Christoph Dette[1]
Siegfried Ebel[1]
Shigeru Terabe[2]

[1] **Institute of Pharmacy and Food Chemistry, Würzburg**
[2] **Faculty of Science, Himeji Institute of Technology, Hyogo**

Neutral and anionic cyclodextrins in capillary zone electrophoresis: Enantiomeric separation of ephedrine and related compounds*

Two methods for the enantiomeric separation of chiral ephedrine alkaloids (ephedrine, methylephedrine, methylpseudoephedrine and norephedrine) by capillary zone electrophoresis in uncoated capillaries were developed. Both methods were optimized to more than 100 000 theoretical plate numbers. The first method used a neutral cyclodextrin (CD) derivative: heptakis (2,6–di-*O*-methyl)-β-cyclodextrin at an acidic pH of 2.5 (20 mM phosphate buffer) at an 18 mM concentration. The second used a newly developed acidic CD derivative, the tetrakis[6-*O*-(4-sulfobutyl)]-β-cyclodextrin sodium salt. The benefits of this new reagent for the chiral separation are a wide range of basic pH available for the enantiomeric resolution. It is also useful for other cases, *e.g.* preventing adsorption without additives.

1 Introduction

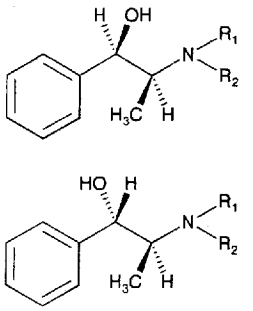
The analysis of different enantiomeric forms of chiral molecules is an area of increasing importance. Although optical resolution by CE is a relatively new technique, it has made much progress in recent years. The chiral separation in CE is mostly obtained by adding chiral selectors, *e.g.* chiral metal complexes [1], cyclodextrins (CD) [2], and chiral surfactants [3], to the running buffer. Thereby the resolution of enantiomers occurs due to either a difference in the formation constant of the host-guest complex or a difference in the distribution coefficient. The former is the case through the use of cyclodextrin, which was introduced by Fanali [2]. Since then a majority of separations have been obtained with neutral CD derivatives, mostly at acidic pH and in coated capillaries. Under these conditions the electroosmotic flow is suppressed, which yields higher resolution. The use of charged CD derivatives is reported by Terabe *et al.* [4]. The CD was derivatized with ethylene diamine and was used for the separation of dansylated amino acids. An acidic CD, carboxymethyl-β-cyclodextrin, is used by Schmitt and Engelhardt [5] for the separation of Dimetiden, a pharmaceutical with a basic amino group. This CD was first used in CE in 1985 [6]; however, the acidic CD derivative was not used as a chiral selector but as a moving "stationary" phase, like a surfactant, for the separation of neutral aromatic isomers.

This work presents the chiral separation in uncoated capillaries with the help of a neutral β-CD or a new acidic β-CD derivative. Four pairs of ephedrine alkaloids (Fig. 1) are chosen as examples. These alkaloids were well investigated beforehand [2, 7]. However, coated capillaries were used for the chiral separation and no combination of chiral and diastereomeric separation is reached. The ephedrine alkaloids have different pharmacological effects, *e.g.* ephedrine is used for broncho dilatation, norephedrine as an antiadiposita. Therefore, in addition to their chiral separation a distinction is desirable between these alkaloids during quality control.

2 Materials and methods

2.1 Instrumentation and reagents

A Beckman P/ACE 2000, equipped with a UV detector operated at 214 nm, was used. The electrophoretic experiments were performed in an uncoated fused-silica capillary with 300 mm effective length to the detector (370 mm total length) and 50 μm ID (Polymicro, Phoenix, A2, USA) with 15 kV as applied voltage at a constant temperature of 30°C. The injection time was 4 s with 34.5 hPa (= 0.5 psi). These standard conditions were used if not otherwise mentioned. (1*S*,2*R*)(+)-ephedrine*HCl(+E), (1*S*,2*S*)(+)-pseudoephedrine (+PE), (1*R*,2*S*)(−)-norephedrine (−NE) were obtained from Fluka (Buchs, Switzer-



R_1	R_2	substance
H	H	-NE
H	CH_3	-E
CH_3	CH_3	-ME
H	CH_3	-PE
CH_3	CH_3	-MPE

Figure 1. Structure of (−)-isomer of the compounds, E, ephedrine, NE, norephedrine, ME, methylephedrine, PE, pseudoephedrine, MPE, methylpseudoephedrine.

