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Multidimensional liquid chromatography of opioid peptides: fluorogenic labelling, retention prediction and separation optimization*

L.M. NICHOLSON,‖ H.B. PATEL,** F. KRISTJANSSON,‡** S.C. CROWLEY JR,‡§ K. DAVE,** J.F. STOBAUGH** and C.M. RILEY†**

‖*Fort Hays State University, 600 Park Street, Hays, KS 67601-4099, USA*
** *Department of Pharmaceutical Chemistry and the Center for Bioanalytical Research, Malott Hall, University of Kansas, Lawrence, KS 66045-2504, USA*

Abstract: The ultra-trace analysis of opioid peptides in biological samples can be achieved by multidimensional liquid chromatography with pre-column fluorogenic derivatization with naphthalene-2,3-dicarboxaldehyde in the presence of cyanide ion. However, in order to take full advantage of the high sensitivity possible with detectors based on laser-induced fluorescence or chemiluminescence, each component of the analytical method must be carefully optimized. In this study, strategies are presented for the prediction of retention time and the optimization of separations of derivatized opioid peptides in multidimensional LC systems.

Keywords: *Opioid peptides; liquid chromatography; column switching; retention prediction; optimization; pre-column derivatization; naphthalene-2,3-dicarboxaldehyde/cyanide; fluorescence detection.*

Introduction

The quantitative determination of opioid peptides in biological fluids and tissues constitutes a significant bioanalytical challenge, because exceptionally low concentrations of the analytes have to be determined against a high background of potentially interfering compounds. For example, the concentration of met-enkephalin (ME) in various regions of the mammalian brain ranges 100–1000 pmol g^{-1} [1] and the circulating concentration of ME in human plasma is approximately 100 fmol ml^{-1} [2]. In contrast, the concentrations of the amino acids, which can interfere with methods of analysis involving chemical derivatization, are many orders of magnitude higher [3, 4]. For example, Shiekhatter *et al.* [3] have shown that the extracellular concentrations of amino acids in the caudate region of the rat brain range from 4.5 nmol ml^{-1} for phenylalanine to 91.2 nmol ml^{-1} for taurine, and Tossman *et al.* [4] have shown that tissue levels of amino acids in this region of the rat brain range from 600 nmol ml^{-1} for alanine to 20 μmol ml^{-1} for taurine.

Several techniques have been described for the quantitative determination of the opioid peptides in biological samples, including radioreceptor assays [5], liquid chromatography with detection by mass spectrometry (LC–MS) or off-line radioimmunoassay (LC–RIA) [6]. Recently, two groups [8–12] have described LC methodology with fluorescence detection for the determination of ME and leucine-enkephalin (LE) in various regions of the rat brain. Kai and co-workers [8–11] have presented a gradient-LC method for the determination of small enkephalins in rat brain parts, based on fluorogenic derivatization of the tyrosyl residue with 1,2-diamino-4,5-methoxybenzene [13]. Mifune *et al.* [12] have described an alternative method for the determination of ME, and ME in the striatum of the rat brain, that employs fluorogenic derivatization (Fig. 1) with naphthalene-2,3-dicarboxaldehyde in the presence of cyanide ion (NDA/CN).

One of the problems associated with the NDA/CN reagent is the production of side-products arising from the reaction of NDA and the cyanide ion [15]. Unfortunately, many of these compounds are fluorescent and can

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†Author to whom correspondence should be addressed.
‡Present address: Delta Ltd, Reykjavikurvegi, Iceland.
§Present address: Boehringer-Ingelheim Animal Health, St Joseph, MO, USA.

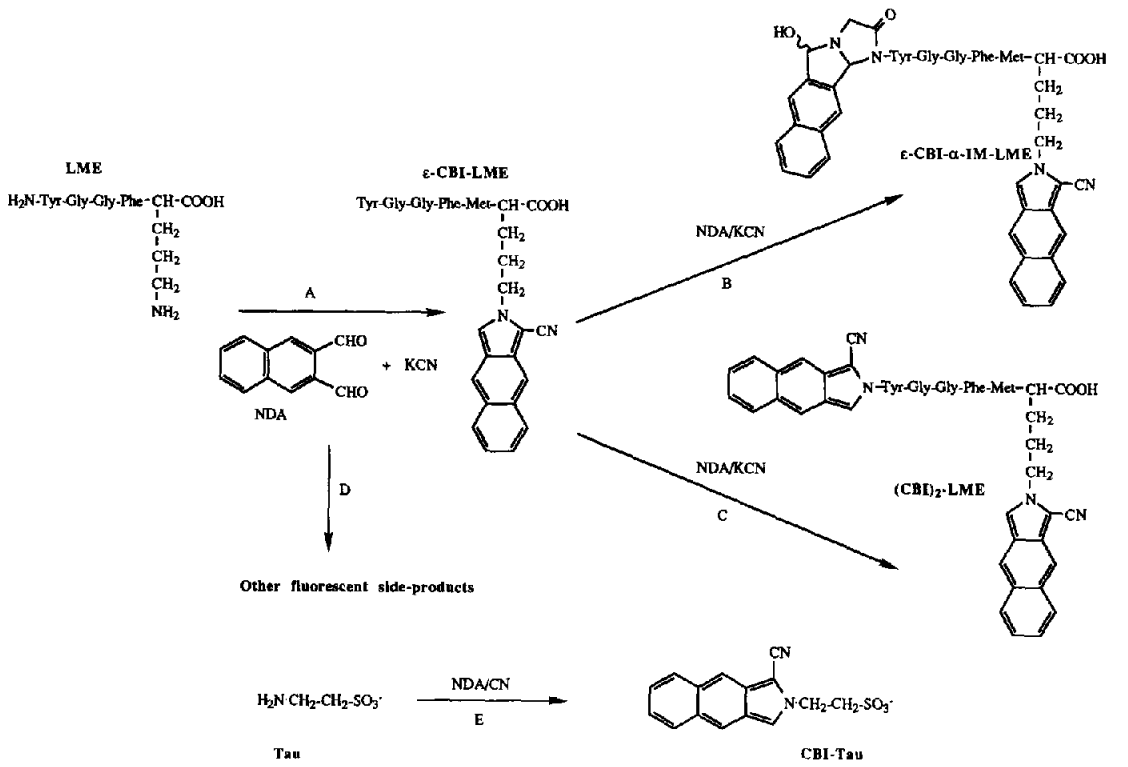

Figure 1
Potential reactions of the opioid peptide LME and the amino acid taurine (Tau) with NDA/CN.

interfere with the LC analysis of the compounds of interest. A second problem of NDA/CN is that associated with the derivatization of peptides with two or more primary amino groups, (i.e. those that in addition to the terminal α-amino group also contain one or more lysyl residues, cf. Fig. 1).

