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Practical approach to the miniaturization of chiral separations in liquid chromatography using packed fused-silica capillary columns

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ABSTRACT

A micro liquid chromatographic system was evaluated and the possibility of transferring some established methods of chiral separation to this system was examined. Fused-silica columns (0.32 mm I.D.) were slurry packed using an ordinary isocratic high-performance liquid chromatographic (HPLC) pump. Reversed-phase C_{18} columns with an average reduced plate height of 3.5 could be packed reproducibly. The chiral selectors chosen were β-cyclodextrin, used as a mobile phase additive, bovine serum albumin adsorbed on silica and a Pirkle phase, covalently bound L-dinitrobenzoyl-phenylglycine. Enantiomeric separations gave results in good agreement with those obtained on conventional HPLC columns.

INTRODUCTION

The use of liquid chromatographic (LC) columns with small inner diameters has increased in the last few years. Since the pioneering work of Scott and Kucera [1] on microbore columns and of Tsuda and Novotny [2], Ishii *et al.* [3] and Yang [4] on packed fused-silica capillary columns, a number of reports have been published describing different kinds of microcolumns in LC. For routine use, slurry-packed capillary columns, often also referred to as micro-LC columns [5], seem to attract the most interest [6–8]. The advantages and disadvantages of micro-LC have been discussed in detail by, *e.g.*, Novotny [9] and Verzele *et al.* [5].

An area where the use of micro-LC columns is of particular interest is in the separation of enantiomers. Much research has been focused on chiral separations, especially in the pharmaceutical industry, and interest is growing with the demands on documentation of drug safety. Optical isomers differ markedly in bioavailability, biological activity and metabolism [10]. A racemic mixture is therefore to be considered as a mixture of two different substances. Consequently, it is of great importance to determine the relative abundance of each isomer, possibly through stereoselective LC. Some chiral selectors in LC have been extensively studied, *e.g.*, β-cyclodextrin (β-CD) [11], bovine serum albumin (BSA) [12], triacetylcellulose [13] and the so-called Pirkle phases [14], and are also commercially available.

Although most of the work on enantiomeric separations so far has been done using conventional LC, miniaturization of the chromatographic system makes it possible to experiment with new stationary phases that are too valuable to use in larger columns, *e.g.*, monoclonal antibodies or receptor proteins.

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The reduced consumption of solvents also makes stereoselective additives in the mobile phase less costly. Further, the use of long fused-silica columns will make it possible, owing to increased efficiency, to separate stereoisomers with very low chromatographic separation factors (α). The aim of this study was to examine the suitability of micro-LC columns (0.32 mm I.D.) in stereoselective separations. To explore possible new chiral phases, a simple and straightforward method of packing both chiral and non-chiral columns in the laboratory is required. Three established methods for chiral separation were transferred to 0.32 mm I.D. packed fused-silica columns and the results obtained were compared with those previously reported using conventional-sized columns. The methods utilized either β-CD as a mobile phase additive [15], BSA as a stationary phase adsorbed on underivatized silica [16] or a commercially available Pirkle phase, covalently bound L-dinitrobenzoyl-phenylglycine [17], as chiral selectors.

EXPERIMENTAL

Chemicals

Terbutalin, in the (+)- and (−)-forms and the racemate, was a gift from Draco (Lund, Sweden) and (±)-oxazepam was a gift from Kabi-Vitrum (Stockholm, Sweden). (±)-Chlorthalidone, (±)-benzoin and BSA were purchased from Sigma (St. Louis, MO, USA). D,L- and L-tryptophan were obtained from E. Merck (Darmstadt, Germany) and D-tryptophan from United States Biochemical (Cleveland, OH, USA). β-Cyclodextrin was purchased from Stadex (Malmö, Sweden). (±)-2,2,2-Trifluoro-1-(9-anthryl)ethanol was obtained from Serva (Heidelberg, Germany), (±)- and (+)-propranolol from Fluka (Buchs, Switzerland) and α-naphthyl isocyanate from Eastman Kodak (Rochester, NY, USA). All other chemicals used were of analytical-reagent grade and the solvents were of high-performance liquid chromatographic (HPLC) or spectrographic grade. Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Prior to use, the mobile phases were filtered through a 0.45-μm HV filter (Millipore) and degassed by purging with helium for 30 min.

Chromatography

A Jasco (Tokyo, Japan) Model 880-PU reciprocating pump was used for both column preparation and chromatography. The pump is capable of delivering flow-rates in the range 0.001–10 ml/min. In order to obtain low (< 1 μl/min) and reproducible flow-rates under all conditions, a split-flow system was used. A three-way union connected between the pump and the injector divided the flow and the splitting ratio was adjusted by the use of a Whitey (Highland Heights, OH, USA) SS-22R2 micrometering valve. The excess flow was returned to the solvent reservoir. UV detection was performed with a Jasco 875-UV spectrophotometric detector equipped with an optional capillary cell holder. The samples were injected with a Valco (Houston, TX, USA) CI4W internal loop injector with a rotor volume of 0.06, 0.2, 0.5 or 1.0 μl. The optimum detection wavelength for each solute was determined by recording the UV spectrum with a Shimadzu (Kyoto, Japan) UV-160 A spectrophotometer.

