A method (denoted SISCAPA) for quantitation of peptides in complex digests is described. In the method, anti-peptide antibodies immobilized on 100 nanoliter nanoaffinity columns are used to enrich specific peptides along with spiked stable-isotope-labeled internal standards of the same sequence. Upon elution from the anti-peptide antibody supports, electrospray mass spectrometry is used to quantitate the peptides (natural and labeled). In a series of pilot experiments, tryptic test peptides were chosen for four proteins of human plasma (hemopexin, α1 antichymotrypsin, interleukin-6, and tumor necrosis factor-α) from a pool of 10,203 in silico tryptic peptide candidates representing 237 known plasma components. Rabbit polyclonal antibodies raised against the chosen peptide sequences were affinity purified and covalently immobilized on POROS supports. Binding and elution from these supports was shown to provide an average 120-fold enrichment of the antigen peptide relative to others, as measured by selected ion monitoring (SIM) or selected reaction monitoring (SRM) electrospray mass spectrometry. The columns could be recycled with little loss in binding capacity, and generated peptide ion current measurements with cycle-to-cycle coefficients of variation near 5%. Anti-peptide antibody enrichment will contribute to increased sensitivity of MS-based assays, particularly for lower abundance proteins in plasma, and may ultimately allow substitution of a rapid bind/elute process for the time-consuming reverse phase separation now used as a prelude to online MS peptide assays. The method appears suitable for rapid generation of assays for defined proteins, and should find application in the validation of diagnostic protein panels in large sample sets.

Keywords: peptide quantitation • mass spectrometry • anti-peptide antibody • stable isotope • affinity chromatography

1. Introduction

Quantitative measurement of proteins is increasingly important as a means of characterizing complex biological systems, including human cells, tissues, and body fluids. The total abundance or concentration of a protein and the relative levels of its potentially numerous post-translationally modified forms, serve as primary readouts of the multilayered regulatory systems managing the cell and organism. Recent technical advances in proteomics have made it possible to resolve, to detect and to quantitate (in some degree) many proteins at once, confirming that patterns of protein expression are modulated in most biological processes, including disease.

Despite the complexity of these modulations, observations carried out at the protein level are inherently attractive because of the intimate connection of proteins with biological function. The corresponding mRNA’s are also modulated by these processes, though mRNA changes appear to be poorly correlated with protein abundance1–3 and generally give no indication of post-translational events.

Rapidly increasing use of multidimensional methods in the protein discovery phase of proteomics is likely to result in the identification of numerous candidate disease markers in serum and plasma. Multidimensional liquid chromatography (LC) peptide separation methods, when combined with tandem mass spectrometry (MS/MS), provide sufficient separation and specificity to detect thousands of peptides in serum digests,4–6 implying the presence of hundreds of different proteins by association with sequence databases. Similarly, multidimensional chromatography of intact proteins, combined with two-dimensional (2-D) gel electrophoresis and MS/MS have allowed

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the identification of several hundred human serum proteins and their post-translationally modified products. Immunoaffinity methods for the removal of high-abundance proteins such as albumin, immunoglobulins, and transferrin, are helpful in extending the dynamic range of both approaches. However, because the dynamic range of proteins in plasma exceeds 10 orders of magnitude even these multidimensional methods fall short of the sensitivity required to discover novel proteins at the concentrations of known cytokines in plasma. More importantly, the cost and difficulty of these methods currently precludes their use in studies of large numbers of individual samples. This is a significant limitation, since quantification of candidate marker proteins in large numbers of individual samples is required to validate specific proteins, or panels of proteins, for clinical use as diagnostic, prognostic, or surrogate markers.

It has been widely assumed that protein-detection arrays would emerge as the preferred means of quantitating sets of candidate marker proteins, in much the same way that DNA arrays are now widely employed to measure patterns of mRNA expression. Several types of capture reagents can be used to specifically bind an individual protein to a site on such an array, including antibodies (polyclonal, monoclonal, and phage display products) and aptamers (RNA and peptide). Beginning with the classical antibody reagents that have been used in high-sensitivity clinical diagnostic immunoassays, efforts to miniaturize these tests have been underway for more than 10 years. A number of promising technology platforms have emerged, but widespread utility seems to be impeded by a lack of validated antibody pairs used for antigen binding and detection. The developers of high quality clinical immunoarrays have known for some time that the generation of appropriate reagents for each assay is difficult, time-consuming, and thus costly. The extensive optimization currently required to make a single high-sensitivity immunoassay perform well on typical patient samples (e.g., avoidance of interference) presents a major barrier when a panel of assays must be carried out in a single fluid volume, i.e., under the same conditions for all assays. These considerations lead to a classical chicken-and-egg problem, wherein high-quality assays are developed only for “validated” markers of established utility, but the markers can only be validated through use of preexisting high quality assays. This Catch-22 is in part responsible for the remarkable shortfall in new protein markers of disease observed over the past decade.

