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Reversed-phase high-performance liquid chromatography for the determination of haemorphin-like immunoreactivity in human cerebrospinal fluid

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Abstract

The haemorphins are opioid peptides derived form the blood protein haemoglobin. This study was focused on the detection and determination of haemorphin-like immunoreactivity in human cerebrospinal fluid (CSF) by reversed-phase HPLC. For this purpose a SMART System, optimized for micropurification, was applied. Prior to application to HPLC, the peptide fraction of the CSF sample was extracted using a reversed-phase silica gel cartridge (Sep-Pak C_{18}). In the HPLC separation, the peptide-like material associated with haemorphin-7 immunoreactivity was recovered and determined using a UV detector. The tryptophan residue present in the haemorphin sequence allowed UV detection at wavelengths (e.g., 276 nm) where interference with other co-eluting peptides lacking this residue is minimized. Recorded levels of haemorphin-like immunoreactivity were compared with those detected by radioimmunoassay.

1. Introduction

The haemorphins are recently identified peptides with opioid activity, which are enzymatically released from the blood protein haemoglobin [1,2]. In contrast to the "classical" endorphins (e.g., the enkephalins), the haemorphins exhibit a relatively high metabolic stability [3], probably owing to the proline residue next to the N-terminal tyrosine (Table 1). The haemorphins also differ from the enkephalins with regard to their receptor activation profile. Whereas the enkephalins preferentially bind to delta-opioid receptors, the haemorphins are selective for both delta- and mu-receptors [4,5]. In addition, the haemorphins also have affinity for sigma-receptors (2).

An opioid active decapeptide (LVV-haemorphin-7), identical with the sequence 32–41 of the β-chain of haemoglobin, was previously isolated from human ventricular cerebrospinal fluid (CSF) [6,7]. In a recent study, a shorter active fragment of this peptide, haemorphin-7 (Tyr–Pro–Trp–Thr–Gln–Arg–Phe), was tentatively identified in human plasma [8]. Haemorphin-7 was found to increase in the circulation following long-distance running. This increase was paralleled by elevated levels of circulating β-endorphin-

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Table 1
Structures of the haemorphins and related peptides

Compound	Structure
Haemorphin-4	Tyr–Pro–Trp–Thr
Haemorphin-7	Tyr–Pro–Trp–Thr–Gln–Arg–Phe
LVV-haemorphin-4	Leu–Val–Val–Tyr–Pro–Trp–Thr
LVV-haemorphin-7	Leu–Val–Val–Tyr–Pro–Trp–Thr–Gln–Arg–Phe
Leu-enkephalin	Tyr–Gly–Gly–Phe–Leu
Met-enkephalin	Tyr–Gly–Gly–Phe–Met

like immunoreactivity [8]. In animal experiments, the haemorphins were shown to lower the blood pressure, probably through a mechanism where the activity of angiotensin-converting enzyme is inhibited [3].

To explore further the physiological relevance of the haemorphins, the use of highly efficient techniques for their detection and determination is essential. In this study, we applied a new chromatographic system, the SMART System, to detect and determine these peptides in cerebrospinal fluid (CSF). Detected levels of haemorphin-like peptide material in CSF collected from patients with cerebrovascular bleeding were compared with those in control samples. Levels calculated from the HPLC results were compared with those recorded by radioimmunoassay (RIA).

2. Experimental

2.1. Peptides and chemicals

Synthetic haemorphins were prepared by Dr. G. Lindeberg (Department of Medical Immunology, Uppsala University, Sweden), except for haemorphin-4, which was a gift from Dr. V. Brantl (Schlingen, Germany). Prior to use, the synthetic peptides were purified by HPLC [8]. Sep-Pak C_{18} silica gel cartridges were purchased from Waters (Milford, MA, USA). All other chemicals and solvents were of analytical-reagent grade from commercial sources.

2.2. Cerebrospinal fluid material

Ventricular CSF from patients with cerebrovascular bleeding was obtained from the Department of Neurosurgery, Karolinska Hospital (Stockholm, Sweden) and lumbar CSF from controls without cerebral disease was obtained from the Department of Neurology, Umeå University (Umeå, Sweden). Prior to analysis, the CSF samples were stored at −70°C.

