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Relative Quantification of Serum Proteins from Pancreatic Ductal Adenocarcinoma Patients by Stable Isotope Dilution Liquid Chromatography-Mass Spectrometry

Angela Y. Wehr¹, Wei-Ting Hwang², Ian A. Blair¹, and Kenneth H. Yu^{3,*}

¹Centers for Cancer Pharmacology and Excellence in Environmental Toxicology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

²Department of Biostatistics and Epidemiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

³Gastrointestinal Oncology Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA

Abstract

We report an innovative multiplexed liquid chromatography-multiple reaction monitoring/mass spectrometry (LC-MRM/MS)-based assay for rapidly measuring a large number of disease specific protein biomarkers in human serum. Furthermore, this approach uses stable isotope dilution methodology to reliably quantify candidate protein biomarkers. Human serum was diluted using a stable isotope labeled proteome (SILAP) standard prepared from the secretome of pancreatic cell lines, subjected to immunoaffinity removal of the most highly abundant proteins, trypsin digested, and analyzed by LC-MRM/MS. The method was found to be precise, linear, and specific for the relative quantification of 72 proteins when analyte response was normalized to the relevant internal standard (IS) from the SILAP. The method made it possible to determine statistically different concentrations for three proteins (cystatin M, IGF binding protein 7, and villin 2) in control and pancreatic cancer patient samples. This method proves the feasibility of using a SILAP standard in combination with stable isotope dilution LC-MRM/MS analysis of tryptic peptides to compare changes in the concentration of candidate protein biomarkers in human serum.

Keywords

SILAC; SILAP; immunoaffinity depletion; tryptic digestion; LC-MS/MS; serum biomarkers

INTRODUCTION

Many methods have been developed to characterize differential proteomes. Application of these methods has generated vast lists of candidate biomarker proteins which await quantification in human samples for validation.¹⁻⁸ Enzyme-linked immunosorbent assay (ELISA) is the most common method used for the quantification of proteins in serum due to its high sensitivity and specificity. However, this method becomes expensive and labor

*Corresponding Author Kenneth Yu, M.D., Gastrointestinal Oncology Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, 300 East 66th Street, New York, New York 10065 YuK1@mskcc.org.

Supporting Information. Sample Analysis and Supplemental Table 1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

intensive when applied to a large number of proteins and samples. Furthermore, high quality monoclonal antibodies are not available for the vast majority of candidate biomarkers identified. As a result, most biomarker validation studies are limited to single or several proteins.⁹⁻¹² Stable isotope dilution with detection by MS has been the mainstay of bioanalytical analyses for the past two decades¹³⁻¹⁵ due to both the specificity and sensitivity with which this methodology can measure the concentration of small molecules. With developments in MS technology, such an approach has developed the speed and sensitivity to allow for detection of low abundance proteins in serum.^{16,17}

Compared with small molecule LC-MS, the analysis of proteins adds several specific challenges. Intact proteins can be difficult to separate on traditional LC columns, especially those designed to work at the decreased flow rates required to achieve high sensitivity microflow electrospray ionization (ESI). However, the peptides that result from tryptic digestion behave much more like small molecules and are easier to separate by reverse phase (RP) LC with MS-compatible mobile phases. Tryptic peptides terminating in lysine and arginine are also amenable to multiple charging by ESI. This makes it possible to analyze product ions of a lower charge state and higher m/z than their corresponding precursor ions, which helps to minimize background noise in monitored transitions, a key concern when working in a complex matrix like human serum.

The protein load in human serum poses another obstacle. LC columns used with microflow ESI/MS are limited in the amount of protein that can be loaded onto the column. The concentration range of proteins in human serum spans over 10 orders of magnitude¹⁸ and 99% of the protein by mass in serum is made up of 22 highly abundant proteins such as albumin and immunoglobulins.¹⁹ Commercially available immunoaffinity columns can be used to remove these proteins from the serum, reducing the protein concentration and effectively increasing the loading capacity of the column.²⁰⁻²² Stable isotope standards and capture by anti-peptide antibodies (SISCAPA) has been developed as a system to purify peptides, derived from proteins of interest, from the biological matrix.²³ However, antibodies specific to each peptide must first be developed, and thus is limiting in a similar fashion to ELISA.