There is thus a need to develop a bridge technology between laborious and expensive methods of discovery proteomics and the high-throughput but development-intensive systems of clinical diagnostics. Ideally this bridge would provide a means for the rapid creation of sensitive, quantitative protein assays that could be applied to validate modest numbers (tens to hundreds) of protein markers in significant numbers (thousands) of plasma or serum samples representing well-characterized sets of normal and diseased individuals. The number of samples required is high to ensure that the candidate marker or panel is associated with the target disease at convincing statistical certainty, and that it is not associated with similar changes in other disease states.

We believe that a particularly promising avenue toward such a system involves combining mass spectrometry (MS) as a quantitative detector with digestion of plasma to peptides. As used in analytical chemistry to quantitate drug metabolites and other small molecules, MS offers high precision (coefficients of variation (CV) below 5%), a good linear response range (> 10^4) and high sensitivity of detection (less than 1 ng/mL). Precision and reproducibility are generally achieved by the incorporation of an internal standard added at a known concentration, ideally as a stable isotope labeled version of the target molecule. By measuring the ion current (representing the number of ions of a given molecule that pass through the MS to the detector) for both natural and isotopically labeled forms of the same chemical structure, an abundance ratio between natural and labeled versions can be measured and used to calculate the concentration of the natural analyte. The specificity of the method relies on the ability of the MS to detect the desired analyte (and associated standard) from a mixture of instilled molecules based on accurately known masses. A particular advantage of this approach is that it has been widely used for many years at high throughput, specifically to measure drug and metabolite concentrations (including peptide-like molecules) in the plasma. In principle, abundance data as accurate as that obtained by MS measurement of drug metabolites should ultimately be obtainable for tryptic peptides as well.

For this strategy to work, it is necessary to fragment the target proteins into peptides in a reproducible fashion, since intact proteins are often too large for efficient resolution and detection by conventional MS. In addition, given the extreme dynamic range of proteins in plasma, enrichment of the peptides generated from low abundance proteins in plasma will be required if the approach is to have general applicability. Current, peptide enrichment is achieved by one- or two-dimensional chromatography, typically reverse-phase or a combination of ion exchange and reverse phase. However, these methods typically require long gradient elutions (30 min to 12 h per sample) to provide the required enrichment, and thus are not well-suited to the analysis of large numbers of samples. An attractive alternative is the use of specific antibody affinity reagents to capture the desired peptides followed by their quick elution into the mass spectrometer. Such an enrichment method can be applied routinely if the antibody adsorbent is capable of being recycled, a property which has been well-established, at least for polyclonal antibodies and protein ligands.

We have investigated an approach (summarized in Figure 1) that we refer to by the acronym SISCAPA (Stable Isotope Standards with Capture by Anti-Peptide Antibodies). SISCAPA combines the four elements previously described: (1) digestion of a protein sample to peptides; (2) addition of internal standard peptides labeled with stable isotopes; (3) enrichment of low abundance peptides by capture with immobilized antibodies; and (4) quantitation by MS. Various combinations of three of these four elements have been used previously to make useful measurements of selected peptides or proteins in complex mixtures, thereby demonstrating some components of the method. A combination of isotopically labeled peptide standards, antibody capture, and MS quantitation was used in the late 1980s to measure specific naturally occurring neuropeptides in brain tissue, while Gygi has used stable isotope labeled peptides and MS/MS (the AQUA method) to quantitate specific modified peptides in a digest and Scriber used a planar array of phage display antibodies to enrich specific peptides in a simple digest prior to detection by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS. Several groups have used antibody capture to enrich specific
proteins for MS analysis,18 while others have used antibodies to capture peptides such as beta amyloid23 and c-myc peptides.24

Our results demonstrate that the SISCAPA method, by combining all four elements, allows specific enrichment and quantitation of peptides from complex mixtures and thus has potential as an important research tool for development of diagnostic markers in the plasma proteome.

