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MULTI-DIMENSIONAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF OPIOID PEPTIDES FOLLOWING PRE-COLUMN DERIVATIZATION WITH NAPHTHALENE-2,3-DICARBOXALDEHYDE IN THE PRESENCE OF CYANIDE ION

PRELIMINARY RESULTS ON THE DETERMINATION OF LEUCINE- AND METHIONINE-ENKEPHALIN-LIKE FLUORESCENCE IN THE STRIATUM REGION OF THE RAT BRAIN

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SUMMARY

The reversed-phase high-performance liquid chromatography of three synthetic opioid peptides, ^{5}leucine-enkephalin, ^{5}methionine-enkephalin and [D-^{2}alanine]-^{5}methionine enkephalin, has been studied after their pre-column fluorogenic derivatization with naphthalene-2,3-dicarboxaldehyde in the presence of cyanide to the corresponding 1-cyanobenz[*f*]isoindole (CBI) derivatives.

The chromatographic properties of the three synthetic CBI-peptides were characterized using three different stationary phases, ODS Hypersil, CPS Hypersil and Spherisorb Phenyl, eluted with mobile phases containing various concentrations of methanol, tetrahydrofuran or acetonitrile in 26 m*M* trifluoroacetic acid, adjusted to pH 3.5. The data obtained using single chromatographic columns were used to design a multi-dimensional system in which the three synthetic CBI-peptides of interest were transferred as a single fraction from one column to a second. The first column served to separate the peptides from the majority of the material in the samples, and the second column was used to separate the three CBI-peptides from each other. The best separation was achieved in which the first column was Spherisorb Phenyl and the second column was ODS Hypersil. Both columns were eluted with a mobile phase of 45% acetonitrile (v/v) in 26 m*M* trifluoroacetic acid (pH 3.5) at a flow-rate of 1.0 ml/min. The method has been applied to the determination of leucine- and methionine-enkephalin-like fluorescence in the striatum of the rat brain

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INTRODUCTION

The opioid peptides [1] represent an important class of neurotransmitters and the direct chemical analysis of these compounds in biological fluids represents a significant scientific challenge. Techniques that have been described for the determination of the opioid peptides include radioreceptor assays [2,3] and high-performance liquid chromatography (HPLC) with detection by mass spectrometry (MS) [4]. Whereas HPLC fractionation followed by either radioimmunoassay (RIA) [5] or MS [4] methodology possesses the required sensitivity and selectivity, these approaches for the ultra-trace analysis of the opioid peptides are not without their limitations. For example, RIA methods involve the disposal of hazardous waste and MS methods require specialized equipment.

Workers in these and other laboratories [6] are actively engaged in a program of research aimed at developing ultra-sensitive, direct chemical methods of analysis that can be adapted for routine determinations of peptides in biological fluids. Very recently, Kai et al. [6] have presented a method for the determination of Leu-enkephalin (LE) in rat brain parts that uses HPLC and pre-column fluorogenic derivatization with 1,2-diamino-4,5-dimethoxybenzene. Their method [6] provides adequate detection limits for LE in brain parts; however, the other major opioid peptide of interest, Met-enkephalin (ME), was obscured by a large fluorescent interference. Additionally, other fluorescent components in the samples result in chromatographic run times of greater than 60 min [6]. It is proposed here that multi-dimensional HPLC (column switching) [7–9] will be required to achieve the peak capacity needed to separate and quantify the low concentrations of the opioid peptides in relevant biological matrices. The present study is concerned with the investigation of the chromatographic properties of three model enkephalins in reversed-phase HPLC systems. These studies were considered to be an important prerequisite to the application of the technology being developed for the analysis of the enkephalins and other peptides of biomedical interest in biological tissues.

In the present study, conventional fluorescence detectors were used and the enkephalins were derivatized with naphthalene-2,3-dicarboxaldehyde in the presence of cyanide (NDA–CN) (Fig. 1), a rationally designed fluorogenic reagent [10–17] for primary amines, amino acids and small oligopeptides. The NDA–CN reagent was developed from the *o*-phthalaldehyde–thiol (OPA–RSH) system, originally described by Roth [18] for the fluorogenic labeling of primary amines. The NDA–CN system produces fluorescent N-substituted 1-cyanobenz[*f*]isoindoles (CBI) with small peptides [13,16] (Fig. 1) and was used in this study for the labeling of the α-amino group of the three solutes of interest.

The overall objective of this program of research is to combine the derivatization procedure shown in Fig. 1 with on-line chemiluminescence (CL) [15,16] and laser-induced fluorescence (LIF) [14] in an HPLC system for the

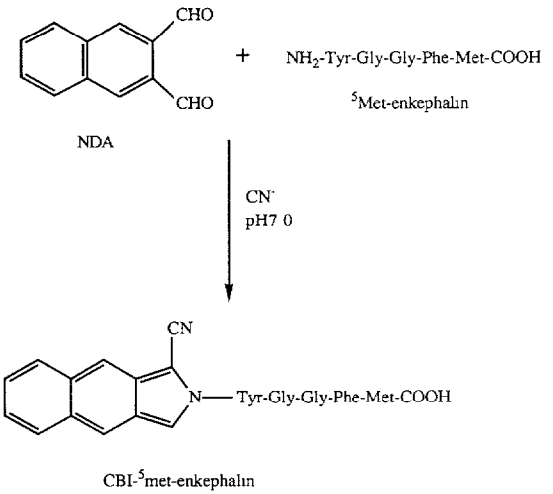

Fig. 1. Fluorogenic reaction of Met-enkephalin with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide ion to produce an N-substituted 1-cyanobenz[*f*]isoindole (CBI-Met-enkephalin).

ultra-trace analysis of a wide range of synthetic and naturally occurring peptides. During the course of these investigations, it has become clear that, if the high sensitivities of CL and LIF are to be exploited, then a highly efficient separation technique will be required. In the present study, conventional fluorescence detection was used, because the main purpose was to characterize the chromatography of the chosen synthetic peptides, LE and ME.

