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Reversed-phase ion-pair liquid chromatography of the angiotensins

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ABSTRACT

The isocratic reversed-phase liquid chromatography of the angiotensins and a number of their synthetic analogues is described. Complete separation of 10 out of 12 peptides was achieved through a solvent optimization strategy with a total analysis time of about 20 min. The retention behavior of the angiotensins studied was described in terms of the hydrophobic contribution of their amino acid residues; there was good correlation between predicted and experimental retention for those peptides that were retained by a common mechanism. However, because ion-pair chromatography was required for good peak symmetry, retention was substantially modulated by the presence of acidic and basic residues. The limit of detection of these peptides was 3–5 pmol by UV absorbance at 214 nm. For those peptides containing a primary amino group the detection limit was improved by two orders of magnitude by fluorogenic derivatization with naphthalene-2,3-dicarboxaldehyde/cyanide to the corresponding N-substituted 1-cyanobenz[*f*]isoindole (CBI) derivatives. The contribution of the CBI ring system to retention was also investigated.

INTRODUCTION

Extensive literature exists on the involvement of the peripheral and central renin/angiotensin system (RAS) in the regulation of blood pressure and aldosterone secretion [1,2]. Therefore in order to understand and investigate the RAS, the identification and quantification of the RAS components and synthetic agonists and antagonists of the angiotensins (ATs) are very important. Classical analytical approaches included radioimmunoassay or radiochemical methods [3]. Subsequently, separation techniques, such as polyacrylamide gel electrophoresis, isoelectric focusing and paper chromatography, have been employed for the analysis of native ATs and their synthetic analogues [4]. Complete resolution of closely similar title peptides, however, was not always possible.

The introduction of reversed-phase liquid chromatography initiated a renewed interest in this field, not only for the ATs but for peptides in general. Liquid chromatographic (LC) separations of the ATs have been achieved, conventionally, by linear-gradient analysis, but poor reproducibility of retention time is a common problem [5–10]. Additionally, gradient analysis can limit the choice of the reagents (salts, ion-pair reagents) that may be employed to modify peak shape and retention characteristics. Isocratic methods are generally more flexible, placing fewer restric-

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tions on the choice of the mobile phase additives and a number of such methods for the analysis of the ATs have been reported [9–14]. However, these methods either lack the desired selectivity or give very poor peak shape. Furthermore, the retention of peptides can be sensitive to very small changes in mobile phase composition and optimization of isocratic separations by the traditional trial and error method can be very tedious. Finally, polar peptides, such as ATs, exhibit non-linear relationships between the logarithm of capacity factor ($\ln k'$) and the volume fraction (φ) of the organic modifier in mobile phase (see, *e.g.*, refs. 15–17), and this has been attributed to the influence of both hydrophobic and silanophilic interactions. As well as making the optimization of separations a complex process, the interactions with residual silanol groups can give rise to assymetric peaks [15–17].

The present study was prompted by the lack of adequate isocratic methodology for the reversed-phase LC of the ATs and the objectives were firstly to investigate the factors influencing the isocratic reversed-phase LC of the ATs (Table I) and secondly to develop a strategy for the optimization of their retention.

EXPERIMENTAL

Chemicals and reagents

Synthetic angiotensin-I (AT-10, peptide content 77.6%), angiotensin-II (AT-20, 83.8%), angiotensin-III (AT-30, 96%) and the other analogues (AT-21–AT-29, Table I), were purchased from Peninsula Labs. (Belmont, CA, U.S.A.). Methanol, acetonitrile and tetrahydrofuran were obtained from Fisher Scientific (Fair Lane, NJ, U.S.A.). HPLC-grade 1-pentane-, 1-hexane-, 1-heptane- and 1-octanesulphonic acid sodium salts and 1-hexane- and 1-octanesulphate sodium salts were obtained from Eastman-Kodak (Rochester, NY, U.S.A.). High-purity water from Milli-Q water

TABLE I

AMINO ACID SEQUENCES, THEORETICAL RETENTION CONSTANTS (ΣD_j) AND CAPACITY FACTORS OF THE ANGIOTENSIN PEPTIDES AND THEIR ANALOGUES

ΣD_j values taken from Sasagawa *et al.* [25]. Capacity factor under optimized solvent conditions, mobile phase C'.

Amino acid sequence	Code	ΣD_j	k'	Name
Asp-Arg-Val-Tyr-Val-His-Pro-Phe	AT-22	4.88	1.13	
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	AT-20	5.88	1.59	Angiotensin-II
Sar-Arg-Val-Tyr-Val-His-Pro-Ala	AT-29	4.03	2.18	Saralasin
Sar-Arg-Val-Tyr-Ile-His-Pro-Gly	AT-25	5.12	2.88	
Sar-Arg-Val-Tyr-Ile-His-Pro-Thr	AT-27	5.02	2.99	
Sar-Arg-Val-Tyr-Ile-His-Pro-Ala	AT-24	5.03	3.04	
Asn-Arg-Val-Tyr-Val-His-Pro-Phe	AT-23	4.33	3.56	
Sar-Arg-Val-Tyr-Ile-His-Pro-Ile	AT-28	6.28	5.55	
Sar-Arg-Val-Tyr-Ile-His-Pro-Leu	AT-26	6.24	6.63	
Sar-Arg-Val-Tyr-Ile-His-Pro-Phe	AT-21	6.61	8.72	
Arg-Val-Tyr-Ile-His-Pro-Phe	AT-30	5.78	10.85	Angiotensin-III
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	AT-10	7.56	13.82	Angiotensin-I

