Serum Concentrations of Prostate-Specific Antigen Measured Using Immune Extraction, Trypsin Digestion, and Tandem Mass Spectrometry Quantification of LSEPAELTDAVK Peptide

Eric W. Klee, PhD; Olga P. Bondar, PhD; Marcia K. Goodmanson, AA; Sergey A. Trushin, PhD; Eric J. Bergstralh, BS, MS; Ravinder J. Singh, MD; N. Leigh Anderson, PhD; George G. Klee, MD, PhD

Context.—Prostate-specific antigen (PSA) is a widely used blood test for detection and monitoring of prostate disease. Many clinicians assume that all test methods produce essentially the same results. However, in spite of efforts to standardize this biomarker, major PSA assay differences still exist. The development of a traceable reference method for measuring serum levels of PSA could help harmonize PSA immunoassays. The measurement of PSA is complicated by its multiple binding proteins. The active form of PSA is a chymotrypsin-like enzyme that forms complexes with circulating protease inhibitors such as antichymotrypsin (ACT) and α2-macroglobulin (A2M). Because of the interference of A2M binding with PSA epitope recognition, PSA bound by A2M is not detected by most immunoassays. Therefore, potential reference methods for harmonizing PSA immunoassays should detect only those forms of PSA that are measured by the immunoassays. Of the PSA that is in circulation and detected by immunoassay, approximately 80% to 90% is complexed with ACT, and 10% to 20% circulates in its free or unbound state. Prostate-specific antigen has multiple antigenic sites and the various commercial immunoassays use antisera that may bind to different epitopes. Theoretically, these use differences in antiserum specificity could lead to differences in the measured values for circulating PSA.

We developed a strategy to use a mass spectrometry (MS) assay to measure PSA concentrations that measures only the immune-reactive forms. Prostate-specific antigen is immune extracted from serum using antibodies directed to different PSA epitopes. This process extracts the same forms of free and ACT-complexed PSA that are measured in immunoassays, but does not extract A2M-PSA. The extracts are trypsin digested and the LSEPAELTDAVK (LSE) peptide...
is quantitated on liquid chromatography–tandem MS. This peptide is unique to PSA and is not subjected to posttranslation phosphorylation or glycosylation. The MS assay is normalized with the World Health Organization (WHO) 96/670 reference standard, which provides traceability. The advantages of traceability in laboratory medicine are well articulated in the review article by Vesper and Thienpont.4

Our choice of peptide for PSA quantitation is the same as the peptide used by other investigators, but our extraction method gives results that harmonize better with immunoassays. Fortin et al10 developed an LSE peptide MS assay that used trypsin proteolysis of whole serum followed by enrichment of the peptides on a solid phase column. Their system correlated well with an immunoassay when they measured PSA spiked into female serum, but they did not measure male sera with endogenous PSA. One would expect that their system would overrecover PSA in male sera, because the digestion of whole serum would include the LSE peptide from PSA bound to A2M. Shi and colleagues11 reported a mass spectrometry method using the LSE peptide that could measure PSA in spiked female serum. Their system used an IgY14 depletion column to remove 14 high-abundance proteins. Only 13% of the spiked PSA remained after depletion. They also reported measurements based on IVGGWECEK peptide in non-depleted sera. With that assay, they found the MS recovered 3.55 times the immunoassay. They attributed this over-recovery to the potential differences in the PSA epitopes recognized by the immunoassay compared with the total PSA measured by MS. Liu and coworkers12 reported a MS PSA assay that used prior immune-affinity depletion. Their system used tandem immuno-affinity depletion: IgY14 LC10 column followed by a Super Mix LCS column (Sigma-Aldrich, St Louis, Missouri). They quantitated both the LSE and the IVGGWECEK peptides. They also reported that up to 85% of the spiked PSA was codepleted with the proteins with the depletion columns and attributed this to the removal of the PSA-ACT complex. When they used their standards made by spiking PSA into female serum to compensate for the depletion losses, their MS assay over-recovered compared with immunoassay by a factor of 2.8-fold. They attributed this difference to potential loss of spiked PSA due to binding to other serum proteins such as A2M.

The procedure described in this paper uses immune extraction to overcome the specificity problems and presents a validated MS procedure that could be used as a reference standard to help harmonize PSA immunoassays.