EXPERIMENTAL

Chemicals and reagents

ME (Tyr-Gly-Gly-Phe-Met), LE (Tyr-Gly-Gly-Phe-Leu) and [D-^{2}Ala]-D-^{5}Met-enkephalin (Tyr-[D]-Ala-Gly-Phe-Met, [A]ME) were obtained from Sigma (St. Louis, MO, U.S.A.) and were used as received. The amino acid composition of each synthetic peptide was confirmed by an independent laboratory (Dr. Charles Decedue, Director, Enzyme Laboratory, University of Kansas, Lawrence, KS, U.S.A.). The NDA was obtained from Oread Labs. (Lawrence, KS, U.S.A.) and was used as received. Methanol, acetonitrile and tetrahydrofuran (THF), used in the preparation of mobile phases, were HPLC grade and were obtained from Fisher Scientific, (St. Louis, MO, U.S.A.). Distilled, deionized water was used throughout and the other chemicals were of the highest purity available and used as received from various sources.

Stock solutions (5 m*M*) of NDA were prepared in acetonitrile on a weekly basis in low actinic (red) glassware and were protected from light in a refrigerator (4°C). Stock solutions (10 m*M*) of potassium cyanide were prepared

in water as required. Standard stock solutions of [A]ME (500 μM) were prepared in 0.1 M acetic acid. Standard stock solutions of LE and ME (both 500 μM) were prepared in water. Further standard solutions of the peptides were prepared by serial dilutions of the stock solutions.

Liquid chromatography

The following HPLC columns were used in these investigations: ODS Hypersil (C_{18}), CPS Hypersil (CN) and Spherisorb Phenyl (each 5 μm, 150×4.6 mm I.D.). The phenyl and C_{18} columns were packed in the upward direction [19] using chloroform as the slurry solvent and methanol as the packing solvent. The CN column and the bulk packings for the other two columns were purchased from Keystone Scientific (State College, PA, U.S.A.).

All the chromatographic equipment was obtained from Shimadzu Instruments (Baltimore, MD, U.S.A.). The single-column system consisted of a Shimadzu Model LC-6A pump, a Rheodyne Model 7125 fixed-loop injector (20 μl) and a Shimadzu Model SPD-6AV UV–VIS spectrophotometer set at 420 nm. The peaks were recorded with a Shimadzu Model C-R3A Chromatopak data system.

The components of the column-switching system (Fig. 2) were two Shimadzu Model LC-6A pumps, two Shimadzu Model FCV-2AH high-pressure switching valves, a Shimadzu Model CTO-6A column oven and a Shimadzu Model RF-530 fluorimetric detector (xenon lamp; $\lambda_{ex}=420$ nm; $\lambda_{em}=490$ nm).

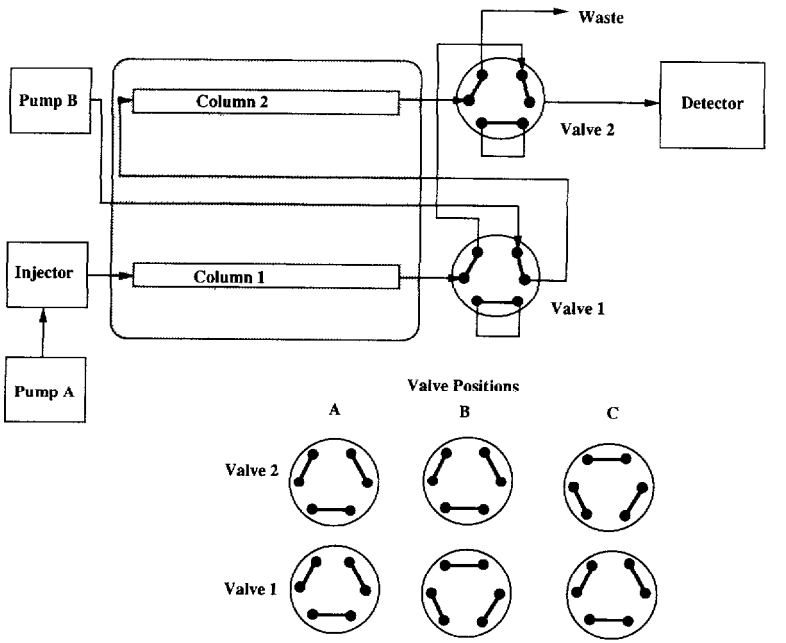

Fig. 2. Column-switching system used in these investigations.

Injections were made with either a Rheodyne Model 7125 fixed-loop (20 μl) injector or a Shimadzu Model SIL-6A autoinjector. Data reduction was achieved using a Shimadzu Model C-R4A Chromatopak data system. All these devices and the switching events were controlled by a Shimadzu Model SCL-6A system controller.

Preparation of rat striatum samples

Samples of striatum were obtained from the brain of male Wistar rats (200–250 g) and were processed according to the method described previously [6]. Those readers interested in the precise details of the extraction procedure are referred to the original work of Kai et al. [6] and only a brief summary of the procedure, highlighting the differences, is provided here. Samples of rat striatum (about 0.2 g) were homogenized with 10% (v/v) hydrochloric acid and 500 pmol of ME (internal standard) were added in a volume of 25 μl. If standard curves were being prepared, homogenates were prepared from tissue, which had been allowed to stand at room temperature for sufficient time to allow complete degradation of the peptides, that is, sufficient time for their peak heights to have decreased below the limits of detection. When preparing these standard curves, 0, 50, 125, 250, 375 and 500 pmol of LE and ME were both added to the homogenate in a total volume of 50 μl. Thereafter, the procedure was exactly as described by Kai et al. [6], with the exception that the final residue was reconstituted in 540 μl of phosphate buffer (pH 6.8, 100 m*M*) and derivatized with NDA–CN.