Experimental

Chemicals and reagents

The following opioid peptides were obtained from Sigma (St Louis, MO, USA) and were used as received: ^{5}Met-enkephalin (ME, Tyr-Gly-Gly-Phe-Met), ^{5}Leu-enkephalin (LE, Tyr-Gly-Gly-Phe-Leu), D-^{2}Ala-^{5}Met-enkephalin (Tyr-D-Ala-Gly-Phe-Met, AME) and ^{6}Lys-^{5}Met-enkephalin (Tyr-Ala-Gly-Phe-Met-Lys, LME). The NDA was obtained from Oread Laboratories (Lawrence, KS, USA) and was used as received. The acetonitrile and methanol were of LC grade and were obtained from Fisher Scientific (St Louis, MO, USA). Distilled, deionized water was used throughout. The other chemicals were of the highest purity available and used as received from various sources.

Stock solutions

Stock solutions (250 μM and 5 mM) of NDA were prepared in acetonitrile on a weekly basis in low-actinic (red) glassware and were protected from light in a refrigerator at 4°C [12] when not in use. Aqueous stock solutions (5 mM) of KCN, ascorbic acid (200 mM), taurine (200 mM), phosphate buffer (pH 6.8, 25 mM) and borate buffer (pH 10.0, 6.5 mM) were prepared as required. Standard aqueous stock solutions of ME (25 μM), LE (25 μM), AME (25 μM) and LME (20 μM) were prepared in 10 mM ascorbic acid and were stored at −20°C when not in use.

Liquid chromatography

The following columns were used in these investigations: ODS Hypersil (C_{18}), Hypersil (CN) and Spherisorb Phenyl (phenyl) (each 5 μm, 150 × 4.6 mm i.d.). The phenyl and C_{18} columns were packed in the upward direction 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, USA).

The chromatographic system described previously by Mifune *et al.* [12] was used in these investigations. This column-switching system consisted of two Shimadzu Model LC-6A pumps, two Shimadzu Model FCV-2AH high-pressure switching valves, a Shimadzu Model CTO-6A column oven and Shimadzu Model RF-530 fluorometric detector (xenon lamp; λ_{ex}, 420 nm; λ_{em}, 490 nm). Injections (20 μl) were made with a Shimadzu Model SIL-6A autoinjector and 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. All chromatographic separations were conducted at 30.0 ± 0.1°C and a flow rate of 1 ml min^{-1}. The mobile phase consisted of trifluoroacetic acid (TFA) (25 mM, adjusted to pH 3.5 with 1.0 M KOH) and varying concentrations of acetonitrile added as an organic modifier, as described below.

Derivatizationn procedures

The four peptides were converted to their corresponding fluorescent *N*-substituted-1-cyanobenz[f]isoindoles (CBI) derivatives by reaction with NDA in the presence of potassium cyanide (Fig. 1). LME was derivatized in a borate buffer at pH 10.0 and the remaining peptides were derivatized in a phosphate buffer at pH 6.8. LME was derivatized by mixing the following solutions in order to give a final volume of 1 ml (the final concentrations of each component are given in parentheses): 25 μl, 200 mM ascorbic acid (5 mM); 20 μl, 20 mM LME (400 mM); 20 μl, 5 mM KCN; 130 μl, 50 mM borate buffer (6.5 mM); 780 μl, acetonitrile–water (8:2, v/v) and 20 μl of 5 mM NDA (100 μM). These solutions were mixed by inversion and incubated on ice (4°C) for 20 min. The reaction was then quenched by the addition of 5 μl of 200 mM taurine (1 mM), mixing and then incubating at 4°C for a further 30 min.

ME, LE and AME were derivatized together in the same solution by mixing, in order: 50 μl, 10 mM ascorbic acid (500 μM); 50 μl, 25 μM AME; 100 μl of a solution containing 25 μM of both LE and ME (1 μM); 500 μM KCN (50 μM); 50 μl acetonitrile–water (5:95, v/v); 500 μl, 50 mM phosphate buffer (pH 6.8) (25 mM); and 200 μl of 250 μM NDA (50 μM). These solutions were mixed by inversion and incubated on ice (4°C) for 20 min. The reaction was then quenched by adding 50 μl of 20 mM taurine (1 mM), mixing and then incubating at 4°C for 10 min. To prepare a mixture of all four peptides, 1 part of the CBI–LME solution was mixed with 5 parts of solution of the other three CBI-derivatives to give final concentrations of CBI–LME (66.7 mM), CBI–ME (333 nM), CBI–LE (333 nM) and CBI–AME (167 nM).

Kinetic experiments

When studying the kinetics of CBI–LME formation and degradation, the derivatization procedure described above was modified by the addition of different concentrations of either acetonitrile or methanol. In these cases, the total volume of the solution was maintained at 1 ml by the addition of water. Aliquots (50 μl) of the unquenched reaction mixture were removed at various times after the reaction had been initiated by the addition of NDA. For the purposes of these kinetic experiments, the chromatographic separation (Fig. 2) was accomplished on the phenyl column with a mobile phase of acetonitrile–TFA (pH 3.5, 25 mM) (55:45, v/v).

Results and Discussion

Derivatization of ^{6}lysine-^{5}methionine-enkephalin (LME)

Figure 1 exemplifies a number of unresolved issues concerning the general application of NDA/CN to the pre-column derivatization of peptides. Figure 2 also serves to highlight a particular problem in the derivatization of lysyl-peptides that contain two or more potential sites of reaction. Recent experience [12, 14, 15] with NDA/CN has shown that both the rate and the yield of the reaction are highest when the pH of the solution is equal to the pK_a of the amine to be derivatized. Therefore in the present study, the peptides containing only one primary α-amino group at the *N*-terminus (LE, ME and AME) were derivatized at pH 6.8. The lysine-containing peptide LME was reacted with NDA/CN at a pH of 10.0, which corresponds approximately to the pK_a of the ε-amino group on the side-chain.

