Use of an Immunoaffinity-Mass Spectrometry-based Approach for the Quantification of Protein Biomarkers from Serum Samples of Lung Cancer Patients*

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It is a challenging task to verify and quantify potential biomarkers expressed at elevated levels in sera from cancer patients. An immunoaffinity-mass spectrometry-based approach has been developed using antibodies to enrich proteins of interest from sera followed by mass spectrometry-based quantification. Antibodies specific to the protein of interest were immobilized to hydrazide resin via the carbohydrate moiety on the Fc region of the antibody. Captured proteins were eluted, reduced, alkylated, and digested with trypsin. Peptides were analyzed by LC coupled with multiple reaction monitoring approach, and quantification was achieved by the addition of stable isotope-labeled (heavy) standard peptides. Using this methodology, we were able to achieve a linear response from 15 to 250 ng/ml for carcinoembryonic antigen (CEA), a known tumor biomarker. Moreover we observed elevated levels of CEA in sera samples from lung cancer patients that to our knowledge is the first time that circulating CEA has been detected by mass spectrometry-based analysis. This approach was further applied to potential protein biomarkers discovered from tumor cell lines and tumor tissues. A linear response was obtained from a multiplex spiking experiment in normal human sera for secretory leukocyte peptidase inhibitor (4–500 ng/ml), tissue factor pathway inhibitor (TFPI) (42–1000 ng/ml), tissue factor pathway inhibitor 2 (TFPI2) (2–250 ng/ml), and metalloproteinase inhibitor 1 (TIMP1) (430–1000 ng/ml). A replicate experiment for a single concentration value yielded a relative coefficient of variation better than 11% for TFPI, secretory leukocyte peptidase inhibitor, and TFPI2. The expression level of the proteins in lung cancer patient sera was assayed by an immunoaffinity-multiple reaction monitoring method, and the results were comparable with those obtained from ELISA. This immunoaffinity-mass spectrometry-based quantification approach thus provides a specific and accurate assay for verifying the expression of potential biomarkers in patient serum samples especially for those proteins for which the necessary reagents for ELISA development are unavailable. Molecular & Cellular Proteomics 7: 1974-1982, 2008.

Biomarkers have attracted significant attention for disease diagnosis, prognosis, and therapeutic response monitoring in recent years (1). The development of clinical biomarkers involves discovery, verification, and validation. Progress in genomics and proteomics has led to a large number of potential biomarkers (2–4). However, the promise of these discoveries requires proper verification and validation in the disease of interest (5). The ELISA has been the established platform for quantification of proteins in sera. However, for many of these newly identified potential biomarkers there are no ELISA kits available, and development of an ELISA kit is time-consuming (6), making it impractical to examine large panels of biomarkers. Although ELISA remains the “gold standard” for clinical applications, mass spectrometry-based quantification analysis has been shown as a possible intermediate technology in the validation process to prioritize candidates for ELISA development (7–12).

Mass spectrometry has been used for quantification of small molecules (drug metabolites, hormones, pesticides, etc.) with high throughput and relatively low CVs1 for many years (13). Most assays use ESI followed by MS/MS analysis along with a stable isotope-labeled internal standard. These measurements have been multiplexed to provide more than 25 analyses in a single assay (14).

In contrast, accurate quantification of proteins and peptides in sera or plasma by mass spectrometry is a challenging problem because of the complexity and large dynamic range

1 The abbreviations used are: CV, coefficient of variation; MRM, multiple reaction monitoring; CEA, carcinoembryonic antigen; SLPI, secretory leukocyte peptidase inhibitor; TFPI, tissue factor pathway inhibitor; TFPI2, tissue factor pathway inhibitor 2; TIMP1, metalloproteinase inhibitor 1; LOD, limit of detection; MARS, Multiple Affinity Removal System; GuHCl, guanidine HCl; DPBS, Dulbecco’s PBS; SISCAPA, stable isotope standards and capture by anti-peptide antibodies.
of the matrix (15). Significant advances have been made in the identification of serum or plasma proteins using mass spectrometry-based techniques (16). The use of traditional survey scans to attempt to detect all the proteins in a sample has been shown to be limited in sensitivity and demonstrates bias toward the higher abundance proteins. Several methods have been reported for using the protein survey data to imply relative quantification by incorporating data base identification score and number of peptides observed (17, 18). The fact that every peptide has a different mass spectrometric response as well as different extraction and cleanup efficiencies makes these methods less accurate.

An alternative is the targeted analysis- or candidate-based approach. In this approach the researcher optimizes the assay to detect and quantify only the peptides (proteins) of interest. The use of this methodology results in an increase in the sensitivity and precision of detecting proteins in plasma or sera by mass spectrometry (19). Anderson and Hunter (19) have used a targeted method, multiple reaction monitoring (MRM), to quantify up to 50 proteins simultaneously in sera from which the six most abundant proteins were removed. The protein of lowest concentration observed using this process was ~0.67 μg/ml for L-selectin, a 33-kDa protein.

