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Enantioseparations in normal- and reversed-phase nano-high-performance liquid chromatography and capillary electrochromatography using polyacrylamide and polysaccharide derivatives as chiral stationary phases

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Abstract

Enantioseparations of chiral compounds with different structures (β-blockers, benzodiazepines, diuretica, etc.) were performed in fused-silica capillaries packed with silica gel which was modified by covalent attachment of poly-N-acryloyl-L-phenylalanineethylester (Chiraspher®) or by coating with cellulose tris(3,5-dimethylphenylcarbamate). Three different separation modes, normal- and reversed-phase nano-HPLC and capillary electrochromatography (CEC) with and without pressure-assistance were performed in these capillaries using essentially the same experimental set-up. This allows a more reliable comparison of the different techniques. Nano-HPLC separations in the normal-phase mode gave relatively high peak efficiency compared to HPLC in regular size columns. However, under the experimental conditions of this study no significant gain in separation efficiency could be obtained by changing from pressure-driven to electrically-driven migration of the solutes.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Electrochromatography; Nano-high-performance liquid chromatography; Polyacrylamide derivatives; Polysaccharide derivatives

1. Introduction

Polyacrylamide and polysaccharide derivatives have been used as chiral stationary phases (CSPs) for high-performance liquid chromatography (HPLC) enantioseparations [1,2]. While enantioseparations with polyacrylamide CSPs are mainly studied with organic mobile phases, successful enantioseparations with polysaccharide CSPs were also reported under reversed-phase conditions [1,3–7]. A miniaturization of chiral separations allows not only a reduction of the expensive chiral packing-materials and high-purity organic solvents, but also makes the analytical process less hazardous for the environment and easier compatible to coupling with a mass spectrometer.

One technique for a miniaturization of chiral separations is capillary electrophoresis (CE) [8], which offers advantages such as high peak efficiency, compatibility with biological samples, rob-

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ustness, versatility, low cost etc. Many chiral selectors such as cyclodextrins (CD), metal complexes and crown ethers previously studied in HPLC were successfully transferred to chiral CE. However, such a transfer may not always be possible. This may be due to the limited solubility of a HPLC-chiral selector in the aqueous CE buffers, a high detector response of the selector, or a reduced chiral recognition under the typical CE conditions. In such cases, capillary electrochromatography (CEC) is the only alternative for using CSPs which have been successfully used in HPLC. Due to the electrically-driven flow, higher plate numbers may be achieved. In addition, as compared to CE, HPLC offers some advantages such as a better reproducibility of data and stationary beds. Thus, CEC [8–10] appears to be a promising approach for chiral separations [8,10–26]. This technique can be realized in two basic modes: CEC in wall-coated open tubular capillaries which was proposed by Mayer and Schurig [11] and CEC in capillaries packed with CSPs [21–26].

The goal of the present study was to compare the applicability of polyacrylamide and polysaccharide derivatives as CSPs in nano-HPLC (normal- and reversed-phase) to CEC.

2. Experimental

2.1. Chemicals and reagents

The racemic compounds (Fig. 1) were from different commercial sources and used without further purification. Phenylthiohydantoin (PTH) derivatives of DL-leucine and DL-methionine were prepared as described [27]. Microcrystalline cellulose (Avicel), wide pore silica gel (LiChrospher 1000, 5 μm), sodium citrate and a Hibar® Chiraspher® HPLC column (250×4 mm) used for chiral separations were purchased from Merck (Darmstadt, Germany). 3,5-Dimethylphenylisocyanate was supplied from Aldrich (Daisenhofen, Germany); (3-aminopropyl)-triethoxysilane and sodium perchlorate were purchased from Fluka (Buchs, Switzerland). The polyacrylamide-type CSP (Fig. 2a) was prepared by radical copolymerisation of N-acryloyl-L-phenylalanineethylester with silica gel previously modified by covalent attachment of methacryloyl groups as described [28]. This packing material (Chiraspher®) was kindly provided by Dr. Michael Schulte (Merck, Darmstadt, Germany).

Before coating with cellulose tris(3,5-dimethylphenylcarbamate) (Fig. 2b), wide-pore silica gel (LiChrospher 1000, 5 μm) was silanized using (3-aminopropyl)-triethoxysilane in benzene in the presence of a catalytic amount of dry pyridine at 80°C. Preparation of cellulose tris(3,5-dimethylphenylcarbamate) (Fig. 2b) was performed by reaction of the corresponding isocyanate with microcrystalline cellulose in dry pyridine at 80°C overnight [29] and isolated as methanol insoluble fraction (yield approx. 95%). The structure was confirmed by ^{1}H-NMR spectroscopy and elemental analysis. Cellulose tris(3,5-dimethylphenylcarbamate) was dissolved in tetrahydrofurane and coated 20% (w/w) on previously aminosilanized wide pore silica gel by a static technique.

2-Propanol, *n*-hexane, benzene, pyridine, tetrahydrofurane, methanol and diethylamine were purchased from J.T. Baker (Deventer, The Netherlands). Acetonitrile was from Scharlau (Barcelona, Spain).

2.2. Preparation of capillary columns

Fused-silica capillaries of 100 μm I.D. from Polymicro Technologies (Phoenix, AZ, USA) were used. The inlet-end of the capillary was connected to a HPLC-precolumn (4.6×50 mm) which served as reservoir for the slurry of the packing material in *n*-hexane/2-propanol 90/10 (v/v). A commercially available HPLC column frit was connected to the outlet-end of the capillary in order to retain the packing material. The slurry of the packing material was ultrasonicated in a water-bath (15 min) and transferred into the reservoir. The system was closed tightly, pressure up to 400 bar was applied using a Knauer pneumatic pump (Knauer, Berlin, Germany) and maintained for 1 h. After complete reduction of the residual pressure (3–4 h), bidistilled water was pumped through the packed bed for 30 min [30]. The outlet and inlet frits were sintered by local heating of the packed bed for approx. 40 s for the polyacrylamide-type CSP and 10 s for the polysaccharide-type CSP using a heating coil (700–800°C). The packed capillaries prepared according to this

