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Studying Protein Phosphorylation in Low MW CSF Fractions with capLC–ICPMS and nanoLC–CHIP–ITMS for Identification of Phosphoproteins

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Abstract

An initial study of protein phosphorylation in human cerebral spinal fluid (CSF) is described. CSF is an important body fluid for study of proteins and metabolites and may lead to the ultimate development of molecular markers to predict neurological diseases or their complications, such as in the case of hemorrhagic stroke. The use of capillary liquid chromatography coupled to inductively coupled plasma mass spectrometry (capLC–ICPMS) for screening using ³¹P as the internal elemental tag atom at ultratrace levels, in combination with molecular mass spectrometry using Spectrum Mill and MASCOT database search engines for peptide identification, is a novel approach in its application to CSF relevant phosphopeptides and phosphorylated proteins. CapLC–ICPMS combined with nano liquid chromatography electrospray ionization, ion trap mass spectrometry (nanoLC–CHIP/ITMS), was utilized for initial experiments with CSF. Specific low-level screening for ³¹P containing compounds is accomplished, and nanoLC–CHIP/ITMS provided the corresponding peptide information and subsequent protein identifications. The fractions containing ³¹P from screening by the capLC–ICPMS were collected offline and analyzed separately with nanoLC–CHIP/ITMS. Synthetic phosphopeptides were used to test the method and to estimate lowest quantifiable limits for phosphorus. Tryptically digested β-casein was then used to demonstrate the viability of the methodology for the complex CSF matrix from hemorrhagic stroke patients while also analyzing for native phosphopeptides in the CSF.

Keywords

cerebrospinal fluid; metallomics; capLC–ICPMS; nanoLC–CHIP/ITMS; hemorrhagic stroke

Introduction

In eukaryotes, protein phosphorylation is likely the most important biological regulatory event.¹ Many enzymes and receptors are turned on and off by phosphorylation and dephosphorylation. Through major advancements in mass spectrometry, there has been significant progress made in the identification of proteins and peptides, as well as determining phosphorylation sites in proteins, and in this study for application to an important sample type, cerebral spinal fluid (CSF). For biological samples, one of the most difficult aspects of using mass spectrometry techniques is the preparation necessary before the MS instrument comes in contact with the sample. Sample preparations, acid or enzymatic digestions, preconcentration or not, and the types of chromatographic separations to be considered are some of the important issues that must be well thought out prior to MS sample introduction.²

Recently there have been increasing numbers of studies using element specific mass spectrometry, ICPMS (inductively coupled plasma mass spectrometry), as a powerful tool for determining phosphorylation sites of peptides and proteins.^{3–10} Becker and colleagues have utilized laser ablation ICPMS in combination with molecular MS for diagnostic information about metals in the brain.^{11,12} Most of these methods take advantage of coupling capillary liquid chromatography with inductively coupled plasma-mass spectrometry (capLC-ICPMS). By using element-specific ICPMS detectors, screening for ³¹P phosphorus-containing compounds in the sample is possible. Typically, analysis of phosphopeptides using analytical scale hydrophobicity based separations implementing high levels of organic modifier (>20% v/v) are incompatible with ICPMS at standard flow rates of ca. 1 mL min⁻¹. However, using the low flow rate of capLC (≤10 μL min⁻¹) enables the use of up to 100% organic solvents.⁴ Additionally, the small injection volumes (≤10 μL) are also beneficial for biological samples that may be difficult to obtain (e.g., CSF). Ideally, nanoLC-ICPMS may exploit these advantages even more so, but the lack of a commercially available chromatography/ICPMS interface complicates this method, thus capLC-ICPMS was chosen for ³¹P specific analysis.