Sample complexity remains a major problem using this targeted approach and severely restricts the limits of detection (LODs) and quantification. Without extensive fractionation many proteins have an LOD in the 1–10 μg/ml range using MRM technology, whereas potential biomarkers of greatest interest are often in the low ng/ml range (10). To improve the detection limits to the required sensitivities, one needs to dramatically reduce the complexity of the sera samples. One method is to remove highly abundant proteins using affinity columns such as the Agilent MARS column (20). Removal of the most abundant proteins results in a lower limit of detection, but it becomes obvious that the next set of abundant proteins becomes the limiting factor (21). The use of extensive sample fractionation has extended the limits of detection of plasma and sera samples, but this is impractical for routine analysis of large numbers of samples because of the requirement for high sample volume, extensive mass spectrometry analysis time, and data processing required (22).

Anderson et al. (23) reported a method using stable isotope standards and capture by anti-peptide antibodies (SISCAPA) for quantifying peptides in complex digests. This approach combines the enrichment by antibody and specificity by mass spectrometric analysis (MRM). Whiteaker et al. (24) have shown that anti-peptide antibodies can be used to enrich the peptides of interest from digested sera that have had the abundant proteins removed. In their experiments they saw enrichment on the order of 10³ for the peptides of interest. Many groups have enriched small proteins and polypeptides in the 20–60-amino acid range with size exclusion chromatography or reverse phase chromatography (25–27). The authors were able to analyze the sample without enzymatic digestion because of the size of the polypeptides. For proteins and polypeptides larger than this, identification and quantification is difficult without digestion. Berna et al. (28) used an immunoprecipitation methodology to quantify myosin light chain in rat serum (limit of quantification, 0.073 nM or 1.6 ng/ml) as they were unable to successfully use an ELISA. Recently Keshishian et al. (29) reported a work flow of optimized sample processing followed by MRM analysis for plasma proteins. Low ng/ml quantitation for several proteins in plasma was achieved.

In the current study, an immunoaffinity-mass spectrometry-based approach was developed that uses antibodies to enrich multiple proteins of interest from sera followed by mass spectrometry-based quantification. Using this approach, we were able to observe CEA in serum for the first time by a mass spectrometry-based method, demonstrating the ability to quantify several potential protein biomarkers in sera in the low ng/ml range. We believe the immunoaffinity-MRM approach provides an attractive methodology to prioritize biomarkers for further assay development.

**EXPERIMENTAL PROCEDURES**

Reagents—Chemical reagents were obtained from Sigma except for mammalian protein extraction reagent and guanidine HCl (GuHCl), which were purchased from Pierce. Modified trypsin was purchased from Promega (Madison, WI). TIMP1, SLPI, TFPI, and TFPI2 antibodies and recombinant proteins were acquired from R&D Systems (Minneapolis, MN); CEA antibodies were purchased from Abcam (Cambridge, MA); and the recombinant protein was from United States Biological Inc. (Swampscott, MA). ELISA kits for TIMP1 and SLPI were purchased from R&D Systems, kits for CEA were from Biomeda (Foster City, CA), and kits for TFPI were from American Diagnostica (Stamford, CT). ELISAs were performed according to the suppliers’ recommendations. Isotope-labeled peptides were purchased from Sigma-Genosys with either a heavy lysine (+8 Da) or heavy arginine (+10 Da).

Lung cancer patient and age-matched normal sera were obtained from Clinical Research Center of Cape Cod (West Yarmouth, MA). Normal sera were purchased from Equitech-Bio (Kerryville, TX) and pooled for the spiking experiment.

**Antibody Binding**—Where several antibodies were available, the antibody recommended by the manufacturer for immunoprecipitation or ELISA was chosen; otherwise polyclonal antibodies were preferred. Antibodies were immobilized on hydrazide beads (Bio-Rad, catalog number 153-6047) according to the manufacturer’s protocol with minor modifications. Antibodies were dissolved or buffer-exchanged into DPBS. Carbohydrates on the antibodies were oxidized by incubating the antibody solution with NaIO₄ (15 mM) for 1 h at room temperature in the dark with gentle shaking. After oxidation, glycerol was added to quench the excess NaIO₄. The antibodies were desalted and buffer-exchanged into 0.1 M NaOAc, pH 5.5.

Antibodies were incubated overnight with hydrazide beads at 4 °C with gentle shaking. An aliquot was collected before and after binding for determination of binding efficiency by protein assay. After immobilization, the active sites on the resin were blocked with glyceraldehyde and washed several times with DPBS to remove any excess non-bound antibody. After determining the binding efficiency, the immobilized resins for all antibodies were combined and aliquoted such that ~10 μg of each immobilized antibody was used for each sample.
Immunooaffinity-MRM for Biomarker Quantification

Protein Binding, Elution, and Digestion—100 μl of sera was diluted 10-fold with M-per (Pierce), and NaCl was added to a final concentration of 150 mM. Serum was incubated with the immobilized antibody resin overnight at 4 °C with gentle shaking. Unbound proteins were collected after incubation. The immobilized resin was washed three times with 1 ml of DPBS, and the protein was eluted with 2 × 100 μl of 4.0 M GuHCl, 0.1 M Tris, pH 8.