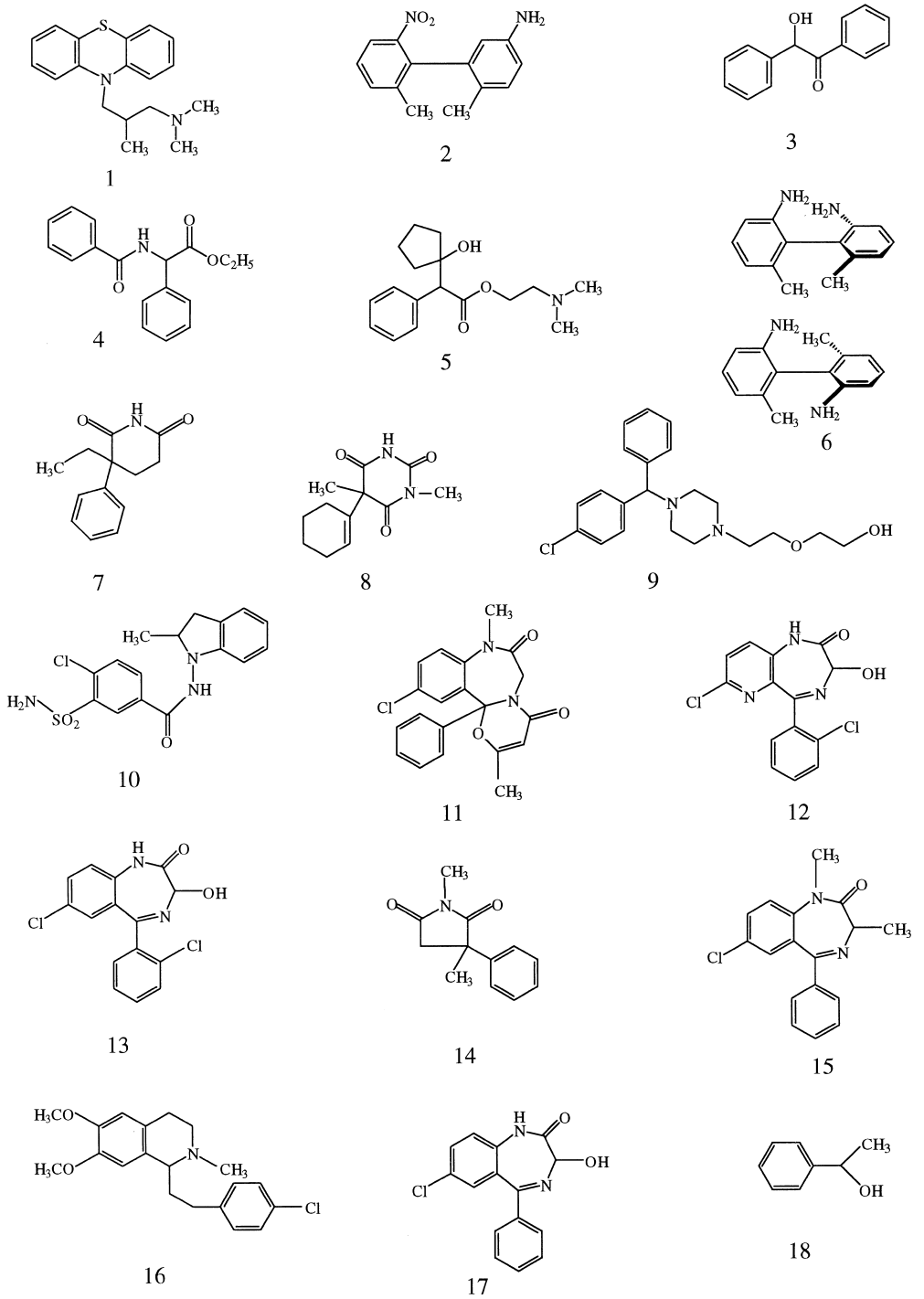

Fig. 1. Structures of the chiral analytes (1) alimemazine, (2) 3′-amino-6,6′-dimethyl-2-nitrobiphenyl, (3) benzoin, (4) N-benzoyl-phenyl-glycin-ethylester, (5) cyclopentolate, (6) 2,2′-diamino-6,6′-dimethylbiphenyl, (7) glutethimide, (8) hexobarbital, (9) hydroxyzine, (10) indapamide, (11) ketazolam, (12) lopirazepam, (13) lorazepam, (14) mesuximide, (15) 3-methyldiazepam, (16) metofoline, (17) oxazepam, (18) 1-phenylethanol, (19) pindolole, (20) piprozoline, (21) propranolol, (22) *trans*-stilbene oxide, (23) trimipramine, (24) verapamil, (25) warfarin, (26) penflutizide, (27) bendroflumethiazide, (28) paraflutizide, (29) chlorthalidone, (30) PTH-leucine, (31) PTH-methionine.

