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Journal of Chromatography A, 848 (1999) 375–385

JOURNAL OF CHROMATOGRAPHY A

Enantiomeric separations by capillary electrochromatography using a macrocyclic antibiotic chiral stationary phase

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Received 5 February 1999; received in revised form 6 April 1999; accepted 9 April 1999

Abstract

Racemic mixtures of tryptophan and dinitrobenzoyl leucine have been successfully resolved by capillary electrochromatography (CEC) using the macrocyclic antibiotic teicoplanin, covalently bonded to a 5 μm silica support. Modification of a previously published packing procedure was required to pack reliable capillaries, capable of performing enantiomeric separations. Good levels of enantioselectivity were obtained in all cases, with optimised separations being performed in less than 6 min. Retention times, resolution and reproducibility are discussed.

Keywords: Chiral stationary phases, electrochromatography; Enantiomer separation; Electrochromatography; Tryptophan; Dinitrobenzoyl leucine; Macrocyclic antibiotics; Antibiotics

1. Introduction

Due to cost and efficacy reasons, many chiral pharmaceutical preparations are commonly administered as racemic mixtures. Often both enantiomers do not possess the same physiological properties and, consequently, the need for analytical methods that discriminate between the two isomeric forms is very important. The ability to separate chiral molecules is one of the most active areas of analytical chemistry. Chromatographic methods typically employed for chiral separations include high-performance liquid chromatography (HPLC) [1], gas chromatography (GC) [2] and, more recently, capillary electrophoresis (CE) [3–6].

Capillary electrochromatography (CEC) is a high-performance chromatography technique, which has attracted immense attention over recent years [7–14], with several review articles [15–17] detailing the technique's progress. In essence, CEC is a hybrid of HPLC and capillary zone electrophoresis (CZE). The stationary phase can be fixed on particulate material, commonly silica beads packed inside the capillary column [7–10] or fixed on the walls of the open tube [11–14]. This technique utilises electroosmotic flow (EOF) as a means to transport solvent (and consequently the solutes) through the capillary. EOF is the bulk flow of liquid through a capillary and is a consequence of the surface charge on the interior wall and, in CEC, is predominantly the charge on the packing material [18,19]. CEC possesses significant

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00496-3

advantages over HPLC since the velocity flow profile in the capillary is near plug-like for an electrically driven system, although Rathore and Horváth [20] describe deviations from the flat flow profile depending on factors such as the zeta potential at the packing surface, conductivities in the packed and unpacked region and charge differences between the surface and the stationary phase. This is in contrast to that generated by a hydraulic pump, which yields a parabolic flow profile due to the shear force at the wall.

In CEC, chiral separations are performed by either chiral-selector-coated capillaries, chiral mobile phase additives or by packing the capillary with an HPLC-type chiral stationary phase [21–30]. To date, there have been a limited number of publications demonstrating chiral separations by CEC. Enantiomers of non-steroidal anti-inflammatory drugs were separated by employing a capillary coated with an immobilised dimethylpolysiloxane containing chemically bonded permethylated cyclodextrin (Chiral-Dex) [21]. Efficiencies of 30 000 were obtained for a film thickness of 0.2 μm, however, a dramatic loss of efficiency was observed when the film thickness was increased. Li and Lloyd [22–24] and Lelièvre et al. [25] studied β-cyclodextrin and related derivatives bonded onto silica particles. Resolution of a number of compounds was demonstrated, a variety of which were of pharmaceutical interest [25]. Successful separation of the neutral enantiomers of chlorthalidone (R_s<1.4) were achieved by the addition of hydroxypropyl-β-cyclodextrin (HP-β-CD) to the mobile phase. Enhanced resolution (R_s<3.4) and a reduction in analysis time was also achieved when the same cyclodextrin derivative was bonded to 5 μm silica particles [25]. Chiral stationary phases based on Naproxen and Whelk-O material were immobilised on 3 μm silica supports by Wolf et al. [26], who demonstrated impressive resolution of a range of neutral analytes. A weak anion-exchanger stationary phase originally applied to HPLC separations was used by Lämmerhofer and Lindner [27] to separate amino acids by CEC. This chiral weak anion-exchanger was bonded to 5 μm silica particles, but, unfortunately, high efficiencies were not obtained. A recent review article described capillaries filled with a monolithic, superporous imprinted polymer obtained by an in-situ polymerisation process. This type of phase has selectivity for a predetermined ligand or class of compounds [28]. Vancomycin, another macrocyclic antibiotic has been applied to CEC and has been successful in the separation of a number of compounds of pharmaceutical interest [29]. Barbiturates such as hexobarbital and mephobarbital were successfully baseline resolved on a permethyl-β-cyclodextrin [30] bonded to 10 μm silica, and efficiencies up to 43 500 N/m were observed.

The concept of utilising macrocyclic antibiotics as chiral stationary phases for liquid chromatography was first introduced by Armstrong et al. in 1994 [31]. The initial use of teicoplanin as a chiral stationary phase was reported in 1995 [32], the amphoteric glycopeptide being covalently linked to 5 μm silica gel. Teicoplanin (Fig. 1) contains 20 chiral centres surrounding four cavities A, B, C and D. These cavities allow for various interactions, including inclusion complexation. In addition, hydrogen donor and acceptor sites are readily available to further facilitate analyte–selector interaction. Macrocyclic antibiotics have proved very successful for a number of HPLC chiral separations in either normal phase, reversed-phase or polar organic mode [31–34]. HPLC separations using vancomycin as a stationary phase proved to be successful for separating N-carbobenzyloxy tryptophan (N-CBZ–tryptophan) and dinitrobenzoyl leucine with selectivities of 1.04 and 1.10, respectively [31]. Separation of β-methyl tryptophan was shown by Peter et al. [34] using a 25-cm teicoplanin column in reversed-phase HPLC mode. Analysis times were less than 15 min, however, baseline resolution was not obtained in this case and a temperature of 1.5°C was required.

