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Reversed-phase liquid chromatography of the opioid peptides — 2. Quantitative structure–retention relationships and isocratic retention prediction*†

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Abstract: The ability of Snyder's theory of linear gradient elution to predict the starting isocratic reversed-phase LC conditions (k' = 4–10) for the opioid peptides was investigated. The errors in predicting the concentration of acetonitrile (Φ) required to elute the peptides with a k' value of 4 were high, ranging from 13.5 to 38.1%. At k' = 10 the errors were generally reduced to less than 20%. This analysis was repeated with the same peptides after conversion to their fluorescent 1-cyanobenz[f]isoindoles (CBIs) by reaction with naphthalene-2,3-dicarboxaldehyde/cyanide. For the CBI derivatives, the errors in predicting the required concentration of acetonitrile for isocratic elution were markedly reduced and ranged from 0 to 14.3 for k' = 4 and 0 to 11.9% for k' = 10. The errors in the model in predicting the required isocratic mobile phase accurately were attributed to a mixed mechanism of retention involving solvophobic and silanophilic interactions and leading to non-linear relationships between log k' and Φ. Even when the errors in predicting the required value of Φ were relatively high, the Snyder approach was found to be very useful in predicting the initial starting conditions for the reversed-phase LC of the native opioid peptides as well as their fluorescence CBI derivatives.

Keywords: *Peptides; opioids; reversed-phase liquid chromatography; pre-column derivatization; naphthalene-2,3-dicarboxaldehyde/cyanide; fluorescence detection; retention prediction; fragmental constants.*

Introduction

Advances in the understanding of the biological action of peptides at the molecular level have resulted in enormous interest in peptides as medicinal agents. In addition, advances in genetic engineering have made feasible the commercial production of peptides [1, 2]. This is reflected by over 150 recombinant protein product candidates being in either Phase I clinical trials or beyond, about a dozen having received FDA approval [3].

Liquid chromatography (LC) is one of the most important techniques for the analysis of peptides. Liquid chromatography utilizing reversed phases chemically bonded to silica microparticles, has emerged during the past 15 years as a very rapid and selective method for the separation of peptides. Although this technique is extremely powerful, method development can be very laborious, time-consuming and selection of the optimum mobile phase is not a trivial process [4]. The conditions for isocratic elution are usually selected by trial-and-error and the overall success of this approach is strongly dependent on the experience and expertise of the chromatographer involved. Not only can this trial-and-error method be frustrating but it may also be unsuitable when there is limited sample available.

Recently, Mifune *et al.* [5] have demonstrated the value of multidimensional liquid chromatgraphy (LC–LC) for the resolution and determination of the opioid peptides leucine-enkephalin and methionine-enkephalin in rat striatum, following pre-column fluorogenic derivatization with the nathphalene-2,3-carboxaldehyde/cyanide reagent [6, 7] to the corresponding 1-cyanobenz[f]isoindoles (CBIs). Subsequently, Nicholson *et al.* [8] described a theory of retention prediction and optimization strategies for the LC–LC of peptides using the enkephalins as model com-

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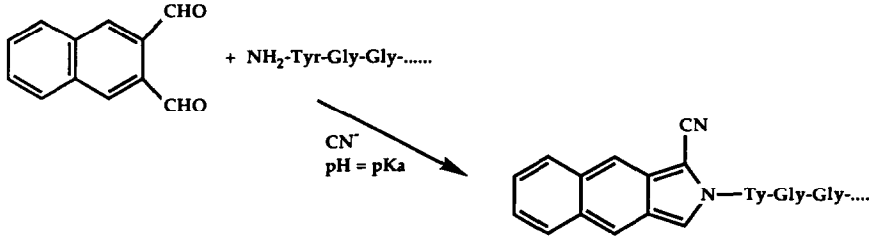
pounds. The main deficiency of the LC–LC optimization strategy described previously was that it still required the identification of the initial starting conditions for each of the columns to be used in the multidimensional mode (i.e. mobile-phase conditions that would provide a k' value in the range 4–10). The present work is concerned with the application of the existing optimization theory described by Snyder and co-workers [9–13] to the isocratic separation of the opioid peptides and their derivatives. The previous work on LC–LC was restricted to four model opioid peptides. In the present study, the number of compounds under investigation has been expanded to include the 21 enkephalin-related peptides shown in Table 1.

Several computer-aided systems have been developed to assist in method development and eluant optimization in isocratic liquid chromatographic (LC) separations [14–16]. These approaches are still in their infancy but as the knowledge of the physico-chemical processes involved in the separation increases, the utility of these systems will improve. The subject of eluent optimization has been discussed in a number of detailed review articles by Berridge [15], Schoenmakers and Mulholland [17], Snyder and Stadalius [18] and Massart *et al.* [19]. However, almost all optimization approaches need a set of initial starting conditions.

Some of the optimization procedures that have been applied to chromatographic problems include simplex techniques, steepest ascent method and factorial experimental design for the optimization of chromatographic separations [15, 17, 19]. Each of these methods of optimization assumes that the simplified model of the given system used is adequate for solving the separation problem; therein lie the shortcomings of all these methods.