system (Millipore, Bedford, MA, U.S.A.) was used for all solutions and mobile phase preparations. Naphthalene-2,3-dicarboxaldehyde (NDA) was obtained from Oread Labs. (Lawrence, KS, U.S.A.). Reagent-grade sodium cyanide was purchased from Matheson, Coleman and Hall (Norwood, Cincinnati, OH, U.S.A.). All other chemicals were of reagent grade.

Liquid chromatography

The chromatography was performed with a modular system which comprised a Shimadzu LC-6A liquid chromatograph (Shimadzu, Columbia, MD, U.S.A.), a SIL-6A autoinjector (Shimadzu), a SCL-6A system controller (Shimadzu), a UV-SPD6AV monitor (Shimadzu), RF-535 fluorescence monitor (Shimadzu), an Omniscribe chart recorder (Houston Instruments, Houston, TX, U.S.A.) and either an CR-6A or an CR-3A Chromatopac computing integrator (Shimadzu).

The ATs were dissolved in phosphate buffer (pH 2.5, 0.1 *M*) and injected (50 μl) into an ODS Hypersil column (5 μm, 150 × 4.6 mm I.D.) (Keystone, State College, PA, U.S.A.). The column temperature was controlled (40 ± 0.1°C) by placing it in an oven (CTO-6A, Shimadzu) and the column effluent was monitored by either UV absorbance (214 nm) or fluorescence (excitation wavelength 420 nm, emission wavelength 490 nm). The autoinjector sample tray was maintained at 6–8°C with an RM6 sample cooling unit (Brinkmann, Westbury, NY, U.S.A.). The flow-rate was varied between 1.0 and 2.0 ml/min.

The chromatographic investigations involved the establishment of the initial conditions for the isocratic separation of the ATs followed by the optimization of the separation using a modified mixture design statistical technique. The initial conditions were established by investigating the influence of the following factors on retention (k') and peak asymmetry (A_s): solvent type (methanol, acetonitrile and tetrahydrofuran) and concentration (φ = 0.2–0.6), buffer type [0.1 *M*; perchlorate, phosphate and trifluoroacetate, pH (2.5, 3.5, 4.5 and 6.0)] and ion pair reagent type (1-pentane; 1-hexane; 1-heptane- and 1-octanesulphonic acid sodium salt and 1-hexane-, 1-octane-sodium sulphate) and concentration (5, 10, 15, 20 and 30 m*M*).

From these initial experiments, three isoelutropic mobile phases were established (A, B and C) in which the peptides of interest eluted as virtually symmetrical peaks ($A_s < 1.2$) with a k' range of approximately 1–20. The aqueous component of the three mobile phases (solvent X) was a phosphate buffer (pH 6.0; 100 m*M*) containing sufficient 1-octanesulphonic acid sodium salt to give a final mobile phase concentration of 30 m*M*. The compositions of the three isoelutropic mobile phases were as follows: (A) X–acetonitrile (75:25, v/v), (B) X–methanol (51:49, v/v), and (C) X–tetrahydrofuran (81.5:18.5, v/v). The isoelutropic plane defined by the triangle A:B:C was then interrogated using a modified mixture design statistical technique to obtain the optimum conditions for the resolution of the 12 ATs of interest.

Derivatization with NDA/CN

Peptides AT-20, -22 and -23 were converted to their corresponding fluorescent N-substituted 1-cyanobenz[*f*]isoindoles (CBI-ATs) by reaction with naphthalene-2,3-dicarboxaldehyde/cyanide (NDA/CN) [18–21]. The reaction was conducted by mixing, in order, 20 μl aqueous peptide solution (25 μM), 50 μl ascorbic acid (200 m*M* in water), 100 μl NaCN (10 m*M* in water), 200 μl NDA (5 m*M* in acetonitrile) and

540 μl phosphate buffer (100 m*M*, pH 7.0). The solution was mixed by inversion and stored over an ice bath (0–4°C) for 20 min. A 50-μl volume of an aqueous taurine solution (200 m*M*) was then added to quench the reaction [21].

RESULTS AND DISCUSSION

Establishment of initial conditions

To establish the initial conditions, the effects of pH, buffer salts and various anionic ion-pairing agents on the retention and peak shape of the AT were investigated in a systematic fashion using acetonitrile as the organic modifier. The objective was to obtain symmetrical peaks and retention with a k' range of 1–20. Pronounced peak tailing ($A_s > 5$) was observed for all the ATs when they were eluted with mobile phases containing various concentrations of acetonitrile in sodium perchlorate–perchloric acid, trifluoroacetic acid–sodium hydroxide and phosphoric acid–sodium hydroxide buffers (each 0.1 *M*) in the pH range of 2.5 to 3.5. A slight improvement in peak shape was achieved by adjustment of the pH of the mobile phase to a value of 6 with a phosphate buffer (0.1 *M*); however, the problem of poor peak shape was ultimately resolved by the use of ion-pair chromatography [22,23].