Since many of the molecules initially analysed, including chlorthalidone, pentobarbital, bendroflumethazide and trimeprazine did not resolve, the focus of this paper will be directed on the separations of the antidepressant drug tryptophan and the derivatised amino acid, dinitrobenzoyl leucine. The macrocyclic antibiotic teicoplanin bonded to silica is used as the chiral stationary phase, which is packed into 100 μm I.D. capillaries using the modified packing technique discussed below. The reproducibility of this revised procedure will be discussed.

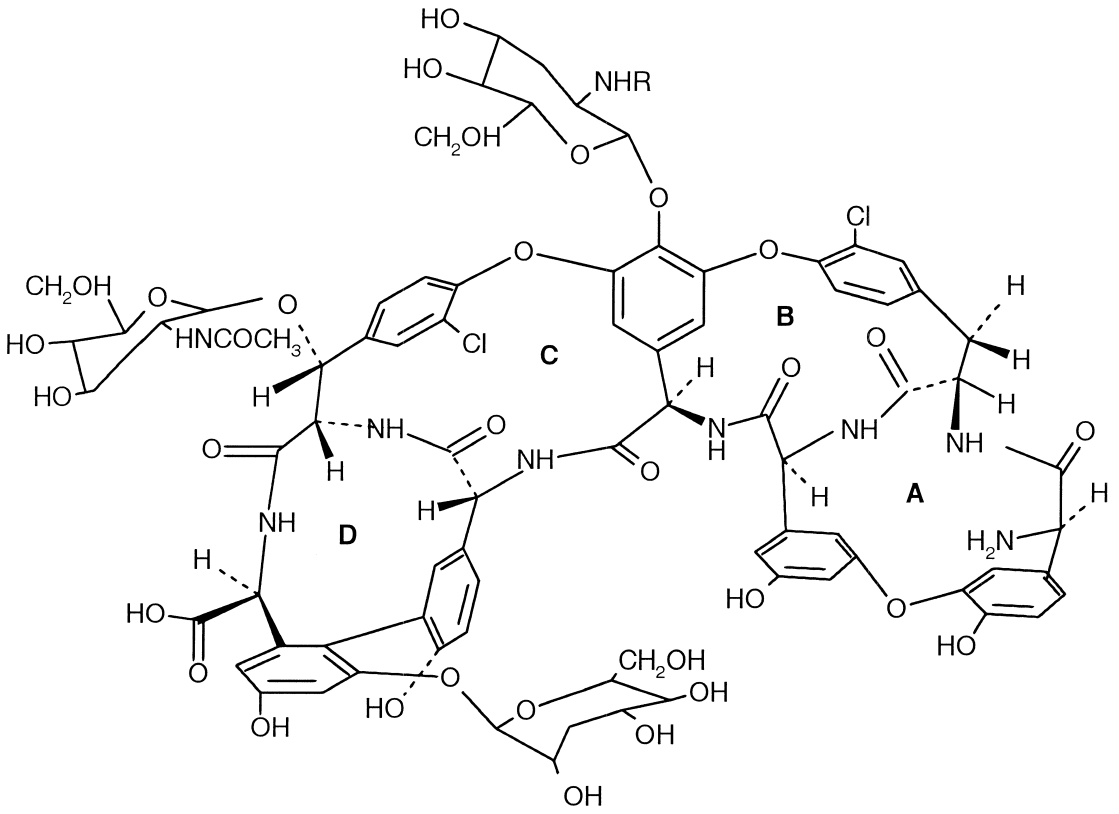

Fig. 1. Structure of teicoplanin.

2. Experimental

Electrochromatography was performed on a Hewlett Packard 3D CE system with a diode-array UV detector (Palo Alto, CA, USA). A run voltage of 30 kV was used throughout the study and the capillary was maintained at 25°C (unless otherwise stated). In order to prevent outgassing, presumed to be generated at the frits [35], a pressure of 10 bar was applied to both the inlet and outlet ends of the capillary. A fused-silica capillary (100 μm I.D.) was purchased from Composite Metal Services (Hallow, UK). Windows and frits were fabricated using an Innovatech ACF electrical burner (Stevenage, UK). This instrument has 12 modes that control the burn temperature and time. Frits were prepared using mode 5, which has a pre-burn of 300°C for 5 s followed by a further burn of 400°C for 5 s; in order to prepare the window, the polyimide was removed using mode 8, which has a pre-burn of 350°C for 5 s followed by a further burn of 450°C also for 5 s. Injections were performed electrokinetically for 5 s at 10 kV.