As noted in an important commentary by Diamandis, problems with pre-analytical, analytical, and post-analytical study design can lead to serious misinterpretations and to generation of data that could be highly misleading.²⁴ In particular, processes of digestion and immunoaffinity removal of abundant proteins can be highly variable and both have been associated with poor precision which can confound studies intended to compare the concentration of an analyte across different samples.^{25,26} Dilution with a stable isotope labeled IS is commonly used in LC-MS assays to correct for losses during sample preparation or variability in chromatographic separation and ionization efficiency. Our group has previously reported the use of a labeled proteome standard,^{3,17,27} prepared by stable isotope labeling by amino acids in cell culture (SILAC)-based methodology,^{28,29} to normalize proteomic profiling. Termed stable isotope labeled proteome (SILAP), the current study represents a novel implementation of this approach. The SILAP standard was prepared from proteins secreted by human pancreatic cell lines in culture, allowing for the relative quantification of a panel of pancreas specific protein biomarkers in serum. Here we report the development and validation of a quantitative method, using relevant stable isotope labeled ISs to normalize the analyte response in human serum and its use for the relative quantification of serum proteins in pancreatic ductal adenocarcinoma (PDAC) patients.

EXPERIMENTAL PROCEDURES

Cell Culture

The human PDAC cell line CAPAN-2 and the immortalized human pancreatic stellate cell (PSC) line RLT-PSC³⁰ were cultured according to standard practice using Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO) supplemented with 10 % fetal bovine serum (Sigma) and 1 % antibiotic (penicillin/streptomycin, Invitrogen, Grand Island, NY). SILAC labeling was achieved using leucine and lysine free DMEM supplemented with ¹³C, ¹⁵N-labeled leucine and lysine (Cambridge Isotopes, Andover, MA), 10 % dialyzed fetal bovine serum (Sigma), and 1% antibiotic (penicillin/streptomycin, Invitrogen). Cells were passaged a minimum of 7 times to achieve amino acid labeling of > 99.0 %. Secreted proteins were harvested by allowing cells to grow to 80 % confluence, washing with phosphate buffered saline (Invitrogen), and incubating the cells with serum-free SILAC media at 37 °C for 48 h. This media was collected, passed through a 0.22 µm filter, and stored at -80 °C.

Immunoaffinity Removal of Abundant Proteins

Human serum was first diluted with SILAP internal standards (IS) prior to processing with either the IgY-14 LC2 column (Seppro®, Sigma) alone, or in tandem with the SuperMix column (Seppro®, Sigma), according to manufacturer instructions. To each serum sample (45 µL), the RLT-PSC SILAP (40 µg protein) and the CAPAN-2 SILAP (16 µg protein) were added. Each sample was then diluted 1:4 with dilution buffer (Seppro®, Sigma) centrifuged through a 0.45 µm filter, then injected onto the immunoaffinity column(s). Dilution buffer was used to elute the flow-through, or low-abundance, fraction which was monitored at 280 nm, collected, and stored on ice. The bound, or high-abundance, fraction was eluted with stripping buffer (Seppro®, Sigma), collected, and stored on ice. The columns were regenerated with neutralization buffer (Seppro®, Sigma). The protein was then concentrated using 3 kDa MW cutoff spin-filters (Millipore, Billerica, MA), and precipitated using methanol-chloroform extraction.

Tryptic digestion

The precipitated protein mixture was dissolved in a small volume of 6 M urea, 2 M thiourea. Following solubilization, the sample was diluted 10-fold with 25 mM ammonium bicarbonate, reduced in 5 mM dithiothreitol for 45 min at 60 °C, alkylated in 15 mM iodoacetamide for 30 min at room temperature in the dark, and digested using sequencing grade trypsin (Promega, Madison, WI) at a ratio of 1:20 by weight at 37 °C overnight.