Since the elemental information obtained from capLC-ICPMS is known to be complementary to molecular information from electrospray MS, utilizing the two approaches will provide additional information to solve complex problems.¹³ Following the recent trend to move to nanoscale experiments for proteomics studies, this approach should be advantageous and applicable, whereby capLC-ICPMS fractions are collected and then run directly into the ESI-MSⁿ system. The low detection capabilities of nanoscale ESI-MSⁿ is also attractive because of the small amount of sample required and usually available with CSF, which is usually difficult to obtain, and then only in small volumes under heterogeneous collection conditions. Such small amounts have inhibited CSF based studies for assessing biochemical pathways and/or signaling mechanisms, as well as for studying molecular markers or molecular level events associated with neurological diseases.

The microfluidic (on a chip) nano-ESI-MS system has the ability to obtain qualitative information from complex biological samples.^{14–17} These studies demonstrated that nanoLC-CHIP-ESI-MS would be a good instrumental partner in conjunction with the ICPMS for ³¹P screening.

While nanoLC-ICPMS has been studied^{9,18–22} for detection of synthetic phosphopeptides,⁹ synthetic thyroxine,⁹ and selenium compounds,^{9,23} these initial experiments, with the planned inclusion of ITMS, go only as far as the capLC-ICPMS. Giusti et al. have also reported the use of nanoLC coupled to ICPMS for investigating selenium species prior to, or, in parallel with ESI-MS analysis.^{19,22}

A proof-of-concept experiment was performed to test the usefulness of using capLC-ICPMS for the screening of CSF phosphopeptides by ³¹P detection, fraction collection, then the use of

nanoLC-CHIP/ITMS for molecular weight information. Spectrum Mill and MASCOT database search engines were used for identification of phosphopeptides. Encouraging results were obtained in the detection of phosphopeptides in CSF showing promise that this methodology could be used for screening phosphopeptides to suggest possible phosphorylated proteins and ultimately relating some of these to brain diseases such as Alzheimer's, Parkinson's and hemorrhagic stroke, an aneurysmal subarachnoid hemorrhage, SAH. This hemorrhagic stroke incident can then be further complicated with the onset of a cerebral vasospasm, a condition wherein arteries in the subarachnoid space between the skull and the brain, constrict (in effect, a blockage) leading to serious debilitation or death. These patients are believed to have unknown substance(s) that are thought to alter vascular signaling mechanisms, possibly *via* phosphorylation pathways. To our knowledge this is the first example of using capLC-ICPMS for elemental screening in combination with the nanoLC-CHIP/ITMS system for molecular identification in CSF, which is enhanced by the putative utility concerning diagnostics/prognostics and understanding disease mechanisms.

Experimental Section

Chemicals and Standards

The HPLC solvents, water and acetonitrile (ACN), were of high purity and purchased from Burdick and Jackson (Muskegon, MI). Sequence grade modified trypsin and the acetic acid buffer were both from Promega (Madison, WI). Formic acid (FA) was purchased from Agilent (U.S.A.). Dithiothreitol, Iodoacetamide, and β -casein-90%+ were all purchased from Sigma-Aldrich (St. Louis, MO). Ammonium bicarbonate was from Fisher (NJ), and urea was from Mallinckrodt Baker (Canada). Synthetic phosphopeptides Pp60 c-src and P60 c-src were purchased from American Peptide Company (Sunnyvale, CA). CSF from patient samples was obtained, with all the required approvals for collecting and using human specimens, from Professors Joe Clark and Gail Pyne-Geithman of the Neurology Department at the University of Cincinnati, OH. Compliance documents are on file in the Vontz Center for Molecular studies at the University of Cincinnati.

