY14/SuperMix depletion.

Application to clinical serum specimens and correlation of SRM and immunoassay results

To demonstrate the capability of the LC-SRM-MS workflows to detect low concentration serum biomarker candidates in actual clinical samples, and to compare the results from SRM with immunoassay, the current “gold standard” in clinical laboratories, an initial set of 9 clinical serum samples with known total PSA concentration ranging from 0.3–18.9 ng/mL by immunoassay (obtained from I. Thompson and R. Leach) were processed separately by IgY14 depletion, and analyzed by LC-SRM-MS in three technical replicates given the sample size limitation. PSA was reproducibly detected in 8 of 9 samples; the CVs were in the range of 2.6–23.8%. Our SRM method was unable to detect PSA in a single sample with a total PSA of 0.3 ng/mL, measured by immunoassay. The total PSA concentrations calculated using the IgY14-SRM calibration curve equation ($y = 0.0064x + 0.0255$) and total PSA levels measured by immunoassay for the 8 serum samples showed a high level of correlation ($R^2=0.95$; see Figure 3).

However, the absolute total PSA concentrations calculated using the total PSA calibration curve equation obtained with the nominal PSA spike-in levels were on average 2.8-fold higher than that provided by immunoassay (Figure 3). This is probably due to the loss of spiked-in PSA from binding/complexation to other serum proteins, e.g., A2M³⁵. To confirm this, the initial calibration curve samples spiked with different concentrations of PSA protein, but without any further processing, were measured by immunoassay for total and free PSA concentrations. Indeed, the immunoassay test consistently provided total PSA concentrations of only approximately 40% of the nominal spike-in levels for all calibration curve samples (Table 1). This was also in total agreement with previous report that 60% of immunoreactivity of seminal fluid PSA can be lost after incubation with female serum due to complexation to A2M; binding to ACT, on the other hand, had almost no effect on signal loss for the immunoassays³⁵.

Likewise, the PSA-A2M complexes would not be detected by SRM-MS, because A2M is selectively removed during IgY14 depletion in our workflows (see Figure 1). This also explained why the overall recovery for the total PSA workflow was <25.5%. Therefore, under these specific circumstances immunoassay measurements of the PSA-spiked calibration curve samples are necessary to accurately calculate the correlation between our SRM results and the immunoassay measurements of the clinical samples. We constructed calibration curves by regressing SRM measured total and free PSA values on immunoassay measured values via the standard linear model for IgY14-SRM ($y = 0.0182x + 0.0180$) and IgY14/SuperMix-SRM ($y = 0.1922x + 0.0571$) (see Figure 4). Values obtained via Deming regression to log-one-plus-SRM values, incorporating a log-one-plus-predictor produced nearly identical results for IgY14-SRM ($y = 0.0186x + 0.0162$) and IgY14/SuperMix-SRM ($y = 0.1912x + 0.0592$), both with a similarly small magnitude of differences in standard errors. The relationship of the SRM total PSA concentrations calculated using these new equations and corresponding immunoassay results for the above 8 clinical samples was then SRM-MS = 1.0122 immunoassay - 0.2343 ($R^2 = 0.95$), and the percentage of absolute total PSA measurement error was smaller than 32% (data not shown). Based on the immunoassay results for total and free PSA in the calibration curve serum samples, the LOD and LOQ for PSA measurement using the LC-SRM-MS workflows in actual clinical samples were 0.80 ng/mL and 2.03 ng/mL, respectively, for total PSA, and 0.31 ng/mL and 0.86 ng/mL, respectively, for free PSA (Table 1). In the PSA spike-in experiments, the overall recovery for the total and free PSA workflows was determined to be >18.8% and >10.8%, respectively. However, if the recovery was calculated based on known losses of PSA to complexation with A2M (60% loss), the calculated percent recoveries of the entire workflow (depletion, digestion and clean-up) for measuring total and free PSA in clinical samples would be substantially higher, i.e., $18.8\%/40\% = 47.0\%$ for total PSA and $10.8\%/(40\% \times 41\%) = 65.9\%$ for free PSA.

Another independent set of 8 clinical serum samples with known total PSA (1.99–36.17 ng/mL) and free PSA (0.51–5.40 ng/mL) concentrations determined by immunoassay (obtained from D. Chan and L. Sokoll) were also analyzed using the IgY14-SRM and IgY14/SuperMix-SRM workflows and the new calibration curves. The results were summarized in Table 2. The linear equations for the correlation of the SRM and immunoassay results were SRM-MS = 0.9020 immunoassay + 0.6963 with $R^2 = 0.99$ for total PSA and SRM-MS = 0.6177 immunoassay + 0.4755 with $R^2 = 0.93$ for free PSA (see Figure 5A and 5B). The percentages of absolute measurement difference were <17% and <32% for total PSA and free PSA respectively (with a single outlier; see Table 2). Using the total PSA calibration curve equation ($y = 0.0182x + 0.0180$) to calculate the PSA concentration for the IgY14/SuperMix-depleted samples, and then comparing these values with the total PSA results from the immunoassays, the correlation actually decreased significantly (linear equation was

SRM-MS = 0.3372 immunoassay + 15.9580 with $R^2 = 0.14$; see Figure 5C), which is consistent with measurement of free but not total PSA following IgY14/SuperMix-depletion.

