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Fingerprinting of molecular components in individual human cerebrospinal fluid samples with a new micropurification system

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ABSTRACT

This paper reports a rapid and sensitive microtechnique for fingerprinting as little as 20 μl of cerebrospinal fluid (CSF) from individual humans. Different molecular components were also isolated from the fluid. The SMART system, a new system optimized for high-recovery micropurification, was used for this purpose. The CSF sample, obtained by lumbar puncture, was applied directly into the system, and the patterns recorded for different individuals under various physiological conditions were compared. The results indicate that the procedure provides a powerful tool for the identification or recovery of CSF components and may also be of importance for diagnostic use.

INTRODUCTION

The access of highly efficient procedures for the separation and analysis of bioactive molecules is of fundamental importance in many areas of biomedical research. A particular need concerns research focussed on bioactive compounds present in the central nervous system (CNS). Substances of interest in the CNS are those involved in neurotransmission or neuromodulation, such as neuropeptides and monoamines. These compounds are present in the tissues of the CNS at relatively low concentration and highly sensitive techniques are necessary for their detection. A procedure in frequent use for the analysis and separation of neuroactive peptide is reversed-phase high-performance liquid chromatography (RP-HPLC). This technique has been successfully used to purify a number of neuropeptides present in the CNS and also in peripheral tissues (*e.g.* refs. 1–5) because of its high resolution power and comparatively short time of analysis.

In previous studies, we have used RP-HPLC to study opioid peptides in the cerebrospinal fluid (CSF) [6–10]. In this fluid, most peptides are present at very low

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concentrations and it was therefore necessary to combine the HPLC analyses with radioreceptor or radioimmunoassays. Furthermore, prior to HPLC the CSF sample requires concentration. In RP-HPLC analysis of other non-opioid peptides present in CSF it is possible to use spectrophotometrical methods for their detection [11]. However, a concentration step is also required in this case.

In this paper, we describe the use of the SMART system for the analysis of molecular constituents in human CSF. This new system is optimized for micropurification and microanalysis of biomolecules in samples of different origins. It may, therefore, provide new possibilities for the analysis and recovery of minute amount of peptides from crude tissue extracts or various body fluids. Here, the SMART system was used for the development of a procedure for fingerprint analysis of molecular components in individual human CSF samples.

EXPERIMENTAL

Chemicals and peptides

Standard peptides used in this study were purchased from Bachem (Bubendorf, Switzerland). The monoamines and their derivatives or metabolites were from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade from commercial sources.

Cerebrospinal fluid material

Lumbar CSF was obtained from women who volunteered for a study of CSF endorphins during pregnancy and in the puerperium [12]. Samples were frozen immediately following collection and stored at −70°C.

Chromatography

CSF samples (20–200 μl) from different individuals were analyzed by the SMART system (Pharmacia LKB Biotechnology, Uppsala, Sweden). The computer-controlled micropreparative chromatography system was equipped with a dual titanium cylinder syringe pump, a temperature-controlled (4°C to room temperature) separation unit containing a dual chamber dynamic mixer (30 + 30 μl), a six-port injection valve, a column holder, in-built detector cells for UV and conductivity measurement, and a fraction collector [13]. The UV monitor was UV-MII with 214-nm optics. Conductivity was measured using the in-built gradient monitor. The conductivity scale was set by calibration with eluent A (100%) and eluent B (0%); A, 0.14% trifluoroacetic acid (TFA); B, 0.12% TFA in 60% acetonitrile. Control of the system and evaluation of results was done with SMART Manager software. The column was μRPC C_2/C_{18}, PC 3.2/3 (particle size 3 μm, 120 Å; 30 × 3.2 mm I.D.). Elution was achieved with a linear gradient of acetonitrile (0–60%) containing TFA at a flow-rate of 240 μl/min.