Correspondence: C. Dette, Institute of Pharmacy and Food Chemistry, Am Hubland, D–97074 Würzburg, Germany
Professor S. Terabe, Himeji Institute of Technology, Kamigori, Hyogo 678–12, Japan

Abbreviations: **CD,** cyclodextrin; **β-CD-SBE-4,** tetrakis [6-*O*-(4-sulfobutyl)]-β-cyclodextrin sodium salt; **DM-β-CD,** heptakis(2,6-di-*O*-methyl)-β-cyclodextrin; **E,** ephedrine; **HPC,** hydroxypropylcellulose; **ME,** methylephedrine; **MPE,** methylpseudoephedrine; **NE,** norephedrine; **PE,** pseudoephedrine; **TBAB,** tetrabutylammonium bromide

* Dedicated to Prof. Dr. H. J. Roth (Tübingen, Germany) on the occasion of his 65th birthday

0173-0835/94/0606-0799 $5.00+.25/0

land). (1*S*,2*S*)(+)-methylpseudoephedrine (+MPE), (1*R*,2*R*)(−)-methylpseudoephedrine (−MPE), (1*S*,2*R*)(+)-norephedrine (+NE), (1*S*,2*R*)(+)-methylephedrine (+ME), (1*R*,2*S*)(−)-methylephedrine (−ME) were obtained from Aldrich, (Steinheim, Germany). (1*R*,2*S*)(−)-ephedrine (−E) were obtained from Merck (Darmstadt, Germany); heptakis (2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD) was obtained from Nascalai Tesque (Kyoto, Japan); tetrakis[6-*O*-(4-sulfobutyl)]-β-cyclodextrin sodium salt (β-CD-SBE-4) was delivered by the Center of Drug Delivery Research, Higuchi Bioscience Center (The University of Kansas, Lawrence, KS, USA). All other reagents and solvents of analytical grade were deliverd by Wako (Osaka, Japan) or Nasclai.

2.2 Procedure

Every new capillary was flushed with 0.1 M NaOH for 30 min, then 10 min with a running buffer, followed by a 20 min separation with 15 kV as an equilibration step. After changing the buffer a similar procedure was carried out with a 10 min rinse with 0.1 M NaOH, a 5 min rinse with the new buffer and a 20 min equilibration with 15 kV.

Between the runs at basic pH the capillary was rinsed 1 min with the buffer. At acidic pH an additional rinse step of 1 min was performed with 0.1 M NaOH before rinsing with buffer. The resolution (R) and the efficiency (N) are calculated using the following equations:

$$R = \frac{1.18(t_2-t_1)}{(w_1+w_2)} \quad (1)$$

$$N = 5.54\left(\frac{t}{w}\right)^2 \quad (2)$$

where t and w denote the migration time at the peak maximum and the peak width at half height, respectively. The indices are the two enantiomers.