2. Materials and Methods

2.1 Selection of Peptides. The protein sequences of 237 proteins known to occur in plasma9 were digested with trypsin in silico to yield a database of 10 203 possible peptides. A series of parameters was then computed for each peptide to estimate its effectiveness as a hapten for B-cell responses, and its performance in MS. An aggregate pass/fail result was derived for each peptide based on the following criteria: (1) the length between 8 and 14 amino acids (optimum size to use as haptens); (2) a Hopp–Woods hydrophilicity between −0.5 and 0.5 (to satisfy properties of B-cell epitopes); (3) mass greater than 800 amu (to facilitate detection by electrospray ionization–MS); (4) no Cys, Met, or Trp residues (to avoid chemical modifications); and (5) no post-translational modifications or polymorphisms listed in the Swiss-Prot database (to minimize occurrence of variant forms). Monitor peptides were selected for four proteins: two of moderate abundance (hemopexin (Hx) and α₁-antichymotrypsin (AAC)) which should be readily detectable in digests, and two of low abundance (interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)) which should not be easily detected in normal plasma and should thus serve as negative controls (Table 1). Several peptides from each of these proteins passed the selection criteria: three for Hx, three for AAC, two for IL-6, and four for TNF-α, and one peptide containing a Pro residue was selected from each protein in order to increase potential immunogenicity. Finally, each candidate peptide was verified as unique by searching the human genome database using the Ensembl genome browser (http://www.ensemble.org/). These selected peptides were designated Nat 2–5 (for natural peptides 2–5).

2.2 Anti-Peptide Antibodies. The tryptic peptide sequences (Nat 2–5) were synthesized with a C-terminal extension (Gly-Ser-Gly-Cys) to provide a cysteine thiol spaced away from the immunizing sequence by a relatively nonantigenic linker (Gly-Ser-Gly). This thiol was used to covalently attach the peptides to protein carriers for immunization, to microwell plates for enzyme-linked immunosorbent assays (ELISA), to agarose supports for antibody affinity purification, and to a fluorescent chromophore for characterization of immobilized antibody supports by flow cytometry (see the preceding paper by Pearson et al.). Because no Cys-containing tryptic peptides were selected, the position of the attachment was unique. The synthetic immunizing peptides were designated IMM 2–5 (Table 2). One peptide (IMM 6 from TNF-α) was also synthesized with an N-terminal extension (Cys-Gly-Ser-Gly) to yield a database of 10 203 possible peptides. A series

Table 1. Plasma Protein Targets

<table>
<thead>
<tr>
<th>protein identity</th>
<th>Swiss-Prot accession no.</th>
<th>protein mass (daltons)</th>
<th>normal range concentration in human serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>interleukin-6 (IL-6)</td>
<td>P05231</td>
<td>23 718</td>
<td>&lt;10 pg/mL</td>
</tr>
<tr>
<td>hemopexin (Hx)</td>
<td>P02790</td>
<td>51 676</td>
<td>0.5–1.15 mg/mL</td>
</tr>
<tr>
<td>α₁-antichymotrypsin (AAC)</td>
<td>P01011</td>
<td>47 650</td>
<td>0.3–0.6 mg/mL</td>
</tr>
<tr>
<td>tumor necrosis factor-α (TNF-α)</td>
<td>P0375</td>
<td>25 644</td>
<td>&lt;10 pg/mL</td>
</tr>
</tbody>
</table>

Table 2. Peptide Haptens (Antigens) Used for Immunization and Fluorescent Labeling

<table>
<thead>
<tr>
<th>protein peptide</th>
<th>identification code</th>
</tr>
</thead>
<tbody>
<tr>
<td>interleukin-6 (IL-6)</td>
<td>EAIAENNLPKGSGC</td>
</tr>
<tr>
<td>hemopexin (Hx)</td>
<td>NFPSVDAFGRDCGSG</td>
</tr>
<tr>
<td>α₁-antichymotrypsin (AAC)</td>
<td>GSGCSG</td>
</tr>
<tr>
<td>tumor necrosis factor-α (TNF-α)</td>
<td>IMM4 Ab 4</td>
</tr>
<tr>
<td>tumor necrosis factor-α (TNF-α)</td>
<td>IMM5 Ab 5</td>
</tr>
</tbody>
</table>

Figure 1. Schematic diagram of the SISCAPA method for peptide quantitation.
peptides were synthesized by Cell Signaling Technology Inc., instructions, for use on streptavidin-coupled POROS beads (see below).

2.3 Labeled Peptides. Stable isotope standard (SIS) monitor peptides were synthesized by Cell Signaling Technology Inc., Beverly, MA and each contained a single fully 13C-substituted amino acid near the C-terminus (Table 3). The peptides were designated SIS 2–5 and corresponded to the natural peptides Nat 2–5. Peptide SIS 3 (labeled on Phe) was 10 atomic mass units (AMU) heavier than the natural peptide (Nat 3), and the other SIS:Nat pairs differed by 6 AMU due to incorporation of a single 13C-substituted Pro or Val. Peptides were quantitated by acid hydrolysis and amino acid analysis (AAA Services Inc., Boring, OR) for use as quantitative standards. Mixture of the five SIS peptides were prepared in 100 mM ammonium acetate, pH 7.5, and most experiments used a mixture with equal stoichiometry (40 nM each).

