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Short communication

Capillary liquid chromatography–fast atom bombardment mass spectrometry using a high-resolving cation exchanger, based on a continuous chromatographic matrix Application to studies on neuropeptide peptidases

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Abstract

Hyphenated mass spectrometric techniques such as LC–MS are advantageous over standard MS methods, because they provide increased sensitivity and minimize signal suppression by other compounds present in the reaction mixture. Recently, we have introduced so-called continuous beds, and applied this technique to prepare a 0.32 mm I.D. cation-exchange capillary column, in order to separate the reaction product substance P(1–7) after proteolytic cleavage of substance P by an endopeptidase recovered from human cerebrospinal fluid. The use of a volatile buffer for elution provides very good flow stability. Ion-exchange microcolumns may be particularly useful for the separation of those peptides that co-elute in reversed-phase chromatography because the separation mechanisms of these two methods are different.

1. Introduction

Detailed characterization of proteolytic activity acting on neuropeptides requires, among other approaches, the isolation of the enzyme to apparent homogeneity and in-vitro analysis of the cleavage products. Present techniques, e.g. radioimmunoassay, are time-consuming and lack structural specificity. Moreover, certain peptide fragments, unknown a priori and released by enzyme action, may not be detected during analysis. Therefore, mass spectrometry (MS) and MS–MS, in combination with liquid chromatography, may provide a rapid and structure-specific identification of the released products [1].

It has been suggested that substance P endopeptidase (SPE), an enzyme present in cerebrospinal fluid, is involved in the generation of the N-terminal heptapeptide from substance P (SP). It has been shown that SP(1–7) decreases blood pressure, inhibits grooming behaviour and antagonizes SP-induced aversive behaviour in mice [2] and produces naloxone-reversible anal-

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gesia [3]. Further studies on SPE require improved procedures for the detection and identification of the peptide products generated by its action.

When complex mixtures of peptides are to be measured, it is worthwhile to separate the peptides (at least partially) on a capillary column before MS analysis. Recently, we have developed a novel type of stationary phase, the so-called continuous bed [1,4,5], which is advantageous over standard, silica-based material. It is also cheap and easy to prepare. Because the gel is covalently bound to the capillary wall, the columns do not require any frit and bleeding of the stationary phase is almost negligible, even at high pH. Fused-silica capillary columns were packed with a cation exchanger and used for on-line LC–MS separation of substance P fragments.

Recently, we have shown that reversed-phase capillary columns based on a continuous matrix can be applied successfully for on-line separation and mass spectral detection of peptide mixtures [1]. The aim of this paper is to test further the potential application of such columns in biomedical research, in particular in combination with mass spectrometry serving as a sensitive and structure-specific detection method.

2. Experimental

2.1. Chemicals

All peptides used in this study were purchased from Bachem (Bubendorf, Switzerland) and their purity was tested by reversed-phase (RP) HPLC and fast-atom bombardment mass spectrometry (FAB-MS). Fused-silica capillary tubing was from Alltech (Deerfield, IL, USA).

2.2. Enzymatic reaction

The SP-converting enzyme was purified to apparent homogeneity according to a method described previously [6]. The reaction was initiated by addition of 0.1 μg of SP(1–11) and the incubation volume was adjusted to 40 μl with 20 m*M* Tris-HCl buffer (pH 7.8). The reaction was quenched by rapid freezing and the mixture was stored for further analysis.

2.3. Capillary columns with continuous beds

The preparation of the cation-exchange columns (150 × 0.32 mm I.D.) has been described previously using acrylic acid as a ligand [7]. The column back-pressure did not exceed 1.7 MPa at flow-rates up to 3 μl/min.

2.4. Fast-atom bombardment mass spectrometry

The Finnigan MAT 95q (Finnigan MAT, Bremen, Germany), a double focusing mass spectrometer was used in this work [8]. In order to achieve maximal sensitivity, analysis was performed with fully opened entrance and exit slits (resolution around 500). A standard continuous-flow FAB interface equipped with a stainless-steel probe tip (Finnigan MAT) was used for the experiments [9].

