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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC–RADIORECEPTOR ASSAY OF HUMAN CEREBROSPINAL FLUID NEUROPEPTIDES

MAXIMIZING RECOVERY OF PICOMOLES OF PEPTIDES AND MINIMIZING MEMORY

DANXIA LIU

The Charles B. Stout Neuroscience Mass Spectrometry Laboratory, The University of Tennessee, 800 Madison Avenue, Memphis, TN 38163 (U.S.A.)

and

DOMINIC M. DESIDERIO*

The Charles B. Stout Neuroscience Mass Spectrometry Laboratory and Departments of Neurology and Biochemistry, The University of Tennessee, 800 Madison Avenue, Memphis, TN 38163 (U.S.A.)*

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SUMMARY

Reversed-phase high-performance liquid chromatography (RP-HPLC) is used to analyze neuropeptides in human cerebrospinal fluid (CSF) obtained from patients suffering from lower-back pain. Because CSF contains only femtomole to picomole amounts of those peptides, it is important to minimize any sample memory effect, while maximizing peptide recovery and avoiding any potential artifactual peak formation during chromatography. This study describes the phenomenon of active site occupancy by peptides on the RP-HPLC column, which is crucial when studying CSF where femtomole to picomole amounts of neuropeptides could be lost. Knowledge of those basic chromatographic factors is important whenever biologic extracts of peptides are applied to and eluted from an RP-HPLC column and then detected off-line with a radioreceptor assay, which is sensitive at the picomole level.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) [1,2] is one of the most effective methods available for the fast and efficient separation of endogenous peptides [3,4] that are obtained from extracts of biological tissues

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and fluids [5–8]. Detection methods that are commonly employed to monitor the presence of those separated peptides in the HPLC effluent include UV, fluorescence, radioimmunoassay (RIA) [8], radioreceptorassay (RRA) [3–6,9–12], bioassay, and mass spectrometry (MS) [3,4,11]. Detection sensitivities of these methods range from the femtomole to nanomole levels [3], and each has its own level of specificity [4]. For example, MS monitors a primary structural parameter (molecular mass, amino acid sequence) of the peptide; all of the other methods monitor only a secondary structural parameter such as peptide bond absorption, fluorescence, or interaction of the peptide with a receptor located on an antibody, in a receptor-enriched preparation, or on a biological tissue preparation (ileum, vas deferens).

The three known opioid peptidergic precursor molecules produce approximately thirty individual opioid neuropeptides [13], and many of those peptides manifest their individual biological activity after interaction with their putatively unique receptor [14,15]. The tachykinin family, which includes the undecapeptide amide substance P [16], is also important. Because of the complicated network of numerous peptides and of four peptide families [17], high-performance separation and detection methods are required.

RP-HPLC methods are very powerful techniques for peptide separations and they are used in many laboratories around the world. RP-HPLC has been used to purify peptides from tissue extracts such as the pituitary [3,4,6,7], where nanomoles of peptides occur. Conversely, for several neuroanatomical reasons, cerebrospinal fluid (CSF) is an appropriate biological fluid in which to measure neuropeptides [10,12]. Furthermore, even though CSF contains femtomole to picomole amounts of peptides, CSF is very "clean" when compared to tissue extracts and minimal if any pre-HPLC preparation of sample is required.

The purpose of this research was to determine the difference, if any, of opioid peptides in the CSF of patients with lower-back pain and the difference in opioid peptides before and after drug treatment. Because of these experimental constraints, we could not combine several CSF samples, which is the most often used method to provide a sufficient amount of peptides for separation and analysis. Instead, more attention had to be paid to the special problems caused by these low concentrations of peptides.

EXPERIMENTAL

Source of CSF samples

Samples of CSF are obtained from patients with lower-back pain who are under clinical investigation [18].

