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Coupling of a Vented Column with Splitless NanoRPLC-ESI-MS for the Improved Separation and Detection of Brain Natriuretic Peptide-32 and its Proteolytic Peptides

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Abstract

The circulating concentration of a biomarker for congestive heart failure, Brain (B-type) Natriuretic Peptide (BNP-32), is measured using ELISA based assays in order to rapidly diagnose and monitor disease progression. The lack of molecular specificity afforded by these assays has recently come into question as emerging studies indicate there are potentially multiple heterogeneous forms of BNP in circulation with immunoreactive capabilities. In order to better understand the molecular biology of BNP-32 as it relates to congestive heart failure, it would thus be advantageous to use a detection platform such as Fourier transform ion cyclotron resonance mass spectrometry. This high resolving power mass spectrometer can provide unparalleled molecular specificity and can facilitate identification and characterization of the various molecular forms across all disease states.

Unfortunately, BNP circulates at low concentrations (as low as 3 fmoles/mL). Thus, it will require a collaborative effort from a number of orthogonal front-end technologies to overcome the disconnect between the practical detection limits of this instrument platform and the physiological levels of BNP-32 and its alternative molecular forms. Herein, we begin optimization of these front-end techniques by first enhancing the conditions for online nanoLC-ESI-MS separations of BNP-32 and its proteolytic fragments. Through extensive analysis of various chromatographic parameters we determined that Michrom Magic C8 stationary phase used in conjunction with a continuous, vented column configuration provided advanced chromatographic performance for the nano-flow separations involving intact BNP-32 and its associated tryptic peptides. Furthermore, conditions for the tryptic digestion of BNP-32 were also studied. We demonstrate that the use of free cysteine as an alkylation quenching agent and a secondary digestion within the digestion scheme can provide targeted tryptic peptides with increased abundances. Combined, these data will serve to further augment the detection of BNP-32 by LC-MS.

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Keywords

Vented Column; BNP-32; Splitless nanoLC; Tryptic Digestion

1. Introduction

Produced primarily within the cardiac atria and ventricles, Brain Natriuretic Peptide (BNP) exists as a pre-propeptide that is further processed to a 108 amino acid prohormone (proBNP). The proBNP is enzymatically cleaved to form a biologically active 32 amino acid C-terminal peptide (BNP-32) and a biologically inactive 76 amino acid N-terminal peptide (NT-proBNP). [1] In patients experiencing congestive heart failure (CHF), it is generally believed that these peptides (BNP-32 and NT-proBNP) are up-regulated and released into circulation where BNP-32 induces natriuresis.[1] Hence, both BNP-32 and NT-proBNP are currently used to diagnose and monitor the severity of CHF. To date, however, very little molecular level information is known about the endogenous levels and molecular forms of BNP as they relate to the existence and severity of CHF.

This lack of knowledge in molecular forms and circulating levels of BNP is based in large part on the technological challenges associated with analyzing BNP because it circulates at low concentrations and potentially in multiple forms. Median BNP-32 concentrations range between 3 fmoles/mL and 323 fmoles/mL for healthy and New York Heart Association (NYHA) Class IV CHF patients, respectively.[2] However, these levels, which are already low, assume that the only form of BNP being detected by the POCTs is BNP-32. Observations that several molecular forms of BNP circulate in the body have resulted from studies involving traditional bioanalytical techniques such as radioimmunoassay[3–5], size exclusion chromatography[6], and 1D-gels combined with western blotting.[7] Evidence suggests the existence of degradation products of BNP-32[3,4,8,9], unprocessed proBNP[3–5], and “high molecular weight BNP”. [3,7,10–12] Our group recently reported the first molecularly specific evidence that the commonly used BNP POCTs were not quantifying endogenous BNP-32 [13], while another study demonstrated the presence of endogenous proBNP in an immunoaffinity purification assay using antibodies specific for NT-proBNP.[14] Considering the presence of heterogeneous forms of BNP and their low circulating abundances, both high sensitivity and specificity are crucial for identification and characterization of these various forms within healthy and CHF inflicted patients.