LC-tandem mass spectrometry (MS/MS) analysis

Peptides were acidified to 0.1 % formic acid and desalted with disposable C18 spin filters (Nest Group, Southborough, MA). Peptides were separated by reversed phase chromatography using a C18 column (0.3 × 150 mm, 3 µm, 200 Å C18AQ, Michrom Bioresources, Auburn, CA) on a microflow HPLC system (Eksigent, Dublin, CA). After a 5 min isocratic hold at 95 % mobile phase A (2 % acetonitrile, 0.1 % formic acid), a linear gradient increasing mobile phase B (95 % acetonitrile, 0.1 % formic acid) from 5 to 60 % over 55 min at a flow rate of 5 µl/min was used to elute peptides online to a TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a CaptiveSpray™ ion source (Michrom Bioresources Inc., Auburn, CA, USA), and used under positive ESI conditions. Raw files were imported to SkyLine,^{31,32} and integration was manually verified as occurring at the correct retention time for the three transitions/peptide and the three transitions/labeled peptide.

Sample Analysis

Human serum samples were collected in accordance with University of Pennsylvania Institutional Review Board approved protocols. Whole blood samples were collected preoperatively in glass tubes without additive (10 mL BD Vacutainer™ No Additive, BD, Franklin Lakes, NJ) and allowed to clot at room temperature for 40 min. Serum was separated by centrifugation at 1500 rpm for 15 min. Aliquots of serum (1 mL) were taken and stored at -80 °C until ready for use. The time from collection to frozen storage was no more than 60 min. Relevant clinical information was collected and linked to individual serum samples, but was de-identified before submitting for analysis. Samples were matched for age, race, and gender. They were classified as PDAC if the patient was determined to have cytopathological or surgical pathological confirmation of pancreatic adenocarcinoma as performed by GI pathologists at the Hospital of the University of Pennsylvania. Control samples were obtained from patients with a distant history of solid tumor malignancy deemed cured based upon greater than five year interval free of disease based on CT scans. Furthermore, greater than 2 year follow up with CT scans following collection of blood sample confirmed no measurable recurrence of disease. Samples were analyzed in batches; each batch contained a mixture of PDAC and control samples. Samples were assigned a unique identifier to blind the analyst during processing. Statistical analysis was conducted using Prism 5 (GraphPad Software, Inc., La Jolla, CA). A two-tailed, unpaired *t*-test with Welch's correction for unequal variances, and a confidence interval of 95 % was used to determine statistical significance.

Results

Development of MRM/MS Transition List

Peptide sequences were selected from a spectral library generated in house^{3,33}. Please see respective manuscripts for detailed methodologies used to acquire and characterize the pancreatic cancer and stellate cell line derived peptides. Briefly, the SILAC labeled secreted proteome of CAPAN-2 cell lines were previously separated by extensive multidimensional chromatography followed by acquisition of MS/MS spectra using a standard data dependent approach on an ion trap based Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FT). Spectra from the RLT-PSC secreted proteome was acquired using a standard two-dimensional LC-MS/MS approach on an ion trap (LTQ) mass spectrometer. See respective manuscripts for detailed information regarding acquisition parameters, proteins and peptides. The peptide list was filtered to remove those redundant to more than one protein, lacking lysine and leucine, singly charged, possessing a SEQUEST Xcorr score less than 2, or a precursor mass below 450 or above 1900. The spectral library was then used to generate a transition list for a selected number of filtered peptides. Three transitions were selected per peptide using the 3 most intense y-ions with *m/z* greater than the precursor *m/z*. Based on this list of transitions, an IS transition list was created using the calculated mass of each peptide assuming [¹³C,¹⁵N] labeling of all lysine and leucine residues. The MRM transition list is provided as Supplemental Table 1.