Chirobiotic T (5 μm) was a gift from Dr. Denise Wallworth, BAS Technocol (Stockport, UK) and was supplied by Astec (Whippany, NJ, USA). Silica (5 μm) was obtained from Hypersil (Runcorn, UK). Sodium silicate, tryptophan and dinitrobenzoyl leucine were purchased from Sigma–Aldrich (Poole, UK). Acetonitrile, acetone, methanol, ethanol, disodium hydrogen phosphate, sodium dihydrogen phosphate and orthophosphoric acid were all purchased from BDH (Poole, UK). All reagents were of HPLC grade. All solutions were prepared using ultra-pure water from an Elga Maxima system (High Wycombe, UK). Mobile phases were prepared by mixing organic solvent with buffer that had previously been adjusted to the desired pH with phosphoric acid. The concentration of the samples was 2 mg/ml

dissolved in 50:50 (v/v) organic solvent–water and these were sonicated for 10 min prior to use.

Capillaries of 100 μm I.D. were packed with 5 μm Chirobiotic T particles using a modified procedure, similar to that developed by Smith and Evans [7] and this will be discussed in detail later. Columns were 24.5 cm to the detector, with a total length of 33 cm. An electrical burner was used to prepare the windows and frits. Once the capillary was packed, it was equilibrated for 30 min or until a stable current and baseline were achieved.

3. Results and discussion

The considerable experience obtained in our laboratory for the packing of capillary columns has lead to the conclusion that the packing pressure should be as high as possible without causing damage to the stationary phase. For standard reversed-phase packing material, the pressure has been optimised to 690 bar. However, the optimum packing pressure for the teicoplanin stationary phase was found to be 480 bar. Capillaries packed at a pressure $<$480 bar were shown to give inferior capillaries with respect to efficiency and column lifetime.

It was found that the packing procedure was crucial to the lifetime and overall success of the column. Even though the first attempts at enantiomeric separations of tryptophan and dinitrobenzoyl leucine were always successful, over a period of time, loss of resolution and an increase in current, from 4 to 20 μA, was observed. When this increase in current was experienced, viewing of the packed capillary under the microscope showed that the stationary phase had migrated past the window and a large void had formed. The problem was identified as a consequence of producing the mid-frit from the teicoplanin stationary phase. It was found that the mid-frit was difficult to fabricate from the teicoplanin phase and often gave way under the application of voltage and pressure. The revised packing procedure already practised for the packing of polymeric stationary phases such as Develosil, Asahipack ODP and J-Sphere M80 allows for sintering of silica particles at the mid-frit, thereby producing a much more stable frit [36]. The first step of the packing procedure and one of the most important involves the formation of an end-frit. This is prepared from 5 μm silica wetted with 3:1 (v/v) water–sodium silicate. A small plug of silica paste is tapped into the end of the 100 μm capillary and, using an electrical burner, the particles are sintered (see Experimental for temperature and time details). The capillary is attached to a high pressure pump (previously described [7]) and a suspension of the stationary phase is then pumped into the capillary at a pressure of ~480 bar against this silica end frit. The slurry is kept in solution by the use of an ultrasonic probe (previously detailed [9]). When the packed section reached 23.5 cm, the ultrasonic probe was turned off and the high pressure was slowly released. The chamber of the ultrasonic probe was then filled with a slurry of silica and this was packed into the capillary ($\geq$1.5 cm). The capillary was then left for at least 1 h to pump through with water and thereby remove the acetone. Next, a second retaining frit was made from the silica at a distance of 24.5 cm from the end frit. By reversing the capillary, the unwanted silica was pumped out, leaving an empty section. A small section of the polyimide coating (~3 mm) was then removed immediately after the retaining frit (see Experimental for temperature and time details), thus allowing for on-column detection. A diagrammatic outline of the process is described in Fig. 2. Operation of these new capillaries always produced stable frits and prevented unpacking of the capillary when pressure and voltage were applied.

Our experiences of CEC indicate that acetonitrile should be chosen as the initial modifier because it results in a higher EOF than various other solvents and has a high dielectric constant and low viscosity [37]. The amino acids tryptophan and dinitrobenzoyl leucine (Fig. 3) were used to study the potential of teicoplanin as a chiral stationary phase for CEC, operated in the reversed-phase mode. Equilibration was performed by ramping the voltage up to 30 kV and holding for 30 min or until a stable current and baseline were achieved.

The initial composition of acetonitrile–2 m*M* Na_2HPO_4, pH 7.0 (70:30, v/v) was chosen as the mobile phase since it had proved successful as a starting point for other separations performed in our laboratory. Under these conditions, tryptophan was successfully resolved in under 4 min using a 5 μm Chirobiotic T (teicoplanin) stationary phase, as