Liquid chromatography (LC) coupled to electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR-MS) is a uniquely suited bioanalytical instrument platform for identifying novel peptides and high molecular weight proteins such as BNP with high specificity. However, before BNP can be characterized by ESI-FT-ICR-MS, the gap must be closed between the detection limits of the instrument and the physiological levels of BNP in plasma. Therefore, it is crucial to develop and optimize front-end technologies to achieve a high level of sensitivity and low limits of detection (LOD) for quantifying (absolutely) and comprehensively characterizing endogenous forms of BNP by mass spectrometry. To aid in accomplishing this we have developed a platform for splitless nanoLC separations of intact BNP-32 as well as its tryptic digest for detection with ESI-FT-ICR-MS.

Prior to reverse phase (RP) LC separations, cleanup of proteomic samples is often performed online by loading first onto a pre- or trap column and, subsequently washing the proteins/peptides with low organic solvent. The trap column is then placed in-line with the analytical column where the retained protein material is eluted off the trap and injected onto the analytical column for separation. Micro-flow rates are generally employed during the loading/washing step in order to expedite the entire process, as well as to minimize the diffusional peak

broadening that occurs in transferring samples from the sample loop and onto the trap column. [15–18] Conversely, nano-flow rates are utilized during the injection/separation step in order to afford better sensitivity with ESI-MS detection.[19–22]

Two general column configurations have been developed to perform nanoLC-ESI-MS analysis of proteomic samples with online sample cleanup; the discontinuous and continuous, vented column configurations. First developed by Licklider *et al.*[23], the latter is suggested to provide superior chromatographic performance due to its minimal inter-column dead volume. Guzzetta and Chien performed a direct comparison between the two configurations during their analysis of a triphasic trap column.[24] Their novel trap column was implemented in both configurations and evaluated for use in an online MuDPIT analysis of a human plasma digest. Although they reported there was no qualitative difference in the chromatograms produced by either configuration, they demonstrated that the continuous, vented configuration identified 10% – 16% more peptides than did the discontinuous configuration. Importantly, the authors concluded this gain in peptide coverage predominantly benefited low abundant proteins. Our work expounds upon Guzzetta and Chien's work and demonstrates, statistically, the superior chromatographic performance afforded by the continuous, vented column configuration for separations involving BNP-32.

Furthermore, we have evaluated conditions for the tryptic digestion of BNP-32 in order to more thoroughly produce its targeted tryptic peptides and increase their overall abundance for detection by nanoLC-MS. A complete protein digestion, free of non-specific modifications, is essential for rapid positive identification by mass spectrometry. Reduction and alkylation of disulfide bonds can assist in unfolding the protein making interior amino acid residues more accessible to the digestion enzyme. Using excessive quantities of alkylating reagent or allowing the reaction to proceed extensively can result in non-specific modifications to non-cysteinyll residues within proteins.[25,26] When using iodoacetamide (IAM) as the alkylating reagent, as employed in our experiments, these carbamidomethyl (cam) modifications remain as the protein is digested, which can generate overalkylated peptides and complicate identification. [27–29]

Overalkylation of proteins/peptides has the potential for negatively affecting their absolute abundance. When reduction and alkylation are employed, there is potential for non-cysteinyll residues within the target peptide to be alkylated non-specifically. This would distribute the target peptide's abundance into multiple mass to charge (m/z) channels, or multiple alkylation states, even for target peptides that do not contain cysteines. Incomplete digestions can also disperse peptide abundances between different cleavage states (i.e., no missed cleavages, one missed cleavage, two missed cleavages, etc.). Overalkylation and incomplete digestion would be particularly detrimental when attempting to quantify low abundant proteins, like BNP-32.

In order to further increase the value of mass spectral measurement of different BNP-32 species (oxidized, reduced and alkylated, and trypsinized), we provide a benchmark for the separation of BNP-32. To enhance the overall sensitivity of detection by nanoLC-ESI-MS, two column configurations, as well as several stationary phases were analyzed to provide optimal chromatographic efficiency. Additionally, digestion parameters were investigated to eliminate overalkylation and missed cleavages, and to increase the overall abundance of targeted BNP-32 tryptic peptides.

