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Journal of Chromatography A, 777 (1997) 41–45

JOURNAL OF CHROMATOGRAPHY A

Short communication

Rapid analysis of endogenous LVV-hemorphin-7 in cerebrospinal fluid by size-exclusion chromatography and electrospray ionization mass spectrometry

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Abstract

LVV-hemorphin-7 is a peptide with opioid-like activity which is recognized by μ and σ receptors. The sequence, derived from β-, γ-, δ- or ε- chains of human hemoglobin has been found in cerebrospinal fluid (CSF) of patients suffering from cerebrovascular bleedings, but not in CSF from healthy individuals. Procedures for isolation and identification of neuropeptides in body fluids and tissue extracts are often laborious and time-consuming. Additionally, extraction yield tends to be low after several chromatographic steps. In this study, we developed a rapid technique to analyze LVV-hemorphin-7 in CSF fluid from a patient with cerebrovascular bleedings using a combination of size-exclusion chromatography and electrospray ionization mass spectrometry. The methodology described utilizes small quantities of CSF (0.3–0.5 ml) and can be completed within a few hours. The proposed strategy opens possibilities for direct measurement of endogenous peptides in small volumes of body fluids. © 1997 Elsevier Science B.V.

Keywords: LVV-Hemorphin-7; Peptides; Hemorphins

1. Introduction

Recent studies have shown the presence of several new neuropeptides possessing opioid-like activity and containing sequences which are not related to any of the three classical opioid peptide precursors, proopiomelanocortin (POMC), proenkephalin and prodynorphin. This group includes β-casomorphins [1] released from β-casein, cytochropins [2] derived from cytochrome *b* and hemorphins [3] identical to the sequence 32–41 of the β-chain of human hemoglobin. The sequence of LVV-hemorphin-7 (Leu–Val–Val–Tyr–Pro–Trp–Thr–Gln–Arg–Phe) has been identified in cerebrospinal fluid (CSF) from patients with cerebrovascular bleedings [4] and its structure was verified by fast atom bombardment mass spectrometry (FAB-MS) [5]. The previously developed isolation procedure including several chromatographic steps, is laborious and the recovery of endogenous material was relatively low. Here, we present another approach which involves rapid pre-separation of the low-molecular-mass CSF compo-

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PII S0021-9673(97)00277-X

nents followed by direct analysis by electrospray ionization mass spectrometry (ESI-MS). This technique may be further applied for the analysis of other components of body fluids [6,7].

2. Materials and methods

Ventricular CSF from a patient with intra-cerebral bleeding was obtained from the Department of Neurosurgery, Karolinska Hospital (Stockholm, Sweden). The CSF was divided into 0.5-ml aliquots and stored frozen at −80°C until assayed [5]. Solvents used for chromatography and mass spectrometry were of HPLC-grade. Other chemicals were of the research-grade and came from Sigma–Aldrich Sweden (Stockholm, Sweden).

2.1. Ultrafiltration of cerebrospinal fluid

A 300-μl aliquot of human CSF was acidified with 100 μl of 0.1 *M* formic acid and filtered through the Ultrafree-MC filter units (Millipore, Bedford, MA) with an M_r 5000 cut-off membrane, according to the manufacturer's recommendations. The filtrate was concentrated in a vacuum centrifuge and redissolved in 75 μl of 30% acetonitrile (ACN) in water, supplemented with 0.1% trifluoroacetic acid (TFA).

2.2. Chromatographic procedure

The size-exclusion column Superdex Peptide PC, 300×3.2 mm (Pharmacia Biotech, Uppsala Sweden), capable of separating molecules of M_r 100–7000, was installed in the SMART system optimized for micropurifications (Pharmacia Biotech). Fifty microliters of the concentrated low-molecular-mass CSF material were injected directly into the Superdex Peptide column. Elution was performed isocratically with 30% MeCN in water supplemented with 0.1% TFA at a flow-rate of 100 μl min^{-1} [8]. One-minute fractions were collected and stored frozen at −80°C.