Column preparation

Column blanks were prepared from fused-silica capillaries according to a previously described method [18], with minor modifications. Capillaries of 0.32 mm I.D. (Scientific Glass Engineering, Ringwood, Australia) were cut into lengths between 200 and 500 mm using a capillary cleaving tool (Supelco, Bellefonte, PA, USA). The column end frit was made from a GF/D glass-fibre filter (Whatman, Maidstone, UK) by rotating the column while pressing its end gently against the filter disc. The filter was then pushed 10 mm into the column with a short length (150 mm) of 0.10 mm I.D. connceting capillary. Finally, the capillaries were fixed with epoxy glue. In order to achieve a connection between the column and the slurry reservoir that could withstand the high packing pressures, a short length of 1/16 in. O.D. and 0.5 mm I.D. stainless-steel tubing was threaded over the column and fixed with epoxy 2 mm below the top (Fig. 1). The slurry reservoir consisted of a 20 mm × 3 mm I.D. stainless-steel column (volume *ca.* 0.14 ml) (Upchurch Scientific, Oak Harbor, WA, USA). A funnel was made out of Kel-F and inserted in the reservoir to facilitate the delivery of the slurry (Fig. 2).

Reversed-phase columns were packed using either Nucleosil 100-5 C_{18} (5 μm, 100 Å, 350 m^2/g)

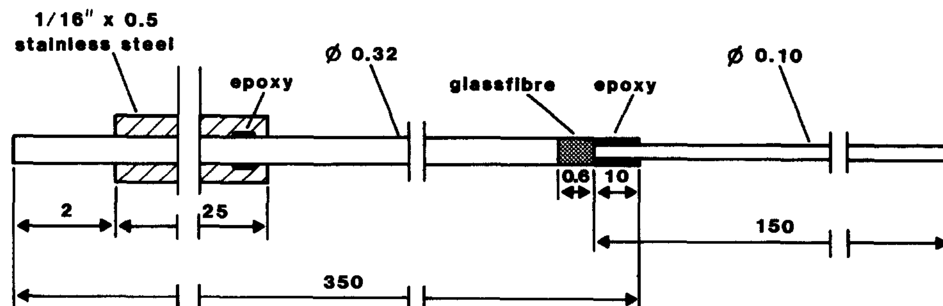

Fig. 1. Schematic diagram of the fused-silica column blank. All dimensions are in millimetres unless indicated otherwise. ø = Diameter; ″ = inch.

(Macherey–Nagel, Düren, Germany), Nucleosil 120-3 C_{18} (3 μm, 120 Å, 200 m^2/g) or Spherisorb ODS 2 (5 μm, 80 Å, 220 m^2/g) (Phase Separations, Norwalk, CT, USA) and columns containing underivatized silica were prepared using Nucleosil (5 μm, 300 Å, 120 m^2/g) (Macherey–Nagel). The Pirkle phase columns were packed with Daltosil 100 L-DNB-phenylglycine (4 μm, 90 Å, 300 m^2/g) (Serva). The packing material was suspended in carbon tetrachloride–2-propanol (50:50, v/v) for reversed-phase material and in chloroform–methanol (2:1, v/v) for underivatized silica and Pirkle phase material. The slurry concentration was 27% (w/w). After sonication for 2 min, the slurry was transferred to the reservoir. The pump, with acetonitrile as displacing medium (methanol for underivatized silica and Pirkle phases), was started immediately with an initial pressure of zero and a flow-rate of 0.5 ml/min. Within 1 min the pressure had risen to 400 atm and the flow-rate was successively decreased to obtain a stabilized pressure of 450 atm. After 30 min, the pump was turned off and the pressure returned to zero. The column was then cut off below the steel capillary and mounted on the injector using a polyimide ferrule and liner (Valco). The reversed-phase columns were conditioned overnight with mobile phase [acetonitrile–water (80:20, v/v)] and then submitted to a column performance test. The test mixture contained toluene (0.12 mg/ml), biphenyl (0.12 mg/ml) and anthracene (0.18 mg/