2.4 Plasma Digestion. A tryptic digest of whole human plasma (from TWP) was prepared as follows: proteins were denatured by addition of 9 volumes of 6 M guanidinium HCl/50 mM Tris-HCl/10 mM dithiothreitol and incubation for 2 h at 60 °C followed by dilution to <1 M guanidinium HCl by addition of 50 mM NaHCO3. The denatured proteins were concentrated to 1/6 vol using Centricon YM-10 spin column concentrators (10 000 MW cutoff; Millipore, Bedford, MA). Trypsin digestion was performed by the addition of sequencing grade modified trypsin (Promega, Madison, WI) to the retentate at a 1:80 ratio (trypsin:plasma proteins) and the reaction was incubated overnight at 37 °C. The digestion mixture was spin filtered using Centricon YM-3 filters (3000 MW cutoff; Millipore, Bedford, MA) to remove undigested proteins, including trypsin, and the filtrate was applied to a prewetted Sep-Pak C18 column (Waters Associates, Milford, MA). Peptides were eluted with 50% acetonitrile/0.1% formic acid and dried using a Speed Vac Concentrator (Savant, Hicksville, NY). The pellets were rehydrated with 20 μL of 100 mM ammonium acetate.

2.5 Antibody Immobilization on POROS Supports. In some experiments, biotinylated, affinity-purified antibodies were exposed to streptavidin-coupled BAA0 Self-Pack POROS beads (Applied Biosystems, Foster City, CA) in suspension for 3 h, washed and used without covalent cross-linking. To achieve a more oriented binding, the affinity-purified IgGs were bound covalently to Self-Pack POROS 20G Protein G beads in suspension, and then covalently cross-linked. The coupling method and characterization of the immunoabsorbents are described in detail in the preceding paper in Pearson et al.

2.6 Construction of Capillary Affinity Columns. Nanobore antibody affinity columns were prepared in 100 μm ID (360 μm OD) fused silica capillaries either containing an integral frit (Integrafit, New Objective Inc., Woburn, MA) or using an Upchurch (Oak Harbor, WA) M520 inline microfilter fitting with 0.5 μm PEEK filter as frit. The fused silica tubing was mated to Upchurch PEEK fittings through use of PEEK tubing sleeves of appropriate dimensions. Antibody-coupled POROS matrices were packed into the capillaries (~80 nl/cm bed volume) using a 1000 psi/g helium pressurized bomb and averaged 1 cm in bed length.

With binding capacities for immunoglobulin G (IgG) of approximately 19 and 30 mg/mL of packed beads (for streptavidin-POROS and protein G POROS supports respectively), very small column volumes are required to bind 100 fmol of a very high affinity ligand (only 0.5 nL for protein G POROS). We elected to “oversize” the columns for ease of packing. Using 100 μm ID (360 μm OD) fused silica capillaries, 1.0 cm columns had volumes in the range of approximately 80 nl, and theoretical binding capacities (if fully saturated with IgG) of 16 pmol of ligand. Flow rates during loading, washing and elution were at least 4 μl/min, or approximately 50 cm/min for 100 μm ID columns, sufficient flow velocities to achieve full perfusion of the POROS support. Using this flow regime, a sample fluid element was exposed to the immobilized antibody for a total of approximately 1–3 s.

2.7 Liquid Chromatography. A chromatography setup (Figure 2, components manufactured by LC Packings, Sunnyvale, CA) similar to that used for 2-D LC/MS was used to test affinity columns. Peptide samples (5 μL) from a capillary autosampler (Famos; LC Packings) were injected onto the antibody column (installed between ports 2 and 9 of valve B of the SwitchOS valve unit) and washed with 100 mM ammonium acetate buffer, after which the antibody column was switched inline with a 300 μm × 1 mm Pepmap C18 trap cartridge (LC Packings), and the antibody-bound peptides eluted with a 5 or 10 μl injection of 1% acetic acid in water. Once the peptides were captured by the C18 trap, this was switched in line with a 75 μm × 15 cm Pepmap C18 analytical column (LC Packings) and eluted by a 150 nl/min acetonitrile (ACN) gradient of ACN/formic acid in water (0 to 60% ACN over 33 min, followed by a 2 min ramp to 80% ACN) directly into the electrospray ionization (ESI) source of the MS. The eluent (1% acetic acid in water) was chosen with the objective of preserving the integrity of the immobilized antibodies, thus facilitating column recycling. More than 90% of the bound peptide was eluted by a single 5 μL injection (~60 column volumes) of acid. The pre-MS peptide separation thus included both immunoaffinity and reverse-phase chromatographic steps.