**PROTOCOLS FOR IMMUNE EXTRACTION, TRYP SIN DIGESTION, AND MS MEASUREMENT OF PSA**

An outline of the preanalytic processing is shown in Figure 1. More details about these methods are included in a related manuscript.13 A combination of 3 monoclonal anti-PSA antisera, which are directed to different PSA epitopes, was used for immune extraction of PSA from serum. These monoclonal antibodies were obtained from suppliers of commercial PSA reagents: PSA36 (epitope 6b) from Fujirebio Diagnostics (Malvern, Pennsylvania), H50 (epitope 3a) from Abbott Laboratories (Abbott Park, Illinois), and PSM773 (epitope 5c) from Beckman Coulter (Chaska, Minnesota). These antisera are used in the capture phase of commercial automated PSA assays. The 3 monoclonal antibodies used for this extraction are not commercially available, but a commercial polyclonal anti-PSA antibody was shown to provide very similar results. Polyclonal anti-PSA antiserum from Dako (Carpinteria, California; catalog No. A0562) was used for the alternative method of immune extraction. Each of the antibodies was labeled with biotin and coupled to magnetic beads using the Pierce Sulfo-NHS-LC-Biotin kit (Thermo Fisher Scientific, Rockford, Illinois). These antisera were titrated to determine concentrations adequate to extract PSA from standards and human serum. The Beckman Access immunoassay was used to determine extraction efficiencies. A mixture of the antibody-coated beads was made by mixing the 3 bead sets to yield a blend with 2 μg PSA36 antibody, 1 μg PSM773 antibody, and 2 μg H50 antibody. The amount of Dako polyclonal antisera that provided equivalent extraction was 10 μg. These beads were used to immune extract PSA from 400 μL of serum. The volume of extracts was adjusted to 800 μL with phosphate-buffered saline, 0.01% Zwittergent (EMD Millipore, Billerica, Massachusetts), for overnight incubation at 4°C on a rotator. Using a magnetic separator, the unbound fraction was removed. The beads were washed 2 times with 100 μL of phosphate-buffered saline, 0.01% Zwittergent; transferred to a new tube; and washed a third time, leaving the beads dry after the final wash in preparation for the digestion with trypsin.

The extracted protein–antibody–bead complexes were washed, denatured, and trypsin digested. The trypsin was bovine pancreas TPCK treated (catalog No. T-1426-1G, Sigma-Aldrich). All digests were performed with extracted PSA bound to magnetic beads on a shaker at 1400 rpm. Samples were denatured with 20 μL of 6 M urea and reduced with 30 mM dithiothreitol for 1 hour at 60°C on the shaker. Samples were alkylated with iodoacetamide at a concentration of 60 mM for 45 minutes in the dark at room temperature on the shaker. After reduction and alkylation the samples were diluted to 100 μL with 50 mM NH4HCO3, pH 8, to lower the urea concentration to 1.2 M, and 1 μg of trypsin was added for 4 hours digestion at 37°C on the shaker. The reaction was stopped with 2 μL of formic acid and the supernatant containing the peptides of interest was recovered using a magnetic separator.

**PreAnalytic Processing Protocol**

- Label anti-PSA antibodies with biotin using Pierce kit
- Couple antibodies to strept-avidin magnetic beads
- Immune-extract PSA from 400 μL serum
- Wash bead-protein complexes 3 times with PBS + 0.01% Zwittergent
- Deature with urea and reduce with DTT for 1 hour
- Alkylate with iodoacetamide for 45 minutes
- Neutralize with NH4HCO3, and add 1 μg trypsin for 4 hour at 37°C
- Transfer to new tube and stop enzyme with formic acid
- Add internal isotope standard and bring to 100 μL for mass spectrometry