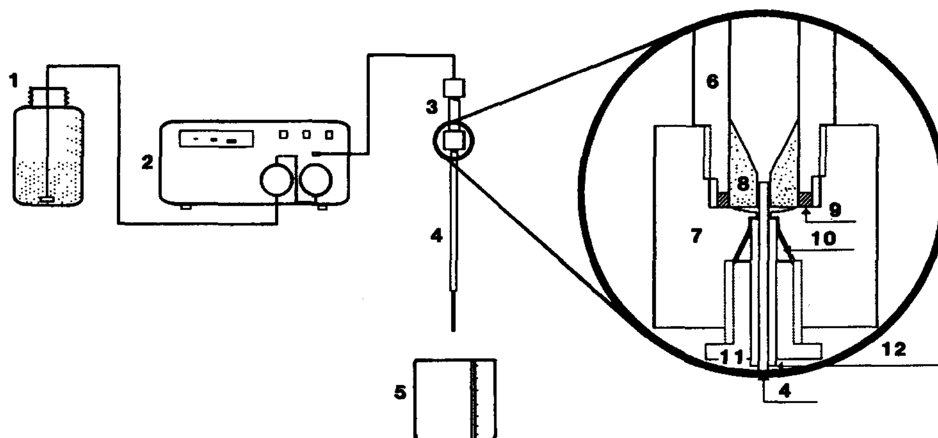

Fig. 2. Set-up for the packing of capillary columns. 1 = Solvent reservoir; 2 = pump; 3 = slurry reservoir; 4 = fused-silica column; 5 = waste; 6 = column tubing (3 mm I.D.); 7 = column end-fitting; 8 = funnel (Kel-F); 9 = seal (PEEK); 10 = ferrule (stainless steel); 11 = nut; 12 = 1/16 in. I.D. tubing (stainless steel).

ml) dissolved in acetonitrile–water (80:20, v/v). The Pirkle phase columns were conditioned with 2-propanol followed by mobile phase [n-hexane–2-propanol (95:5, v/v)].

Adsorption of BSA

Columns with underivatized silica were packed as described above. After washing with *ca.* 50 column volumes of water and equilibration with the same volume of 0.05 *M* phosphate buffer (pH 5.0), sodium nitrite was injected. The eluted peak was symmetrical and showed an efficiency of about 4000 theoretical plates/m. The immobilization of BSA by adsorption was performed as described previously [16]. A solution of 1 mg/ml BSA in 0.05 *M* phosphate buffer (pH 5.0) was pumped through the column until breakthrough of BSA was detected at 280 nm. The column was then equilibrated with BSA-free buffer until a stable UV baseline was obtained. From the point of breakthrough the amount of BSA adsorbed on the column was calculated.

Calculations

Experimentally, the retention time of a non-retained compound, t_0, was determined by injecting either sodium nitrite solution or water or by observing the first baseline disturbance. The plate number N was calculated according to the equation $N = 5.54\ (t_R/w_{0.5})^2$, where t_R is the retention time and $w_{0.5}$ is the band width at half-height. The capacity factor, k', the plate height, H, the reduced plate height, h, and the asymmetry factor, A_s, were all calculated using standard equations [19]. The flow resistance parameter ϕ was calculated as $\phi = (\Delta p t_0 d_p^2)/(L^2 \eta)$ where Δp is the pressure drop, d_p is the average particle diameter, L is the column length and η is the dynamic viscosity of the mobile phase. The separation impedance, E, is defined as $E = h^2\phi$. The total column porosity, ε, was calculated from $\varepsilon = (4Ft_0)/(\pi d_c^2 L)$, where F is the volumetric flow-rate and d_c is the column inner diameter. The stereoselectivity, α, was calculated as k'_2/k'_1 and the equation $R_s = 2(t_{R2} - t_{R2})/(w_1 + w_2)$, where w is the band width, was used in the calculation of the resolution of wholly and partially resolved peaks [19]. This method of determining the resolution might give erroneous results with poorly resolved peaks, but with R_s exceeding unity the error is negligible.

RESULTS AND DISCUSSION

Instrumentation

The Jasco Model 880-PU pump is designed with a lower flow-rate limit of 1 μl/min. However, the use of mobile phases with high concentrations of β-cyclodextrin led to deterioration of the plunger seals and check valves of the pump. Although this was not noticeable at high flow-rates (*e.g.*, >0.5 ml/min), it prevented a steady mobile phase delivery near the lower flow-rate limit (1 μl/min). This problem was circumvented by the use of a split-flow system. Such a system also permits the use of pumps with a higher minimum flow-rate (*e.g.*, 0.01 ml/min). Further, a split-flow system makes it possible to deliver flow-rates well under 1 μl/min provided that the back-pressure is sufficiently high. The use of a metering valve instead of a dummy column to set the splitting ratio makes the adjustment of the pressure (or flow-rate) easier and exchange of the mobile phase faster.

The standard 8-μl flow cell of the detector is far too large for a micro-LC system. In order to avoid excessive band broadening, the volume of the detector cell must be well under 1 μl. Low cell volumes can be obtained by so-called on-column detection [4,5]. This means mounting the connecting capillary directly in the light path as close as possible to the column end frit. However, after removal of the polyimide coating, the fused-silica capillary becomes very fragile and the risk of breakage is obvious. A more convenient method, although the increased dead volume gives a slightly higher band broadening contribution, is to use a separate capillary tube mounted in the detector to which the column outlet can be connected via a piece of PTFE tubing. The influence of the dead volume of this connection on the overall efficiency is shown in Table I. In all instances the direction of light is perpendicular to the solvent flow. As can be seen, the use of a wider capillary (0.32 mm I.D.) as a measuring cell reduces the efficiency and increases the peak asymmetry compared with the case where detection is made directly on the 0.10 mm I.D. connecting capillary. The limit of detection, however, is decreased fourfold. Reducing the inner diameter of the connecting capillary to 0.05 mm allows the use of a 0.20-mm flow cell without any loss of efficiency. The increased path length provides

TABLE I

EFFECT ON COLUMN PERFORMANCE OF DIFFERENT CONNECTIONS BETWEEN THE COLUMN AND DETECTOR

Column, 440 mm × 0.32 mm I.D. packed with Nucleosil 100-5 C_{18}; mobile phase, acetonitrile–water (80:20, v/v); sample, anthracene, 0.18 mg/ml in mobile phase; volume injected, 60 nl; detection wavelength, 215 nm; flow-rate, 2.0 μl/min.