3 Results and discussion

3.1 Neutral β-CD derivative

The three main cyclodextrins, the α-, β- and γ-CD, differ in the size of their truncated cavity. These differences occur because of the number of glucose units for α-, β- and γ-CD with 6, 7 and 8 units, respectively. A rough guide for the choice of CD derivative is that enantiomeric separation with α-CD is mainly successful for compounds without an aromatic ring, with β-CD for compounds with one aromatic ring and with γ-CD for compounds with multiple rings. This was also true when the enantiomeric pairs of ephedrine (E), norephedrine (NE), methylephedrine (ME) and methylpseudoephedrine (MPE) were separated. Neither α-CD nor γ-CD, added to a 20 mM phosphate buffer, pH 2.5, gave any chiral separation, but β-CD did. The disadvantage of β-CD is its insufficient solubility. About 16 mM are soluble in water. High concentrations of urea (> 1 M) must be used additionally if more β-CD is necessary. Heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD) is a widespread β-CD derivative, which has already been used in several separations. Because of the derivatization not only the solubility is much better (> 400 mM) but also a higher separation factor in enantiomeric resolution can be provided. Responsible for the latter is a deeper cavity and modified selectivity. Improving the condition to 18 mM of DM-β-CD in 20 mM phosphate buffer, pH 2.5, the resolution of ±E, ±NE and ±MPE is 0.88, 1.09 and 2.39, respectively. ±E was not baseline separated. The efficiency was insufficient, with N of approximately 24000. Fanali [2] obtained a higher efficiency for ±E with similar conditions in a coated capillary. The enantiomeric separation clearly deteriorated, changing the pH to pH 3.0. Belder and Schomburg [8] showed that the dynamic coating of the capillary by suitable polymers, *e.g.* cellulose derivatives, improves peak shape and chiral resolution. Therefore hydroxypropyl cellulose (HPC) was added to the buffer in an optimized concentration of 0.1%. This resulted in a slight improvement (N about 33000), but the efficiency remained insufficient.

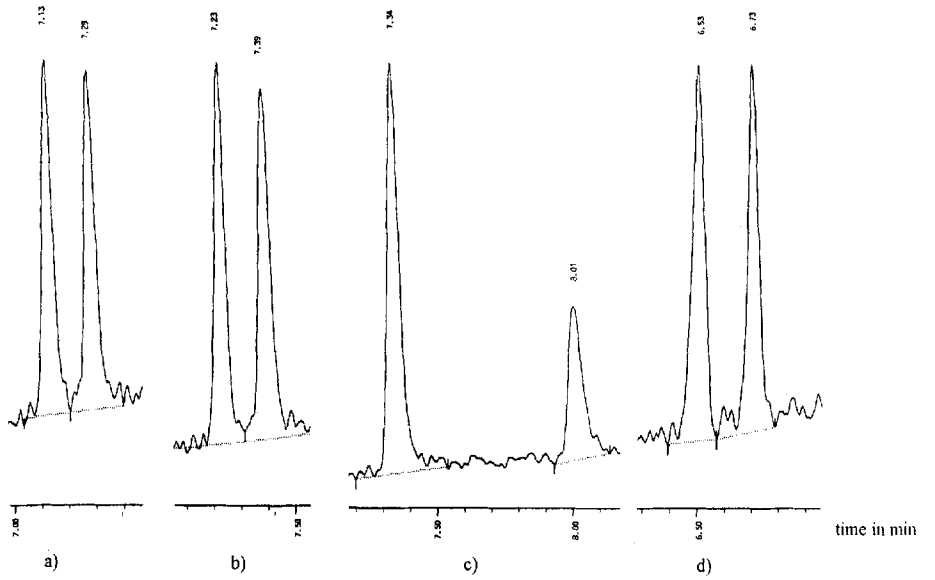


Figure 2. Separation of (a) ±E, (b) ±ME, (c) ±MPE, (d) ±NE. Order of migration: in each case (−)-isomer before (+)-isomer. Capillary: 300 mm effective length to the detector, T = 30°C, V = 15 kV. Buffer: 20 mM phosphate, pH 2.5, plus 18 mM DM-β-CD, 10 mM TBAB and 0.1% HPC.

