ELSEVIER

Journal of Chromatography B, 687 (1996) 303–336

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

Cyclodextrins and enantiomeric separations of drugs by liquid chromatography and capillary electrophoresis: basic principles and new developments

Françoise Bressolle[a,*], Michel Audran[b], Tuyet-Nga Pham[b], Jean-Jacques Vallon[c]

[a]*Laboratoire de Pharmacocinétique, Faculté de Pharmacie, Université de Montpellier I, Montpellier, France*
[b]*Laboratoire de Biophysique, Faculté de Pharmacie, Université de Montpellier I, Montpellier, France*
[c]*Laboratoire de Biochimie, Pharmaco-Toxicologie et Analyse des Traces, Hôpital Edouard Herriot et Laboratoire de Chimie Analytique III, Faculté de Pharmacie, 69437 Lyon Cedex 03, France*

Received 9 December 1995; revised 29 May 1996; accepted 5 June 1996

Abstract

Investigation of individual drug enantiomers is required in pharmacokinetic and pharmacodynamic studies of drugs with a chiral centre. Cyclodextrins (CDs) are extensively used in high-performance liquid chromatography as stationary phases bonded to a solid support or as mobile phase additives in HPLC and capillary electrophoresis (CE) for the separation of chiral compounds. We describe here the basis for the liquid chromatographic and capillary electrophoretic resolution of drug enantiomers and the factors affecting their enantiomeric separation. This review covers the use of CDs and some of their derivatives in studies of compounds of pharmacological interest.

Keywords: Reviews; Enantiomer separation; Cyclodextrins; Drugs

Contents

*Corresponding author.

0378-4347/96/$15.00
PII S0378-4347(96)00263-0

1. Introduction

Current regulatory guidelines stress the importance of investigating drug enantiomers in pharmacokinetic and pharmacodynamic studies of drugs with a chiral centre [1–3]. However, in order to study these properties, the initial step involves the development of a suitable method for their separation. The separation of enantiomers can be achieved in different ways: (i) derivatization of enantiomers with a chiral reagent prior to the chromatographic analysis, (ii) addition of the chiral reagent to the chromatographic mobile phase to form diastereomeric adducts, ion pairs, metal complexes, etc., with the enantiomers, (iii) the use of chiral selectors: either natural chiral macromolecules (proteins, carbohydrates or their derivatives), cyclodextrins (CDs) or small chiral molecules covalently or ionically bonded to the stationary phase. One widely used group of chiral selectors is CDs. Indeed, CDs are extensively used in high-performance liquid chromatography (HPLC) as stationary phases bonded to a solid support [chiral stationary phase, CSP], or as mobile phase additives in HPLC and capillary electrophoresis (CE). CE in its different forms is a powerful analytical technique that has become a realistic alternative to the liquid chromatographic methods. CDs impart enantioselectivity to the chromatographic system by formation of transient diastereomeric complexes with the analyte enantiomers [4–6].

In the present study, factors involved in liquid chromatographic and capillary electrophoretic resolution of enantiomers are reviewed. Examples of the actual use of CDs and some of their derivatives in analytical chemistry of drugs is presented.

2. CDs and principle of chiral recognition

2.1. Structure of CDs

2.1.1. Native CDs

CDs are cyclic, non-reducing oligosaccharides consisting of D-glucopyranose units bonded through α-1,4-linkages. Dextrins, the products of partial hydrolysis of starch, can give cyclic dextrins under

the action of CD-glycosyl-transferases. The smallest is the α-CD (six glucose units), followed by β-CD (seven units), and γ-CD (eight units). All of them are crystalline, non hygroscopic substances, produced industrially [7]. In CDs, the sugars adopt a $^4C_1$ chair conformation and orient themselves so that the molecule forms a toroidal/hollow truncated cone structure. A variety of water-soluble and insoluble compounds can fit into this cavity to form inclusion complexes [8,9]. Physical and chemical characteristics of native CDs are given in Table 1 [7,10]. However, Claire Myles et al. [11] in a computer graphics study of β-CD crystal structures gave slightly different values for the native β-CD cavity diameter, 0.734 nm (0.728 nm for inclusion complexes of β-CD). The chemical structure and the approximate geometric dimensions of the conical cylinders are illustrated in Fig. 1. β-CD is the most easily available of the CDs; however, it shows an anomalously low solubility in aqueous–organic solvents (18.5 g/l at 25°C) [2], which is a serious limitation [7,12]. The use of urea in the eluent enhances solubility [13] but leads to problems with baseline stability and high viscosity [2,14,15]. An alternative way to improve the solubility of CDs is through derivatization of the hydroxyl groups.

For different reasons (price, availability, cavity dimensions in which most of common solute molecules can fit closely, etc.), β-CD represents 95% of all produced and consumed CDs.

2.1.2. Modified CDs

In the CDs every glucopyranose unit has three free OH groups, two of which (on C_2 and C_3) are

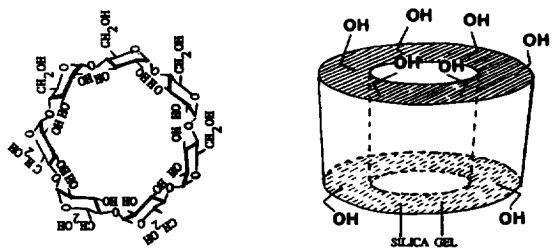


Fig. 1. Structure of β-CD.

secondary and one (on C_6) primary [7]. In β-CD, 21 hydroxyl groups can be modified by substituting the hydrogen atom or the hydroxyl group by a wide variety of groups like: alkyl-, hydroxyalkyl-, amino-, thio-, glucosyl-, maltosyl-, methyl-, hydroxyethyl-, hydroxypropyl-, acetyl-, etc. The aim of such derivatizations may be: (i) to improve the solubility of the CD derivative; (ii) to improve the fit, and/or the association between the CD and its guest, with concomitant stabilization of the guest, thus reducing its reactivity, mobility; (iii) to attach specific (catalytic) groups to the binding site (i.e., in enzyme modeling); or (iv) to form insoluble, immobilized CD-containing structures, polymers, e.g., for chromatographic purposes [7].

From the thousands of CD derivatives described, those actually produced, standardized and available are highly water-soluble methylated and 2-hydroxypropylated β-CDs (Table 1). Among them, the *heptakis* (2,6-di-*O*-methyl β-CD (DM) and 2-hydroxypropylated β-CD (HPβ-CD)) are crystalline products soluble in cold water but insoluble in hot water. Acetylated, naphtylethylcarbamoylated, 3,5-dimethylphenycarbamoylated and paratoluyl ester β-

Table 1
Physical and chemical characteristics of native and some derivatized cyclodextrins (CDs)

	α-CD	β-CD	γ-CD	Methylated β-CD		2-Hydroxypropylated β-CD	
				RAMEB	DM	HPBCD-2-7	HPBCD-4-6
Molecular mass	972	1135	1297	1317	1331	1292	1384
Number of glucose units	6	7	8	7	7	7	7
Cavity volume (nm^3)	1.74	2.62	4.27	–	–	–	–
External diameter (nm)	1.37	1.53	1.69	–	–	–	1.53
Cavity diameter (nm)	0.57	0.78	0.95	–	–	–	0.78
Depth (nm)	0.79	0.79	0.79		1.0-1.1		
Solubility in water, g% (w/v at 25°C)	14.5	1.85	23.2	>100	>50	>33	>33
Number of subtituents/CD unit	–	–	–	~13	14	~2.7	~4.6

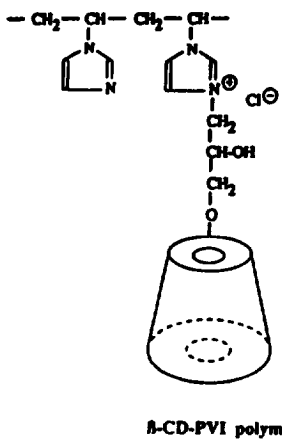
RAMEB: randomly methylated β-CD; DM: *heptakis* (2,6)-di-*O*-methyl β-CD. Data from Szejtli [7].

CD are available in silica bonded forms; permethylated bonded α, β or γ-CD, are also available from manufacturers (Table 2 and Fig. 2). All of these bonded CDs are derivatized at their OH groups outside of the cavity (Fig. 1) [7].

Claire Myles et al. [11] gave cavity diameters of 0.75 nm for monosubstituted β-CD, of 0.74 nm for disubstituted and of 0.72 nm for trisubstituted β-CD. The authors indicate that the basic conformation of the molecule remains approximately the same for natural, mono-substituted and partially permethylated β-CDs with major changes observed only in the case of full permethylation. As a consequence, no significant perturbations caused by inclusion of the guest molecule can be observed.

Table 2
Most common substitutents used in derivatized CD

Substitutents	
$-CH_3$	Methylated β-CD
$-C(=O)-CH_3$	Acetylated α-, β- and γ-CD
$-CH_2-COOH$	Carboxymethylated β-CD
$-CH_2-CH(OH)-CH_3$	Hydroxypropylated β-CD (S or RS)
$-CO-NH-CH(CH_3)$ + naphthyl	Naphthylethyl carbamate β-CD (R or S)
$-CO-NH-$ + 3,5-dimethylphenyl	3,5-Dimethylphenyl carbamate β-CD
$-CO-$ + $-CH_3$	*p*-Toluoyl β-CD
$-NH-CH(CH_3)$ + phenyl	α-Methylbenzylamine modified β-CD (S)
$-NH-CH(CH_3)$ + naphthyl	Naphthylethylamine modified β-CD
$-NH(CH_2)_2-NH-$ + pyridyl	Pyridylethylenediamine β-CD
$-NH(CH_2)_2-NH-$ + nitropyridyl	Nitropyridylethylene diamine β-CD
$-CO-NH-$ + phenyl	Phenyl carbamate β-CD
$-CH_2-CHOH-CH_2-N^+(CH_3)\,Cl^-$	Cationic β-CD



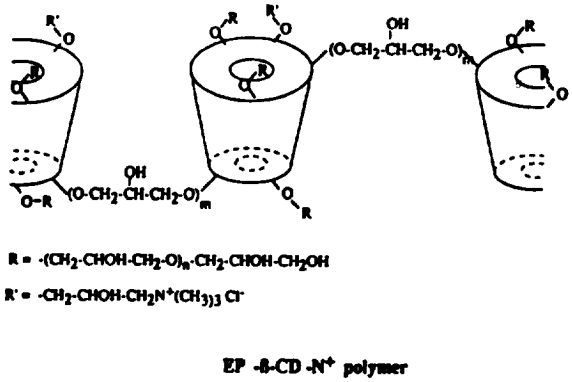


Fig. 2. Structure of β-CD polymers. Data from Thuaud et al. [30].

2.2. Chiral recognition by CDs

The β-CD molecule, containing 35 stereogenic centres is the best sized complex former. Guest solutes can interact via Van der Waals forces with its hydrophobic cavity. In most cases the binding in the cavity is too symmetrical to induce large enantioselectivity and other points are necessary to achieve chiral recognition. β-CD has 14 secondary OH groups (on carbons 2 and 3 of the glucose units) around the opening of the cavity, seven clockwise and seven counter-clockwise; and at the opposite rim it has seven primary hydroxyl groups (on carbon 6 of the glucose units) [16]. Consequently, hydroxyl groups can give hydrogen bonding with the guest enantiomer. The interior of the cavity (Fig. 1)

contains two rings of C–H groups with a ring of glycosidic oxygens in between. As a result, the cavity is relatively hydrophobic while the external faces are hydrophilic [17].

Complexation will occur if: (i) there is steric compatibility between the CD cavity and the guest molecule (but only a part of the guest molecule can also be included), (ii) the affinity of the guest molecule for the CD cavity is higher than for the other components present (i.e., solvent). Host–guest hydrophobic interactions, Van der Waals forces and hydrogen bonding occur independently or in combination to explain encapsulation by CDs. The stoichiometry of inclusion compounds is usually 1:1 (host–guest polar ratio). However, complexes can be made of two or more guests (especially with the large γ-CD cavity) or of several CD molecules by inclusion of different parts of a large guest. Moreover, the size of the CD is a factor in chiral recognition. Indeed, the use of different size CDs (α, β and γ) resulted in very different enantioselectivities [18].

2.3. CDs and chiral separation

Fig. 3 illustrates schematically the inclusion of a simple phenyl group with *ortho*, *meta*, and *para* substituents. The separation of enantiomers is based both on the formation of transient diastereomers with the chiral centre of CDs, and on the difference between their stability constants [19,20]. According to Wainer and Drayer [21], there are few basic structural rules that a compound should meet in order for the enantiomers to be separated on CDs. They are the following: (i) the compound should not be so large that it cannot at least partially enter the CD cavity; (ii) the chiral centre should be near the opening of the CD cavity; (iii) the compound should have an aromatic system α or β to the chiral centre, but occasionally the ring can be γ to the chiral centre; and finally (iv) there could be the potential for hydrogen bonding at or near the centre of chirality.

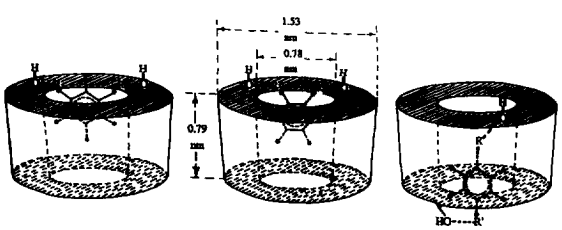


Fig. 3. Schemes of inclusion in β-CD of a simple phenyl group with: (a) *ortho*, (b) *meta* and (c) *para* substituents.

Amstrong et al. [22], comparing β-CD complexes of propranolol and warfarin, have improved the understanding and application of the chiral interactions with β-CD.

2.4. CDs and structural features of solutes

Furuta and Nakazawa [23] studied the mechanism of the chiral recognition of uniconazole by β- and γ-CD columns. Substitutions on the benzene ring, most probably included in the CD cavity, did not modify the enantioselectivity but only the chiral recognition on both β- and γ-CD columns. Larger substituent groups (i.e., terbutyl) fit better into the γ-CD cavity, and the smaller methyl and isopropyl groups allow chiral separation on β-CD but are too small for a good fit with the γ-CD cavity. A three-point interaction is necessary for the chiral recognition: hydrophobic interaction in the CD cavity, and two hydrogen bondings with the hydroxyl groups at the opening of the CD.

Carmilleri et al. [24] studied 17 chiral compounds derived from the amino alkyl phosphonic acid structure for their retention and resolution on an acetylated β-CD bonded phase. Retention and resolution appeared to increase with an increase in the distance between the chiral carbon and the amino group. Substituents on the benzene ring only influenced the retention time by varying the hydrophobicity of the aromatic ring.

Casy et al. [25] investigated by HPLC and ^{1}H NMR the chiral recognition of 17 thromboxane antagonists by β-CD bonded phases (Cyclobond I) and β-CD as mobile phase additive. Evidence of formation of 1:1 inclusion complex was given, as well as the proof that both aromatic moieties (phenol and pyridyl) can penetrate β-CD. For the native CD bonded phase, usually a minimum of two hydrogen bonding groups are necessary for enantiomeric separation. In the case of derivatized CD stationary phases, multi-ring π systems can substitute one hydrogen bonding group [25].

3. Use of CDs in HPLC

3.1. Use of CD stationary phases

3.1.1. Synthesis of CD stationary phases

CSPs are categorized under several different types of chromatography including brush type, also called Pirkle CSP, inclusion and affinity. CDs represent a major portion of the inclusion-type of columns. Columns with native or derivatized CD stationary phases have been developed [26–29]. Recently, β-CD polymers immobilized on porous silica have been synthesized (Fig. 2, Refs. [30–32]).