Parameter	A	B	C
Connection:			
Length × I.D. (mm)	120 × 0.10	120 × 0.10	120 × 0.05
Volume (μl)	1.10	1.10	0.39
Cell:			
Length × I.D.[a] (mm)	3 × 0.10[b]	3 × 0.32	3 × 0.20
Volume (μl)	0.024	0.24	0.094
k'	4.00	4.01	4.16
N (plates/m)	50 200	48 200	50 600
A_s	1.0	1.1	1.1
Limit of detection[c] (ppm)	6.1	1.6	2.5

[a] The inner diameter of the cell equals the pathlength of the light.
[b] Measured directly on the 0.10 mm I.D. connecting capillary.
[c] Limit of detection = 2/signal-to-noise ratio.

a 2.5-fold increase in detectability. Narrow connecting capillaries (<0.05 mm I.D.) can be used in lengths up to a few decimetres without any apparent reduction of the overall efficiency [20]. The geometry of the flow cell, on the other hand, has a great influence on the overall efficiency and care should be taken to avoid unswept volumes in the connection between the cell and the capillary (Fig. 3).

Micro-LC system

The performance characteristics of five reversed-phase columns are listed in Table II. Although the value of the reduced plate height, h, shows some variation and is higher than 2, which is usually considered to be the minimum value [9], it is clear that columns of reasonable efficiency can be packed without difficulty using an ordinary isocratic HPLC pump. The column resistance parameter, ϕ, is at or below the lower limit of the typical range 500–1000 whereas the separation impedance [21] ranges from 4000 to 8000, mainly owing to the influence of high reduced plate heights. The value of the total porosity, ε, is below the expected value of 0.8–0.9 for porous silica [6,22]. The discrepancy indicates that the nitrite ion used as a dead-time marker is in fact being partly excluded from the pores of the silica. Nevertheless the value can be used in comparisons between different columns. A typical test chromatogram is shown in Fig. 4. Reversed-phase C_{18} columns packed in lengths between 100 and 450 mm showed similar reduced plate heights.

Van Deemter plots, H *vs.* u, from three different columns and/or mobile phases are shown in Fig. 5. As can be seen, the use of low-viscosity mobile phases and small particle sizes is favourable owing to the increase in the optimum linear velocity. A high linear velocity and hence a high volumetric flow-rate

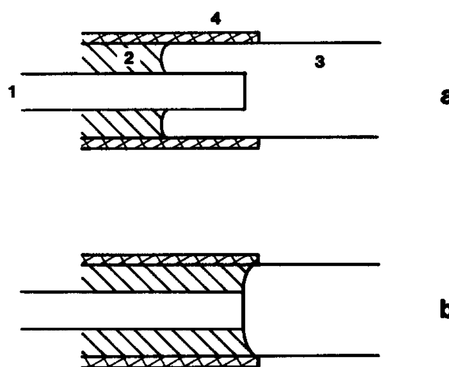

Fig. 3. Connection between connecting capillary and the flow cell in the detector. 1 = Connecting capillary; 2 = epoxy glue; 3 = flow-cell capillary; 4 = polyimide coating. The connection shown in (a) should be avoided owing to the formation of unswept volumes. The low unswept volume in (b) is obtained by withdrawing the connecting capillary through the partially hardened epoxy.

TABLE II

COLUMN PERFORMANCE CHARACTERISTICS

Column, 0.32 mm I.D.; stationary phase, (A–C) Nucleosil 100-5 C_{18}, (D and E) Spherisorb 5 μm C_{18}; mobile phase, acetonitrile–water (80:20, v/v); sample, anthracene, 0.18 mg/ml in mobile phase; volume injected, 60 nl; detection wavelength, 215 nm; flow-rate, 1.5 μl/min.

Column	h	ϕ	E	ε
A	3.47	445	5356	0.454
B	3.32	370	4077	0.431
C	4.22	465	8283	0.510
D	3.19	532	5423	0.394
E	3.50	324	3970	0.371
Mean	3.54	427	5422	0.432
S.D.	0.40	81.6	1740	0.054
R.S.D. (%)	11.3	19.1	32.1	12.5

give shorter analysis times and less stringent demands on the lower flow-rate limit and the flow stability of the solvent-delivery system.

Fig. 4. Test chromatogram showing the resolution of toluene (first eluted), biphenyl and anthracene (last eluted). Column, 200 mm × 0.32 mm I.D. packed with Spherisorb 5 μm C_{18}; mobile phase, acetonitrile–water (80:20, v/v); flow-rate, 1.8 μl/min; back-pressure, 18 kg/cm^2; a.u.f.s., 0.04; volume injected, 60 nl; sample concentration, toluene 0.12, biphenyl 0.12, anthracene 0.18 mg/ml; UV detection at 215 nm. N (anthracene) = 13 260.