A standard mixture of heavy isotope-labeled peptides for the analytes (200 fmol/peptide) and 12 quality control peptides (~2 pmol/peptide to monitor MS and LC performance from their individual m/z and elution profiles) were added to the eluted protein solution. Samples were reduced with DTT (5 mM) for 30 min at 60 °C and alkylated with iodoacetamide (20 mM) for 1 h at room temperature in the dark. Excess iodoacetamide was quenched by adding an additional aliquot of DTT. The sample was then diluted to 0.8 M GuHCl with 0.1 M NH₄HCO₃, pH 8, and digested with trypsin overnight at 37 °C. The sample was desalted using a 1-ml Oasis hydrophilic-lipophilic balance cartridge (Waters, Milford, MA) and dried in a SpeedVac (Thermo-Savant, Wallingford, MA). The samples were dissolved in 0.1% formic acid by gently vortexing for 30 s prior to LC-MRM analysis. 10% of the sample was injected on the 150-mm column, and 5% was loaded on the 75-μm × 150-mm column.

LC-MRM Analysis—LC-MRM was performed on an Agilent (Santa Clara, CA) 1100 LC system equipped with a 150-μm × 50-mm C₁₈ Everest trap column and a 75-μm × 150-mm or a 150-μm × 150-mm C₁₈ Everest analytical column (Vydac, Deerfield, IL). The analytes were loaded onto the trapping column and eluted using a gradient of 3–30% acetonitrile with 0.1% formic acid at a flow rate of 300 nl/min or 1.5 μl/min over 90 min. MRMA data were collected on a 4000 QTRAP instrument (Applied Biosystems, Foster City, CA) equipped with a NanoSpray II source. LC-MRM conditions were optimized for each peptide using the heavy isotope peptide standards. MRM transitions were acquired at unit resolution in both the Q1 and Q3 quadrupoles with 100-ms dwell time. Quantification was accomplished using the Analyst QS 2.0 software package, and the areas of all analytes were normalized to the signals of their heavy stable isotope analogues.

RESULTS AND DISCUSSION

The large numbers of potential biomarker candidates being discovered from proteomics studies have overwhelmed current verification capabilities. Many of these potential candidates have no commercially available ELISA kits, resulting in no accurate way to quantify the protein levels in sera. Many candidates have antibodies available that have not been studied extensively for specificity and sensitivity and may not be suitable for ELISA measurements. Routine quantification of these biomarkers in sera is essential for the characterization of these candidates for diagnostic applications. Mass spectrometry is capable of quantifying proteins present in μg/ml and higher concentrations; however, many of the traditional biomarker candidates (e.g. CEA and prostate-specific antigen) are present in the ng/ml range. The complexity and the large dynamic range of proteins in sera make quantification in the ng/ml range by mass spectrometry a challenging task.

Our approach was to use antibody affinity for the enrichment of the proteins of interest. We demonstrate that the use of immobilized antibodies to enrich the potential biomarker candidates followed by MRM mass spectrometry for quantification with stable isotope-labeled internal standards leads to the ability to quantify proteins in the ng/ml range in sera (Fig. 1).

Antibody Conjugation—Antibodies for each protein were obtained from commercial sources, and immobilization was achieved as described under “Experimental Procedures.” Binding efficiencies ranged from 60 to 95% and were taken into account when mixing the antibodies together for multiplexing so that ~10 μg of antibody/protein was used in each experiment. Antibodies with lower specificities are acceptable in these experiments, increasing the number of antibodies available for this approach. Utilizing this approach takes advantage of the high affinity of the antibody to capture and therefore enrich antigens that are in very low abundance in sera and the extreme specificity of the MRM technology.

Selection of Peptides and Transitions for Quantification—The selection of the peptides used for quantification of the proteins of interest is critical. For candidates where recombinant protein was available, we digested the protein and de-
terminated the peptides that produced the best signal in an LC/MS experiment. Several peptides were chosen for each protein based on the intensity of the extracted ion chromatogram and their fragmentation pattern in MS/MS mode. The ideal peptide should have several (three to five) intense fragments (transitions) and a minimal number of less intense fragments. This need differs from the requirements of a peptide for identification purposes where more fragments provide greater confidence for data base matching. In contrast, for an MRM experiment, fewer peptide fragments result in a greater signal for each transition. The peptides chosen were analyzed in an MRM experiment with digested recombinant protein based on the intensity of the extracted ion chromatogram to ensure that they were unique to the protein of interest. The peptide for each protein meeting these criteria was selected for synthesis with heavy isotope incorporation. If the biomarker candidate did not have recombinant protein available, selection of peptides and transitions could be accomplished by using peptides observed in discovery experiments, identified from open source data bases (30, 31), or derived from the multiple reaction monitoring-initiated detection and sequencing (MIDAS) work flow (32, 33).