Development of LC Retention Time Schedule

The MRM transition IS list was split into smaller lists each composed of no more than 90 transitions. Each list was then used to analyze the IS, monitoring all transitions for the duration of the chromatographic run. Using a dwell time of 10 ms, the cycle time was maintained at < 1 sec. SkyLine software was used to identify the correct peak and retention time for each peptide based on the co-elution of all three transitions as well as predicted elution time. SkyLine software was used to generate a scheduled method, encompassing the entire list of transitions, both analyte and IS, using a four min window for each peptide. A four min window provided a maximal overlap of 180 transitions, corresponding to a cycle

time of 1.8 sec (Figure 1A). Analysis of the effect of dwell time on peak area revealed that no further gain is achieved beyond a dwell time of 20 ms, yet the dwell time can be reduced to 10 ms without significant loss of signal (Figure 1B). At a dwell time of 10 ms, no difference in peak area was seen when varying the number of points across the peak from 10 to 50 (Figure 1C). Thus, a scheduled method utilizing a dwell time of 10 ms and 10 points per peak, as provided by the scheduled method resulting in a 1.8 sec cycle time with a chromatographic method producing 18 sec wide peaks, was deemed acceptable.

Development of Low Abundance Protein Enrichment

Two immunoaffinity methods were compared for removal of abundant proteins, both based on the Seppro® Protein Depletion System (Sigma, St. Louis, MO). The IgY-14 LC column alone, designed to remove the 14 highest abundant proteins (HAP) present in human plasma, was compared with the IgY-14 LC column used in tandem with the SuperMix LC column, designed to remove a large subset of moderately abundant proteins (MAP) present in human plasma. To test the ability of the immunoaffinity column(s) to selectively remove only the highly and moderately abundant proteins from serum, the enzyme matrix metalloproteinase 2 (MMP2), a protein not known to be present in either the HAP or MAP proteomes, was used as a surrogate. MMP2 was added to human serum and processed by each LC removal method. MMP2 was only detected in the unbound flow-through fraction of each method as demonstrated by both Western blot and LC-MS/MS analysis (Figure 2A). MMP2 was not detected by LC-MS/MS in any of the HAP fractions. Some interference was observed in the LC-MRM/MS chromatogram which eluted close to the peptide MMP2 peptide VDAAFNWSK (VDA) when using the IgY-14 column alone (Figure 2B) compared with the combined IgY-14/SuperMix columns (Figure 2C). However, for the MMP2 peptide AFQVWSDVTPLR (AFQ), the IgY-14 (Figure 2D) and combined IgY-14/SuperMix columns (Figure 2E) generated very similar LC-MRM/MS chromatograms. There was no inference in the LC-MRM/MS chromatograms from the bound fraction when either method was used (Figures 2B-E). A method, which involved the IgY-14 column alone was chosen because the analytical procedure was much simpler and a final workflow (Scheme 1) was adopted. Elements of the process were then validated with respect to precision, linearity, sensitivity, and specificity.

LC-MS Validation

A tryptic digest of both labeled and unlabeled proteins derived from the cell culture secretome was analyzed by LC-MRM/MS in triplicate. The precision of the peak area response was plotted as a function of several factors including retention time, peak area, peak width, and precursor m/z . Peak area (Figure 3A) and precursor m/z (Figure 3) had the most striking effects. When precision was plotted as a function of peak area it became clear that below a peak area of 10,000, the percent relative standard deviation (% RSD) was consistently above 15 % (Figure 3A). Similarly, when plotting precision as a function of parent m/z , the % RSD was above 15 % for parent m/z below 500, and with the exception of one analyte, below 15 % when the precursor m/z was above 600 (Figure 3B). These data were used to drive the selection of peptides for further analysis. The linearity of the response was assessed by preparing a tryptic digest of MMP2, which was then diluted over a range of 2 to 100 fmol and spiked with digested SILAP IS. The peptide response was linear over the range of 10 to 100 fmol of MMP2 carried through the assay with the SILAP standard for VDAAFNWSK (VDA, Figure 3C) and AFQVWSDVTPLR (AFQ, data not shown). The LC-MRM/MS response was measureable for both peptides with 10 fmol of MMP2 (Figure 3D).