Instrumentation

Capillary-LC-ICPMS—An Agilent Technologies (Agilent Technologies, Santa Clara, CA) model 1200 series capillary liquid chromatograph consisting of a binary pump, degasser, chilled autosampler, and a variable wavelength UV detector was used for the capLC separation. The capillary scale separations were performed using an Agilent Zorbax 300 SB-C₁₈ column (5 μ m, 150 mm $\times$ 0.5 mm) (Agilent Technologies, Santa Clara, CA). The capLC was interfaced to the Agilent 7500ce ICPMS (Agilent Technologies, Santa Clara, CA) through commercially available PEEK-coated silica tubing. The PEEK tubing from the capLC was connected to the commercially available DS-5 capillary nebulizer (Cetac Technologies, Omaha, NE). The gradient system used to separate the phosphopeptides was the following: A and B (A: 99.9% H₂O, 0.1% Formic Acid, FA; B: 89.9% acetonitrile, ACN, 10% H₂O, 0.1% FA, (% are v/v)) at a flow rate of 10.0 μ L/min with the following gradient conditions: 0–5 min, 0% B; 5–25 min, 40% B; 25–32 min, 45% B; 32–47 min, 45% B; 47–49 min, 70% B; 49–55 min, 0% B. The following ICPMS conditions were used; Rf power, 1550 W; nebulizer carrier gas pressure, 90 psi; Sampling depth, 7–8 mm; collision/reaction cell flow rate, 3.8 mL/min He; Octopole bias, –18V; Quadrupole bias, –16V for a net +2V energy discrimination barrier.

NanoLC-CHIP/ITMS—All electrospray experiments were done on an Agilent 6300 Series HPLC-CHIP/Ion Trap XCT system (Agilent Technologies, Santa Clara, CA). The 1200 LC equipped with both a capillary and nano pump was used for loading and flushing the chip nanocolumn. A more detailed description of the HPLC-CHIP system has already been published.²⁴ The chip used, contained a Zorbax 300SB C₁₈ enrichment column (4 mm $\times$ 75

μm , 5 μm) and a Zorbax 300SB C₁₈ analytical column (43 mm $\times$ 75 μm , 5 μm). Sample loading onto the enrichment column was set at a flow rate of 4 $\mu\text{L}/\text{min}$ with a 97:3 ratio of solvent A and B (A: 100% H₂O, 0.1% FA; B: 90% ACN, 10% H₂O, 0.1% FA). After loading the enrichment column, the flush is switched by on-chip microfluidics to the analytical column at a flow rate of 0.4 $\mu\text{L}/\text{min}$ with the following gradient conditions: 0–5 min, 3% B; 5–25 min, 40% B; 25.01–32 min, 45% B; 32–47 min, 45% B; 47–49 min, 70% B; 49.01–55 min, 0% B; followed by 10 min for column re-equilibration.

The following MS conditions were used; 4 L nitrogen/min drying gas at a temperature of 300 °C; MS capillary voltage: 1900 V; skimmer: 30.0 V; capillary exit: 100.0 V; trap drive: 85.0 V; two averages were taken for each precursor ion. The target number of ions was 500 000 with a maximum accumulation time of 150 ms. The MS scan range was 50–2200 m/z in standard-enhanced scan mode. Fragmentation conditions for MS² (almost always used in this study, with a few MS³); number of precursor ions per cycle: 5; fragmentation amplitude: 1.30 V; spectra were actively excluded for fragmentation: after 2 spectra and released after 1 min. ESI-MS, MS/ESI-MS², and MS/ESI-MS³ experiments were completed.

Peptide Identification

Database searching was performed on Spectrum Mill (Rev. A. 03.02) (Agilent Technologies, Santa Clara, CA) and MASCOT (Matrix Science, London, UK). The selected parameters used for the searches were: data searched against the Swiss-Port database, taxonomy: mammal, enzyme: (trypsin for casein and none selected for the CSF samples), 2 missed cleavages, mass tolerance of precursor ions and product ions $\pm$ 0.8 Da, variable modification: phosphorylation (threonine, serine, tyrosine or T, S, Y, respectively). For database searching with Spectrum Mill, the autovalidation guidelines were adapted from previous studies.^{25,26} The identified peptides were searched using autovalidation criteria; minimum score for fragmentation of 1⁺, 2⁺, and 3⁺ were 8, with a score peak intensity (SPI) of at least 70%. However, for peptides with a 2⁺ or 3⁺ fragmentation and an SPI of 80+%, a score of greater than 6 was considered. For MASCOT, the scores have to be reported as a significant hit, as reported by MASCOT (a significant hit in MASCOT has an ion score that exceeds the identity threshold).