2.8 Mass Spectrometry. The chromatography system was coupled to either a Q-STAR Pulsar QqTOF mass spectrometer or a Q-TRAP triple quadrupole/linear ion trap mass spectrometer (LC/MS/MS system) both from Applied Biosystems (Foster City, CA). Analyst software (Applied Biosystems) was used to control both instrument setups. The Q-STAR was calibrated with two reference materials (cesium iodide and sex pheromone inhibitor) each day, whereas the Q-TRAP was calibrated once per week.
The four synthetic, isotope-labeled SIS peptides were the other at the C-terminus. peptide, one designed to be anchored at the N-terminus and purity of $^{13}$C in the labeled amino acids was not 100.0%. LC/MS/MS was also used to determine the fragmentation patterns of each peptide and confirm the expected sequences. High intensity (efficiently produced) fragments were identified in the MS/MS spectrum of each monitor peptide (Table 4). Collision energy and declustering potential parameters for optimal production of these product ions were selected on an ABI Q-TRAP mass spectrometer using automatic features of the Analyst software. In general, the fragmentation patterns observed for these peptides in the Q-STAR and Q-TRAP instruments were very similar.

### 3.3 Antisera and Affinity-Purified Antibodies

Indirect ELISA titers of rabbit antisera collected 38 days after immunization were obtained using the immunizing peptide-carrier constructs as solid-phase adsorbed antigens. Titers ranged between 1:22 000 and 1:70 000 for each antisera, compared to <50 for all preimmunization bleeds (not shown). For each immunogen, two bleeds (52 and 56 days post-initial immunization) were pooled from the rabbit with the higher titer first bleed and used for affinity purification of antibodies. Affinity-purified antibodies were analyzed by 1-D gel electrophoresis and quantitated by determination of absorbance at OD$_{280}$ nm. Affinity purification from ~44 mL serum yielded between 4 and 19 mg of purified antibody specific for the various peptide antigens.

### 3.4 1-D MS Quantitation: Selected Ion Monitoring

In an initial set of experiments, we generated an immunoaffinity support carrying immobilized Ab3 (specific for Nat 3 peptide from hemopexin) by first biotinylating the affinity purified antibody preparation and then coupling it to POROS streptavidin beads. A nanoaffinity column made with this support was then evaluated using a multidimensional chromatography setup (Figure 2) to test the specificity and efficiency of peptide binding. The detection of bound and eluted peptides was accomplished on a Q-STAR Pulsar mass spectrometer by following the ion current in specific narrow mass ranges (~0.25 amu wide) encompassing the dominant monoisotopic form of each SIS and Nat peptide (selected ion monitoring: SIM). Figure 3 shows the ability of the LC/MS to detect separately each of the four SIS peptides from a mixture (sample injected directly onto the C18 trap, bypassing the antibody column, as a positive control). The individual SIS peptides differed in retention time as well as mass, allowing unequivocal identification. The SIS peptides also varied in detection efficiency, but all showed strong signals in the Q-STAR at loadings of 24–48 fmol. Integrated counts within the specified mass windows (trace B in Figure 3) accounted for about half the total ion current due to each peptide (peaks in trace A), with the remaining counts distributed over the minor $^{13}$C isotopic forms as expected.

We next investigated the peptides bound by Ab 3 from the mixture of SIS peptides. The antibody column selectively bound and released peptide SIS 3 (Figure 3 trace C), as expected, since it has the same core sequence as the Ab 3 peptide antigen IMM 3. In this case, the antibody column bound 50% of the applied SIS 3 peptide (as estimated by integrated ion current), 2.7% of peptide SIS 5 and no detectable SIS 2 or SIS 4 peptides. Using
a column of ~100 nL and a flow rate of 4 µL/min, any given volume element of the sample was in contact with antibody for only ~1.5 s, which may be too short to allow efficient peptide binding and hence accounting for less than 100% recovery of the “correct” peptide. A bigger challenge was presented when an unfractiionated tryptic digest of human plasma spiked with SIS peptide standards was applied to the antibody column, followed by a wash and acetic acid elution (Figure 4). Both the natural (Nat 3; peak A) and labeled (SIS 3; peak C) versions of the targeted Hx peptide were bound by the antibody. These two peptides have the same chemical structure and elute at the same time from the reverse phase column, but they differ in mass (Nat 3 shows the expected decrement of 5 amu relative to SIS 3, half the 10 amu isotopic difference because the ion is doubly charged; z = 2). A quantitative ratio (Nat 3/SIS 3) of 7.5 was obtained.