Synthetic peptides

Methionine enkephalin (ME=YGGFM), leucine enkephalin (LE=YGGFL), LE-Arg, ME-Arg-Phe, α-neo-endorphin, ME-Arg-Gly-Leu, substance P, and six dynorphin A fragments (1-7, 1-8, 1-9, 1-10, 1-13, and 1-17) were purchased from Sigma (St. Louis, MO, U.S.A.), and dynorphin A fragment 1-12 and β-endorphin from Peninsular (Torrance, CA, U.S.A.). The peptides were used without further

TABLE I

GRADIENTS FOR ELUTION OF HUMAN CSF PEPTIDES FROM AN RP-HPLC COLUMN

Gradient	Time (min)	Acetonitrile (%)	Rate of change (%/min)
A. New column wash	0	10	–
	300	100	0.3
	500	100	0
B. Eluting gradients	0	10	–
1. Program 1 (80 min)	18	15	0.28
	48	18	0.10
	72	30	0.50
	80	100	8.75
2. Program 2 (17 min)	97	100	0
C. New gradients	0	10	–
	18	15	0.28
1. Program 3 (112 min, 120 fractions)	48	18	0.10
	72	30	0.50
	92	60	1.5
	112	100	2.0
2. Program 4 (40 min)	152	100	0
3. Program 5 (60 min)	152	100	–
Reverse	212	10	−1.5

purification. Each peptide was dissolved in methanolic triethylamine formate (TEAF) (50:50, v:v) to produce a concentration of 0.5 $\mu g/\mu l$ of each peptide.

High-performance liquid chromatography

A Varian microprocessor-driven pump system was used with a C_{18} reversed-phase HPLC packing (85 Å pore size diameter; 10 μm particle diameter) in a stainless-steel analytical column (150×4.6 mm I.D.). Peptides were monitored with a UV detector set at 200 nm; a fraction collector (LKB) collected 1-min fractions during gradient elution [3]; the flow-rate was 1.5 ml/min [19].

Table I shows the gradients used in this research. Gradient B has been used for most of our RP-HPLC separations [3–6,9,18–20]. Gradients A and C were developed for this present research; basically, they supplement the first 72 min of gradient B using a volatile TEAF buffer [21].

Profiling of opioid receptor activity

For the RRA that was used as the detection system following RP-HPLC, a receptor-rich P_2 preparation from a canine limbic system was employed [22]. RRA [14,15] detected the presence of opioid receptor activity in each one of the 90 fractions by using HPLC-purified [^{3}H]etorphine [23]. This ligand was selected because it competes with endogenous opioid peptides for binding to several different types of opioid receptors that are located in the limbic system P_2 preparation.

Following pre-incubation (45 min, 37°C) of the P_2 receptor-enriched preparation, sample and competing tritiated ligand were added, and the mixture was

incubated (0°C, 2.0 h). Unbound radiolabel was removed by rapid filtering through a cell harvester (Skatron, Sterling, VA, U.S.A.), and the radioactivity that remained on the filter was measured. Total binding (T) was measured in the absence of and non-specific binding (NS) in the presence of the "cold" ligand ME. Specific binding (S) was calculated as $T-NS$. Specific binding measured in each HPLC fraction was compared to S of known amounts of ME from a calibration curve, and therefore the opioid receptoractivity measured in each one of the 90 fractions was expressed as pmol ME equivalents per ml CSF, which was plotted for each HPLC fraction [18].

RESULTS

The examples discussed below collect representative data from several areas: RRA blanks before and after standards are injected; several CSF samples and subsequent blanks; a blank following a column elution of a CSF sample using the routine gradient B (Table I); and several CSF samples and subsequent blanks; one blank was collected after eluting the samples with a new gradient C (programs 3 and 4) and the other with gradient C (programs 3–5) to separate a sample.

Washing of a new HPLC column

When a new HPLC column is purchased, it is necessary to wash it with the very shallow gradient A over a long period of time to provide sufficient time for column equilibration and to ensure removal of any interfering materials. Generally, two washes with gradient A are sufficient to give a background of ≤ 1 pmol ME equivalents.