Derivatization procedure

The peptides were converted to their corresponding CBI derivatives (Fig. 1) as follows: 20 μl of Me and/or LE (0–500 pmol in 20 μl) were added to a freshly prepared mixture of [A]ME (500 pmol, 20 μl), ascorbic acid (50 μl, 200 m*M*), potassium cyanide (100 μl, 10 m*M*), NDA (200 μl, 5 m*M*) and phosphate buffer (540 μl, 100 m*M*), pH 6.8). After shaking by hand for about 30 s, the reaction was allowed to proceed for 20 min over an ice bath (0–4 °C) and then quenched by the addition of taurine (2-aminoethanesulfonic acid, 50 μl, 200 m*M*). The solutions were stored for a further 10 min over an ice bath (0–4 °C) to allow the complete reaction of the residual NDA–CN with taurine. Solutions of the derivatized peptides could be stored for up to 6 h on ice (0–4 °C) and protected from light by wrapping the sample vials with aluminum foil. Alternatively, the samples could be stored in the tray of the autosampler provided it was refrigerated at 0–4 °C and protected from light.

RESULTS AND DISCUSSION

Chromatographic properties of CBI-LE and CBI-ME

Initially, the retention behavior of CBI-LE and CBI-ME was studied using three different stationary phases (C_{18}, phenyl and CN) and three different

organic modifiers (acetonitrile, methanol and THF) added to the mobile phase. These studies were performed at ambient temperature (approximately 22°C) and the aqueous component of the mobile phase was 26 m*M* trifluoroacetic acid (TFA), adjusted to pH 3.5 with potassium hydroxide. The effects of organic modifier type and concentration on the retention of CBI-LE and CBI-ME on each of the three columns were studied and the results are summarized in Figs. 3 and 4. In all cases, the retention of CBI-LE and CBI-ME decreased with increasing concentration of organic modifier. However, the retention properties of the three columns for the analytes were dependent on the nature of the organic modifier in the mobile phase (Figs. 3 and 4). When the organic modifier was methanol, then the order of retention of CBI-LE and CBI-ME was C_{18} > phenyl > CN (Fig. 3). In contrast, when the mobile phase modifier was acetonitrile or THF, then retention was much less dependent on the nature of the stationary phase (Fig. 3).

Fig. 4 explores the differences in the chromatographic properties of the three

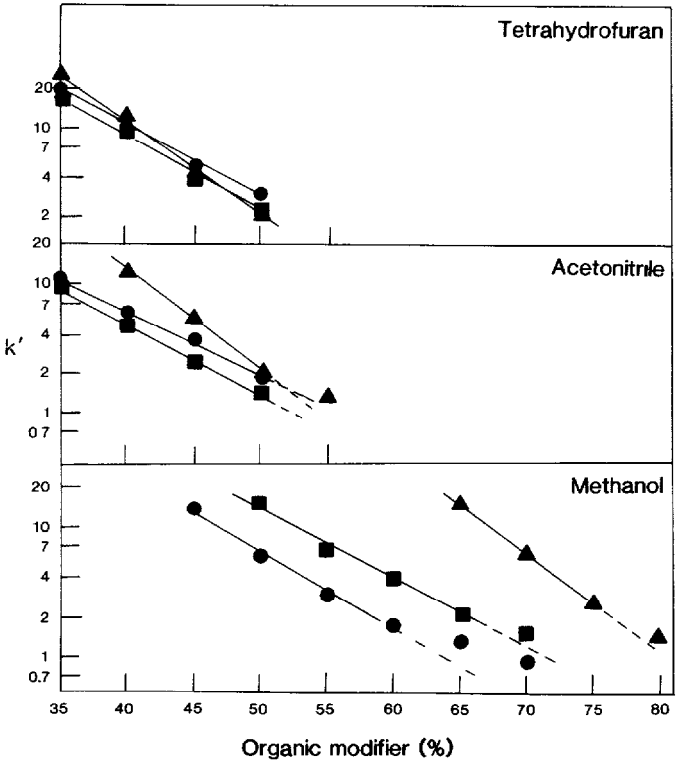

Fig. 3 Relationship between the capacity ratio (k') of CBI-LE and the concentration of tetrahydrofuran, acetonitrile and methanol, using three different stationary phases. Stationary phases: ODS Hypersil (▲), Spherisorb phenyl (■) and CPS Hypersil (●). Mobile phases: acetonitrile, methanol or tetrahydrofuran in 26.5 m*M* TFA (pH 3.5). Temperature: ambient.