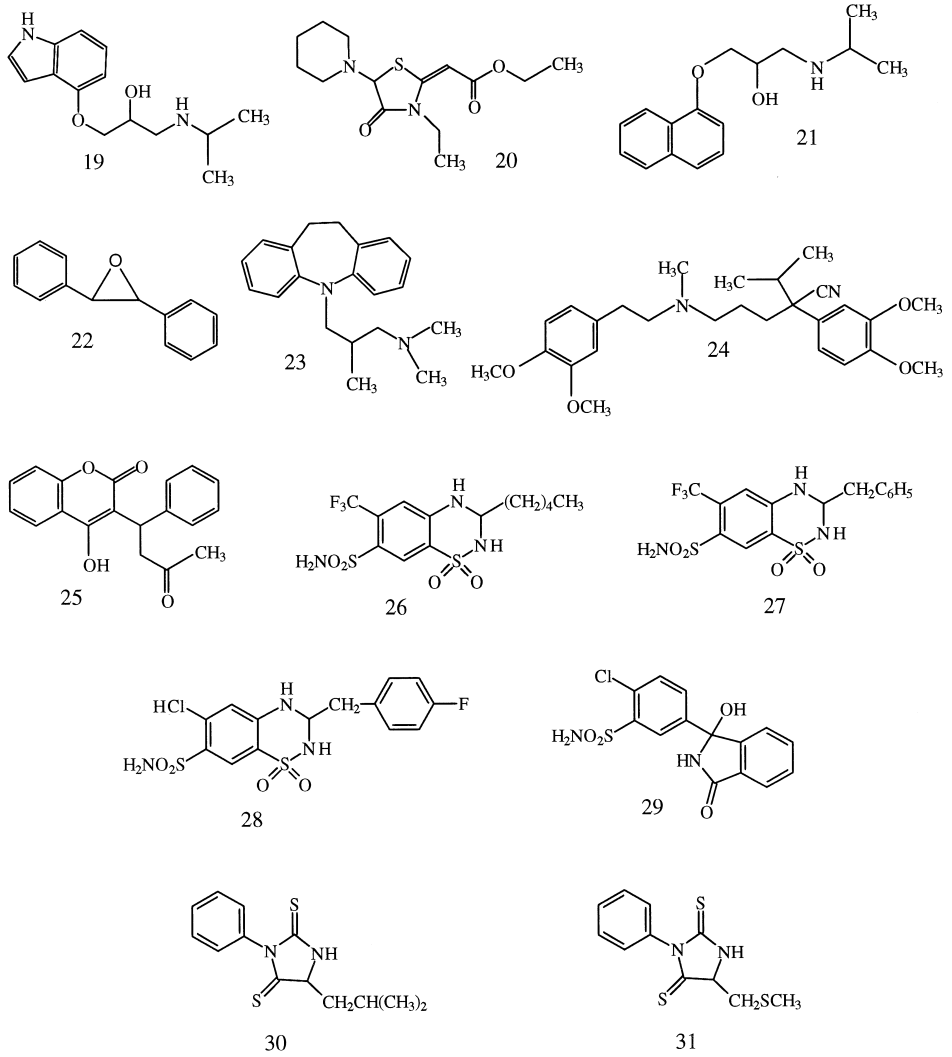

Fig. 1. (*continued*)

technique were used for nano-HPLC and CEC separations.

2.3. Nano-HPLC in normal-phase mode

Nano-HPLC separations in normal-phase mode were performed using a HP ^{3D}CE (Hewlett-Packard, Waldbronn, Germany) capillary electrophoresis instrument. The total length of the capillary was 30 cm. The length of the packed bed was 10 cm in the case of the polyacrylamide-type CSP and 22 cm in the case of the polysaccharide-type CSP. Sample injection and separation were carried out by high pressure (10–12 bar). On-capillary detection was performed with the diode-array UV-detector.

2.4. Nano-HPLC in aqueous buffers, pressure-assisted CEC and CEC

Nano-HPLC in aqueous buffers and CEC experiments were performed with a HP ^{3D}CE capillary electrophoresis equipment in the case of the polyacrylamide-type CSP and with a CE-system Grom-100 (Grom, Herrenberg, Germany) combined with a

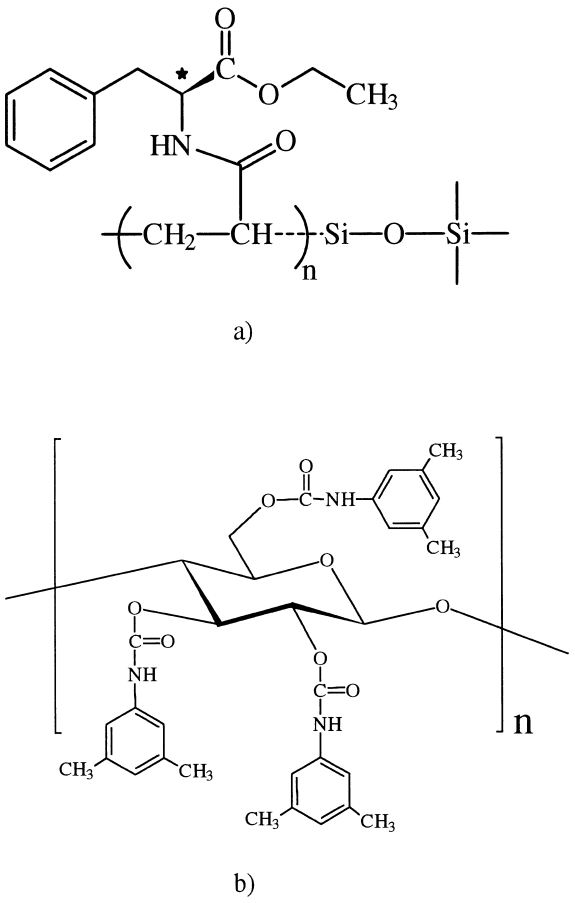

Fig. 2. Structures of Chiraspher® (a) and cellulose *tris*(3,5-dimethylphenylcarbamate) (Chiracel-OD) (b).

Merck Hitachi L-6000 pump (Merck, Darmstadt, Germany) and a home-made flow-splitter in the case of the polysaccharide-type CSP. The overall length of the capillaries was 30 cm and 40 cm, respectively. The length of the packed bed was 10 cm and 22 cm, respectively.

3. Results and discussion

3.1. Normal-phase nano-HPLC

Miniaturized chiral separations using polysaccharide-type CSPs in narrow-bore [31–33] and capillary columns [34] have been reported previously. However, special micro-HPLC injection loops and micro-size detection cells were applied in these experimental set-ups. In this study an attempt was made to perform nano-HPLC enantioseparations using a commercially available capillary electrophoresis equipment.