2. Experimental

2.1. Materials

HPLC-grade acetonitrile and water were purchased from Burdick and Jackson (Muskegon, MI). Formic acid, oxidized BNP-32, ammonium bicarbonate, iodoacetamide, trypsin, and free

cysteine were from Sigma-Aldrich (St. Louis, MO) and dithiothreitol (DTT) from BioRad (Hercules, CA). Zwittergent 3–16 detergent was purchased from Calbiochem (La Jolla, CA). The 75 μm i.d. PicoTip capillary column with 15 μm emitter tip and the fritted 75 μm i.d. IntegraFrit capillary were purchased from New Objective (Woburn, MA). Reverse phase packing material used for in-house column packing were as follows: 5 μm , 200Å silica Magic C18AQ and Magic C8 (Michrom BioResources, Auburn, CA), 5 μm Targa C18 120Å silica (Higgins Analytical, Mountain View, CA), 5 μm mRP C18 >1000Å silica (Agilent, Palo Alto, CA), and 4 μm Jupiter Proteo C12 90Å silica (Phenomenex, Torrance, CA). A custom packed Magic C18AQ OPTI-PAK trap cartridge was purchased from Optimize Technologies (Oregon City, OR). All Valco fittings, including the stainless steel (SS) tee and the 10-port valve, were purchased from VICI Valco Instruments Co. Inc. (Houston, TX) or ChromTech (Apple Valley, MN).

2.2. Column Packing

To prepare the analytical columns, 75 μm i.d. PicoTip emitter columns were packed using a methanolic slurry of stationary phase (~5 mg/ml). The column was attached to a pressurized cell (bomb), packed and cut to the desired length (~15 cm). A 15 cm column length resulted in intermediate back pressures and was sufficient for the purpose of our evaluations. The columns were inspected under a stereomicroscope to ensure a 90° cut and then fitted to either a SS zero dead volume (ZDV) fitting for use in the discontinuous column configuration, or a SS tee for use in continuous, vented column configuration. All “dummy” columns were prepared in identical fashion using 75 μm i.d. IntegraFrit capillaries.

To pack the trap columns for the vented column configuration, a 75 μm i.d. fused silica capillary was connected to the SS tee, in-line with a packed analytical column. A 75 μm i.d. IntegraFrit capillary was connected to the venting outlet on the SS tee, with the fritted end nearest the tee. The fused silica capillary trap column was then connected to the bomb and packed to the desired length (~5 cm) with a methanolic slurry of stationary phase identical to that within the analytical column. This packing method, which permitted the methanol to exit via the vent rather than the analytical column, allowed for rapid packing of the void within the SS tee as well as the desired length of the trap column. Care was taken to ensure proper tight packing of the stationary phase within the tee by repeatedly depressurizing and pressurizing the bomb during the packing process. Also to ensuring proper packing within all filled parts, the trap and analytical columns were connected to the nanoLC in their respective configurations and equilibrated by flushing with 80% B, 50% B, and 2% B for 2 hours, 2 hours, and 1 hour respectively. If the tee was not packed efficiently while attached to the bomb, a small displacement of the stationary phase from the trap column into the tee was observed during the equilibration process (~15 mm).

2.3 Column Configurations

The terms continuous and discontinuous were first coined by Guzzetta and Chien[24] and are used herein to differentiate between two general column configurations. Discontinuous is used to indicate a configuration in which the trap and analytical columns are separated by a switching valve. Continuous denotes a configuration where the two columns are always in-line with one another, as is the case in the vented configuration. For both configurations, a 6 port valve located on the Eksigent autosampler was used for aspiration of the sample into a 10 μL sample loop and, subsequent, fast loading/washing of the sample onto the trap columns.

The discontinuous column configuration (Figure 1A) has a long history of use in proteomics [15–18] and is briefly described here for direct comparison to the continuous, vented column configuration. In the load position, samples were introduced onto a custom packed OPTI-PAK trap column (250 nL) and washed using a high-flow aqueous solvent from Channel 1. The

valve was then switched to the inject position, where the analytes retained within the trap were eluted into a transfer line and onto the analytical column via the nano-flow gradient from Channel 2. In assembling this configuration, care was taken to minimize the length of the transfer line so as to reduce the dead volume between the trap and analytical column.

