Vaters

ENHANCED IDENTIFICATION OF TRACE-LEVEL PEPTIDES FROM COMPLEX PROTEIN DIGESTS USING THE **IONALYTICS[™] SELECTRA[™] IN LC/MS EXPERIMENTS**

Karin Venne¹, Eric Binneil¹, David. A. Barnett² and Pierre Thibault¹ Caprion Pharmaceuticals Inc., Montreal, Canada Ionalytics Corporation, Ottowa, Canada

Introduction

The lonalytics[™] Selectra[™] is an ion-filtering device providing additional, orthogonal separation prior to LC/MS and LC/MS/MS experiments, and is fully integrated with a range of Waters® Micromass® mass spectrometers. The lonalytics Selectra uses FAIMS (high-field asymmetric waveform ion mobility spectrometry) technology to separate gas-phase ions based on changes in mobility that occur as a function of their electric field strength. As a result, the use of FAIMS with Electrospray MS and MS/MS analyses on Waters Micromass MS systems provides an easy route to enhance the detection and characterization of proteins, peptides, various other biomolecules and small molecules.

Protein identification using nano-LC/MS/MS provides reliable sequencing information for low femtomole levels of in-gel digests. However, this task is more challenging for sub-femtomole peptide levels as precursor detection is often compromised by the presence of chemical noise associated with the ionization of the mobile phase constituents and column bleed. This application note describes how researchers at Caprion Pharmaceuticals (Montreal, Canada) used the lonalytics Selectra integrated with a Waters Micromass Q-Tof[™] mass spectrometer to help identify these trace level peptides.



and the integrated lonalytics Selectra.



ENHANCED IDENTIFICATION OF TRACE-LEVEL PEPTIDES FROM COMPLEX PROTEIN DIGESTS USING THE IONALYTICS SELECTRA IN LC-MS EXPERIMENTS

Karine Venne¹, Eric Bonneil¹, David A. Barnett² and Pierre Thibault¹ ¹Caprion Pharmaceuticals Inc., Montreal, Canada ² Ionalytics Corporation, Ottawa, Canada

> Protein identification using nano-LC-MS/MS provides reliable sequencing information for low femtomole levels of in-gel digests. However, this task is more challenging for subfemtomole peptide levels as precursor detection is often compromised by the presence of chemical noise associated with the ionization of the mobile phase constituents and column bleed.

> The Ionalytics Selectra[™] provides a simple mechanism for reduction of this interfering chemical noise. Using FAIMS (high-field asymmetric waveform ion mobility spectrometry) technology, the Ionalytics Selectra separates gas-phase ions based on changes in mobility that occur as a function of electric field strength. The Ionalytics Selectra operates at ambient conditions and is easily installed between the existing atmospheric pressure ionization source and the inlet aperture of the mass spectrometer without venting of the vacuum system.

The FAIMS technology uses a high voltage asymmetric waveform applied to a set of electrodes. This waveform is composed of a high voltage component called the *dispersion voltage* (DV) and an opposite polarity low voltage component. Application of the waveform causes ions introduced between the electrodes to oscillate. Differences in an ion's mobility under low- and high-field conditions will cause the ion to travel different distances during the two phases of the waveform. Over time, the ion will drift towards one of the two electrodes. The ion drift can be overcome by applying a low DC voltage, called the *compensation voltage* (CV), to one of the two electrodes. A unique ion-focusing mechanism ensures that the selectivity offered by the CV is accompanied by high analyte transmission efficiency.

Improvements in sensitivity and S/N using the lonalytics Selectra are described for tryptic digests of standard proteins and post-nuclear membrane extracts from U937 differentiated macrophages.

EXPERIMENTAL:

Protein Extracts

Protein standards phosphorylase b, cytochrome c, alcohol dehydrogenase (ADH), bovine serum albumin (BSA) and lactalbumin were obtained from Sigma Chemicals (St. Louis, MO) and used without further purification.

The pro-monocyte cell line U937 (ATCC CRL-1593.2) was grown in 10% fetal calf serum (FCS) supplemented RPMI-1640 with glutamine and penicillin/streptamycin up to a density of 2 million cells/ml. Cells were then used as "monocytes" or differentiated into "macrophages" by exposing them to 25 nM of phorbol ester (PMA) for 48 hours. Cells were harvested, washed, disrupted by cavitation in 200 mM ammonium bicarbonate, and centrifuged (first at 1000 g and then at 100 000 g) to remove nuclei and cell debris. This extraction procedure produced a post-nuclear membrane (PNM) enriched in total membrane proteins. Protein concentrations were obtained using bicinchoninic acid (BCA).