The addition of alkylsulphates and -sulphonates to the mobile phase increased the retention of the oppositely charged ATs and, consistent with previous observations (*e.g.*, refs. 22 and 24) retention increased with increasing concentration (5–30 m*M*) and hydrophobicity (chain length, $n = 5$–8) of the ion-pair reagent. More importantly the peak shape of the solutes improved dramatically with the addition of the ion-pairing agent to the mobile phase [23]. The improvement in peak shape was related to the concentration and chain length of the ion-pair reagent; and A_s values of less than 1.2 were achieved with 30 m*M* octanesulphate sodium salt in the aqueous component of the mobile phase. (Throughout these ion-pair experiments, the pH of the aqueous component of the mobile phase was maintained at a value of 6.0 with a phosphate buffer (0.1 *M*) and the volume fraction of the organic modifier, acetonitrile, was fixed at 0.25.) These initial experiments established mobile phase A as the optimum binary solvent system for the resolution of the 12 ATs, which eluted between $k' = 1.47$ (AT-22) and 21.6 (AT-10) with good column efficiency ($N = 20\,000$–$40\,000$ plates/m) and excellent symmetry ($A_s < 1.2$). The composition of mobile phase A was X–acetonitrile (75:25, v/v), where the aqueous component of the mobile phases (X) was a phosphate buffer (pH 6.0; 100 m*M*) containing sufficient 1-octanesulphonic acid sodium salt to give a final mobile phase concentration of 30 m*M*.

To reduce the consumption of the peptides, these experiments were conducted with two peptides AT-29 and AT-10, which, according to their theoretical retention constants (ΣD_j, Table I [25]), were expected to be the first and last analytes to elute, respectively. Peptide AT-30 was correctly predicted to be the last peptide to elute. However, AT-20 and -22 eluted before peptide AT-29, which was, in fact, the third peptide to elute. Nevertheless, the ΣD_j values did allow the initial conditions for the separation of the 12 peptides to be established by studying the two analytes with the largest and the smallest values of ΣD_j. An explanation for the earlier than expected elution of AT-20 and -22 is provided below, in the section *Quantitative structure–retention relationships.*

Solvent optimization

The first step in the solvent optimization was to establish that mobile phases B and C containing methanol ($\varphi = 0.49$) and tetrahydrofuran ($\varphi = 0.185$), respectively, were isoelutropic with mobile phase A (in these and subsequent experiments the composition of the aqueous solvent X was the same as in mobile phase A). The three mobile phases (A, B and C) defined an isoelutropic plane (k' for AT-10 $\approx$ 20) as shown in Fig. 1. Fig. 2 shows the isochronal chromatograms corresponding to three isoelutropic mobile phases A, B and C.

The second phase of the optimization procedure was to explore the isoelutropic plane (A:B:C) to establish the optimum quaternary mixture (acetonitrile–methanol–