2.5. Liquid chromatography–mass spectrometry

A scheme of the hyphenated system is presented in Fig. 1. The syringe pump (Model 140A, Applied Biosystems, Foster City, CA, USA) has been constructed such that a linear gradient of ammonium acetate buffer was delivered at a flow-rate of 3 μl/min without split-

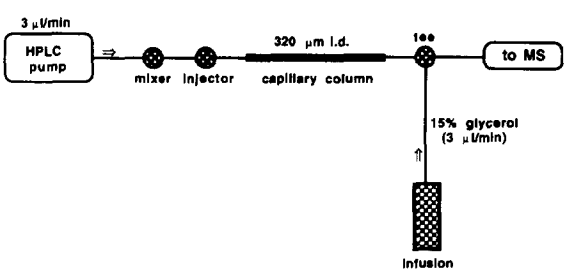

Fig. 1. Schematic diagram of the capillary LC–FAB-MS assembly. The chromatographic column was a cation-exchange continuous bed with an inside diameter of 0.32 mm.

ting. In order to avoid large dead volumes, the original gradient mixer was replaced with a zero-dead-volume mixing tee, mounted directly before the injector.

The volume of the sample loop (5 μl) was chosen such that it could accommodate dilute samples with a relatively large volume. The make-up solution (15% glycerol) was added to the post-column flow via a liquid junction made of a zero-dead-volume tee. The flow-rate of the matrix was maintained at 3 μl/min with the aid of a syringe infusion pump. A 50 μm I.D. fused-silica capillary was also used for all connections to the continuous-flow FAB interface.

The HPLC solvents were 50 m*M* ammonium acetate (pH 5.1) (solvent A) and 500 m*M* ammonium acetate (pH 5.1) (solvent B). Separations were performed using gradient elution starting with an isocratic run at 0%B for 5 min, followed by a linear increase of solvent B from 0 to 50% in 20 min and from 50 to 70% in 10 min. The chromatographic run was ended by elution of the column with another linear gradient from 70 to 0% B in 5 min. The flow-rate through the capillary was maintained at 3 μl/min.

3. Results

We used a capillary with a cation-exchange polymer to test whether such columns are suitable for on-line mass spectrometry and for the analysis of peptide products formed after proteolysis.

Selected-ion chromatograms are presented in Fig. 2, where substance P(1–7) was clearly separated from SP(1–11) on the ion-exchange capillary column. The selected-ion chromatogram taken at *m/z* 1364.0 indicates the presence of an additional signal, which originates from the ammonium adduct of the SP(1–11). This adduct was formed due to the presence of the ammonium acetate buffer used for the chromatographic separation. It is worth noting that only SP(1–11) and not the heptapeptide has an ammonium group attached to the molecule. Proba-

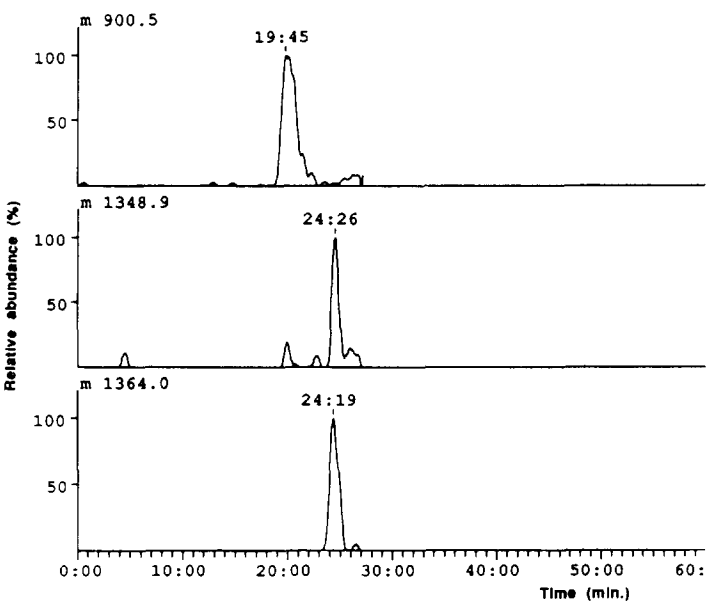

Fig. 2. Selected-ion chromatograms of substance P(1–7) at *m/z* 900.5 (expected mass: 900.5), substance P at *m/z* 1348.9 (expected mass: 1347.7) and substance P with an adducted ammonium group at *m/z* 1364.0 (expected mass: 1365.7). The peptides were separated by micro chromatography on a cation-exchange continuous bed.

bly the amidated C-terminal of SP(1–11) provides a site of stronger attachment than its fragment (1–7) lacking this modification. It is not very likely that SP(1–11) could undergo oxidation at the C-terminal methionine residue during an in-vitro experiment. Our previous experience suggests that incubation in other buffers (e.g. Tris-HCl) does not cause a shift in the molecular mass of SP. The partial mass spectra of both the substrate and a major product released after enzymatic conversion, are shown in Fig. 3.