In addition, several other peptides of interest bound to the column. One was a partial digestion product of Hx (Figure 4, peak B) that contained the Nat 3 sequence plus two more N-terminal amino acids (a doubly charged ion at 767.9 amu). This peptide gave a slightly higher ion current than Nat 3, suggesting that it was more abundant and indicating that tryptic digestion of the hemopexin in plasma was incomplete, a result confirmed by 1-dimensional polyacrylamide gel analysis (not shown). Another peptide (doubly charged at 773.9 amu, and thus 12 amu heavier than the 767.9 peptide) accounted for ~40% of the ion current of a peak at 45.8 min (labeled 472.23 in Figure 4) and yielded the same y-ion fragmentation as the Hx peptide of 767.9 amu. However its b-ions and N-terminal Trp immonium ion were all 12 amu heavier, suggesting that it is a version of peptide 767.9 with a +12 amu modification to the N-terminal Trp residue.

The most abundant non-Hx peptides were derived from albumin, and include peptides of mass 949.9 and 633.7 (+2 and +3 ions of peptide RHPFYAPELFFAK, together making up the large peak at 49.2 min), 871.97 (peptide HPFYAPELFFAK at 50.5 min) and 507.29 (LVAASQALGL, the C-terminal albumin peptide, at 43 min). Another prominent non-Hx peptide was a fragment of Apo A-I lipoprotein (QGLLPVLESFK) that shares an internal dipeptide sequence (PV) with Nat 3. This dipeptide may constitute a cross-reacting portion of an epitope recognized by the antibody. The Apo A-I peptide has a mass almost identical to that of SIS 3, but differs in elution time from the C18 column. The identities of the major bound peptides were confirmed by MS/MS.

Studies of repeated elution cycles after a single sample load indicated that 90–95% of the bound SIS 3 peptide was eluted by a single exposure to 5 µL of 1% acetic acid. Repeated load: elute cycles established that the ion current observed for SIS 3 (selecting mass and elution time in SIM) was very reproducible, with a CV of approximately 5% (data not shown). No deterioration of binding capacity was observed over 5–10 cycles, although this was not a strict test of antibody survival because the column capacity was initially much larger than would be saturated by the amounts of peptide applied.

3.5 Characterization of Oriented Antibody Supports. Chemical cross-linking of affinity-purified rabbit antibodies to POROS Protein G beads had previously been shown to result in high capacity affinity supports capable of surviving hundreds of cycles of protein binding and acid elution. Such supports are likely to have higher capacity as well since the antibody is first oriented by binding of the Fc domain to Protein G. This procedure was used successfully with all five affinity-purified rabbit anti-peptide antibody preparations in the current work. The affinity matrices were first characterized by flow cytometry...
prior to use in nanoaffinity capillary columns upstream of mass spectrometry. All of the antibody supports showed high capacity binding, with the exception of the adsorbent made with Ab 6 (raised to the N-terminal-linked TNF-α peptide IMM 6). This was not used in the MS experiments.

3.6 “2-D” MS/MS Quantitation: Selected Reaction Monitoring. To increase the specificity of the measurement of the intended peptides, we used selected reaction monitoring (SRM) to quantitate specific fragment ions of the isotope-labeled and natural monitor peptides (Table 4). The chromatography system used above was transferred to a triple quadrupole MS incorporating an ion trap in Q3 (ABI Q TRAP), and the yield of the selected product ions (high-mass y-series ions in this case) was optimized by systematically varying collision energy and declustering potential. The optimized parameters for fragmenting each peptide (Table 4) were then used in a scanning multiple SRM method for LC-MS/MS sample analysis that cycled through 200 ms measurements of either all 8 peptides (SIS 2–5 and Nat 2–5) or only the 4 SIS peptides (SIS 2–5), depending on the sample type. Both quadrupole mass filters (Q1 and Q3) were set to low resolution (~4 amu windows) to maximize sensitivity. The sensitivity of the detection system for the SIS peptides in SRM mode was examined by injecting a series of dilutions of the SIS peptide mixture directly onto the C18 trap cartridge, bypassing the immunoaffinity column (data not shown). The lower limit of detection averaged approximately 2 fmol and the response was approximately linear in the 2–200 fmol region, with decreasing slope near 2000 fmol.