**Figure 1.** Preanlytic processing protocol. Zwittergent is a detergent from EMD Millipore (Billerica, Massachusetts). Abbreviations: DTT, dithiothreitol; PBS, phosphate-buffered saline; PSA, prostate-specific antigen.
The LSE tryptic peptide from this digested protein was quantitated using liquid chromatography–tandem MS. Prior to measurement, a stable isotopic form of this peptide was added as an internal standard to assure tube-to-tube consistency. This peptide was synthesized in the Mayo Proteomics Research Center (Rochester, Minnesota) on an ACT 396 Multiple Peptide Synthesizer (Advanced ChemTech, Louisville, Kentucky), using recommended procedures for DIC (1,3-diisopropylcarbodiimide) activation and coupling. The LSE tryptic peptide was quantitated based on the double-charged precursor ion at 639.92 m/z, amu (atomic mass unit) and single-charge transitions at 949.5 m/z, amu and 475.4 m/z, amu on an API 5000 spectrometer with the internal standard at 639.9 m/z, amu and 949.5 m/z, amu. The assay was calibrated with 7 standards made from female sera spiked with 90% PSA-ACT (No. P0625; Scripps Laboratories, San Diego, California) and 10% free PSA (No. 1344-SE; R&D Systems, Minneapolis, Minnesota). This 90:10 ratio of bound to free is the same as recommended by the International Federation of Clinical Chemistry and used in the WHO preparation.14,15 A reference standard made from WHO 96/670 at 10 ng/mL was run with each assay and the results from each assay were adjusted to provide 100% recovery to assure traceability. Absolute quantitation was performed by liquid chromatography–tandem MS using a CTC Analytics HTC PAL auto sampler (LEAP Technologies, Carrboro, North Carolina), a Shimadzu 10-AD binary pumping system (Shimadzu Scientific Instruments, Columbia, Maryland), and an API 5000 triple quadruple mass spectrometer (Applied Biosystems, Foster City, California). For each run a total of 100 µL of sample was injected onto a 50 × 2.1-mm TARGA C18 column (Higgins Analytical, Mountain View, California) and run at 250 µL/min for 30 minutes. The gradient used consisted of solvent A (water, 0.1% formic acid) and solvent B (methanol, 0.1% formic acid) starting at 5% B for 2 minutes, increasing to 95% B during 22 minutes, holding at 95% B for 2 minutes, decreasing back to 5% B in 1 minute, and then holding for 1 minute (total time 30 minutes). The API 5000 instrument source parameters are shown in Table 1.

**METHOD VALIDATION STUDIES**

The efficiency of immune extraction was assessed by comparing the postextraction immunoassay PSA concentrations (Beckman Access) with the initial values using the 7 standards and 5 serum pools. The extraction efficiency was calculated as the percentage of PSA removed by extraction; that is, the initial PSA minus the residual PSA compared with the original PSA. The completeness of the trypsin digestion was monitored using spiked specimens. Assay precision was evaluated using 2 commercial quality controls (CLINIQA, San Marcos, California) and 3 serum pools. Separate aliquots of each of these controls were extracted twice for each assay, with the first aliquot measured at the beginning and the second aliquot run at the end for 9 assays.

Method comparisons were performed using frozen aliquots of serum samples collected from men participating

---

**Table 1. LSEPAELTDAVK (LSE) Peptide and Mass Spectrometry Measurement Characteristics**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Transitions</th>
<th>Declustering Potential</th>
<th>Entrance Potential</th>
<th>Collision Energy</th>
<th>Collision Exit Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSE transition</td>
<td>636.8^++/943.48 m/z</td>
<td>60 V</td>
<td>10 V</td>
<td>27 eV</td>
<td>40 V</td>
</tr>
<tr>
<td>LSE transition 2</td>
<td>636.8^+!/472.37^+ m/z</td>
<td>60 V</td>
<td>10 V</td>
<td>27 eV</td>
<td>27 V</td>
</tr>
</tbody>
</table>

**Figure 2.** Mass spectrometry spectrum of the LSEPAELTDAVK peptide and daughter fragments. The mass to charge ratio is designated as m/z.
in an internal review board–approved study with the Mayo Clinic Prostate SPORE research program. One hundred samples were from men undergoing prostate biopsy, with 50 having no evidence of cancer and 50 having cancer on their biopsies. These test results were used both for comparing analytic results and for evaluating diagnostic accuracy for separating men with prostate cancer from men with benign prostate disease. In addition, 5 samples were collected from 5 men who were being treated for advanced prostate cancer as part of the Prostate SPORE program. These specimens were used to compare the tracking performance of the immunoassays and the MS assay.