Sample Preparation

For estimation of detection limits and test of method, 1 mg synthetic phosphopeptides Pp60 c-src and P60 c-src were dissolved in 1 mL H₂O, and working solutions of decreasing concentration were prepared from the appropriate dilutions of the stock solution with water when required.

The 90% purity β -casein was not purified before use, so some alpha form is expected. Tryptic digestion of β -casein was performed as follows: 1 mg of β -casein was dissolved in 1 mL of water. A 20 μL solution of 0.4 M ammonium bicarbonate (pH 7.5) and 8 M urea was added to the dissolved casein. Then 5 μL of 45 mM dithiothreitol was added, and the solution was incubated at 50 °C for 15 min to reduce the protein. After the solution was cooled to room temperature, 5 μL of 100 mM idoacetamide was added, after which, it was left in the dark at room temperature for 15 min. For digestion, 20 μg of trypsin was dissolved in 200 μL of 50 mM acetic acid (as a resuspension buffer). From this solution, 5 μL of the trypsin solution was added to the protein solution and then incubated at 37 °C overnight. To inhibit the trypsin activity, the samples were placed in the freezer and left frozen until used.

Samples of CSF were received frozen at –80 °C. After thawing on ice, CSF samples were pooled from a population that had experienced SAH. Aliquots of 200 μL from each of four samples were pooled together for a total of 800 μL . Samples were then loaded into 5 kDa spin concentrators (Agilent, Wilmington, DE), spun at 5000g for 20 min at 4 °C, using a Sorvall

RC-5B refrigerated superspeed centrifuge (Dupont Instruments) in order to remove the most abundant proteins in the CSF such as albumin, transferrin, and immunoglobulins as well as other high molecular weight species and proteins. The eluted sample was collected (designated as the <5 kDa sample) as the retained portion was then the >5 kDa sample and not used in this study. The samples were passed through 0.22 μ m filters (Agilent Technologies, Santa Clara, CA) prior to LC separation. Immediately following preparation, with the aim of minimizing degradation, samples were injected into the capLC-ICPMS.

Results and Discussion

Analysis of Standards for Method Confirmation. Detection Limits of Synthetic Phosphopeptides by CapLC-ICPMS

Detection limits for ^{31}P by capLC-ICPMS were done using synthetic phosphopeptides Pp60 c-src and P60 c-src. Both phosphopeptides were studied over a range of concentrations to test the sensitivity and linearity. The capLC gradient discussed previously was used and R^2 values for Pp60 c-src and P60 c-src were determined to be 0.988 and 0.995, respectively. Estimated (observable) ^{31}P detection limits (ca. $2\times$ the background signal at the appropriate m/z) were 5 $\mu\text{g/L}$ and 10 $\mu\text{g/L}$ for Pp60 and P60, respectively, based on a 1 μL injection. These would be estimated absolute detection limits of 5pg (160 fmol) and 10 pg (320 fmol), respectively. (These capLC-ICPMS results are included in the Supporting Information as Supplement 1.)

NanoLC-CHIP/ITMS Results for Synthetic Peptides

A peptide fraction from the capLC showed a base peak chromatogram (BPC) with one major peak at 17.4 min, and a mass of 749.6^+ corresponding to the parent ion in the MS. MS^2 results give 3 fragments that would be suspected with the small phosphopeptide P60c-src, m/z values of 277.2, 334.2, 577.2. The 3 fragments are all from the MH^{+1} and confirm the P60 peptide. Similar confirmation was obtained with the other peptide.

Analysis of Casein. CapLC-ICPMS Results

After confirming the method with synthetic peptides, β -casein was used as the model protein to ensure confidence in the method.^{4,5} Data were taken for casein spiked into both water and into CSF. While the spike amount overloaded the column and broadened the peaks, only an early eluting small and narrow peak was collected several times for submission to nanoLC-CHIP-ITMS.

