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# Nanospray mass spectrometry for identification of peptides. Application of a novel interface

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**Electrospray ionization mass spectrometry is a powerful tool for identification of biomolecules such as peptides, proteins, oligosaccharides and neurotransmitters. Recent development of the nanospray techniques, applied at ultralow flow-rates, allowed a sensitive analysis of compounds at femto/attomolar level. Here, we present application of a novel nanospray device for the analysis and fragmentation of peptides with high sensitivity on a sector instrument. The lowest applied flow-rate of the mobile phase was maintained at 50 nl/min with a sample load of 90 fmol. Nanospray also provided a complete analysis of 500 nl of the sample for over 10 min, including sequencing of as little as 40 pmol of a substance. Such analysis provides full structural information necessary to identify the molecules.**

One of the most challenging tasks in biochemistry and biotechnology is discovery and identification of endogenous molecules at sub-femtomolar level. Among various techniques, mass spectrometry [1] had an immense impact on biotechnology and drug development. Electrospray ionization mass spectrometry [2, 3] offers several advantages as compared to other analytical methods, such as: improved speed, structural information and simultaneous analysis of mixtures. In that respect, mass spectrometry, in combination with tandem mass spectrometry (MS/MS, CID), provides unambiguous identi-

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**Abbreviations:** CID, collision-induced dissociation; ESI-MS, electro-spray ionization mass spectrometry; FFFR, first field-free region; Fmoc, 9-fluorenylmethoxy-carbonyl; LCQ, ion trap mass spectrometer; MHC, major histocompatibility complex; MS/MS, tandem mass spectrometry.

fication of biomolecules. Recent years brought significant progress in ultrasensitive technology, thus making possible analysis of the content of single cell [4]. Data were also reported describing analysis of hemoglobin obtained from 10 red blood cells [5] and characterization of peptides bound to the major histocompatibility complex (MHC) molecules [6]. Here, we intended to apply a novel nanospray device, designed for the Finnigan's LCQ mass spectrometer, for sensitive sequencing of peptides at femtomolar level.

## MATERIALS AND METHODS

***Chemicals.*** Nociceptin/orphanin fragment [1–6] was synthesized using solid-phase Fmoc method as described previously [7]. Gramicidin S and Leucine-enkephalin (Leu-enk) were obtained from Sigma-Aldrich (Poznań, Poland). Purity of the peptides was tested by the reversed-phase HPLC and electrospray ionization mass spectrometry (ESI/MS). Solvents were of the HPLC-grade (Merck, Warszawa, Poland) and were used without additional filtration. All other reagents were of the research grade and came from various commercial sources.

***Mass spectrometry.*** A Finnigan MAT 95S (Finnigan MAT, Bremen, Germany), double-focusing instrument with reversed geometry (B, E; where B = magnetic field, and E = electric field) was applied. The instrument was equipped with an electrospray source (ESI) which was tuned using gramicidin S at a concentration of 4 pmol/μl. Basic operation of the system was described in our previous papers [8, 9].

***Tandem mass spectrometry (MS/MS).*** High-energy collision-induced dissociations (CID) at constant B/E ratio, were performed in the collision cell located in the 1-st field-free region of the instrument (FFFR). Helium was used as a collision gas and intensity of the parent ion was adjusted to approximately 30% of its initial abundance.

Nomenclature of Roepstorff & Fohlman [10] was applied for fragments assignment.

***Nanospray interface.*** The PicoTip adapter LTQ-ADP (New Objective, Inc., Cambridge, MA, U.S.A.) was mounted in the electrospray flange according to the manufacturer's recommendations. Briefly, the standard ESI source head was removed and a mounting block for the nanospray was attached. All connections were made with Microtight HPLC fittings (Upchurch Scientific, Oak Harbor, WA, U.S.A.). Transfer line between injector and an interface was prepared from a 20 cm long fused-silica capillary (50 μm I.D., 150 μm O.D.). A gold-coated tip (2.5 cm long PicoTip) made of a fused-silica was mounted at the front of the device. The tip was tapered at the top to a diameter of 8 μm (I.D.). The distance between the top of the tip and a heated capillary was about 5 mm which allowed to obtain a stable spray. The set-up scheme of the system is presented in Fig. 1.