Tryptic digestion of the mixture of standard proteins was performed as follows: each protein was diluted in 100 mM ammonium bicarbonate (pH 8.1) to a concentration of 1 μ g/ μ l. Trypsin was added (enz/prot: 1/25) and the digestion proceeded overnight at 37°C. The tryptic digests were then pooled and diluted with 0.2% formic acid and 5% acetonitrile to a concentration of 10 fmol/ μ l.

Protein extracts obtained from U937 cells were prepared in aliquots of 100 µg of PNM and each was treated with DNAse and diluted in 100 mM sodium carbonate (pH 11.0). The sample was centrifuged at 14 000 g and the pellet was resuspended in 100 mM ammonium bicarbonate (pH 8.1) and digested overnight with trypsin (enz/prot: 1/25) at 37°C. The sample was then reduced with tri (2-carboxyethyl) phosphine (TCEP) following digestion.

Mass Spectrometry

Mass spectrometry analyses were conducted using a Waters/Micromass Q-Tof micro™ or Q-Tof Ultima™ (Manchester, UK) equipped with a modular Waters® CapLC[®] liquid chromatograph. The LC-MS system consisted of a C-18 Symmetry precolumn (5 mm x 300 µm, Waters) and an analytical column (10 cm x 150 µm i.d., Jupiter 5 µm C18 particles, Torrance, CA). Tryptic digests were injected on the column, and peptide elution was achieved using a linear gradient of 10-60% acetonitrile (0.2% formic acid) in 60 minutes. All MS/MS spectra were obtained using data-dependent experiments with Ar as a collision gas.

The lonalytics Selectra electrodes were installed adjacent to the sampling cone of the mass spectrometer. The installation did not require venting of the instrument. The dispersion voltage was set at -4000 V and a gas mixture of 1/1 nitrogen/helium was used as the desolvation/carrier gas.



Figure 1. Total ion current (TIC) spectra obtained for LC-MS analyses of a tryptic digest of a mixture of five proteins (20 fmol/inj.). Top panel: conventional nano-LC-MS analysis. Bottom panel: nano-LC-MS analysis with the lonalytics Selectra implemented (CV = -15.5 V). Inset in both panels are the extracted MS at 11 minutes. Data was acquired on a Q-Tof micro.

RESULTS:

Two LC-MS separations of a tryptic digest from a mixture of five proteins (20 fmol/inj.) are presented in Figure 1. The top panel shows a total-ion-chromatogram (m/z 300-1600) collected without the Ionalytics Selectra, while the bottom panel shows the LC run collected with the Ionalytics Selectra installed between the ion source and the mass spectrometer. Each panel includes an extracted mass spectrum taken at a retention time of approximately 11 minutes. The TIC profile obtained for the conventional nano-LC-MS analysis (top) shows a relatively high baseline profile compared with that of the corresponding analysis using the Ionalytics Selectra at a CV of -15.5 V (bottom). The higher ion counts observed in the conventional nano-LC-MS analysis are attributed to singly charged solvent ions, ions associated with column bleed, and

other non-specific interferences. In contrast, the mass spectrum extracted from the lonalytics Selectra analysis shows a reduction of background ions since the majority of these ions are transmitted through the lonalytics Selectra electrodes under different voltage conditions.

Figure 2 shows ion chromatograms, reconstructed from the analysis in Figure 1, for three doubly charged ions: m/z 714.0 (HLQIIYEINQR) from phosphorylase b, m/z 584.9 (TGPNLHGLFGR) from cytochrome c, and m/z 536.3 (EKDIVGAVLK) from ADH. These ions were observed in the conventional nano-LC-MS analysis (left panels) to have S/N values below 10:1. When the lonalytics Selectra was implemented, these same ions showed S/N values that were 6- to 12-fold higher (right panels). These increases in S/N are the result of both higher ion

intensity and lower background signal than obtained from the conventional nano-LC-MS analysis. The extracted mass spectra obtained for the doubly charged peptide ion at m/z 584.9 are presented in the bottom panels. The conventional nanoelectrospray mass spectrum has several peaks that are more intense than m/z 584.9, many of which arise from singly charged ions. The mass spectrum acquired when the Ionalytics Selectra was implemented (right panel) is greatly simplified. The majority of the singly charged background species observed in the conventional nanoelectrospray mass spectrum have been removed. Several doubly charged ions are easily identified, and have higher S/N ratios when compared with the conventional nanoelectrospray mass spectrum.