As shown in Figs. 3 and 4 and Tables 1 and 2, efficient chiral separations can be obtained using capillaries packed with Chiraspher® or with cellulose tris(3,5-dimethylphenylcarbamate) immobilized on silica gel. The higher separation efficiency observed in packed capillary columns compared to regular size columns are in good agreement with theoretical considerations [35–37] and experimental data observed in previous studies for achiral separations

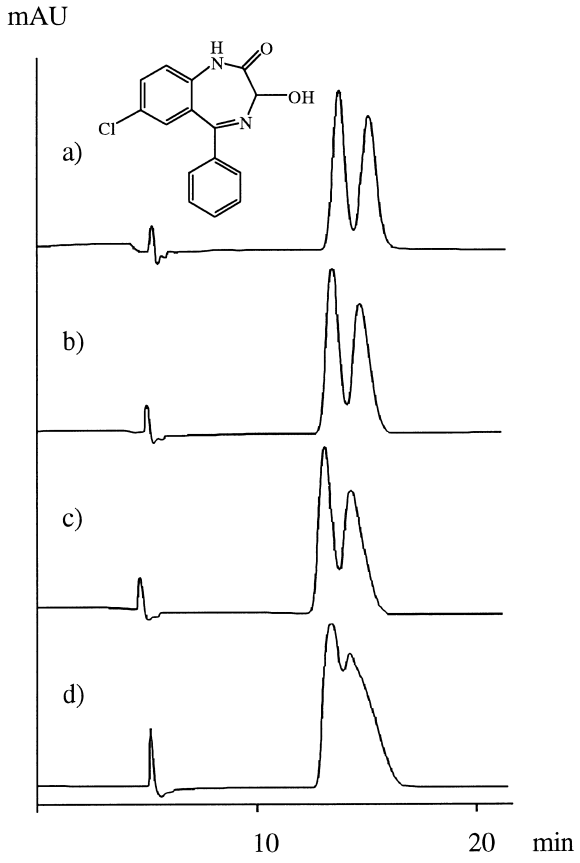

Fig. 3. Effect of the injection time on the enantioseparation of oxazepam in a capillary column (100 μm×22.5 cm effective length/10 cm packed bed length/30 cm total length) packed with Chiraspher®, 5 μm. Mobile phase: *n*-hexane/dioxane/2-propanol 70/25/5 (v/v/v). Applied pressure: 12 bar. Injection time: (a) 0.6 s; (b) 1.8 s; (c) 3 s; (d) 6 s. UV-detection at 214 nm.

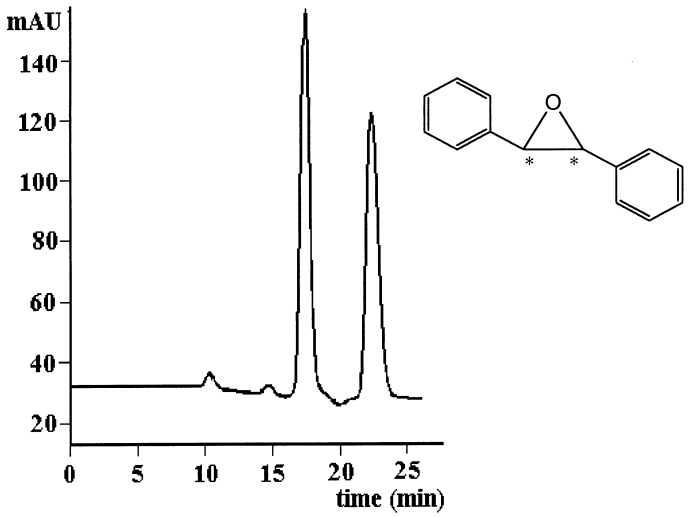

Fig. 4. Nano-HPLC enantioseparation of trans-stilbene oxide in a capillary column (100 μm×22 cm effective length/30.5 cm total length) packed with cellulose tris(3,5-dimethylphenylcarbamate)-coated silica gel, 5 μm. Mobile phase: *n*-hexane/2-propanol 90/10 (v/v). Applied pressure: 12 bar inlet; UV-detection at 214 nm.

[38,39]. The elimination of extra column volumes due to on-capillary sample injection and detection may also contribute to the observed improvement of the separation efficiency. The high efficiency separations may be performed with a pressure gradient as low as 10–12 bar. The analysis times were not always comparable in regular size and capillary columns. This may also contribute to the apparent difference in the separation efficiencies using these two types of columns. It should be noted that overloading the column may cause severe adverse effects on the separation in miniaturized columns as shown in Fig. 3. A similar effect for achiral separations was noted recently [40].

As these preliminary studies show, separations in the capillary format can be an attractive alternative to chiral separations using regular size columns. The main incentives of capillary columns include: (a) a higher column efficiency achieved at moderately low pressure gradients; (b) a drastically reduced con-

Table 1
Enantioseparations on Chiraspher® material in common-size (a) and nano- (b) normal-phase HPLC

Racemate	Concentration (mg/ml^{-1})	Mobile phase (v/v/v)	System	k'_1	k'_2	α
Oxazepam	1	70/25/5	a	8.40	9.80	1.20
			b	1.63	1.89	1.16
Lopirazepam	1	60/39/1	a	8.06	9.74	1.20
			b	3.10	3.86	1.25
Lorazepam	1	65/34/1	a	5.38	6.24	1.16
			b	1.41	1.60	1.13
Penflutizide	2	55/44/1	a	7.53	8.86	1.20
			b	2.55	2.95	1.16
Chlorthalidone	1	50/50/0	a	12.30	15.00	1.20
			b	2.02	2.29	1.13

Mobile phase: *n*-hexane/dioxane/2-propanol.
System a: Hibar® Chiraspher® (250×4 mm), flow: 1 ml/min
System b: 100 μm I.D., packed bed length: 10 cm, total length: 30 cm, applied pressure 12 bar inlet.