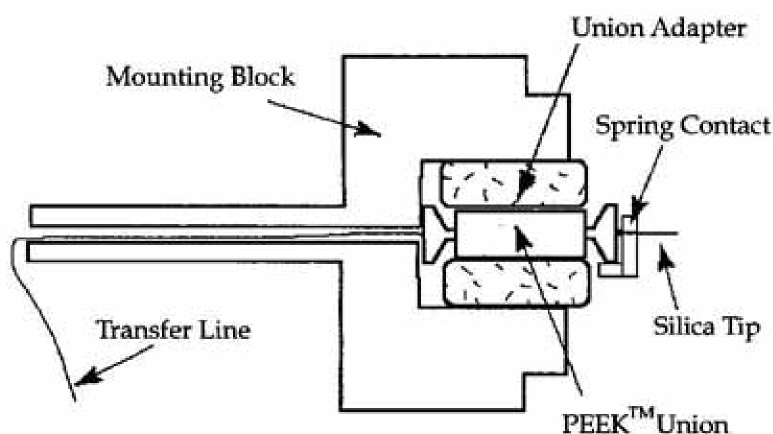


**Figure 1. Schematic diagram of the nanospray set-up installed on the Finnigan MAT 95S sector instrument.**

(Obtained with permission from Dr. G. Valaskovic, New Objectives, Inc.).

Mobile phase consisted of 30% methanol, supplemented with 0.1% formic acid and was provided by a syringe pump 22 (Harvard Apparatus, South Natick, MA, U.S.A.). Flow-rate was maintained at 50-150 nl/min. Electrical adjustments for the nanospray optimization were similar to those used for standard electrospray analysis. The only exception was the value of the high voltage applied to the gold-coated tip, which was decreased to 1.0-1.2 kV as compared to 2.5 kV for standard settings. Under such conditions a stable spray was maintained for the entire working day (about 10 h). The tip was occasionally inspected under a light microscope to examine the metal coating and possible clogging.

Samples, dissolved in the mobile phase, were injected *via* a Valco four-port injector with a 500 nl internal loop. Theoretically, such sample volume should provide a signal lasting at least 10 min at a flow-rate of 50 nl/min, which is sufficient for complete analysis including determination of the molecular ion, and for peptide sequencing. In fact, the signal lasted for a longer time due to the slight adsorption of the sample on the capillary walls (tailing effect).

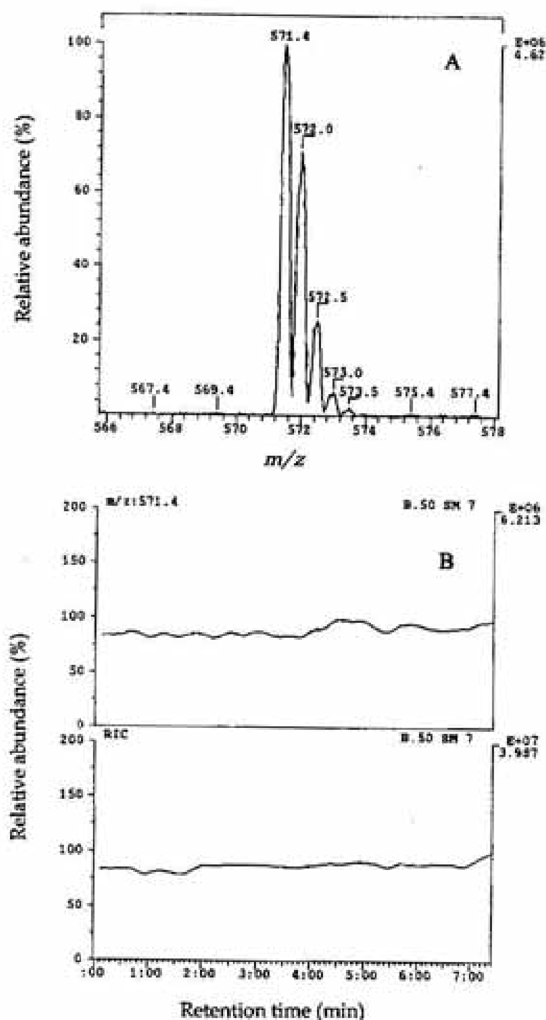


**Figure 2. (A) Partial electrospray mass spectrum of gramicidin S, used for optimization and tuning of the nanospray interface at a flow-rate of 50 nl/min.**

The distance between particular ions indicates the presence of a doubly-charged signal; (B) Signal stability of gramicidin S after optimization of the source. Selected Ion Current at *m/z* of 571.4 belongs to gramicidin S (top panel) and a Total Ion Current (bottom panel).