Nanochip LC Results for Std. β -Casein

Digested β -casein fractions were collected off-line and no additional sample preparation was performed prior to the fraction being injected into the CHIP system. The data from the nanoLC-CHIP system were then searched using Spectrum Mill and MASCOT to help us assess the viability of the data outcomes seen thus far. The casein results with these comparisons are given in Supplement 2 of the Supporting Information.

Analysis of Native CSF for <5 kDa Fractions. CapLC-ICPMS Results

Before fractions were collected, the reproducibility of the CSF samples was tested. Since CSF is a biological sample, there were concerns about possible degradation. Even though precautions were taken, for example, keeping the samples on ice and making fresh samples before experiments, it was necessary to test the reproducibility of this method using the same CSF sample three times. There were virtually no differences seen when the same sample was left in the autosampler at room temperature and injected three times for a 60 min experimental time, plus an additional five minutes for sample injection and solvent fast equilibration change.

Two of the capLC peaks were broader than optimal, suggesting the injection amount could easily have been reduced along with mobile phase modifications, if only these largest peaks were of interest. However, since we first wanted to demonstrate the utility of the method for low levels or low copy numbers of proteins, we ran the samples as indicated to not lose the smallest peaks, while still obtaining viable data. Current experiments are now studying further the largest peaks.

One reason that <5 kDa was chosen is because hemorrhagic CSF contains blood proteins such as hemoglobin, albumin and others. A large majority of these are much heavier than 5 kDa, so it was decided to focus on the small molecular weight proteins in hopes of minimizing higher molecular weight (MW) interference or contamination. In this application, CSF fractions from capLC were collected (plasma offline) after being screened for ^{31}P by capLC-ICPMS and analyzed on the nanoLC-CHIP/ITMS. These offline fractions are shown in Figure 1–Figure 4. We chose CSF to develop and optimize these methods because there is a critical need for a better understanding of CSF diagnostics following subarachnoid hemorrhage, SAH. It is relatively clear that CSF changes post SAH, leading to the neurovascular complications that cause patient decline. It is also relatively clear that vascular smooth muscle in the brain is very dependent upon its environment, including the CSF, for controlling contraction and relaxation.^{27–29} Thus, an understanding of phosphorylated proteins and phosphoproteins in the CSF, post SAH (even those proteins with few copies, could be an important avenue to better understand the post SAH complications and improve its diagnosis. Future studies using these methods comparing CSF from clinically evident post SAH vasospasm patients and patients without vasospasm could be employed to identify proteins and/or pathways involved in this debilitating pathology.

NanochipLC Results

From the capLC-ICPMS results, the CSF was collected in 5 fractions (four of these are shown in Figure 1–Figure 4, in view of space constraints) The individual fractions were collected off-line as previously discussed, and analyzed on the nanoLC-CHIP/ITMS. Shown in Figure 1–Figure 4 are the capLC-ICPMS ^{31}P traces with the fraction collected highlighted in gray. The BPC is calculated by using the intensity of the most intense ion in the mass spectrum. BPCs generally showed better S/N ratios compared to TICs (total ion chromatograms) in this study. From the fraction analyzed on nanoLC-CHIP/ITMS, Spectrum Mill returned peptide information as shown in the figures (results are all shown that satisfied the interpretation criteria discussed above and in supplement 2) of the Supporting Information. All of the figures below are from the <5 kDa fraction of cerebral vasospasm samples, and only the phosphorylation results are shown. There were about an equal number of results reported for phosphopeptides without phosphorylation modifications (these account for missing line numbers in the tables).

In Figure 1, fraction 1 from the capLC-ICPMS experiment is further investigated by nanoLC-ITMS. Both line numbers 1 and 2 as shown in the table above indicate phosphorylated peptides matched with good scores (SPI > 70) and have retention times considered reasonable for the fraction collected from the capLC-ICPMS. The first phosphopeptide matched, shown in line 1 shows two phosphorylations on threonine and serine, and is matched to the protein serine/threonine-protein kinase MAK. This type of protein catalyzes the phosphorylation of serine or threonine residues on target proteins by using ATP as a phosphate donor.³⁰