2.3. Mass spectrometry

A Finnigan MAT 95Q (Finnigan MAT, Bremen, Germany) hybrid double-focusing B-E-Q instrument was used in this study. The mass spectrometer was equipped with an ESI source. Mobile phase consisted of 30% MeCN in water with 0.1% formic acid and entered the ESI interface at a flow-rate of 30 μl min^{-1}. Nitrogen was applied as a sheath gas at a pressure of ~3.5 bar and the high voltage of the sprayer was adjusted to 2.7 kV. Samples (5 μl of each fraction) were introduced via a Valco injector and analyzed in a positive-ion mode at a full acceleration voltage of 5 kV as described previously [8]. Calculations of the molecular masses was performed with the PEPMATCH software, supplied by the manufacturer.

3. Results

Low-molecular-mass components present in human CSF, filtered through a M_r 5000 membrane were applied on the size-exclusion Superdex Peptide column. Components having a molecular mass below

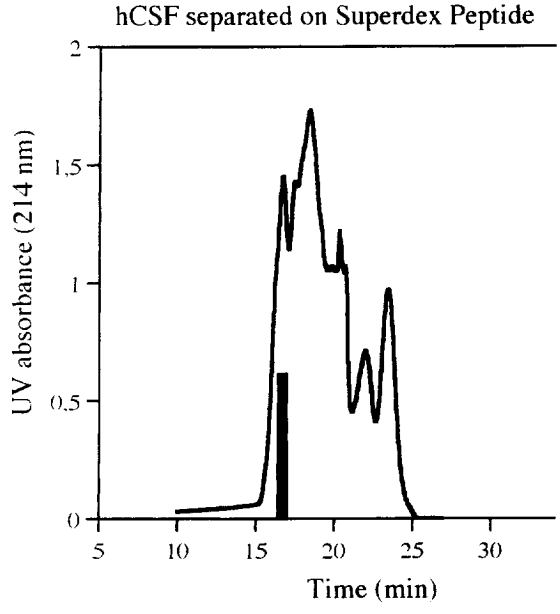

Fig. 1. Size-exclusion chromatography of the low-molecular-mass fraction of human CSF (hCSF) from patient with intra-cerebral bleeding. CSF sample was filtered through the M_r 5000 PVDF membrane filter, concentrated to 75 μl and injected on the Superdex Peptide column (300×3.2 mm) connected to the SMART system. Elution was performed with 30% aqueous acetonitrile with 0.1% TFA at a flow-rate of 100 μl min^{-1}. Fraction containing LVV-hemorphin-7 is denoted by a bar.