RESULTS

Fig. 1 illustrates the UV pattern recorded from chromatographic analysis of a CSF sample (20 μl) collected from a woman in late pregnancy. It is obvious from the figure that the present technique provides an efficient separation of a number of

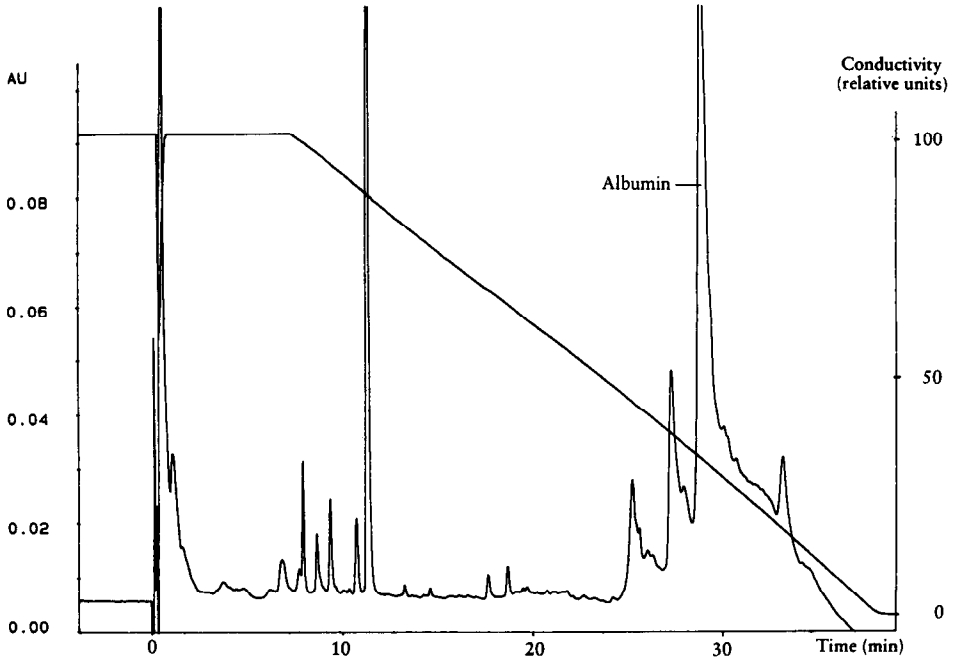

Fig. 1. Reversed-phase separation with the SMART system of human CSF (20 μl) collected from a woman at late pregnancy. The UV profile was recorded at 214 nm and the true gradient (descending solid line) was monitored by on-line conductivity measurement in the system. The main peak was identified electrophoretically as albumin and the adjacent (earlier-eluting) peak as α-microglobulin. Eluent A: 0.14% trifluoroacetic acid (TFA). Eluent B: 0.12% TFA in 60% acetonitrile. For further details, see text.

TABLE I

RETENTION TIMES RECORDED FOR VARIOUS NEUROPEPTIDES, MONOAMINES AND THEIR METABOLITES

Substance[a]	Retention time (min)
Dopamine	2.5
MOPEG	5.7
Serotonin	7.4
5-HIAA	10.6
HVA	11.4
SP (1–7)	13.4
Met-enkaphalin	16.6
Dynorphin B	18.8
SP	20.1

[a] MOPEG = MHPG = 3-methoxy-4-hydroxyphenylglycol (noradrenaline metabolite); 5-HIAA = 5-hydroxyindoleacetic acid (serotonin metabolite); HVA = homovanillic acid (dopamine metabolite); SP = substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2); Met-enkephalin = Tyr-Gly-Gly-Phe-Met; dynorphin B = Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr.