Figure 2(A) shows that a single peak is produced when NDA/CN is reacted with a 1000-fold molar excess of the peptide (LME). Because the conditions were chosen which would greatly favour reaction at the ε-amino group ($pK_a \approx$ pH = 10), the single peak in

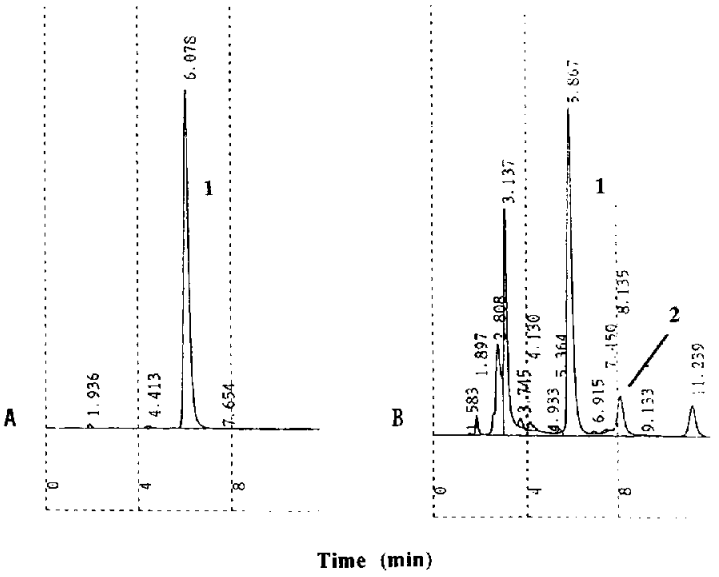

Figure 2
Chromatograms obtained after: (A) reacting NDA/CN with a 1000-fold excess of LME; and (B) reacting LME with a 1000-fold excess of NDA/CN. Chromatographic conditions: Spherisorb Phenyl (5 μm, 150 × 4.6 mm i.d.) eluted with acetonitrile–TFA (pH 3.5, 25 mM) (55:45, v/v) at a flow rate of 1.0 ml min^{-1} and 30.0 ± 0.1°C. Peaks 1 and 2 were not present in the derivatization blanks and have been attributed to the reaction of LME and NDA/CN. The relationships between the peak areas of 1 and 2 and time are shown in Figs 3 and 4.

Fig. 2(A) was attributed to the ϵ-amine-labelled CBI-derivative (ϵ-CBI–LME). In contrast, when more realistic analytical conditions were used for the derivatization of LME (i.e. 1000-fold excess of NDA/CN), several peaks were seen in the chromatogram (Fig. 2B) in addition to the single peak seen in Fig. 2(A). Peaks 1 and 2 (Fig. 2B) were not seen in the blank and were attributed to the reaction of LME with NDA/CN. The remaining fluorescent peaks in the chromatogram were attributed to side products of NDA/CN that were produced in the absence of an added amine. Both chromatograms shown in Fig. 2 were injected within 2 min of initiation of the reaction, and if the reaction was allowed to proceed for longer periods of time, then the peaks heights of the side-products increased continuously.

Figures 2 and 3 show that irrespective of whether the analyte (LME) or the reagent (NDA/CN) was in excess, the reaction of LME with NDA/CN was essentially instantaneous. With limiting NDA/CN, the 10% decrease in the area of peak 1 attributed to ϵ-CBI–α-IM-LME after 6 h was explained by the gradual oxidation of the methionine group [12]. By contrast, when the reagent was in excess, peak 1 declined exponentially with a half-life of approximately 25 min. The decrease in the height of peak 1 was accompanied by an increase in the height of peak 2 (Fig. 2B), which was attributed to the reaction of the α-amino group of ϵ-CBI–LME with NDA/CN.

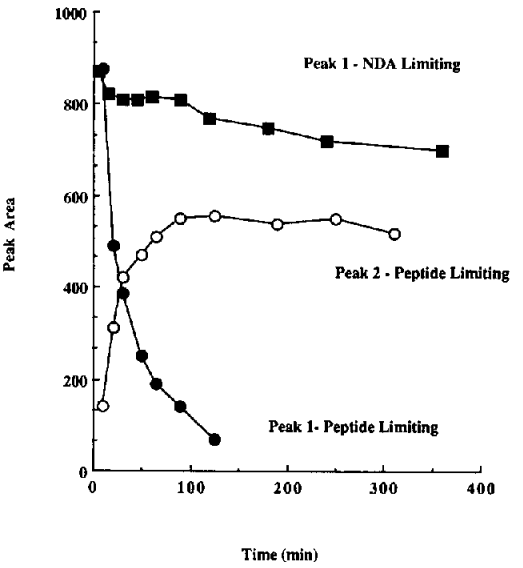

Figure 3
Kinetics of the reaction of the opioid peptide LME with NDA/CN under the conditions of either: (A) excess peptide (squares); or (B) excess NDA/CN (circles) with reference to the data shown in Fig. 2. With excess peptide, only peak 1 (■) was detected. With excess NDA/CN, peak 1 (●) was detected initially and then converted to peak 2 (○).

One structure that could be assigned to peak 2 is the bis-CBI-derivative of LME [i.e. $(CBI)_2$–LME]. However, de Montigny *et al.* [14] have shown that under alkaline conditions (pH ≫ pK_a), the α-amino group of the peptide will react with NDA to produce a non-fluorescent imidazoyl derivative. Therefore, a more likely structure for peak 2 is the ε-CBI–α-imidazoyl-derivative (ε-CBI–α-IM-LME) shown in Fig. 1. Interestingly, the area of peak 2 at the end of the reaction (Fig. 3) was only 60% of the initial area of peak 1. This reduction in response was attributed to quenching of fluorescence arising from intramolecular interactions between the polyaromatic ring systems of either $(CBI)_2$–LME or ε-CBI–α-IM-LME.

The precise structural assignment of peak 2 was considered to be beyond the scope of this study, because the primary focus was the practical application of the NDA/CN reagent system to the pre-column derivatization of the peptides. Nevertheless, this aspect of NDA/CN chemistry is the subject of extensive research in the authors' laboratories, for which the results will be presented at a later date. Irrespective of its structure, the production of the bis-derivative, as well as the production of the side-products arising from the reagent itself, could be prevented by the addition of a quenching reagent such as taurine (Fig. 1). Under the experimental conditions shown in Figs 2 and 3 (pH 10.0, 20% acetonitrile), the production of peak 1 and its subsequent conversion to peak 2 were too rapid to allow the addition of taurine in a timely enough fashion to permit the reproducible trapping of the analyte in its mono-CBI form. On the other hand, the reactions leading to the production of peak 2 were too slow and by the time they were complete (ca. 3 h) the production of side-products from the reagent itself was substantial. Additionally, the area of peak 2 was less than that of peak 1. For these reasons, allowing the reaction to proceed to completion was not realistic.