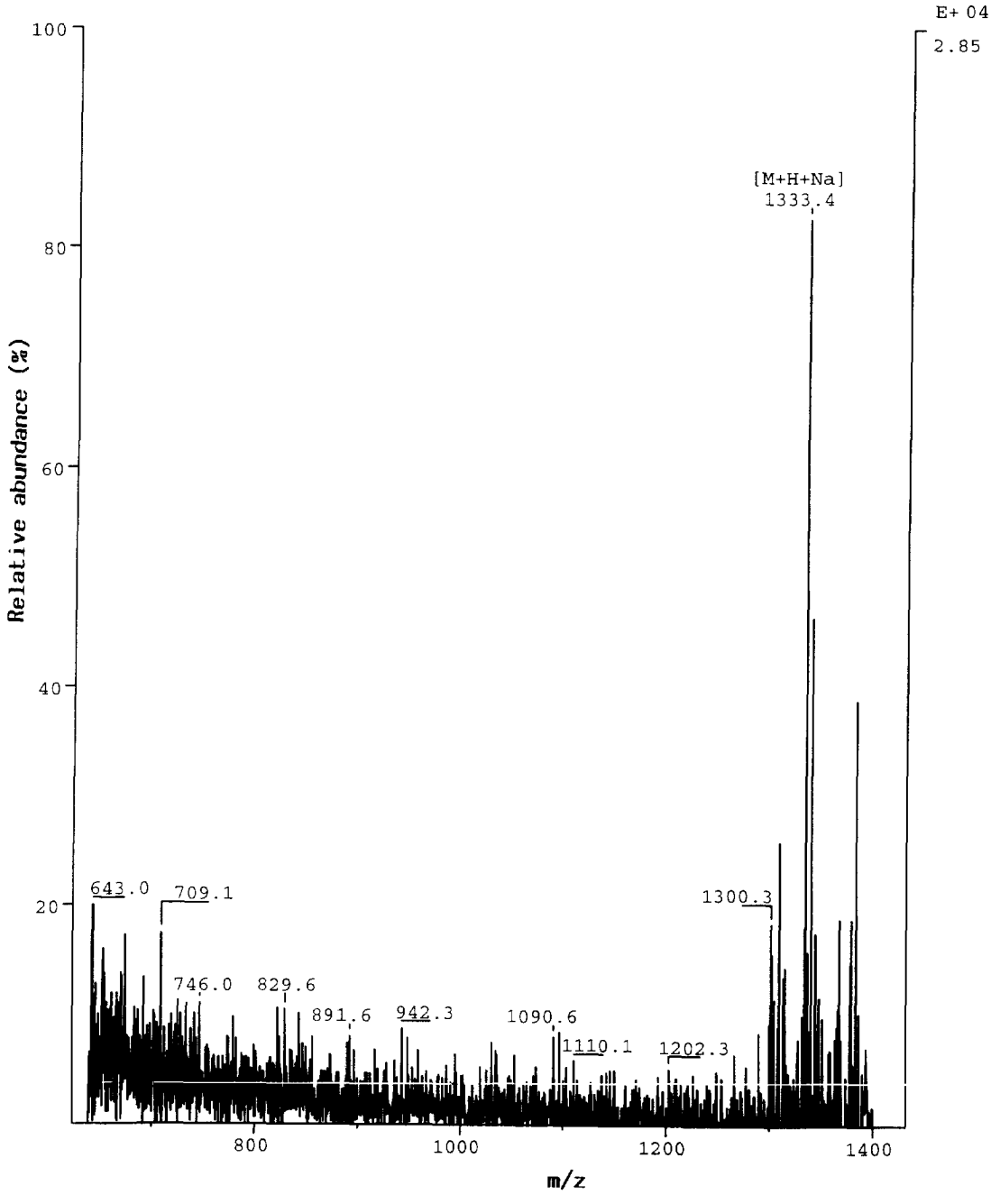

Fig. 2. Analysis of the fraction (No. 16) separated on the Superdex Peptide size exclusion column and containing LVV-hemorphin-7. Partial electrospray mass spectrum taken in a positive-ion mode showing the major peak at m/z of 1333.4 corresponding to the Na adduct of the decapeptide.

5000 were pre-separated into several fractions as shown in Fig. 1. Each fraction (between 15 and 25) was directly analyzed by ESI-MS. The void volume of the column was 1.1 ml, as calibrated with various peptide standards [8,9]. Thus, the decapeptide was expected to elute in a volume of 1.6 ml. Fig. 2 shows the partial mass spectrum with a peak at *m/z* of 1333.4, corresponding to the Na adduct ion of LVV-hemorphin-7 and eluted in fraction 16 (1.6 ml) as indicated with a bar in Fig. 1. An ion at *m/z* 1309 belonging to the protonated molecule is also observed. Deconvolution of the spectrum in Fig. 3 reveals the molecular mass 1332.5 which corresponds to the calculated mass of Na adduct of the decapeptide.

4. Discussion

We applied ESI-MS spectrometry in combination with a chromatographic method for rapid tracing of the hemoglobin fragment LVV-hemorphin-7, naturally occurring in the CSF from individuals with cerebrovascular bleeding but which is not detectable in CSF from healthy persons [4]. The peptide was rapidly isolated from the contaminating high-molecu-