To further validate the external calibration approach and demonstrate the utility of the SRM assay, total PSA concentrations in a blinded set of 33 clinical serum samples (obtained from D. Chan and L. Sokoll) were also analyzed by LC-SRM-MS, and the data unblinding and analysis were completed at NCI (the SRM and immunoassay data are available in Supplementary Table 1). Ten of the 33 samples were analyzed in process replicates while the other 23 samples were analyzed in SRM technical replicates due to sample size limitation. Even though almost half of the samples had total PSA concentrations that were outside of the calibration curve concentration range (0.80–17.77 ng/mL, as determined by immunoassay), a linear relationship of SRM-MS = 1.2046 immunoassay + 0.8618 with $R^2 = 0.90$ was still obtained (Figure 6). The raw data were also analyzed using a second linear model in which each replicate was treated as a separate observation in a linear mixed effects model with a random intercept within each process replicate accounting for the process replicate means. This allowed an analysis of variance, which demonstrated that 97% of the total variation was due to process replicate, with the remaining 3% due to technical replicate, even though only 10 of the 33 samples have process replicates. In addition the regression analysis was repeated using the above mentioned log-Deming regression, producing again, virtually identical results (SRM-MS = 1.2232 immunoassay + 0.5901) with a similarly small magnitude of differences in standard errors. This blinded study demonstrated good sensitivity and robustness of the SRM assay; with the use of the same PSA calibrant for external calibration, the correlation between SRM and immunoassay and the accuracy of SRM assay are expected to increase further.

Percent free PSA (%fPSA)

Using the SRM assays developed in this study, %fPSA values can readily be obtained without the requirement for specific PSA antibodies. As expected, the calculated %fPSA using SRM values was fairly consistent ($44 \pm 6\%$; or $41 \pm 2\%$ using immunoassay values) in all the calibration curve samples (i.e., IgY14/SuperMix vs. IgY14). The %fPSA level of approximately 40% in the spike-in samples (Table 1) probably reflected the use of a single PSA source (purified PSA from human seminal fluid), as opposed to the physiological factors that can affect the %fPSA levels *in vivo*. In contrast, %fPSA varies significantly from 6% to 48% in the set of 8 clinical serum samples for which both total and free PSA SRM measurements were performed, and did not correlate with the total PSA level; the percentage of absolute difference for the determination of %fPSA using SRM and immunoassay was $< 38.7\%$ except for one sample (see Table 2 for details). Percent free PSA is known to be independent of total PSA levels, and measurement of %fPSA can improve the differentiation between benign prostate hyperplasia and prostate cancer^{36, 37}. However, since clinical outcome information is not available for the set of 8 samples tested here, we do not know whether these %fPSA values correlated with diagnosis or outcome. Nevertheless, the total and free PSA SRM assays developed in this study perform comparably to conventional methods based on affinity reagents, and therefore, they could conceivably be used for future preliminary investigations of analyte performance prior to the development of immunoassays.

Conclusions

Given its high sensitivity, specificity, and reproducibility, immunoassay has been the “gold standard” in clinical laboratories for measuring protein biomarkers for a variety of clinical applications. However, *de novo* development of new immunoassays is associated with substantial cost and long development lead time. Stable isotope dilution coupled to LC-SRM-MS provides an attractive alternative for sensitive, accurate, and multiplexed

measurement of protein biomarkers, particularly in early phases of biomarker development, prior to the investment in immunoassay development. In this study, SRM assays and two different immunoaffinity depletion-based workflows were developed for the first time for highly sensitive and accurate measurement of both total and free PSA in serum without the need for special PSA antibodies. The validity of these two independent workflows for total and free PSA measurements was confirmed by the differences in overall PSA recovery and SRM-MS signal intensity, and correlation to corresponding immunoassay results. Protein quantification at the low ng/mL level was achieved for both total (LOQ 2.03 ng/mL) and free (LOQ 0.86 ng/mL) PSA measurements in clinical serum samples. Moreover, good correlations (R^2 ranging from 0.90 to 0.99) between the SRM and the immunoassay results were obtained for several independent clinical serum sample sets, demonstrating the robustness of these SRM PSA assays. In addition, the strategy demonstrated here can readily be extended in the future to other desirable protein targets in complex biofluids that are characterized by similar bound and free interactions, without the need to develop specific antibodies to the free and bound forms, providing an effective workflow for efficient screening on numerous candidate biomarkers in human plasma or serum.