Figures 4 and 5 show that the reactions of NDA/CN with LME giving rise to peaks 1 and 2 can be slowed significantly by the addition of either methanol or acetonitrile. In addition, Figs 4 and 5 show that these reductions in rate can be achieved without influencing the yield (maximum height) of the more analytically useful product, peak 1. Accordingly, the optimum solvent system for the derivatization of LME to ε-CBI–LME (peak 1) was acetonitrile–borate buffer (pH

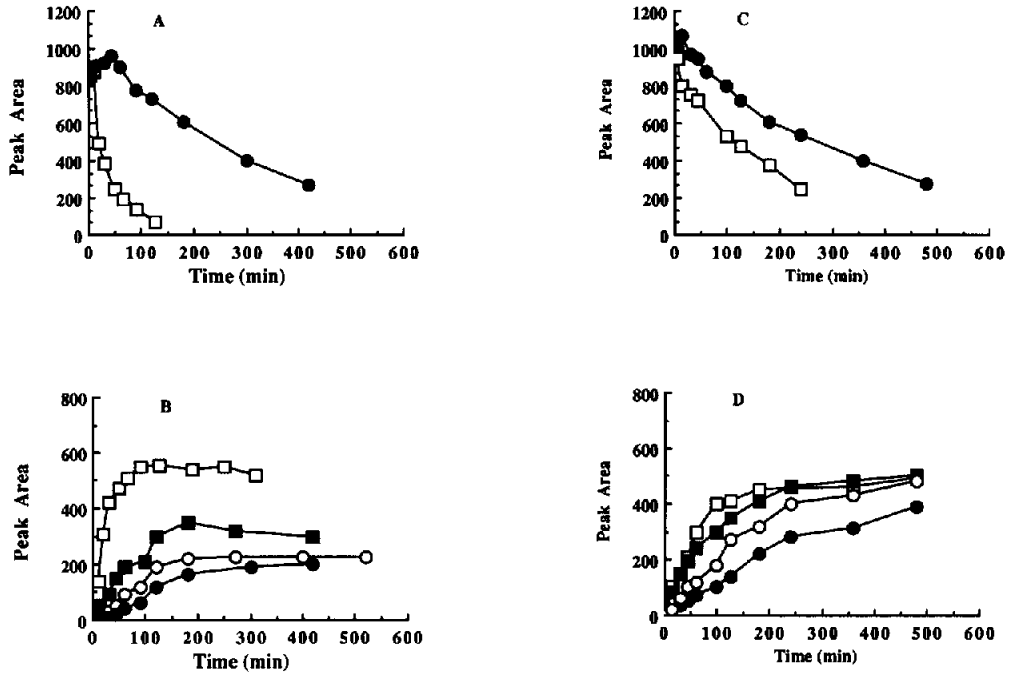

Figure 4
Effects of acetonitrile (A and B) and methanol (C and D) on the kinetics of peaks 1 [(ε-CBI–LME) and 2 (ε-CBI–α-IM-LME or $(CBI)_2$–LME)] shown in Fig. 2. The concentrations of the organic solvents were: 20 (●); 40 (○); 60 (■); and 80% (□) (v/v). Some overlapping data have been omitted for clarity.

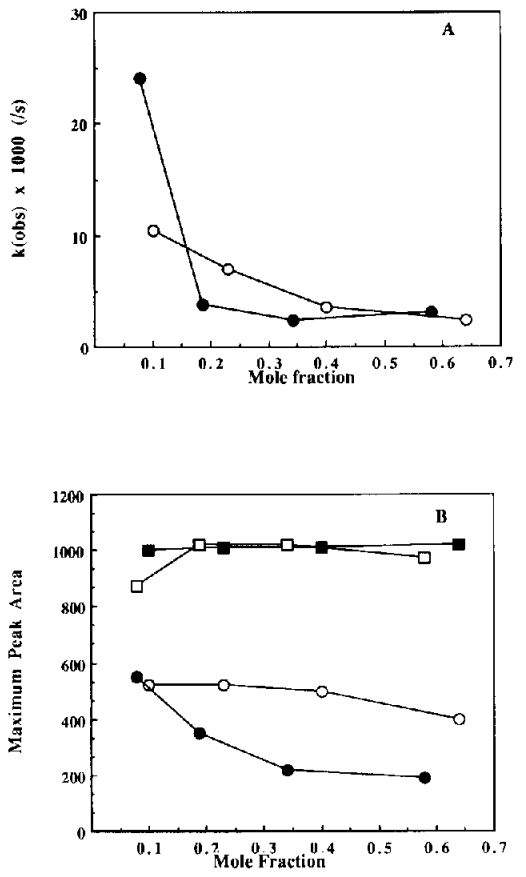

Figure 5
Effects of acetonitrile (closed symbols) and methanol (open symbols) on: (A) the pseudo-first order (k_{obs}) degradation of peak 1 (ε-CBI–LME); and (B) the yield of peaks 1 (ε-CBI–LME) and peak 2 shown in Fig. 2.

10) (8:2, v/v). The reaction of LME with NDA/CN was allowed to proceed for 20 min, after which it was quenched by the addition of taurine. The production of side-products could be further minimized by conducting the reaction at 4°C [12].

Chromatographic investigations

Quenching of the NDA/CN reagent with taurine resulted in the production of CBI–taurine, which has very similar spectroscopic properties to the analytes of interest [14] (λ_{ex}, 420 nm; λ_{em}, 490 nm). Being more polar, CBI–taurine tends to elute close to the solvent front under the reversed-phase LC conditions that are generally suitable for the retention of the CBI-derivatives of the opioid peptides LE, ME, AME and LME [12]. Unfortunately, the concentration of CBI–taurine in the final solution is much greater than those of the peptides being determined and the very large peak arising from CBI–taurine often obscures the analytes of interest. Recently, Mifune *et al.* [12] have demonstrated the value of multidimensional reversed-phase LC (i.e. with column switching) for the resolution of three opioid peptides ME, AME and LE (as their CBI-derivatives) from a large excess of CBI–taurine. In that procedure, the CBI-peptides were resolved from the CBI–taurine on Spherisorb Phenyl or CPS Hypersil columns and then switched to an ODS Hypersil column where they were resolved from each other. The higher peak capacity of the multidimensional approach compared with a single-column system also provided the resolution required for the determination of LE and ME (as their CBI-derivatives) in the striatum region of the rat brain, using CBI–AME as an internal standard [12].