The point of zero charge for the fused silica is approximately pH 2.0 [9]; therefore the surface still has a slightly negative charge. At pH 2.5 the compounds are protonated and positively charged. For that reason the compounds were strongly adsorped at the capillary wall, which can be recognized by distinct peak tailing and poor efficiency. The adsorption on the capillary wall is an electrostatic attraction. By adding a small amount of cationic substance, competition occurs between the analytes and this substance about the points of attraction. If the concentration of the positive substance is high enough, analytes will not be adsorped anymore. Nishi *et al.* [10] used tetraalkylammonium salts to reduce a similar electrostatic attraction between cationic analytes and anionic micelles. Therefore tetrabutylammonium bromide (TBAB) was added to the buffer at an optimized concentration of 10 mM. The resolution and efficiency improved significantly (N about 130 000). A combination of the optimized additives gave a further improvement (N about 145 000). The enantiomeric separation of ±E, ±NE, ±MPE and ±ME is brilliant for each pair, with resolutions of 1.87, 2.17, 6.53 and 1.84, respectively (Fig. 2). In each case the order of migration is (−)-isomer before (+)-isomer. Unfortunately a mixture of all nine substances, +PE was also added, was not separable under these conditions. ±E, −MPE, ±ME and +PE had nearly the same electrophoretic mobility. They migrated within one broad peak.

3.2 Anionic β-CD derivative

CDs, which are modified with negative groups, provide two features. They are still chiral selectors and have their own electrophoretic mobility (μ_{ep}) opposite (positive) to the electroosmotic flow. The latter could mean a better resolution (see Eq. 3) [11], when analytes with an opposite charge to the CD are used. In this case the average electrophoretic mobility ($\bar{\mu}_{ep}$) will become more negative; the denominator therefore will become smaller, whereas the difference in electrophoretic mobilities ($\Delta\mu_{ep}$) will remain almost the same in comparison with the use of neutral CDs.

$$R = \frac{1}{4}\left(\frac{V}{2D}\right)^{\frac{1}{2}}\left(\frac{l}{L}\right)^{\frac{1}{2}}\frac{\Delta\mu_{ep}}{(\mu_{eo}+\bar{\mu}_{ep})^{\frac{1}{2}}} \qquad (3)$$

V, D, l, L are the applied voltage, the diffusion coefficient, the effective length and the total length of the capillary, respectively. As a result, the third term will increase. If the absolute values of μ_{eo} and $\bar{\mu}_{ep}$ are nearly equal the denominator will be minimized. The complex of the analyte with the negative CD derivative will have a negative μ_{ep} and therefore also a negative $\bar{\mu}_{ep}$. For that reason a basic pH is necessary for good resolution.

β-CD-SBE-4 is a new β-CD derivative with a molecular weight of M_r 1683.3. Four butyl chains, each with a sulfo group at one end, are connected by ether bridges to four of the seven OH groups at the primary hydroxyl rim (Fig. 3). β-CD-SBE-4 has much better solubility in water than β-CD and higher concentrations than 40 mM are feasible. First β-CD-SBE-4 was added at a 20 mM to a 20 mM phosphate buffer, pH 2.5. As predicted above, no separation was achieved. Then 20 mM β-CD-SBE-4 was added to 20 mM borate buffers of different pH (pH 9.25, 9.5, 9.75, 10.0). None of the buffers gave a separation for ±NE, but ±E, ±MPE and ±ME were separated. Thereby the calculated resolution remained the same for ±E and ±ME and slightly decreased for ±MPE toward higher pH. Nevertheless, the efficiency improved toward higher pH. The pK_a value of the analytes are around 9.5. For this reason the analytes change their charge over the used range of pH from positive to more or less neutral. A weaker electrostatic attraction to the capillary wall results. The decrease in resolution can be compensated by a higher concentration of β-CD-SBE-4 (Fig. 4). Also ±NE was nearly separated by using 40 mM of β-CD-SBE-4. The β-CD-SBE-4 provides washing effects, because it has the same charge as the capillary wall and a structure like detergents with a hydrophobic cavity and a polar and acidic sulfo group. Additionally, more β-CD-SBE-4 is available to interact with the analytes. Both effects further minimize the remaining electrostatic attraction. Therefore N increases significantly in addition to the better resolution.