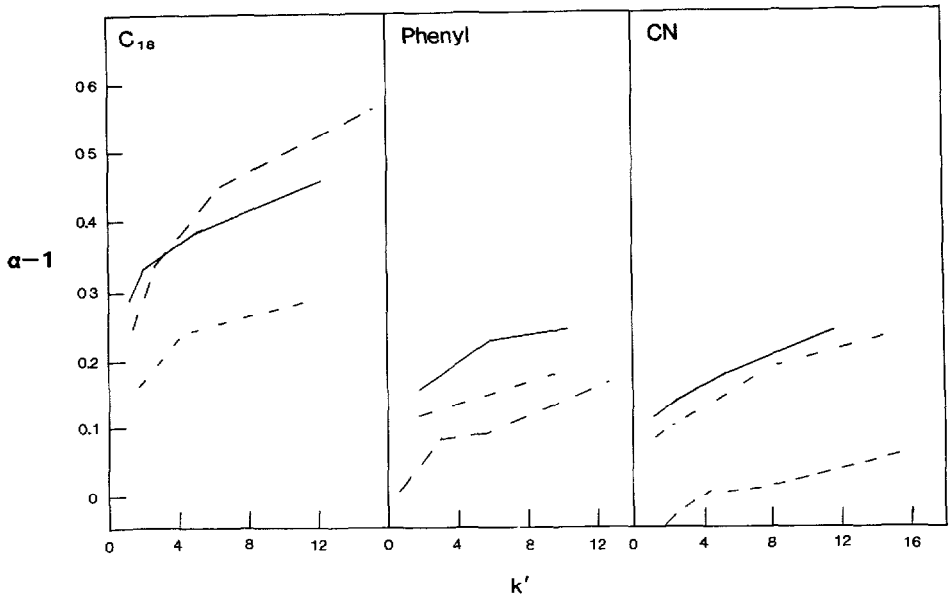

Fig. 4. Relationship between solute selectivity (eqn. 1) and the capacity ratio (k') of CBI-LE using the chromatographic conditions shown in Fig. 3. Mobile phases: acetonitrile (– – –), methanol (——) or tetrahydrofuran (-·-) in 26 m*M* TFA (pH 3.5).

columns and the three mobile phase modifiers, showing the relationships between solute selectivity (α-1) and retention (k'), where α is defined as:

$$\alpha = \frac{k'_{LE}}{k'_{ME}} \quad (1)$$

In virtually every chromatographic system studied, retention was related to solute hydrophobicity such that CBI-LE eluted after CBI-ME. The few exceptions to this were situations in which the retention times of both derivatives were relatively short ($k' < 3$).

For each column studied, solute selectivity increased with increasing retention, that is decreasing concentration of organic modifier (Fig. 4). To normalize for retention, the selectivity data have been plotted against k' in Fig. 4, which shows that selectivity was influenced by the nature of both the stationary phase and the organic modifier in the mobile phase. Fig. 4 shows that the C_{18} column provided the greatest solute selectivity, irrespective of the nature of the organic modifier added to the mobile phase. In contrast, the CN and phenyl columns were much less capable of detecting differences in solute hydrophobicity, and for a given organic modifier, the CN column was less selective than the phenyl column (Fig. 4.). It is interesting to note that acetonitrile provided the greatest selectivity on the C_{18} column and the lowest selectivity on the phenyl and CN columns, compared with the other two modifiers stud-

ied. These latter observations were particularly useful in the rational design of the conditions for a multi-dimensional system.

Multi-dimensional system

The investigations described up to this point were restricted to the analysis of synthetic CBI-LE and CBI-ME. In the column-switching experiments, [A]ME was included because it lies between CBI-ME and CBI-LE in hydrophobicity and was a potential internal standard. Additionally, subsequent studies were conducted at $30 \pm 1\,^{\circ}C$, compared with ambient temperature, because it was found that thermostating the column improved the reproducibility of retention times. By reducing the variability in retention times due to fluctuations in ambient temperature, the size of the fraction and the number of potential interferences transferred from column 1 to column 2 could be kept to minimum. The column-switching system used in these studies (Fig. 2) employed two high-pressure valves and was designed so that the eluent from either column 1 or column 2 could be monitored. In this way the retention times and widths of peaks eluting from column 1 could be measured accurately.

When column switching is to be used for the determination of a single component in a complex matrix, it may be necessary to maximize the resolution on both columns. However, if two or more components in the mixture are to be measured, then a somewhat different approach may be needed if resolution is to be achieved in a reasonable analysis time. The most obvious approach of switching each compound of interest in turn from the first column to the second will probably result in impractical analysis times if more than three or four components are to be measured. This study attempts to address this problem using three synthetic peptides, LE, ME and [A]ME, and the results here will provide guidelines for the separation of more complex mixtures containing a larger number of analytes.

The approach taken was to identify chromatographic conditions, using the results from single-column systems, that would permit a class separation of synthetic LE, ME and [A]ME (as their CBI derivatives) from the matrix on column 1 and then to separate these three peptides from each other on column 2. At this point, the primary objective was to achieve complete separation of the three CBI-peptides from each other and from the side products of the derivatization reaction. It will be shown later that the rational optimization of the chromatography of synthetic CBI-ME, CBL-LE and CBI-[A]ME also identified the conditions that may permit the determination of ME and LE in the striatum region of the rat brain.

The problem of side-products of the NDA–CN reagent is demonstrated in Fig. 5. Fig. 5 shows the resolution of these contaminants from CBI-LE, CBI-ME and CBI-[A]ME, which are only partially resolved from each other. These side-products are believed to arise from the reaction of NDA with the cyanide ion and occur parallel with the formation of the CBI derivatives [17]. These