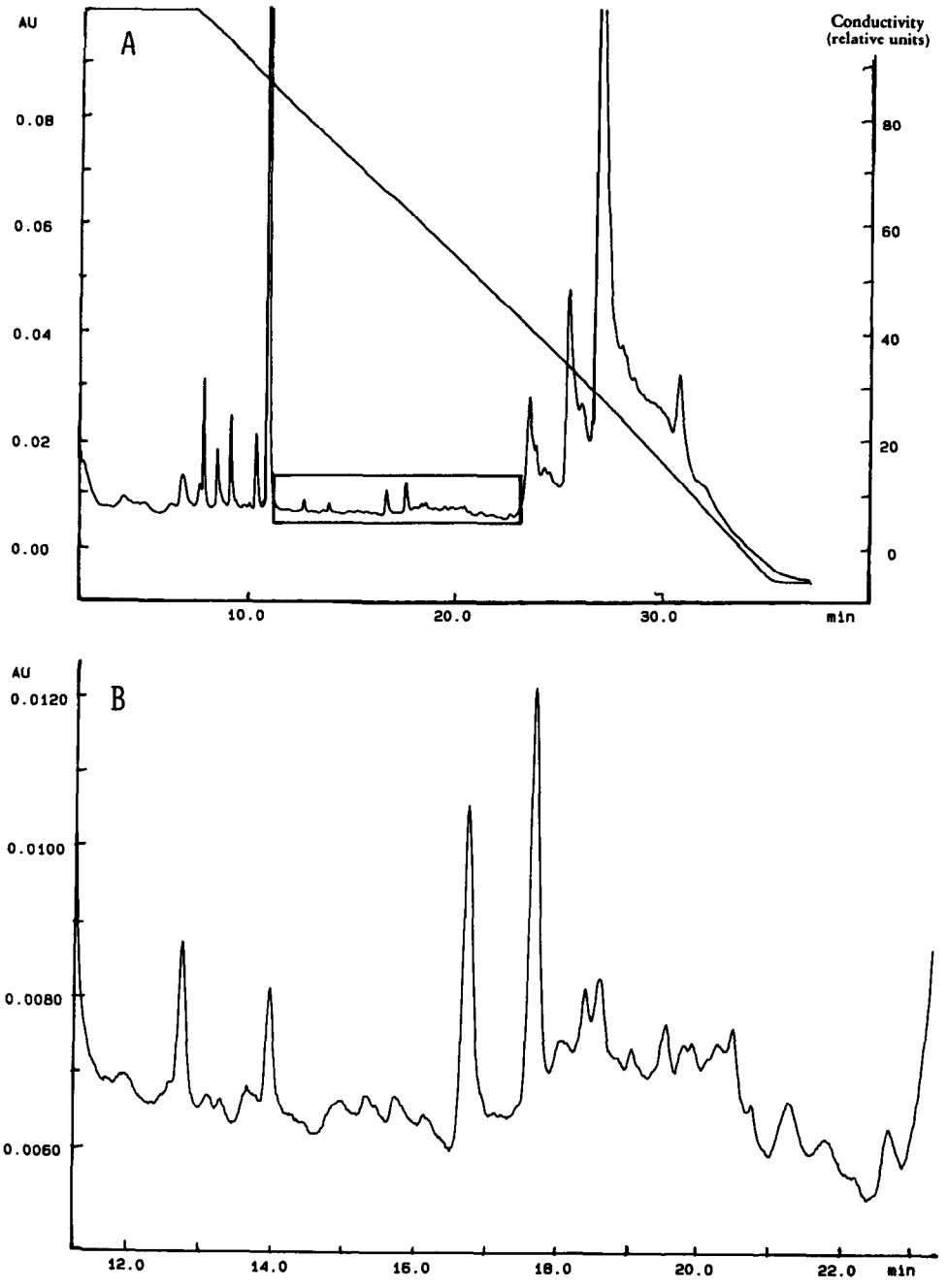

Fig. 2. (A) Analysis of human CSF (20 μl), collected from a woman at late pregnancy, by the SMART system using conditions for reversed-phase separation. The UV profile was recorded at 214 nm and the true gradient (descending solid line) was monitored as before. (B) Computer-enhanced enlargement of the boxed area seen in the upper panel. For separation conditions, see text and the legend to Fig. 1.