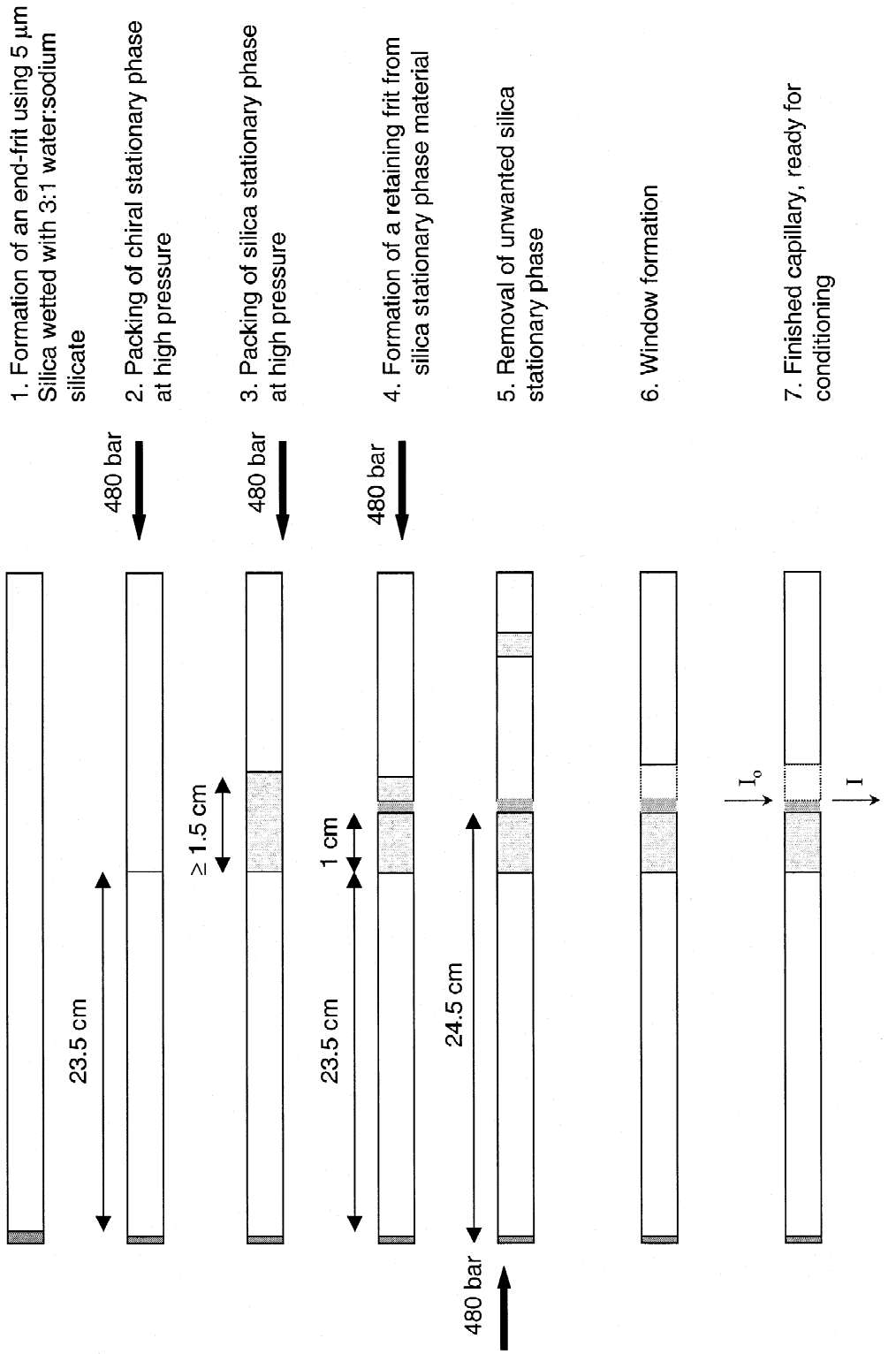

Fig. 2. Modified method for packing capillary columns.

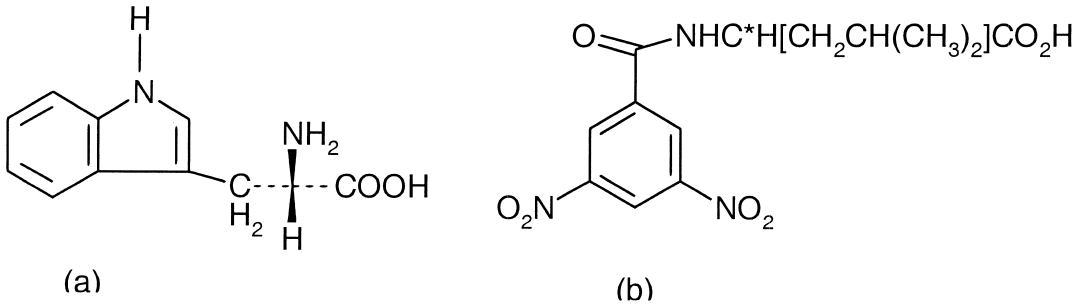

Fig. 3. Structures of the enantiomeric analytes (a) tryptophan and (b) dinitrobenzoyl leucine.

shown in Fig. 4. Excellent injection-to-injection retention time repeatability was observed. Using an Excel spreadsheet, the % RSD was calculated to be <0.87 at 25°C ($n=5$). This data is shown in Table 1 and were obtained for a set of repeat injections. Injection-to-injection repeatability from day-to-day will be investigated at a later date. However, present indications are that the long term stability of these columns is high. As expected, the less retained analyte showed higher chromatographic efficiency (up to 35 500 N/m) than the more retained antipode. This is primarily due to reduced diffusion. The high efficiencies typically expected from packed capillary CEC were not realised in this study, unlike the results achieved by Wolf et al. [26]. One explanation of the poor efficiencies obtained could be due to the use of 5 μm stationary phase particles, however, other factors such as pH and viscosity of the mobile phase would also effect efficiency values. The use of much smaller particles would lead to a more densely packed and uniform column and, as a result, a decrease in eddy diffusion (A). A reduction of A in