2.3. Sample preparation

CSF samples (10 ml) were thawed (the samples were kept in a cold room at 5°C) and eluted through Sep-Pak C_{18} cartridges for reversed-phase (RP) extraction. Prior to sample application, the cartridge was successively washed with 5 ml of methanol, 5 ml of methanol containing 0.04% trifluoroacetic acid (TFA) and finally with 10 ml of distilled water containing 0.04% TFA. Following sample application, the cartridge was washed with 4 ml of distilled water and subsequently with 4 ml of 20% methanol before elution with 4 ml of 60% methanol (all solutions containing 0.04% TFA). The eluate obtained at a methanol concentration of 60% was evaporated in a vacuum concentrator (Savant Vac, Hicksville, NY, USA) and collected for further analysis by HPLC or radioimmunoassay. In calibration elutions, synthetic haemorphins were added to control CSF and subjected to the same extraction procedure.

2.4. Chromatography

RP-HPLC was performed using a SMART System (Pharmacia–LKB Biotechnology, Uppsala, Sweden). The computer-controlled system was equipped with a micro-precision pump, SepUnit, micro-fraction collector and built-in detector cells for UV (μPeak Monitor with variable-wavelength monitor) and conductivity measurements. The conductivity scale was set by calibration with eluent A (100%) and eluent B (0%): A, 0.14% TFA; B, 0.13% TFA in 60% acetonitrile. The column used was a μRPC C_2/C_{18} SC 2.1/10 (particle size 3 μm, 120 Å) (100 mm × 2.1 mm I.D.). Elution was carried out with a gradient of acetonitrile (0–60% over 40 min) maintaining a flow-rate of 100 μl/min. Prior to injection, the sample (100 μl) was centrifuged at 10 000 *g* for 1 min. Collected fractions (100 μl) were dried in a Savant Vac concentrator and assayed by RIA.

2.5. Radioimmunoassay

The RIA for haemorphin-7 was based on the charcoal adsorption technique and was conducted as described in a previous paper [8]. Briefly, antibodies were raised in rabbits and the iodinated petide was used as a tracer. Cross-reaction in the RIA and LVV-haemorphin-7 was 100% and for LVV-haemorphin-6 and shorter fragments of the peptide it was less than 1%. The detection limit of the RIA was 4 fmol per tube.

3. Results

The SMART System used in this study for RP-HPLC, was optimized for the separation of various haemorphin structures. As shown in Fig. 1, the technique appeared to be highly efficient for resolving the synthetic haemorphin-4 and haemorphin-7 and also their N-terminal elongations LVV-haemorphin-4 and LVV-haemorphin-7, respectively.

Prior to HPLC of haemorphin-like peptide material in CSF, the samples were extracted on Sep-Pak C_{18} cartridges. In calibration elutions the synthetic peptides were added at different concentrations (0.01, 0.05, 0.1 and 0.2 μg/ml) to a control CSF sample (10 ml) containing no or negligible amounts of the native peptides. By this approach, it was possible to determine the recovery of the extraction procedure. Table 2 summarizes data obtained from these recovery studies. It was found that on adding synthetic peptides in concentrations of 0.1 μg/ml (total amount 1 μg), their recovery following HPLC ranged from 44 to 76%. The recovery seems relatively low, but it is known from several

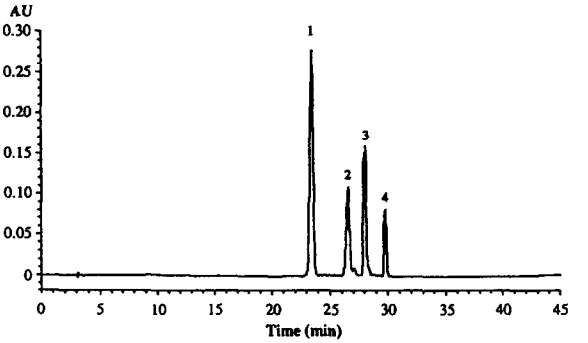

Fig. 1. RP-HPLC using the SMART System of synthetic haemorphins. Elution was carried out using a linear gradient of acetonitrile (0–60% over 40 min) at a flow-rate of 100 μl/min. The UV absorbance was recorded at 276 nm. For further details, see text. Peaks: 1 = haemorphin-4 (1 μg); 2 = haemorphin-7 (1 μg); 3 = LVV-haemorphin-4 (4 μg); 4 = LVV-haemorphin 7 (1 μg).

Table 2
Recovery of synthetic haemorphins added to control CSF (1 μg in 10 ml) following Sep-Pak C_{18} extraction and RP-HPLC

Peptide	Recovery (%)[a]
Haemorphin-4	44
Haemorphin-7	56
LVV-haemorphin-4	52
LVV-haemorphin-7	76

[a] Values are means from three separate experiments.

studies that losses of hydrophobic peptides often occur during Sep-Pak extraction [9].