**Case Study: CEA—** CEA was one of the first tumor-associated antigens identified and is expressed in nearly 50% of all human tumors (34). In this study we examined the detection sensitivity of the immunoaffinity-MRM methodology for this known marker. CEA is a highly glycosylated protein with a molecular mass around 180 kDa. The molecular mass based on the primary amino acid sequence is 77 kDa, meaning that greater than 100 kDa is due to glycosylation and/or other modifications. The majority of the 28 potential N-glycosylation sites in CEA are occupied by oligosaccharide chains (35). The large molecular mass and high degree of glycosylation along with the low concentration in normal sera (∼1 ng/ml) make the detection and quantification of CEA by mass spectrometry a very difficult challenge. To date, there is no report of detection of CEA in sera by a mass spectrometry-based profiling experiment. Therefore, we chose CEA for a case study to test this platform.

To determine the sensitivity of the immunoaffinity-MRM platform, recombinant CEA was spiked into pooled normal sera and serially diluted from 250 to 15 ng/ml. CEA antibody resins were mixed together and aliquoted such that ∼15 μg of each antibody was used. A plot showing CEA concentration versus the area ratio of the light to heavy peptide is shown in Fig. 2A. The limit of detection was determined to be ∼15 ng/ml (signal to noise ratio of 3 for light peptide). An expression level for CEA of 5 ng/ml is routinely used as a cutoff for normal expression in sera (36). Next expression data from the immunoaffinity-MRM experiments comparing CEA levels in four late stage lung cancer sera with four normal sera were collected and compared with data from ELISA analysis of the same sera samples (Fig. 2B). The immunoaffinity-MRM methodology shows a significant signal above normal only for the 112 ng/ml sample (tumor sample B). The concentration for tumor samples A, C, and D (6, 23, and 26 ng/ml, respectively) is around the limit of detection for the immunoaffinity-MRM approach. To our knowledge this is the first time that CEA has been detected or quantified in sera or plasma of cancer patients by mass spectrometry-based analysis. These data for CEA suggest that this method has the ability to detect a protein in the ng/ml range from a complex background of proteins in serum. Through continued improvement in the capture and recovery efficiency we believe that the limit of detection for CEA can be lowered such that CEA can be detected in normal sera samples. Alternatively the method can be utilized as a qualitative filter for a high level of CEA (or other cancer antigens) in sera samples to prioritize the candidate list for assay development.

**Multiplexed Assay for Potential Protein Biomarkers—** To evaluate the significant number of biomarker candidates and to identify candidates with diagnostic characteristics, a process must be capable of evaluating multiple markers at a time. Because serum samples may be limited, the process must also minimize sample consumption. Our immunoaffinity-MRM

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**Table I Peptide sequences and transitions**

| Protein | Peptide | m/z precursor | Transition selected fragment m/z of fragment |
|---------|---------|---------------|-------------------------------------------|
| TFPI    | FFFNIFTR | 546.3         | y5 650.5, y6 797.7, y7 944.7              |
| SLPI    | CLDPVDTPNPTLR | 692.8       | y7 800.5, y9 996.7, y10 1111.6            |
| TFPI2   | YYDRTGQSCR | 525.6         | y72+ 486, y92+ 625, y102+ 706.5           |
| CEA     | CETQNPVSAR | 581.3         | y5 539.3, y6 643.3, y8 872.5              |
| TIMP1   | EPGLCWQSLR | 673.9         | y6 790.7, y7 950.4, y9 1120.6             |

* The third transition for TIMP1 was not used for quantification due to interference for the heavy peptide.
approach was tested for use in a multiplex mode by measuring several proteins in sera at the same time. We have previously established a robust and comprehensive process for discovering potential protein biomarkers that are overexpressed in cancer cell lines and tissues (37). As a proof of concept, four of the proteins (TFPI, TFPI2, SLPI, and TIMP1) identified from this platform were selected for multiplexing the immunoaffinity-MRM quantification. All four proteins had recombinant protein and antibodies commercially available, and the molecular mass of these proteins ranged from 14 to 41 kDa (Table II). Three of the proteins (TFPI, SLPI, and TIMP1) were chosen because they have commercially available ELISA kits for verifying the results from the immunoaffinity-MRM method. These three proteins have a wide range of concentrations in our pooled normal sera as determined by ELISA (TIMP1, 140 ng/ml; TFPI, 56 ng/ml; and SLPI, 1 ng/ml), providing a comprehensive test of the sensitivity of the platform. These proteins therefore are a realistic representation of the diagnostic candidates and the available reagents one could encounter in a discovery effort.