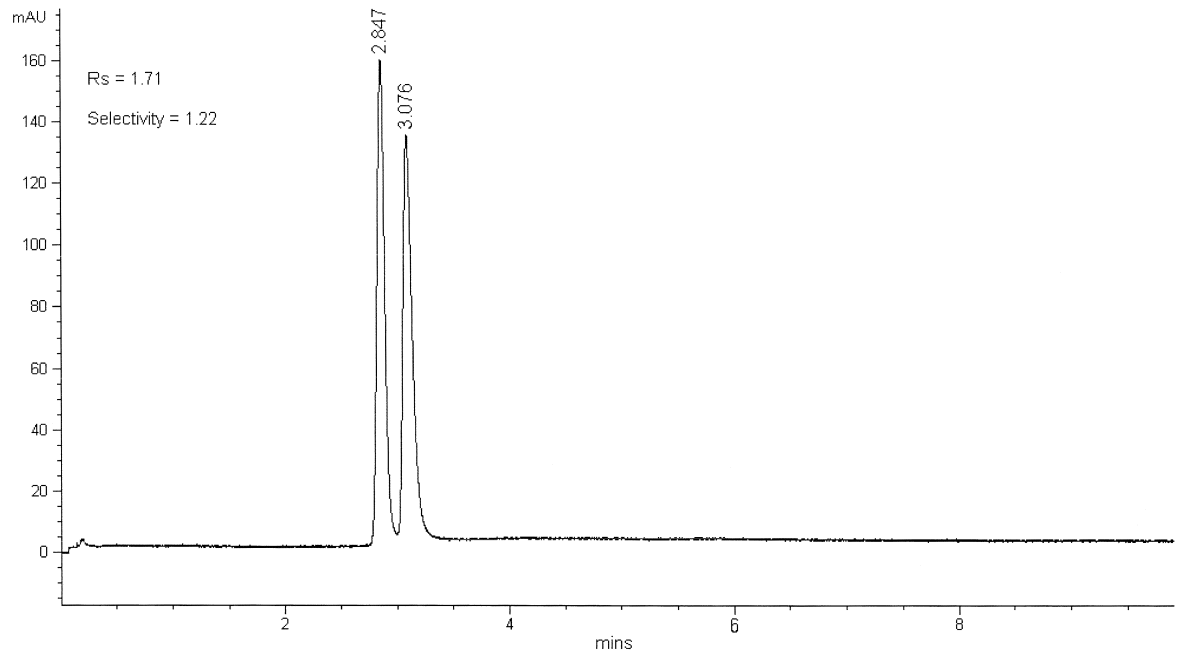

Fig. 4. CEC separation of tryptophan enantiomers on Chirobiotic T. Capillary: effective length, 24.5 cm; total length, 33 cm; 100 μm I.D. Conditions: acetonitrile–2 mM Na_2HPO_4, pH 7 (70:30, v/v); 30 kV; 25°C; injection at 10 kV for 5 s; UV detection at 214 nm. Analyte concentration: 2 mg/ml in acetonitrile–water (50:50, v/v).

Table 1
Injection-to-injection repeatability of retention time and resolution[a]

Injection	t_{R1}	t_{R2}	R_s	N_1/m	N_2/m
1	2.854	3.084	1.69	34 637	28 624
2	2.859	3.087	1.70	34 755	29 834
3	2.847	3.076	1.71	34 861	29 612
4	2.814	3.038	1.69	34 453	29 457
5	2.807	3.031	1.70	35 510	29 906
Mean	2.85	3.06	1.70	34 843	29 487
RSD (%)	0.85	0.87	0.49	1.15	1.74

[a] Relative standard deviations (RSDs %) of retention time and resolution were calculated from the enantioseparation of tryptophan.

Table 2
Column-to-column reproducibility of the packing procedure[a]

Column no.	t_{R1}	t_{R2}	R_s	N_1/m
1	4.285	4.589	1.52	34 841
2	3.348	3.709	2.10	31 873
3	3.506	3.799	1.66	30 804
4	4.268	4.669	1.61	23 465
5	3.481	3.805	1.77	27 400
6	3.199	3.505	1.85	28 571
7	2.836	3.063	1.698	34 843
Mean	3.56	3.88	1.74	28 828
RSD (%)	15.11	14.80	10.87	13.88

[a] Relative standard deviations (RSDs %) of retention time and resolution were calculated from the enantioseparation of tryptophan.

the height equivalent to a theoretical plate (H) equation (Eq. 1) would result in a lower minimum in the van Deemter plot.

$$H = Ad_p\left(\frac{ud_p}{D_m}\right)^{1/3} + \frac{B}{u} + \frac{Cud_p^2}{D_m} \quad (1)$$

where B is the longitudinal diffusion, C is resistance to mass transfer, u is the mean linear velocity, D_m is the solute diffusion coefficient in the mobile phase and d_p is the average particle diameter. The contribution to band broadening from the C term can also be reduced by the use of small diameter particles. The resolution factor (R_s) was calculated using Eq. (2);

$$R_s = \frac{1.18(t_{R_2} - t_{R_1})}{w_{1_{1/2}} + w_{2_{1/2}}} \quad (2)$$

where t_{R_1} and $t_{R_2}$are the retention times of enantiomers 1 and 2, respectively, $w_{1_{1/2}}$and $w_{2_{1/2}}$ are the widths of the respective peaks at half height. The number of theoretical plates (N) was calculated using Eq. (3);

$$N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2 \quad (3)$$

A number of capillaries were prepared for this study and a statistical evaluation of the data from the analysis of tryptophan is shown in Table 2. Column-to-column reproducibility was >15% RSD ($n=7$). In theory, the RSD value could be reduced if greater control over the packing stage was achieved. For instance, it was reasonably difficult to pack the chiral phase exactly to 23.5 cm. Other problems included variation of the frit porosity between columns. However, resolution of the enantiomers was always achieved under these conditions and the frits appeared stable during these studies.