Digestion Validation

The SILAP IS was spiked into an unlabeled protein sample, derived from the cell culture secreted proteome, to test the precision of the trypsin digestion. From this pool, 5 replicates were trypsin digested and analyzed by LC-MRM/MS. Using analyte peak area alone, the % RSD was less < 20 % for all proteins. When normalized using the IS peak area, the % RSD of the analysis < 15 % for all proteins (Figure 4A) which conforms to commonly observed acceptance criteria for bioanalytical methods. Linearity of the digestion was tested by spiking a fixed amount of the SILAP standard into increasing amounts of authentic MMP2 in the range 10 to 250 fmol. Samples were then trypsin digested and analyzed by LC-MRM/MS. The response was found to be linear across the range of 10 to 250 fmol (Figure 4B). Replicate analysis at the lower limit of quantification (LLOQ) of 10 fmol (Figure 4C) resulted in a % RSD less than 20 % for both peptides which conforms to bioanalytical acceptance criteria.^{34,35}

Validation of Immunoaffinity Removal of Abundant Proteins

Five replicates of serum mixed with SILAP IS were processed by LC immunoaffinity removal of the HAP (IgY-14), trypsin digested, and analyzed by LC/MS. The precision of the peak areas and peak area ratios for 26 endogenous proteins present in the serum is plotted in Figure 5A. The precision of the analyte area alone is unacceptable for many proteins, with % RSD > 20 %. However, when the peak areas are normalized to the IS, the % RSD of the ratio was < 20 % for all proteins (Figure 5A). Similar data were obtained for the other 46 proteins that were analyzed (data not shown). Linearity of the analysis was determined by spiking SILAP IS into a 10 – 60 µL serum curve. The curve was then processed by LC immunoaffinity removal of the HAP (IgY-14), trypsin digested, and analyzed by LC-MS. The area ratio response for one protein is given as an example (Figure 5B). The curves for all proteins were linear with r^2 values all above 0.8. To determine the specificity of the analyte response, the response of the endogenous protein in serum was compared to the response in the SILAP IS (Figure 5C). Similarly, the specificity of the IS response was determined by comparing the response of the heavy protein in the IS to the response in blank serum lacking IS (Figure 5D). The IS does not interfere with the analyte signal, nor does the serum possess any compounds interfering with the IS. This finding was consistent for all proteins. The interference was determined to be < 5 % for all proteins (data not shown).

Sample Analysis

Details regarding serum samples analysis and statistical considerations can be found in the Supporting Materials Section.

DISCUSSION

A series of developmental studies has provided a powerful workflow for reliably interrogating biological fluids for candidate disease biomarkers. The workflow consists of depletion, digestion, chromatographic separation and stable isotope dilution LC-MRM/MS analysis (Scheme 1). A spectral library was developed in house for the derivation of the MRM/MS transition list. All of the peptides that were monitored had been previously been identified by LC-MS/MS in our laboratory. This approach made it possible to analyze peptides known to endure the processes of digestion, chromatographic separation, and ionization well, which may have conferred an advantage during the validation of these processes. While monitoring only peptides derived from disease specific cell line libraries is a potential strength of this approach, if specific proteins of interest are not present in the labeled standard, monitoring is not possible. Methods have been developed to circumvent this limitation, for example, by synthesizing a labeled peptide standard, an approach termed

AQUA^{36,37}, or by synthesizing the intact labeled protein.³⁸ These methods for generating a labeled standard of interest can be integrated into the workflow we describe here.

Three transitions were monitored for both the labeled and unlabeled peptides. Comparing quantification conducted using either the single most abundant transition, or the sum of all three, gave comparable results with respect to precision, linearity, and specificity (data not shown). The use of six transitions per peptide (three for the labeled peptide, three for unlabeled peptide) did make possible the accurate identification of chromatographic retention time. This step was critical to LC-MRM/MS window scheduling, and peak integration (Figure 1). Scheduling reduced the number of concurrent transitions enabling a shorter cycle time and thus a sufficient number of points per peak for proper peak integration. Finally, a SILAP standard was used to refine the transition list prior to analysis of analytes (Scheme 1), ensuring that each analyte would possess a solidly performing corresponding IS.