In order to reduce the extra-column band broadening due to dispersion in the injector, it is important to keep the injection volume low. If the sample is dissolved in a solvent identical with the mobile phase the column performance rapidly deteriorates with increasing injection volume (Table III). The use of larger injection volumes is possible, however, if an injection medium of lower solvent strength is used. Using this technique, up to 1 μl has been injected with only a minor reduction in the plate number. However, there is a tendency towards an increased peak asymmetry and an upward shift of the capacity factor.

Small injection volumes generally requires concentrated samples which in turn increases the risk of column overload. Table IV shows the effect on the capacity factor (k'), plate number (N) and asymmetry factor (A_s) of increasing the sample concentration. As expected, both k' and N are affected by increased sample concentrations whereas A_s remains almost unaffected. Plotting N against log (sample concentration) reveals a break point around 1 mg/ml corresponding to $5 \cdot 10^{-6}$ g solute/g packing material. In the evaluation of the microcolumns the sample concentration is generally kept five to ten times below this value.

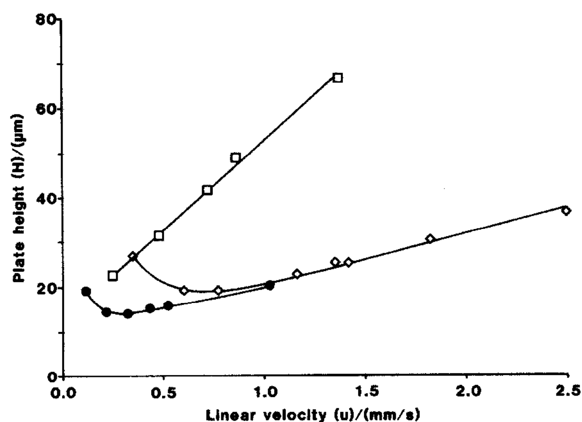

Fig. 5. Comparison between van Deemter plots from three different columns. Column, 200 mm × 0.32 mm I.D. packed with Nucleosil C_{18}; solute, biphenyl 0.12 mg/ml in mobile phase; UV detection at 215 nm. Mobile phase and particle diameter: column I (□) = methanol–water (70:30, v/v) and 5 μm; column II (◇) = acetonitrile–water (80:20, v/v) and 5 μm; column III (●) = methanol–water (70:30, v/v) and 3 μm.

TABLE III

EFFECT ON COLUMN PEFORMANCE OF DIFFERENT INJECTION VOLUMES AND SAMPLE SOLVENTS

Column, 255 mm × 0.32 mm I.D. packed with Nucleosil 100-5 C_{18}; mobile phase, acetonitrile–water (80:20, v/v); sample, biphenyl in mobile phase; amount injected, 6 ng; detection wavelength, 215 nm; flow-rate, 2.0 μl/min.

Sample solvent[a]	60 nl			500 nl			1000 nl		
	k'	N	A_s	k'	N	A_s	k'	N	A_s
80:20	2.13	12 500	1.0	2.04	6 400	1.3	1.76	3 000	1.5
40:60	2.11	12 000	1.0	2.15	11 800	1.1	2.22	10 500	1.1
20:80	2.15	11 100	1.1	2.20	11 100	1.1	2.24	10 800	1.2

[a] Acetonitrile–water (v/v).

Chiral separation

Microcolumns are well suited for stereoselective separations with β-cyclodextrin as a chiral mobile phase additive. ODS reversed-phase columns of high efficiency can be prepared according to the above method and a low volumetric flow-rate reduces the consumption of the additive. In the separation of (±)-terbutalin with β-CD as a chiral mobile phase additive, the retention time (t_R) and the separation factor (α) are dependent on the β-CD concentration (Fig. 6, Table V). The values of α and R_s are in good agreement with those reported previously [15]. The separation of the enantiomers of chlorthalidone is shown in Fig. 7. The relatively high lipophilicity of the substance results in a long retention time in spite of the high content of organic modifier.

BSA adsorbed on underivatized silica was used as a chiral stationary phase. The amount of BSA adsorbed was calculated from the breakthrough curve and found to be 1.3 mg. With about 12 mg of silica in the column, the adsorption is *ca.* 0.1 g/g, which is in good agreement with a previous report [16].