In studies of CSF samples from patients with cerebrovascular bleedings, the content of haemorphin-like immunoreactivity varied depending on the individual diagnosis. A patient with a subdural haematoma exhibited comparatively higher levels of peptides co-eluting with haemorphin-4, haemorphin-7 and LVV-haemorphin-4 (Fig. 2), whereas one patient with subarachnoid bleeding only showed a modest increase in UV-absorbing material co-eluting with LVV-haemorphin-7 (Fig. 3). This latter UV peak or shoulder was also found to be associated with LVV-haemorphin-7-like immunoreactivity, although its peak was seen at a retention time of 29.6 min instead of the 29.8 min found for the synthetic LVV-haemorphin-7. However, repetitive chromatography showed that these divergences were within the precision of the instrument. Analysis of samples from other patients with identical diagnosis gave similar HPLC patterns to those shown in Figs. 2 and 3 (data not shown). In control patients, the levels of haemorphins were very low, although detectable at least in one patient (Fig. 4). In both Figs. 3 and 4 it was possible to observe UV-absorbing peaks associated with haemorphin-like material only when the chromatograms were expanded.

The level of the separated haemorphin-like

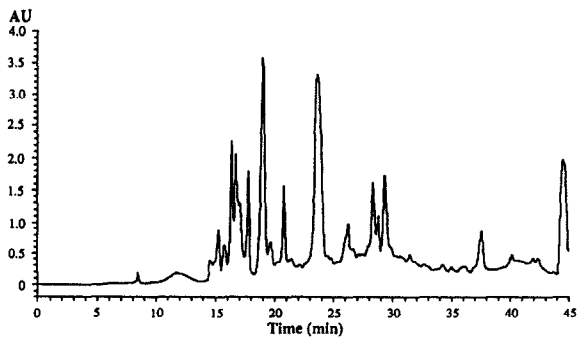

Fig. 2. RP-HPLC of a concentrated CSF sample (10 ml) from a patient with a subdural haematoma. Separation conditions as in Fig. 1. The UV absorbtion at 276 nm was recorded and 100-μl fractions were collected and dried for RIA. Haemorphin-7-like immunoreactivity and LVV-haemorphin-7-like immunoreactivity were eluted in fractions 27 and 30, respectively. For further details, see text.

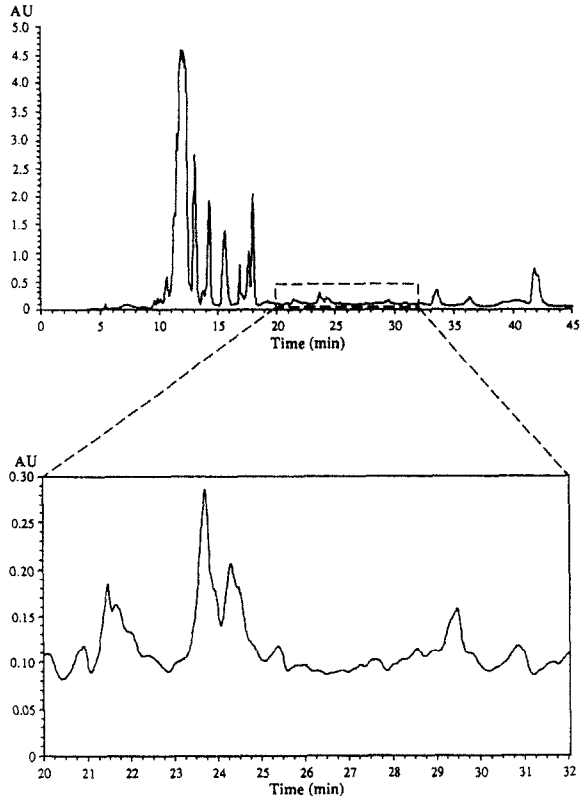

Fig. 3. RP-HPLC using the SMART System of a concentrated CSF sample (10 ml) from a patient with subarachnoid bleeding. Conditions as in Figs. 1 and 2. Fractions of 100 μl were collected and dried before testing by RIA. Haemorphin-7-like immunoreactivity and LVV-haemorphin-7-like immunoreactivity were eluted in fractions 27 and 30, respectively. At the bottom, part of the chromatogram is expanded to shown peaks in the area were the synthetic haemorphins were observed to elute.

CSF components (Figs. 2–4) was determined by recording the area under the curve (AUC). A calibration graph was established for each synthetic peptide, which was added to a control CSF sample at different concentrations. A sample for each peptide concentration was run through the Sep-Pak C_{18} cartridge and analysed by HPLC, where the AUC was recorded.