A limitation in development of the depletion method was the lack of authentic protein standards. MMP2 was selected as surrogate for all other proteins of interest based on its presence in the SILAP IS, as well as its enzymatic activity and the availability of sensitive and specific antibodies. By selecting an enzymatically active protein, the chances of acquiring a recombinant protein with the correct primary sequence and structure were enhanced. The commercially available recombinant MMP2 was efficiently separated from the HAP and recovered from the unbound fraction during depletion (Figure 2). However, it cannot be reasonably expected that all proteins selected for our analysis would behave analogously to MMP2. The apparent MS response for any given peptide might be reduced by either loss of signal, or an excess of noise. For example, a reduction in signal may occur if a protein were lost during depletion, due to either nonspecific binding to the column or association with highly or moderately abundant plasma proteins.^{39,40} Conversely, the apparent signal of some lower abundance proteins might benefit from the reduction in noise resulting from tandem depletion of the MAP and HAP. A balance must be struck between analyte loss and background reduction, which may only properly be determined by analyzing each protein individually. However, such an analysis would be excessively costly given the preliminary nature of the study. Thus, a surrogate protein was chosen during development, in the anticipation that it would approximate the behavior of the average analyte.

When studying the sources of imprecision in the LC-MS analysis, the determination that retention time had little effect on precision could be expected due to the well crafted scheduled method developed with dwell time and cycle time in mind (Figure 1). The poor precision below 10,000 area counts can be expected as well since low intensity analytes often suffer from poor precision. These data helped establish 10,000 area counts as the lower limit of detection for the Vantage TSQ MS (Figure 1A). However, the observation that precision was improved above precursor m/z 600 was somewhat surprising (Figure 1B)) and could reflect either the performance of the Vantage TSQ MS or the impact of background noise as fewer small molecules might interfere at this elevated m/z . Overall, the LC-MS analysis performed well with respect to precision and linearity (Figures 5A and 5B).

The reproducibility and linearity of the trypsin digestion was surprisingly good (Figure 4B). Previous studies have shown that trypsin digestion can vary in the degree of completion, confounding the use of peptide internal standards.²⁶ Furthermore, recombinant proteins from non-mammalian systems are often incorrectly folded and so undergo differential trypsin hydrolysis compared with the endogenous mammalian serum proteins.^{41,42} Normalization using the intact protein SILAP IS clearly improved the precision and was responsible for bringing the analysis into conformance with the commonly accepted criteria of %RSD less

than 15% (Figure 4A). However, even without normalization, the digestion was remarkably reproducible. This could be due to use of the spectral library in forming the peptide list, which is likely enriched for stable and easily digested proteins.

Validation of the depletion method was anticipated to be the most problematic and highlighted the importance of the SILAP standard. Without normalization, the precision of the analyte area was unacceptable for many proteins (Figure 5A). However, this was corrected when normalized to the IS. This can be attributed to the ability of the intact protein in the SILAP standard to mimic the endogenous analyte. Since the proteins in the SILAP standard are all native proteins produced in non-transduced human cells, they all possess the correct primary sequence and are properly folded and post-translationally modified, enabling them to properly track each analyte during depletion and digestion, a feat not possible with recombinant or synthetic proteins or peptides. A major limitation in the use of stable isotope labeled tryptic peptides ISs (AQUA peptides)^{36,37} is that they are not affected by differences in extraction and digestion efficiency in the same way as the intact proteins. This can lead to inaccurate quantification even though excellent standard curves can be generated from the unlabeled peptides. For example only two peptides from serum ApoA1 provide reproducible results when used for quantification of the protein in serum.⁴³ The linear range as determined by the current analysis was small, spanning less than an order of magnitude (Figure 5B). However, it was expected that the endogenous protein levels to vary by less than 4-fold, so this range was considered to be suitable for the required analyses.

The specificity of the analysis was excellent and most likely due to the use of precursor ions with m/z above 600 and product ions with m/z greater than the relevant precursor ion (Figure 3B). It is important to note the importance of ensuring the isotopic purity of the SILAP standard. If the cells are not passaged sufficiently in the heavy media, contamination with unlabeled protein could lead to high background in the analyte channel, reducing the ability to detect changes in abundance (Figure 5D). A limitation of this study was the lack of protein standards with known concentrations, preventing the assessment of accuracy and a definitive lower limit of quantification. However, by studying the endogenous proteins present in the serum samples, it was possible to determine the precision, linearity, and specificity of the method, which was sufficient for the relative quantification of proteins necessary to evaluate potential biomarkers.