The lack of widespread application of optimization approaches may most probably be due

Table 1
Structures of the opioid peptides used in this study and their derivatization with naphthalene-2,3-dicarboxaldehyde/cyanide (NDA/CN). Primary-amine containing residues available for derivatization are shown in bold



Peptide	Amino-acid sequence	Trivial name
1	**Tyr**	Tyrosine
2	**Tyr**-Gly	
3	**Tyr**-Gly-Gly	
4	**Gly**-Gly-Phe	
5	**Tyr**-Gly-Gly-Phe-Met	Methionine-enkephalin
6	**Tyr**-Gly-Gly-Phe-Met-**Lys**	
7	**Tyr**-Gly-Gly-Phe-Met-Arg	
8	**Tyr**-Gly-Gly-Phe-Met-Arg-Arg	
9	**Tyr**-Gly-Gly-Phe-Met-Arg-Phe	
10	**Tyr**-Gly-Gly-Phe-Met-Arg-Gly-Leu	
11	**Tyr**-Gly-Gly-Phe-Met-Arg-Arg-Val-NH_2	Metaphinamide
12	**Tyr**-Gly-Gly-Phe-Met-Thr-Ser-Glu-**Lys**-Ser-Gln-Thr-Pro-Leu-Val-Thr-	α-Endorphin
13	**Tyr**-Gly-Gly-Phe-Met-Thr-Ser-Glu-**Lys**-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-**Lys**-Asn-Ala-Tyr-**Lys**-**Lys**-Gly-Glu	β-Endorphin
14	**Tyr**-Gly-Gly-Phe-Leu	Leucine-enkephalin
15	**Tyr**-Gly-Gly-Phe-Leu-Arg-**Lys**-Tyr-Pro	β-Neo-endorphin
16	**Tyr**-Gly-Gly-Phe-Leu-Arg-**Lys**-Tyr-Pro-**Lys**	α-Neo-endorphin
17	**Tyr**-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-**Lys**-Val-Val-Thr	Dynorphin B
18	**Tyr**-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-**Lys**-Leu-**Lys**-Trp-Asp-Asn-Gln	Dynorphin A
19	**Tyr**-Gly-Gly-Phe-Met[O]	Methionine-enkephalin sulphoxide
20	**Tyr**-[D]-Ala-Gly-Phe-Met	
21	**Tyr**-Gly-Gly-Phe-Met[O]$_2$	Methionine-enkephalin sulphone

to lack of experience in these systems, which do require a significant understanding of the theory of chromatography. The expertise required to increase the acceptability of these optimization procedures is now being incorporated in expert systems. Schoenmakers and co-workers [20, 21] have recently developed an expert system, ESPRIT that also incorporates instrumental constraints, so that full use is made of the instrumentation as well as the separation method.

Massart and co-workers [22] have recently reported rules which are incorporated into an expert system, called LABEL, for the selection of LC methods in pharmaceutical analysis. LABEL uses a cyanopropyl column which can be used in both normal-phase (NP) and reversed-phase (RP) modes. The success rate using these rules for 44 pharmaceutical preparations was reported to be 82%.

The expert systems being developed for chromatographic optimization at present are only used for relatively small molecules and it may be many years before such systems will be available for more complex solutes, such as peptides and proteins. The main difficulty with the prediction of optimum separation conditions for peptides and proteins compared with smaller organic solutes is their high molecular weight, tertiary structure, and the presence of multiple polar and apolar functional groups. The structural complexity of peptides and proteins can lead to numerous and complex solute–solvent, solute–stationary phase and solute–solute interactions and, consequently, unpredictable retention behaviour. One approach that has been described for the optimization of isocratic reversed-phase separation of simple peptides, is that described by Snyder and co-workers [10]. This approach involves the prediction of optimum isocratic conditions, based on two or more linear gradient runs. The advantage, which may also be the weakness of this approach, is that it requires no knowledge of the solute structure and it also assumes a single retention mechanism (solvophobic interactions). The latter assumption is particularly important because the theory assumes a simple linear relationship between the logarithm of the chromatographic capacity factor (k') and the proportion of organic modifier in the mobile phase (Φ).

This theory has been tested with simple solutes, for example substituted benzene derivatives, as well as larger molecules including simple peptides. However, it has not been applied to multi-functional peptides, such as the opioids, and one of the primary objectives of this study was to test the applicability of this theory to these solutes. Although not necessary for the application of this theory to simple solutes, an understanding of retention mechanisms for peptides and the relationship between structure and retention will probably be necessary for the application of this theory to more complex solutes such as the opioid peptides.

Experimental

Materials

All the chemicals used were the highest quality available and were used as received. Peptides (Table 1) were purchased from Sigma Chemical Co. (St Louis, MO, USA) and Peninsula Labs Inc. (Belmont, CA, USA). Mobile phases were filtered using 0.45 μm nylon membrane filters prior to mixing.

Apparatus

Shimadzu pumps (LC-6A), auto-injector (SIL-6A), column oven (CTO-6A), UV–vis spectrophotometric (SPD-6AV) and fluorescence HPLC monitor (RF-530) were used. A Shimadzu C-R3A Chromatopac with a floppy disk drive (FDD-1A) was used for data collection and reduction. The samples in the auto-injector were kept at 4°C using a Brinkmann Lauda Refrigerating Circulator model RMT-6. Chromatographic columns were packed using an upward slurry-packing procedure [23]. Drylab I® was used to predict the isocratic conditions from gradient data.

Procedures

Column dead time (t_o) determination. When using UV detection, the t_o values were determined by injecting a weaker solvent than the mobile phase and timing the subsequent disturbance in the baseline. When using fluorescence detection, t_o was determined by injecting CBI-taurine with pure acetonitrile as the mobile phase.

Dwell time (t_D) determination. The dwell time, t_D was determined in the absence of a column using water as the solvent A and methanol as the solvent B. The pump B was turned on after 4.9 min subsequent to the start of the run and the delay in the deflection

observed in the baseline was measured. The difference between the programmed start of pump B and the time the deflection occurred was taken as the t_D. The mean value (±SD, $n = 3$) was determined to be 2.13 ± 0.03 min for this system. The manufacturer's specification for the internal volume of the high-pressure mixing chamber was 1.90 ml.