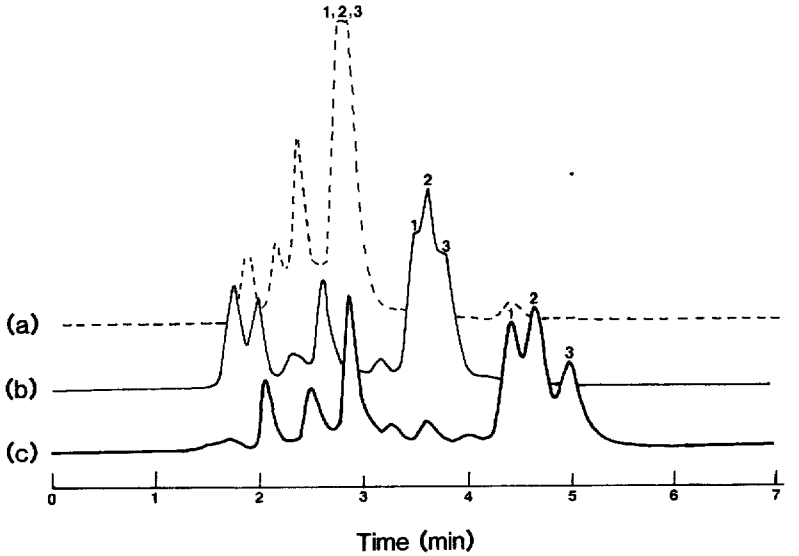

Fig. 5. Effect of acetonitrile concentration on the separation of (1) CBI-ME, (2) CBI-[A]ME and (3) CBI-LE on Spherisorb phenyl. Stationary phase: Spherisorb phenyl (5 μm, 150 mm $\times$ 4.6 mm I.D.). Mobile phases. (a) 50% acetonitrile, (b) 45% acetonitrile and (c) 40% acetonitrile in 26 m*M* TFA (pH 3.5). Flow-rate: 1.0 ml/min. Temperature: 30 $\pm$ 1 °C. The concentrations of the peptides added to the derivatization medium were approximately 25 μM, and taurine was not included in the procedure.

side-products can be minimized by quenching the reaction with an excess of taurine after completing the derivatization; however, they cannot be eliminated totally and require chromatographic resolution [18]. Quenching of side-reactions with taurine removes the excess NDA and cyanide and produces CBI-taurine, which then has to separated from the analytes. Generally, CBI-taurine elutes at the solvent front in most systems useful for the HPLC of CBI-peptides (Fig. 6); however, because its concentration is much higher than those of the analytes it can still present problems. The peak seen at the solvent front in some of the multi-dimensional separations shown in Figs. 7 and 8 represents CBI-taurine transferred from the first column.

The results illustrated in Figs. 3 and 4 revealed three key chromatographic properties of CBI-ME and CBI-LE, and these properties proved useful in the design of a multi-dimensional system. Firstly, the C_{18} column was much more retentive than the other two studied. Secondly, solute selectivity was much less on the CN and phenyl columns than on the C_{18} column. Finally, acetonitrile produced the highest selectivity on the C_{18} column and the lowest selectivity on the other two columns. Based on these observations, the multi-dimensional systems chosen for further investigation utilized ODS Hypersil as column 2 and either Spherisorb Phenyl or CPS Hypersil as column 1. Aceto-

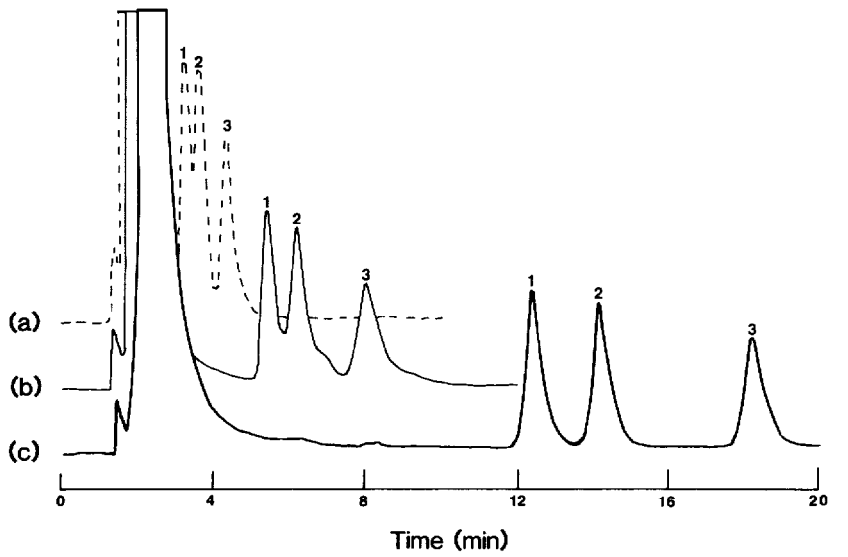

Fig. 6 Effect of acetonitrile concentration on the separation of (1) CBI-ME, (2) CBI-[A]ME and (3) CBI-LE on ODS Hypersil. Stationary phase: ODS Hypersil (5 μm, 150 mm$\times$4.6 mm, I.D.). Mobile phases: (a) 50% acetonitrile, (b) 45% acetonitrile and (c) 40% acetonitrile in 26 m*M* TFA (pH 3.5) Flow-rate: 1.0 ml/min. Temperature: 30$\pm$1°C. The concentrations of the peptides added to the derivatization medium were approximately 25 μ*M*. Taurine was included in the procedure and is the large peak eluting early in each chromatogram.

nitrile was chosen as the organic modifier used for the mobile phases eluting both columns. In addition to the selectivity advantages provided by acetonitrile, using the same modifier for both columns eliminated the problems of solvent demixing that can occur in multi-dimensional systems [7]. By using a CN or phenyl column as the stationary phase for column 1, the three solutes of interest could be transferred to column 2 in a single fraction (Figs. 5, 7 and 8). Complete resolution of the peaks from each other was then achieved on column 2, due to its higher selectivity (Figs. 6–8).