The α and R_s values of the separations obtained for D,L-tryptophan, (±)-benzoin and (±)-oxazepam (Fig. 8, Table VI) are similar to those reported previously [16,23] using 1.6 and 4.6 mm I.D. stainless-steel columns. As shown in Fig. 8a, the separation of D- and L-tryptophan is highly dependent on the pH of the mobile phase. A pH of at least 7 is required to obtain reasonable selectivity. A further increase in pH leads to an even better separation. Unfortunately, the increased alkalinity also leads to breakdown of the silica and desorption of the BSA. The separation of (±)-benzoin (Fig. 8b), on the other hand, is almost independent of the pH of the mobile phase in the range 5–7 (not shown). The enantiomers of the benzodiazepine derivative oxazepam rapidly racemize in aqueous solutions [24]. This leads to coalescence, the formation of a diffuse zone between the two separated peaks containing a mixture of the enantiomers. This is particularly pronounced for separations with long retention times and results in an absence of baseline separation in spite of an apparently high resolution. Although increasing the flow-rate reduces the efficiency of the column, the resulting reduced intra-

TABLE IV

EFFECT ON CAPACITY FACTOR (k'), PLATE NUMBER (N) AND ASYMMETRY FACTOR (A_s) OF INCREASING SAMPLE AMOUNT

Column, 260 mm × 0.32 mm I.D. packed with Nucleosil 100-5 C_{18}; mobile phase, acetonitrile–water (80:20, v/v); detection wavelength, 215 nm; sample, biphenyl in mobile phase; injection volume, 60 nl; flow-rate, 2.0 μl/min.

Concentration (mg/ml)	Amount injected (μg)	k'	N	A_s
0.05	0.003	2.14	15 000	1.1
0.1	0.006	2.13	14 900	1.1
0.5	0.03	2.08	14 500	1.0
1.0	0.06	2.02	14 300	1.1
2.0	0.12	1.97	13 900	1.0
5.0	0.30	1.88	13 100	1.0
10	0.60	1.86	12 400	0.9

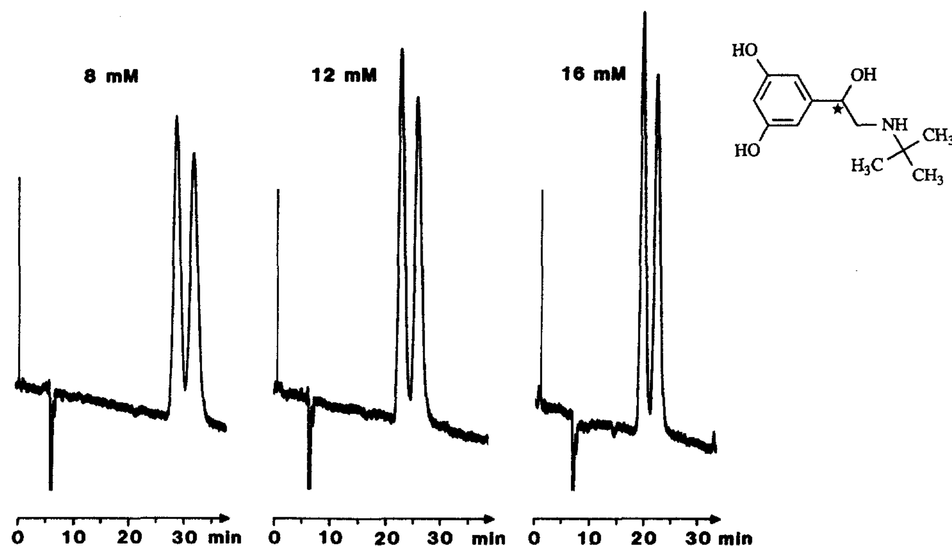

Fig. 6. Chromatograms showing the resolution of (±)-terbutalin with different concentrations of β-cyclodextrin in the mobile phase. Column, 190 mm × 0.32 mm I.D. packed with Nucleosil 100-5 C_{18}; mobile phase, 0.05 *M* ammonium acetate (pH 6.0) with 2% methanol as modifier; flow-rate, 1.8 μl/min; volume injected, 60 nl of a 0.75 m*M* solution in water; UV detection at 215 nm.

column racemization results in a better separation (Fig. 8c).

In contrast to the previous two phases, the Pirkle phase L-DNB-phenylglycine is of the normal-phase type and runs with a hexane–isopropanol mixture as mobile phase. The Pirkle phase in question has been used in the separation of a large number of different substances such as aryl alcohols, aryl sulphoxides, bi-β-naphthol derivatives and β-amino alcohols. As solutes 2,2,2-trifluoro-1-(9-anthryl)ethanol, [17] and the naphthyl isocyanate derivative of propranolol [25] were chosen (Fig. 9, Table VII). A 100 mm × 4 mm I.D. stainless-steel column was packed and used analogously to the capillary columns using the same batch of L-DNB-phenylglycine. The separation of (±)-2,2,2-trifluoro-1-(9-anthryl)ethanol yielded results similar to those reported in Table VII (k'_1 = 1.89, α = 1.28).

TABLE V

CHROMATOGRAPHIC DATA FROM FIG. 6

Chiral separation of (±)-terbutalin with β-cyclodextrin (β-CD) as chiral mobile phase additive.

[β-CD] (m*M*)	k'_1 [a]	k'_2	α	R_s	N_1
8	4.14	4.70	1.14	1.17	2300
12	3.00	3.53	1.18	1.39	2100
16	2.31	2.78	1.20	1.38	2000

[a] (+)-Terbutalin.