These types of phosphorylations may cause changes in the function of the protein. The second phosphopeptide matched shows phosphorylation only on serine and is from the C-type lectin domain family 9 member A protein.³¹

Figure 2 shows 10 peptide matches for Fraction 2 using the Spectrum Mill database searching tool with the five phosphorylated peptides shown and including phosphorylations at all three

possible sites; serine (S), threonine (T), and tyrosine (Y). All the scores shown satisfy or exceed the search criteria indicated above. Fraction 2 is the least resolved fraction and the subject of current experiments involving varying injection volumes and mobile phase changes. Nevertheless, useful information was obtained from this fraction. From repeated experience it is noted here that the collected fraction, when analyzed on the CHIP column, elutes a few minutes prior to the retention time seen using the capLC-ICPMS, as might be expected with parameter changes from column to chip. However, these elution time differences are unimportant to the database search.

The Ubiquitin protein has been linked to major human neurodegenerative diseases; Alzheimer's and Parkinson's.³² It is shown for CSF from the vasospasm condition in line 5 of the table above. Ubiquitin is an intermediate signaling molecule that will target other proteins for trafficking or degradation and has been proposed to be involved in the significance of proteolytic degradation of tissue proteins, such as the heart during failure between compensated to decompensated heart failure.³³ Also another Ubiquitin protein was matched and shown in line 10 of the table. The Lutheran blood group glycoprotein (shown on line 2) is known to mediate intracellular signaling and is thought to be in very low concentrations in the brain.³¹ It is also directly associated with the basal layer of cells in epithelia and the endothelium of blood vessel walls.³¹ Nibrin (shown on line 7) function is involved with the maintenance of chromosome integrity and plays a critical role in the cellular response to DNA damage.³¹ As indicated above, fraction 2 is under continuing study.

Fraction 3 (Figure 3) had four phosphorylation matches on serine and threonine corresponding to various proteins. The SPI and Spectrum Intensity scores are good. Line numbers 6, 7, and 8 in the above table describe peptides which are singly phosphorylated on serine, while line 2 describes a single phosphorylation on threonine and is the highest scoring phosphopeptide match (Score > 9 with SPI of almost 90). This protein match, Fatty acid synthase, is of high interest because there have been studies linking fatty acids and the cerebral vasospasm sample types. The hypothesis is that accumulation in CSF of free fatty acids may play a role in the development of vasospasm after a SAH.³⁴

Figure 4 indicates that peaks corresponding to lines 3, 6, and 7 in the table below were phosphorylated on serine and threonine. A particularly interesting peptide and protein report is on line 7 showing high SPI and Spectrum Intensity. Adrenergic agonists can bind to this particular adrenergic receptor triggering a cell response. In the case of SAH with vasospasm complications, such agonists are used to treat vasospasm and the appearance of the adrenergic receptor protein may be significant. This could be indicating failure of the system or a partial response of the system to the vasospasm.

Clearly there is much work remaining as the data reported include only representative data of the <5 kDa fraction of the cerebral vasospasm sample, while the higher MW portions plus the nonstroke and SAH nonvasospasm samples need to be studied before protein or peptide comparisons can be made among the three sample types. Further, if the initial cross sample comparisons show interesting differences, more samples will need to be analyzed, sufficient to establish statistical validity for molecular differences in the three sample types.

Native CSF Discussion

Other results from capLC-ICPMS clearly and repeatedly show that there are many phosphorus containing compounds in the CSF in the <5 kDa fraction. This is illustrated in Figure 5, which is a set of capLC-ICPMS chromatograms of the SAH vasospasm (red), SAH nonvasospasm (green), and normal nondiseased sample types (black). It shows the differences in ³¹P containing species across the three sample types for the lowest copy numbers of proteins or peptides that were detectable. It is understood that by lowering the injection volumes and

making some mobile phase modifications, the number of peaks below 25 min should be markedly enhanced and allow analysis for the higher concentration of phosphorus species.