Four antibodies (Ab 2–5), immobilized on POROS Protein G matrices and shown to be effective immunoadsorbents by flow cytometry (see the preceding paper by Pearson et al.), were then characterized for retention of the four SIS peptides (SIS 2–5). A mixture of 200 fmol of each of the SIS peptides was applied to each single-antibody nanoaffinity column (~100 nL) in a separate experiment. Following peptide application, the column was washed with 100 mM ammonium acetate buffer, bound peptides were eluted with a 10 μL injection of 1% acetic acid onto the C18 trap and the peptides were eluted from the trap and resolved on an analytical C18 column by a 24 min gradient of 0–60% ACN. The result was a series of traces across the LC gradient of specific parent/product molecular measurements. In each case, the use of SRM eliminated confounding signals, showed a low background and yielded a single peak for each peptide in the LC gradient. As a consequence, the interference observed in SIM mode due, for example, to the QGLLPVLESFK peptide of Apo A-I having a mass similar to that of Nat 3, was eliminated. Each column showed substantial enrichment of the “correct” peptide (Figure 5, Table 5), with average enrichments (ratio of the antigen peptide to a nonantigen peptide ion current) ranging from 16 to 325. The average of these enrichments across all pairs was 120: the average

![Figure 4. MS/MS analysis of peptides bound by Ab 3 from a mixture of SIS calibrants and a tryptic digest of whole human plasma. Beneath the total ion chromatogram in the upper right are three traces of ion currents observed in 0.25 amu windows centered at 610.8, 767.9, and 615.8 amu, respectively. Peak A (a portion of whose MS/MS fragmentation pattern is shown on the left as panel A) is the Nat 3 peptide derived from Hx in the digest. Peak B is an incomplete tryptic peptide including Nat 3. Peak C is SIS3, the isotopically labeled version of the Nat 3 peptide. Peak D is a peptide derived from plasma Apo A-I lipoprotein, having nearly the same mass as SIS 3.](image-url)
antigen peptide gave 120-fold higher signal than the average nonantigen peptide after capture and elution.

The cross-reaction between Ab 2 and fluorescent peptide IMM 4 (the Alexa Fluor 488 conjugate of IMM 4), observed by flow cytometry (see the preceding paper by Pearson et al.), was not observed by MS, using the same antibody support and unconjugated SIS 4 peptide, suggesting that the cross-reaction was likely to be an artifact caused by a structural change in the peptide upon conjugation. Surprisingly, the specificity of each column for the “correct” peptide increased after the first 1–2 acid elution cycles. It is interesting that an increased binding capacity for the “correct” peptide was also seen after recycling trials during the initial characterization of the POROS Protein G immunoaffinity adsorbents performed by flow cytometry (see the preceding paper by Pearson et al.).

When each of the four antibody columns was exposed to a tryptic digest of human plasma and the bound peptides examined using an 8-way SRM (4 SIS and 4 Nat peptides), only two of the natural peptides were bound: Nat 3 (derived from Hx) and Nat 4 (from AAC). The immunoaffinity columns specific for Nat 2 and Nat 5 (from IL-6 and TNFα respectively) did not bind enough of these peptides to be detected by MS, as expected due to their low abundance in normal human plasma. A series of five repeat binding and elution cycles using Ab 3 showed no evidence of a decline in capacity and gave a CV of 6% on the integrated SRM peak of SIS 3 peptide from run to run (Figure 6).

4. Discussion

We have demonstrated that polyclonal anti-peptide antibody nanocolumns can enrich specific peptides from a mixture, and that the enriched peptides can be quantitated by mass spectrometry in relation to stable isotope labeled synthetic peptides as internal standards. This process forms the basis of the SISCAPA method, which will be useful in establishing high-throughput assays for candidate disease marker proteins in plasma, other body fluids, and tissues.

The SISCAPA method is designed for selected proteins of known sequence and thus addresses a goal different from that of survey proteomics, which generally aims at an unbiased representation of as many sample proteins as possible. By focusing on protein or peptide analytes selected in advance, the SISCAPA approach exchanges global coverage for the possibility of increased analytical precision and throughput: it attempts to extend proteomics from the protein survey domain into the realm of classical (and very successful) analytical chemistry. In principle, the method provides high sensitivity and precision, while requiring minimal MS machine time per measurement. From a practical viewpoint, the use of a single-step elution of peptide from an antibody (essentially an injection event) in place of a gradient elution, may also allow simplification of the fluid handling system, with attendant improvement in robustness.

Only two specific reagents are required for each assay: the internal standard peptide and the peptide-binding antibody. The stable isotope labeled synthetic peptide (typically 8–15 amino acids in length and having the sequence of the monitor peptide chosen to represent the protein from which it is derived in the digest) can be made by automated peptide synthesis. Such peptides can be made commercially at reasonable cost, given the low (fmol to pmol) consumption involved. Raising anti-peptide antibodies (e.g., in rabbits) is now common practice using synthetic peptide immunogens (typically as a substitute for a whole protein that is difficult to obtain) and such reagents can also be produced commercially. Since the anti-peptide antibody in the SISCAPA method is used only for enrichment and does not take a direct part in the quantitation...
(which is performed by MS), the properties of the antibodies are probably not as critical as they are for antibodies in immunoassays.