Concentrations of β-CD-SBE-4 higher than 40 mM will be an object of further investigation. Following the data of Fig. 4 a better resolution should be possible with an increase of the β-CD-SBE-4 concentration. This assumption can be supported by the following. Wren [12] pointed out that the addition of organic solvents to a CD concentration higher than the optimum can have a

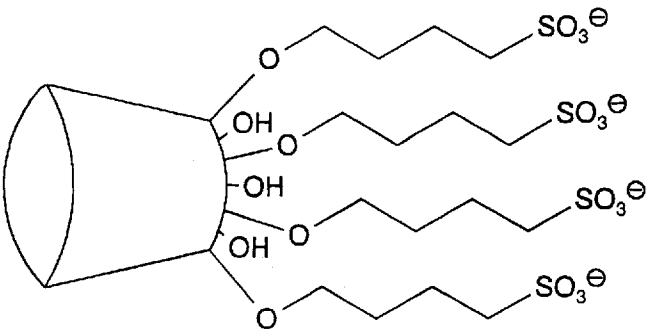


Figure 3. Structure of β-CD-SBE-4.

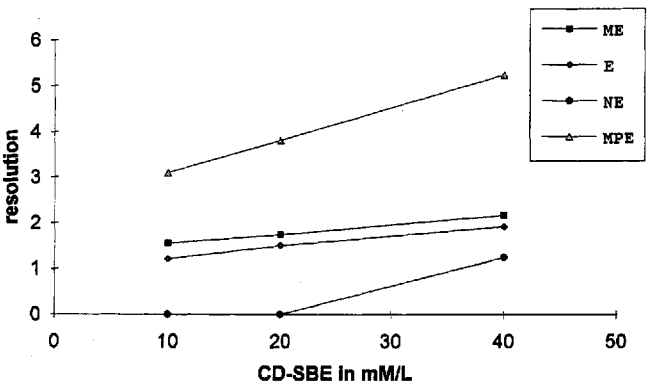


Figure 4. Dependence of the concentration of β-CD-SBE-4 on the resolution of the pairs of enantiomers. Buffer: 20 mM borate, pH 10.0.