The complexity of multidimensional LC systems and the difficulties posed in developing rational optimization strategies are illustrated in Fig. 6. In addition to the stationary phase type and temperature, at least five mobile phase variables (organic-modifier type, organic-modifier concentration, pH, buffer type and buffer concentration) may have to be considered in the optimization of a single-column reversed-phase system. If ion-pair chromatography were to be considered, then pairing-ion type and concentration would have to be added to the list, giving a total of eight variables. In a multidimensional system the number of possible variables, which determines the complexity of the system and the time required for optimization, is equal to the

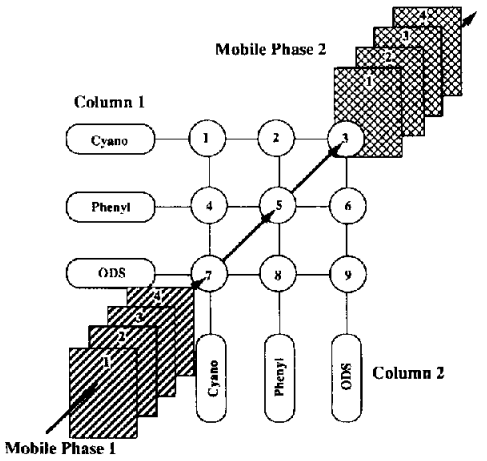

Figure 6
Summary of the various combinations of mobile phase and stationary phases possible in a two-column system.

product of the number of variables available in each single-column system. In the earlier report [12], Mifune *et al.* have shown that the data generated from experiments on the individual columns (Spherisorb Phenyl, CPS Hypersil and ODS Hypersil) could be used to optimize the separation of the CBI-peptides in a multidimensional, two-column system. However, the previous approach was empirical and the objectives of this study were to develop: (a) a theory for the prediction of retention time in a two-column system from data derived from measurements made on the individual columns; and (b) a method of data analysis for the optimization of multidimensional, two-column separations.

The same three reversed-phase columns [Spherisorb Phenyl, ODS Hypersil (C_{18}) and CPS Hypersil (CN)] were used throughout. The columns were eluted with mobile phases of trifluoroacetic acid (TFA) (25 mM, pH 3.5) containing various concentrations of acetonitrile. All the experiments were conducted at 30.0 ± 0.1°C.

Prediction of retention in multidimensional liquid chromatography

Initially, the retention of CBI–ME, CBI–AMI and CBI–LME was characterized by determining their capacity ratios on three single columns eluted with at least four concentrations of acetonitrile (Table 1). The data obtained on the individual columns were then used to predict the retention times of the four peptides in 28 multidimensional systems, chosen in random fashion. The retention data obtained in the single-column experiments were fitted to equation (1) by polynomial regression using the software "CricketGraph" and a Macintosh IICX (Apple Computers) personal computer:

$$\ln k' = B_1\Phi + B_2\Phi^2 + \ln k'_o, \qquad (1)$$

where Φ is the volume fraction of acetonitrile in the mobile phase. The values of the coefficients B_1, B_2 in the $\ln k'_o$ (equation 1) for each solute on each column are shown in Table 1. The squared coefficient of correlation (r^2) was >0.999 for each fit. Evaluation of the intercept terms, $\ln k'_o$, revealed that the order of retention of the solutes on the three columns was C_{18} > phenyl > CN. Evaluation of the values for the B_1 terms permitted the sensitivity of the retention of solutes to change in acetonitrile concentration to be determined. This analysis revealed that retention of the solutes was most sensitive to change in concentration of acetonitrile on the C_{18} column. The sensitivity of retention to change in acetonitrile was similar on the other two columns studied. Theoretically, these results suggest that variability of retention time arising from repetitive preparation of mobile phase would be the same for the CN and phenyl columns, and lower on these two columns than on the C_{18} column.

Two different methods were used to compare the retention of the four solutes and the selectivity of the three stationary phases with respect to the CBI-peptides (Table 2). The first method involved comparison of retention and selectivity for the three columns when eluted with the same mobile phase (Φ = 0.4). This analysis revealed that the order of retention for the solutes on the three columns was C_{18} > phenyl > CN, consistent with the previous analysis of the intercept terms, $\ln k'_o$. Because selectivity increased with increasing retention, comparison of the selectivity of the three phases was achieved by normalizing the retention to a k' value of 5 for CBI–AME. Normalization of the retention revealed that the selectivity of the three phases differed for the four CBI-derivatives studied. In particular, the three α values describing the separation of all the four components were very similar on the phenyl column. In contrast similar α values were obtained for the separation of the first three components on the cyano and C_{18} phases. However, a much larger value of α was obtained for the separation of CBI–AME and CBI–LME on the CN column compared with

Table 1
Regression coefficients (equation 1) describing the retention of four synthetic peptides on three stationary phases

Stationary phase	Solute	$\ln k'_o$*	B_1*	B_2*
CN	CBI–ME	8.92	−30.75	24.68
	CBI–AME	7.68	−23.59	15.34
	CBI–LE	8.56	−27.00	19.16
	CBI–LME	9.83	−30.99	23.55
Phenyl	CBI–ME	10.69	−34.70	27.11
	CBI–AME	10.73	−34.23	26.35
	CBI–LE	11.20	−35.21	26.80
	CBI–LME	11.34	−35.73	27.98
C_{18}	CBI–ME	20.25	−70.17	61.39
	CBI–AME	20.85	−71.99	63.22
	CBI–LE	21.50	−71.99	63.22
	CBI–LME	19.49	−65.02	55.02

*As defined in Table 1.