Chromatography. The mobile phase was prepared by mixing appropriate volumes of the organic modifier and the aqueous buffer. This particular method of preparing mobile phase was used because the isocratic mobile phase compositions were prepared by using two pumps (which mixed the solvents by volume). The aqueous component of the mobile phase (solvent A) was 0.1% v/v trifluoroacetic acid (TFA). Linear gradients were run from 2 to 62% acetonitrile and from 40 to 100% acetonitrile for the opioid peptides and their CBI-derivatives, respectively, unless stated otherwise. Various ramps were utilized. The injection volume was 20 μl and the flow rate was 1 ml min^{-1} in all these experiments.

Detection of the opioid peptides was by UV absorbance (210 nm). Fluorescence was used for the detection of the CBI-peptides. Excitation was achieved with a xenon lamp at 420 nm and emission was collected at 490 nm.

Fluorogenic derivatization of the opioid peptides. The selected opioid peptides were converted to their corresponding fluorescent N-substituted-1-cyanobenz[f]isoindole (CBI) derivatives by reaction with NDA in the presence of sodium cyanide (Table 1). Lysyl-containing peptides 6 and 15 were derivatized in a borate buffer at pH 10.0 and the remaining peptides 5, 7, 11, 14, 19 and 20 were derivatized in phosphate buffer at pH 6.8. Selective derivatization of the α- and ϵ-amino groups was carried out as follows. Peptides 5, 7, 11, 14, 19 and 20 were derivatized at the α-amino group by the following procedure: 50 μl ascorbic acid (200 mM), 5 μl peptide solution, 10 μl NaCN (10 mM), 820 μl phosphate buffer, pH 6.8 (50 mM), 100 μl acetonitrile and 10 μl NDA in acetonitrile (5 mM) were mixed in the order listed. The resultant mixture was mixed thoroughly by inversion and incubated on ice (4°C) for 20 min. The reaction was quenched by the addition of 5 μl of taurine (200 mM). The mixture was incubated for a further 10 min at 4°C. The concentrations in parentheses are the initial concentrations of the components in the reaction. Peptides 6 and 15 were derivatized at the ϵ-amino group by the following procedure: 10 μl ascorbic acid (200 mM), 5 μl peptide solution (0.5 mg ml^{-1}), 10 μl NaCN (10 mM), 150 μl sodium borate buffer, pH 10.0 (50 mM), 800 μl acetonitrile and 20 μl NDA in acetonitrile (5 mM) were mixed in that order. The resultant mixture was thoroughly mixed and incubated on ice (4°C) for 20 min. The reaction was quenched by the addition of 5 μl of taurine (200 mM). The mixture was incubated for a further 30 min at 4°C. The concentrations in parentheses are the initial concentrations of the components in the reaction.

Results and Discussion

Theory

This work makes extensive use of the theory of gradient elution and isocratic retention prediction described by Snyder and co-workers [9–13]. In this section, the basic equations will be described. Those wishing to read more of this subject are referred to the original literature [24] and to Snyder's work [9, 10, 25, 26]. The theory of gradient elution and isocratic retention prediction makes the important initial assumption that the logarithm of the capacity factor (log k') is linearly related to the volume fraction of organic modifier (Φ) in the aqueous mobile phase:

$$\log k' = \log k_w - S\Phi \qquad (1)$$

where S is the slope or solvent sensitivity factor and k'_w is the capacity ratio in the completely aqueous mobile phase (i.e. 100% solvent A when $\Phi = 0$). Equation (1) tends to hold true for simple, low molecular weight, organic compounds but substantial deviations from linearity have been reported for more complex molecules such as peptides and proteins [5, 10, 27, 28]. These deviations from linearity are generally caused by mixed retention mechanisms, changes in conformation (tertiary structure), or both, and can be accommodated by the use of a quadratic equation relating log k' and Φ. Unfortunately, the isocratic retention prediction theory cannot easily accommodate such a quadratic equation. One of the main purposes of the present work was to explore the influence of the non-linear retention of the opioid peptides and their CBI-derivatives [5]

(Table 1) on the ability of the Snyder theory of gradient elution to prediction isocratic retention conditions. The fundamental relationship describing retention in any gradient elution system is given in equation (2):

$$\int_0^{V_g} \frac{dV}{V_a} = 1 \qquad (2)$$

where V_g is the corrected retention volume of the band, dV refers to a differential volume of mobile phase that passes through the band centre during its migration along the column, and V_a is the instantaneous retention volume. In linear gradient elution, the instantaneous capacity ratio (k_t) is related to time (t) and the gradient steepness parameter (b) by:

$$\log k_t = \log k_o - b(t/t_o) \qquad (3)$$

where k_o is the instantaneous capacity ratio of the solute at the start of the run (i.e. at $t = 0$). The gradient steepness parameter is related to the gradient slope ($\Delta\Phi/t_G$), the flow rate (F) and the solvent sensitivity factor [equation (1)] by the equation:

$$b = [\Delta\Phi S V_m/t_G F]. \qquad (4)$$

The equation describing the elution time of a solute under linear gradient conditions (t_g) is obtained [equation (5)] by combining equations (1)–(4):

$$t_g = \frac{V_m}{bF} \log (2.303 k_o b + 1) + t_o + t_D. \qquad (5)$$

The gradient dwell time (t_D) depends on the instrumentation used to generate the gradient and can be obtained by break-through experiments. If elution times (t_{g1}, t_{g2}, etc.) are obtained at two or more run times (t_{g1}, t_{g2}, etc.) then it follows that:

$$t_{g1} = \left(\frac{t_o}{b_1}\right) \log (2.303 k_o b_1 + 1) + t_o + t_D \qquad (6)$$

$$t_{g2} = \left(\frac{t_o}{b_2}\right) \log (2.303 k_o b_2 + 1) + t_o + t_D \qquad (7)$$

and

$$b_1/b_2 = \beta = t_{G2}/t_{G1}. \qquad (8)$$

Equations (6)–(8) comprise three equations in three unknowns (k_o, b_1 and b_2) that can be solved numerically with commercially available software (Drylab®) to obtain k_o and b_1. Subsequently, S can be obtained from equation (4) and k_w can be obtained from equation (1).