Table 2
Enantioseparation in normal-phase nano-HPLC using capillary columns packed with tris(3,5-dimethylphenylcarbamate) of cellulose coated on silica gel

Racemate	Mobile phase	Applied pressure	t_1 (min)	t_2 (min)	k'_1	k'_2	α	R_s	N_1	N_2
Benzoin	a	12 bar	23.90	31.60	1.95	2.90	1.49	3.17	1925	2060
Benzoyl-phenyl-glycin-ethylester	a	12 bar	24.40	30.42	2.01	2.76	1.37	2.18	1575	1500
2,2′-Diamino-6,6′-dimethylbiphenyl	a	12 bar	17.92	23.48	1.21	1.90	1.60	2.61	1600	1475
3′-Amino-6,6′-dimethyl-2-nitrobiphenyl	a	12 bar	17.40	22.43	1.15	1.77	1.54	3.29	2770	2630
Cyclopentolate	a	10 bar	9.50	12.00	0.17	0.48	2.83	2.74	2060	2030
Glutethimide	d	12 bar	18.50	20.10	1.28	1.48	1.15	0.92	3015	1470
Hexobarbital	a	12 bar	22.99	27.32	1.84	2.37	1.29	0.94	530	310
Mesuximide	b	12 bar	20.30	24.40	1.51	2.01	1.34	1.71	1735	1300
Metofoline	a	12 bar	18.10	25.20	1.23	2.11	1.71	2.92	1200	1150
L-Phenylethanol	a	12 bar	17.45	19.53	1.15	1.41	1.22	1.23	1620	1895
Piprozoline	d	10 bar	22.40	29.00	1.76	2.58	1.46	3.20	2510	2250
Propranolol	c	12 bar	24.10	30.20	1.98	2.73	1.38	2.23	1600	1620
trans-Stilbene oxide	a	10 bar	16.30	20.30	1.01	1.51	1.49	3.24	4265	3540
Troeger's base	a	10 bar	14.73	16.62	0.82	1.05	1.28	1.28	1759	1862

[a] *n*-Hexane/2-propanol 90/10 (v/v).
[b] *n*-Hexane/2-propanol 85/15 (v/v).
[c] *n*-Hexane/2-propanol/diethylamine 90/10/0.1 (v/v/v).
[d] Methanol.

sumption of increasingly expensive or environmentally hazardous mobile phases and (c) new detection possibilities with extremely low column flow-rates [36]. In addition, nano-HPLC enantioseparations may be performed using commercially available CE-instruments which are equipped with a moderately high internal or external pressure source.

3.2. Reversed-phase nano-HPLC

Reversed-phase nano-HPLC enantioseparations can be performed in the same capillaries (Figs. 5 and 6 and Tables 3 and 4) that were used for normal-phase enantioseparations. However, a higher pressure-gradient is required for these separations and,

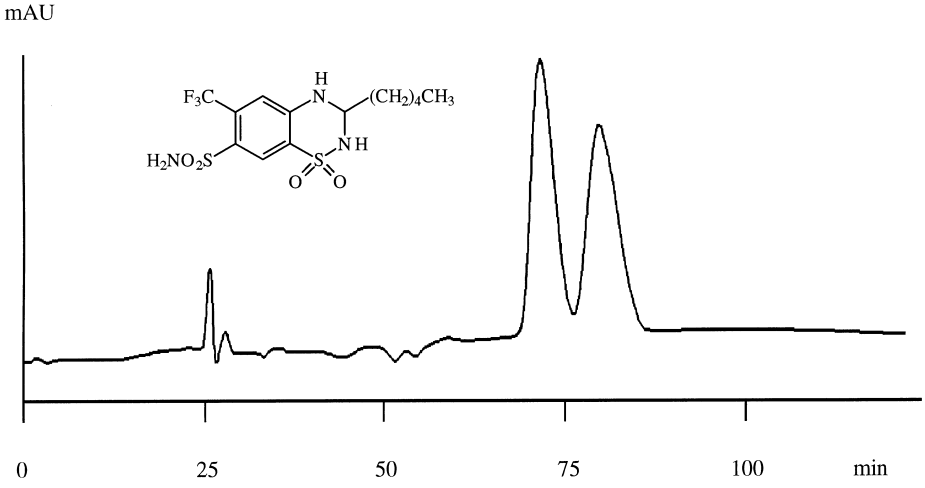

Fig. 5. RP nano-HPLC enantioseparation of penflutizide in a capillary column (100 μm×22.5 cm effective length/10 cm packed bed length/30 cm total length) packed with Chiraspher®, 5 μm. Mobile phase: methanol/water 60/40 (v/v). Applied pressure: 12 bar inlet/4 bar outlet. UV-detection at 214 nm.

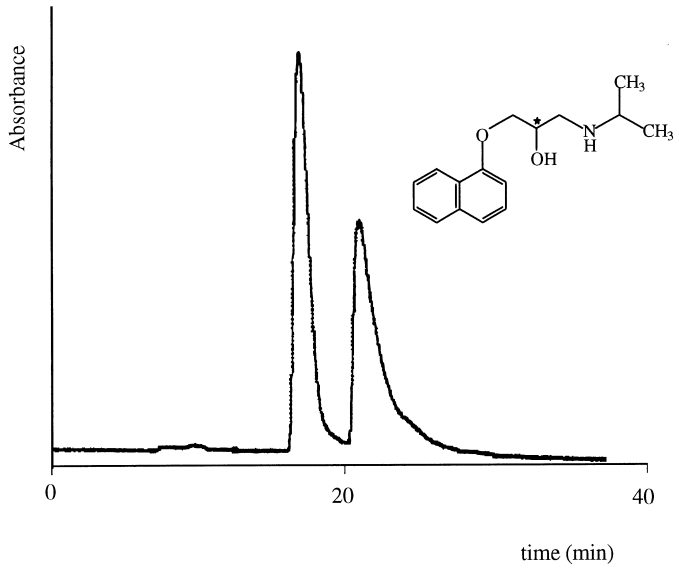

Fig. 6. RP nano-HPLC enantioseparation of propranolol in a capillary column (100 μm×22 cm effective length/40 cm total length) packed with cellulose tris(3,5-dimethylphenylcarbamate)-coated silica gel, 5 μm. Mobile phase: 1 *M* – $NaClO_4$/acetonitrile (60/40, v/v). Applied pressure: 30 bar. UV-detection at 214 nm.

therefore, they were performed in capillaries with a shorter packed bed length, or alternatively, using a modified Grom capillary electrophoresis system. Plate numbers obtained in reversed-phase nano-HPLC were comparable or higher (15–25%) to regular size columns (data not shown).