CONCLUSIONS

This work has shown that 0.32 mm I.D. slurry-packed fused-silica capillary columns can be packed using an ordinary isocratic HPLC pump as a packing unit. The same pump was also used together with a UV detector equipped with an optional capillary cell holder in a chromatographic system on which the packed columns were tested. Efficient reversed-phase C_{18} columns were reproducibly packed and showed an average reduced plate height of 3.5. Enantiomeric separations on these columns with β-cyclodextrin as a chiral mobile phase additive gave results similar to those reported for conventional columns under the same conditions. This correlation was also found with columns using either BSA or the Pirkle phase L-DNB-phenylglycine as a chiral stationary phase. In our opinion, almost every method originally developed for use on a conventional HPLC system can be transferred with little effort to a micro-LC system such as that described here. This switch to micro-LC in routine analyses leads to

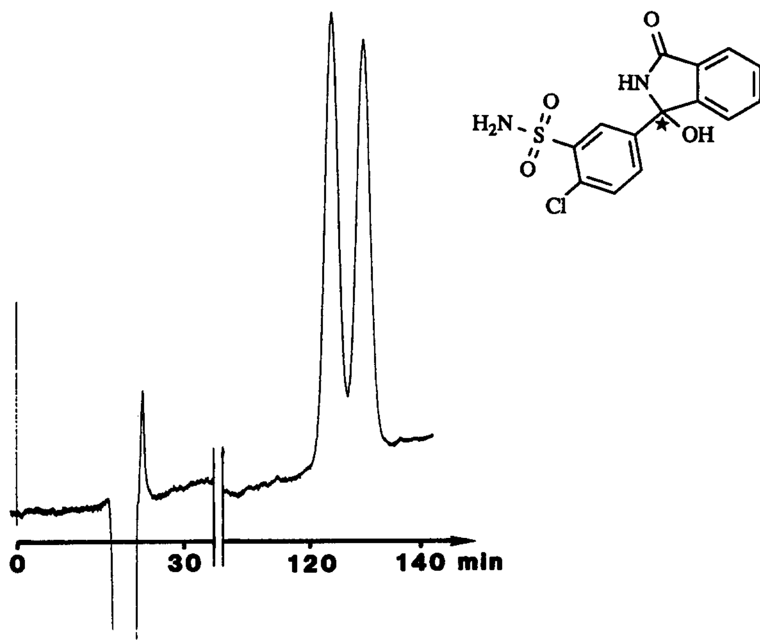

Fig. 7. Resolution of (±)-chlorthalidone on a 300 mm × 0.32 mm I.D. column packed with Nucleosil 100-5 C_{18}. Mobile phase, 8.4 m*M* β-cyclodextrin in 0.05 *M* ammonium acetate (pH 4.7) with 20% of ethanol as modifier; flow-rate, 1.1 μl/min; volume injected, 200 nl of a 0.01 mg/ml solution in water; UV detection at 218 nm. k'_1 = 6.32; k'_2 = 6.65; α = 1.05; R_s = 1.19: N_1 = 13 300.