Gradient elution of the native opioid peptides

The gradient behaviour of the selected opioid peptides was investigated on three stationary phases: ODS Hypersil (ODS), CPS Hypersil (CN) and Phenyl Hypersil (phenyl). The selectivity of methanol and acetonitrile as organic modifier was also studied. The gradient data for the combination of ODS and acetonitrile were used to predict the isocratic behaviour of a selection of the opioid peptides. The chromatographic properties of the CBI-derivatives of the selected opioid peptides were also investigated. Each stationary phase was eluted with a linear gradient of acetonitrile in 0.1% v/v trifluoroacetic acid (TFA). In all cases, the initial and the final conditions were 2 and 62%, respectively. The gradient elution time for each solute (t_g) was determined using a gradient run-time of 30 min and the results are given in Table 2. A similar experiment was also conducted for the ODS column using methanol as the organic modifier. However, this organic modifier was not investigated extensively because significant baseline drift arising from end-absorption of the methanol at 210 nm made collection of retention data difficult. The initial and the final conditions for methanol gradient were 30 and 80%, respectively.

Mobile phase and stationary phase selectivities

The ODS column was determined to be the most useful for further investigation because all the 18 peptides were retained by this column. In contrast, peptides 1–4 eluted at the solvent front on the phenyl column, peptides 1–3 eluted at the solvent front on the cyano column, and peptides 13, 16 and 18 were completely retained on the cyano column. The high affinity of peptides 13, 16 and 18 for the cyano bonded phase was attributed to the strong interactions between the arginyl groups and the residual silanols [27–29].

Even though the combination of an ODS column and an acetonitrile-based eluent was more generally useful for the separation of the opioid peptides than the other combinations studied, it was of interest to compare the

Table 2
Gradient retention times (t_g) for a number of opioid peptides on three stationary phases, using mobile phases containing either acetonitrile (MeCN) or methanol (MeOH)

Peptide*	Gradient retention time† (min)			
	ODS/MeCN	ODS/MeOH	Phenyl/MeCN	CN/MeCN
1	5.5	—‡	—‡	—‡
2	6.08	—‡	—‡	—‡
3	2.84	—‡	—‡	—‡
4	14.34	—‡	—‡	5.20
5	15.45	—‡	8.53	13.70
6	15.33	—‡	10.35	17.51
7	15.80	—‡	11.75	18.56
8	16.30	10.29	13.78	26.30
9	20.61	13.52	16.84	25.05
10	19.59	12.87	15.79	22.86
11	17.46	9.69	16.07	27.63
12	18.50	12.92	16.58	20.15
13	29.12	22.30	29.51	—§
14	21.21	13.36	12.58	19.56
15	21.74	14.29	19.41	29.54
16	20.83	13.73	19.31	—§
17	23.21	16.34	22.48	35.64
18	29.58	17.47	25.54	—§

*See Table 1.
†Mean of at least two determinations. All values were within ±5% of the mean.
‡Unretained.
§Completely retained.

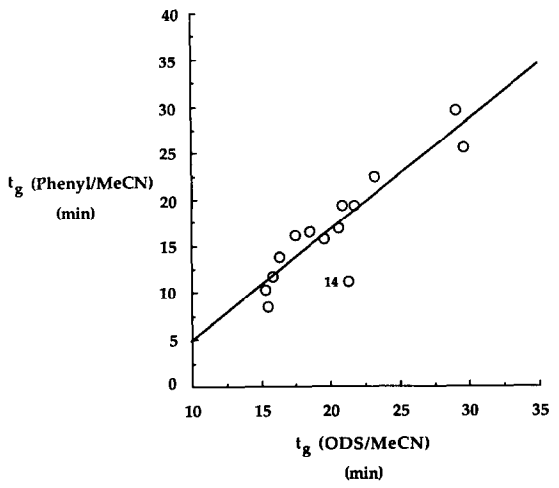


Figure 1
Comparison of the retention times (t_g) of the opioid peptides on phenyl and ODS columns eluted with a linear acetonitrile gradient. The symbols represent experimental data and the regression line has been drawn according to equation (11). The data point corresponding to peptide 14 has been marked on the graph.