Two approaches have been used to attach CD to the silica. The first relies on the covalent bonding of CDs (α-, β- and γ- or derivatized CDs) to silica beads via several spacer arms (Refs. [28,33–38], Fig. 1). This method can be generally classified into three main types: (i) a spacer arm is grafted onto silica gel and the CD is reacted with the terminal group of the spacer arm, (ii) the reactive group of the spacer arm coupled to a CD molecule is reacted with silanol groups on the surface of silica gel, (iii) part of the spacer arm is coupled to silica gel and another part to the CD and immobilization consists in reaction of these two parts. These supports exhibit excellent properties. A method allowing direct analysis of serum based on the use of a β-CD bonded phase which incorporates diol as well as CD moieties has been described [39]. Recently, a silica gel with chemically bonded peralkylated β-CDs, using the second approach for immobilization has been reported [38]. The second type of packing consists of adsorption on silica of β-CD polymers prepared either by condensation of β-CD with bifunctional reagent (epichlorohydrin) (EP-β-CD-N^+ polymer) [30,40], or by grafting a monosubstituted β-CD derivative onto a linear polymer (polyvinylimidazole) (PV1-β-CD) [30,31].

The use of CD bonded phase columns can be performed in three different modes: reversed-phase, normal-phase, and polar-organic modes [9,18,41].

3.1.2. Chiral separations on native CDs in reversed-phase

The reversed-phase mode employs hydro-organic mobile phases (aqueous buffers with a few percent of organic modifier such as methanol, acetonitrile, ethanol, DMSO or DMF). The chiral recognition mechanism is the result of inclusion complex formation between the hydrophobic moiety of the analyte and the relatively non-polar interior of the CD cavity [42–44]. The organic modifier competes with the solute for the CD cavity. The amount of organic modifier (present in the cavity and displaced by the analyte) controls the retention of the solutes [30]. Therefore, the greater the percentage of modifier the more easily a solute is displaced from the CD resulting in shorter retention times. As the weakest organic modifier, methanol is recommended, followed by acetonitrile and ethanol. Because acetonitrile and ethanol have a much greater affinity for the CD cavity than methanol, one needs less of these modifiers to obtain comparable retention times [45]. The two main problems are that the interior cavity is not quite non-polar because of glycosidic oxygens, and that both ends of the cavity are open to solvent. Therefore only those guest molecules which have large hydrophobic groups and appropriate shape can form strong inclusion complexes with it. To overcome these problems, the binding forces were increased by modifying the CDs through derivatization of the hydroxy groups [17,46,47]. Enantioselectivity with the derivatized CD bonded phase is thought to involve not only inclusion complex formation but also additional interactions between the analyte and CD substituents.

Florance and Konteatis [48] studied on β- and γ-CD columns the relevance of the distance of the chiral centre from the phenyl ring for chiral resolution of a series of cyclic and linear dipeptides containing aromatic amino acids. These authors concluded that the proximity of the chiral centre to the 2′- and 3′-hydroxyls at the opening of the CD cavity is important for resolution of enantiomers, and that the presence of an aromatic group is beneficial but not essential for the formation of an inclusion complex.

Macaudiere et al. [49] studied the influence of pH, nature and content of the organic modifier, and phosphate buffer concentration in the chiral resolution of 3-thienylcyclohexyl glycolic acid (TCGA) on a β-CD bonded phase. The selectivity is improved by the use of methanol instead of acetonitrile; retention is governed by (i) methanol content, (ii) the pH of the mobile phase and (iii) buffer concen-

tration. Both the acidic and the alcohol groups of TCGA play a major role in selectivity. A total loss of selectivity is observed for the methyl ester of TCGA and low values are obtained for the thienylcyclohexylacetic acid and for the diol solute. The cyclohexyl group appears to be preferentially included; a smaller group (cyclopentenyl) gives lower selectivity and retention. The addition of a methylene group between the chiral centre and the cyclohexyl moiety, induces a free rotation of the solute that results in a loss of selectivity. Chiral recognition involves: (i) interaction with the secondary hydroxyl groups of the CD cavity, followed by (ii) inclusion complex formation. The authors concluded that the influence of secondary hydroxyl groups of the CD rim was clearly established, in addition to the influence of solute functional moieties on selectivity.

3.1.3. Chiral separations on native CDs in normal-phase

In the normal-phase mode (i.e, using nonpolar solvents such as hexane, toluene, ethanol, 2-propanol, etc.) the least polar component of the mobile phases occupies the CD cavity and cannot be easily displaced by most solutes. Solute retention, therefore, is not due to inclusion complex formation but rather to adsorption of the solute at the outside of the CD [42,50]. In these systems, high enantioselectivity towards many classes of chiral compounds was observed. The eluents consist mainly of neat acetonitrile with small amounts of triethylamine, glacial acetic acid and methanol as modifiers. The chiral recognition arises from stereoselective hydrogen bonding between donor and acceptor sites of the analyte with the secondary hydroxyl groups at the opening of the CD cavity [44,50]. The strength of the interaction between the CD stationary phase and the solute, and therefore the retention and stereoselectivity, are determined mainly by the structure of the analyte and the competitive interaction of mobile phase components.

The use of non-aqueous eluents in conjunction with CD phases offers several advantages over water-rich systems, including faster equilibration of the column, more stable baseline and more sensitive fluorescence detection due to the lack of quenching effects which occur in aqueous solutions.

3.1.4. Chiral separations on native CDs in polar-organic mode

The mobile phase system is composed of acetonitrile with small portions of hydrogen bonding modifiers (i.e., methanol, acetic acid, and triethylamine). The chiral recognition mechanism for compounds separated in this mode is thought to involve solute hydrogen bonding with the secondary hydroxyl group on the CD through a size dependent interaction in which the solute acts like a cover on the CD. Dipole stacking and steric factors are also relevant. For instance, when the hydrogen on a secondary amine is replaced by a methyl group, enantioselectivity and retention are decreased or lost (e.g., oxazepam and temazepam). This suggests that the acidic hydrogen bonding group (i.e., proton donor) may be important to the chiral recognition mechanism [18]. The retention and enantioselectivity mechanisms are probably due to hydrogen bonding and dipolar interactions. Using several drugs (anticholinergics, β-adrenergic blockers, non-steroidal anti-inflammatories, α-adrenergic agonists, calcium channel blockers, antihyperlipoproteinemics, diuretics, sedative–hypnotic–anxiolytic agents, pesticides and herbicides) Chang et al. [18] studied mobile phase and stationary phase effects.

Mobile phase effects. Since acetonitrile does not compete with solutes for hydrogen bonding interactions with CD hydroxyl groups, retention can be excessive for molecules with multiple hydrogen bonding sites. Therefore, small quantities of methanol (methanol can hydrogen bond both to the CD stationary phase and to the analyte) are added to the mobile phase to reduce retention. However, a large excess of methanol can negate chiral recognition in many cases [18]. Very small amounts of triethylamine together with glacial acetic acid ($\leq 1\%$) are also added to the mobile phase.

Most of the compounds resolved using this type of mobile phase contain amine functionalities, while a few have carboxylic acid or phenolic moieties. Chang et al. [18] reported that the ratio of triethylamine to glacial acetic acid controls the degree to which these ionisable solutes are protonated or deprotonated. The optimization of this ratio is essential for optimizing selectivity [41].

Effect of native CD stationary phases. β-Adrenergic blocking agents have been separated in the polar-organic mode on native β- and γ-CD bonded stationary phases [41], while for the most part they were not separated in the reversed-phase or in the normal-phase modes. Hydrogen bonding could be responsible for enantioselectivity and retention. The use of different sized CDs (α, β and γ) resulted in different enantioselectivities. Thus, the size of the CD is a factor in chiral recognition [18].

3.1.5. Chiral separations on derivatized CD stationary phases

Enantiomeric compounds are not always resolved satisfactorily using native CD columns. Therefore, CD stationary phases were modified with linkers with potent hydrophilic or hydrophobic interactions (i.e., clusters of phenyl groups) to enhance chiral recognition. The phenyl groups are bound to the hydroxyl groups on both the smaller and larger openings of the CD cone; the phenyl groups sticking out of the larger opening can interact with hydrophobic parts of the analytes in the outside of the cone. If this hydrophobic part of the solute is achiral, the hydrophobic interaction may regulate the fitness of the chiral part to the chiral recognizing centre inside the cone.

In the reversed-phase mode, as native CDs cannot resolve amino acid derivatives, the major contribution to the overall enantioselectivity of the derivatized CD stationary phases is thought to be due to additional interaction of the analyte with the CD substituents [51]. On the (*R*)-, (*S*)-, and (*S,R*)-phenethylcarbamate-β-CD the chirality of CD substituents does not contribute to the overall enantiorecognition, but seems to be more important for nonstereospecific π-π interactions. The naphtyl moiety [(*S*)-(−)-1-(1-naphthyl)ethylcarbamate-β-CD] exhibits larger hydrophobic surface for π-π interactions than the phenyl group [52].

Recently, it has been described that the use of peralkylated (methyl or propyl) chemically bonded β-CDs immobilized on silica gel allows a good separation of racemates of some drugs (i.e., hexobarbital) [38].

Thuaud et al. [30] and Thuaud and Sebille [31] studied the chromatographic separation of barbiturate and thiobarbiturate enantiomers by using two types of stationary phases: native β-CD or hydroxypropyl-β-CD residues linked to silica and β-CD containing polymers adsorbed onto silica. The sulfur atom at the C-2 position of the pyrimidine ring enhances the affinity of the solutes for the CD CSPs, and the occurrence of a polar interaction involving this atom leads to a better fitting of thiobarbiturates with the β-CD cavities. The authors concluded that the chiral recognition is mainly dependent on the hydroxypropyl substituents in the β-CD cavity; the enantiorecognition is the highest with a thiocarbonyl instead of a carbonyl group and when substituents at the C-5 position have an adequate size (Fig. 4).

In the normal-phase mode, the CD cavity being occupied by the least polar component of the mobile phase, interactions occur only with the outside groups of the CD by a π-π mechanism (i.e., between phenethyl, naphtylallyl and carbamoylated groups and hydrophobic part of the chiral solutes) [51]. The configuration of the CD groups plays an important role in enantioselectivity [52].

Nakatsu and Stalcup [53] studied a naphtylethylcarbamoyl γ-CD bonded phase to resolve a number of enantiomeric pairs of 3,5-dinitrobenzoyl derivatized amino acids in normal-phase HPLC. Selectivity is increased as the aliphatic carbon chain length increases, while with aromatic amino acids, the inverse is observed. The highest selectivities are obtained when the aromatic rings are closer to the stereogenic centre. With aromatic amino acids, elution orders differ between naphtyl γ and β-CD phases indicating some still unexplained mechanism in chiral recognition.

Hargitai et al. [54] studied, under normal-phase conditions, the enantioselectivity of thirteen chiral stationary phases containing 3,5-dimethylphenylcarbamate-functionalized β-CD, chemically bonded to silica. The degree of substitution of carbamate groups on β-CD and the amount of immobilized carbamoylated β-CD seem to be the most important factors for the enantioselectivity. The highest selectivities were obtained on complete carbamoylated β-CD CSPs.

In the polar-organic mode, beside the native β-CD, the two most useful columns allowing enantiomeric resolution are the acetylated- and the (*R*)-naphthylethylcarbamate-β-CD stationary phases [18]

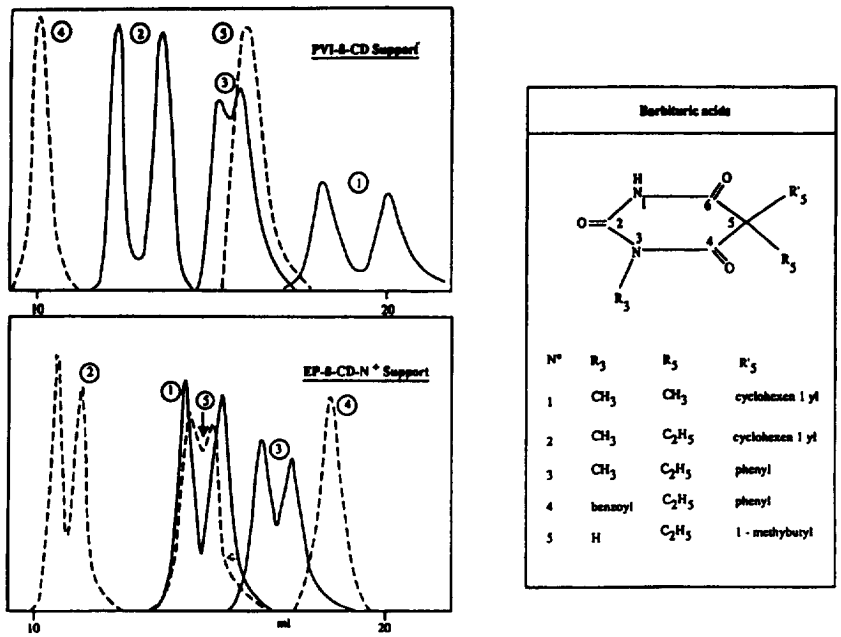


Fig. 4. Separation of barbituric acid enantiomers on a 30 cm column: the compositions of the methanol–0.1 *M* (pH 4) phosphate mobile phases used with PVI-β-CD and EP-β-CD-N^+ were 30:70 and 40:60 (v/v), respectively. Data from Thuaud et al. [30].

offering two different interaction substituents (ester and carbamate).

In the polar-organic mode (99% methanol–1% acetic acid, v/v) enantioselectivity is not dependent on the nature (phenyl or naphtyl) of the substituent, except if the solutes bear a π basic naphtyl moiety (i.e., dansyl derivatives) responsible for repulsive interactions with the naphtyl groups on the CD with consecutive lower chiral recognition. Enantiorecognition is governed mainly by the CD moiety, with reduced selectivities originating in the very low number of hydroxyls available for stereoselective hydrogen bonding [52].

A multimodal behaviour can also be observed on derivatized CD columns. Hilton et al. [52], studying the performances of phenethyl and naphtylethylcarbamate CD bonded phases, concluded that carbamoylation of bonded CD allows two types of chiral interactions (with the CD cavity or the chiral substituent). The resultant selectivity depends on the polarity of the mobile phase. In hydro-organic solvents, the carbamoylated β-CD phases behave similarly to native CD phases; enantioseparation takes place due to interactions with the CD cavity. In the polar-organic mode, π-complex-hydrogen bonding (Pirkle-type) with the external CD substituents are responsible of the enantioseparation. The authors concluded that carbamoyl β-CD columns yield a multimodal behaviour and therefore offer a wide applicability to resolve chiral compounds.

Vandenbosch et al. [55] evaluated the difference in the chiral recognition mechanism of *S*-naphthylethylcarbamate derivative of β-CD in normal-phase and reversed-phase modes. These authors used the dinitrobenzoic acid phenylethyl-amide as model compound. In the reversed-phase mode, the enantiomers of this amide with π-acidic character cannot be separated, while a good separation is obtained in the normal-phase mode. In this mode, the CD cavity is probably filled with the hydrophobic mobile phase, so that inclusion complexes are not favoured, which implies that the naphthyl substituent, which has a π-basic character, may become more important in the retention and the chiral discrimination mechanism.

Several multiple-interaction chiral stationary phase have been developed by Li and Purdy [17]. These stationary phases contain a hydrophobic cavity ca-

pable of inclusion complexation, aromatic groups capable of π-π interaction, polar hydroxyl groups capable of hydrogen-bonding with the polar functional groups of the solute, and bulky non-polar groups providing steric repulsion, Van der Waals interaction, and/or conformational control. The direct separation of enantiomers of a wide variety of chiral compounds are reported in3 Table 3. For most solutes, the retention time on these phases is much longer than the retention time on the β-CD stationary phase.