The continuous, vented column configuration has been described previously for use in split-flow nanoLC separations[23], but is described here using splitless-nanoLC technology (Figure 1B). Briefly, samples were loaded onto the self-packed trap column and washed with high-flow solvent via Channel 1 while in the load positions. Impurities from the trap exited by means of a fritted capillary which was vented to the atmosphere in this position (i.e., vented column). When the valve was switched to the inject position, the vented column closed, directing the nano-flow gradient from Channel 2 through the trap and directly onto the in-line, analytical column. Additionally, the continuous, vented column configuration was evaluated with and without the use a packed bed of stationary phase (i.e., “dummy” column) in-line of Channel 2 during the sample loading/washing steps. When in place, flow from Channel 2 was diverted onto the “dummy” column in order to maintain pressure on the nano-flow pump heads with the valve in the load position. When the “dummy” column was not present in the continuous configuration, solvent from Channel 2 was sent directly to waste during this step by means of an empty capillary.

2.4. Sample Preparation

Aliquots containing 6.25 ng of native, oxidized BNP-32 in 200 mM ammonium bicarbonate buffer (pH~8) were dried down and stored at -20°C . For the reduced and alkylated BNP-32 sample, an aliquot of native BNP-32 was reconstituted with 25 μL buffer ($\sim 0.25\text{ }\mu\text{g/mL}$). To the protein solution, enough 0.5 mM DTT was added such that the molar ratio of cysteine residues:DTT was 1:5. The solution was vortexed, incubated for 1 hour at 37°C , and cooled to room temperature. Enough 5 mM IAM was added to the reduced BNP-32 solution such that the molar ratio of thiol:IAM was 1:5, accounting for the 2 thiols on each DTT. The solution was vortexed and incubated for 1 hour at 37°C in the dark. To quench the alkylation, enough DTT or free cysteine was added to give a 10:1 molar ratio of quenching reagent:IAM, and this was allowed to react for 30 minutes at room temperature. Reduced and alkylated BNP-32 samples were diluted, fractionated, and dried down for storage at -20°C .

For preparation of the BNP-32 digest, reduction, alkylation, and alkylation quenching were preformed as described above. To the alkylated BNP-32 solution, 0.01 mg/mL trypsin was added to give a 1:50 ratio (w/w) of trypsin:BNP-32. This solution was vortexed and incubated for 4–20 hours at 37°C . A secondary digest to reduce missed cleavages was performed on one digest sample with trypsin using the same conditions as described above. Additional trypsin was added after 4 hours and the solution was incubated further for 2 hours. The solutions were diluted, fractionated, and dried down for storage at -20°C until later use. Prior to LC-MS experiments, aliquots were reconstituted and diluted in 0.001% Zwittergent 3–16 detergent such that a 10- μL injection would contain 1–2 pmol of BNP-32.

2.5. Online nanoLC-MS

NanoLC-ESI-FT-ICR-MS measurements were made utilizing an Eksigent (Dublin, CA) nanoLC-2D system equipped with an autosampler (Dublin, CA), coupled to a hybrid LTQ-FT Ultra mass spectrometer from Thermo Scientific, Inc (San Jose, CA). The LTQ-FT was equipped with an actively shielded 7-Tesla superconducting magnet from Oxford Instruments (Concord, MA).

Mobile phase A was water/acetonitrile/formic acid (98/2/0.1% by volume) and mobile phase B was acetonitrile/water/formic acid (90/10/0.1% by volume). In both column configurations,

a 10 μL sample injection ($\sim 1\text{--}2$ pmol) was loaded from the sample loop onto the respective trap column at 3 $\mu\text{L}/\text{min}$ by way of a 15 μL metered injection of 2% B from Channel 1. The analytical separations were run on the nano-flow pump at 500 nL/min initially maintaining 2% B. After 5 min, an increase to 10% B over 2 min preceded a linear gradient to 50% B over 40 min. After ramping up to 95% B over 3 min and holding for 5 min, the initial conditions were reinstated over 2 min and held 8 min for re-equilibration. A column heater was used for experiments with the mRP C18 and Jupiter Proteo C12 stationary phases maintaining a temperature of 80 $^{\circ}\text{C}$ and 40 $^{\circ}\text{C}$, respectively, as recommended by the manufacturers.

The MS method contained four events; precursor scan within the ICR cell followed by three data dependent tandem MS scans of the first, second, and third most abundant peaks in the ion trap. The AGC limit was set at 5×10^5 and the mass resolving power for m/z 400 was 100,000_{fwhm}. For tandem MS the AGC limit within the ion trap was 1×10^4 . The instrument was externally calibrated according to the manufacturer's protocol.