From a broad perspective, it is impractical to develop new immunoassays for verifying hundreds of emerging biomarker candidates, thus creating a severe bottleneck in the biomarker pipeline. In contrast SRM is particularly suitable for pre-clinical screening/prioritization of the biomarker candidates derived from the broad discovery studies, because it is much easier to configure the assays and the cost is much lower. Another important feature of SRM is its strong multiplexing capability (e.g., hundreds of peptides in a single analysis). Although typically it is not common to multiplex large numbers of analytes together in a diagnostic laboratory unless they are being utilized for an in vitro diagnostic multivariate index assay, this unique multiplexing capability provides an invaluable tool for *de novo* biomarker discovery and verification. The time and expense of developing immunoassays can then be reserved for the most highly promising biomarker candidates, prior to use in large-scale clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Experimental workflows for quantification of total and free PSA in clinical serum samples using SRM and external calibration. The calibration curves were obtained by spiking PSA protein in a pooled female serum from healthy donors at 9 different concentrations ranging from 0.5 to 50 ng/mL, followed by immunoaffinity depletion (IgY14 for total PSA; IgY14/SuperMix for free PSA), trypsin digestion, SPE C18 clean-up, internal standard peptide spike-in, and LC-SRM-MS analysis.

2A

2B

Figure 2.

Calibration curves of transition 636.8/943.5 monitored for peptide LSEPAELTDAVK derived from PSA. The spiking concentrations were 0.5, 1, 1.5, 2.5, 5, 7.5, 10, 25, and 50 ng/mL for both the IgY14 (A) and IgY14/SuperMix (B) based workflows for measurements of total and free PSA, respectively. Three process replicates were performed for each data point.

Figure 3.
Correlation of SRM (IgY14 workflow) and immunoassay results of total PSA measurements for a set of 8 clinical serum samples from the University of Texas Health Science Center.

4A

4B

Figure 4. Re-constructed calibration curves for (A) total and (B) free PSA measurements using the IgY14 and IgY14/SuperMix based workflows, respectively. The PSA concentrations measured by the total and free PSA immunoassays, instead of the nominal PSA spiking levels, were used here, considering the significant loss due to complexation to A2M.

5A

5B

5C

Figure 5.

Correlation of SRM and immunoassay results of a set of 8 clinical serum samples from the Johns Hopkins Medical Institutions. The results were obtained by using the re-constructed calibration curves for both (A) total and (B) free PSA measurements. The PSA values calculated for the IgY14/SuperMix-depleted samples using the total PSA calibration curve equation do not correlate with the total PSA values provided by the immunoassays (C).

Figure 6. Correlation of SRM and immunoassay results of total PSA measurements for a set of 33 blinded clinical serum samples from the Johns Hopkins Medical Institutions.

Table 1

Immunoassay results for total and free PSA in the calibration curve samples (a pooled female serum sample with purified human seminal fluid PSA spiked in; before immunoaffinity depletion).

PSA spike-in level (ng/mL)	total PSA (ng/mL)	free PSA (ng/mL)	%fPSA
0	0.14	0.03	21.4%
0.5	0.33	0.12	36.4%
1	0.56	0.22	39.3%
1.5	0.80	0.31	38.8%
2.5	1.06	0.43	40.6%
5	2.03	0.86	42.4%
7.5	3.10	1.31	42.3%
10	4.18	1.74	41.6%
25	9.52	3.79	39.8%
50	17.77	6.95	39.1%

Table 2
Summary of LC-SRM-MS and immunoassay analyses of a set of 8 clinical serum samples for both total and free PSA.

ID	tPSA by SRM (ng/mL)	%CV	tPSA by immunoassay (ng/mL)	%error (tPSA)	tPSA by SRM (ng/mL)	%CV	fPSA by immunoassay (ng/mL)	%error (fPSA)	%tPSA (immunoassay)	%fPSA (SRM)	%error (%fPSA)
1	3.93	9.5	3.63	8.4	1.32	7.9	1.46	-9.8	40.2	33.5	-16.8
2	2.24	8.7	1.99	12.8	1.09	4.7	0.51	113.3	25.6	48.5	89.1
3	15.58	5.2	13.57	14.8	3.80	2.5	5.4	-29.6	39.8	24.4	-38.7
4	5.04	12.7	6.03	-16.4	1.01	8.4	1.13	-10.5	18.7	20.1	7.1
5	3.84	11.5	3.66	4.9	0.52	2.9	0.76	-31.5	20.8	13.6	-34.7
6	32.34	2.9	36.17	-10.6	1.82	1.6	2.34	-22.4	6.5	5.6	-13.2
7	19.43	8.4	20.06	-3.2	1.96	5.9	2.37	-17.4	11.8	10.1	-14.8
8	6.09	3.4	6.82	-10.7	1.88	1.4	1.56	20.8	22.9	30.9	35.3