Particularly noteworthy are the comparisons between the normal sample and the diseased samples where differences are highlighted for the lower concentration fractions. These differences need to be investigated in future studies to determine the molecular nature of those differences and if they are significant to the disease. For example, the peak at 22 min in the normal sample disappears in the diseased samples. For the diseased samples, a peak appears at 27 min but is not part of the normal sample chromatogram. For the peak at 33.5 min, the diseased samples show ^{31}P containing species, while the normal sample does not. It is important to be able to identify these differences, even at these lowest levels. Additionally, a study is now in progress with the larger peaks being further separated to more fully analyze the higher level ^{31}P containing species.

The Spectrum Mill results did not always provide a good match for a phosphopeptide. It is possible that some of these ^{31}P peaks seen are from other phosphorus compounds/metabolites in the CSF (the database was not instructed to search for phosphorylated peptides only). For example, phosphate elutes at the beginning of the capLC-ICPMS chromatogram. For this phosphate compound there was a standard and known retention time; therefore, data were not collected for that fraction. Since protein and peptide standards for every phosphate containing protein and peptide in CSF are not available, retention time matching cannot be done for all the phosphorus peaks seen in the chromatogram. Because the only searching tools that were available at the time of this study were Spectrum Mill and MASCOT, only the possible phosphor-peptides and proteins available from these have been discussed. At this time, the higher scoring matches are taken with confidence, particularly in view of the casein results, while the lower scoring matches may not be valid, and further work needs to be done to confirm these as well as to search for lower MW metabolites.

Conclusions

The sensitivity of the capLC-ICPMS in combination with the nanoLC-CHIP/ITMS is effective at characterizing phosphopeptides, even in highly complex matrices such as CSF. By using this method, which combines capLC-ICPMS with element specific detection for ^{31}P , or other elemental screening, and nanoLC-CHIP/ITMS with a database search for molecular weight information, peptide and protein identification can be done. This demonstrates an important extension to previously reported methods in the field of phosphopeptide/protein phosphorylation analysis. At this stage, the concept appears to be valid, and with future studies important findings should come forth relative to CSF associated with various human health conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
 ^{31}P capLC-ICPMS of <5 kDa vasospastic CSF. Fraction 1 was collected and run on the nanoLC-CHIP/ITMS, and the corresponding results are from Spectrum Mill.

Figure 2.
 ^{31}P capLC-ICPMS of <5 kDa vasospastic CSF. Fraction 2 was collected and run on the nanoLC-CHIP/ITMS, and the corresponding results are from Spectrum Mill.

#	Score	SPI %	Spectrum Intensity	Variable Sites	Sequence and Modifications	RT min	MH+; Matched (Da)	Accession #	Protein name
2	9.61	89.5	3.52e +006	T251t	(A)GtNTDGSKE(Q) t:Phosphorylated T	35.04	908.396	P19096	Fatty acid synthase (EC 2.3.1.85)
6	7.06	92.0	4.15e +006	S181s	(V)GMDSSYLsVK(E) s:Phosphorylated S	19.36	1086.514	Q9H161	Homeobox protein aristaless-like 4 Homo sapiens
7	6.96	81.2	2.60e +006	S1038s	(G)FFNSPSTSRPTLLs(S) s:Phosphorylated S	19.25	1553.796	QO1279	Epidermal growth factor receptor precursor (EC2.7.10.1) Mouse
8	6.66	95.5	1.53e +006	S299s	(A)NSHSNTIGsAV(C) s:Phosphorylated S	19.26	1086.517	P54760	Ephrin type-B receptor 4 precursor (EC2.7.10.1) Tyrosine-protein kinase Human

Figure 3.
CSF with SAH vasospasm showing fraction 3 from capLC-ICPMS taken to the nanoLC-CHIP
CHIP-ITMS for phosphorylation analysis.

Figure 4. CSF with SAH vasospasm showing fraction 3 from capLC-ICPMS taken to the nanoLC-CHIP-ITMS CHIP-ITMS for phosphorylation analysis.

Figure 5. CapLC-ICPMS study across the three sample types and showing difference in ^{31}P species among them.