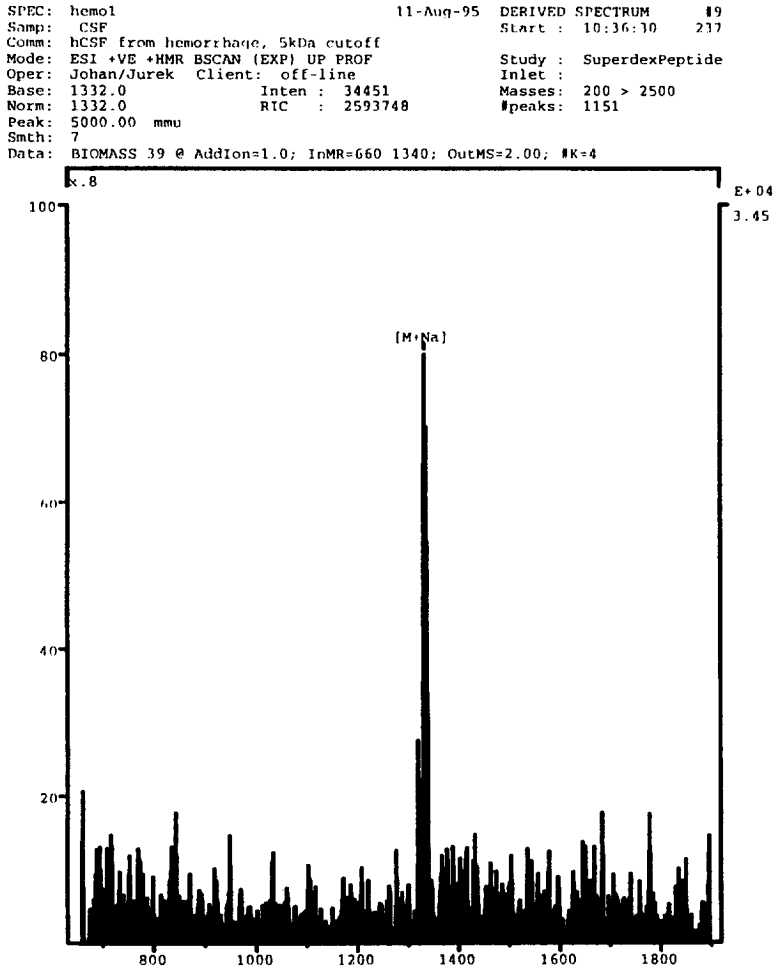

Fig. 3. Deconvoluted partial mass spectrum of the content of fraction No. 16 (Fig. 1). The calculated mass corresponds to the calculated mass of LVV-hemorphin-7.

lar-mass components and salts by ultrafiltration and further separation on the novel size-exclusion HPLC column connected to the SMART system [8,9]. Molecular information obtained by mass spectrometry corresponds to the calculated mass of LVV-hemorphin-7. The data obtained here were previously confirmed by Edman degradation [4] and by sequencing using FAB-MS [5,10]. The decapeptide was found to be very abundant in the CSF from patient suffering from cerebrovascular bleeding [4] and its content in this body fluid was estimated at 115–300 pmol ml^{-1} using specific radioimmunoassay. Thus, small (300 μl) aliquots of the fluid used in this study contain between 35–90 pmol of endogenous material, a quantity easily accomplished by modern electrospray interfaces [11,12]. It is worth noting that the major component of the LVV-hemorphin-7 detected by the ESI-MS consisted of a sodium adduct. Attraction of the Na was, however, expected, because the size exclusion technique is unable to deplete molecules with metal ions.

Separation of the CSF components was achieved using a novel size-exclusion column connected to the SMART micropurification system. The advantages of this approach were discussed in our previous papers [8,9]. The technique supplied additional verification of the molecular mass after spectra deconvolution because complex composition of the biological samples may sometimes obscure interpretation of the electrospray data, resulting in false assignment of the molecular mass. Moreover, separation efficiency and, thus, sensitivity of the method has been significantly improved to greater than 85% while the preparation time was reduced from about 1 week to 4 h. Peptide fractions were separated at much better resolution compared to low-pressure chromatography systems. Using the modified procedure we were able to trace the naturally occurring peptide in the body fluid in an efficient manner which, in future, also opens the possibility for quantitative studies of endogenous peptides using minute amounts of precious biological material.

Acknowledgments

This work was supported by the Swedish Alcohol Research Fund, the KBN grant 1147/IA/158/95 and the Swedish Medical Research Council (Grant 9459).

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