Initially, experiments were conducted using the CN phase as column 1, but this column was abandoned in favor of phenyl, which gave a better separation of the CBI-peptides from CBI-taurine. Having established the best combination of columns, the mobile phase conditions were then optimized by investigating the effects of acetonitrile concentrations used in the eluents for both columns and the results are shown in Fig. 7. In these experiments, nine chromatograms of CBI-ME, CBI-LE and CBI-[A]ME were obtained by eluting the columns with all combinations of 40, 45 and 50% acetonitrile in 26 m*M* TFA (pH 3.5). In all cases, only the eluent fraction containing the peaks of interest was transferred from column 1 to column 2 (Fig. 7). As expected, the overall retention times decreased with increasing concentrations of acetoni-

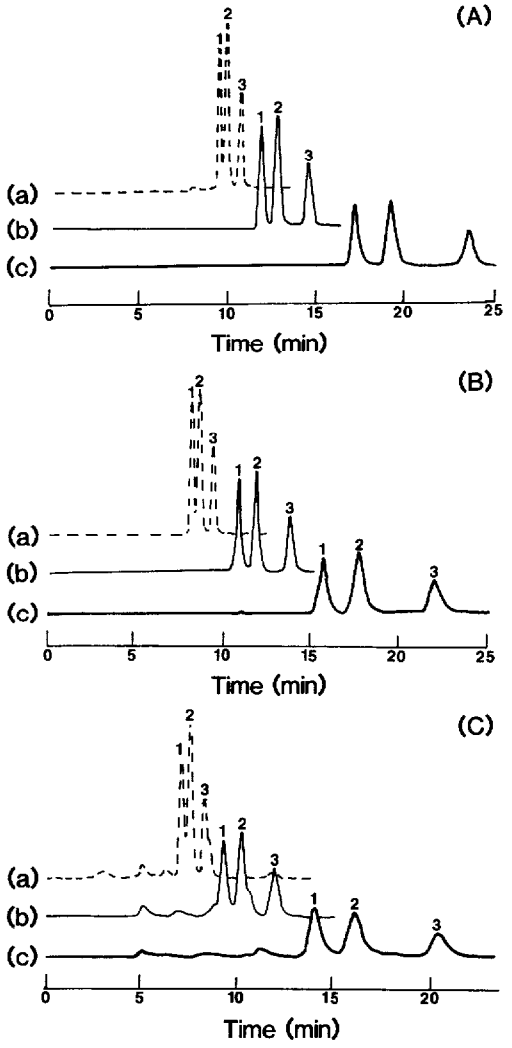

Fig. 7. Effect of acetonitrile concentration on the separation of (1) CBI-ME, (2) CBI-[A]ME and (3) CBI-LE using the column-switching system shown in Fig. 2. Column 1: Spherisorb Phenyl (5 μm, 150 mm × 4.6 mm I.D.); mobile phases for column 1 and switching times (in parentheses): (A) 40% acetonitrile (4.1–5.2 min), (B) 45% acetonitrile (3.2–4.1 min) and (C) 50% acetonitrile (2.6–3.1 min) in 26 m*M* TFA (pH 3.5). Column 2: ODS Hypersil (5 μm, 150 mm × 4.6 mm I.D.); mobile phases for column 2: (a) 50% acetonitrile, (b) 45% acetonitrile and (c) 40% acetonitrile in 26 m*M* TFA (pH 3.5). Flow-rates were 1.0 ml/min for each column. Temperature: 30 ± 1°C. The concentrations of the peptides added to the derivatization medium were approximately 25 μM, and taurine was not included in the procedure.

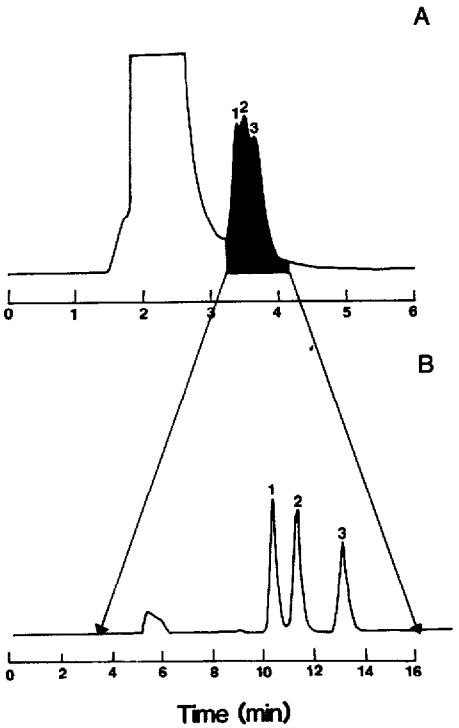

Fig. 8. Optimized separation of (1) CBI-ME, (2) CBI-[A]ME and (3) CBI-LE using the column-switching system shown in Fig. 2. Column 1: Spherisorb Phenyl (5 μm, 150 mm$\times$4.6 mm I.D.). Column 2: ODS Hypersil (5 μm, 150 mm$\times$4.6 mm I.D.). Both columns were eluted with 45% acetonitrile in 26.5 m*M* TFA (pH 3.5) at a flow rate of 1.0 ml/min. Temperature: 30 0$\pm$0.1°C. The concentrations of the peptides added to the derivatization medium were approximately 25 μ*M*. Taurine was included in the procedure and represents the large peak eluting early in the upper chromatogram.

trile used for the elution of either column. More importantly, however, the concentration of acetonitrile used to elute column 1 had a significant effect on the overall resolution of the CBI- peptides from each other and the number of components in the original matrix, which were ultimately transferred to column 2. Provided that the concentration of acetonitrile used to elute column 1 was 40 or 45%, then the three CBI-peptides could be transferred selectively to column 2. If the concentration of acetonitrile used to elute column 1 was equal to or greater than 50%, then a substantial number of components, some of which interfere with the compounds of interest, were transferred to column 2 (Fig. 7C).

The concentration of acetonitrile in the mobile phase eluting column 1 also influenced the peak shape. Provided that the acetonitrile concentration eluting from column 1 was equal to or less than that used to elute column 2, then no

loss in column efficiency occurred on the second column. This observation can be attributed to zone compression of the band at the head of column 2 [20]. The optimum multi-dimensional separation of CBI-LE, CBI-ME and CBI-[A]ME is shown in Fig. 8 and was achieved when both columns were eluted with 45% acetonitrile in TFA (26 m*M*, pH 3.5). It is interesting to note that baseline resolution of the three CBI-peptides could be achieved using column switching with sharper peaks and a shorter analysis time of 14 min compared with the 20 min required using the C_{18} column alone. In the latter case, an eluent containing 40% acetonitrile in 26 m*M* TFA (pH 3.5) was required for baseline resolution (Fig. 6).