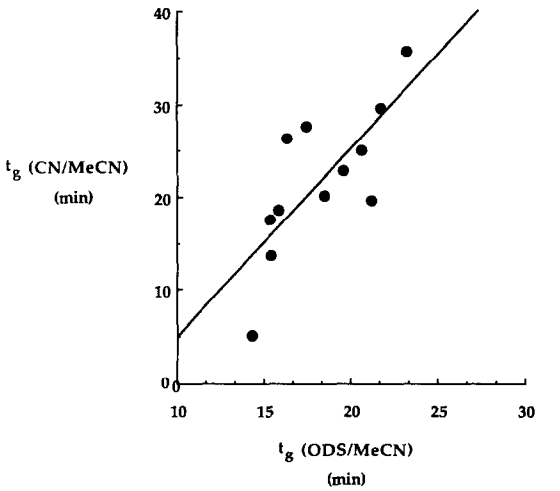


Figure 2
Comparison of the linear gradient retention times (t_g) of the opioid peptides on CN and ODS columns eluted with a linear acetonitrile gradient. The symbols represent experimental data and the regression line has been drawn according to equation (10).

selectivity of each of the other two columns with that of the ODS column, because these other columns may have a specific application in the separation of particular opioid peptides. The selectivities of the columns were compared by plotting the gradient retention time (t_g) for opioid peptides on either the CN or the phenyl column against the corresponding values of t_g on the ODS column (Figs 1 and 2). The least squares regression analyses of these empirical relationships are given by equations (9) and (10) respectively:

Phenyl vs ODS:

$$t_g \text{ (phenyl)} = 1.18\, t_g \text{ (ODS)} - 6.97 \quad (r = 0.921); \qquad (9)$$

CN vs ODS:

$$t_g\ (\text{CN}) = 2.02\ t_g\ (\text{ODS}) - 15.23 \quad (r = 0.748). \qquad (10)$$

Considering first the comparison of the phenyl and ODS columns [equation (9)], the general relationship between the retention times on the two columns is good ($r = 0.921$) but is clearly perturbed by one data point corresponding to peptide 14 (Fig. 1). It is not clear why this particular peptide is so much less retained on the phenyl column than on the ODS column; however, if this data point is omitted, the correlation between the t_g values on the two columns is improved substantially [$r = 0.958$, equation (11)]:
Phenyl vs ODS (revised):

$$t_g\ (\text{phenyl}) = 1.20\ t_g\ (\text{ODS}) - 6.94 \quad (r = 0.958). \qquad (11)$$

The high correlation coefficient [equation (11)] is indicative of a common retention mechanism for the opioid peptides on the phenyl and ODS columns. Although nothing can be said from these analyses about the retention mechanism, this aspect of the work will be discussed later. However, some information with respect to relative selectivity can be obtained from the slope and intercept of the relationships. A value for the slope greater than 1 for this relationship [equation (11)] indicates that the selectivity of the phenyl phase is generally more sensitive to change in structure than that of the ODS phase. On the other hand, the large negative intercept means that the overall retention power of the phenyl column is lower than for the ODS column. The higher selectivity of the phenyl column combined with its lower overall retention power meant that tyrosine and the three small peptides 2, 3 and 4 were unretained on this phase. In contrast, tyrosine and peptides 2, 3, and 4 were adequately retained on the ODS column.

The relatively poor correlation between the retention times (t_g) of the peptides on the CN column and the ODS column was attributed to significantly different retention mechanisms operating on the two columns. The hydrophobicity of the CN phase was much less than that of the ODS phase. However, 12 of the 18 peptides studied were more retained on the CN column than they were on the ODS column. Each of these more strongly retained peptides contained one or more basic amino acid residues (arginine or lysine) and the increased retention on the CN phase was attributed to stronger interactions with the more accessible residual silanols [27, 28]. Three peptides 13, 16 and 18 which contained three or more basic residues did not elute from the CN column under the conditions studied, consistent with there being very strong interactions between the basic residues and the silanol residues. In contrast, tyrosine and peptides 2, 3, 4, 5 and 14, which did not contain any basic residue, eluted more rapidly from the CN column than from the ODS column, consistent with the lower hydrophobicity of the CN phase and the absence of silanophilic interactions.

The final analysis conducted on these data was the composition of the selectivity of acetonitrile- and methanol-based gradients on the ODS column (Fig. 3). The gradient retention of all the peptides was lower with the methanol-based gradient than with the acetonitrile gradient, but this can be attributed to the higher initial concentration of methanol used, rather than its solvent strength, which is known to be lower than that of acetonitrile [30]. The regression analyses [equation (12)] comparing the t_g values of the opioid peptides, revealed that the selectivities of these two organic modifiers were very similar ($r = 0.924$):
MeCN vs MeOH:

$$t_g\ (\text{MeOH}) = 0.75\ t_g\ (\text{MeCN}) - 2.05 \quad (r = 0.924). \qquad (12)$$

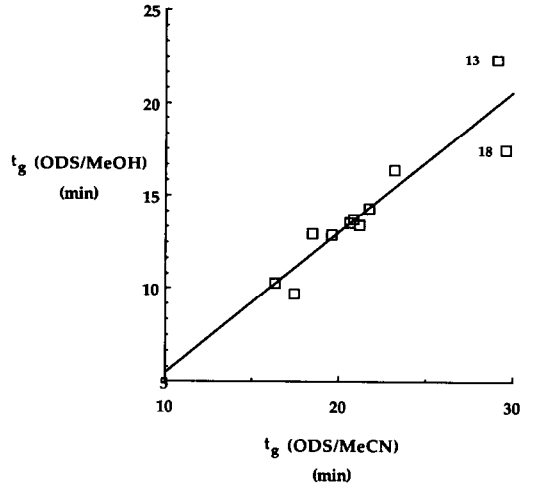


Figure 3
Comparison of the retention times (t_g) of the opioid peptides on the ODS column with linear methanol or acetonitrile gradients. The symbols represent experimental data and the regression line has been drawn according to equation (12).

In fact, the relationship is only perturbed by two large peptides, β-endorphin (peptide 13) and dynorphin A (peptide 18), which have 31 and 17 amino acid residues, respectively. There is no obvious explanation in the different chromatographic behaviour of these two peptides. Clearly, however, β-endorphin and dynorphin A would be more readily separated on the ODS column with a methanol-based gradient than one based on acetonitrile.