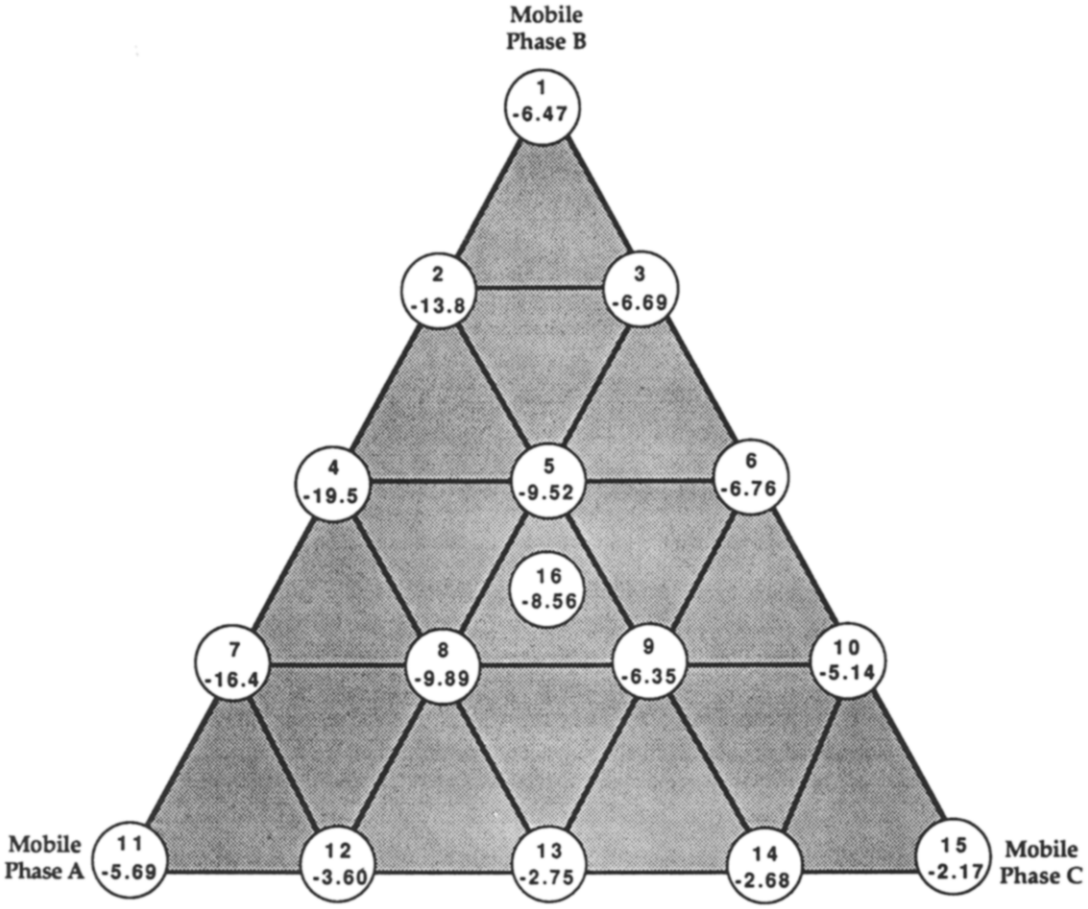

Fig. 1. Modified mixture design statistical technique. A 5-level pseudo-factorial experimental design was used in which pseudo-solvents A, B and C were combined in different proportions. Each node (1 to 16) represents a combination of these solvents. Chromatographic optimization function (COF) values corresponding to each solvent composition (nodes 1–16) are shown in the circles and in Table II. The aqueous component of the mobile phases (solvent X) was a phosphate buffer (pH 6.0; 100 m*M*) containing sufficient 1-octanesulphonic acid sodium salt to give a final mobile phase concentration of 30 m*M*. Column: ODS Hypersil (5 μm, 150 $\times$ 4.6 mm I.D.). Temperature: 40 $\pm$ 0.1°C.

tetrahydrofuran–solvent X) for the resolution of the solutes. Employing these initial conditions, a 16-point experiment (Fig. 1) was conducted so that a modified mixture design statistical technique with bi-dimensional interpolation could be employed to identify the optimum conditions [26]. The quality of each chromatogram was judged by the calculation of the chromatographic optimization function (COF) [26–29]:

$$\mathrm{COF} = \alpha \sum_{i=1}^{p-1} \ln\left(\frac{R_{s_i}}{R_{s_{di}}}\right) + \beta(t_{\max} - t_{\exp}) - \gamma(n - p) \qquad (1)$$