Data obtained by RIA confirmed the results showed in Figs. 2–4. Thus, haemorphin-7 was confirmed as a major component in the patient with a subdural haematoma. This patient exhibited comparatively low levels of LVV-haemor-

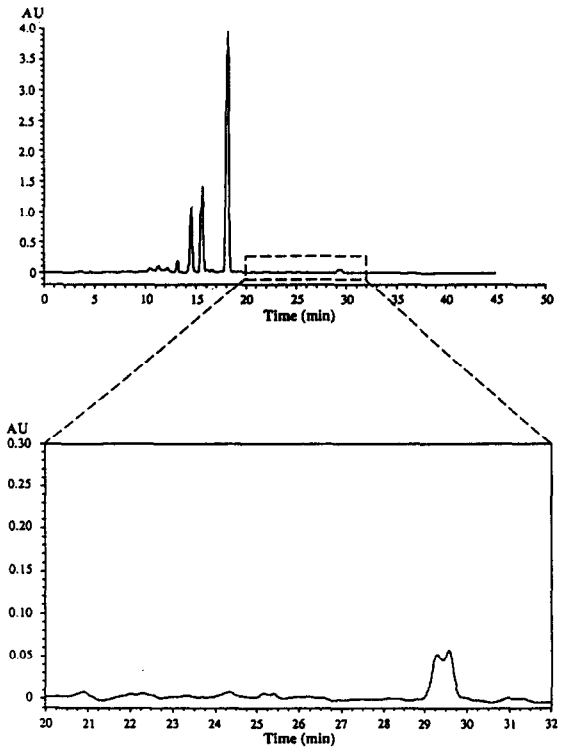

Fig. 4. RP-HPLC using the SMART System of a concentrated control CSF sample (10 ml). Conditions as in Figs. 1–3. Fractions of 100 μl were dried and taken for analysis by RIA. Haemorphin-7 and LVV-haemorphin-7-like immunoreactivity were eluted in fractions 27 and 30, respectively.

phin-7. The patients with subarachnoid bleeding, shown in Fig. 3, and the control subject contained the highest levels of haemorphin-7-like immunoreactivity, although in control samples immunoreactive material co-eluting with LVV-haemorphin-7 was also found. No RIA recognizing haemorphin-4 or LVV-haemorphin-4 was used in this study.

4. Discussion

RP-HPLC is frequently applied in studies of neuroactive peptides. However, so far very few studies have been published in which the technique combined with UV detection has been successfully used for the determination of peptides present in the CSF. One major reason for this may be that the level of these peptides in this fluid is very low and the procedure has to be combined with RIA or radioreceptor assay [9]. Another reason is that most instruments used for HPLC do not allow the recovery and detection of any peptide below certain levels. The SMART System is a recently developed chromatographic technique that fulfils some important requirements for efficient peptide separation and recovery. With this system, peptides and other biological molecules can be detected at levels below 5 pmol [10]. In previous studies in our laboratory this system was applied to probe molecular components in CSF from pregnant and puerperal women [10]. The technique was also useful for the separation and detection of monoamines and their metabolites. The system was further applied to determine CSF peptides and proteins in the rat [11]. In a recent study, we were able to isolate an opioid peptide for sequence determination from human milk [12].

This study indicates the potential of this HPLC system for the determination of the haemorphin-like immunoreactivity in human CSF. Although in this work the number of patients studied was limited, we believe that this approach may be useful as a complement to the clinical scores used in the diagnosis of patients with cerebrovascular bleeding. Further, the appearance of peptides known to act on opioid receptors is of interest with regard to some of the symptoms related to patients with the actual disorder. The presence of haemorphin-7 [13], LVV-haemorphin-4 [14], LVV-haemorphin-6 [2] and LVV-haemorphin-7 [6,7] in tissue extracts and body fluids suggests a sequential degradation of the β-chain of the blood protein haemoglobin. The release of LVV-haemorphin-7 is indicative of the action of a chymotrypsin-like enzyme. The shorter haemorphin structures may result from further degradation of this peptide as indicated in a previous study [3].

In conclusion, the study has demonstrated the usefulness of the SMART System for the recovery and determination of haemorphin-7-like material in CSF from patients with cerebrovascular bleeding. The technique may be applicable not only to the determination of the haemor-

phins in CSF, but possibly also to the determination of these compounds in plasma, where they are known to be released during certain physiological [8] or perhaps also pathophysiological conditions.

Acknowledgements

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