The selection criteria used here to choose tryptic peptides for SISCAPA (including size, hydrophilicity, absence of Cys, Trp, Met, and presence of Pro) were not terribly stringent, yielding candidates from 84% of 237 proteins known to occur in plasma. Nevertheless, these criteria yielded peptides that ionized well (detection limits near 2 fmol in the MS systems used) and generated antibodies capable of significantly enriching the target peptide (between 16- and 325-fold) in four of four cases examined. It therefore seems likely that at least one good SISCAPA peptide (with good MS performance and yielding good antibodies) can be obtained for almost any desired candidate protein.

The rabbit polyclonal antibodies we used proved to be effective, even though neither the immunization protocol nor the affinity purification process was optimized for recyclable binding of tryptic peptides. Each had limited specificity, and captured other peptides in addition to the target (antigen) peptide. This was evident from the fact that small amounts of inappropriate SIS peptides bound to each antibody, which limited the overall average enrichment to an average 120-fold. This value agrees with the results obtained by flow cytometry with fluorescent peptides in the preceding paper by Pearson et al. In the case of one antibody (Ab3) we characterized a series of nontarget peptides bound from a tryptic digest of plasma, including several albumin peptides and a peptide from apo A–I lipoprotein. A large majority of the bound albumin peptide was comprised of HPYFYAPELLFFAK and the related apo A–I peptide shares a dipeptide sequence (PV) with the target sequence, providing a potential rationale for its weak binding.

It is clear that the performance of the antibodies can be substantially improved. On the basis of an enrichment averaging 100-fold for the peptides examined here, we would expect polyclonal antibodies made in this manner to contribute about 2 orders of magnitude to the sensitivity of a given MS platform. However, given the high concentration of antibody binding sites attainable on the POROS supports we used here (10^-5 M), it should be possible to bind >90% of a peptide for which the antibody has an affinity of 10^6 M^-1, and 99.99% of a peptide for which the affinity is 10^9, effectively independent of the peptide concentration (assuming of course that the antibody is not saturated). Although we did not characterize the elution profile of the bound peptide, experience with protein ligands on similar POROS-antibody columns8 showed a sub-column-volume elution volume, which would suggest that peptide was eluted in <100 nL from our columns (and correspondingly smaller volumes for smaller columns). Using 10 fmol as a clearly detectable peptide abundance in such a volume, then efficient antibody capture of a peptide from a digest of 10 nL plasma would allow detection of a 40 kd protein present at 40 ng/mL. Better sensitivity than this would require improved mass spectrometry. Antibodies of higher specificity and affinity would be ideal for antigen capture and release, and for practical reasons it would be useful if they were able to withstand recycling as well (to allow use with multiple samples). Despite the small amounts required, it would also be beneficial to have an unlimited source of antibodies that are amenable to standardization. All of these attributes could be best satisfied with properly selected monoclonal antibodies, an approach we are pursuing for use in the present system.

Tryptic digestion protocols require similar optimization. We observed several partial digestion products that diminished signal from the expected ions, and their presence, if not eliminated, will have to be calibrated if peptide abundances are to serve as effective protein abundance surrogates. At this stage, little quantitative optimization of peptide yield has been undertaken, and this will be a requirement in the development of any of the proposed MS quantitation methods. Given effective monitoring tools, we expect a robust and automatable protocol can be developed.

The rapidly increasing resolution and sensitivity of MS methods will improve the effectiveness of SISCAPA as well. We have shown that two-dimensional MS selection of both peptide and fragment masses (SRM) allows effective rejection of a nontarget peptide having a mass very close to that of a SIS peptide (e.g., the Apo A–I peptide having the same mass as SIS 3). In the case of the SRM of the nine SIS + Nat peptide sequences used here, each could be observed specifically in a digest with no nontarget peptide peaks detectable in the LC chromatogram. The primary limitation of the SRM approach (the limitation in the number of multiplexed peptides detectable in a short time) could be addressed by a sufficiently high-resolution single-dimension (SIM) approach such as Fourier transform ion cyclotron resonance (FT-ICR) MS, where achievable resolutions of > 100 000 may provide the same level of peptide specificity as SRM with similar dynamic range.

We believe that continued development of the body of technology used in the SISCAPA method should be given high priority, given the importance of protein marker validation in translating proteomic marker discoveries into clinically useful tests.

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References


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