3.3. Pressure-assisted CEC and CEC

An electrically-driven migration mechanism instead of a pressure-driven mechanism may offer the following advantages: an inherently higher peak efficiency due to the plug-like profile of the electrically-driven flow and the possibility to decrease the particle size of the packing material (i.e. increase the plate number) without a drastic increase of the pressure drop.

For charged analytes, the electroosmotic flow (EOF) may be assisted by the electrophoretic mobility of the analyte. However, the EOF becomes the

Table 3
Enantioseparations on Chiraspher®material in (a) common-size and (b) nano- reversed-phase HPLC[c]

Racemate	System	t_0 (min)	t_1 (min)	t_2 (min)	k_1'	k_2'	α	N_1	N_2	R_S
Penflutizide	a	2.40	10.88	12.38	3.53	4.16	1.18	690	740	0.87
	b	25.84	71.44	79.34	1.76	2.07	1.18	2290	2090	1.27
Bendroflumethiazide	a	2.25	13.43	15.30	4.97	5.80	1.17	1500	1400	1.21
	b	42.21	91.60	103.19	1.17	1.44	1.23	1410	1045	1.04
Paraflutizide	a	2.27	15.10	16.32	5.65	6.19	1.10	1455	1195	0.45
	b	40.46	101.54	111.92	1.51	1.77	1.17	1600	1250	0.84
PTH-Leucine	a	2.30	8.01	8.60	2.48	2.74	1.10	2330	1695	0.72
	b	33.75	54.85	57.75	1.12	1.25	1.12	2980	2365	0.79
PTH-Methionine	a	2.30	8.20	8.74	2.57	2.80	1.09	1525	1735	0.68
	b	23.63	56.16	63.61	1.09	1.19	1.09	2865	2495	0.62

[a] Hibar® Chiraspher® (250×4 mm), flow: 1 ml/min
[b] 100 μm I.D., packed bed length: 10 cm, total length: 30 cm, applied pressure: 12 bar inlet/4 bar outlet.
[c] Mobile phase:methanol/water 60/40.

Table 4
Enantioseparation in reversed-phase nano-HPLC using capillary columns packed with tris(3,5-dimethylphenylcarbamate) of cellulose coated on silica gel

Racemate	Mobile phase	Applied pressure	t_1 (min)	t_2 (min)	k'_1	k'_2	α	R_s	N_1	N_2
Alimemazine	a	28 bar	33.00	35.10	3.02	3.28	1.08	0.98	3860	2215
Benzoin	b	44 bar	31.39	36.60	4.14	5.00	1.21	1.41	1395	1315
Hydroxyzine	a	28 bar	21.00	24.26	1.56	1.96	1.25	0.92	2030	1040
Indapamide	b	44 bar	28.86	37.80	3.73	5.20	1.39	1.74	1205	660
Ketazolam	c	44 bar	25.17	29.22	3.13	3.79	1.21	0.59	340	210
Lorazepam	c	44 bar	11.57	20.57	0.90	2.37	2.65	2.60	355	330
3-Methyldiazepam	c	44 bar	24.55	34.34	3.02	4.63	1.53	1.84	630	430
Lopirazepam	c	44 bar	7.23	9.99	0.19	0.64	3.44	1.65	670	480
Oxazepam	c	44 bar	16.21	27.23	1.66	3.46	2.11	2.75	525	670
Pindolol	a	28 bar	13.75	25.05	0.68	2.05	3.03	2.98	835	330
Propranolol	a	28 bar	18.95	23.02	1.31	1.81	1.38	1.64	1430	810
Trimipramine	a	28 bar	36.20	42.30	3.41	4.16	1.22	1.91	3280	3190
Verapamil	b	43 bar	16.18	19.20	1.65	2.15	1.30	1.48	2035	985
Warfarin	c	40 bar	20.72	29.47	2.34	3.75	1.60	1.91	645	500

[a] 1 *M* – $NaClO_4$/acetonitrile 60/40 (v/v).
[b] 0.5 *M* – $NaClO_4$/acetonitrile 60/40 (v/v).
[c] 0.25 *M* – $NaClO_4$/acetonitrile 60/40 (v/v).

major electrical driving force for neutral analytes in the absence of a pressure drop in packed columns. In order to estimate the contribution of the EOF to the migration of analytes through the polyacrylamide- and polysaccharide-packed capillaries, the migration times of the most likely non-retained neutral markers

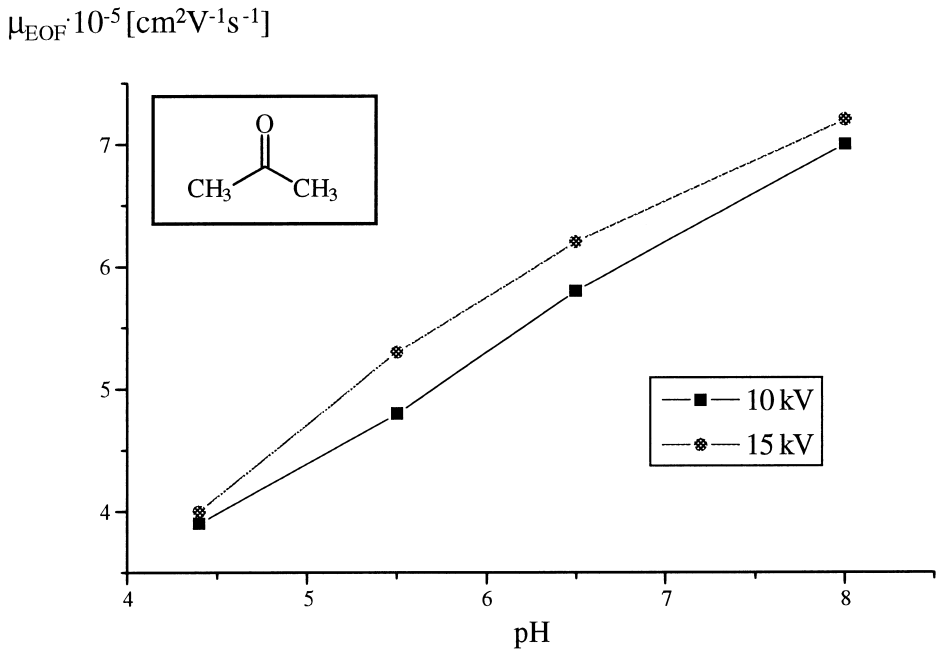

Fig. 7. Dependence of the EOF on the applied voltage and pH in a capillary column (100 μm×22.5 cm eff. length/10 cm packed bed length/30 cm total length) packed with Chiraspher®, 5 μm. Mobile phase: acetonitrile/50 m*M* NaH_2PO_4, pH 8.0, 40/60 (v/v).