It may be surprising that three of the serum proteins were reduced in the PDAC patients compared with controls (Figure 6A, Table 1). Given the tumor cell culture model used and the presence of tumors in the PDAC patients it might have been expected that an increase in protein expression would have occurred. Interestingly, a similar finding was made recently by Matsubara *et al.* who showed that CXC chemokine ligand 7 was down-regulated in the plasma of PDAC patients when compared with controls.¹¹ Therefore, it is somewhat simplistic to assume that all protein expression and secretion into the circulation will be increased in cancer patients. There are many processes, which would account for this observation including differences in rates of secretion from tissues and/or increased proteolysis. Therefore, the serum biomarkers reflect only the circulating steady-state concentrations and do not necessarily reflect biological processes in tumor tissue.

However, downregulation or silencing of Cystatin M has been implicated in a number of malignancies.⁴⁴⁻⁴⁶ Loss of IGF binding protein 7 expression has been reported to be a critical step in the development of human tumors, including melanoma, colon and thyroid cancers.⁴⁷ IGF binding protein 7 is a putative tumor suppressor gene. In lung cancer, IGF binding protein 7 has been shown to be a target gene of p53 and inactivated by hypermethylation.⁴⁸ Villin-2, also known as ezrin, has been implicated as being upregulated in pancreatic cancer tissue.⁴⁹ It remains unclear what mechanisms result in lower serum

levels of the protein in the patient samples studied. Importantly, demonstration of systemic changes in levels of these proteins has not previously been reported in pancreatic cancer.

The final method that was developed performed reproducibly for the duration of sample analysis. Of the 72 proteins quantified in 40 serum samples, 3 showed a significant difference in normalized response when comparing PDAC with control samples (Table 1). No significant differences were observed unless the values were normalized to the relevant IS (Figure 6A). The simultaneous analysis of 72 proteins made it possible to quickly evaluate the list of potential biomarkers to identify those appropriate for further analysis. This method was sufficiently rapid and inexpensive to permit the analysis of many proteins, while retaining the rigor required to achieve statistical significance.

In summary, the method that was developed has shown the feasibility of using stable isotope dilution LC-MRM/MS analysis of tryptic peptides to compare differences in the concentration of endogenous proteins in human serum for the selection of potential biomarkers for further validation. Moreover, the thorough investigation of the method illustrates the power of the SILAP standard to enhance method performance. This SILAP-based method can easily be expanded to cover a larger set of proteins or validated for the absolute quantification of multiple proteins simply through the addition of a standard curve derived from authentic protein standards. This precise, linear, and specific method could have far reaching implications in the study of many human diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AFQ	AFQVWSDVTPLR peptide
ELISA	Enzyme-linked immunosorbent assay
ESI	electrospray ionization
HAP	highest abundant proteins
IS	internal standard
LC	liquid chromatography
MAP	moderately abundant proteins
MMP	matrix metalloproteinase
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
PDAC	pancreatic ductal adenocarcinoma
PSC	pancreatic stellate cell
RP	reversed phase
SILAC	stable isotope labeling by amino acids in cell culture
SILAP	stable isotope labeled proteome
SISCAPA	Stable isotope standards and capture by anti-peptide antibodies

VDA

VDAAFNWSK peptide

Figure 1. LC-MRM/MS transition list development. (A) LC retention time window. A four min window provided a maximal overlap of 180 transitions, corresponding to a cycle time of 1.8 sec. (B) A dwell time of 10 ms could be used without significant loss of signal. (C) No difference in peak area was observed when varying the number of points across the peak from 10 to 50. Tryptic peptides used were VDA and AFQ from MMP2.

Figure 2.