## RESULTS

Optimization of the nanospray source was performed with gramicidin S. Partial mass spectrum of this cyclic peptide solution (4 pmol/$\mu$l) that was pumped at a flow-rate of 50 nl/min, is shown in Fig. 2A. Isotopic distribution of the doubly-charged ion of gramicidin S at *m/z* of 571.4 is clearly visible, due to the higher resolution given by the sector instrument as compared to the ion-trap or quadrupole detectors. The distance between particular ions (Fig. 2A) gives also a clue as to the molecular mass of the analyzed compound. In this case, the average distance is equal to 0.5 mass unit, thus additionally verifying that this signal is that of a doubly-charged ion.

Stable spray is crucial for performing a reliable analysis. Therefore, we investigated the relationship between intensity of the ion current and applied high voltage. The optimal setting was dependent on the flow-rate of the mobile phase and on the internal diameter of the capillary tip, and was set experimentally.

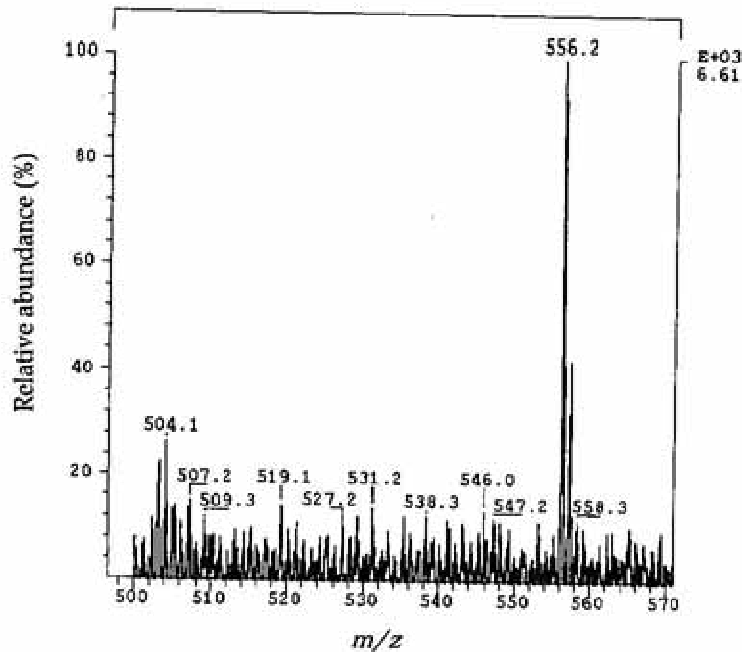


**Figure 3. Nanospray spectrum of Leu-enkephalin after injection of 90 fmol of peptide.**

Data represent a single scan lasting 5 s which corresponds to 750 amol of the sample applied.

Stability of the system operating at 50 nl/min is presented in Fig. 2B where the Total Ion Current (bottom panel) and the Selected Ion Current (top panel) were recorded. This parameter did not vary by more than ±10% for at least 1 h.

Leucine-enkephalin was applied to test sensitivity of the nanospray system. The results are presented in Fig. 3 where 90 fmol of this peptide was injected into the instrument and the signal lasted for over 10 min in the instrument. The narrow scanning range was applied in order to increase sensitivity of measurements. The presented spectrum represents a single scan (lasting 5 s) which corresponds to 750 amol of the sample applied.

Peptide sequencing was demonstrated using nociceptin/orphanin FQ fragment [1-6]. This newly discovered peptide is involved in pain transmission and shows sequence similarity to dynorphins. The amount of peptide used for this experiment was 80 pmol and the flow-rate was set at 150 nl/min. The general fragmentation scheme for peptides (Fig. 4) was assigned according to the nomenclature given by Roepstorff & Fohlman [10]. Figure 5 shows the electrospray tandem mass spectrum (daughter scan) of the parent ion at *m/z* of 584.6, corresponding to the molecular ion of nociceptin [1-6]. All identified fragments are labeled along the spectrum. Additionally, the fragment ions are collected in Table 1. As similar experiment was performed using a lower amount of the sample, 17 pmol, and the obtained information, as shown in Fig. 6, was still sufficient to reveal the sequence of the analyzed peptide.