acetone and thiourea, were measured as a function of the applied voltage. As shown in Figs. 7 and 8, a significant cathodic EOF could be observed in both kinds of capillaries. However, it seems that the EOF in the sorbent bed of the polysaccharide-packed capillary is directed to the anode, some optimization of the buffer was required, because it was difficult to perform CEC in polysaccharide-packed capillaries with a mixture of acetonitrile and phosphate buffer. The latter caused high currents and bubble formation.

The separation of the enantiomers of bendroflumethiazide in pressure-assisted CEC is shown in Fig. 9. The migration times of the enantiomers decrease drastically with increasing voltage, whereas the separation selectivity remains almost constant. The shorter analysis time may be considered as an advantage of pressure-assisted CEC compared to the HPLC-mode.

The effect of the applied voltage on the separation of the enantiomers of indapamide with polysaccharide-type CSP packed capillaries was also studied (Fig. 10). The application of a voltage of 10–15 kV in addition to the pressure contributed positively to the separation. However, at higher voltages the separation efficiency was decreased due to the short analysis time and perhaps the adverse effect of the Joule heating.

The dependence of the enantioseparation of bendroflumethiazide on the organic modifier was studied in the case of Chiraspher®-packed capillaries. As shown in Fig. 11, acetonitrile as organic modifier was advantageous to methanol. This is consistent with the superiority of acetonitrile in CEC as buffer modifier observed previously for achiral separations [9].

In summary, this study shows the feasibility of the miniaturization of chiral separations using polyacrylamide- and polysaccharide-type CSPs. High efficiency enantioseparations in normal-phase nano-HPLC may be performed at a relatively low pressure (10–12 bar) using commercially available capillary

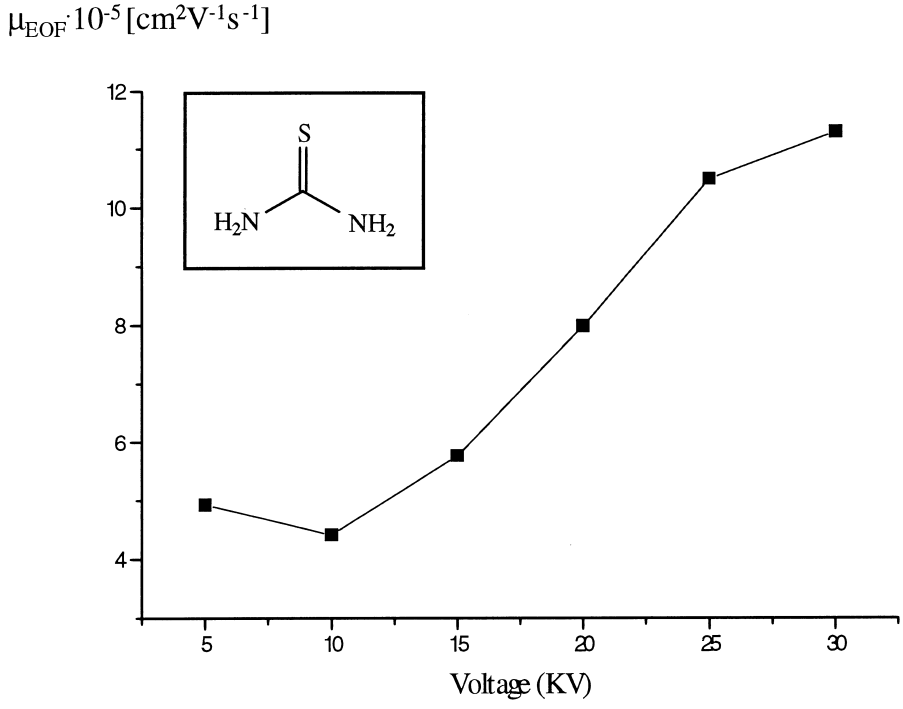

Fig. 8. Effect of the applied voltage on migration time of thiourea through the capillary column (100 μm×22 cm effective length/40 cm total length), packed with cellulose tris(3,5-dimethylphenylcarbamate)-coated silica gel, 5 μm. Applied pressure: 25 bar. Mobile phase: 20 m*M* sodium citrate (pH 7.0)/acetonitrile 55/45 (v/v).

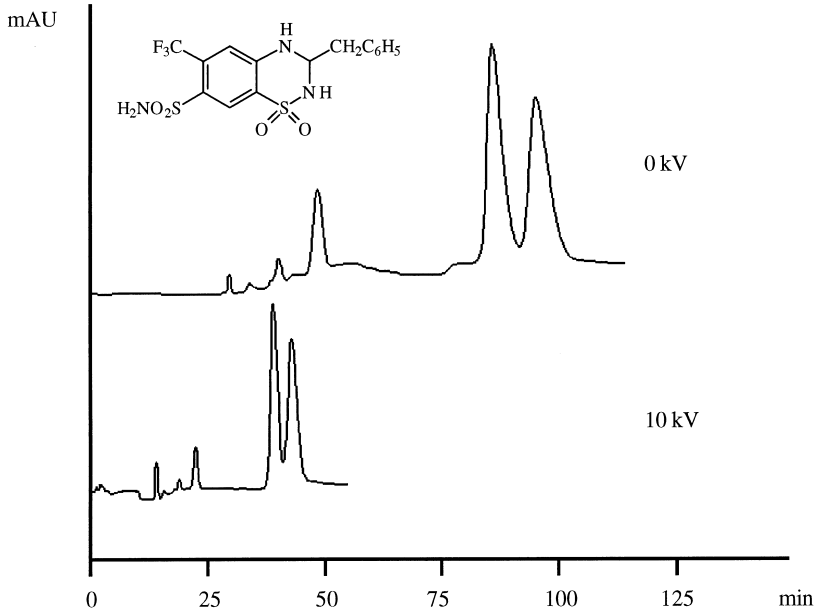

Fig. 9. Effect of the applied voltage on enantioseparation of bendroflumethiazide. Mobile phase: methanol/50 m*M* NaH_2PO_4 60/40 (v/v). Applied pressure: 12 bar inlet/4 bar outlet. Conditions as in Fig. 7.