2.6. Data Analysis

RAW data files were processed by SEQUEST through Bioworks (Thermo Fisher Scientific, Inc.) to confirm peptide sequences, missed cleavages, and overalkylation. Using the Xcalibur software package (Thermo Fisher Scientific, Inc.), extracted ion chromatograms (XIC) of oxidized BNP-32 and reduced and alkylated BNP-32 at m/z windows of 577.46–578.46 and 596.97–597.97, respectively, were manually analyzed to determine retention times, peak widths, and peak asymmetries. Identical data treatment was performed using XIC of the tryptic peptides, ISSSSGLGCK, KMDRISSSSGLGCK, MVQSGSGCFGR, SPKMVQSGSGCFGR, and ISSSGLGCKVLR, at m/z windows, 498.23–498.25, 509.24–509.26, 549.74–549.76, 470.88–470.90, and 455.24–455.26, respectively. Additionally, the XIC for the overalkylated tryptic peptide, KMDRISSSSGLGCK, was generated with an m/z window of 527.76–528.76. MATLAB Software version 2006 developed by The MathWorks (Natick, Massachusetts) was used to prepare notched box plots for analysis of chromatographic efficiencies between column configurations.

3. Results and Discussion

3.1. Optimization of BNP-32 Separations

Optimizing chromatographic performance is crucial for the detection and quantification of low abundant proteins within complex media. When employing LC-ESI-MS, separations play an important role in determining the LOD because coeluting species can suppress the ionization of one another as they compete for charge within the electrospray droplet. By increasing the peak capacity of a separation method, the probability that multiple species will coelute decreases. This, in turn, reduces competition for charge, increases ionization efficiencies, and lowers the overall LOD. A chromatographic figure of merit that directly affects peak capacity and consequently the LOD is the system's efficiency.

Chromatographic efficiency, which is a measure of the overall chromatographic performance of a system, is determined by the number of theoretical plates (N) in a system. In order to estimate N , Foley and Dorsey developed an exponentially modified Gaussian model featuring retention time (t_R), peak width at 10% height ($W_{10\%}$), and peak asymmetry (B/A).[30]

$$N = \frac{41.7 \left(\frac{t_R}{W_{10\%}} \right)^2}{\frac{B}{A} + 1.25} \quad (1)$$

At $W_{10\%}$ for a tailing peak, A represents the time difference between the front of the peak and the overall retention time. B is calculated as the difference in time between the retention time and the tailing end of the peak. All peaks analyzed here were asymmetric in nature, justifying the use of the skewed peak model (equation 1). Furthermore, because the columns used in this study were packed to slightly different lengths (L), ranging between 14.15–14.65 cm, it was necessary to normalize the number of theoretical plates. A common chromatographic figure of merit, known as the plate height (H), [31,32] allowed for this normalization, with smaller plate heights indicating greater column efficiencies.

$$H = \frac{L}{N} \quad (2)$$

The ideal chromatographic system would provide a broad retention window for our tryptic peptides, while providing narrow, symmetric, and well resolved peaks. In general, the system with the greatest peak capacity for our range of target peptides would provide the smallest value of H with adequate peak spacing.

3.1.1 Determining Optimal Stationary Phases—Our group has previously utilized two different stationary phases for analysis of BNP-32. Targa C18 was employed in quantifying recovered BNP-32 from a dual-antibody immunoaffinity purification assay [13], while Magic C18AQ was used for analysis of BNP-32 alkylated with iodoacetamide versus 2-iodo-N-octylacetamide. [33] However, the optimal stationary phase or phases for the various molecular forms of BNP have yet to be determined and established as standard protocol for separations. Here five stationary phases were evaluated for the RPLC separation of intact BNP-32 (oxidized or reduced and alkylated), as well as its associated tryptic peptides. The stationary phases were chosen based on their potential for either bottom-up or top-down proteomic applications. Each form of BNP-32 was run in triplicate on all five stationary phases utilizing the continuous, vented column configuration with a “dummy” column (see Figure 1A). Average column efficiencies provided means to determine the optimal packing material for both intact and tryptic BNP-32 species.