Isocratic retention prediction

Having established the combination of an ODS column and acetonitrile gradient as the most suitable chromatographic system for the reversed-phase LC of the opioid peptides, further experiments were conducted to test the applicability of gradient elution for the prediction of the isocratic elution behaviour. These experiments were conducted with eight peptides (5, 6, 7, 11, 14, 15, 19, 20 and 21) that were chosen as being representative of the large group of peptides studied (Table 1). These peptides were also studied as their corresponding CBI-derivatives. By studying the peptides underivatized and as their CBI-derivatives, it was therefore possible to obtain information on the contribution of the CBI ring system to chromatographic retention properties.

Retention times (t_g) were obtained for each solute under linear gradient conditions at run times (t_G) of 20 and 30 min. The respective initial and final conditions were 2 and 62% acetonitrile for the underivatized peptides and 40 and 100% for the CBI-derivatives, except for the CBI-derivative of peptide 19. The initial and final conditions were 20 and 80% acetonitrile for chromatography of CBI-peptide 19. The values of S and k'_w were then calculated from equations (1) and (6)–(8) and the measured retention times (Table 3). From the values of S and k'_w, the concentration of acetonitrile required to elute the analytes (Φ_{cal}) with k' values of 4 or 10 were calculated from equation (1). The results are shown in Table 4.

Table 4 also gives the observed concentrations of acetonitrile (Φ_{obs}) that were required to elute the analytes with the same k' values. By comparing the observed and calculated values of Φ required to elute the peptides with a particular k' value (4 or 10), a number of conclusions can be drawn with respect to the applicability of Snyder's gradient technique for the prediction of isocratic conditions. Table 4 shows that the errors in predicting the concentration of acetonitrile required to elute the underivatized opioid peptide with a k' of 4 ranged from 13.5 (peptide 15) to 38.1% (peptide 19). Except for peptide 19, the errors in predicting the Φ value for a k' of 10 were generally much less than those in predicting the Φ value for k' of 4; also, they were less than 10% for five of the nine peptides studied. The error in predicting the required concentration of acetonitrile to elute peptide 19, was 48% for $k' = 10$, compared with 38% for $k' = 4$.

The introduction of the hydrophobic CBI ring system to the peptide resulted in a substantial improvement in the ability of the model to predict the isocratic mobile phase composition required for a given k' value. Furthermore, there was no difference in the

Table 3
Predicted values of S and log k'_w for native and CBI-derivatives of opioid peptides

Peptide*	Native peptide†				CBI-derivative‡			
	t_{g1}	t_{g2}	$-S$	log k'_w	t_{g1}	t_{g2}	$-S$	log k'_w
5	15.2	18.7	4.29	1.81	8.69	11.9	7.93	4.01
6	12.4	15.3	4.22	1.55	10.8	16.4	6.13	3.56
7	12.9	15.8	4.11	1.58	10.1	15.5	7.99	4.36
11	15.7	17.5	2.05	1.39	14.2	22.4	3.69	2.71
14	16.2	21.2	3.68	1.88	9.9	14.1	6.23	3.44
15	17.1	21.7	4.29	2.18	13.4	21.4	4.37	3.01
19	12.4	15.0	2.34	1.59	13.3	23.7	5.11	2.76
20	15.6	19.3	3.99	1.91	9.6	12.7	5.07	2.81

*See Table 1.

†Column ODS Hypersil; mobile phase: acetonitrile–trifluoroacetic acid (0.1%) (0.02–0.62:0.98–0.38, v/v); gradient times: $t_{G1} = 20$, $t_{G2} = 30$ min; flow rate 1 ml min^{-1}.

‡Column ODS Hypersil; mobile phase: acetonitrile–trifluoroacetic acid (0.1%) (0.40–1.00:0.60–0.00, v/v); gradient times: $t_{G1} = 20$, $t_{G2} = 60$ min; flow rate 1 ml min^{-1}; except for peptide 19, where the mobile phase was: acetonitrile–trifluoroacetic acid (0.1%) (0.40–1.00:0.60–0.00, v/v).

Table 4
Calculated and observed values of Φ for k' = 4 or 10 for various native opioid peptides and their CBI derivatives

Peptide	k' = 4			k' = 10		
	Φ_{calc}*	Φ_{obs}†	Error (%)	Φ_{calc}*	Φ_{obs}†	Error‡ (%)
Native peptides						
5	0.28	0.23	17.7	0.19	0.17	10.5
6	0.23	0.16	30.4	0.13	0.13	0.0
7	0.24	0.18	25.0	0.14	0.14	0.0
11	0.38	0.31	18.4	0.19	0.20	−5.3
14	0.35	0.28	20.0	0.24	0.22	8.3
15	0.37	0.32	13.5	0.28	0.26	7.1
19	0.42	0.26	38.1	0.25	0.13	48.0
20	0.33	0.25	24.2	0.23	0.20	13.0
CBI-derivatives						
5	0.46	0.42	2.3	0.41	0.37	2.6
6	0.48	0.45	6.3	0.44	0.39	7.1
7	0.47	0.43	8.5	0.42	0.37	11.9
11	0.57	0.56	1.8	0.46	0.47	−2.2
14	0.45	0.45	0.0	0.39	0.40	−2.6
15	0.55	0.54	1.8	0.46	0.46	0.00
19	0.42	0.36	14.3	0.34	0.32	5.9
20	0.44	0.43	2.3	0.36	0.38	−5.6

* Value of Φ calculated from equation (1) using the coefficients in Table 2.
† Experimentally determined value of Φ.
‡ $[(\Phi_{calc} - \Phi_{obs})/\Phi_{calc}] \times 100\%$.

errors associated with the prediction of k' = 4 and k' = 10 for the CBI-derivatives of the selected opioid peptides. For both k' = 4 and k' = 10 for all but one of the eight peptides, the concentration of acetonitrile required (Φ_{obs}) were predicted within 10%.