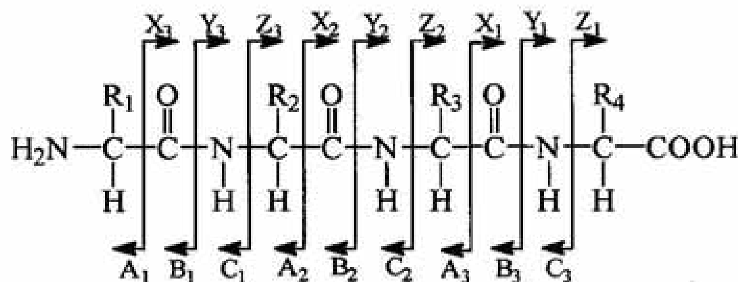


**Figure 4. Peptide fragmentation nomenclature, according to Roepstorff & Fohlman [10].**

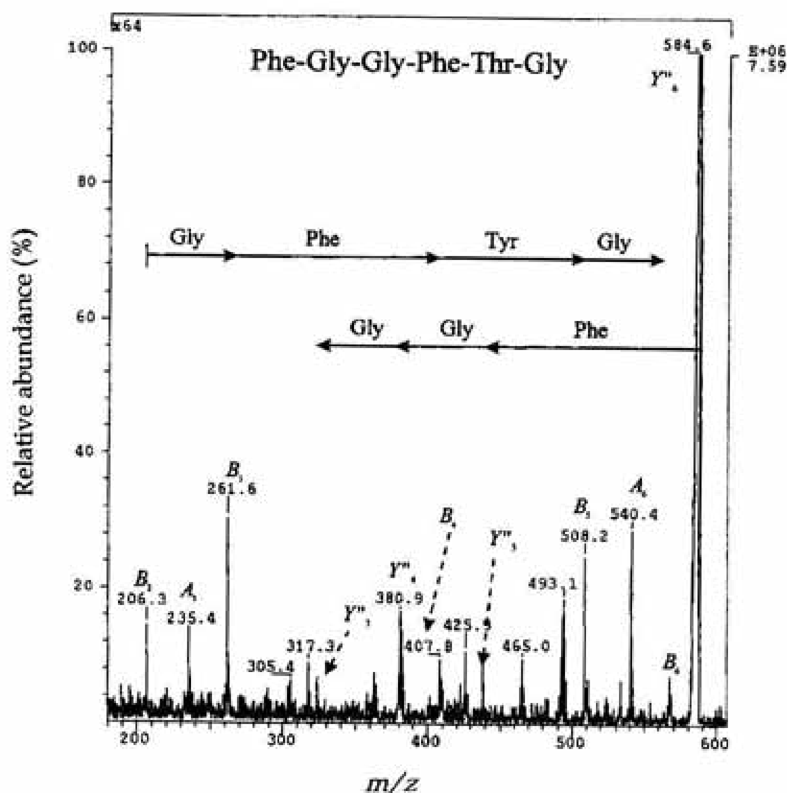


**Figure 5. Tandem mass spectrometry of 80 pmol of nociceptin [1–6].**

Experiment was performed in the 1-st field-free region of the instrument (constant B/E). Flow-rate was maintained at 150 nl/min. Fragment ions are assigned along the spectrum.

## DISCUSSION

A novel nanospray interface, designed for the LCQ ion-trap mass spectrometer, was successfully operated on the sector instrument. Application of this nanospray device has not been published yet, either connected to the LCQ instrument, or mounted to the magnetic spectrometers. Therefore, the aim of this paper was to test the novel device and to apply it for sensitive analysis of a peptide. Decreased flow-rate between 50–150 nl/min provided more efficient ionization of the analyte, thus assuring higher sensitivity of analysis [11, 12]. In addition, the long-lasting signal in the instrument makes possible sequencing at femtomolar level. In this respect, such a device is ideal for identification of molecules of endogenous origin [6, 11]. Standard electrospray interfaces require addition of the make-up flow to stabilize the electrospray signal. This, in turn, significantly decreases sensitivity of the measurements due to sample dilution in the source. In this paper we demonstrated that analysis of as little as 90 fmol of the peptide at a signal-to-noise ratio better than 1:5 is possible. Such improved sensitivity and applied ultralow flow-rates make the nanospray device compatible with capillary zone electrophoresis and capillary liquid chromatography. Moreover, application of the lower flow-rates also provides a much more efficient ionization of the sample which, in turn, leads to improved sensitivity [13]. Thus, nanospray devices become a powerful tool for identification of biomolecules, and the amino acid sequence data can be compared with the databases available on the Internet for complete identification of protein sequences [14].