All the specimens were measured on 2 separate MS runs with the 3 antibody extraction system and on a third MS run with the polyclonal extraction system. All the specimens also had PSA measurements on the Roche (Indianapolis, Indiana) cobas and Beckman Access immunoassays. Cross plots and linear regression statistics were used to compare the measurements. Plots were also made comparing the immunoassays with the MS for the 5 serial collections from 5 patients with advanced prostate cancer. The clinical utility of the MS assay was compared with the utility of the immunoassays for separating men with prostate cancer versus benign prostate disease using receiver operating characteristic curve analysis (SAS, Cary, North Carolina).

In addition, to facilitate transfer of the liquid chromatography–tandem MS assigned values to future assays, a panel of 20 specimens was established. Twenty aliquots of deidentified single-donor specimens collected with the Prostate SPORE program were frozen at –80°C. Each of these specimens was measured in triplicate on 3 separate MS assays using the 3-antibody extraction system and was measured on the Roche cobas and Beckman Access immunoassays.

RESULTS

The extraction systems worked very well, with extraction efficiencies from 99.4% to 98.6% with an average of 99.2% for the 3-antibody extraction and from 99.7% to 98.2% with an average of 99.1% for the polyclonal extraction. The trypsin digestion also performed well, with recoveries ranging from 95% to 99% and an average recovery of 98%. The interassay precision for the MS triple antibody extraction assay using the 2 commercial controls varied from 6.0% at 4.1 ng/mL to 5.4% at 27.7 ng/mL. The serum pools had CVs of 8.6%, 4.3%, and 3.6% at levels 1.5, 5.3, and 10.2 ng/mL.

The MS spectrum of the LSE peptide and its fragments is shown in Figure 2. The primary peptide gave a strong signal without interference at 636.8 m/z. Three daughter fragments gave signals at 472.3, 943.49, and 1072.47. The second fragment was used for quantitation because it was by far the strongest.

Comparisons of the 2 MS assays and the 2 immunoassays are shown in Figure 3. All the methods are highly correlated. Figure 3, A, shows that the MS with the polyclonal extraction antibody worked well and correlated well with the MS using the 3-antibody extraction system. The linear coefficient of correlation was 0.994 and the regression slope was 0.981, with a 95% confidence interval of 0.965 to 1.002. Figure 3, B and C, shows the MS assay with prior antibody extraction correlated well with both the Roche cobas assay (correlation coefficient = 0.988) and the Beckman Access assay (correlation coefficient = 0.995). The Roche assay gave slightly lower results (regression slope = 0.984; 95% confidence interval, 0.957–1.011), whereas the Beckman assay gave results that were higher (regression slope = 1.080; 95% confidence interval, 1.060–1.100). If the Beckman results were adjusted down by 22% to compensate for the difference between Beckman Coulter’s Hybritech calibrator and the WHO standard, then the Beckman results would be 14% lower than the MS system. Figure 4, A through E, shows that the MS method closely tracks the serial PSA changes shown with the 2 immunoassays. A comparison of the clinical utility of these 3 assays for separating men with prostate cancer versus benign prostate disease using receiver operating characteristic curve analysis (SAS, Cary, North Carolina).
operating characteristic curve analysis is shown in Figure 5. We found no statistical differences among the 3 methods, with areas under the curve for separation of cancer from benign prostate disease equal to 0.6774 for our MS assay, 0.6852 for the Roche cobas immunoassay, and 0.6759 for the Beckman Access immunoassay.

The MS values for the 3-antibody LSE peptide results for the 20-member panel of serum specimens are shown in Table 2. The panel results span the PSA range from 2.08 to 39.7 ng/mL. The specimens were measured on 3 assays using separate extractions for each assay. The across-assay CVs ranged from 1.02% to 8.46%. These panel results were

Figure 4. A through E, Serial tracking comparisons of prostate-specific antigen (PSA) measurements for 5 samples collected over time from 5 men with advanced cancer. Each sample was measured with mass spectrometry (MS) and 2 immunoassays. The MS measurements were made with PSA that was extracted from the serum using a combination of 3 anti-PSA monoclonal antibodies (MS-3 Antibody Extract). The immunoassay measurements using Roche cobas immunoassay (Roche Diagnostics, Indianapolis, Indiana) are shown with diamond symbols. The immunoassay measurements using the Beckman Access immunoassay (Beckman Coulter, Chaska, Minnesota) are shown with square symbols. Each of the series of samples tracked similarly with all 3 methods.