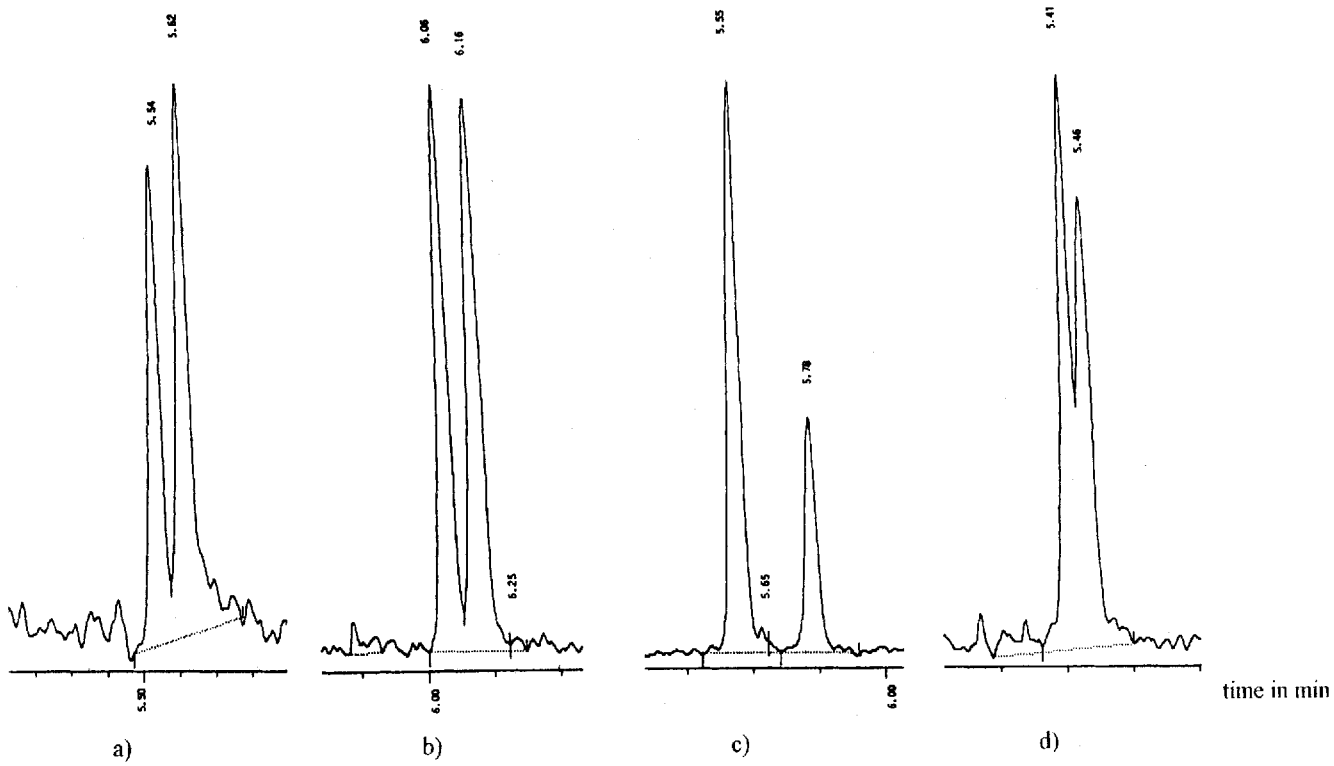


Figure 5. Separation of (a) ±E, (b) ±ME, (c) ±MPE, (d) ±NE. Order of migration for ±E and ±NE is (–)-isomer before (+)-isomer, for ±MPE and ±ME (+)-isomer before (–)-isomer. Buffer: 20 mM borate, pH 10.0, containing 40 mM β-CD-SBE-4; other conditions as in Fig. 2.

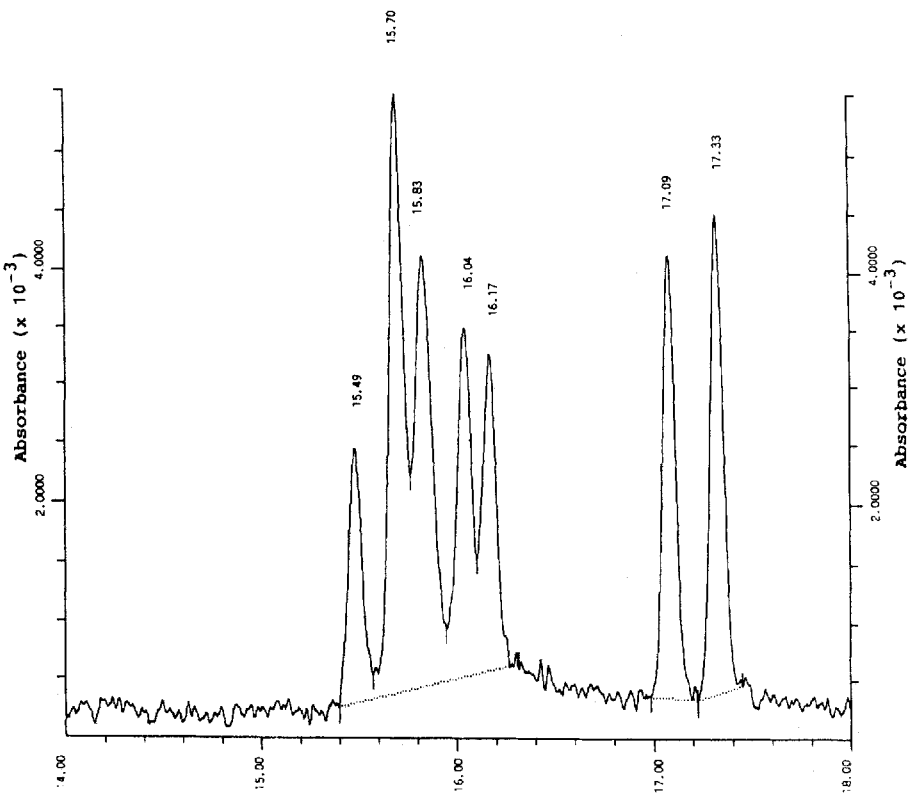


Figure 6. Separation of a mixture of the four pairs of enantiomers and +PE. Order of migration: +PE (t_M = 15.49 min), –NE/+MPE (15.70), +NE/–E (15.83), +E (16.04), –MPE (16.17), +ME (17.09), –ME (17.33). Capillary: 500 mm effective length to the detector, 50 μm ID, T = 26°C; other conditions as in Fig. 5; *x*-axis: time in min.