To ensure that the column was clean, TEAF (500 μl) was injected, gradient B was run, and 45 fractions (each fraction equals 2 min) were collected. These 45 fractions are defined as "blank". Opioid receptor activity was measured in each fraction.

Saturation of sites on the ODS particles that actively bind opioid peptides

It is well known from gas chromatographic (GC) and gas chromatographic–mass spectrometric (GC–MS) studies [24] that chromatographic columns (GC,LC) contain binding sites for polar or chemically derivatized biological molecules. Indeed, these sites appear so specific that ^{1}H and ^{2}H forms of particular chemically derivatized molecules will separate chromatographically.

These active sites also exist on RP-HPLC columns, and must, therefore, be occupied by peptide before the femtomole to picomole amounts of CSF peptides can be determined by HPLC. This occupancy problem is not so critical (if at all) for pituitary and some other tissues as it is for CSF samples. If specific care of preparation is not taken, several injections of a biological extract onto a non-prepared column are required before the endogenous amount of that peptide could be detected [24]. Indeed, at first, no response is found for initial injections of biologic extracts; then, a low response is observed; and finally, subsequent injec-

tions yield increasing amounts of detected compound until saturation of active sites occurs and a plateau is attained.

To demonstrate the necessity of pre-occupying active sites with the peptides that will be analyzed in biological extracts, a recovery study was carried out. First, a known amount of ME (12.5 pmol) was injected in triplicate onto a previously unused RP-HPLC column. The peptide was eluted, collected, and analyzed by RRA. The average reading of those three measurements (4.5, 5.1, 3.1 pmol) was 4.2 pmol, which corresponds to a recovery of 34%. Then, ME was injected (2 μg = 3.5 nmol, three times) onto the column to saturate the active sites, and eluted. Last, 12.5 pmol ME were injected again and the ME was determined (11.2 and 9.0 pmol) to be 10.1 pmol. The second recovery was 81%, which was a significant improvement over the first recovery of 34%. The column was washed twice with gradient B, and the second wash was collected and the blank was determined by RRA. That blank showed only baseline receptor activity. After those two washes, 12.5 pmol ME were injected again in triplicate and ME was determined by RRA (8.8, 6.7, and 9.6 pmol), which averaged to 8.4 pmol; those data indicate a 67% recovery. Whereas the two washes decrease the recovery from 81 to 67%, those data also clearly demonstrate the need to saturate active sites on the HPLC column with the target peptide.

In most HPLC applications, reference solutions are used to determine retention times. However, that injection requires proof that those standards are completely eluted from the column and do not affect analysis of the next sample. This concern is especially pertinent for analysis of femtomole to picomole levels of compounds. To investigate that phenomenon, a mixture of standard peptides (2 nmol of each peptide) was injected and eluted with gradient B. Then, a blank of 45 HPLC fractions was collected. Gradient B was used to separate the standards and to collect the blank. The results from standard elution/blank determination experiments (triplicate) demonstrate that gradient B cleans the column very well. The above experiment indicates that injecting nanomole amounts of peptide standards to calibrate retention times and to presaturate the active sites on the column does not yield any opioid receptor activity in the subsequent HPLC blank, offering increased confidence that no interference occurs with subsequent samples after eluting standards with gradient B.

We define an acceptable background level as 1 pmol ME equivalent of receptor activity, which does not necessarily mean that 1 pmol of a peptide has been found, but rather that 1 pmol is the limit of detection sensitivity for that particular RRA.

RP-HPLC–RRA analysis of human CSF samples

Each sample of human CSF (4 ml) was lyophilized, and the residue was dissolved in TEAF (500 μl). The TEAF solution was centrifuged (Clay Adams table centrifuge, Parsippany, NJ, U.S.A.), and the supernatant analyzed by RP-HPLC. Peptides in CSF were eluted using gradient B, 90 fractions were collected (1 fraction per min), and each fraction was analyzed for content of opioid receptor activity. Fig. 1 shows an RP-HPLC–RRA profile of a human CSF sample.