Table 3
Optical resolution of enantiomers using multiple-interaction chiral stationary phases[a]

Solute	R_s
(a) Methylbenzylamine-modified β-CD	
Dansylamino acids	
Norleucine	1.01
Aspartic acid	0.90
Serine	0.80
Leucine	1.30
Valine	1.10
Norvaline	0.74
Glutamic acid	0.75
Methionine	0.75
Threonine	1.20
Phenylalanine	0.80
DNP-amino acids	
DNP-DL-α-amino-*n*-butyric acid	0.70
DNP-DL-norvaline	1.20
DNP-DL-norleucine	1.83
DNP-DL-α-amino-*n*-caprylic acid	1.62
DNP-DL-methionine sulfone	0.90
DNP-DL-methionine	2.57
DNP-DL-ethionine	2.37
DNP-DL-citrulline	0.80
DNP-DL-glutamic acid	0.70
Other chiral compounds	
3-Indolelactylaspartic acid	1.36
Phenylalanine	1.50
Carbobenzyloxyalanine	0.75
N-Phthaloylamine	2.80
Indoline-2-carboxylic acid	3.0
3-Indolelactic acid	1.36
4-Methoxymandelic acid	1.01
Mandelic acid	3.33
2-Phenylpropanediol	1.01
2-Phenylpropionic acid	1.26
Suprofen	0.90
(b) Naphthylethylamine-modified β-CD	
Trimeperazine	1.21
Promethazine	3.67
Ethopropazine	4.00

R_s: Resolution factor.
[a] Data from Li and Purdy [17].

3.1.6. *Pre-column achiral derivatization before CD-bonded HPLC*

Although CDs or their derivatives covalently bonded to silica are extremely effective selectors for a wide variety of enantiomers even without derivatization, in many instances it is necessary to derivatize the chiral compounds, especially if they do not contain an aromatic group [20,26,45].

Derivatization of chiral primary and secondary amines with suitable achiral anhydrides results in highly absorbent or fluorescent compounds suitable for HPLC enantiomeric separation. The high selectivity and efficiency of the system coupled with the aromatic chromophores (i.e., 1,8-naphthalic anhydride) enabled enantiomeric purity determinations of functionalized amines at trace levels. The retention parameters, the elution order, and the selectivity obtained on native β-CD bonded phase depend very strongly on the derivatizing agent used (Fig. 5) [50]. Since the derivatization reaction provides not only an aromatic moiety for easy photometric detection but also a free carboxylic group capable of hydrogen bond formation with β-CD stationary phase, both the

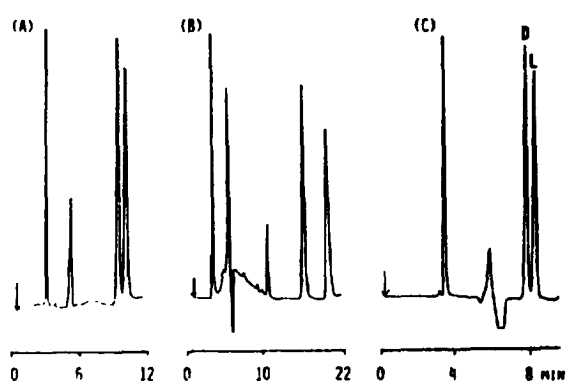


Fig. 5. Enantioseparation of amino compounds after derivatization on β-CD column using a polar organic mobile phase (acetonitrile–triethylamine–acetic acid, 1000:4:2, v/v). The derivatizing agents were (A) Phthalic anhydride derivative of 2-ethylhexylamine; (B) diphenylmaleic anhydride derivative of 2-methylpiperidine; (C) 3-nitrophthalic anhydride of phenylamine methyl ester. Data from Pawlowska et al. [50].

chiral amine and achiral anhydride moiety are closely associated with CD. The enantioseparation is affected by the size of the aromatic substituent.

Pre-column derivatizations are usually investigated using the dansyl [20,56], 3,4-dinitrobenzoyl [20,57], or α-phthalaldehyde [20,58] derivative agents. Other derivatizations such as with naphthalene-2,3-dicarboxaldehyde-cyanide reagent to form highly fluorescent derivatives have been described [20,59]. Other reagents for the derivatization of amino acids into highly fluorescent compounds are: 9-fluorenylmethoxycarbonyl glycine chloride (FMOC-Gly-Cl), 9-fluorenylmethyl chloroformate (FMOC) [20,51,60,61] and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [20,51]. It has been reported that the chiral recognition mechanism for a number of FMOC- and AQC-derivatized amino acids on derivatized β-CD [44,60] or native α-, β- and γ-CD bonded phases [44] is dependent on water concentration in the mobile phase. Both methanol and water diminish the hydrogen bonding interaction between the solute and the CD due to the competitive adsorption and solvation effects of these molecules. Hydrogen bonding solvents have considerable influence on both retention and enantioselectivity. Unlike the native CD bonded phases, the derivatized CD phases exhibited enantioselectivity towards some of the AQC functionalized amino acids in water-rich systems [51]. Separation of FMOC-functional imino acids done in the polar-organic mode are generally preferable to those in hydro-organic mobile phases. The *R*-(−)-(1-naphthyl)-ethyl carbamoylated-β-CD column is generally more selective for these compounds than the acetylated-β-CD column [60].

Rizzi et al. [62] compared the enantioseparation on native β-CD stationary phase of amphetamine, methamphetamine and ring-substituted amphetamines (MDA and MDMA) with the separation on classical RP8 phase of diastereomers formed with phenylisothiocyanate (PITC) or AQC or naphtylisothiocyanate (NITC) or N-α (2-4 dinitro 5-fluorophenyl)-L-alanin-amide (MARFEY's reagent) (Table

Table 4
Enantioseparation of non-derivatized amphetamines on β-CD column and of derivatized amphetamines on RP-8 columns

	Non-derivatized[1]		PITC[2]		NITC[3]		AQC[4]		MARFEY's derivatives[5]	
	k_2	α	k_2	α	k_2	α	k_2	α	k_2	α
Amphetamine	3.22	1.06	2.43	1.34	2.01	1.17	3.92	1.30	14.1	1.08
Metamphetamine	5.65	1.07	2.72	1.30	2.19	1.10	3.60	1.29	11.8	1.08
4-Hydroxyamphetamine	4.85[a]	1.08[a]	2.49	1.32	1.90	1.11	4.10	1.33	5.6	1.31
4-Methoxyamphetamine	5.60	1.05	2.56	1.36	1.88	1.13	4.02	1.34	12.2	1.00
3,4-MDMA	6.86[b]	1.09[b]	3.22	1.21	1.77	1.21	6.04[c]	1.00[c]	5.84	1.23
3,4-MDA	7.78[b]	1.10[b]	3.38	1.21	1.96	1.20	5.48[c]	1.00[c]	12.6	1.58
2,5-Dimethoxyamphetamine	2.37	1.06	*	*	*	*	3.65[d]	1.21[d]	19.2	1.08
2,5-Dimethoxymethamphetamine	2.77	1.05	*	*	*	*	1.61[d]	1.07[d]	11.2	1.18
4-bromo-2,5-dimethoxyamphetamine	0.25	<1.02	*	*	*	*	2.04[d]	1.10[d]	28.2	1.00
4-Ethyl-2,5-dimethoxyamphetamine	0.20	1.00	*	*	*	*			14.4[e]	1.04[e]

PITC, phenyl isothiocyanate; NITC, naphthyl isothiocyanate; AQC, *cis*-aminoquinolyl-*N*-hydroxy-succinimidyl carbamate; MARFEY's, *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide; MDMA, methylenedioxymetamphetamine; MDA, methylenedioxyamphetamine; k_2: retention factor of the second eluted enantiomer, α: enantioselectivity coefficient.

[1] Mobile phase: 0.1 *M* ammonium acetate pH 7–methanol (95:5; 90:10[a]; 85:15[b], v/v).

[2,3] Mobile phase: 0.1 *M* ammonium acetate 0.1% TEA pH 5.5–methanol (40:60, v/v), temperature 7°C; flow-rate 0.4 ml/min.

[4] Mobile phase: 0.1 *M* ammonium acetate 0.1% TEA pH 5.5–methanol (50:50 v/v), temperature 20°C; flow-rate 0.5 ml/min.

[5] (RP-8) column; mobile phase: 0.1 *M* ammonium acetate 0.1% TEA pH 5.5–methanol (45:55, v/v), temperature 20°C; flow-rate 1.0 ml/min.

[c] Methanol 40% (v/v).

[d] Temperature 10°C; methanol 40% (v/v).

[e] Methanol 60% (v/v).

* Very low *k*.

Data from Rizzi et al. [61].

4). AQC derivatization which is completed in a few seconds, is the procedure of choice for most of the amphetamines.

Another example is the derivatization of penicillamine with the *o*-phthalaldehyde-2-mercaptoethanol reagent [20,63].

In some cases, the interactions with native β-CD CSP gave insufficient enantioseparation. This problem was overcome by using derivatized CD CSPs to provide additional adsorption (binding) sites. For example, carbamoylated β-CD columns, compared to the native β-CD CSPs, enhanced enantioselectivity of aromatic substituted amines. Likewise, permethylated β-CD in polysiloxane (one in 60 silicon atoms in the polymer chain carries a CD moiety), coated on porous silica, is an interesting alternative to the usual chemical bonding of CD to silica and subsequent end-capping of the polar sites on the support [64]. This new CSP has been used for several barbiturates.

3.1.7. Use of displacers in CD preparative HPLC

Quintero et al. [65] synthesized various chiral displacers derived from mandelic acid to be used in preparative chiral displacement chromatography for the isolation of stereoisomers on α-CD CSPs. The displacers must satisfy the following requirements: high solubility in the carrier solvent to form concentrated displacer solutions, competitive and strong adsorption on the CSP and should allow easy and fast regeneration of the column. In the case of Pirkle-type stationary phases, a generic displacer structure was identified, based on the synthesis of 3,5-dinitrobenzoyl esters of alcohols, 3,5-dinitrophenylcarbamates of alcohols, and *N*-3,5-dinitrophenylamidoethyl-1-alkanoates. For α-CD CSPs, the displacers must contain a phenyl anchor group which fits the cavity of α-CD, a section that can form multiple hydrogen bonds with the secondary hydroxyl groups of α-CD (carboxyl and carbonyl groups) and a solubility-adjusting tail section which regulates displacer solubility (alkanoate group of variable chain length). Various derivatives of mandelic acid were tested as to their capacity factor and adsorption isotherm: both parameters increase regularly with the length of the alkanoate chain, and thus cover a range a broad enough to be of use for actual displacement chromatographic applications on Cyclobond II columns.

3.2. Use of CD added to the mobile phase

Chromatographic separations using CDs as mobile phase modifiers are largely based on the selectivity of formation of inclusion complexes. The chromatographic properties are a function of the stability of these complexes. Selectivity and stability are the two driving forces acting synergistically and directly related to the properties of the guest and to the interactions with the CD [2,66–68]. Enantiomers may show different inclusion complex stabilities based on differences in hydrophobic and hydrogen bonding interactions with the β-CD [69]. A major drawback in the use of CDs in the mobile phase is the necessity of high CD concentrations (near the solubility limit) in order to obtain the chromatographic resolution. This problem can be overcome by the use of the more soluble methylated β-CDs but they need a long time to reach the equilibrium of a dynamic coating of the stationary phase. Roussel and Favrou [2] proposed the use of a carboxymethylated β-CD and a new cationic β-CD derivative as chiral additives. The cationic β-CD is far more soluble in water than free β-CD and no dynamic coating is required. It presents definite advantages over the use of other commonly used CDs. Retention and resolution may be controlled owing to the wide range of solubility of the cationic β-CD. In general, the enantioselectivities observed are at least as good as those for native β-CD under the same conditions and are potentially improved by using higher concentrations. Cationic β-CD has been used in the enantioseparation of eight amino acid derivatives (phenylthiohydantoins and methylthiohydantoins) and of several chiral drugs (hexobarbital, mephobarbital, terbutaline, chlorthalidone) [2].

Permethylated β-CD, owing to their strong adsorption on ODS columns, forms a chiral stationary phase generated dynamically, allowing separation of chiral barbiturates [70].

Several authors studied CDs as chiral mobile phase additives in reversed-phase [20,71–75]. Many factors seem to be responsible for the separation, including the type of CD used [76,77], its concentration in the mobile phase [76–78], the type of

mobile phase [76–79] and the temperature of the separation process [76,77].

3.2.1. Inclusion complex equilibria

According to Walhagen and Edholm [79], retention in systems containing β-CD as a chiral mobile phase additive can be described as follows:

$$\frac{1}{k} = \frac{1}{k_0} + \frac{K_f[CD]_T^N}{k_0} \quad (1)$$

where K_f is the apparent formation constant for the inclusion complex and k_0 is the retention factor in the absence of CD; k is the retention factor of the sample solute with a concentration $[CD]_T$ of CD in the mobile phase, and N is the stoichiometry of the complex. A plot of $1/k$ versus $[CD]_T^N$ will be a straight line through the origin with a slope of K_f/k_0. This treatment assumes that CD and complexed analyte are not retained by the stationary phase. When the stoichiometry of the complexes is known (from several chromatographic runs with different CD concentrations), only two chromatographic analyses are useful: one to determine k_0 and the other to determine k at a known CD concentration.

3.2.2. Influence of the type and concentration of CDs

These factors have been widely studied [20,80–82]. It has been found that α-CD may be primarily applied to the separation of small molecules (one-ring systems), while β- and γ-CDs are suitable for the resolution of compounds with larger molecular size (2–4 ring systems).

Shimada et al. [83] report the HPLC separation of baclofen after derivatization into various diastereomers and using α, β, or γ-CD in the mobile phase. Although conventional reversed-phase HPLC allowed separation of the diastereomers, the addition of CD to the mobile phase improved the separations. Depending on the type of derivative, α, β, or γ-CD gave the best results.

Lamparczyk et al. [77] studying the separation of norgestel enantiomers in the reversed-phase mode, observed a decreased retention factor with increasing β-CD concentrations but no chiral resolution over the whole range of β-CD concentrations (0–16 mM). The observed effect suggests that the guest-CD complexes being nearly unadsorbed, are not exchanged from the mobile phase.

Eto and Noda [84], using short (25 or 50 mm) ODS cartridge columns, obtained most satisfactory results when the mobile phase contained 11.2 nM β-CD in a 94:6 (v/v) mixture of 33.3 nM of KH_2PO_4 and methanol. Using 10 nM phosphate buffer (pH 7) and 2-methyl-2-propanol in the mobile phase, Pullen et al. [69] showed that the separation factor of two racemic cycloheptaindole derivatives varied as a function of the β-CD concentration.

Chatjigakis et al. [85] studied the retention properties (retention factor) and solubility of natural and glycosylated CDs in methanol–water and acetonitrile–water HPLC mobile phase binary mixtures. A linear relationship between the volume fraction of the organic solvent and the logarithm of the capacity factor is observed. β-CDs are definitely the most hydrophobic, followed by glycosylated (glycosyl or galactosyl) β-CDs in which derivatizations increase the hydrophilic character. For α and γ-CDs, opposite hydrophobicities were measured by HPLC, depending whether methanol or acetonitrile mixtures are used. Hydrophobicity measured in methanol–water systems correlates better (γ-CD more hydrophilic than α-CD) with the solubility in water than when measured in acetonitrile water systems.

3.2.3. Influence of the stationary phases

Some authors found that CDs are not adsorbed on the surface of either polar or apolar stationary phases [20,80,81]. The polarity and the type of stationary phases play important roles in the retention, separation selectivity and efficiency. The same resolving power was found on various octadecylsilica columns at constant eluent composition with entirely different retardations [20,80].

Nowakowski et al. [86] in a systematic study of the retention mechanism of native α-, β- or γ-CDs in the reversed-phase mode, showed the existence of different behaviours for every CD towards the C_{18} stationary phase: γ-CD is markedly less retained than the smaller β-CD and is nearly as strongly retained as the much smaller α-CD, suggesting that α-CD has a larger effective hydrophobic surface area. Molecular modeling calculations confirm important differences in interaction energies of the CDs with the

octadecyl alkyl group. α-CD can form a single strongly bound complex and has a higher retention, while β- and γ-CDs form several weaker complexes. The interaction energy of the water network with γ-CD is much stronger than with α- and β-CDs and explains the lower retention and lower apparent hydrophobic surface area of γ-CD.