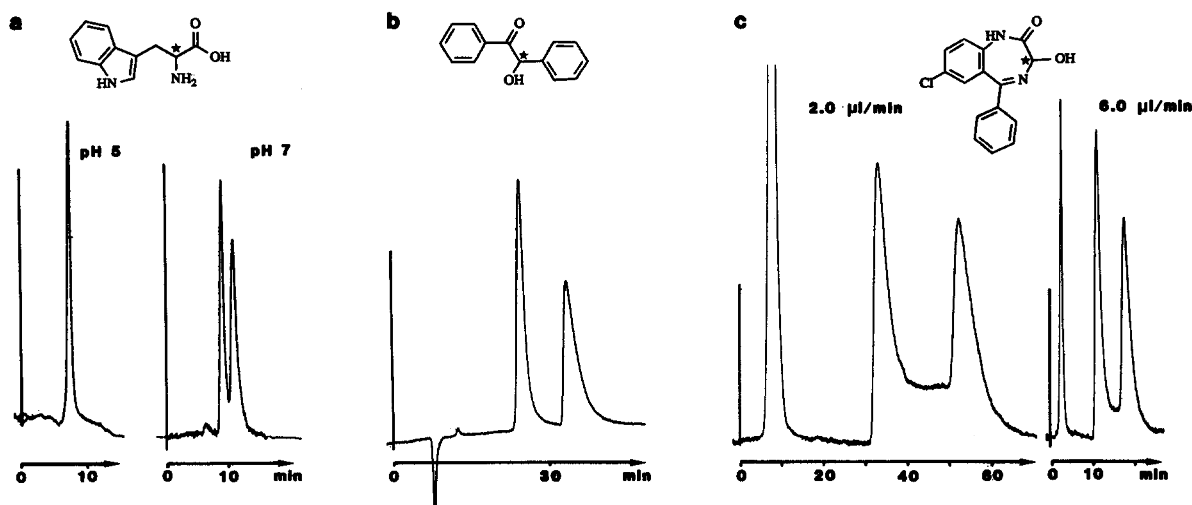

Fig. 8. Resolution of (a) D,L-tryptophan, (b) (±)-benzoin and (c) (±)-oxazepam on a 250 mm × 0.32 mm I.D. column packed with 5-μm BSA–silica. (a) Mobile phase, 0.05 *M* phosphate buffer (pH 5.0 and 7.0) with 2% of *n*-propanol as modifier; flow-rate, 2.5 μl/min; volume injected, 200 nl of a 0.02 mg/ml solution in the mobile phase; UV detection at 279 nm. (b) Mobile phase, 0.05 *M* phosphate buffer (pH 5.0) with 2% of *n*-propanol as modifier; flow-rate, 2.4 μl/min; volume injected, 200 nl of a 0.02 mg/ml solution in water–methanol (98:2, v/v); UV detection at 279 nm. (c) Mobile phase, 0.05 *M* phosphate buffer (pH 7.0) with 2% of *n*-propanol as modifier; flow-rate, 2.0 and 6.0 μl/min; volume injected, 200 nl of a 0.02 mg/ml solution in water–methanol (75:25, v/v); UV detection at 249 nm.

TABLE VI

CHROMATOGRAPHIC DATA FROM FIG. 8

Chiral separation on a BSA column.

Substance	pH	k'_1	k'_2	α	R_s	N_1
Tryptophan	5	0.10	–	–	–	1400
	7	0.28[a]	0.55	1.95	1.20	1100
Benzoin	5	2.37	3.61	1.52	2.45	1600
Oxazepam	7[b]	3.40	5.84	1.72	2.6	450
	7[c]	3.69	6.40	1.73	2.1	360

[a] D-Tryptophan.
[b] 2 μl/min.
[c] 6 μl/min.

a drastic reduction in the costs of solvent purchase and disposal.

It should be stressed, however, that although micro-LC generally shows a higher mass sensitivity than conventional HPLC, its concentration sensitivity is poorer owing to the small injection volumes. Quantitative analysis at low concentrations can therefore be more difficult to perform using microcolumns. Furthermore, microcolumns are usually operated at lower signal-to-noise ratios and problems with detector noise and drift are therefore more likely to occur. However, this issue is a subject for further research.

Micro-LC is likely to open up new possibilities in

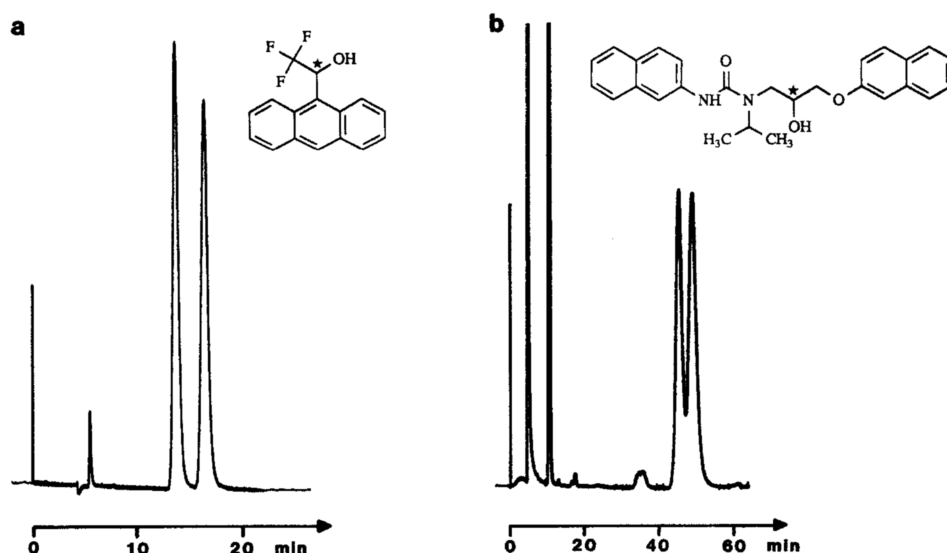

Fig. 9. Resolution of (a) (±)-2,2,2-trifluoro-1-(9-anthryl)ethanol and (b) (±)-propranolol as its naphthyl isocyanate derivative on a 260 mm × 0.32 mm I.D. column packed with 4-μm L-DNB-phenylglycine. (a) Mobile phase, *n*-hexane–2-propanol (95:5, v/v); flow-rate, 1.9 μl/min; volume injected, 60 nl of a 0.08 mg/ml solution in 2-propanol; UV detection at 253 nm. (b) Mobile phase, *n*-hexane–2-propanol–acetonitrile (90:10:2, v/v/v); flow-rate, 1.9 μl/min; volume injected, 60 nl of a 2-propanol-diluted reaction mixture (less than 1.5 m*M*); UV detection at 214 nm.

TABLE VII

CHROMATOGRAPHIC DATA FROM FIG. 9

Chiral separation on a Pirkle column.

Substance	Mobile phase[a] (v/v/v)	k'_1	k'_2	α	R_s	N_1
Tri-F[b]	95:5:–	2.22	2.87	1.29	2.54	3200
Propranolol	90:10:2	12.0[c]	13.3	1.11	1.12	2500

[a] Mobile phase: *n*-hexane–isopropanol–acetonitrile.
[b] 2,2,2-Trifluoro-1-(9-anthryl)ethanol.
[c] (+)-Propranolol.

the development of separation methods utilizing expensive or rare mobile and stationary phases such as monoclonal antibodies or receptor proteins.

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