Limits of detection were determined for each of the four proteins. Recombinant proteins (TFPI, TFPI2, SLPI, and TIMP1) were spiked into pooled normal sera and were serially diluted. The dilution scheme was designed based on their endogenous concentration in normal sera for each protein. Concentrations of the proteins in the pooled normal sera were determined by ELISA (Table II). A plot of the area ratio of the light peptide to heavy peptide showed a linear correlation for all peptides from the highest concentrations to the limit of detection or the endogenous level in the pooled normal sera (SLPI: \[ y = 0.0487x + 0.0795, R^2 = 0.9993; \] TFPI: \[ y = 0.0083x + 0.3488, R^2 = 0.995; \] TFPI2: \[ y = 0.0013x + 0.0013, R^2 = 0.9997; \] TIMP1: \[ y = 0.0234x + 10.051, R^2 = 0.9874. \] TFPI and TIMP1 have significant endogenous concentrations in sera causing the non-zero intercept. From the slopes and intercepts the endogenous levels in the pooled normal sera can be calculated. The endogenous concentration for TFPI and SLPI determined by immunoaffinity-MRM (42 and 1.6 ng/ml, respectively) matched very well with the ELISA data (TFPI, 56 ng/ml; and SLPI, 1 ng/ml). The calculated endogenous concentration of TIMP1 was 430 ng/ml. The difference between ELISA (140 ng/ml) and MRM data for TIMP1 could be due to the fact that the capture antibodies or protein standard used may be different from those used in the ELISA. The detection limits for SLPI and TFPI2 in sera were determined to be less than 4 and 2 ng/ml, respectively, from the dilution curves (Table II).

To determine the reproducibility of the immunoaffinity-MRM analysis, the CV for each protein was determined. A single point from the dilution curves was repeated four times, and a relative CV of ~5% for SLPI (62.5 ng/ml) and TFPI2 (31.25 ng/ml) and ~11% for TFPI (125 ng/ml) was achieved. Therefore, the performance of the assay is quite reproducible. The peptide used from TFPI had the greatest CV at 11%. The peptide from TFPI, however, elutes close to the end of the LC gradient where there may also be contaminants eluting, resulting in a lower reproducibility for the electrospray cond-

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**Fig. 2.** A, a plot of the ratio of the area of the light peptide (CEA spiked into sera) to the heavy peptide (synthetic internal standard) from 15 to 250 ng/ml. The limit of detection for CEA is ~15 ng/ml. B, expression levels of CEA in late stage lung cancer patient sera (A–D) assayed by the immunoaffinity-MRM experiments (bottom) and ELISA (top) are shown. The dotted line on the ELISA graph is the limit of detection for the immunoaffinity-MRM experiment. L, light; H, heavy.
tions. This suggests that the hydrophobicity of peptides is another important consideration that may need to be taken into account when choosing them for protein quantification.

Levels of the four proteins (SLPI, TFPI, TFPI2, and TIMP1) were studied in a set of 12 late stage lung cancer serum samples and 12 normal serum samples using the immunoaffinity-MRM methodology. Antibodies for the four proteins (one antibody per protein) were immobilized on the resin, mixed, and aliquoted. The mixed antibody resin (10 μg/antibody) was incubated with 0.1 ml of sera diluted 10-fold with binding buffer to a final volume of 1 ml. Each sample was processed as described under “Experimental Procedures.” Three aliquots of the patient and normal sera were analyzed, two aliquots using the same batch of antibody resin and a third aliquot using antibody resin prepared at an earlier time and with antibodies from different lots. Each sample was analyzed in duplicate by LC-MRM.

Fig. 3 shows the levels of TIMP1, TFPI, and SLPI in late stage lung cancer sera samples measured by ELISA (A) and immunoaffinity-MRM (B). The concentration of TFPI2 in the late stage lung cancer sera was below the detectable limit, 2 ng/ml. The levels determined using MRM and ELISA are well

**TABLE II**

| Protein | Peptide       | LOD in sera | ELISA pooled normal | S/N for most intense transition at LOD/normal sera | Molecular mass |
|---------|---------------|-------------|---------------------|--------------------------------------------------|---------------|
| TFPI    | FFFNiFTTR     | <4          | 1                   | 1000                                             | 41            |
| SLPI    | CLDPVDTPNPTTR | n/a         | 56                  | 1000                                             | 14            |
| TFPI2   | YYDRTYQSC     | <2          | 1                   | 1000                                             | 32            |
| CEA     | CETNPSAR      | 15          | 1                   | 1000                                             | 180           |
| TIMP1   | EPGLCTQSLR    | n/a         | 140                 | 1000                                             | 24            |