The usefulness of coupled-column chromatography for separation of drug enantiomers in biological fluids has been performed by using the first achiral column for the separation of the enantiomers from endogenous materials, followed by enantiomeric separation on another achiral column using CD in the mobile phase [20,79].

3.2.4. Influence of the composition of the mobile phase

Using γ-CD in the mobile phase, stronger inclusion complex formation and more effective enantioseparation can be achieved by decreasing the organic solvent content in the eluent [20,81,87]. The suitable concentration of organic modifier depends on the polarity of the stationary phase; by increasing the concentration, the retention factors of the solute complexes decrease similarly to those with ordinary reversed-phase systems.

The elution order of the compounds is not altered and the selectivity is not modified by the nature of the organic solvent [20,88]. The solutes and the organic solvents compete for the preferred location in the hydrophobic cavity, which results in various degrees of interaction of the compounds to be tested with CDs.

An interesting possibility to increase enantioseparations combines CD inclusion complex formation with ion-pair chromatography [20,89].

Fell et al. [90] studied the utility of applying the simplex approach for optimization in chiral HPLC as compared with response surface mapping. Three column systems including a β-CD column were tested for the resolution of several benzodiazepine enantiomers. β-CDs as a mobile phase additive were also studied. Another approach to optimize the mobile phase composition with respect to peak resolution and analysis time using a two-level full factorial design has been proposed by Valiente Barderas and Duprat [91].

3.2.5. Influence of the temperature

The effect of elevated column temperature on the separation characteristics (retention factors, selectivity and resolution) is unpredictable [15,20,88].

Lamparczyk et al. [77] studied the effect of temperature on the resolution of enantiomers of norgestel and have shown that a linear Van't Hoff behaviour, common to both enantiomers, is observed in the range between 70–40°C. In this range the degree of complexation with β-CD for both enantiomers is very low and stereoselectivity is not observed. The temperature at which the deviation from Van't Hoff behaviour, together with chiral separation, begins is 40°C; below this temperature the inclusion mechanism starts to be important. When the temperature decreases in the sub-ambient region the retention also decreases. The best chiral separation is achieved in the range from −5 to 0°C. The enantioselectivity in this temperature range can be interpreted as being due to slower rotation of the guest and host molecules and hence a steric fit is possible.

3.2.6. Influence of pH and ionic strength

If the guest compound contains ionizable functional group(s), the inclusion complex formation is affected by the pH and the ionic strength. In absence of ionizable groups these effects are not observed [20,82].

Retention of basic compounds on silica under reversed-phase conditions is a complex function of pH, and the organic solvent and buffer concentrations [69,92]. Retention mechanisms are a combination of ion-exchange interactions with ionized silanol groups and hydrophobic interactions with siloxane groups on the silica surface [69,93]. Using two racemic cycloheptaindole derivatives as model compounds, Pullen et al. [69] have shown that the separation factor was unaffected by sodium ion concentration but modified by the pH.

Chiral separations with porous graphitic carbon as the achiral stationary phase and β-CD in the mobile phase enable poorly ionizable drugs to be investigated at the extreme end of the pH range [20,94].

Sueyasu et al. [95] developed a simple and accurate HPLC method for measuring thiamylal enantiomers in serum using reversed-phase conditions and a mobile phase containing β-CD. The

enantioseparation was not influenced by a change in pH over the range 3.0–4.6. The hydrophobic cavity of β-CD can selectively include the non-ionic species. Thus, thiamylal ($pK_a = 7.5$) under acidic conditions, is almost non-ionic, allowing inclusion complex formation.

3.2.7. *Influence of organic modifiers*

In the reversed-phase mode, alcohol modifier is necessary for formation of inclusion complexes [68,96]. The presence of organic modifiers reduces enantioselectivity induced by β-CD in the mobile phase [5,97]. Low eluent modifier levels are therefore employed. It is necessary then to use a column packing of low hydrophobicity, in order to obtain reasonable retention values. Another significant factor is CD solubility that is reduced by addition of methanol, but enhanced by the presence of the other modifiers used.

Organic amine modifiers should be used in the reversed-phase mode to improve peak symmetry [69]. Indeed, addition of triethylamine to the mobile phase improves peak shape but has little effect on selectivity [5].

3.3. *Use of CDs for the quantitation of drugs in biological fluids*

The separation and the quantification of individual enantiomers in biological material required high selectivity firstly, to discriminate the analytes from matrix components and secondly, to separate the optical isomers. This is especially pronounced at low concentration of the analyte. It was pointed out that many of the chiral chromatographic systems developed for pure substances are not useful directly in bioanalysis. On chemically bonded CD-phases only composition changes of the mobile phase can be used to adjust selectivity. In a recent paper, Katagi et al. [98] studied the optimum composition of the mobile phase using methanol and acetonitrile as modifiers to separate enantiomers of methamphetamine and its main metabolites amphetamine and *p*-hydroxymethamphetamine in human urine (Fig. 6). In contrast, when CD is added to the mobile phase two more possibilities exist: (1) the concentration of CD can be changed and (2) the nature of the stationary phase can be altered. If stationary

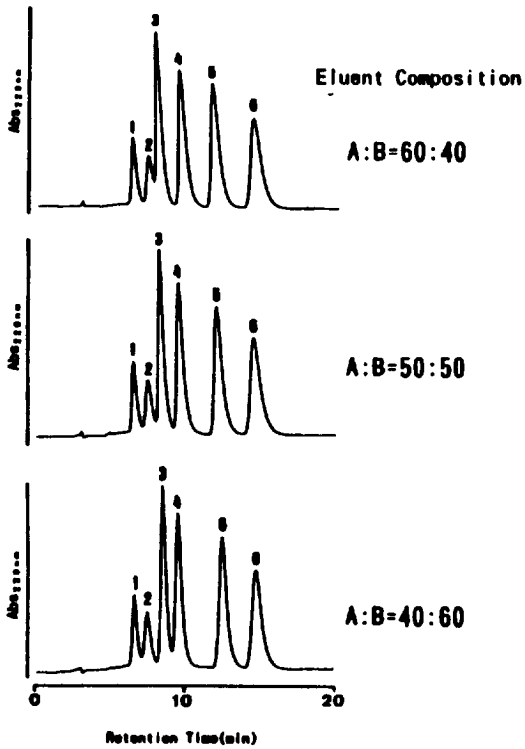


Fig. 6. Chromatograms obtained for spiked urine with a mixture of A and B as an eluent. Eluent A: methanol–50 m*M* potassium phosphate buffer (pH 6.0) (60:40, v/v). Eluent B: acetonitrile–50 m*M* potassium phosphate buffer (pH 6.0) (20:80, v/v). Column: β-CD phenylcarbamate-bonded silica column. Peaks: 1 = D-*p*-hydroxymethamphetamine; 2 = L-*p*-hydroxymethamphetamine; 3 = D-amphetamine; 4 = L-amphetamine; 5 = D-methamphetamine; 6 = L-methamphetamine. Data from Katagi et al. [98]

phases with different functionalities can be used great improvements toward the matrix components can be obtained. Consequently, coupled column chromatography offers a great potential in separation and quantification of optical isomers in biosamples [79].

Some of the recent applications of the use of CDs as CSP or added to the mobile phase for the quantitation of drugs in biological fluids are summarized in Table 5. Examples of chromatograms are given in Fig. 7.

Most of authors use a previous step to eliminate the endogenous substances, either by liquid–liquid [3,99,101,104,106–109], by liquid–solid [79,84, 95,98,103,111,112] extraction or by column-switch-

Table 5
Some enantiomeric separations in biological fluids based on complexation with CDs using HPLC

Enantiomers separated	Type of CD (Stationary phase)	Mode	Detection	Biological sample	Ref.
(a) *Use of CD stationary phases*					
trans-6,6a,7,10,10a,11-Hexahydro-8,9-dimethyl-11-Oxodibenz[*b*,*e*]oxepin-3-acetic acid	β-CD (Cyclobond I)	RPM	UV	Plasma	[3]
Ibuprofen	β-CD (Cyclobond I)	RPM	UV	Plasma, urine, bile	[99]
Chlorpheniramine maleate, chlorthalidone, hexobarbital, mephobarbital	β-CD (Cyclobond I)	RPM	UV	Serum	[39]
Atenolol	Polyphenyl carbamate β-CD(Cyclobond)	RPM	Fluorimetry	Plasma	[100]
Warfarin, 6-OH-warfarin, 7-OH-warfarin	β-CD	POM	UV	Plasma	[101]
Ibuprofen	β-CD (Cyclobond)	RPM	UV	Plasma	[102]
Salsolinol	β-CD-OH (Nucleodex)	RPM	Electrochemistry	Plasma	[103]
Moguisteine metabolites	β-CD (Cyclobond I)	RPM	UV	Plasma and urine	[104]
Xanthine oxidase inhibitor (BOF-4272)[a]	β-CD (Ultron ED-CD)	RPM	UV	Serum albumin	[105]
Propranolol	β-CD (Cyclobond I)	POM	Fluorimetry	Plasma and urine	[9]
Citalopram and desmethylated metabolites	Acetylated β-CD (Cyclobond)	RPM	Fluorimetry	Plasma	[106]
Thioridazine	Acetylated β-CD (Cyclobond)	RPM	Fluorimetry	Plasma	[107]
Salsolinol and *N*-methylsalsolinol	β-CD (Chiradex)	RPM	Electrochemistry	Brain	[108]
SCH 39304	β-CD (Cyclobond I)	RPM	UV	Plasma	[109]
Methamphetamine, amphetamine and *p*-hydroxymethamphetamine	β-CD phenylcarbamate (Ultron ES-PhCD)	RPM	Mass spectrometry	Urine	[98]
(b) *Use of CD added to the mobile phase*					
Renin inhibitor RO-42-5892/001 after dansylation	β-CD (Dimethyloctyl silica)	RPM	UV	Plasma	[110]
Chlorthalidone	β-CD (Phenylpropyl silica)	RPM	UV	Whole blood	[79]
Terbutalin	β-CD (Octadecyl silica)		Amperometry	Plasma	
5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	β-CD (TSK-gel ODS 80)	RPM	UV	Hepatocytes	[111]
Mephenytoin	β-CD (Supelcosil LC-8)	RPM	UV	Urine	[112]
Barbiturates and hydantoins	β-CD (ODS column)	RPM	UV	Serum	[84]
5-(*p*-Hydroxyphenyl)-5-phenylhydantoin, mephobarbital, 10,11-dihydroxy-10,11-dihydrocarbamazepine	β-CD (ODS column)	RPM	UV	Serum	[113]
Pinacidil and pinacidil-pyridine-*N*-oxide	β-CD (ODS column)	RPM	UV	Plasma, urine and liver slices	[114]
Azepinoindole	β-CD (silica stationary phase)	RPM	UV	Plasma	[115]
Thiamydal	β-CD (TSK-gel ODS 80)	RPM	UV	Serum	[95]

[a] HPLC frontal analysis with on-line HPLC system; RPM, reversed-phase mode; POM, polar-organic mode.

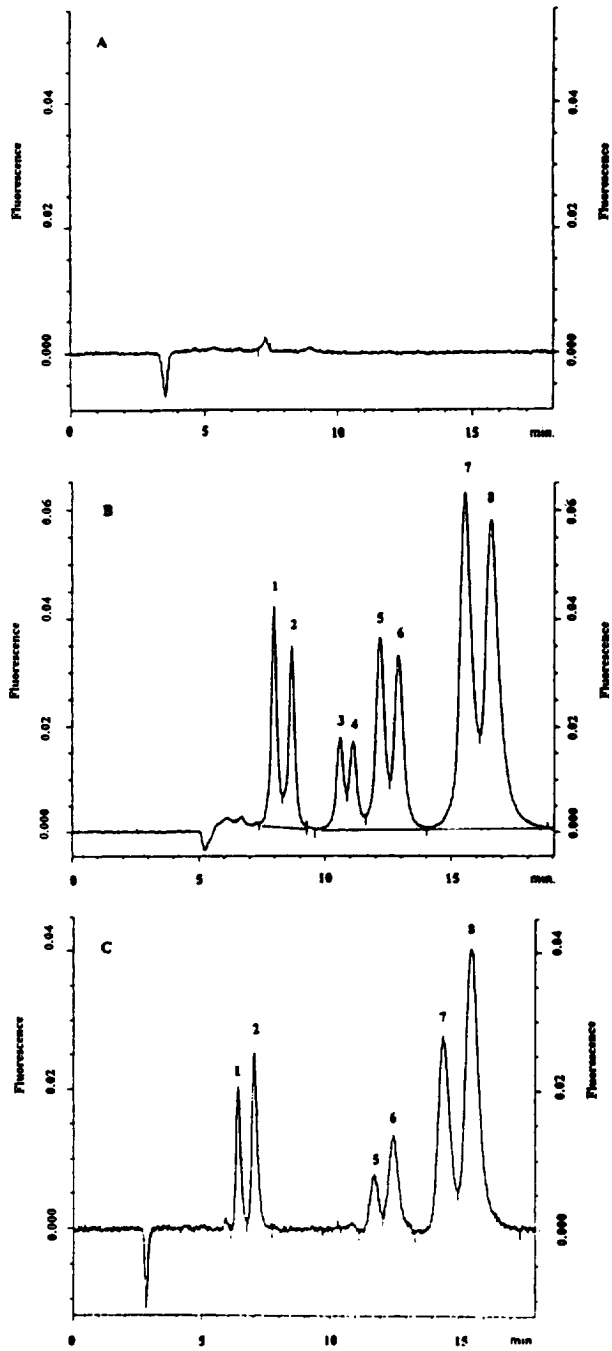


Fig. 7. Typical chromatograms of extracts of (A) blank plasma sample; (B) plasma sample spiked with citalopram (250 ng/ml), desmethylated citalopram (100 ng/ml), and didesmethylated citalopram (60 ng/ml); (C) chromatogram from a patient (steady-state conditions). Peaks: 1 = desmethylbenzoctamine; 2 = benzoctamine; 3 = *S*-(+)-didesmethylated citalopram; 4 = *R*-(−)-didesmethylated citalopram; 5 = *S*-(+)-desmethylated citalopram; 6 = *R*-(−)-desmethylated citalopram; 7 = *S*-(+)-citalopram; 8 = *R*-(−)-citalopram. Column: Acetylated β-CD. Data from Rochat et al. [106]

ing technique [110,113]. Nevertheless such preliminary steps are time consuming or expensive.

Techniques allowing direct injection of biological media without prior deproteinisation have been described. An on line HPLC system which combines a high-performance frontal analysis column (diol–silica column) and a chiral separation column (β-CD immobilized silica column) has been used by Shibukawa et al. [105] to determine the concentration of unbound enantiomers of a new xanthine oxidase inhibitor in human, bovine and rat serum albumins after direct injection into the diol–silica column. Some authors [100] have proposed an achiral–chiral coupled-column HPLC technique: the drug is first separated from endogenous substances or metabolites on an achiral column, then the drug fraction of the eluent is transferred into a chiral column. The same authors also proposed the direct injection of plasma in an hydrophilic size-exclusion gel column for the deproteinisation, followed by an ODS silica column for the concentration of atenolol fractions and a new chiral β-CD column for the enantioseparation. For the same purpose, Stalcup and Williams [39] used a particular mixed β-CD diol-type chiral column; the diol functionality mitigates the deleterious effects of the anionic silica surface which contributes to denature the proteins, thereby producing a column compatible with serum proteins. With the conditions used, proteins elute with the void volume while the smaller chiral analytes are retained. Other CD-based phases, including the hydroxypropylated-β-CD as well as the α- and β-CD phases, synthesized using the same protocol, may also be amenable to direct determination of the enantiocomposition of chiral analytes in serum.