Table 2
Retention data for the CBI-derivatives of four opioid peptides on three stationary phases, under conditions of (A) constant mobile phase composition ($\Phi = 0.4$) and (B) normalized retention

Stationary phase solute	k' ($\Phi = 0.4$)	α	k' ($k'_{CBI-AME} = 5$)*	α
Cyano				
CBI–ME	1.77	1.12	4.48	1.12
CBI–AME	1.99	1.15	5.00	1.19
CBI–LE	2.29	1.31	5.93	1.55
CBI–LME	2.99		9.21	
Phenyl				
CBI–ME	3.16	1.12	4.48	1.12
CBI–AME	3.53	1.17	5.00	1.19
CBI–LE	4.08	1.13	5.93	1.12
CBI–LME	4.60		6.68	
C_{18}				
CBI–ME	7.41	1.15	4.36	1.15
CBI–AME	8.54	1.19	5.00	1.13
CBI–LME	10.17	1.59	5.63	1.70
CBI–LE	16.17		9.58	

*Cyano, $\Phi = 0.327$; phenyl, $\Phi = 0.374$; C_{18}, $\Phi = 0.428$.
k' values calculated from equation (1) with the coefficients from Table 1.

that on the phenyl column, and the retention order of these two compounds was reversed on the C_{18} column compared with that of the other two.

The retention data obtained in single-column systems (Table 1) were then used to develop a general theory that would predict the retention times of the solutes in a multi-dimensional system. The retention times of the solutes were determined in 27 different column-switching systems. These systems were chosen at random and the only restriction placed on the arrangement of columns was that the peaks were always switched to a more retentive column, because previous experience had shown [12] that this was necessary to ensure complete zone compression. Accordingly, the following column arrangements were studied with a variety of mobile phases: cyano→phenyl, cyano→C_{18} and phenyl→C_{18}. The four solutes were initially injected as a mixture onto the first column to determine the switching-time window. They were then re-injected and switched as a single fraction to the second column as they eluted from first column. The peaks were tracked, if necessary, by injecting separately or in pairs.

Initial attempts to predict the retention of the CBI-peptides in the column-switching system involved a simple assumption that the overall retention time (t_{tot}) was equal to the sum of the retention times on the individual columns (t_{11} and t_{22}), plus the time spent in the switching valve and associated tubing (t_{ext}), i.e.

$$t_{tot} = t_{11} + t_{22} + t_{ext}. \quad (2)$$

Analysis of the data acquired according to equation (2) gave the following relationships between the observed ($t_{tot,obs}$) and predicted ($t_{tot,pred}$) retention times:

$$t_{tot,obs} = 0.933t_{tot,pred} + 1.64, \quad r^2 = 0.940 \text{ (all solutes)}, \quad (3)$$

$$t_{tot,obs} = 0.841t_{tot,pred} + 2.52, \quad r^2 = 0.815 \text{ (CBI–ME)}, \quad (4)$$

$$t_{tot,obs} = 0.994t_{tot,pred} + 1.05, \quad r^2 = 0.991 \text{ (CBI–LME)}. \quad (5)$$

Analysis of the data for the individual solutes, according to equation (2), revealed that the greatest contribution to the error between the observed and predicted retention time was associated with CBI–ME (equation 4), whilst the smallest error was associated with CBI–LME (equation 5). Equations 4 and 5 and Fig. 7 show that the errors in using equation (2) for the prediction of retention were related to the order in which the solutes were transferred from the first column to the second and not necessarily to the final elution order. Thus the largest errors (approaching 30%) were associ-

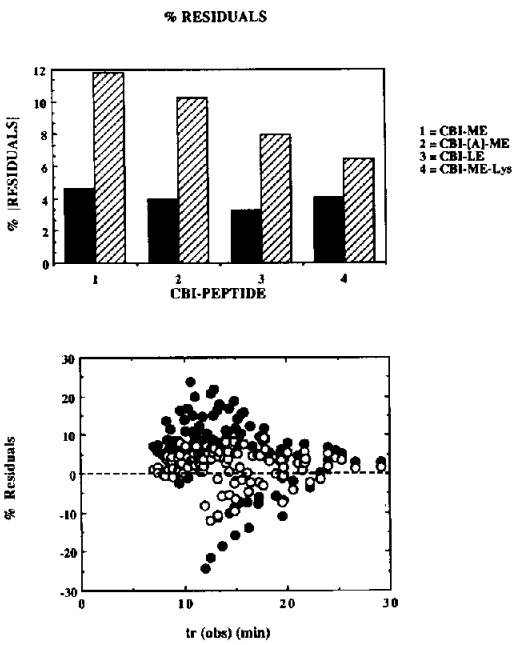

Figure 7
Errors in the prediction of the retention times of the four CBI-derivatives of ME, LE, AME and LME in various LC–LC systems. The upper panel shows the relative errors in the predictions, for all the solutes, arising from the use of equations 6 (closed) and 8 (open). The lower panel shows the means of the absolute values of the relative errors in the predictions, arising from the use of equations 6 (hatched) and 8 (closed), plotted against peptide elution number.

ated with predicting the retention of CBI–ME, whereas the smallest errors (<8%) were associated with predicting the retention of CBI–LME. The source of these errors was identified by the analysis of the processes that occur during the column-to-column transfer of several components in a single fraction ("heart-cutting") (Fig. 8). Because the components were partially resolved on the first column, not all the components were transferred to the second column at the same time. Therefore the first component was eluted for a significant period of time on the second column with mobile phase from the first column (t_{21}). On the other hand, as soon as the last peak was transferred, the mobile phase was switched and it was eluted from the second column almost entirely under the influence of the second mobile phase ($t_{22} \gg t_{21}$). Taking these effects into account, equation (6) may be more appropriate for the prediction of retention:

$$t_{tot} = t_{11} + t_{22} + t_{21} + t_{ext}, \qquad (6)$$

where t_{21} and t_{22} are the residence times spent

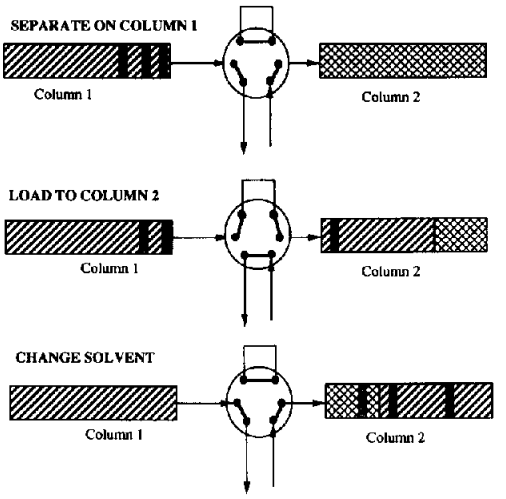

Figure 8
Diagram showing the changes in solvent composition that take place during transfer of three peaks ("heart-cut") from column 1 to column 2 in a two-column system.

by the solute on column 2 under the influence of mobile phases 1 and 2, respectively.