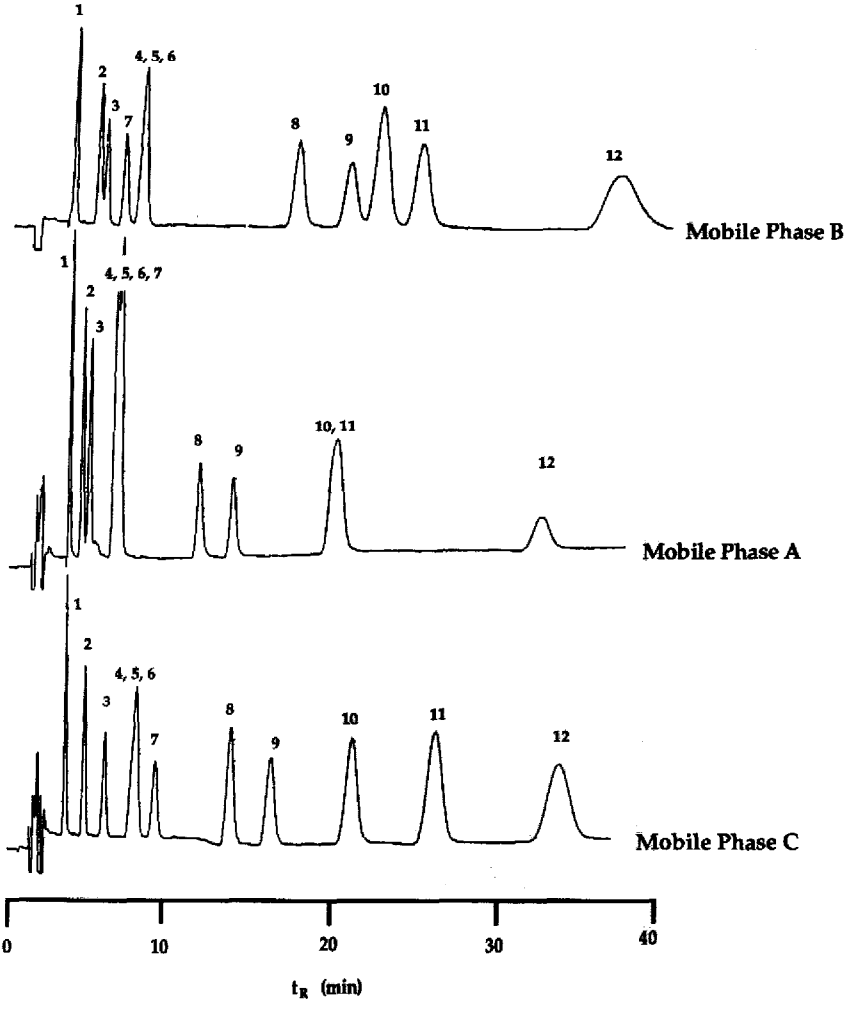

Fig. 2. Isochronal chromatograms corresponding to the three isoelutropic solvents A, B and C. Column: ODS Hypersil (5 μm, 150 × 4.6 mm I.D.). Mobile phases A, B and C as in text. Temperature: 40 ± 0.1°C. Peaks: 1 = AT-22; 2 = AT-20; 3 = AT-29; 4 = AT-24; 5 = AT-25; 6 = AT-27; 7 = AT-23; 8 = AT-28; 9 = AT-26; 10 = AT-21; 11 = AT-30; 12 = AT-10.

where R_{s_i} is the resolution factor for peak-pair i and $i + 1$, $R_{s_{di}}$ is the desired resolution for peak-pair i and $i + 1$ (in this case 1.8 for each pair), t_{exp} is the experimental analysis itime (*i.e.*, retention time, t_R, of last peak, p), t_{max} is the maximum desired analysis time (25 min), p is the actual number of peaks in the chromatogram and n is the expected number of peaks in the chromatogram (12). The weighting factors for the separation term (α), the analysis time term (β) and the total number of peaks term (γ) were arbitrarily assigned values of 1, 0.2 and 3, respectively. The COF values for the 16 chromatograms are shown in Fig. 1. The maximum COF value was obtained for node number 15, which corresponds to optimum mobile phase C.

The isochronal separations of the 12 peptides for mobile phase A, B and C are shown in Fig. 2. A further appreciation of the influence of organic modifier on the overall resolution can also be obtained from the resolution map in Fig. 3. It is clear that mobile phases containing tetrahydrofuran (C) or acetonitrile (B) were preferable to those containing methanol (A) because of the separation of AT-21 (peak 10) from AT-30 (peak 11) and the separation of AT-23 from AT-24, -25 and -27. The latter peptides (AT-24, -25 and -27, peaks 4, 5 and 6) were unresolved in all the mobile phases investigated (Fig. 2). Mobile phase C was superior to mobile phase B primarily because of the better separation that was achieved for AT-22, -20 and -29 (peaks 1–3) and the overall resolution of AT-28, -26, -21, -30 and -10 (peaks 8–12).

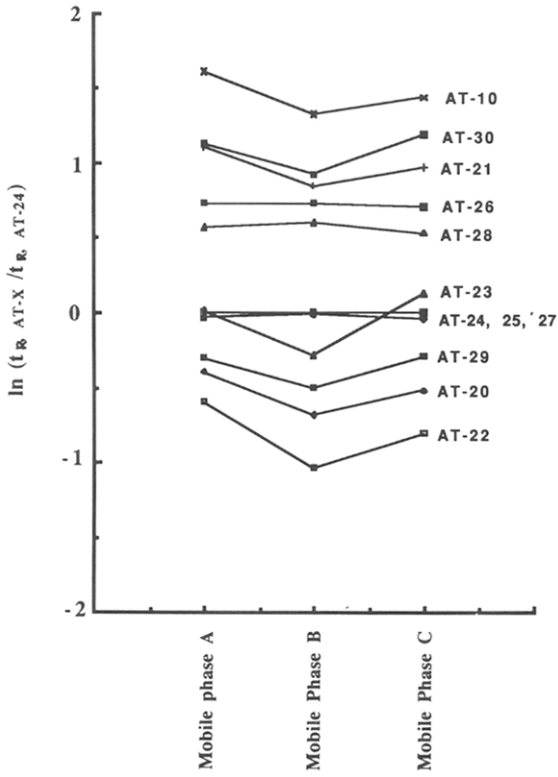

Fig. 3. Resolution map showing the retention times of the 12 ATs with the three isoelutropic solvents A, B and C (see text).

The final step in the optimization procedure was to increase the volume fraction of tetrahydrofuran from 0.185 to 0.190 (solvent C'). This decreased the overall analysis time from 30.6 to 21.2 min (Fig. 4) without altering the selectivity previously achieved. This optimization effort allowed isocratic separation of 10 of the 12 closely related AT peptides (Table I) which has not been previously reported. In addition, the peak shapes ($A_s < 1.2$) and the column efficiencies are much better than previous attempts at isocratic resolution of these peptides [5–14].