4. Use of CDs in capillary electrophoresis (CE)

The use of CE for the separation of enantiomers has recently been reviewed [116–120]. In direct methods, based on the differences in effective mobilities of enantiomers in a chiral background electrolyte (BGE), resulting from different stabilities of transient complex of enantiomers with the chiral selector, CDs and derivatives are the most widely used. The applicability of different modes of capillary electrophoretic techniques using CD or CD derivatives as chiral selector is reported in Table 6.

Table 6
Modes of capillary electrophoretic techniques using CD or CD derivatives as chiral selector

Mode	Applicability
Capillary zone electrophoresis	Very good
Micellar electrokinetic chromatography	Very good
Electrochromatography	Good
Capillary gel electrophoresis	Good
Capillary isotachophoresis	Not very good

4.1. Chiral resolution in capillary zone electrophoresis (CZE)

4.1.1. Principle

When a charged solute forms a complex with a neutral CD, its charge/mass ratio, and thus its mobility decreases. The free or uncomplexed species migrates as it would in the absence of CD. The chiral separation occurs by indirect means. The mobilities of each enantiomer, free or complexed, are identical to one another. Differences in the equilibrium constants determine the ratio of free/complexed material. If the equilibrium constants are sufficiently different among enantiomers, separation will occur. Starting with the general resolution equation for CE

$$R_s = 0.177\Delta\mu_{ep}\sqrt{\frac{EL}{(\mu_{ep}+\mu_{eo})D_m}} \quad (2)$$

where R_s is the resolution, $\Delta\mu$ is the difference in mobility between the enantiomers, E is the field strength, L is the capillary length to detector, μ_{ep} is the average mobility, μ_{eo} is the electroosmotic mobility and D_m is the diffusion coefficient. Wren et al. [118] and Wren and Rowe [121] calculated $\Delta\mu$ and R_s. The mobility of the first (μ_a) and the second (μ_b) enantiomers is equal to:

$$\mu_a = \frac{\mu_1 + \mu_2 K_1[C]}{1 + K_1[C]} \quad (3)$$

$$\mu_b = \frac{\mu_1 + \mu_2 K_2[C]}{1 + K_2[C]} \quad (4)$$

where μ_1 and μ_2 are the mobility of the uncomplexed and of the complexed solute, respectively, C is the concentration of the chiral selector and K_1 and K_2 are the equilibrium constants. This shows that a solute's apparent mobility is influenced by the proportion of time spent as complexed material. The

difference between the electrophoretic mobilities of the two enantiomers, $\Delta\mu = \mu_a - \mu_b$, can be calculated from the following relationship:

$$\Delta\mu = \frac{(\mu_1 - \mu_2)(K_1 - K_2)[C]}{1 + (K_1 + K_2)[C] + K_1K_2[C]^2} \quad (5)$$

From Eq. 4, we can see that if $\mu_1 = \mu_2$ or $K_1 = K_2$, then $\Delta\mu = 0$. If $[C]$ approaches zero or is very large, $\Delta\mu$ approaches zero as well. The greater the affinity of the solute for the selector (large K), the lower the optimal selector concentration. Therefore, both the solute and the type of CD selected influence the final result.

The optimal concentration can be calculated from:

$$[C]_{opt} = \frac{1}{\sqrt{K_1K_2}} \quad (6)$$

Further advances in optimization theory were made by Rawjee et al. [122,123] when they found that solute charge influences the complexation equilibrium constant. This means that pH is another variable that may have to be optimized when developing a separation. The authors proved that for certain solutes, the order of elution of the enantiomers may be pH-dependent.

4.1.2. Factors affecting chiral separations

Influence of CD type. Higher affinity of one of the stereoisomers for the CD is a result of a better steric orientation in the CD analyte complex. This leads to a longer incorporation time of this stereoisomer in the CD cavity, and therefore to a lower mobility in the electrophoretic system.

Changes in the size and/or the substituents on the CD result in alteration of enantioselective properties. Numerous authors have clearly shown that large differences in chiral resolution are generally obtained for a given analyte by using different CDs.

An inclusion complex must be formed although the ability of a molecule to form an inclusion complex with a CD does not cause enantiodiscrimination per se. Thus, the effect of the cavity size must be studied.

In a work on enantioselective separations of α-amino acid derivatives in CD modified CZE, Lindner et al. [124] showed that there was a direct relation between spatial expansion (aromatic size) of the amino acid N protection group and the inner diameter of the CD cavity. Voluminous fluorescent derivatives (DNS, FMOC) showed best resolution with γ-CDs, whereas the smaller chromophores of dinitrophenyl (DNP), dinitrobenzoyl (DNB), carboxybenzyl (CBZ) and AQC could be efficiently separated with β-CD derivatives. Similarly, only γ-CD were able to resolve FMOC-D/L carnitine enantiomers [125].

The effect of the cavity size on enantiomeric resolution has been demonstrated by many other authors [126–131]

Garrison et al. [132] studied the separation of phenoxy acid herbicides and their enantiomers. Racemic dichlorprop, the phenoxy acid with the smallest effective molecular diameter, is separated by the α-CD which has the smallest cavity size, but not by the β-CD, which may simply be too large for differential complexation. The fact that the dichlorprop racemate is separated by DM-β-CD, which has the same inner diameter as β-CD, implies an additional complexation mechanism. Indeed, besides the inclusion complex, two other points of interaction are believed to involve two of the substituents on the stereogenic center, interacting with the chiral environment on the surface of the CD toroid.

Changes in the substituents on the native selector result in alterations in enantioselective properties. This fact is well known and used to achieve a good resolution in enantiomeric separations. Several papers on CZE deal with the selection of the appropriate CD type for enantiomeric resolution [127,133–139].

Methyl-β-CDs have been extensively employed in CE chiral separations, often demonstrating enhanced resolutions over the parent macrolide [140].

Wren and Rowe [141] stated that in the presence of methyl-β-CD, hydrophobicity would be the major force driving complexation in a series of β-blockers.

St Pierre and Sentell [135] showed, regarding enantiometric separation of terbutaline, that better enantioselectivity was achieved with HP-β-CD than with β-CD because the hydroxyl on the HP-β-CD propyl group was motionally less restricted than the rim hydroxyls in β-CD.

When the guest molecules are prevented from penetrating deep into the CD cavity, interaction between the rim of the CD and the guest molecule

could become more favorable or less favorable. For propranolol [135] resolution is better with HP-β-CD than with methyl-β-CD. Because of the size of the rigid naphtalene ring with respect to the diameter of the β-CD cavity, propranolol would fit snugly inside the CD cavity. With the addition of a propyl spacer to the rim of β-CD, the rigidity of the cavity decreases, which will allow improved inclusion of the naphtalene group, but secondary interactions between the hydroxyl on the propyl spacer and the hydroxyl and amine groups off of the chiral center of propranolol are enhanced.

Matchett et al. [139], studied the enantioresolution of propranolol and four derivatives, and showed that of the three modified CDs investigated, hydroxyethyl-β-CD provided the largest chiral resolution values for all five analytes. This may be linked to its extended hydrogen bonding chains on the CD rim.

In a study of enantiomeric separation of fluoxine (Fl) and norfluoxine, Piperaki et al. [138] calculated binding constants of three Fl-CD complexes: Fl-β-CD, Fl-methyl-β-CD and Fl-HP-β-CD. The binding constant with methyl-β-CD was about 10% lower than with native CD, and Fl-HP-β-CD is seen to have a very low binding constant in comparison with Fl-β-CD. In this case bulky group introduced at the cavity rim might sterically interface with Fl inclusion into the cavity.

The use of charged CDs in chiral separations by CE can expand the resolution window in comparison with neutral CDs. As demonstrated by Schmitt and Engelhardt [142], they offer much higher flexibility in optimizing separation problems. They can be used in an uncharged mode for ionic enantiomers with enhanced selectivity because of high hydrogen bonding capability. In the charged mode they are suitable for the separation of non ionic enantiomers. In the latter mode, they offer additionally electrostatic interaction via ion pairing with analytes of opposite charges. This has a large influence on selectivity and resolution. As demonstrated by Chankvetadze et al. [143], anionic CD derivatives, such as sulfobutyl ether-β-CD, exhibit a chiral recognition not only towards positively charged and neutral analytes, but also negatively charged analytes. Thus, the role of coulombic interactions in the host–guest complexation using CD-host does not seem to be critical.

Influence of CD concentration. The resolution and the migration terms of analytes are strongly influenced by the CD concentration [144–148]. At the low concentration extreme, there is not enough chiral selector available to form complexes and, therefore, no separation of enantiomers is possible. At the high concentration extreme (experimentally limited by solubility of chiral selector), both enantiomers are completely complexed and no separation of enantiomers is possible in this case either. Only in the midrange of concentration will an adequate separation occur.

Baumy et al. [149] studied the effect of β-CD concentrations on the migration time and on the chiral resolution of 5-methoxy-3-(di-*n*-propylamino)chroman and 5-hydroxy-3-(di-*n*-propylamino)chroman enantiomers on a large range of β-CD concentrations: from 1 μ*M* to 100 m*M* (above 16 m*M* CD's concentration urea 8 *M* was added to increase β-CD solubility) (Fig. 8). As the β-CD concentration increases, the free enantiomer fraction becomes smaller and consequently the electrophoretic mobility is reduced. At the micromolar level of β-CD concentrations (1–100 μ*M*) no enantioseparation occurred because the benzopyran derivative enantiomers were not complexed. At millimolar β-CD concentrations (1–5 m*M*), the electrophoretic mobility of each enantiomer decreases with increasing complexation percentage, and a baseline resolution of the two enantiomers was achieved. As expected, there was an optimum β-CD concentration which led to optimum enantioselectivity. Further increase in β-CD concentration results in a decrease in resolution. Thus, the resolution generally increases with increasing concentrations up to a certain point where a maximum is reached. Further increase in the CD concentration can result in a slow or rapid decrease in resolution, again depending on the chiral compound and CD type [144].

The concentration of CD at maximum resolution is dependent on the chiral compound being separated and on the type of CD used. The higher the affinity of the enantiomer for the selector, the lower the optimum selector concentration which have a high affinity for the selector. This fact was confirmed by Bechet et al. [150]. However, studying the resolution of five chiral basic pharmaceuticals, St Pierre and Sentell [135] showed that even when low concen-

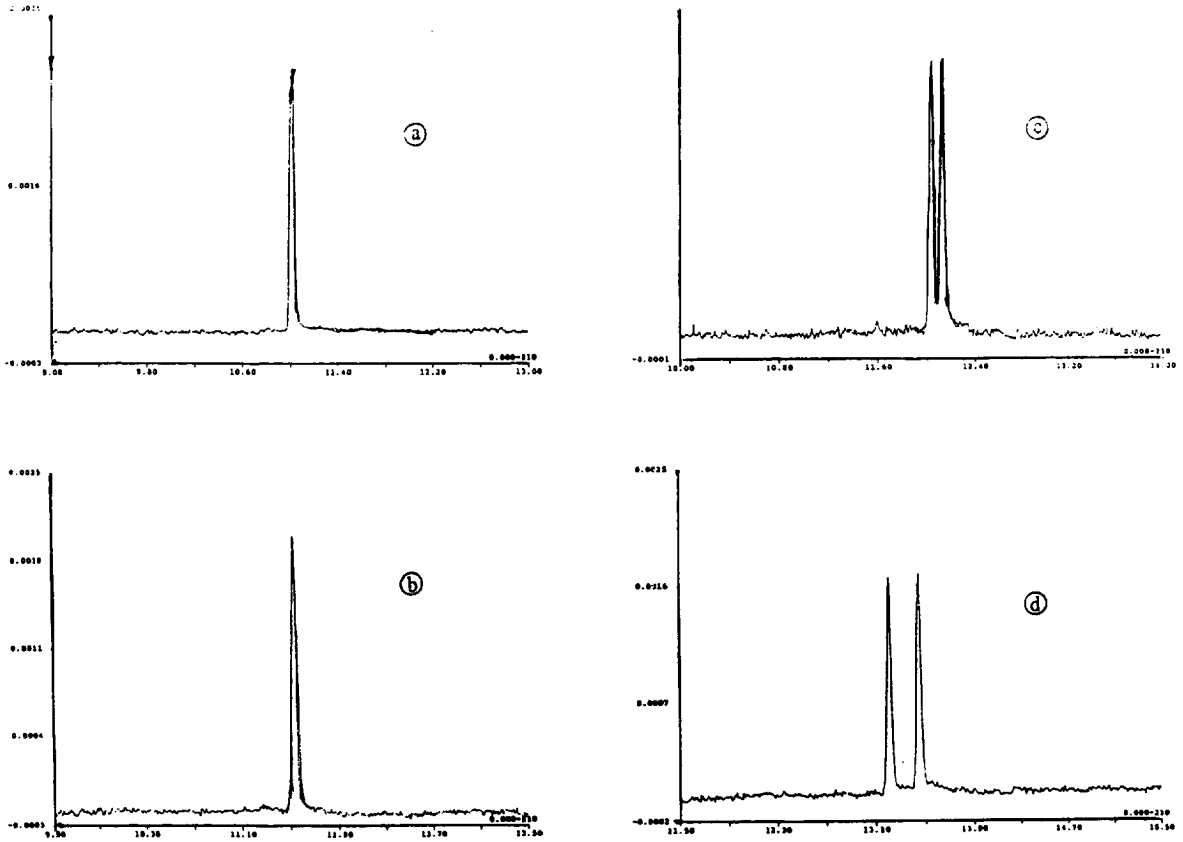


Fig. 8. Effect of β-CD concentration on the enantioseparation of 5-OH-3-(di-*n*-propylamino)chroman. Fused-silica capillary column, 70 cm×50 μm I.D.: applied voltage, +18 kV; buffer, 50 m*M* phosphate–borate (pH 7.0)–β-CD–8 *M* urea; detection at 210 nm; temperature, 25 °C. β-CD concentration: (a) 1 μ*M*; (b) 100 μ*M*; (c) 1 m*M*; (d) 5 m*M*. Data from Baumy et al. [149].

trations of CDs were examined (1, 3, 6 m*M*), enantiomeric separation for atropine and metropolol, which have a high affinity for the selector, could not be achieved. Two possible explanations have been proposed: either the difference in CD binding between the enantiomers may be too small, or even though there are strong interactions between the solute and the CDs, they are not enantioselective.

As the CD concentration increases, the mean migration times become longer but the number of theoretical plates seems only slightly affected by a change in CD concentration. Palmarsdottir [127] observed an almost linear relationship for all the chiral compounds studied. However, Heuermann and Blaschke [151] showed that the observed migration times for dimethindene and four possible metabolites in the concentration range 88–90 m*M* HP-β-CD exhibited a non linear increase. They explained this by the fact that high CD concentrations result in dimerization of CD, leading to a loss of selectivity and hence shorter migration time.

Degree of substitution. In a separation of optical isomers of nine organic acids by CZE using HP-β-CD with a degree of substitution between 3.0 and 7.3 [(2-hydroxy)propyl groups/CD molecule], Valko et al. [152] showed that the degree of substitution (DS) had a significant influence on the resolution of the enantiomers. In some cases, the chiral recognition could be completely lacking at a DS value but using the same type of CD at another DS the separation of the enantiomers may be possible.

Richard and Bopp [153] compared various sources of HP-β-CD on the separation of the *R* and *S*

enantiomers of a drug and a potential impurity. In all cases, the *R* enantiomer was separated from the *S* enantiomer. In some cases, however, one of the enantiomers of an impurity was not completely separated from the trace *R* enantiomer of the drug making difficult the determination of chiral purity of the drug. Further investigations showed only very minor variation in the lot-to-lot consistency from one supplier but definite variation with the degree of substitution.