The designed nanospray interface is easy to use. Care should be taken to avoid non-filtered solutions which may cause clogging of the capillary tip. We have found, that operation of this device mounted on the sector instrument is more flexible, because of the X-Y manipulator mounted on the electrospray flange. This manipulator, which is designed by the manufacturer (i.e. Finnigan), allows fine adjustment of the spray, thus contributing to the increased sensitivity of analysis.

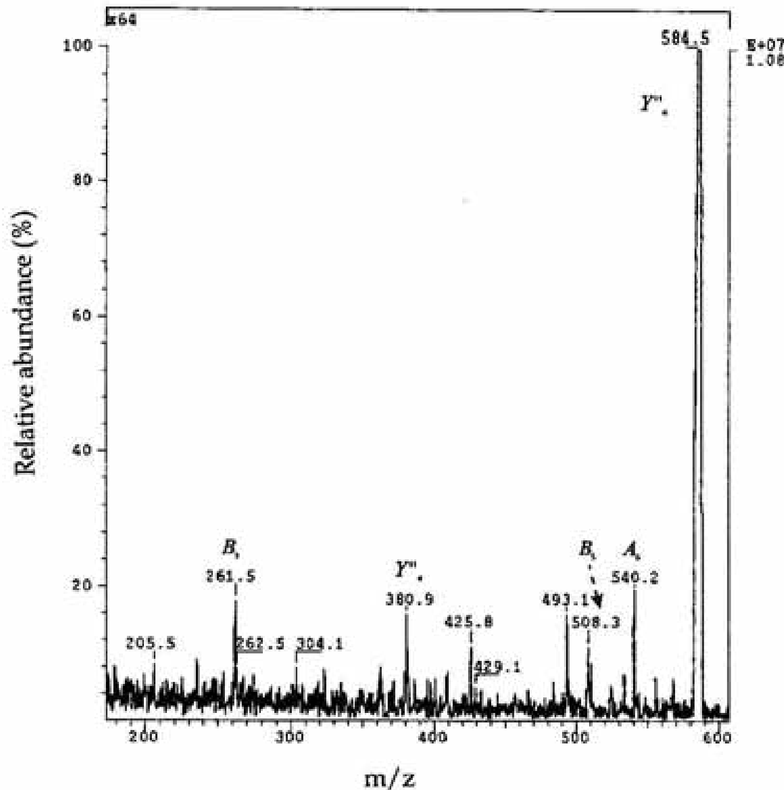


**Figure 6. Tandem mass spectrum of 17 pmol of nociceptin [1–6].**

Experimental details were identical to those described in the legend to Fig. 3.

**Table 1. Major MS/MS fragments of nociceptin [1–6].**

Values (*m/z*) marked in bold represent sequence ions detected along the electrospray mass spectrum (data taken from Fig. 5). For details concerning fragmentation nomenclature see Fig. 4.

| No. | Seq | A | B | Y'' | No. |
|---|---|---|---|---|---|
| 1 | Phe | 120.1 | 148.1 | **585.3** | 6 |
| 2 | Gly | 177.1 | **205.1** | **438.2** | 5 |
| 3 | Gly | **234.1** | **262.1** | **381.2** | 4 |
| 4 | Phe | 381.2 | **409.2** | **324.2** | 3 |
| 5 | Thr | 482.2 | **510.2** | 177.1 | 2 |
| 6 | Gly | **539.3** | **567.3** | 76.0 | 1 |

The distance between the sprayer tip and the heated capillary is not very critical, but should be kept between 5–7 mm. The important feature of the Pico Tip adapter is its approximately 10-fold lower price, as compared to the interfaces produced by the manufacturers of mass spectrometers. Moreover, even less experienced users can quickly take advantage of the ready-made adapter, without making efforts at designing their own constructions.

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