A clean column is required after separating peptides in every CSF sample, otherwise subsequent data are compromised and firm conclusions cannot be reached

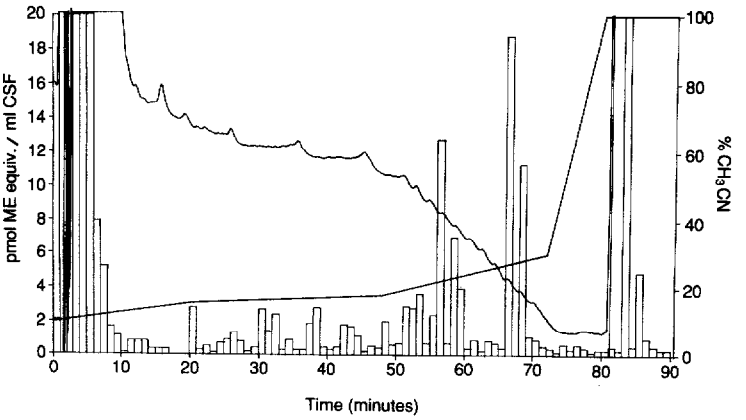

Fig. 1. RP-HPLC-RRA profile obtained with gradient B from human CSF sample A. The chromatogram indicates UV absorption; gradient B is displayed as percentage acetonitrile; TEAF is the buffer; RRA data are shown as vertical bars.

on drug effects or on the nature of a patient's CSF peptides. To determine whether the column was sufficiently clean after a CSF sample was eluted, the opioid receptor activity in the 45 fractions of a subsequent blank was measured. Fig. 2 is an example of such a blank using gradient B after separating a sample; some receptor activity was found. The time used for program 2 (gradient B) was increased from 30 min to 2 h; four different times of program 2 were used for that gradient, and five blanks were collected after eluting five samples. All of those blanks indicated that the column was not cleaned sufficiently. Even after collecting two blanks after one CSF sample, the second blank was not clean, especially in the fraction 84 area (for 90 fractions). Then, programs 3 and 4 (gradient C) were used and 120 1-min fractions were collected. Fig. 3A contains the HPLC–RRA data for a CSF sample, and Fig. 3B the subsequent second blank. The RRA background of the second blank after separating the sample is still unacceptable.

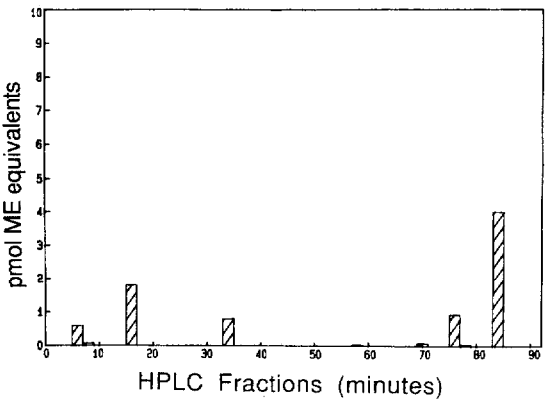

Fig. 2. RP-HPLC-RRA profile of a blank obtained after elution of human CSF sample A with gradient B.