The solution to equation (6) was obtained by the application of the theory of stepwise-gradient elution described by Jandera and Churáček [16], who showed that the corrected retention volume (V'_r) of a solute on a column eluted sequentially with n solvents of volume V_i could be described by:

$$V'_r = \sum_{i=1}^{n-1} V_i\left(\frac{k'_i - k'_n}{k'_i}\right) + V_o k'_n. \qquad (7)$$

Substituting equation (7) into equation (6) and rewriting in terms of capacity ratios gives 8, which describes the overall retention of a solute in a two-column-switching system:

$$t_{tot} = t_{o1}(1 + k'_{11}) + t_s\left(\frac{k'_{12} - k'_{22}}{k'_{12}}\right) + t_{o2}(1 + k'_{22}) + t_{ext}, \qquad (8)$$

where t_s is the width of the switching window (in units of time). When the retention data were re-analysed according to equation (8), the agreement between the observed and predicted values of t_{tot} was excellent:

$$t_{tot,obs} = 1.001\ t_{tot,pred} + 0.376, \quad r^2 = 0.986 \text{ (all solutes)} \qquad (9)$$

Moreover, Fig. 7 and equations (10) and (11) show that when the data were re-analysed

according to equation (8) the errors were randomly distributed and were independent of the order in which the peaks were transferred from the first column to the second:

$$t_{tot,obs} = 1.000\ t_{tot,pred} + 0.352, \quad r^2 = 0.959\ (CBI\text{–}ME), \tag{10}$$

$$t_{tot,obs} = 0.998\ t_{tot,pred} + 0.465, \quad r^2 = 0.991\ (CBI\text{–}LME). \tag{11}$$

The regression analyses described in equations (9–11) were obtained assuming a value of zero for t_{ext} (equation 6). The values of the intercept terms in equations (9–11) of 0.352–0.465 min were in excellent agreement with the experimentally determined value for t_{ext} of 0.40 min.

Optimization strategies for mulitidimensional separations

Optimization of separations on single LC columns has been the subject of intense research and several mathematical approaches have been described [17, 18]. By contrast, very little has been published [12] on the quantitative optimization of multidimensional system. Mifune *et al.* [12] have developed a method for the optimization of the multidimensional separation of opioid peptides on the basis of data generated from single-column experiments. However, their method was empirical and tedious. In the present study, a simple equation (equation 12) for the optimization of multidimensional separations was developed and tested using the retention data generated in the previous section on retention prediction. The multidimensional chromatographic optimization function (COF) was defined by:

$$COF = \sum_{i=1}^{n-1} A \ln\left[\left(\frac{N^{0.5}}{4\ R_s}\right)\left(\frac{k^*_{i+1}}{1 + k^*_i}\right)\right] + B(k^*_{max} - k^*_n), \tag{12}$$

where N and R_s are the average number of theoretical plates for the four peaks, and the required resolution (1.5) between the ith peak and the next peak to elute ($i + 1$), respectively. A and B are weighting factors with arbitrarily assigned values of 1.0 and 0.05, respectively. The rationale for the choice of this particular COF (equation 12) was to allow the description of the overall quality of the multidimensional separations with respect to both the resolution of the peaks (first term in equation 12) and the overall analysis time (second term equation 12). The apparent capacity ratio (k^*) in the multidimensional system was defined by:

$$k^* = \left(\frac{t_1 + t_2}{t_{o1} + t_{o2}}\right) - 1, \tag{13}$$

where t_1 and t_2 are the retention times on the two columns, and t_{o1} and t_{o2} are the void times for the two columns. The maximum acceptable capacity ratio (k^*_{max}) was arbitrarily assigned a value of 10.0 (equation 12).

The property of equation (12) to predict the optimum conditions for the multidimensional separations was tested by substituting the retention data for the four derivatized peptides, CBI–ME, CBI–ME, CBI–AME and CBI–LME, described previously. When the data were presented in tabular form, it was difficult to discern any obvious relationship between COF and composition of the chromatographic systems. However, when the data were presented in a polar coordinate system (COF maps in Figs 9 and 10), clearer pictures of the relationships between COF and mobile phase composition and COF and column combinations emerged.

Figure 9 is a COF map showing the optimum column configuration for the LC–LC separation of four opioid peptides as their CBI-derivatives (for simplicity the actual COF values were re-scaled in linear fashion so that a value of 4.9 was assigned to the largest COF and a value of 0 was assigned to the smallest COF). The three columns are shown as concentric circles, with the various combinations of columns displayed in an anti-clockwise fashion according to increasing COF value. The square symbol indicates the first column and the circle indicates the second column. Accordingly, Fig. 9 shows that the best combination of columns was CPS Hypersil for column 1 and Spherisorb Phenyl for column 2, because this combination accounts for all the data points in the 4th quadrant of the COF map (COF = 3.75–5.00). Similarly, the data in the 4th quadrant of the COF map in Fig. 10 indicate that the best separations will be obtained if the two columns are eluted with 35 to 45% acetonitrile in TFA (pH 3.5, 25 mM). Figure 11 shows representative separations of the four CBI-peptides in two multidimensional systems, both employing CPS Hypersil as column 1 and Spherisorb Phenyl as column 2,

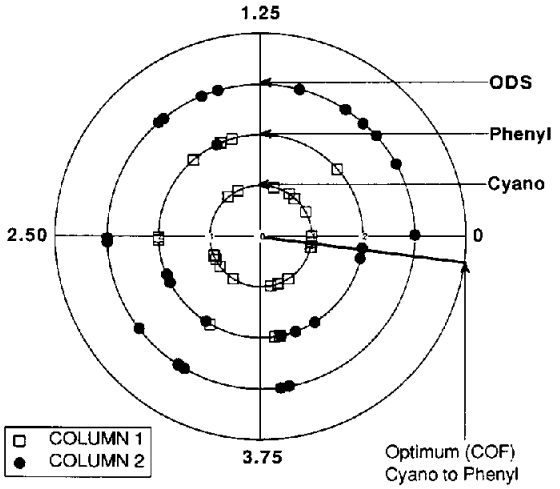

Figure 9
COF map, showing the optimum column configuration for the LC–LC separation of four opioid peptides as their CBI-derivatives. The three columns are shown as concentric circles, with the various combinations of columns displayed in an anti-clockwise fashion according to increasing COF value. The square symbol indicates the first column and the circle indicates the second column.