Influence of analyte shape. The influence of the shape of the analyte on migration time and enantiomer resolution has been exemplified by tryptophan derivatives [116,154], some 2-aryl propionic acid non-steroidal anti-inflammatory drugs [137], propranolol and analogues [139], and some chiral pharmaceuticals containing the imadazole moiety [130].

Influence of analyte concentration. Rickard and Bopp [153] examined resolution tailing and peak separation as a function of the sample concentration and volume injected. The data indicated that the resolution was essentially constant at low sample concentrations, but decreased extensively at high sample concentrations. The change in resolution with sample concentration was even greater than its change with the CD concentration. In contrast to the behaviour observed with changes in CD concentration, the peak separation remained nearly constant but the peaks became much more asymmetrical when the sample concentration was varied. The authors concluded that the molar ratio, analyte concentration and sample volume interacted to give differences in resolution, peak shape and peak separation, and that there were optimum combinations of CD concentration with moderate sample concentrations and injection volumes that gave the best separation.

Effect of organic modifiers. These modifiers influence several parameters, e.g., the viscosity of the BGE, the solubility of the analytes and the EOF. Their important role in CE enantioseparation was first reported by Fanali [155]. Some authors obtained the separation of propranolol enantiomers by adding 30% of methanol to the BGE, while others used methanol to increase selectivity and resolution [124,132,150,155–157] (Fig. 9). One disadvantage of methanol addition was the increase in migration time for all optical isomers: addition of 20% methanol more than doubled the migration time for optical isomers.

The existence of one optimum concentration of the organic modifier (methanol, acetonitrile, 2-propanol) for chiral resolution of imidazole derivatives has been shown by Chankvetadze et al. [130]. However, in some cases modifier addition could lead to a loss in selectivity as reported by Rogan et al. [158] for the enantiomeric resolution of salbutamol and its chiral and achiral related impurities, and by Nishi et al. [131] for the separation of enantiomers of trimetoquinol hydrochloride related substances and some other drugs.

Matchett et al. [139] have found that 30% methanol has a beneficial effect on enantiomeric resolution of propranolol and four derivatives with hydroxyethyl-β-CD and Ac-β-CD, yet it produced both higher and lower resolutions using methyl-β-CD dependent on the analyte structure.

Upon addition of organic modifier there is an improvement of the analyte solubility in the electrolyte during migration, and a decrease of the equilibrium constant and thus of affinity of the enantiomers for the CD. The optimum CD concentration should then become higher when organic

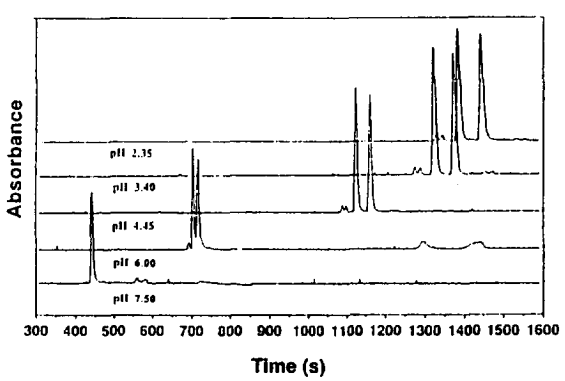


Fig. 9. Resolution enhancement by the addition of organic solvent, demonstrated for 4-hydroxy-2,7-dimethoxy-2H-1,4-benzoxazin-3(4H)-one. To the basic buffer (20 m*M* borate pH 9.3–20 m*M* β-CD) have been added: (a) 0% methanol; (b) 10% methanol; (c) 20% methanol; (d) 10% acetonitrile; (e) 25% acetonitrile. Data from Thunecke et al. [157].

modifier is added to the buffer. Therefore, at CD concentrations equal to or below the optimum value for a modifier free buffer, the addition of modifier should lead to a decrease of the apparent mobility difference between the enantiomers and hence of the chiral resolution. The addition of organic modifiers also results in a decrease in EOF probably via interaction of the modifier with the capillary wall thus altering charge and hydrophobicity and consequently changing potential as a driving force of EOF. A consequence is an increase in migration time which can assist resolution.

Organic additives proved to be a useful tool to increase resolution and sometimes even to make a separation possible, in particular with strongly binding analytes, which always results in prolonged analysis time due to lower EOF.

Surfactants such as cellulose derivatives (hydroxypropylmethyl cellulose) or large non polar substances (hexadecytrilammonium bromide) could be added to reduce the EOF and, thus provide a longer time to discriminate between the diastereoisomeric complexes and give a better separation [153].

Effect of BGE. Selection of an appropriate buffer (BGE) is often critical in enantiomeric separations. Buffer components, ionic strength and pH are all important variables.

The concentration of the BGE can have a pronounced effect on resolution. Increasing buffer strength or ionic strength will modify the hydrophobic interactions of the enantiomers with the CD. However, it is not clear how this change will, by itself, improve the selectivity or efficiency of the separation: maybe by promoting the inclusion of the solute hydrophobic moiety into the interior cavity of CD. On the other hand, increasing the buffer concentration reduces the EOF because it reduces its driving force, the zeta potential, and consequently causes an increase in migration times. Longer migration times, in turn, provide a longer time for enantiomer chiral selector discrimination. That is, longer times within the electric field where the two complexes have slightly different mobilities will give a greater absolute separation of the two species.

Additional band broadening during the longer separation will be relatively small so that the principal disadvantage of reduced EOF is an increase in analysis time.

The concentration of BGE can have a pronounced effect on the resolution of optical isomers. Thus, a high ionic strength buffer can generate a better resolution owing to a large improvement in efficiency, whilst the enantioselectivity remains approximately constant [149,150,152,153].

However, Peterson [129] obtained only minor improvements in resolution of chiral drug molecules when the Tris concentration was increased.

In order to evaluate the effect of electrolyte ionic strength during enantioseparation of warfarin, Gareil et al. [144] performed a series of experiments at the optimum pH with 20 m*M* *N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonate (TAPS) buffer containing increasing concentrations of sodium chloride; the chiral resolution increased drastically as the sodium chloride concentration increased. However, this concentration should not much exceed 125 m*M*, otherwise, the electrolyte conductivity would be too high. A still better resolution was obtained with a 100 m*M* phosphate buffer of similar conductivity, but about double the ionic strength (ca. 275 m*M*). TAPS buffer electrolyte of equal concentration but much lower ionic strength (ca. 50 m*M*) gave a much lower resolution.

Thus, the effect of the type of buffer must be investigated. The use of sodium phosphate buffer showed slightly better resolution in enantiomeric separation of amino acid derivatives as compared with borate buffer. The optimum ionic strength studied in a range from 10 to 100 m*M* was found to be 20 m*M* for borate buffer and 70 m*M* for phosphate buffer [124]. Guttman and Cooke [156] used several buffers for the separation of racemic propranolol. They noticed that when phosphate buffer was used, significantly lower resolution values were obtained. This lower resolution might be caused by a drop in efficiency due to the mobility mismatch between the solute and the higher mobility running buffer ions, and the competition between the HPO_4^{2-} ions and the solute molecule for binding to chiral selector.

The maximum usable buffer concentration is limited by the conductivity of the buffer. When the current is large enough that it produces more Joule heat than can be dissipated, the separation efficiency

is compromised. Thus it is advantageous to use low conductivity buffers such as zwitterionic buffers and small diameter capillaries when possible.

pH influences both migration time and chiral resolution. When neutral CDs are used, the compound must be charged for the complex to have a non zero mobility [129,130,153] (Fig. 10).

Increasing the pH of the background electrolyte results in an increased EOF. The change in the apparent mobility of the enantiomers follows the same pattern as the EOF [152].

A high effective mobility of the uncomplexed enantiomers and a low EOF seem favorable for the enantiomeric resolution [127].

Nishi et al. [131] recommended to use a solution of low pH, i.e., under the condition of low velocity of EOF for chiral separation by CD-CZE. However, for some compounds a solution of high pH was better for fast enantioseparation.

Ingelse et al. [154] showed that an increase in buffer pH led to a decrease in the resolution factor of 16 basic compounds studied (bupivacaine, atenolol, metroprotol, isoproterenol, oxprenolol, propranolol, ketamine, ephedrine, norephedrine, epinephrine, norepinephrine, terbutalin and methyl, ethyl and butyl ester of tryptophan), except for terbutalin which showed maximum resolution at pH 3.5. This decrease in resolution with increasing pH was probably due to the increase in the EOF causing a higher apparent mobility of the basic analytes and thus a shorter time of interaction with the chiral selector.

In some cases the resolution of enantiomers show a maximum according to the pH [152,156]. Lindner et al. [124] investigated pH effects over the range of pH 5–9.2 for DNB-, DNP- and AQC-amino acids using sodium phosphate 70 m*M* as background electrolyte and HP-β-CD 30 m*M* as chiral selector. As a result, all separations of DNB, DNP, dansylated (Dns) and FMOC derivatives showed enhanced resolution at pH values 7–9, since the resulting anionic analytes run counter to the EOF and the pseudo-stationary CD phase. AQC derivatives required a low pH (5–7) resulting in a reduction of complex formation. Otherwise, the EOF may be too rapid, resulting in elution of solute before separation has occurred. However, DNP–Phe showed a best separation efficiency at pH 6.

In other cases two pH values were found for optimum resolution values [149,159]. Rawjee et al. [122,123] used the multiple equilibria-based DID

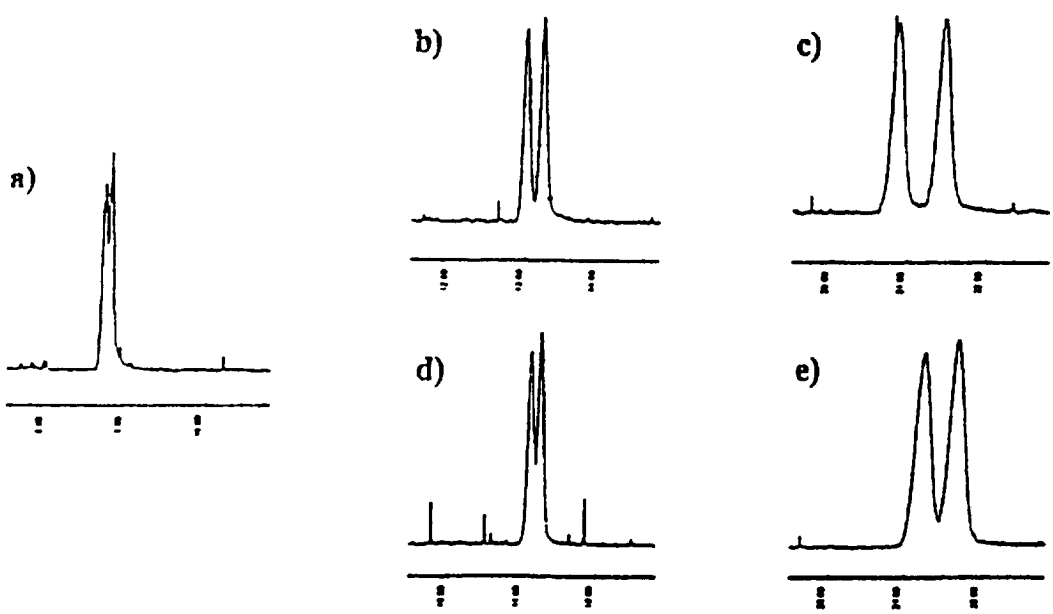


Fig. 10. Separation of racemic mixture of LY248686 using 100 m*M* Tris–phosphate buffers at the indicated pH. Other separation conditions were about 4 m*M* HP-β-CD using a ABI 270HT instrument with a 75 cm (50 cm to the window)×50 μm I.D. silica capillary, 23 kV, 30 °C and 214 nm detection. Data from Rickard and Bopp [153].

selectivity model and the extended peak resolution equation of CE to analyze the possibilities of electrophoretic enantiomer separations of chiral weak bases using HP-β-CD as resolving agent. They have verified experimentally the existence of three separation types for chiral weak bases. In a desionoselective separation (exemplified by atropine), only the non-dissociated enantiomers complex selectively, and resolution is possible in a narrow pH range in the vicinity of the pK_a value. These separations are rugged in terms of the concentration of resolving agent. In an ionoselective separation (exemplified by chloroamphetamine) only the dissociated enantiomers complex selectively. Resolution, displaying a local maximum as a function of the resolving agent concentration, is possible at any pH value that is at least two units below the pK_a. Resolution is also possible in the vicinity of the pK_a value, albeit at high resolving agent concentrations and with a reversed migration order. In a duoselective separation (exemplified by propranolol), both the dissociated and the non dissociated forms of the enantiomers complex selectively with the resolving agent. The resolution surface again has two lobes, affording different migration orders at different pH and resolving agent concentration combinations.

A change of pH permits much greater flexibility when chargeable CDs are used [142,143].

Effect of temperature. Changes in the capillary temperature can also lead to different effects on chiral resolution. Besides a decrease of buffer viscosity and changes in efficiency, an increase in temperature will decrease the formation constant of the complexes between the analyte enantiomers and the chiral selector and increase mobilities of the analytes [127].

Lindner et al. [124] have investigated the effect of temperature ranging from 5 to 60°C on enantiomeric separation of DNB-Phe, DNP-Phe and AQC-Leu. Results revealed an approximately linear relation of ln selectivity vs. $1/T$ in the temperature range from 5 to 40°C. The authors concluded that higher temperatures resulted in a significant change in the migration behaviour and, consequently, in the separation of the diastereomeric complexes as a result of additional predominantly entropy controlled effect.

Guttman and Cooke [156] observed that the resolution of the propranolol enantiomers decreased from 1.9 to 1.1 when temperature increased from 20 to 50°C. The use of lower temperature seems favorable for the solute–chiral selector complexation [160].

Effect of applied voltage. Palmarsdottir [127] has investigated the effect of separation voltage on resolution, mean migration time and efficiency of the separation of terbutalin at different pH values. The efficiency of the separation increased at higher voltage up to a certain level where a slight decrease or a leveling off was observed. The increase was not as pronounced as for efficiency. A similar effect was observed on the resolution of propranolol enantiomers [156].

Migration times can be shortened by increasing the applied voltage, but elevated voltages cause band broadening due to thermal effect which results in a decrease in resolution [124,127,151,156]. A voltage setting of 15–20 kV seems to be a good compromise for sufficient resolution and acceptable speed of analysis.

4.2. CE with charged CDs (CD-EKC)

Electrokinetic chromatography (EKC) utilizes the technique of CZE in combination with the principle of chromatography. It permits the separation of uncharged or neutral analytes [161,162]. In EKC, a pseudo stationary phase or a separation carrier having electric charge is added to the running buffer. It migrates electrophoretically as a solute in CZE, at a velocity different from that of the surrounding aqueous phase. An electrically neutral solute migrates at the velocity of the EOF when it is free from the pseudo stationary phase, while it migrates at the velocity of the pseudo stationary phase when it is incorporated in this phase. Thus, the distribution coefficient of the solute between the pseudo stationary phase and the surrounding aqueous phase determines the relative migration order. Several charged substances have been used as pseudo stationary phases: ionic micelles, CD derivatives having ionic groups, microemulsions and proteins.

CD-EKC used charged CDs which migrate with their own electrophoretic mobilities according to the polarity. The migration order between the enantio-

mer can also be manipulated by selecting natural CDs or charged CDs [163]. CD-EKC permits enantioresolution of neutral compounds as well as ionic compounds.

Terabe [161] and Terabe et al. [164] first used this type of CD for the separation of positional isomers of aromatic compounds. Schmitt and Engelhard [165,166] and Smith [167] used successfully β-CD with carboxy groups for enantioresolution. At high pH, deprotonation of these groups leads to mobility of the negatively charged chiral selector. Positively charged CD derivatives used by Nardi et al. [168] were effective for optical resolution of acidic solutes.

4.3. CD-modified micellar electrokinetic chromatography (CD-MEKC)

It is the most widely used EKC technique for enantioseparation.