**Fig. 3.** The expression levels for TIMP1, SLPI, and TFPI in 12 late stage lung cancer patients sera along with 12 normal sera samples. A, ELISA results from two experiments. B, immunoaffinity-MRM data showing the reproducibility from sample triplicates. C, the correlation of protein expression levels determined using MRM and ELISA. Good correlation was obtained with a linear correlation coefficient of *r* = 0.95 for TIMP1 (*p* value = 1.1 × 10<sup>−12</sup>, 95% confidence interval of 0.89–0.98), *r* = 0.83 for SLPI (*p* value = 5.5 × 10<sup>−7</sup>, 95% confidence interval = 0.64–0.92), and *r* = 0.7 for TFPI (*p* value = 1.6 × 10<sup>−4</sup>, 95% confidence interval = 0.41–0.86). L, light; H, heavy.
correlated with a linear correlation coefficient of $r = 0.95$ for TIMP1 (p value $1.1 \times 10^{-12}$ and 95% confidence interval of 0.89–0.98), $r = 0.83$ for SLPI (p value $5.5 \times 10^{-7}$, 95% confidence interval = 0.64–0.92), and $r = 0.7$ for TFPI (p value $1.6 \times 10^{-4}$, 95% confidence interval = 0.41–0.86). The average CV for the immunoaffinity-MRM analyses is 10.6% with a range from less than 1 to 26%. The samples with the highest CVs were from the TFPI data (15.3%); this is consistent with the higher CV from the single point experiment. These results indicate that this approach has acceptable reproducibility for the verification of potential serum biomarkers.

Previous approaches used for quantification of protein in serum removed abundant proteins using a MARS column followed by LC-MRM analysis (19). To estimate the sensitivity enhancement for the immunoaffinity-MRM method compared with depletion of abundant protein followed by digestion and LC-MRM, we analyzed TIMP1 in the pooled normal sera. The immunoaffinity-MRM platform gives a signal over ~150 times greater (area/area) than the signal using the MARS depletion column followed by LC-MRM (Fig. 4). With the MARS depletion LC-MRM sample, a much longer gradient (200 versus 90 min) was required to reduce ion suppression to observe the signal from the TIMP1 peptide.

In this study we showed that proteins can be quantified in sera in the low ng/ml range with a CV of 11% or less using the immunoaffinity-MRM methodology. This method provides several advantages over the traditional ELISA for protein quantification. Only a single antibody is required, whereas two antibodies are generally needed for ELISA. In addition, the specificity requirement of the antibodies is lower than that for antibodies used for ELISA. Mass spectrometry provides the necessary specificity following capture by an antibody and in essence can be used as the second antibody. The immunoaffinity-MRM approach uses a concept similar to that of the previously reported SISCAPA method (23, 24). However, this technique can be used for detection and quantification of known isoforms or variants (or post-translational modifications) of proteins assuming that the antibody or antibodies used will specifically immunoprecipitate the different isoforms. The major requirement for the antibody is that it should have a relatively high affinity for the specific protein and that it is suited for immunoprecipitation.

Reproducible and complete digestion of proteins is an essential part of most mass spectrometry experiments. This is extremely important for highly glycosylated proteins such as CEA because glycosylation will affect the digestion efficiency. Chen et al. (38) have shown that the use of mass spectrometry-compatible surfactants affects the number of proteins detected in shotgun proteomics experiments. Addition of surfactants may increase the digestion efficiency of the proteins pulled down in the immunoaffinity-MRM experiments and

Fig. 4. **Extracted ion chromatograms for the three transitions of TIMP1 in normal sera.** The top trace shows the signal using the immunoaffinity-MRM method, and the bottom trace shows the signal using the MARS depletion column and MRM. The maxim amount of sample (1 µg) for column capacity was loaded onto the LC column for the MARS depleted sample, whereas 5% of the sample was used for immunoaffinity-MRM analysis. The immunoaffinity-MRM platform results in an ~150 times greater signal (area/area). No signal was observed for the 90-min gradient using MARS depletion followed by MRM. cps, counts/s.
therefore may increase the sensitivity of the experiments. Optimization of the wash, elution, and digestion protocols needs to be addressed in the future because they will affect the overall recovery of the proteins of interest and therefore affect the limit of detection and quantification.

The ability to mix multiple antibody resins to pull several proteins from sera simultaneously can lead to a sample size requirement per protein that is comparable with ELISA experiments. Our results show that this is possible for four proteins, but this method should be capable of measuring large numbers of proteins in one assay similar to the approach by Anderson and Hunter (19). Multiplexing results in the equivalent of less than 10 μl of sample/protein if 10 or more proteins are analyzed at once. Established ELISAs, in general, are more sensitive than the immunoaffinity-MRM method. By continued optimization of the binding, elution, and digestion conditions, we should be able to improve the sensitivity and lower the limits of detection. The optimized conditions should result in a higher recovery of the proteins of interest and lower nonspecific binding, which will allow a greater percentage of the sample to be loaded on the LC column. By optimizing the parameters we should be able to reach detection limits lower than we reported here for many proteins.

In conclusion, we have developed an immunoaffinity-mass spectrometry-based approach using antibodies to enrich multiple proteins of interest from sera followed by LC-MRM-based quantification. As a proof of concept, we have shown that CEA can be detected and quantified in a subset of patient sera. This is the first reported detection of CEA in sera using a mass spectrometry-based approach in serum samples. This method has allowed us to quantify potential protein biomarkers in sera in the low ng/ml range with an acceptable CV.