Effects of temperature

Early in these investigations, it was found that the efficient transfer of the solutes between columns was compromised by small changes in retention times on column 1. The major cause of shifts in retention time was found to be slight changes in ambient temperature. Consequently, the volume of the fraction transferred between columns could be kept to a minimum by thermostating the columns at 30.0 ± 1 °C.

Reaction kinetics and stability of the derivatives

Fig. 9 shows that the reaction is substantially complete (> 95%) after 15 min and that the derivatives are stable for a further 45 min at 0–4 °C (ice bath).

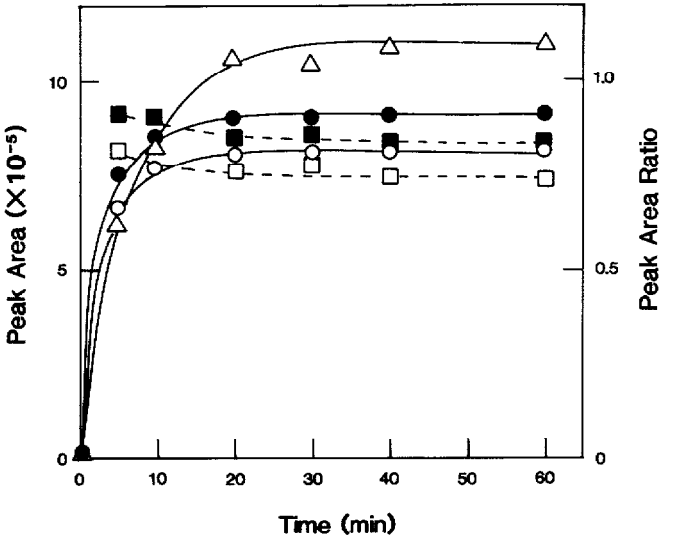

Fig. 9. Formation kinetics of CBI-ME(●), CBI-[A]ME (△) and CBI-LE (○). The peak-height ratios CBI-ME/CBI-[A]ME (■) and CBI-LE/CBI-[A]ME (□) are also shown. Chromatographic and derivatization conditions as Figs. 8 and 1.

The reaction was quenched after 20 min by the addition of excess taurine, which eliminated the residual NDA–CN, without affecting the peak heights of the derivatives (Fig. 9). The reaction of the NDA–CN with taurine was complete within 10 min. However, storage of the CBI derivatives for 5 h at 0–4 °C resulted in significant degradation of the synthetic CBI-ME (25%) and CBI-[A]ME (10%). Because CBI-LE was stable under these conditions, and because the degradation of synthetic CBI-ME and CBI-[A]ME was prevented by the addition of an antioxidant, ascorbic acid, the degradation of CBI-ME and CBI-[A]ME was attributed to the oxidation of the methionine residues to their corresponding sulfoxides. Provided ascorbic acid was added to the solutions, each of the CBI derivatives of LE, ME and [A]ME was stable (less than 2% loss) at 4 °C for at least 6 h.

Determination of LE- and ME-like fluorescence in rat striatum: preliminary results

The use of pre-column derivatization with NDA–CN (Fig. 1) coupled with multi-dimensional chromatography appears to hold great promise for the determination of peptides in biological matrices. The derivatization reaction is rapid (15 min) and the derivatives are stable for at least 6 h. The method is now being applied to the determination of LE and ME in striatum of the rat brain and preliminary results are presented in this communication.

Fig. 10A shows a chromatogram of an extract of rat striatum, after derivatization with NDA–CN, and Fig. 10B shows the same extract after spiking with synthetic samples of CBI-ME and CBI-LE. Both samples illustrated in Fig. 10A and B contain the same concentration of the internal standard, [A]ME. Peaks 1 and 3 in Fig. 10A have the same retention times as authentic samples of synthetic CBI-ME and CBI-LE, respectively, and there is no evidence of any interfering peaks in the chromatograms of striatum extracts. If the striatum samples were not treated with acid immediately after dissection, then peaks 1 and 3 (Fig. 10) disappeared rapidly with time, presumably as a result of enzymatic degradation. It should be noted that it is highly unlikely that peaks 1 and 3 are not representative of endogenous ME and LE, respectively, because their corresponding CBI derivatives are eluted from a multi-dimensional system. For the peaks to have the same retention times as their corresponding standards in the multi-dimensional system, they must have the same retention times in the two different chromatographic systems, which is generally considered to be good evidence for proof of structure. It is also important to note that the fluorescent peaks 1 and 3 (Fig. 10) must contain a primary amino group, because NDA–CN is highly specific for this moiety.

The extraction method for the enkephalins was identical to that described by Kai et al. [6] who used the procedure as part of an HPLC assay for LE in rat brain parts. Results from this present study confirm those obtained previously [6] that the recovery of the small enkephalin-like peptides from rat brain