Quantitative structure–retention relationships

The optimization procedure for the isocratic separation of the 12 ATs was conducted without considering either the structure of the peptides or their retention mechanisms. After identifying the initial conditions, the only variables considered were the nature and the concentrations of the organic modifiers in the mobile phase. The ΣD_j values, which are essentially a measure of solute hydrophobicity were useful in establishing the initial conditions. However, Fig. 5 and eqn. 2 show that the overall relationship between retention and solute hydrophobicity (ΣD_j) was poor:

$$\ln k' = 0.546 \sum D_j - 1.648, \quad r^2 = 0.511 \ (r = 0.715) \tag{2}$$

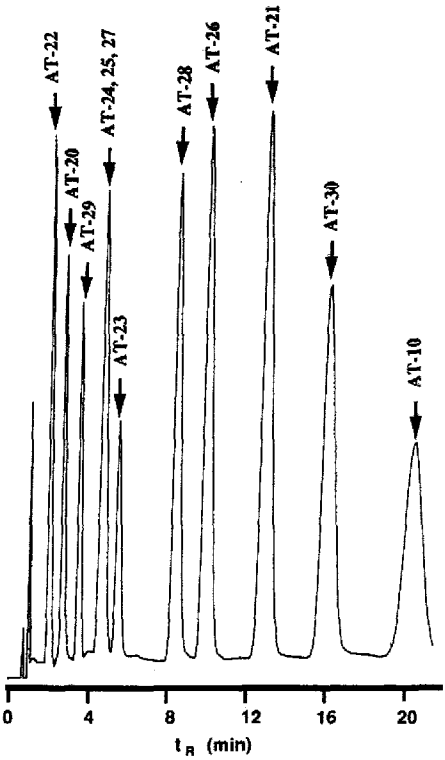

Fig. 4. Separation of ATs under optimized solvent conditions (mobile phase C'). The aqueous component of the mobile phases (solvent X) was a phosphate buffer (pH 6.0; 100 m*M*) containing sufficient 1-octanesulphonic acid sodium salt to give a final mobile phase concentration of 30 m*M*. Column: ODS Hypersil (5 μm, 150 × 4.6 mm I.D.). Temperature: 40 ± 0.1°C.

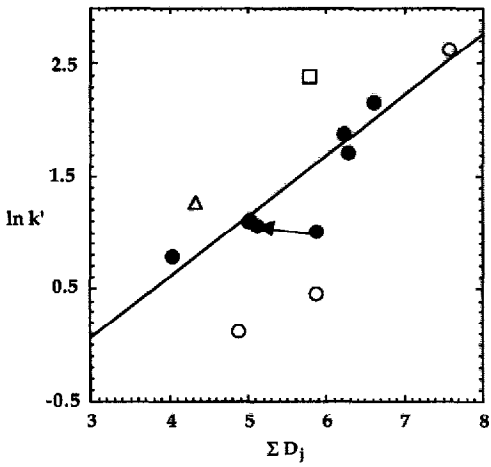

Fig. 5. Relationship between the capacity ratios of the 12 ATs studied and their theoretical retention constants (ΣD_j) (Table I). The different symbols represent peptides with the same N-terminal amino acids as follows: Sar (●, AT-21, -24, -25, -26, -27, -28, -29), Asp (○, AT-10, -20, -22), Asn (□, AT-23), Arg (△, AT-30). The aqueous component of the mobile phases (solvent X) was a phosphate buffer (pH 6.0; 100 m*M*) containing sufficient 1-octanesulphonic acid sodium salt to give a final mobile phase concentration of 30 m*M*. Column: ODS Hypersil (5 μm, 150 × 4.6 mm I.D.). Temperature: 40 ± 0.1°C.

This poor correlation (eqn. 2) was not unexpected because ion-pair chromatography was employed and several of the peptides had different charges arising mainly from differences in the N-terminal amino acid. It has been well established [23,30] that retention in reversed-phase ion-pair LC is markedly influenced by the size of the electrical charge on the solutes. Fig. 5 and eqn. 3 show that there was a very good correlation between the observed retention (ln k') and the theoretical retention constants (ΣD_j) for those peptides, which possessed a sarcosine residue at the N-terminus (AT-21, -24, -25, -26, -27, -28 and -29). It is reasonable to assume that these peptides all possessed the same electrical charge because they all have the same ionizable functional groups.

$$\ln k' = 0.541 \sum D_j - 1.562, \quad r^2 = 0.939 \ (r = 0.969) \tag{3}$$

It is interesting to note that two of the three ATs with an N-terminal aspartic acid (AT-20, -22) residue lie substantially below the regression line defined by the peptides with the N-terminal sarcosine (Fig. 5). In contrast, the data point for the other aspartic acid-containing peptide (AT-10) is very close to the regression line defined by the sarcosine-containing peptides. This observation was explained by the fact that the additional negative charge in AT-10 arising from the aspartic acid residue was cancelled by the additional positive charge from the second histidine residue, which is not present in the other two aspartic acid-containing peptides (AT-20, -22). Although the net electrical charge on the two remaining peptides (AT-23 and -30) was expected to be the same as that on the sarcosine-containing peptides, the retention of AT-23 and -30 was greater than predicted by eqn. 3. This was particularly true for AT-30, which had an N-terminal arginine residue. The retention of AT-30 was about three times

greater than would have been predicted from the regression line generated by the sarcosine-containing peptides.