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REFERENCES

1. Aebersold, R., Anderson, L., Caprioli, R., Druker, B., Hartwell, L., and Smith, R. (2005) Perspective: a program to improve protein biomarker discovery for cancer. J. Proteome Res. 4, 1104–1109

2. Orth, A. P., Batalov, S., Perrone, M., and Chanda, S. K. (2004) The promise of genomics to identify novel therapeutic targets. Expert Opin. Ther. Targets 8, 587–596

3. He, T., Kim, Y. J., Heidbrink, J. L., Moore, P. A., and Ruben, S. M. (2006) Drug target identification and quantitative proteomics. Expert Opin. Drug Discov. 1, 477–489

4. Aebersold, R., and Mann, M. (2003) Mass spectrometry-based proteomics. Nature 422, 198–207

5. Vitzthum, F., Behrens, F., Anderson, N. L., and Shaw, J. H. (2005) Proteomics: from basic research to diagnostic application. A review of requirements and needs. J. Proteome Res. 4, 1086–1097

6. Edwards, R. J., Wrigley, A., Bai, Z., Bateman, M., Russell, H., Murray, S., Lu, H., Taylor, G. W., Boobis, A. R., and Sriskandan, S. (2007) C-terminal antibodies (CTAbs): a simple and broadly applicable approach for the rapid generation of protein-specific antibodies with predefined specificity. Proteomics 7, 1364–1372

7. Ong, S. E., and Mann, M. (2005) Mass spectrometry-based proteomics: a new window for biomarker discovery. Nat. Chem. Biol. 1, 477–489

8. Liao, H., Wu, J., Kuhn, E., Chin, W., Chang, B., Jones, M. D., O’Neil, S., Clauser, K. R., Karl, J., Hasler, F., Roubenoff, R., Zolg, W., and Guild, B. C. (2004) Use of mass spectrometry to identify protein biomarkers of disease severity in the synovial fluid and serum of patients with rheumatoid arthritis. Arthritis Rheum. 50, 3792–3803

9. Kirsch, S., Widart, J., Louette, J., Focant, J. F., and De Pauw, E. (2007) Development of an absolute quantification method targeting growth hormone biomarkers using liquid chromatography coupled to isotope dilution mass spectrometry. J. Chromatogr. A 1153, 300–306

10. Kuhn, E., Wu, J., Karl, J., Liao, H., Zolg, W., and Guild, B. (2004) Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and 13C-labeled peptide standards. Proteomics 4, 1175–1186

11. Immel, D., Greven, S., and Reineiper, P. (2006) Targeted proteomics in biomarker validation: detection and quantification of proteins using a multi-dimensional peptide separation strategy. Proteomics 6, 2947–2958

12. Ackermann, B. L., and Berna, M. J. (2007) Coupling immunoaffinity techniques with MS for quantitative analysis of low-abundance protein biomarkers. Expert Rev. Proteomics 4, 175–186

13. Haieh, Y., and Korfmarcher, W. A. (2006) Increasing speed and throughput when using HPLC-MS/MS systems for drug metabolism and pharmacokinetic screening. Curr. Drug Metab. 7, 479–486

14. Barr, D. B., Barr, J. R., Maggio, V. L., Whitehead, R. D., Melissa, A. S., Whyatt, R. M., and Needham, L. L. (2002) A multi-analyte method for the quantification of contemporary pesticides in human serum and plasma using high-resolution mass spectrometry. J. Chromatogr. B 778, 99–111

15. Anderson, N. L., and Anderson, N. G. (2002) The human plasma proteome: history, character, and diagnostic prospects. Mol. Cell. Proteomics 1, 845–867

16. Ommen, G. S., States, D. J., Adamski, M., Blackwell, T. W., Menon, R., Hennjakob, H., Apeleier, R., Haab, B. B., Simpson, R. J., Eddes, J. S., Kapp, E. A., Moritz, R. L., Chan, D. W., Rai, A. J., Admon, A., Aebersold, R., Eng, J., Hancock, W. S., Hefta, S. A., Meyer, H., Paik, Y. K., Yoo, J. S., Ping, P., Pounds, J., Adkins, J., Qian, X., Wang, R., Wasinger, V., Wu, C. Y., Zhao, X., Zeng, R., Archakov, A., Tsugita, A., Beer, I., Pandey, A., Pisano, M., Andrews, P., Tammen, H., Speicher, D. W., and Hanash, S. M. (2005) Overview of the HUPO Plasma Proteome Project: results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database. Proteomics 5, 3226–3245

17. Zhang, B., VerBerkmoes, N. C., Langston, M. A., Uberbacher, E., Hettich, R. L., and Samatova, N. F. (2006) Detecting differential and correlated protein expression in label-free shotgun proteomics. J. Proteome Res. 5, 2908–2918

18. Liu, H., Sadovyg, R. G., and Yates, J. R., III (2004) A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal. Chem. 76, 4193–4201

19. Anderson, L., and Hunter, C. L. (2006) Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. Mol. Cell. Proteomics 5, 573–588

20. Martosella, J., Zolotar’ina, N., Liu, H., Nicol, G., and Boyes, B. E. (2005) Reversed-phase high-performance liquid chromatographic prefractionation of immunodepleted human serum proteins to enhance mass spectrometry identification of lower-abundant proteins. J. Proteome Res. 4, 1522–1537