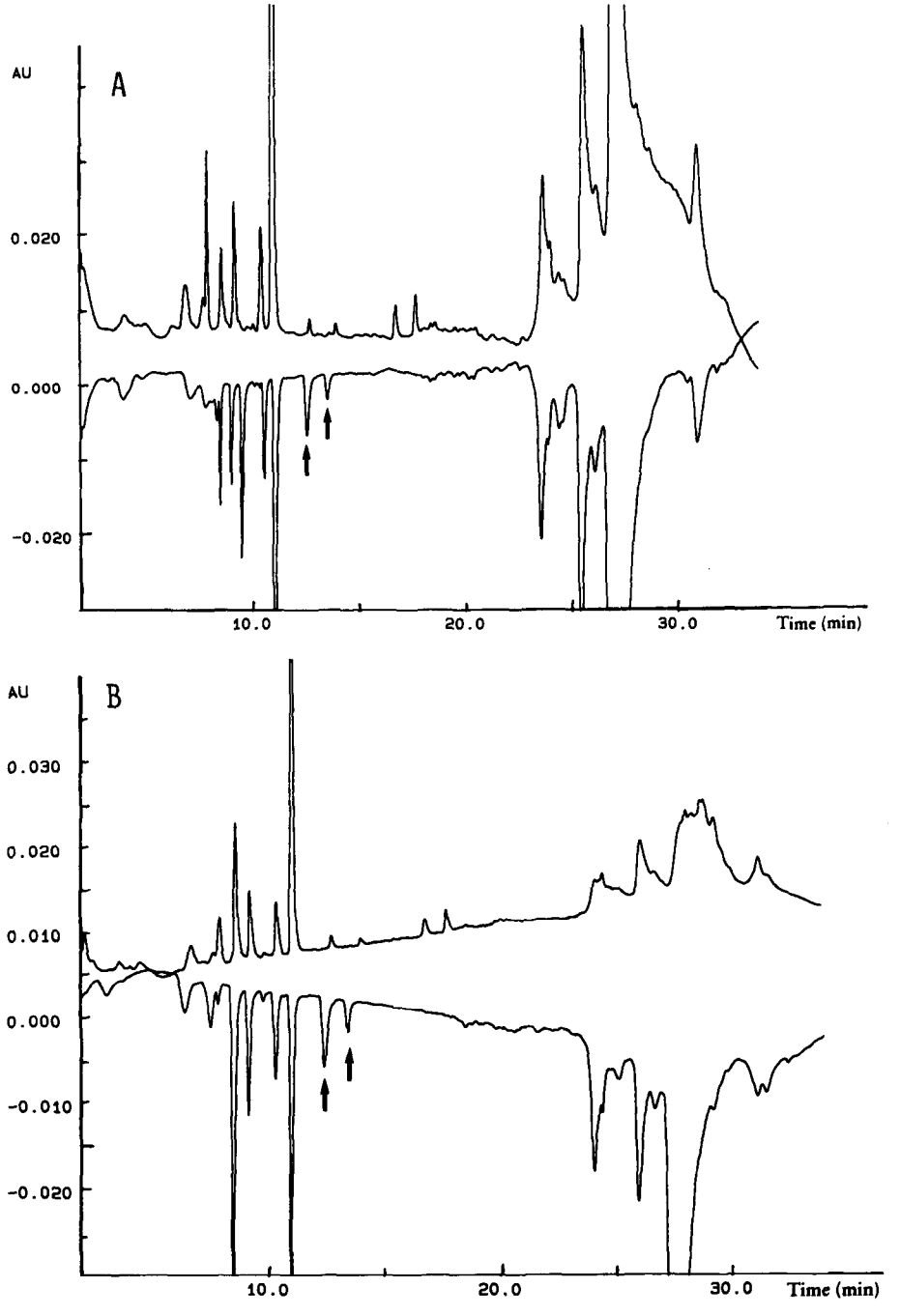

Fig. 3. Fingerprinting of molecular components in CSF obtained from two separate women (A and B), during late pregnancy and the lactation period. The upper curve in each chromatogram is CSF obtained during late pregnancy, whereas the lower curve represents CSF collected during lactation. The mirror images (lower curve) were done with the "shift amplitude" function in SMART Manager. Other conditions were identical to those given in the legend to Fig. 1.