The errors in predicting the required mobile phase composition were attributed to a non-linear relationship between log k' and Φ, arising from the mixed (silanophilic and solvophobic) interactions. Each of the peptides contained at least one functional group (α-amino, ε-amino, guanidino) known to interact strongly with residual silanol, via electrostatic (ion-exchange) interactions [27, 28]. Figure 4 shows the observed relationship between log k' and Φ for peptide 6 and the theoretical relationship obtained from the values of S and k'_w, generated from the gradient experiments. The difference between the theoretical line and the observed line (Fig. 4) clearly demonstrates why the errors in the prediction of Φ are substantially overestimated at lower k' values. This figure also shows that the perfect prediction of the mobile phase composition required to elute peptide 6 with a k' value of 10 is a coincidence which arises from the intersection of the predicted linear and the observed curved (quadratic) relationships.

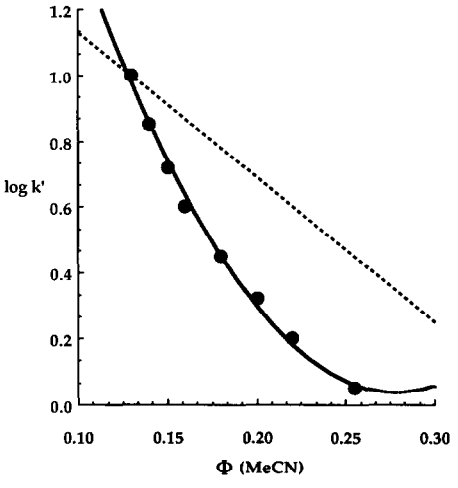


Figure 4
Relationship between log k' of peptide 6 and the volume fraction of acetonitrile (Φ) in the mobile phase. The symbols and solid line represent experimental data obtained under isocratic conditions. The dashed line represents the relationship between log k' and Φ predicted from two linear gradient runs.

The introduction of the bulky CBI ring system appeared to reduce the contribution of the silanophilic interaction to the overall retention and resulted in an essentially linear relationship between log k' and Φ for all solutes studied. This linear relationship be-

tween log k' and Φ for the CBI-peptides explains the excellent agreement that was observed between the predicted and observed isocratic conditions required for a given k' value (Table 4 and Fig. 5).

The reduction in the contribution of the silanophilic interaction appeared to be related to the presence of the CBI ring *per se*, rather than the neutralization of positively charged functional groups, because the introduction of the ring linearized the relationship between log k' and Φ, for all the peptides including those containing arginine or sulphoxide groups, which do not react with NDA/CN.

The interaction between protonated amines and residual silanols is well documented [27, 28]. However, the interaction between sulphoxide groups and residual silanols has not been reported, but is clearly demonstrated by the non-linear relationship between log k' and Φ values for methionine enkaphalin sulphoxide (peptide 19). Figure 6 shows that the retention of underivatized peptide 19 is dominated by silanophilic interaction at Φ greater than 0.4 and that the overall relationship between the log k' and Φ can be fitted to a quadratic equation. The introduction of the CBI ring completely abolishes silanophilic interactions, such that at Φ greater than 0.4, the underivatized peptide was more retained on the column than its CBI-derivative. At Φ less than 0.35, where solvophobic interactions dominate, the CBI-derivative of peptide 19 was more retained than the native peptide, consistent with its greater hydrophobicity.

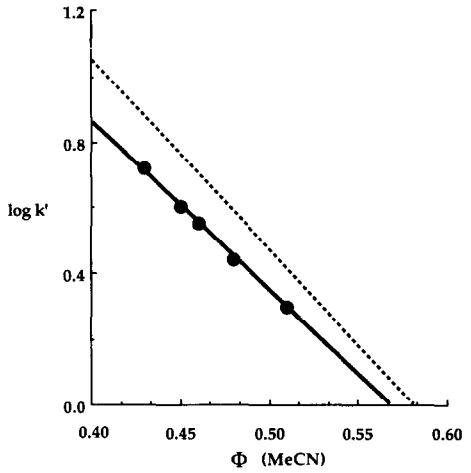


Figure 5
Relationship between log k' for the CBI-derivative of peptide 6 and the volume fraction of acetonitrile (Φ) in the mobile phase. The symbols and solid line represent experimental data obtained under isocratic conditions. The dashed line represents the relationship between log k' and Φ predicted from two linear gradient runs.

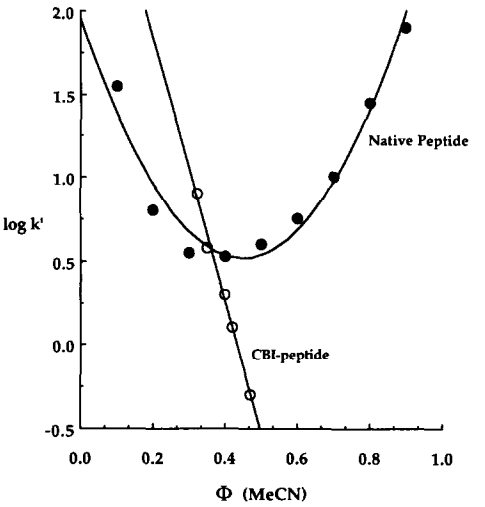


Figure 6
Relationship between log k' of methionine-enkephalin sulphoxide (peptide 19) and its CBI derivative and the volume fraction of acetonitrile (Φ) in the mobile phase. The symbols represent experimental data obtained under isocratic conditions.