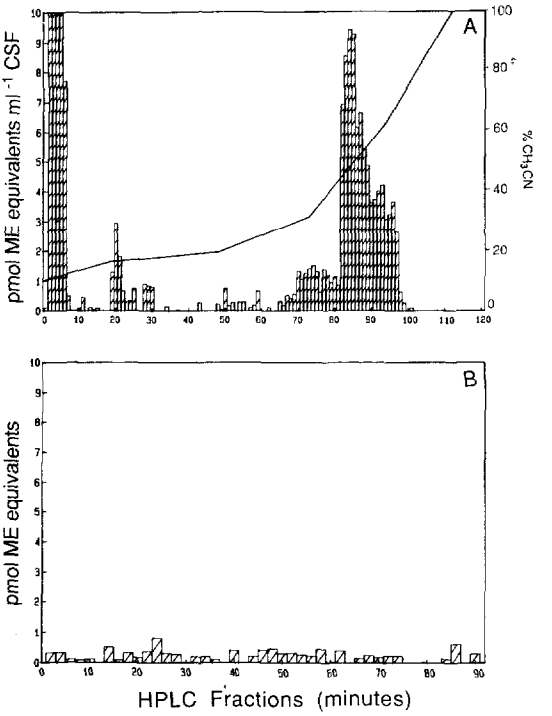

Fig. 3. (A) RP-HPLC-RRA profile obtained from human CSF sample B with gradient C (programs 3 and 4). Gradient C is plotted in same manner as gradient B in Fig. 1. (B) Second RP-HPLC-RRA profile of a blank obtained with gradient B after elution of human CSF sample B with gradient C (programs 3 and 4).

Gradient C was then used to elute the CSF sample. The two blanks obtained after eluting two separate samples both show a clean background, and Fig. 4 displays one of those two experiments. Clearly, comparing Figs. 3B and 4B, these data demonstrate that the shallow reverse program 5 (gradient C) is effective in cleaning and re-equilibrating the RP-HPLC column.

During this study of the CSF from lower-back pain patients, a consistent RRA peak was found at fraction 84 (Fig. 1). Because the increase of the organic modifier, acetonitrile, in that region of the HPLC gradient B was previously so high (8.75%/min; 72 to 100% in 8 min), subsequently it was found necessary to decrease that high rate to maximize the recovery of that (those) peptide(s) and to increase the chromatographic resolution of that (those) endogenous compound(s) that elute in that area. A decrease in the gradient slope was required because originally it was suggested that all peptides would probably elute at lower acetonitrile concentrations. However, it is now realized that longer peptides, precursors or more hydrophobic peptides do elute near fraction 84. The new gradient C increases the resolution of fraction 84 compared to gradient B (Figs. 3A and 4A). Indeed, that peak of receptor activity has become important and is the objective of another study to be reported later.

Because of the need to have a clean RRA background, a separate RP-HPLC

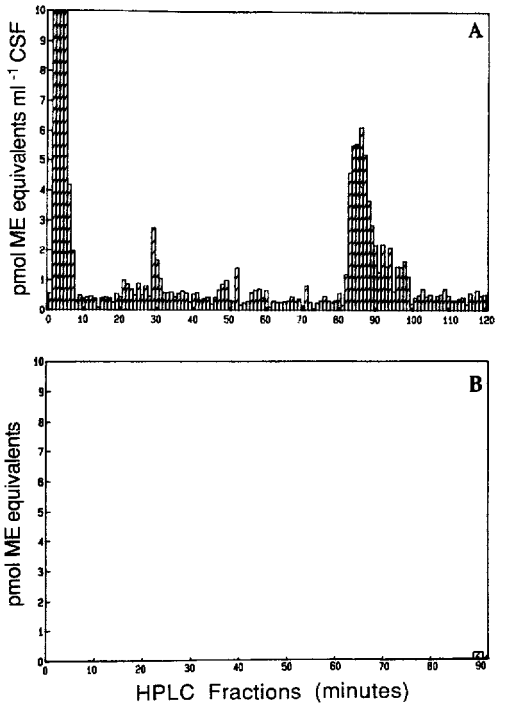

Fig. 4. (A) RP-HPLC-RRA profile obtained from human CSF sample C with gradient C. (B) RP-HPLC-RRA profile of a blank obtained with gradient B after elution of human CSF sample C with gradient C (programs 3–5).

column is now used for each type of biological sample (pituitary tissue, control and tumor; tooth, pulp and decalcified; cornea, CSF) under investigation.

DISCUSSION

Four conclusions are derived from the data presented in this paper.