4.3.1. Principle

There are two major methods used in MEKC for enantiomeric separation: one is MEKC using chiral surfactants and the other is based on CD modified MEKC [169].

CD-MEKC was introduced by Terabe [161]. CD cannot be solubilized into the micelle employed in MEKC because of the hydrophilic nature of the external portion [170] and will not interact with the micelle. It behaves as another phase in comparison with the micelle and reduces the partitioning of the analyte in the non-micellar aqueous phase [171].

In CD-MEKC, the solutes are distributed among three phases: an aqueous phase, the micelle and the CD. Solutes form inclusion complexes with CD based on their size, geometry and physicochemical properties while interactions with micelles are based on solute hydrophobicity [172]. When a solute is incorporated in the micelle, 3 types of interactions are possible: (i) the solute is adsorbed on the surface of the micelle by electrostatic or dipole interactions, (ii) the solute behaves as a co-surfactant by participating in the formation of the micelle, (iii) the solute is incorporated in the core of the micelle [173]. When the solute is included in the CD cavity, it migrates with the electroosmotic velocity and when it is incorporated in the micelle it migrates with the micellar velocity [174].

CD-MEKC has extended MEKC to two useful application domains: separation of hydrophobic compounds [174] and enantiomeric separation of neutral racemic mixtures.

4.3.2. Factors affecting chiral separation

Effects of CD types upon chiral separation. In a micellar solution, a monomeric surfactant, which is in equilibrium with the micelle, exists in the aqueous phase and it can be included by the CD because of the presence of the lipophilic hydrocarbon chain. It may prevent solute from inclusion. CDs having wider cavity (γ-CD) have the capability of including the solute together with the surfactant monomer [170,171,173,175]. Accordingly to all these results, for chiral separation, γ-CD has been found to be generally more effective in CD-MEKC than β-CD and its derivatives which were mainly employed in HPLC [173]. However, exceptions may occur [176].

Effects of CD concentrations upon chiral separation. As in CZE, γ-CD concentrations affect retention time and resolution [170,175–177] (Fig. 11). Optimum chiral selector concentration can be predicted mathematically and varies according to the affinity of each enantiomer for the chiral selector [178].

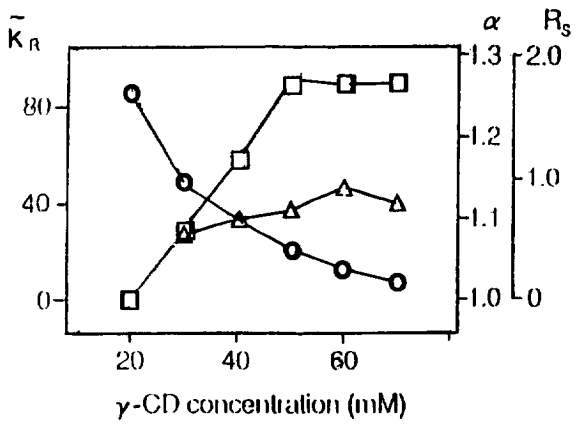


Fig. 11. Effect of γ-CD concentration in the separation solution on (○) the retention factor, k_R (retention factor of the *R*-enantiomers), (△) the separation factor, α, and (□) resolution, R_s. Separation solution, 100 m*M* SDS and 2 *M* urea in 100 m*M* borate buffer (pH 9.0) containing γ-CD. Data from Furuta and Doi [175]

Effects of analyte shape. Furthermore, the same authors studied enantiomeric separation of 14 compounds structurally analogs of diniconazole and uniconazole by CD-MEKC using γ-CD [179]. Effects of substituents on the benzene ring and alkyl groups on chiral recognition were investigated. Enantiomeric separation could be achieved for 13 compounds out of fourteen. Chiral recognition appeared to be significantly affected by the steric environment of the substituents on the benzene ring. From observations after NMR analysis, it is postulated that the benzene ring is included in the CD cavity together with SDS monomer. It plays an important role in the formation of CD complexes and chiral recognition in MEKC. The alkyl group is not included in the CD cavity because the space cavity is already full.

Effects of types and concentration of micelles upon chiral separation. Surfactants can be anionic, cationic, non-ionic, zwitterionic or mixtures of each. Varying physical nature (size, charge, geometry, etc.) of the micelle by different surfactants can yield great changes in selectivity. Those surfactants have to be used above their critical micelle concentration (CMC). Under such conditions, the analytes are believed to incorporate themselves either into the micelles or the CD cavity. Interactions of CD molecules with cationic or anionic surfactants under micellar conditions have been studied by conductometric measurements [180,181]. According to these measurements, CDs induce an increase in the apparent CMC of a surfactant, since the available monomers are partly associated in a complex with CDs. Interactions of surfactants with the outer hydrophobic shells of CDs were also suggested. SDS, an anionic surfactant with a CMC of 8.2 m*M*, is the most widely used [171,175,176].

Recently, a combination of a polymerized micelle, poly(sodium *N*-undecylenyl-D-valinate) [poly(D-SUV)], and γ-CD was used successfully for the first time by Wang and Warner [177]. The polymerized micelle not only possesses normal micellar properties, but also has several advantages over normal micelles: (i) stability was enhanced because of covalent linkage of the surfactant monomers; (ii) there is no critical micellar concentration CMC. Indeed, for normal micelles, the concentration of the surfactant has to be above the CMC in order to be effective, and thus the working concentration range may be very narrow. Thus at very high concentrations of a charged surfactant, excess heat will be generated in the capillary and will inhibit optimal separation; (iii) polymerized micelles are more rigid than normal micelles. Thus interactions between the solute and the polymerized micelle must occur near the surface of the micelles. The mass transfer rate of the solute between a polymerized micelle and the bulk solution should be faster than that of a normal micellar system; which allows penetration of the solute via dynamic equilibrium.

Effects of organic modifiers on chiral separation. It has been reported that the addition of an organic solvent may improve enantioselectivity for some compounds in MEKC. In general, the migration time of the sample increases with an increase in concentration of organic solvents. This is due to a decrease in EOF. Furuta and Doi [175] investigated these effects on chiral separation of diniconazole and uniconazole under optimum operating conditions. Organic solvents added were acetonitrile, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 2-methyl-1-propanol and 2-methyl-2-propanol. Concentrations of each solvent tested were 2% and 5% (v/v). Resolution of both compounds was significantly improved for all organic modifiers and generally increased with increasing concentrations. Enantiomers of uniconazole were only resolved with solutions containing organic solvents. The organic modifier added not only increases the hydrophobicity of the solution and influences the distribution of the solute among the micelle, the aqueous phase and the CD, but can also compete with the solute in forming an inclusion complex with the CD. This indicates that the enantioselectivity by CD would decrease when bulkier and more hydrophobic organic modifiers are used, as their molecules would be expected to form stable inclusion complexes. Authors have emitted the hypothesis that the modifier molecule might be included together with the solute in the CD cavity and play a positive role in filling the space. Tight filling of a molecule to be complexed to a CD cavity is one of the important factors for chiral recognition by CD. Hence, there

might be an optimum content of organic modifier for each compound [170,172,177].

However, the use of acetonitrile as modifier with poly(D-SUV) produces opposite effects to methanol [177]. It is known that acetonitrile can displace solutes from CD cavities which would be expected to reduce the chiral recognition of the γ-CD.

Effects of types and buffer concentrations on chiral separation. Noroski et al. [176] observed no chiral resolution when they used 0.05 *M* sodium phosphate buffer at pH 9.5, or borate buffer at low concentration (less than 0.01 *M*) for the determination of enantiomer of a cholesterol lowering-drug. However, they obtained a large improvement of resolution when increasing borate concentration. Increasing borate concentration from 10 to 150 m*M* highly improved resolution (0 to 6.6). An increase in ionic strength increases the viscosity of the run buffer which slows the EOF and increases migration times. An additional mechanism is suspected. Borate buffer appears essential for chiral resolution. Borate anion may complex with the vicinal hydroxyl group of the analyte. This complex would have a greater net negative charge (−2) and its motion would be retarded due to increased electrophoretic attraction to the anode. Furthermore, since CDs are composed of glucose units containing hydroxyl groups at asymmetric carbons, it is possible that a ternary complex between analyte, CD and borate is formed.

The same results were obtained by other authors. For Furuta and Doi [175], increasing borate buffer from 50 to 100 m*M* increased resolution of diniconazole from 1.08 to 1.49. Wang and Warner [177], with poly(D-SUV). as micellar phase, observed an improvement of enantiomeric resolution and a lengthening of migration times when borate buffer concentrations increased over the range 5–45 m*M*.

Effects of chiral additives on chiral separation. Effect of some chiral additives (sodium D-camphor-10-sulfonate and L-menthoxyacetic acid), which can be included in the CD cavity, on chiral recognition of five drugs was investigated by Nishi and Fukuyama [170]. The separation factors and resolution of the enantiomers were improved with increasing concentrations of the chiral additive up to a certain concentration. A higher concentration reduced resolution because of an increase in retention factor, and thus there was deviation from optimum retention factor. The solute will be included in the γ-CD cavity together with the chiral additive. Interactions of the solute with both the CD and the chiral additive, through the hydrophobic or ionic portions will probably give enhanced enantioselectivity.

Optimum chiral separation will be obtained by adjusting the concentrations of CDs and chiral additives for each enantiomeric solute. The addition of the former reduces the migration times of the solute while the latter increases it.

Effects of addition of urea on chiral separation. For Furuta and Doi [175], addition of urea in a 0–4 *M* concentration range increased enantiomeric resolution of diniconazole from 1.29 to 1.70.

Effects of other operating parameters on chiral separation. The selection of the pH of the buffer solution is important for chiral separation. During their investigation for finding optimum conditions for enantiomeric separation of diniconazole by CD-MEKC, Furuta and Doi [175] found better resolution at pH 9 than at pH 8 or 8.5.

They also increased applied voltage in a range 10–20 kV. A decrease in resolution was observed with increasing voltage. Since an increase in voltage increases the rate of EOF, migration times became shorter because the analytes spend less time interacting with the chiral CD cavity. However, thermal effects due to Joule heating at higher applied voltage can also alter resolution. The same results were found by Noroski et al. [176] for enantiomeric separation of a cholesterol lowering-drug using β-CD.

Furuta and Doi [175] also varied the temperature: by increasing the temperature from 22 to 30°C enantiomeric resolution of diniconazole decreased from 1.6 to 1.20.

Noroski et al. [176] tested the effects of capillary length on chiral resolution of a cholesterol lowering-drug: a longer capillary results in a greater resolution and a longer migration time.

4.4. Use of CDs in isotachophoresis (ITP)

CDs have also been successfully used in ITP for the separation of enantiomers by several authors

[182–186]. A mathematical model describing the electrophoretic migration of strong electrolytes in the presence of neutral complexing agent was proposed also by Dubrovcakova et al. [187]. The model allows the determination of formation constants and mobilities of complex compounds from experimental isotachophoretic data.

4.5. Use of CDs in electrochromatography (EC)

Electrochromatography uses the electroosmotic flow for mobile phase delivery instead of a high pressure pump as in liquid chromatography. Mayer and Schuring [188,189] and Mayer et al. [190] prepared a CD derivative coated capillary tube (Chirasil-Dex) for gas chromatography and supercritical fluid chromatography and this capillary was also used for EC.

Armstrong et al. [191] used a capillary coated with a derivatized β-CD and organohydrosilane copolymer in EC.

4.6. Use of CDs in capillary gel electrophoresis (CGE)

CDs can be immobilized by using a gel. The gel matrix suppresses electroosmosis and serves as a support of the chiral selector. The separation of enantiomers by CD-CGE was successfully utilized by Guttman et al. [192] using β-CD in a polyacrylamide gel matrix and Cruzado and Vigh [193] using acetyl-β-CD with acrylamide in cross-linked and linear gels.

4.7. Use of CDs for the quantitation of drugs in biological fluids

Although capillary electrophoresis using CDs in the running buffer has been shown to provide rapid, inexpensive, and highly efficient analytical methods for the separation of isomers, few reports described its quantitative applications in biological fluids. Applications of the use of CDs for the quantitation of drugs in biological samples are summarized in Table 7. Example of electropherogram of human urine is given in Fig. 12. To eliminate the endogenous compounds liquid–liquid extraction was mostly used [144,194,195,197–199,203]. In the method described by Sheppard et al. [202] no extraction was used to clean up the samples, urine was simply filtered prior to analysis. A method to quantify hexobarbital directly in rat plasma has been described by Francotte et al. [196]. To avoid coelution of the drug enantiomers with protein plasma, the SDS concentration was increased, allowing solubilization of plasma proteins in the SDS micelles, together with the addition of an organic modifier such as methanol. This method has the merit of permitting direct and rapid analysis of drug enantiomers in plasma samples without the need for any pre-treatment such as deproteinization or extraction.

Although very high mass sensitivity detection is

Table 7
Some enantiomeric separations in biological fluids based on complexation with CDs using CE

Enantiomers separated	Type of CD	Mode	Biological sample	Ref.
Bupivacaine and Verapamil	TM-β-CD	CZE	Serum	[194]
Cicletanine	γ-CD	MECK	Plasma	[195]
Warfarin	Methyl-β-CD	CZE	Plasma	[144]
Nonsteroidal antiaromatase drugs and intermediates	α-, β-, γ-CD and DM β-CD	CZE and MECK	Plasma	[196]
Dimethindene and *N*-desmethyl-dimethindene	HP-β-CD	CZE	Urine	[197]
Verapamil and norverapamil	TM-β-CD	CZE	Plasma	[198]
Mephenytoin, 4-hydroxyphenytoin and 4-hydroxymephenytoin	β-CD	MECK	Urine	[199]
Verapamil	TM-β-CD	CZE	Plasma	[200]
Terbutaline, ephedrine bambuterol, brompheniramine, propranolol	Alkyl substituted-β-CD	CZE and ITP	Plasma	[201]
Terbutaline, ephedrine	*Heptakis*-β-CD	CZE	Urine	[202]
Zopiclone and metabolites	β-CD	CZE	Urine and saliva	[203]

DM β-CD = dimethyl β-CD; TM β-CD = trimethyl β-CD; HP β-CD = hydroxypropyl β-CD.