Enantioselectivity (α) was shown to be affected by the type of organic solvent. Fig. 5a shows the separation of tryptophan using a mobile phase of methanol–2 mM Na_2HPO_4, pH 7.0 (80:20, v/v) and Fig. 5b using a mobile phase of ethanol–2 mM Na_2HPO_4, pH 7.0 (80:20, v/v). Enantioselectivity was calculated using Eq. (4).

$$\alpha = \frac{t_{R_2} - t_0}{t_{R_1} - t_0} \quad (4)$$

where t_{R_1} and $t_{R_2}$are the retention times of enantiomers 1 and 2, respectively, t_0 is the retention time of the perturbation seen on the baseline. This is assumed to be equal to the velocity of the EOF. Methanol and ethanol were studied as possible organic modifiers in the mobile phase since the majority of HPLC work published on macrocyclic antibiotics used such solvents [31–34]. Selectivity values of 1.83 and 1.65 were observed for ethanol and methanol, respectively, compared to a value of 1.22 for acetonitrile. As a result of the higher enantioselectivity, greater resolution was achieved when using either methanol or ethanol in the mobile phase. However, the greater resolution was counterbalanced by an increase in run time. Peak shapes could be improved by increasing the column tem-

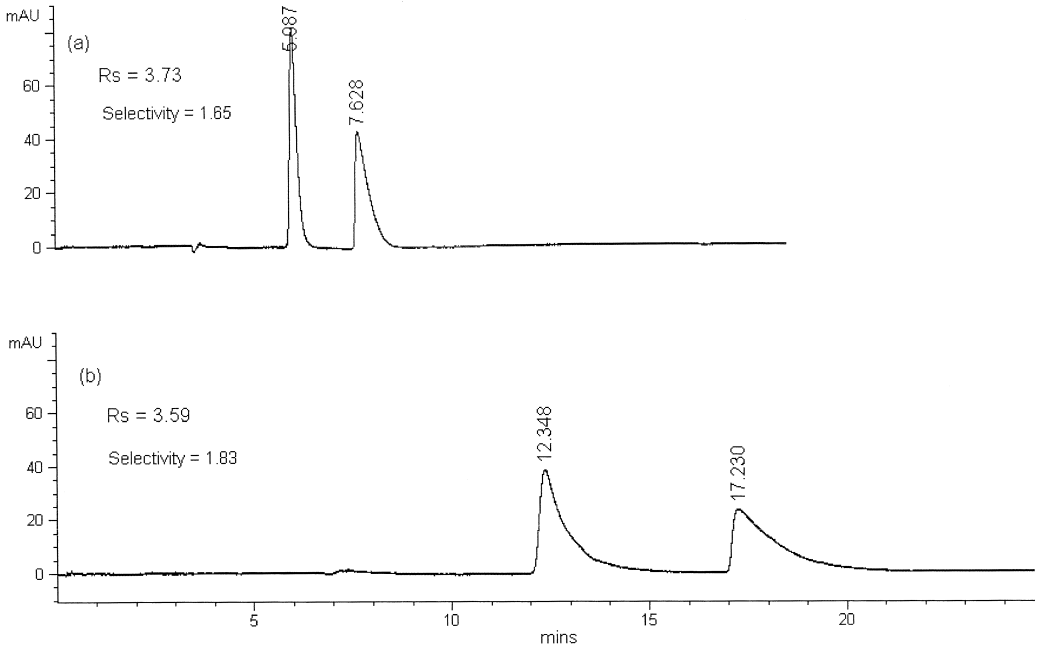

Fig. 5. CEC separation of tryptophan enantiomers on Chirobiotic T. Capillary: effective length, 24.5 cm; total length, 33 cm; 100 μm I.D. Conditions: (a) methanol–2 m*M* Na_2HPO_4, pH 7 (80:20, v/v), (b) ethanol–2 m*M* Na_2HPO_4, pH 7 (80:20, v/v); 30 kV; 25°C; injection at 10 kV for 5 s; UV detection at 214 nm. Analyte concentration: 2 mg/ml in 50:50 (v/v) organic solvent–water.

perature, since an increase in temperature will result in faster mass-transfer kinetics; this study will be reported at a later date. The columns were readily equilibrated between each mobile phase with no obvious loss of resolution. The same stability was also evident when working at low pH. Fig. 6a shows the separation of tryptophan with the mobile phase acetonitrile–5 m*M* NaH_2PO_4, pH 3.5 (70:30, v/v) and Fig. 6b using acetonitrile–5 m*M* NaH_2PO_4, pH 4.0 (70:30, v/v) as the eluant. Resolution values were calculated as 1.60 and 2.39 using Eq. (2). Using a mobile phase adjusted to pH 4.0 produced a faster run time due to an increase in the EOF. The increase in EOF from pH 3.5 to 4.0 could be the result of residual silanol groups present on the stationary phase and in the silica bed, resulting from the packing technique. This phenomenon has been observed with other capillaries packed with the same stationary phase and using this packing technique.