One final observation worthy of comment is the poor resolution of peptides AT-24, -25 and -27, which was accurately predicted by the fragmental retention constants. These three peptides (AT-24, -25 and -27) differ only in the amino acids at the C-terminus and have only very small differences in their theoretical retention constants [25]. The ΣD_j values for Gly, Ala and Thr are 0.22, 0.13 and 0.12, respectively [25].

Fluorescent derivatives of the angiotensins

Using UV absorbance at 214 nm, the detection limit (signal-to-noise ratio 3) for the ATs studied here was 3–5 pmol injected (50 μl). This detection limit is generally inadequate for the determination of endogenous or exogenously administered ATs [30–32]. For example, the concentrations of native angiotensin-I and angiotensin-II in canine cerebrospinal fluid (CSF) are about 1.54 and 6.17 fmol/ml, respectively, as determined by radiolabelled peptides [30]. About ten-fold higher concentrations (10–50 fmol/ml) of these peptides have been reported in human plasma [31]. Recently, deMontigny *et al.* [18] and others [19–21] have described the naphthalene-2,3-dicarboxaldehyde/cyanide reagent system for the fluorogenic derivatization of primary amines. The present study has demonstrated the feasibility of using NDA/CN for the enhancement of detection of the primary amine-containing ATs, using AT-20, -22 and -23 as model compounds. The objectives of the present study were to establish the detection limit of the CBI-ATs and to investigate the contribution of the CBI-ring system to the reversed-phase retention of these peptides.

The ATs (25 μM) were converted to their fluorescent CBI-derivatives using the procedures developed previously for the derivatization of opioid peptides [21] by reaction with an excess of NDA (1 mM) and sodium cyanide (2 mM). The reaction was conducted at a pH of 7.0, which is approximately equal to the pK_a of the terminal amino group and is generally considered to be an optimum condition for the rate and yield of the reaction [18,20]. After reacting for 20 min over an ice bath (0–4°C) the reaction was quenched by the addition of excess taurine (10 mM). Quenching of the reaction was necessary to prevent accumulation of fluorescent degradation products of the NDA/CN reagents system [19–21]. With mobile phase C′ the detection limits of CBI-AT-20, -22 and -23 were 50–100 fmol (signal-to-noise ratio 3) with conventional fluorescence with excitation at 420 nm (xenon lamp) and detection of the emission at 490 nm.

Recently, it has been shown [33–35] that sub-fmol detection limits for CBIs and similar ring systems are possible with the laser-induced fluorescence (LIF) detectors based on the He–Cd laser. However, most of the previous studies [33,34] have dealt with the analysis of amino acids, biogenic amines or protein hydrolysates. Efforts in this laboratory are being devoted towards the application of LC–LIF to analysis of intact peptides in biological samples. Preliminary results on the application of LC–LIF to the analysis of opioid peptides were presented recently [35], and its application to other classes of peptides including the ATs will be presented at a later date.

The contribution of the CBI-ring system to retention was calculated by determining the capacity ratios (Fig. 6 and Table II) of AT-20, -22 and -23 and their corresponding CBI-derivatives on an ODS Hypersil column at 40°C with a mobile

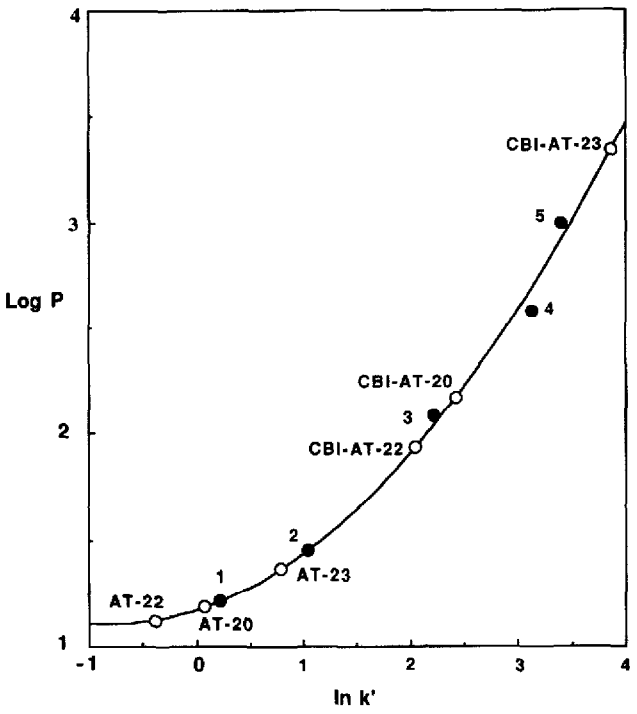

Fig. 6. Calibration plot showing the relationship between the 1-octanol–water partition coefficient (P) and chromatographic retention (k'). The data points (see also Table II) for acetanilide (1), benzaldehyde (2), anisole (3), toluene (4) and bromobenzene (5) represent experimental k' values and literature values of log P [36]. The line has been drawn according to the quadratic eqn. 4. The data points for the ATs and the CBI-ATs represent experimental k' values and values of P calculated from eqn. 5. Column: ODS Hypersil (5 μm, 150 × 4.6 mm I.D.). Mobile phase C': X–tetrahydrofuran (81:19, v/v). The aqueous component of the mobile phases (X) was a phosphate buffer (pH 6.0; 100 mM) containing sufficient 1-octanesulphonic acid sodium salt to give a final mobile phase concentration of 30 mM. Temperature: 40 ± 0.1°C.