Contribution of the CBI-ring system to retention

The final step in the chromatographic characterization of the opioid peptides involved the calculation of the contribution of the CBI ring system to retention. Under normalized solvent conditions, the capacity ratios at Φ(MeCN) = 0.25 were obtained by extrapolation of the quadratic equation [equation (13)] relating log k' and Φ, using the coefficients, S' and D given in Table 5:

$$\log k' = \log k_w - S'\Phi + D\Phi^2. \quad (13)$$

The contribution of the CBI ring system (τ_{CBI}) at Φ(MeCN) = 0.25 was then calculated from equation (14) for each of the eight peptides studied (Table 6):

$$\tau_{CBI} = \log \alpha = \log \left(\frac{k'_{CBI}}{k'_{RNH_2}}\right). \quad (14)$$

Table 6 shows that the contribution of the CBI ring system to the retention of the opioid peptides (except for peptide 19) had a mean value (±SD) of 1.9 (±0.1) under these isocratic conditions. The data (Table 6) were further analysed by relating the logarithm of the capacity ratios to the sum of the retention coefficients of the constituent amino-acid resi-

Table 5
Coefficients ($\log k'_w$, S and D) of the quadratic equation [equation (13)] relating retention ($\log k'$) to the volume fraction of acetonitrile in the mobile phase for eight opioid peptides and their corresponding CBI derivatives

Peptide	Native peptide			CBI-derivative		
	$\log k'_w$	$-S'$	D	$\log k'_w$	$-S'$	D
5	2.28	7.44	0.00	7.00	22.69	17.80
6	3.00	20.13	33.53	4.93	13.60	8.90
7	3.19	20.38	32.92	5.14	14.97	10.38
11	1.91	5.43	3.94	5.98	15.69	10.89
14	3.71	16.08	17.55	6.71	20.25	14.66
15	1.81	0.25	−11.14	6.32	16.92	11.71
19	1.70	6.33	8.11	5.30	17.33	11.74
20	3.63	17.52	22.07	7.98	27.63	24.45

Table 6
Logarithm of the capacity ratios of eight opioid peptides and their corresponding CBI derivatives, and the values of the contribution of the CBI ring system to retention (τ_{CBI})

Peptide	$\log k'_{CBI}$*	$\log k'_{RNH_2}$*	τ_{CBI}†
5	2.44	0.42	2.02
6	2.08	0.07	2.01
7	2.04	0.15	1.89
11	2.74	0.80	1.94
14	2.57	0.78	1.79
15	2.82	1.05	1.77
19	1.70	0.63	1.07
20	2.60	0.63	1.97

*Calculated from equation (13) using the coefficients in Table 5.
†Equation (14).

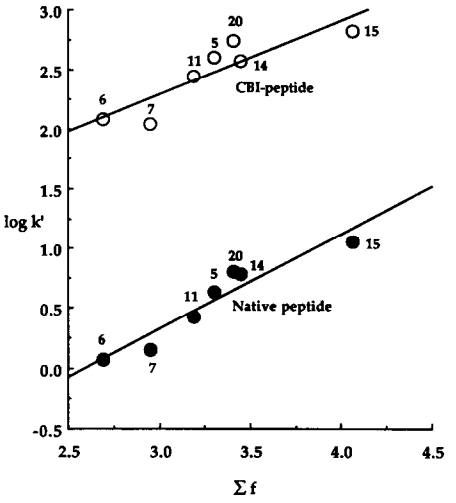


Figure 7
Relationships between the isocratic $\log k'$ values [at Φ(MeCN) = 0.25] of seven opioid peptides, and their CBI derivatives, and the sum of their fragmental retention constants [31]. Each peptide has been identified by the numbers shown in Table 1. The symbols represent experimental data and the lines have been drawn according to equations (14) and (15) for the native and derivatized peptides, respectively.

dues of the peptides (Σf) described by Sasagawa and Teller [31]. This analysis could not be performed for peptide 19 because no suitable value of Σf for the sulphoxide group could be found. The linear relationships between $\log k'$ and Σf for the native peptides and CBI derivatives are shown in Fig. 7 and given by equations (15) and (16), respectively:
native peptides:

$$\log k'[\Phi(\text{MeCN}) = 0.25] = 0.79\,\Sigma f - 2.06 \quad (r = 0.953); \qquad (15)$$

CBI derivatives:

$$\log k'[\Phi(\text{MeCN}) = 0.25] = 0.63\,\Sigma f + 0.93 \quad (r = 0.887). \qquad (16)$$

The good agreements between the slopes of these relationships [equations (15) and (16)] are indicative of a common retention mechanism. Because this analysis was performed at a relatively low value for Φ(MeCN) of 0.25, it was concluded that solvophobic interactions were dominant.

Conclusions

This study has demonstrated the constant contribution of the CBI ring system to the retention of peptides, which means that the retention of CBI-peptides may be predicted from that of the corresponding native peptide. This observation should be particularly useful in peak identification of peptides following pre-column derivatization with NDA/CN. Furthermore, it was also shown that the isocratic conditions [Φ(MeCN)] for the native opioid peptides can be predicted within 12%, provided one is prepared to accept a k' value of 10. Errors in predicting the concentration of acetonitrile to achieve a k' value of 4 for the

opioid peptides were as high as 40%. However, the logarithmic relationship between k' and Φ results in relatively small errors in the observed value of k', within the range $4 \leq k' \leq 10$. Thus the use of linear gradient elution for the prediction of isocratic retention represents a viable approach for the identification of the initial isocratic conditions for both the single-column and multidimensional separations of the opioid peptides and their fluorescent CBI derivatives.

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