positive effect on the apparent mobility difference and hence the resolution. It will have a negative effect when added to a CD concentration lower than the optimum. The addition of 10% MeOH to the 40 mM β-CD-SBE-4 led to a loss in resolution. Hence the optimum of the β-CD-SBE-4 concentration may not yet be reached. Figure 5 illustrates the enantiomeric separation of the four pairs of analytes with 40 mM β-CD-SBE-4 in a 20 mM borate buffer, pH 10.0. Thereby the resolution for ±E, ±NE, ±MPE and ±ME is 1.13, 0.71, 3.08 and 1.27, respectively. The migration order for ±E and ±NE is (−)-isomer before (+)-isomer, whereas for ±MPE and ±ME it is (+)-isomer before (−)-isomer.

The conditions were optimized for a capillary length of 500 mm effective length and a temperature of 26°C, keeping 15 kV as separation voltage. Under these conditions a mixture of the four enantiomeric pairs of ±E, ±NE, ±MPE and ±ME and +PE can be separated with N about 200 000 (Fig. 6). Seven of the nine substances are separated. −E and +NE and also −NE and +MPE migrate in one peak in each case.

4 Concluding remarks

Chiral separations with CD derivatives are also possible in uncoated capillaries. Inclusion of additives like TBAB in combination with HPC can minimize tailing effects at acidic pH when using neutral CD. Anionic CD derivatives like β-CD-SBE-4 should be used at basic pH where they provide features such as their own electrophoretic mobility, a better resolution and capillary cleaning effects. Comparing the two CD derivatives, β-CD-SBE-4 provides a much broader pH range to obtain a sufficient enantiomeric resolution. Therefore it is possible to choose a pH where not only the enantiomeric separation is adequate but also the separation of similar substances is possible.

C. Dette was the recipient of a short-term scholarship of the German Academic Exchange Service (DAAD 574311567/3). He would like to thank the Himeji Institute of Technology for the permission to stay 3 months as a research assistant at the Faculty of Science. Also, the authors are grateful to Professor J.F. Stobaugh and the Center of Drug Delivery Research, The University of Kansas, for the generous gift of β-CD-SBE-4.

Received December 9, 1993

5 References

[1] Gassman, E., Kuo, J. E., Zare, R. N., *Science*, 1985, *230*, 813–814.
[2] Fanali, S., *J. Chromatogr.* 1989, *474*, 441–446.
[3] Terabe, S., Shibata, M., Miyashita, Y., *J. Chromatogr.* 1989, *480*, 403–412.
[4] Terabe, S., *Trends Anal. Chem.* 1989, *8*, 129–134.
[5] Schmitt, T., Engelhardt, H., *J. High Resolut. Chromatogr.* 1993, *16*, 525–529.
[6] Terabe, S., Ozaki, H., Otsuka, K. and Ando, T., *J. Chromatogr.* 1985, *332*, 211–217.
[7] Liu, Y.-M., Sheu, S.-J., *J. Chromatogr.* 1992, *600*, 370–372.
[8] Belder, D., Schomburg, G., *J. High Resolut. Chromatogr.* 1992, *15*, 686–693.
[9] Bolt, G. H., *J. Chem. Phys.* 1957, *61*, 1166–1169.
[10] Nishi, H., Tsumagari, N., Terabe, S., *Anal. Chem.* 1989, *61*, 2434–2439.
[11] Terabe, S., Yashima, T., Tanaka, N., Araki, M., *Anal. Chem.* 1988, *60*, 1673–1677.
[12] Wren, S. A. C., Rowe, R. C., *J. Chromatogr.* 1992, *609*, 363–367.