The Ionalytics Selectra can also be implemented in the analysis of much more complex protein samples, such as that arising from the digest of U937 macrophage cells. The PNM fraction is expected to contain in excess of 5000 proteins at concentrations ranging from 0.01 to 60 nM. The LC-MS analysis of a 500 ng injection of this digest is presented in Figure 3; the conventional nano-LC-MS data is shown in the panel to the left and the nano-LC-MS data collected with the Ionalytics Selectra implemented is presented in the panel to the right. The complexity of this sample is illustrated by the contour profile of m/z vs. retention time vs. intensity, where each dot corresponds to an individual ion. The logarithmic intensity scale shows a progressive increase in abundance for ions as the color transitions from black to bright yellow. The horizontal streaks at m/z 445.1 and 519.1 in the conventional nano-LC-MS analysis correspond to siloxane ion clusters typically observed as chemical noise. The vertical streak at 65 minutes corresponds to non-peptidic







Figure 3. Contour profiles of an LC-MS analysis of a digest of U937 macrophage cells. Panels to the left were collected using conventional nano-LS-MS, and panels to the right included the Ionalytics Selectra (CV = -16 V) in the analysis. Ion abundance increases as the color scale changes from black to yellow. Inset are mass spectra collected for the indicated regions from the contour profile. This data was collected using a Q-Tof Ultima.

singly charged ions associated with column bleed. These peaks are not present in the lonalytics Selectra analysis (right panel) as their transmission through the electrodes requires different electrical conditions.

Inset on each contour profile is a narrow region of the mass spectra for peaks highlighted by the small blue and green boxes. The mass spectra show three doubly charged precursors at m/z 605.8, 608.8 and 629.3 with higher S/N values using the Ionalytics Selectra (right insets) compared with nanoelectrospray (left insets). The enhanced sensitivity offered by the Ionalytics Selectra enabled the acquisition of meaningful MS/MS spectra for these tracelevel peptide ions, as shown in Figure 4. The product ion spectra of the doubly charged ions at m/z 629.3 and 608.8 provided characteristic fragment ions encoding the partial sequence of peptides VMSEFNNNFR and EEPGEDFPAAR corresponding to lysosome-associated membrane glycoprotein 3 (CD63) and Transferrin receptor protein (CD71), respectively. It is noteworthy that CD71 is expressed at levels less than 50 000 copies/ cell, and that immunofluorescence microscopy yielded very faint staining for this protein. It was unambiguously identified in the present





experiment. The doubly charged precursor ion at m/z 605.8 was not successfully identified through a Mascot database search, while BLAST supported the partial sequence assignment VC*QPEGER (modified cysteine residue shown by an asterisk) corresponding to a protein of unknown function.

CONCLUSIONS:

The separation capability of the lonalytics Selectra provides enhanced detection of high attomole to low femtomole amounts of peptides when compared with conventional nanoelectrospray mass spectrometry. This is achieved by higher transmission of targeted ions and by reduction of the chemical noise associated with transient signal arising from the electrospray process and/or column bleed during LC-MS experiments.

KEY BENEFITS OF THE IONALYTICS SELECTRA:

- Improved detection limits
- Reduced background
- Increased selectivity

For more information about the lonalytics Selectra, please contact us:

Ionalytics Corporation Building M50-IPF 1200 Montreal Road Ottawa, Ontario K1A 0R6 Canada

Tel: 613-842-0977 Fax: 613-842-0981

E-mail: info@ionalytics.com

www.ionalytics.com



Information contained in this publication is for reference purposes only. Every effort has been made to ensure that the information contained in this publication is complete and accurate; however, lonalytics assumes no responsibility for and will not be liable for any errors or omissions and shall have no liability for any costs, losses or damages of any kind whatsoever that might result from any use or reliance on the information contained herein.

This publication is not part of any agreement of purchase and sale related to the purchase of any lonalytics Selectra product and must not be relied upon by a purchaser of such product.

Information, descriptions and specifications in this publication are subject to change without notice.

Sales Offices:

ApplicationNOT

For more information about the lonalytics Selectra, please contact: lonalytics Corporation Building M50-IPF, 1200 Montreal Road Ottawa, Ontario K1A OR6, Canada Tel: 613-842-0977 Fax: 613-842-0981 E-mail: info@ionalytics.com www.ionalytics.com

WATERS CORPORATION 34 Maple St. Milford, MA 01757 U.S.A. T: 508 478 2000 F: 508 872 1990 www.waters.com





Waters, Micromass, Q-Tof, CapLC, Q-Tof Ultima and Masslynx are trademarks of Waters Corporation. Ionalytics and Selectra are trademarks of Ionalytics Corporation.

©2004 lonalytics Corporation All Rights Reserved Waters Corporation Printed in the U.S.A. 720000843EN May 2004 LW-PDF