(A) Western blot of MMP2 in human serum. Lanes 1 and 2 display the unbound and bound fractions, respectively derived from the LC IgY-14 column alone. Lanes 3, 4, and 5 contain the unbound, IgY-14 bound, and SuperMix bound fractions from the tandem LC depletion using both the IgY-14 and SuperMix columns. Recombinant MMP2 is shown in lane 6. The amount of protein (μg) in each sample is displayed below the blot. (B) Stable isotope dilution LC-MRM/MS chromatograms displaying the presence of MMP2 Peptides VDA (B,C) and AFQ (D,E) and their corresponding heavy isotope labeled ISs in the unbound (depleted) fractions, but absent in the bound fractions using the IgY-14 column alone (B,D) and in tandem with the SuperMix column (C,E). Assignments of the LC-MRM/MS transitions can be found in Supplemental Table 1.

Figure 3.

The impact of different parameters on precision of analysis. (A) Peak area. (B) Precursor m/z . (C) Linearity of response for MMP2 peptide VDA over the range 10 to 100 fmol protein. (D) Analysis of MP2 peptides VDA and AFQ and their heavy isotope ISs by LC-MRM/MS from 10 fmol of MMP2 carried through the assay. The LC-MR/MS transitions are presented in Supplemental Table 1.

Figure 4.

Precision of trypsin digestion. (A) Precision of digestion was improved when normalized to the internal standard response (solid bars) compared with the non-normalized response (open bars). Data are shown for 26 proteins. (B). The response after digestion remained linear over the range of 10 to 250 fmol for VDA and AFQ. (C). Both VDA and AFQ peptides were readily detected at levels corresponding to 10 fmol MMP2 carried through the assay and quantified by LC-MRM/MS.

Figure 5.

Effect of normalization of the analyte response to the IS response. (A) The precision of depletion was dramatically improved when normalized to the IS response. (B) The response after depletion was linear over the range of 10 to 60 mL serum. (C) The specificity for analysis of insulin-like growth factor binding protein 7 was evident by the lack of response in the IS channel of an LC-MRM/MS chromatogram when no IS was added to the serum sample. (D). The specificity of the insulin-like growth factor binding protein 7 IS was evident by the lack of response in the analyte channel of an the LC-MRM/MS chromatogram from a blank sample to which the IS had been added.

Figure 6. Statistical analysis of selected serum proteins (cystatin M, insulin-like growth factor binding protein 7, and villin 2) from PDAC patients and controls. (A) There were significant differences between PDAC and control patients when the LC-MRM/MS responses were normalized to the relevant IS. (B) There were no significant differences between PDAC and control patients when the LC-MRM/MS responses were not normalized.

Scheme 1.

The final workflow consisted of dilution of the serum sample with SILAP standard, followed by depletion of highly abundant proteins, trypsin digestion, and LC-MRM/MS analysis.

Table 1

Statistical analysis of differential proteins in serum of PDAC and control patients.

Protein	Response	PDAC Mean $\pm$ SEM	Control Mean $\pm$ SEM	Difference between means	95% confidence interval	R squared	p value
Cystatin M	Peak area	17190 $\pm$ 2498	20370 $\pm$ 2202	-3184 $\pm$ 3330	-9936 to 3568	0.0241	0.3452
	Area ratio	0.124 $\pm$ 0.010	0.184 $\pm$ 0.016	-0.060 $\pm$ 0.019	-0.098 to -0.021	0.2391	0.0033
IGF binding protein 7	Peak area	32110 $\pm$ 3565	27140 $\pm$ 3806	4970 $\pm$ 5215	-5602 to 15540	0.0240	0.3467
	Area ratio	0.076 $\pm$ 0.004	0.107 $\pm$ 0.013	-0.030 $\pm$ 0.013	-0.057 to -0.003	0.1955	0.0305
Villin 2	Peak area	7164 $\pm$ 912.1	11850 $\pm$ 1640	-4687 $\pm$ 1877	-8524 to -849.0	0.1770	0.0184
	Area ratio	0.243 $\pm$ 0.017	0.354 $\pm$ 0.032	-0.111 $\pm$ 0.036	-0.186 to -0.037	0.2426	0.0049