Figure 5. Receiver operating characteristic (ROC) curves comparing the efficiency of the immune-extraction mass spectrometry (MS) measurements of prostate-specific antigen with measurements made with the Roche cobas immunoassay (Roche Diagnostics, Indianapolis, Indiana) and the Beckman Access immunoassay (Beckman Coulter, Chaska, Minnesota) from 100 men undergoing prostate biopsy (50 showing prostate cancer and 50 showing no cancer). The areas under the ROC curves (Area) are not statistically different for the 3 methods.
glycoprotein, these extraction systems often remove a portion of the PSA that adheres to the high-abundance proteins along with the proteins and therefore make the measurement system incomplete. Another advantage of our immune extraction is the increase in the relative concentration of PSA caused when the peptides from the PSA in a larger sample of serum are resuspended in a smaller injection volume of buffer for the MS. This increase in concentration helps improve the robustness of the MS assay.

References

Table 2. Reference Control Panel Assigned Values and Precisions Based on Triplicate Extractions and Mass Spectrometry Measurements of LSEPAELTDAVK Peptide After Immune Extraction of Prostate-Specific Antigen (PSA)

<table>
<thead>
<tr>
<th>Code</th>
<th>PSA, ng/mL</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2B233</td>
<td>2.08</td>
<td>1.02</td>
</tr>
<tr>
<td>1C301</td>
<td>2.76</td>
<td>5.48</td>
</tr>
<tr>
<td>3B376</td>
<td>3.65</td>
<td>6.16</td>
</tr>
<tr>
<td>3C472</td>
<td>4.33</td>
<td>6.48</td>
</tr>
<tr>
<td>4B459</td>
<td>4.37</td>
<td>7.62</td>
</tr>
<tr>
<td>2C478</td>
<td>4.46</td>
<td>2.92</td>
</tr>
<tr>
<td>6C712</td>
<td>6.79</td>
<td>7.59</td>
</tr>
<tr>
<td>C100</td>
<td>8.53</td>
<td>6.15</td>
</tr>
<tr>
<td>C110</td>
<td>9.57</td>
<td>1.99</td>
</tr>
<tr>
<td>C125</td>
<td>10.57</td>
<td>2.38</td>
</tr>
<tr>
<td>C140</td>
<td>11.37</td>
<td>1.83</td>
</tr>
<tr>
<td>C150</td>
<td>11.43</td>
<td>2.02</td>
</tr>
<tr>
<td>H135</td>
<td>11.87</td>
<td>4.64</td>
</tr>
<tr>
<td>3H213</td>
<td>18.17</td>
<td>5.11</td>
</tr>
<tr>
<td>C220</td>
<td>18.77</td>
<td>5.34</td>
</tr>
<tr>
<td>C165</td>
<td>18.83</td>
<td>5.68</td>
</tr>
<tr>
<td>C300</td>
<td>21.93</td>
<td>5.77</td>
</tr>
<tr>
<td>H225</td>
<td>25.97</td>
<td>8.46</td>
</tr>
<tr>
<td>4H289</td>
<td>27.70</td>
<td>6.88</td>
</tr>
<tr>
<td>SH405</td>
<td>39.73</td>
<td>6.83</td>
</tr>
</tbody>
</table>

Abbreviation: CV, coefficient of variation.

highly correlated with the Roche cobas and Beckman Access immunoassays, \( R^2 = 0.993 \) and 0.987 respectively.

COMMENT

The MS assays reported in this study are the first potential reference mass spectrometry assays that measure the same forms of PSA as the commercial immunoassays. These assays can reliably quantitate PSA concentrations in male sera that closely match existing immunoassays, and these assays are traceable to the WHO PSA standard. The major advantage of our method is the added specificity related to the immune extraction, which excludes the circulating forms of PSA that are bound to A2M and therefore are not measured by immunoassays. This immune extraction also markedly reduces the amount of protein that needs to be enzyme digested to release the LSE peptide and thereby increases the efficiency of the digestion process. Others have tried to extract the high-abundance proteins to help improve the digestion process. However, because PSA is a glycoprotein, these extraction systems often remove a...