At lower temperatures, the mass-transfer kinetics of the enantiomer–teicoplanin complex are affected. It would be expected that a lower temperature would improve resolution [38]. This was confirmed by a series of experiments, which are graphically represented in Fig. 7. However, at this stage, there is insufficient data to determine the separate effects of temperature on the kinetics and thermodynamics of the separation. Tryptophan was separated over a temperature range of 15–40°C, using the conditions set out in Fig. 4. A working temperature of 25°C was found to be the optimum value to produce baseline resolution in the shortest possible time.

Using a mobile phase of acetonitrile–5 m*M* NaH_2PO_4, pH 2.3 (70:30, v/v), dinitrobenzoyl leucine was near baseline resolved in under 6 min at a controlled temperature of 25°C. A typical example of this is shown in Fig. 8 ($R_s = 1.47$ $\alpha = 1.10$). At pH 7, no enantiomeric resolution was seen. No further optimisation of this separation was attempted at this stage. The unusually high baseline noise experienced in this separation was due to the window slightly shifting out of alignment in the cell. Thus, detection was being performed through an area that is not completely free of particles. Obviously, in Figs. 4–6, this was not the case and, subsequently, detector noise levels are significantly reduced.

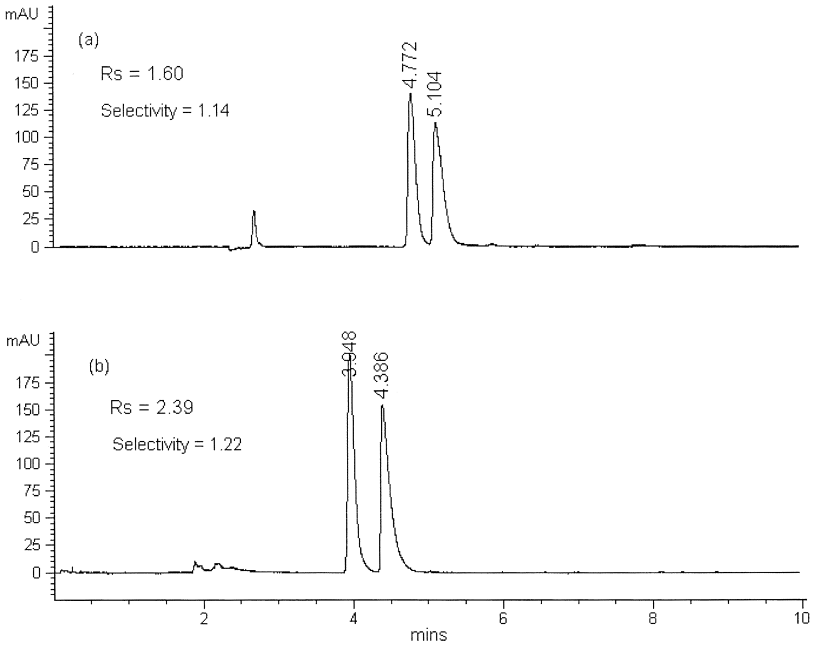

Fig. 6. CEC separation of tryptophan enantiomers on Chirobiotic T. Capillary: effective length, 24.5 cm; total length, 33 cm; 100 μm I.D. Conditions: (a) acetonitrile–5 m*M* NaH_2PO_4, pH 3.5 (70:30, v/v), (b) acetonitrile–5 m*M* NaH_2PO_4, pH 4 (70:30, v/v); 30 kV; 25°C; injection at 10 kV for 5 s; UV detection at 214 nm. Analyte concentration: 2 mg/ml in 50:50 (v/v) acetonitrile–water.