UV-absorbing components present in the fluid. A major component was electrophoretically identified as human serum albumin, whereas a second peak eluting before but adjacent to albumin was identified as α-microglobulin. Several distinct components emerged from the column at retention times between 5 and 15 min. At least two of these components coincided with the monoamine metabolites 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA), as listed in Table I. By the addition of synthetic HVA to the CSF sample, the peak eluting at 11.4 min was significantly increased. Both 5-HIAA and HVA are known to be present in CSF and their levels are the subject of great interest in research on various CNS diseases. Only minor UV peaks are observable in the retention time area corresponding to neuropeptides (*cf.* Fig. 1 and Table I). However, because the instrument allows computer-enhanced enlargement of selected regions in the chromatogram, it was also possible to visualize several UV peaks in this area (Fig. 2). None of these peaks, however, are likely to represent any of those peptides listed in Table I, due to their extremely low levels in the CSF compartment [14]. Runs of standard peptides (Table I) indicated that the procedure allowed UV detection of these synthetic compounds at levels of 10–15 ng. The detection limit for the monoamine and monoamine metabolites used in this study was about ten times lower.

Analysis of samples collected from one woman at term pregnancy and during lactation indicated that the recorded UV pattern remained essentially the same (Fig.

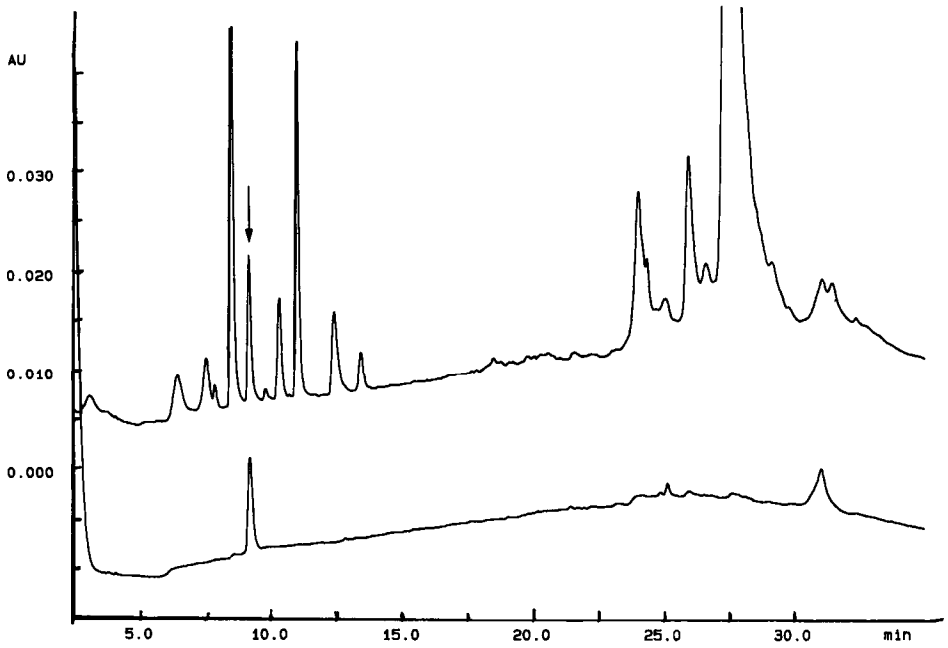

Fig. 4. Isolation and recovery of an unknown component in CSF. The material in the peak marked with an arrow was collected and diluted with eluent A. It was then reinjected onto the same column. The recovery was 78%, as calculated by a comparison of the integrated peak areas (done with SMART Manager). The sample was 20 μl CSF collected from a lactating woman. Other conditions were identical to those used for the experiment shown in Fig. 1.