(1) For the high-sensitivity detection required for analysis of opioid peptides in human CSF, a column-cleaning procedure for a new column was developed. This gradient A is quite shallow (0.3%/min) and long (300 min). Furthermore, a hold at 100% acetonitrile is long (200 min). This procedure effectively removes from the column those eluting materials that have interfered with RRA detection to produce an unacceptably high RRA background. Presumably, that eluting material does not contain opioid peptides (none were injected in our laboratory nor apparently by the column manufacturer), but could have been column "bleed" and/or other material that could interfere mechanically with [^{3}H]etorphine binding to the opioid receptors.

(2) An appropriately cleaned RP-HPLC column with no detectable RRA background contains active sites that have a high specificity and high affinity (pmol) for opioid peptides. This HPLC active site phenomenon is comparable to GC and GC–MS data, where absorption of a carrier is required before analysis of biological levels of compound can be performed. Indeed, ^{1}H forms of com-

pounds separate from corresponding ^2H forms [24]. Occupancy of those active sites is crucial, but proof must also be provided that an acceptable blank is obtained before a subsequent CSF sample is analyzed. Data are presented to demonstrate that a clean RP–HPLC–RRA background is obtained after eluting a mixture of standard peptides (nmol), and that nanomole amounts of opioid peptide carrier increased the recovery by 238% for picomole amounts of ME.

The exact molecular details of this active site peptide binding phenomenon are not well understood, but it may be hypothesized that the active site-bound synthetic peptide may exchange one-for-one with endogenous peptides during the elution of the CSF sample. Experiments with radioisotope-labeled peptides or with stable isotope-labeled peptides could be performed to test that exchange hypothesis, but extreme care must be taken to avoid potential interference with the final HPLC detector. Radiolabels interfere with RIA and RRA detection, whereas stable isotopes interfere with RIA, RRA and MS detection.

(3) New gradients (C) effectively clean and re-equilibrate the RP-HPLC column after eluting a CSF sample (Fig. 4B).

(4) The use of gradient C significantly increases the chromatographic separation and recovery of late-eluting peaks (Figs. 3A and 4A). Those peaks could be due either to opioid peptide precursors or to peptides having increased hydrophobicity.

The patients from whom CSF has been obtained by lumbar puncture were individuals who suffered from lower-back pain [18], and some of those patients were candidates for neurosurgery. This chromatographic study was undertaken to provide accurate information on the opioid peptidergic pathways that may be operative or defective in that patient population and to provide an objective molecular criterion for neurosurgery. Preliminary data have been published elsewhere [18].

We hypothesize that metabolic defects may contribute to the clinical manifestations in several clinical studies [20], and towards a rational experimental solution to that question, information based on the structure of all pertinent receptor-active compounds is needed. That type of pattern information is readily provided by a separation–detection analytical system that monitors a wide range of opioid peptides, and RP-HPLC and RRA, respectively, satisfy those two requirements. Of course, it must be realized that RRA data can be considered to be only semiquantitative at best, and do not convey structural information. Nonetheless, RRA does provide a useful first screen to detect HPLC fractions that contain receptor activity, because subsequent detection techniques such as MS [11] can provide structure information.

We have presented analytical data obtained during the RP-HPLC separation of endogenous peptides in human CSF and the detection of those opioid receptor-active peptides by RRA.

At first glance, these particular experimental points may seem to be trivial and not worth mentioning or describing in very much detail, yet it can be stated from experience that these parameters must be assiduously considered, rationalized, controlled, and monitored when analyzing biological samples that contain peptides at the picomole to femtomole level.

In conclusion, careful experimentation has been undertaken to study opioid peptides in the CSF of patients with lower-back pain. Interesting data in the RP-HPLC–RRA fraction 84 have been found. Careful column cleaning, saturation of active sites with reference compounds, effective separation of CSF peptides, and efficient elution of CSF from the column using gradient C, which contains an additional shallow reverse gradient to carefully clean a column, are described. Overall, our level of confidence in this mode of analysis has been increased following attention to experimental RP-HPLC details.

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