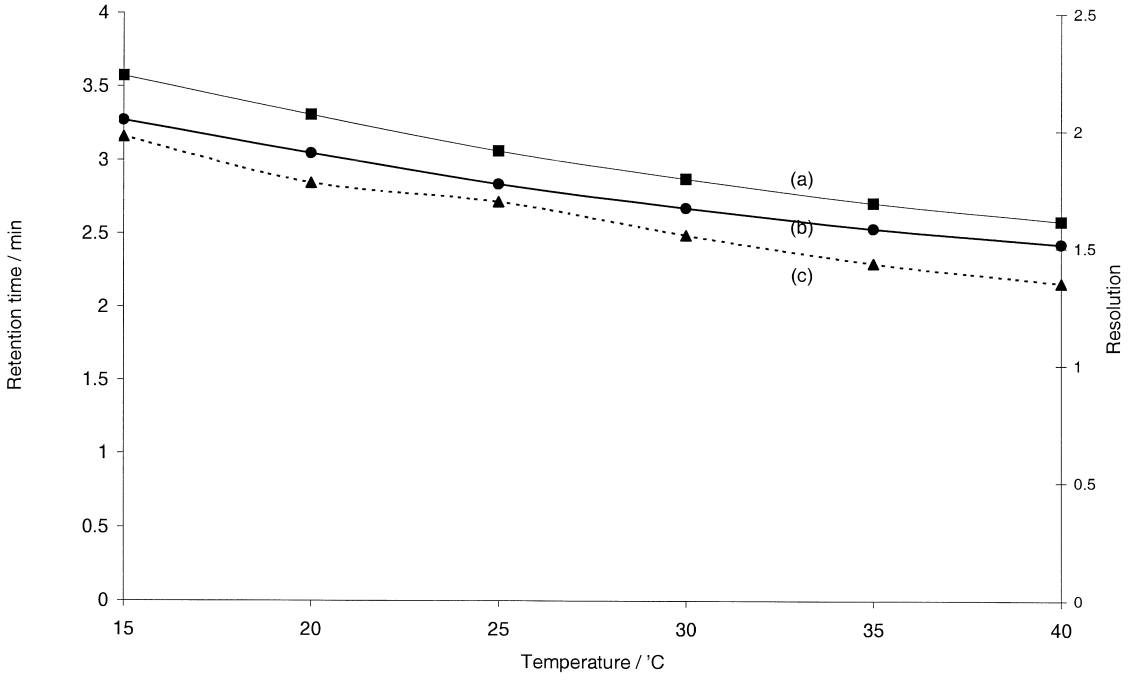

Fig. 7. Effect of temperature on retention time and resolution for tryptophan. Conditions were the same as for Fig. 4. (a) Retention time of enantiomer 1. (b) Retention time of enantiomer 2. (c) Resolution of the enantiomeric separation.

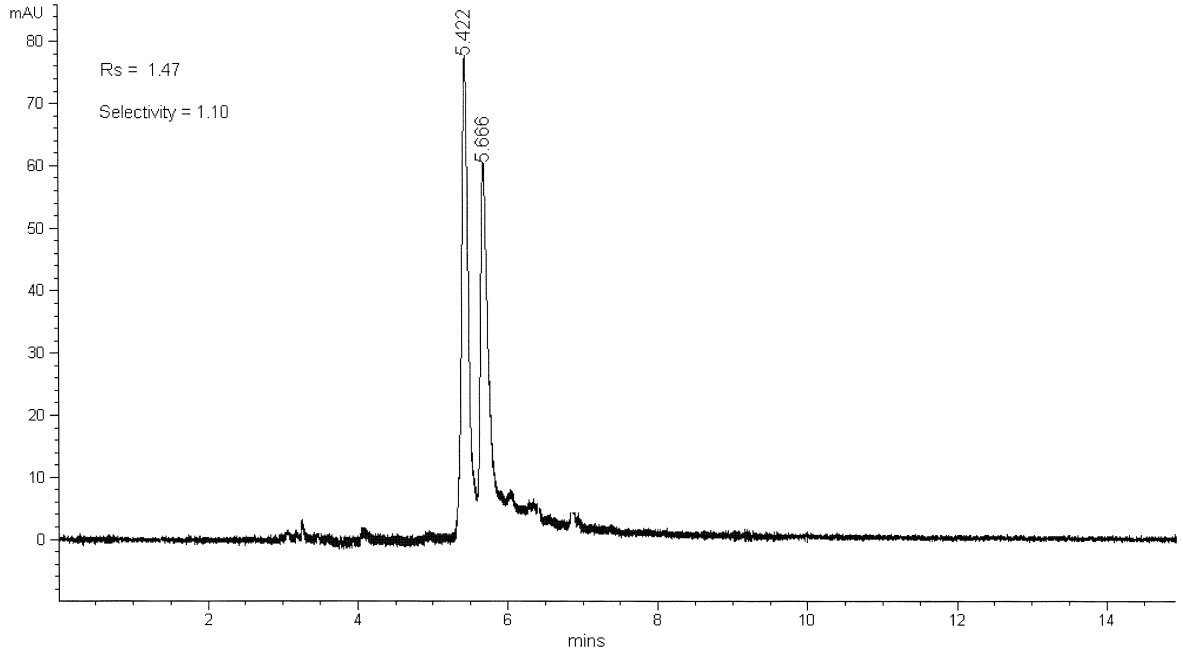

Fig. 8. CEC separation of dinitrobenzoyl leucine enantiomers on Chirobiotic T. Capillary: effective length, 24.5 cm; total length, 33 cm; 100 μm I.D. Conditions: acetonitrile–5 m*M* NaH_2PO_4, pH 2.3 (70:30, v/v); 30 kV; 25°C; injection at 10 kV for 5 s; UV detection at 254 nm. Analyte concentration: 2 mg/ml in 50:50 (v/v) acetonitrile–water.

4. Conclusions

The detailed packing procedure is suitable for any stationary phase that does not produce stable frits, e.g. Develosil, Asahipack ODP and J. Sphere M80. This study shows that the packing of the capillaries is a critical step and, if prepared successfully, would give good resolution of enantiomeric compounds. However, it is difficult to assess the overall performance of the capillary column using only tryptophan. This study has involved the scanning of over 15 analytes, but only tryptophan has been baseline-resolved to date. Further analytes would need to be resolved in order to produce a suitable test mixture in order to evaluate this phase. Good stability within the pH parameters studied was experienced. All columns were found to require only 30 min equilibration and all separations were achieved in under 18 min.

Teicoplanin showed preferred interaction with analytes that had both an amine group and a carboxylic acid group attached to the chiral centre. Further research is underway to investigate this observation. Additional work will attempt to resolve further chiral molecules with teicoplanin as well as other antibiotic stationary phases.

Acknowledgements

The authors would like to acknowledge Zeneca and SmithKline Beecham for funding the analytical centre at I.C. and providing A.C.F's grant, and Dr. D. Wallworth for providing the Chirobiotic T stationary phase. We thank Hewlett Packard for donation of CEC equipment and Dr. P. Ferguson for useful discussions.

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