Shown in Figure 2 are representative XICs for the 6+ charge state of intact (oxidized) BNP-32 separated on each of the five stationary phases. The average H calculated for these packing materials ranged from 2.5 to 110 μm , with the Magic C8 giving the lowest value of $2.5 \pm 0.6 \mu\text{m}$ ($n=3$). Not surprisingly, these quantitative results correspond well to the qualitative appearance of each XIC. In Figure 2, it is readily apparent that the Magic C8 stationary phase provides the most well retained, symmetric, and narrow peak for the separation of BNP-32 in its oxidized form. Results for the reduced and alkylated species mirrored those of the oxidized form on each stationary phase evaluated (data not shown), indicating that Magic C8 is the superior packing material for the separations of intact BNP-32.

For the separation of a BNP-32 digest, H was calculated for five tryptic peptides within each run and averaged to estimate the efficiency across the entire elution window. The five tryptic peptides chosen for this purpose are labeled A–E in Figure 3. Upon visual inspection of the base peak chromatograms for each stationary phase (Figure 3) the Jupiter C12 appears to give the sharpest peak shapes, which is corroborated by the small plate height calculated for this column packing material (see Table 1). However, this same column packing material produces a narrow elution window; indicating poor peak capacity for this stationary phase. The optimal packing material must provide a high level of efficiency while maintaining sufficient peak spacing. Both the Magic C18AQ and Magic C8 appear to meet these qualifications. But for the sake of simplicity, the Michrom Magic C8 packing material is recommended due to its

exceptional chromatographic performance for both the intact forms and tryptic digest of BNP-32.

3.1.2 Evaluation of a Vented Column Configuration—Initially, our attempts at implementing the continuous, vented column configuration utilized an empty capillary to transfer the nano-flow from Channel 2 to waste during the loading/washing steps, rather than the dummy column depicted in Figure 1A. With the empty capillary in place, the nano-flow pumps from Channel 2 experienced drastic pressure differentials (~2500 psi) when switching between the inject and load positions. This resulted in significant delays (>1 min) in achieving the target flow rate at the beginning of each gradient run. To remedy this, we replaced the transfer capillary with a packed bed of stationary phase (i.e. a “dummy” column) so as to provide equivalent pressures between the two valve positions. As a result, the pumps from Channel 2 encountered a much smaller pressure differential (~100 psi) with the dummy column implemented; reducing delay times (~10 sec) for the continuous, vented configuration. In order to achieve near identical pressures, the “dummy” column was packed to a combined length of the trap and analytical columns, using the same inner diameter capillary and stationary phase.

Both continuous, vented configurations (with and without a “dummy” column) were evaluated for their chromatographic performance and compared to the discontinuous configuration using the Magic C18AQ stationary phase. In order to determine the chromatographic efficiencies of the various column configurations, a BNP-32 tryptic digest was injected on each (n=10). All three configurations employed the same 15 cm analytical column for direct comparison of the different configurations. A representative base peak chromatogram for each configuration is shown in Figure 4. The peaks generated by the discontinuous configurations are noticeably wider compared to those in the continuous configurations. Consequently, peaks that are well resolved in both continuous configurations overlap significantly in the discontinuous configuration (e.g. S and T). To quantify these observations, the notched box plots in Figure 5 were constructed using the efficiencies calculated for five tryptic peptides (labeled Q-U in Figure 4 and Figure 5). The box plots located in Figure 5 inset are a composite of all five peptides and give the best representation of each configuration’s efficiency over the entire elution window of the BNP-32 digest. These box plots clearly confirm both continuous, vented column configurations provide significantly more efficient separations of the tryptic peptides compared to the discontinuous configuration. Moreover, the continuous configuration utilizing a “dummy” column gave significantly improved efficiencies compared to the configuration lacking a “dummy” column for two of the five peptides examined. Although these results do not confirm a “dummy” column affords superior chromatography within the continuous configuration, it does indicate a potential benefit. In general, the narrower peak widths and overall better chromatographic efficiencies afforded by the continuous, vented configuration resulted in greater absolute ion abundances with respect to the discontinuous configuration. This indicates the use of the continuous, vented configuration should facilitate detection of BNP-32.