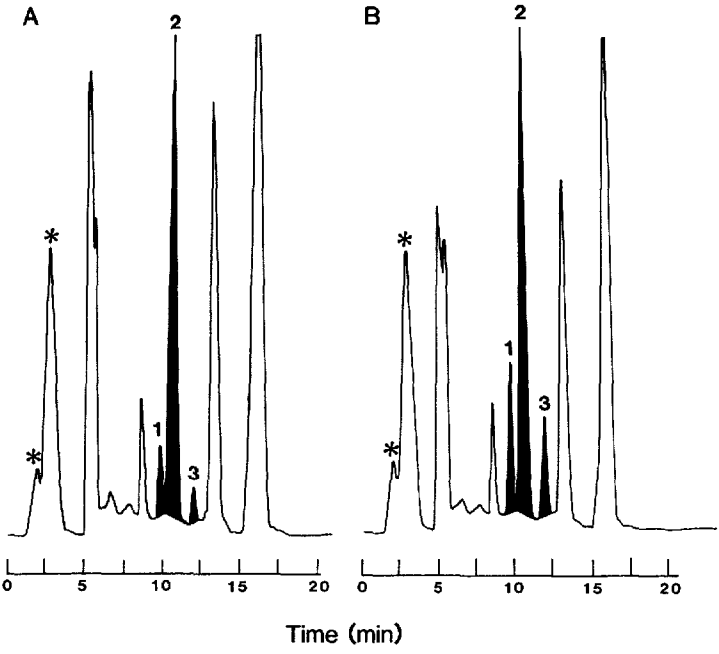

Fig 10 Chromatograms of LE-like fluorescence (1) and ME-like fluorescence (3) in the striatum region of the rat brain. Chromatographic and derivatization conditions as Figs. 8 and 1. Chromatogram A shows apparent endogenous levels of 429 pmol ME and 130 pmol LE per g of wet tissue. Peak 2 corresponds to the CBI derivative of the synthetic peptide, CBI-[A]ME, which was used as internal standard Chromatogram B is the same as chromatogram A except 2 pmol of CBI-ME and 2 pmol of CBI-LE were added to the 20-μl solution immediately prior to injection. The three peaks of interest have been highlighted for clarity. The two peaks seen at the beginning of each chromatogram (*) are actually late eluting components from the previous injection.

parts using this procedure is approximately 80%. In the present study, the recovery was determined by comparing the slope of the calibration curve prepared in spiked brain tissue with the slope obtained from buffered (pH 7.4) aqueous solutions. The present method is linear by peak-height ratio measurements ($n=5$, $r^2=0.998$) in both aqueous standard solutions and spiked striatum homogenates over the range 0–10 pmol of CBI-peptide injected (20 μl injected).

Fig. 10 shows a chromatogram of an extract of rat striatum after derivatization with NDA–CN. The apparent concentration of LE in the striatum (130 pmol/g of wet tissue) found in this present study agrees well with the values reported previously by Kai et al. [6]. The apparent concentration of ME in the striatum was found to be 429 pmol/g of wet tissue. In rat striatum, the limits of detection of LE- and ME-like fluorescence are both 100 fmol (signal-to-noise ratio=3), using an injection volume of 20 μl and a conventional fluorescence detector. This value for the limit of detection corresponds to quan-

tifiable levels of LE- and ME-like fluorescence of approximately 25 pmol/g of wet tissue. No attempts were made to increase the sensitivity by increasing the injection volume since the detection limits are quite sufficient for the simultaneous determination of LE- and ME-like fluorescence in the striatum of the rat brain.

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REFERENCES

1 R.C.A. Frederickson and L.E. Greary, Prog. Neurobiol., 19 (1982) 19.
2 P. Angwin and J.D. Barchas, J. Chromatogr., 231 (1982) 173.
3 H. Sato, Y. Sugiyama, Y. Sawada, T. Iga and M. Hanano, Life Sci., 35 (1984) 1051.
4 D.M. Desiderio, H. Takeshita, H. Onishi, G. Fridland, F.S. Tanzer, C. Wakelyn and C. Dass, NIDA Monogr., 70 (1986) 304.
5 R.W. Hendren, NIDA Monogr., 70 (1986) 255.
6 M. Kai, J. Ishida and Y. Ohkura, J. Chromatogr., 430 (1988) 271.
7 J F.K. Huber, R. van der Linden, E. Ecker and M. Orleans, J. Chromatogr., 83 (1973) 267.
8 C.M. Riley, L.A. Sternson, A.J. Repta and R.W. Siegler, J. Chromatogr., 299 (1982) 373.
9 C.M. Riley, L.A. Sternson and A.J. Repta, J. Pharm. Sci., 72 (1983) 351.
10 P. deMontigny, J.F. Stobaugh, R.S. Givens, R.G Carlson, K. Srinivasachar, L.A. Sternson and T. Higuchi, Anal. Chem., 59 (1987) 1096.
11 W. Ried and H. Boden, Chem. Ber., 89 (1956) 708.
12 R.G. Carlson, K. Srinivasachar, R.S. Givens and B.K. Matuszewski, J. Org. Chem., 51 (1986) 3978.
13 B.K. Matuszewski, R.S. Givens, K. Srinivasachar, R.G. Carlson and T. Higuchi, Anal. Chem., 59 (1987) 1102.
14 M.C. Roach and M.D. Harmony, Anal. Chem., 59 (1987) 411.
15 R.S. Givens, R.L. Schowen, J.F. Stobaugh, T. Kuwana, F. Alvarez, N. Parekh, B.K. Matuszewski, T. Kawasaki, O. Wong, M. Orlovic, H. Chokshi and K. Nakashima, Luminescence Applications in Biological, Chemical, Environmental, and Hydrological Sciences, American Chemical Society, Washington, DC, 1989 p. 127.
16 N. Hanaoka, R.S. Givens, R.L. Schowen and T. Kuwana, Anal. Chem., 60 (1988) 2193.
17 P. de Montigny, C.M. Riley and J.F. Stobaugh, J. Pharm. Biomed. Anal., submitted for publication.
18 M. Roth, Anal. Chem., 43 (1971) 880.
19 P.A. Bristow, P.N. Brittain, C.M. Riley and B.F. Williamson, J. Chromatogr., 131 (1977) 57
20 S A. Tomellini, S.-H. Hsu and R.A. Hartwick, Anal. Chem., 58 (1986) 904.