21. Echan, L. A., Tang, H. Y., Ali-Khan, N., Lee, K., and Speicher, D. W. (2005) Depletion of multiple high-abundance proteins improves protein profiling

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capacities of human serum and plasma. *Proteomics* 5, 3292–3303

22. Qian, W. J., Jacobs, J. M., Liu, T., Camp, D. G., II, and Smith, R. D. (2006) Advances and challenges in liquid chromatography-mass spectrometry-based proteomics profiling for clinical applications. *Mol. Cell. Proteomics* 5, 1727–1744

23. Anderson, N. L., Anderson, N. G., Haines, L. R., Hardie, D. B., Olafson, R. W., and Pearson, T. W. (2004) Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J. Proteome Res.* 3, 235–244

24. Whiteaker, J. R., Zhao, L., Zhang, H. Y., Feng, L. C., Piening, B. D., Anderson, L., and Paulovich, A. G. (2007) Antibody-based enrichment of peptides on magnetic beads for mass-spectrometry-based quantification of serum biomarkers. *Anal. Biochem.* 362, 44–54

25. Kiernan, U. A., Nedelkov, D., and Nelson, R. W. (2006) Multiplexed mass spectrometric immunoassay in biomarker research: a novel approach to the determination of a myocardial infarct. *J. Proteome Res.* 5, 2928–2934

26. Kiernan, U. A., Nedelkov, D., Niederkofler, E. E., Tubbs, K. A., and Nelson, R. W. (2006) High-throughput affinity mass spectrometry. *Methods Mol. Biol.* 328, 141–150

27. Taylor, S. W., Andon, N. L., Bilakovics, J. M., Lowe, C., Hanley, M. R., Pittner, R., and Ghosh, S. S. (2006) Efficient high-throughput discovery of large peptidic hormones and biomarkers. *J. Proteome Res.* 5, 1776–1784

28. Berna, M. J., Zhen, Y., Watson, D. E., Hale, J. E., and Ackermann, B. L. (2007) Strategic use of immunoprecipitation and LC/MS/MS for trace-level protein quantification: myosin light chain 1, a biomarker of cardiac necrosis. *Anal. Chem.* 79, 4199–4205

29. Keshishian, H., Addona, T., Burgess, M., Kuhn, E., and Carr, S. A. (2007) Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. *Mol. Cell. Proteomics* 6, 2212–2229

30. Craig, R., Cortens, J. P., and Beavis, R. C. (2004) Open source system for analyzing, validating, and storing protein identification data. *J. Proteome Res.* 3, 1234–1242

31. Deutsch, E. W., Eng, J. K., Zhang, H., King, N. L., Nesvizhskii, A. I., Lin, B., Lee, H., Yi, E. C., Ossola, R., and Aebersold, R. (2005) Human Plasma PeptideAtlas. *Proteomics* 5, 3497–3500

32. Cox, D. M., Zhong, F., Du, M., Duchoslav, E., Sakuma, T., and McDermott, J. C. (2005) Multiple reaction monitoring as a method for identifying protein posttranslational modifications. *J. Biomol. Tech.* 16, 83–90

33. Unwin, R. D., Griffiths, J. R., Leverentz, M. K., Grallert, A., Hagan, I. M., and Whetton, A. D. (2005) Multiple reaction monitoring to identify sites of protein phosphorylation with high sensitivity. *Mol. Cell. Proteomics* 4, 1134–1144

34. Ballesta, A. M., Molina, R., Filella, X., Jo, J., and Gimenez, N. (1995) Carcinoembryonic antigen in staging and follow-up of patients with solid tumors. *Tumour Biol.* 16, 32–41

35. Krop-Watorek, A., Klopfick, A. G., Czerwinski, M., and Lisowska, E. (2002) Adhesive properties of carcinoembryonic antigen glycoforms expressed in glycosylation-deficient Chinese hamster ovary cell lines. *Acta Biochim. Pol.* 49, 273–283

36. Carpelan-Holmstrom, M., Haglund, C., Kuusela, P., Jarvinen, H., and Roberts, P. J. (1995) Preoperative serum levels of CEA and CA 242 in colorectal cancer. *Br. J. Cancer* 71, 868–872

37. Li, A., Han, M., Parmele, D., Joseloff, E., Nicol, G., Danis, C., Fitzhugh, W., Birse, C., Moore, P., He, T., and Ruben, S. M. (2007) Use of lung cancer cell lines and tissues provides a productive discovery platform for the MS identification of candidate lung cancer biomarkers, in *American Association for Cancer Research (AACR) Special Meeting: Advances in Proteomics in Cancer Research, Amelia Island, Florida, February 27–March 2, 2007*, AACR, Philadelphia

38. Chen, E. I., Cociorva, D., Norris, J. L., and Yates, J. R., III (2007) Optimization of mass spectrometry-compatible surfactants for shotgun proteomics. *J. Proteome Res.* 6, 2529–2538