An explanation for the superior performance of the continuous, vented configurations can be found in the work by Meiring[34] and Yi[35]. Both reports suggested the main advantage of the continuous, vented column configuration over the discontinuous configuration arises from the reduction of inter-column dead volume between the trap and analytical columns. In the discontinuous configuration, analytes eluting off the trap must pass through the switching valve and a transfer capillary before reaching the analytical column. Since this occurs at nano-flow rates, the samples experience a significant amount of dead volume prior to separation, which leads to diffusional broadening of the peaks. However, in the continuous configuration, the only inter-column dead volume appears within the venting tee, which can be eliminated altogether by packing the tee with stationary phase. By creating a continuous bed of stationary

phase between the trap and analytical columns, any inter-column dead volume is eliminated, which translates into improved chromatographic performance and better sensitivity.

3.3. Evaluation of BNP-32 Digestion Protocol

3.3.1 Alkylation Quenching—To prevent overalkylation, an alkylation quenching step can be employed prior to protein digestion. In this step it is assumed that the excess alkylating reagent will react more rapidly with the quenching reagent rather than the non-cysteiny l residues within the protein, thereby preventing overalkylation from occurring during the digestion step and beyond. In order to determine the optimal reagent for quenching alkylation by IAM, two BNP-32 tryptic digestions were performed with two different quenching reagents: DTT and free cysteine. Both digestions employed identical conditions for reduction, alkylation, and digestion, and allowed 30 minutes at room temperature for quenching (see section 2.4). Each digest was subjected to LC-MS analysis and the presence of overalkylated peptides in each was determined manually by exact mass measurements (<5 ppm). The peptides identified in this manner are listed in Table 2. The digestion that employed DTT produced a large number of overalkylated peptides, indicating that DTT did not react effectively with the excess IAM. Conversely, no overalkylated peptides were present in the digest utilizing free cysteine as the quenching reagent, suggesting that IAM reacts more rapidly and more efficiently with free cysteine than with DTT.

3.3.2 Tryptic Digestion—Protein digestion protocols vary widely, recommending enzymatic reactions last between 2 and 24 hours for reaction completion. However, we observed a four hour tryptic digestion of BNP-32 gave no more missed cleavages than one allowed to react for 20 hours (data not shown). This suggests trypsin's enzymatic activity in the digestion of BNP-32 has effectively ended within a four hour digestion period. In order to achieve a more complete digestion, a secondary tryptic digestion was performed after an initial four hour tryptic digestion of BNP-32 and was allowed to proceed two hours. Although tryptic peptides with missed cleavages were present after the secondary tryptic digest, these species occurred less frequently (Figure 6). After a 20 hour digestion, the peptide with one missed cleavage, KMDRISSSSGLGCK, was actually more abundant than the corresponding peptide with no missed cleavage, ISSSSGLGCK. By including a secondary digest, the presence of KMDRISSSSGLGCK was significantly diminished, causing an increase in the abundance of ISSSSGLGCK. An overall decrease in missed cleavages was also observed in the peptide series containing the peptide MVQSGGCFGR. Further optimization of the digestion protocol is required to achieve a complete digestion of BNP-32 and to traverse this protocol to complex mixtures.

4. Conclusions

In these studies, a set of chromatographic parameters were identified that provide a high level of performance for the nanoLC separations of BNP-32 and its various molecular forms. It was observed that the Michrom Magic C8 stationary phase afforded a superior level of performance for the RPLC separations of intact BNP-32 and its tryptic digest. A continuous, vented column configuration was evaluated for automated, online sample cleanup using splitless nanoLC technology. Compared to the more often employed discontinuous configuration, the continuous configuration gave significantly improved chromatographic efficiencies. The inclusion of a “dummy” column within the continuous configuration provided more rapid pressure stabilization and enhanced the chromatographic performance for tryptic peptides. Chromatographic conditions not evaluated here such as flow rates, column lengths, mobile phase compositions, and gradient profiles, could be altered to provide additional enhancement to the nanoLC platform described in this study.