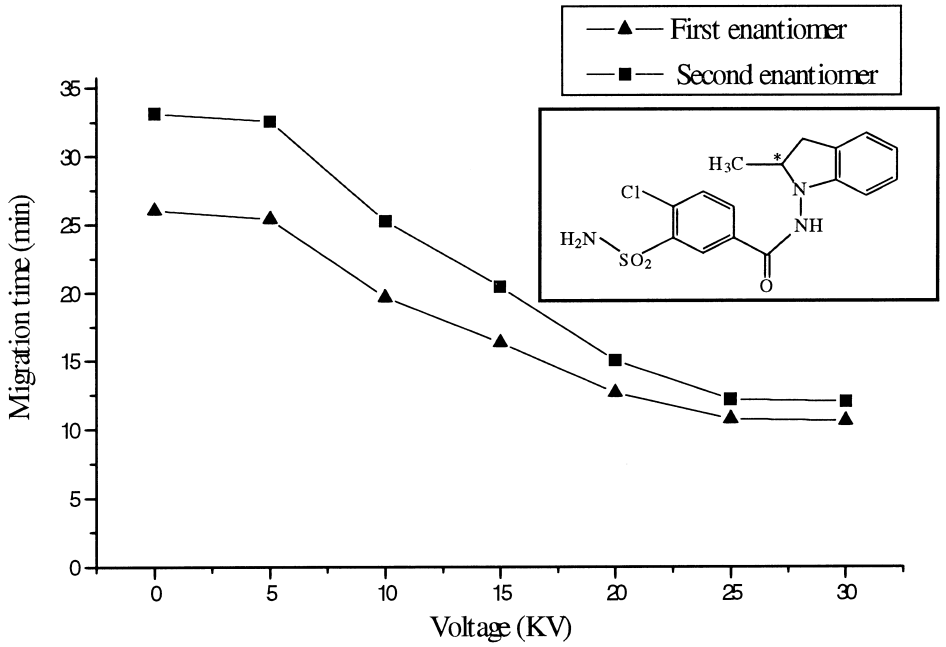

Fig. 10. Effect of the applied voltage on migration time and separation of indapamide enantiomers. Conditions as in Fig. 8.

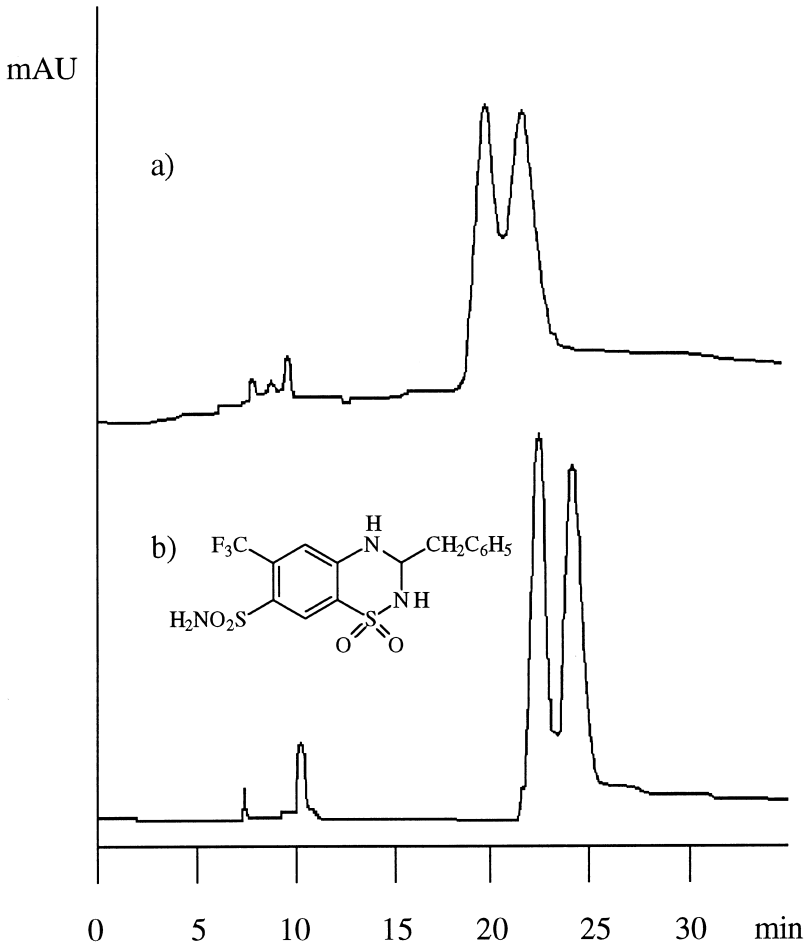

Fig. 11. CEC enantioseparation of bendroflumethiazide in a Chiraspher®-packed capillary using (a) methanol/50 m*M* NaH_2PO_4, pH 8.0, 60/40 (v/v) and (b) acetonitrile/50 m*M* NaH_2PO_4, pH 8.0, 40/60 (v/v). Applied voltage: (a) 20 kV, (b) 12.5 kV; applied pressure: 10 bar on inlet and outlet vial. Other conditions as in Fig. 7.

electrophoresis instruments. Enantioseparations in reversed-phase nano-HPLC, pressure-assisted CEC and CEC without an additional pressure gradient can be carried out using the same capillaries. Further studies are required in order to optimize enantioseparations using the electrically-driven mechanism. Especially the negative influence of column overloading must be taken into consideration.

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