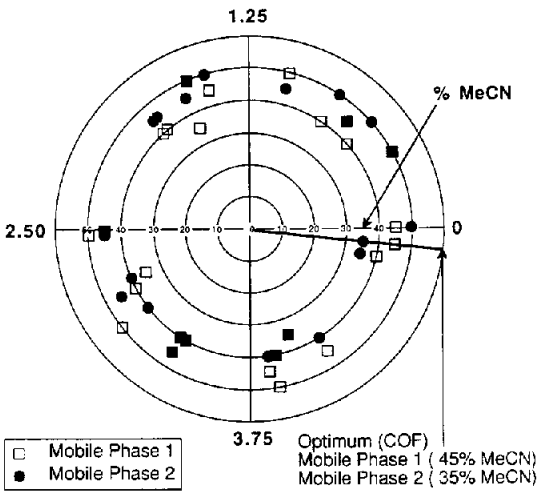

Figure 10
COF map showing the optimum mobile phase combination for the LC–LC separation of four opioid peptides as their CBI-derivatives. The concentrations of acetonitrile in the mobile phases are shown as concentric circles, with the various combinations of mobile phases displayed in an anti-clockwise fashion according to increasing COF value. The square symbol indicates the mobile phase used to elute the first column and the circle indicates the mobile phase used to elute the second column.

and eluted with slightly different concentrations of acetonitrile (35 or 40%), demonstrating the success with which equation (12) could be used to predict the optimum chromatographic conditions.

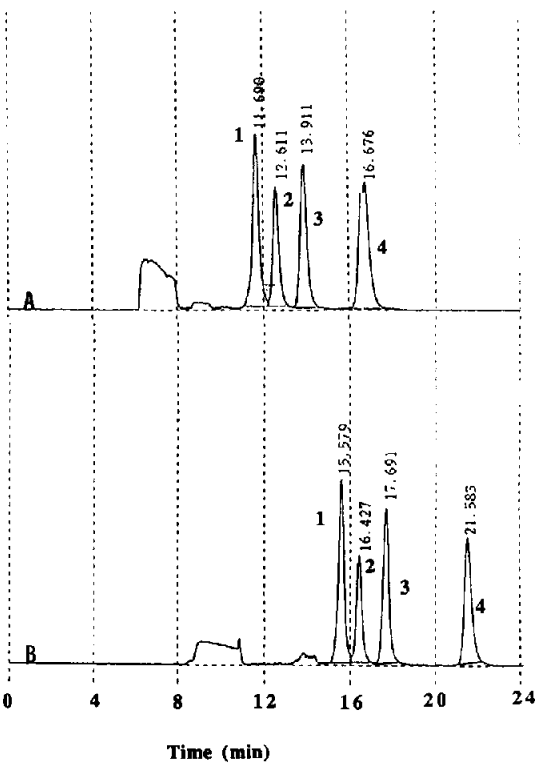

Figure 11
Optimized multidimensional separations of the four CBI-derivatives of ME (1), AME (2), LE (3) and LME (4). Stationary phases: Column 1, CPS Hypersil (5 μm, 150 × 4.6 mm i.d.); column 2, Spherisorb Phenyl (5 μm, 150 × 4.6 mm i.d.). Mobile phases: (A) column 1 eluted with acetonitrile–TFA (pH 3.5, 25 mM) (40:60, v/v), column 2 eluted with acetonitrile–TFA (pH 3.5, 25 mM) (40:60, v/v); (B) column 1 eluted with acetonitrile–TFA (pH 3.5, 25 mM) (35:65, v/v), column 2 eluted with acetonitrile–TFA (pH 3.5, 25 mM) (40:60, v/v). Other chromatographic conditions as Fig. 2.

Conclusions

Pre-column derivatization with NDA/CN combined with multidimensional reversed-phase liquid chromatography provides a basis for the sensitive and selective determination of peptides in biological media [12]. However, each step in the procedure must be optimized if the full potential of this methodology is to be realized. The derivatization kinetics should be fully characterized in terms of rate and yield and the multidimensional chromatography should be optimized in a rational manner. The results of this study have shown that retention times in multidimensional can be predicted rapidly and accurately from measurements made on single columns and that the single-column data can also be used to optimize the multidimensional conditions. Figure 12 pro-

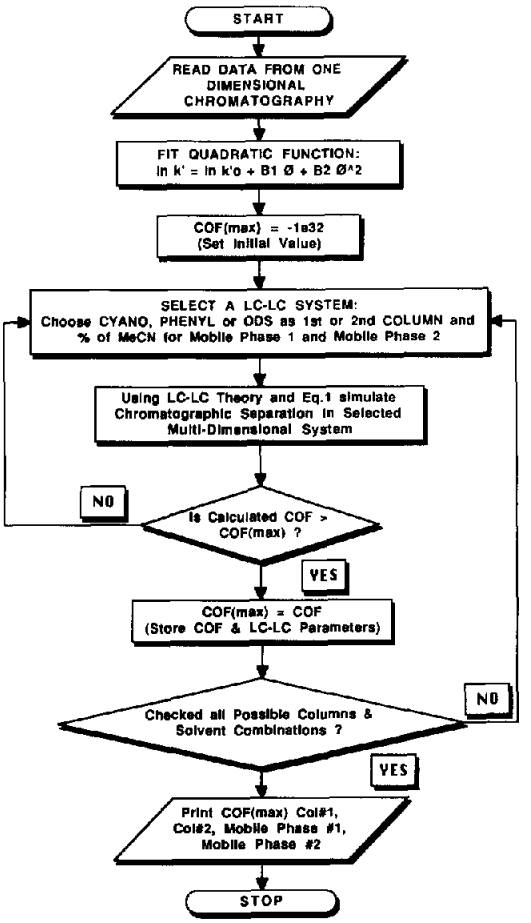

Figure 12
Flow-chart for separation optimization in multidimensional LC–LC systems.

vides a flow-chart that summarizes the steps taken in the optimization processes described here and should prove useful in future applications.

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