4. Discussion

The aim of this work was to test the applicability of the recently developed continuous chromatographic beds to the on-line separation of neuropeptides in conjunction with mass spectrometry. For this purpose we used a cation-exchange microcolumn. To our knowledge, this is the first report on the use of this type of stationary phase for the analysis of neuropeptides by mass spectrometry.

A volatile buffer (ammonium acetate) was

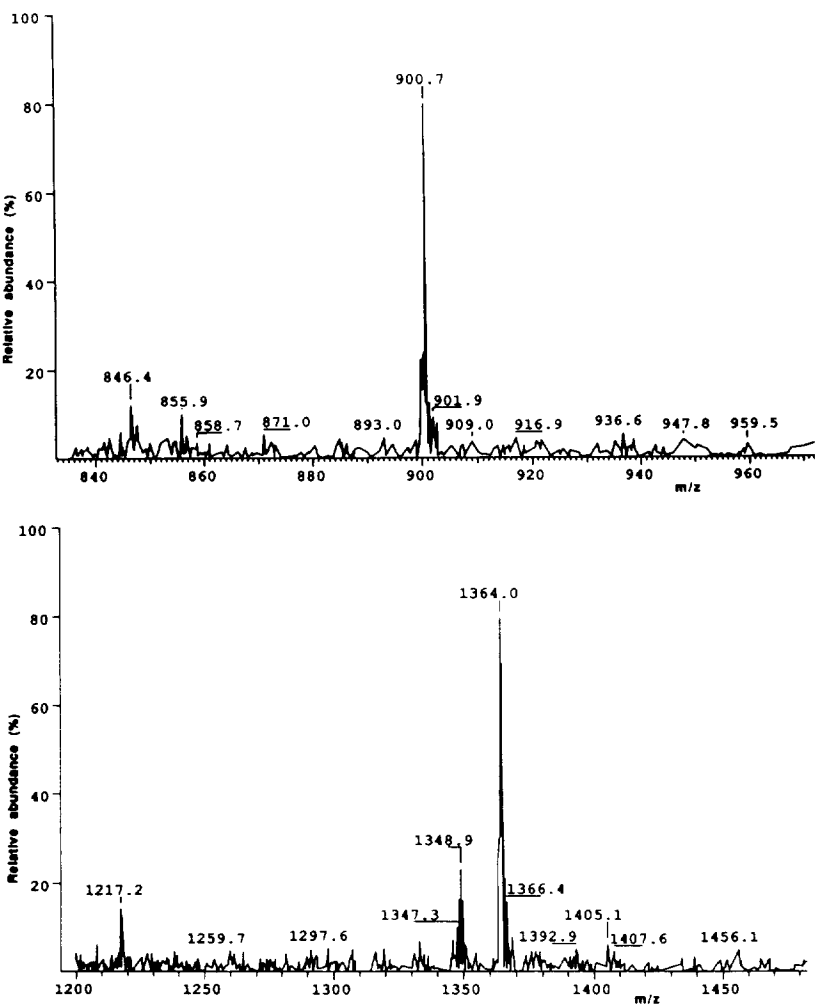

Fig. 3. Partial mass spectra of the separated peptides, taken in the positive-ion mode (magnetic scan). The absolute abundances of the peptides were: $1.9 \cdot 10^4$, $1.6 \cdot 10^3$ and $2.3 \cdot 10^4$ for SP(1–7), SP(1–11) and ammonium adduct SP(1–11), respectively.

used in order to avoid significant contamination of the ion source. Moreover, water-based buffers provide a more stable flow into the instrument compared to some organic solvents, e.g. acetonitrile or methanol. These solvents require the addition of a much higher concentration of glycerol to stabilize the flow, which can only be done at the expense of sensitivity. It is worth noting, that the liquid junction is much easier to handle than a coaxial system, and does not require fine adjustments of capillaries near the probe tip.

The sensitivity of the present system allows to detect substances at the picomolar level (nanograms), which in many cases is necessary for the identification of endogenous peptides and their fragments.

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