phase of solvent X–THF (81:19, v/v). The capacity ratios (k') were converted to apparent partition coefficients (P) by calibration (Table II) of the chromatographic system with five compounds, acetanilide, benzaldehyde, anisole, toluene and bromobenzene, the partition coefficients of which are well established [35]. The experimental k' values were related to the known partition coefficients by a quadratic equation (eqn. 4) from which the apparent partition coefficients of the ATs and their CBI-derivatives were calculated.

$$\log P = 0.103\,(\ln k')^2 + 0.093 \ln k' + 1.125,\ n = 5,\ r^2 = 0.992\ (r = 0.996) \qquad (4)$$

The contribution of the CBI-ring system to the 1-octanol–water partition coefficient (π_{CBI}) was then calculated from:

$$\pi_{CBI} = \log\left(\frac{P_{CBI\text{-}AT}}{P_{AT}}\right) \qquad (5)$$

TABLE II

1-OCTANOL–WATER PARTITION COEFFICIENTS (log P) OF TEST SOLUTES AND THE CHROMATOGRAPHICALLY DERIVED log P VALUES FOR THE ANGIOTENSINS AND THEIR CYANOBENZ[*f*]ISOINDOLE DERIVATIVES

Log P (exp) = logarithm of the 1-octanol–water partition coefficient [36]; log P (calc) = logarithm of the 1-octanol–water partition coefficient calculated from eqn. 4; π_{CBI} = contribution of CBI-ring system to the 1-octanol–water partition coefficient calculated from eqn. 5.

Analyte	Log P (exp)	Log P (calc)	π_{CBI}
Acetanilide	1.21	–	–
Benzaldehyde	1.45	–	–
Anisole	2.08	–	–
Toluene	2.58	–	–
Bromobenzene	2.99	–	–
AT-22	–	1.122	–
AT-20	–	1.184	–
AT-23	–	1.364	–
CBI-AT-22	–	1.937	0.815
CBI-AT-20	–	2.166	0.982
CBI-AT-23	–	3.337	1.97

Very similar values for the contribution of the CBI-ring systems were obtained for CBI-AT-20 (π_{CBI} = 0.982) and CBI-22 (π_{CBI} = 0.815) but these values were substantially lower than the value obtained for CBI-AT-23 (π_{CBI} = 1.97). It should be noted that these values of π_{CBI} actually represent the combined effect of adding a CBI-ring system and the removal of a positively charged amino group. The values obtained here for π_{CBI} may differ from those measured in other chromatographic systems because ion-pair chromatography was used and it is expected that the positively charged amino group will be involved in the retention process via electrostatic interactions with the oppositely charged pairing ion. Nevertheless, the results indicate that the contribution of the CBI-ring system to retention is not constant, at least in ion-pair systems, and that it is influenced by the nature of the amino acid to which it is attached. The fact that the value obtained for π_{CBI} was the same for AT-20 and -22, which both have an aspartic acid residue at the N-terminus, provides some confidence that the retention contribution of the CBI-ring system is constant for a particular amino acid residue, but this needs to be substantiated in future investigations.

CONCLUSIONS

Synthetic and naturally occurring ATs were eluted from an ODS Hypersil column under isocratic conditions with excellent peak shape ($A_s < 1.2$) and good column efficiencies (N = 20 000–40 000 plates/m), provided sodium alkylsulphonates or alkylsulphates were added to the mobile phase to eliminate tailing. Once the initial isocratic conditions were determined, an optimized separation of the AT was achieved by establishing three isoelutropic mobile phases A, B and C containing acetonitrile, methanol or tetrahydrofuran, respectively. The second and final step involved the

exploration of the isoelutropic plane (A:B:C) using a modified mixture design statistical technique with bidimensional interpolation to establish the optimum quaternary mixture (acetonitrile–methanol–tetrahydrofuran–solvent X) for the resolution of the solutes. The main advantage of this approach was that it allowed the optimization of the separation to be achieved with a small number of experiments and the minimum expenditure of potentially expensive peptides. In addition, the separation was optimized in the absence of detailed knowledge of retention mechanisms or the structure of the peptides.

This study also demonstrated that the detectability of those ATs containing a primary amino group may be substantially enhanced by reaction with naphthalene-2,3-dicarboxaldehyde to the corresponding fluorescent cyanobenz[*f*]isoindole (CBI) derivatives. Finally, preliminary data were obtained to indicate that, although the addition of the hydrophobic CBI-ring system enhances the retention of peptides, its contribution to retention depends on both the chromatographic mechanism being employed and the nature of the amino acid to which it has been covalently attached.

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