Additionally, alkylation quenching and digestion conditions were augmented in order to more efficiently produce targeted BNP-32 tryptic peptides and increase their overall abundance. Free cysteine provided a more rapid and thorough quenching of alkylation by IAM and prevented overalkylation. A secondary digestion step notably reduced the amount of missed cleavages in the tryptic digestion of BNP-32. By increasing the duration of the secondary digest, or possibly including a third, the presence of missed cleavages should become negligible. To provide optimal nanoLC-ESI-MS detection sensitivity, these parameters could be used in conjunction with other novel front-end technologies that improve ionization efficiency or ion transfer. Collectively, these studies in chromatographic performance and digestion efficiencies serve to enhance downstream MS detection of BNP-32 and its associated tryptic peptides, as well as other low abundant proteins.

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Figure 1.

Schematics showing the valve configurations for (A) the discontinuous column configuration and (B) the continuous, vented column configuration.

Figure 2.

(A) Secondary structure of BNP-32 in its native, oxidized form with tryptic peptides highlighted. (B) FT-ICR mass spectrum of the 6+ charge state of oxidized BNP-32. (C) Overlays of the XIC of oxidized BNP-32 (577.46–578.46 m/z) for each of the five stationary phases evaluated for separations.

Figure 3.

Base peak chromatograms of a BNP-32 tryptic digest on each stationary phase. The peptides labeled are A) KMDRISSSSGLGC(cam)K, B) ISSSSGLGC(cam)K, C) KMDRISSSSGLGC(cam)K+(cam), D) MVQGSGC(cam)FGR, E) ISSSGLGC(cam)KVLK. Peptide B is not labeled within the mRP C18 chromatogram because it elutes early at ~3.5 minutes.

Figure 4.

Representative base peak chromatograms of a BNP-32 tryptic digest run on each column configuration using Magic C18AQ stationary phase. The five peptides used for estimating chromatographic efficiencies are labeled in each chromatogram. The peptides are Q) ISSSSGLGC(cam)K, R) KMDRISSSSGLGC(cam)K, S) MVQGSGC(cam)FGR, T) SPKMOVQGSGC(cam)FGR, U) ISSSSGLGC(cam)KVLK.

Figure 5.

Notched box plots of chromatographic efficiencies for the three column configurations examined. In ten replicate injections on each configuration, five tryptic peptides (Q–U) were used to determine the number of theoretical plates. The inset plots for each configuration are a composite of all five peptides over all replicates. The “+” denotes an outlier within each distribution and overlapping notches indicate no significant difference between the distributions’ medians.

Figure 6.

Base peak chromatograms of BNP-32 tryptic digests. Relative peak abundances are normalized between chromatograms to the most abundant peptide present. The dotted blue chromatogram represents a standard tryptic digest lasting for twenty hours, while the solid black chromatogram represents a tryptic digest employing a two hour secondary digest after an initial four hour digestion.

Table 1

Average plate heights and selectivities for the five stationary phases evaluated for nanoLC separations of BNP-32 digest.

Stationary Phase	Plate Height (H)
Magic C18	4.3 μm
Targa C18	2.7 μm
mrPC18	29 μm
Magic C8	4.0 μm
Jupiter C12	1.3 μm

Table 2

List of peptides identified within a BNP-32 tryptic digest utilizing DTT as an alkylation quenching reagent. An * indicates an oxidized methionine. In comparison, only the mono-alkylated peptides listed were identified in a BNP-32 tryptic digest utilizing free cysteine as the quenching reagent.

Overalkylated	Mono-alkylated
MVQGSGC(cam)FGR + cam	MVQGSGC(cam)FGR
M*VQGSGC(cam)FGR + cam	M*VQGSGC(cam)FGR
MVQGSGC(cam)FGR + 2cam	MVQGSGC(cam)FGRK
MVQGSGC(cam)FGRK + cam	SPKMVQGSGC(cam)FGR
SPKMVQGSGC(cam)FGR + cam	SPKM*VQGSGC(cam)FGR
SPKM*VQGSGC(cam)FGR + cam	ISSSSGLGC(cam)K
SPKMVQGSGC(cam)FGR + 2cam	ISSSSGLGC(cam)KVLK
ISSSSGLGC(cam)K + cam	MDRISSSSGLGC(cam)K
MDRISSSSGLGC(cam)K + cam	KMDRISSSSGLGC(cam)K
KMDRISSSSGLGCK + cam	KM*DRISSSSGLGC(cam)K
KMDRISSSSGLGCK + 2cam	