3). However, it was possible to distinguish additional peaks that were present in CSF at term pregnancy but not during lactation. Most of these peaks were observed at retention times expected for neuropeptide structures (*cf.* Fig. 3 and Table I). Furthermore, at least two UV-absorbing components (indicated by arrows in the figure) were recorded in samples from women in the lactation period but were not observed in the fluid collected at their term pregnancy (Fig. 3). Other differences in the chromatograms were also observed. The patterns shown in Fig. 3 are also typical for two other women who volunteered for the study (not shown). In samples from the fifth woman in the group of healthy volunteers the additional peaks during lactation were less pronounced. Data also indicated some minor inter-individual differences between the recorded profiles, thus indicating that the pattern is unique for each individual. Apart from the additional peaks discussed above, the inter-relationships between the different components in a single subject seemed to remain over a longer period of time (Fig. 3).

In order to check the recovery of the present technique, individual components present in the CSF were re-analyzed. In the experiment shown in Fig. 4, a CSF component recovered from a 20-μl sample at a retention time of 9 min was re-chromatographed. Based on peak areas, a recovery of 78% was calculated. In studies of nanogram amounts of synthetic peptides a recovery of 80–90% was found. Furthermore, repetitive runs of individual CSF samples indicated a very high reproducibility for the system.

DISCUSSION

In this work, we have used the SMART system to develop a reversed-phase chromatography technique for the analysis and separation of components in human CSF. As the CSF is in constant exchange with the extracellular fluid of the brain and spinal cord, it represents a source of substances that derive from the CNS. Biochemical changes in the CNS, which may occur in chronic pain, psychiatric diseases or in neurologic disorders, may thus be tracable by CSF analysis. For ethical reasons, however, the volume of CSF that can be drawn from patients is limited. The present procedure allows detection and analysis of molecular constituents in the CSF with as little as 20 μl of the fluid. In previous studies, we have used around 2 ml of CSF to screen the peptide pattern by RP-HPLC [11].

In the present study, we have used a new system to analyze UV-absorbing components in human CSF. Several of these components are likely to represent proteins (*e.g.* albumin and α-microglobulin) or smaller peptides, whereas other may be due to molecules of non-peptide structure. The chromatographic patterns shown in, for example, Fig. 3 reveal a characteristic profile typical for the samples analyzed in this study. Predominating components are seen both in the area close to the void volume and in the region of the albumin peak. Attempts to recover and determine the structure of these components are in progress. The structures of the "additional" components seen at term pregnancy and during lactation are of particular interest. In a preliminary experiment, the largest component in the group of peaks eluting early in the gradient (see Fig. 1) was recovered in microgram amounts and was subjected to amino acid analysis. No amino acids above backgrounds levels were detected in this material, indicating that this component is not a peptide or protein. Structure identifi-

cation of this non-peptide compound may, therefore, require other techniques such as NMR and mass spectrometry. For that purpose, additional amounts of this component are necessary, and in on-going work the SMART system will be used for its recovery from considerable larger volumes of CSF.

The characteristic patterns recorded for individual CSF samples also suggest that the present procedure may be useful for the identification of unique substances that may be characteristic for certain physiological or pathophysiological conditions. For instance, in degenerative CNS disorders such as Alzheimers disease, an abnormal function of some peptide systems have been hypothized (*e.g.* refs. 15 and 16). Similarly, the generation of abnormal peptides has been suggested to occur in certain psychiatric diseases, *e.g.* schizophrenia [17] and post-partum psychosis [18]. It is therefore tempting to speculate that it might be possible to identify certain CSF peptides that are characteristic for each of these particular disorders using the present technique. Furthermore, as a consequence of this possibility, the system might also be of importance as a diagnostic tool. In this context, the technique may not only be useful for analysis of peptides or peptide products but also for the analysis of other structures including monoamines and their metabolites.

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