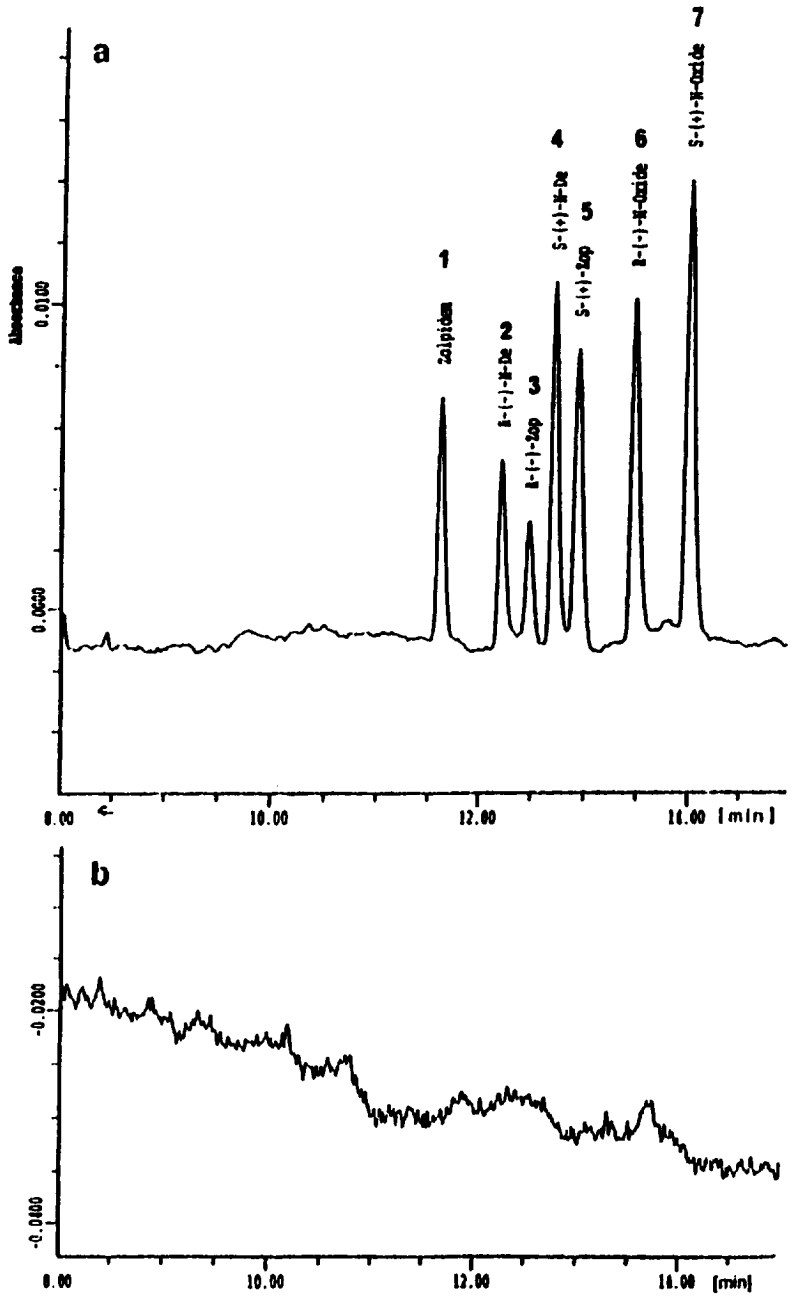


Fig. 12. Electropherogram of (a) human urine 6 h after oral administration of zopiclone and (b) blank urine. Peaks: 1 = internal standard; 2 = *R*-(−)-*N*-desmethylzopiclone; 3 = *R*-(−)-zopiclone; 4 = *S*-(+)-*N*-desmethylzopiclone; 5 = *S*-(+)- zopiclone; 6 = *R*-(−)-zopiclone-*N*-oxide; 7 = *S*-(+)-zopiclone-*N*-oxide. Data from Hempel et al. [203].

obtained in CZE, concentration sensitivity is poor. Thus determination of drugs in biosamples, where concentrations in the range nmol–pmol/l are common, is hampered. Pälmarsdöttir and Edholm [201] used column liquid chromatography for sample pretreatment and pre-concentration on line with CZE. This technique was successfully applied to the enantiomers' separation of terbutaline in plasma.

A new high-performance capillary electrophoresis method (CE/frontal analysis) was developed by Ohara et al. [200] for the enantioselective determination of the unbound concentration of basic drug (verapamil) under plasma protein binding equilibrium. The unbound concentrations of verapamil enantiomers in high concentration of human serum albumin solution could be directly determined. The reliability of this method was confirmed by comparison with a conventional ultrafiltration–chiral HPLC method.

5. Comparison between HPLC and CE

CDs exhibit a great range of desirable properties useful in both HPLC, CZE and MEKC. For example, CDs absorb very little in the UV region. They are stable over a wide pH range, insensitive to light and non toxic. The solubility is sufficient and can be increased by substitution and by addition of urea.

Capillary electrophoresis can be considered as complementary to other analytical techniques such as HPLC for the separation of enantiomers. CE presents the following advantages, (i) the amounts of samples and separation buffer are much less than those used in HPLC, (ii) usually the chiral selector is dissolved in the BGE and thus the use of expensive chiral columns is not required, (iii) relatively high efficiencies are obtained (more than 100 000 theoretical plates), (iv) the capillary is equilibrated in a few minutes. The disadvantages of CE in comparison with HPLC are the lower reproducibility, the poorer sensitivity and the smaller possibility of preparative applications.

Abushoffa and Clark [204] examined methods by HPLC (β-CD or hydroxypropyl-β-CD as mobile phase additive or Cyclobond I or II CSP in the reversed-phase mode) and CE (CZE with β-CD) for resolution of the antischistosmiasis drug oxamniquine in the pharmaceutical preparation. The authors demonstrate the usefulness of CE as a tool to resolve chiral drugs and illustrate its advantages over HPLC in this case.

References

[1] E.J. Ariens, Trends Pharmacol. Sci., 7 (1986) 200.
[2] C. Roussel and A. Favrou, J. Chromatogr. A, 704 (1995) 67.
[3] A.M. Krstulovic, J.M. Gianviti, J.T. Burke and B. Mompon, J. Chromatogr. B, 426 (1988) 417.
[4] A. Cooper and D.D. MacNicol, J. Chem. Soc. Perk. Trans. II, Fasc. 8 (1978) 760.
[5] A.D. Cooper and T.M. Jefferies, J. Chromatogr., 637 (1993) 137.
[6] D.W. Armstrong, J. Liq. Chromatogr., 3 (1980) 895.
[7] J. Szejtli, Med. Res. Rev., 14 (1994) 353.
[8] I.W. Wainer, in I.W. Wainer (Editor), Drug Stereochemistry. Analytical Methods and Pharmacology, Marcel Dekker, New York, NY, 1993, 2nd ed., p. 139.
[9] C. Pham-Huy, B. Radenen, A. Sahui-Gnassi and J.R. Claude, J. Chromatogr. B, 665 (1995) 125.
[10] G.W. Ponder, S.L. Butram, A.G. Adams, C.S. Ramanathan and J.T. Stewart, J. Chromatogr. A, 692 (1995) 173.
[11] A.M. Claire Myles, D.J. Barlow, G. France and M.J. Lawrence, Biochim. Biophys. Acta, 1199 (1994) 27.
[12] T. Takeuchi, H. Asai and D. Ishii, J. Chromatogr., 357 (1986) 409.
[13] D.Y. Pharr, Z.S. Fu, T.K. Smith and W.L. Hinze, Anal. Chem., 61 (1989) 275.
[14] C. Pettersson, T. Arvidsson, A.L. Karlsson and I. Marle, J. Pharm. Biomed. Anal., 4 (1986) 221.
[15] J. Noroski, D. Mayo and J.J. Kirschbaum, J. Pharm. Biomed. Anal., 10 (1992) 447.
[16] D.T. Burns, J. Pharm. Biomed. Anal., 12 (1994) 1.
[17] S. Li and W. Purdy, J. Chromatogr., 625 (1992) 109.
[18] S.C. Chang, G.L. Reid III, S. Chen, C.D. Chang and D.W. Armstrong, Trends Anal. Chem., 12 (1993) 144.
[19] S. Friebe and G.J. Krauss, J. Chromatogr., 598 (1992) 139.
[20] S. Görög and M. Gazdag, J. Chromatogr. B, 659 (1994) 51.
[21] I.W. Wainer and D.E. Drayer, Drug Stereochemistry. Analytical Methods and Pharmacology, Marcel Dekker, New York, NY, 1988.
[22] D.W. Armstrong, T.J. Ward, R.D. Armstrong and T.E. Beesley, Science, 232 (1986) 1132.
[23] R. Furuta and H. Nakazawa, Chromatographia, 35 (1993) 555.
[24] P. Camilleri, C.A. Reid and D.T. Manallack, Chromatographia, 38 (1994) 771.
[25] A.F. Casy, A.D. Cooper, T.M. Jefferies, R.M. Gaskell, D. Greatbanks and R. Pickford, J. Pharm. Biomed. Anal., 9 (1991) 787.
[26] D.W. Armstrong and W. DeMond, J. Chromatogr. Sci,. 22 (1984) 411.

[27] D.W. Armstrong, C.D. Chang and S.H. Lee, J. Chromatogr., 539 (1991) 83.
[28] K. Fujimura, S. Suzuki, K. Hagashi and S. Masuda, Anal. Chem., 6 (1990) 2198.
[29] K. Nakamura, H. Fujima, H. Kitagawa, H. Wada and K. Makino, J. Chromatogr. A, 694 (1995) 111.
[30] N. Thuaud, B. Sebille, A. Deratani, B. Popping and C. Pellet, Chromatographia, 36 (1993) 373.
[31] N. Thuaud and B. Sebille, J. Chromatogr. A, 685 (1994) 15.
[32] G. Crini, G. Torri, B. Martel, L. Janus and M. Morcellet, Chromatographia, 41 (1995) 424.
[33] K. Fujimura, T. Ueda and T. Ando, Anal. Chem., 55 (1983) 336.
[34] Y. Kawaguchi, M. Tanaka, M. Nakae, K. Funazo and T. Shono, Anal. Chem., 55 (1983) 1852.
[35] M. Tanaka, Y. Kawaguchi, M. Nakae, Y. Mizobuchi and T. Shono, J. Chromatogr., 299 (1984) 341
[36] M. Tanaka, J. Okazaki, H. Ikeda and T. Shono, J. Chromatogr., 370 (1986) 293
[37] Y. Kuroda, T. Koto and H. Ogoshi, Bull. Chem. Soc. Jpn., 66 (1993) 1116.
[38] I. Ciucanu and W.A. König, J. Chromatogr. A, 685 (1994) 166.
[39] A.M. Stalcup and K.L. Williams, J. Liq. Chromatogr., 15 (1992) 29.
[40] N. Thuaud, B. Sebille, A. Deratani and G. Lelièvre, J. Chromatogr., 555 (1991) 53.
[41] D.W. Armstrong, S. Chen, C. Chang and S. Chang, J. Liq. Chromatogr., 15 (1992) 545.
[42] D.W. Armstrong, W. DeMond and B.P. Czech, Ann. Chem., 57 (1985) 481.
[43] D.W. Armstrong, J. Liq. Chromatogr., 7 (1984) 353.
[44] J. Zukowski, M. Pawlowska, M. Nagatkina and D.W. Armstrong, J. Chromatogr., 629 (1993) 169.
[45] W.L. Hinze, T.E. Riehl, D.W. Armstrong, W. DeMond, A. Alak and T. Ward, Ann. Chem., 57 (1985) 237.
[46] D.W. Armstrong, A.M. Stalcup, M.L. Holton, J.D. Duncan, J.R. Faulkner and S.C. Chang, Anal. Chem., 2 (1990) 1610.
[47] C.D. Chang and D.W. Armstrong, Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Chicago, IL, March 4–8, 1991, Paper No. 200.
[48] J. Florance and Z. Konteatis, J. Chromatogr., 543 (1991) 299.
[49] P. Macaudiere, M. Caude, R. Rosset and A. Tambuté, J. Chromatogr., 450 (1988) 255.
[50] M. Pawlowska, J. Zukowski and D.W. Armstrong, J. Chromatogr. A, 666 (1994) 485.
[51] M. Pawlowska, S. Chen and D.W. Armstrong, J. Chromatogr., 641 (1993) 257.
[52] M.L. Hilton, S.C. Chang, M.P. Gasper, M. Pawlowska, D.M. Armstrong and A.M. Stalcup, J. Liq. Chromatogr., 16 (1993) 127.
[53] C.N. Nakatsu and A.M. Stalcup, J. Liq. Chromatogr., 16 (1993) 209.
[54] T. Hargitai, Y. Kaida and Y. Okamoto, J. Chromatogr., 628 (1993) 11.
[55] C. Vandenbosch, D.L. Massart and W. Lindner, J. Pharm. Biomed. Anal., 10 (1992) 895.
[56] M. Tanaka, T. Shono, D.Q. Zhu and Y. Kawaguchi, J. Chromatogr., 469 (1989) 429.
[57] A.M. Stalcup, S.C. Chang and D.W. Armstrong, J. Chromatogr., 540 (1991) 113.
[58] M.I. Merino, E. Blanco Gonzales and A. Sanz-Medel, Anal. Chim. Acta, 234 (1990) 127.
[59] A.L.L. Duchateau, G.M.P. Heemels, L.W. Maesen and N.K. de Vries, J. Chromatogr., 603 (1992) 151.
[60] J. Zukowski, M. Pawlowska and D.W. Armstrong, J. Chromatogr., 623 (1992) 33.
[61] D.W. Armstrong, M.P. Gasper, S.H. Lee, N. Ercal and J. Zukowski, Amino Acids, 4 (1993) 402.
[62] A.M. Rizzi, R. Hirz, S. Cladrowa-Runge and H. Jonsson, Chromatographia, 39 (1994) 131.
[63] M.I. Merino, E. Blanco Gonzalez and A. Sanz-Medel, Microchim. Acta, 107 (1992) 73.
[64] V. Schurig, M. Jung, S. Mayer, M. Fluck, S. Negura and H. Jakubetz, J. Chromatogr. A, 694 (1995) 119.
[65] G. Quintero, M. Vo, G. Farkas and G. Vigh, J. Chromatogr. A, 693 (1995) 1.
[66] K. Cabrera and G. Schwinn, Kontakte (Darmstadt), 3 (1989) 3.
[67] K. Cabrera and G. Schwinn, Int. Lab., 20 (1990) 28.
[68] A. Favrou and C. Roussel, in A.R. Hedges (Editor), Minutes 6th Int. Symp. Cyclodextrins, Chicago, April 1992, Editions de la Santé, Paris, 1992, p. 603.
[69] R.H. Pullen, J.J. Brennan and G. Patonay, J. Chromatogr. A, 691 (1995) 187.
[70] J. Zukowski, D. Sybilska, J. Bojarski and J. Szejtli, J. Chromatogr., 436 (1988) 381.
[71] J. Debowski, D. Sybilska and J. Jurczak, J. Chromatogr., 237 (1982) 303.
[72] J. Debowski, D. Sybilska and J. Jurczak, J. Chromatogr., 282 (1983) 83.
[73] J. Debowski, D. Sybilska and J. Jurczak, Chromatographia, 16 (1982) 198.
[74] J. Debowski, J. Jurczak, D. Sybilska and J. Zukowski, J. Chromatogr., 329 (1985) 206.
[75] J. Zukowski, D. Sybilska and J. Bojarski, J. Chromatogr., 364 (1986) 225.
[76] D. Sybilska, M. Asztemborska, A. Bielejewska, J. Kowalczyk, H. Dodziuk, K. Duszczyk, H. Lamparczyk and P. Zarzycki, Chromatographia, 35 (1993) 637.
[77] H. Lamparczyk, P.K. Zarzycki and J. Nowakowska, J. Chromatogr. A, 668 (1994) 413.
[78] H. Lamparczyk, P. Zarzycki, R.J. Ochocka, M. Asztemborska and D. Sybilska, Chromatographia, 31 (1991) 157.
[79] A. Walhagen and L.E. Edholm, Chromatographia, 32 (1991) 215.
[80] M. Gazdag, G. Szepesi and L. Huszär, J. Chromatogr., 351 (1986) 128.
[81] M. Gazdag, G. Szepesi and L. Huszär, J. Chromatogr., 371 (1986) 227.
[82] M. Gazdag, G. Szepesi and L. Huszär, J. Chromatogr., 436 (1988) 31.
[83] K. Shimada, K. Mitamura, M. Morita and K. Hirakata, J. Liq. Chromatogr., 16 (1993) 3311.

[84] S. Eto and H. Noda, J. Chromatogr., 579 (1992) 253.
[85] A.K. Chatjigakis, Ph.J.P. Cardot, A.W. Coleman and H. Parrot-Lopez, Chromatographia, 36 (1993) 174.
[86] R. Nowakowski, P.J.P. Cardot, A.W. Coleman, E. Villard and G. Guiochon, Anal. Chem., 67 (1995) 259.
[87] T. Takeuchi and N. Nagae, J. Chromatogr., 595 (1992) 121.
[88] M. Gazdag, G. Szepesi and K. Mihalyfi, J. Chromatogr., 450 (1988) 145.
[89] G. Szepesi and M. Gazdag, J. Pharm. Biomed. Anal., 6 (1988) 623.
[90] A.F. Fell, T.A.G. Noctor, J.E. Mama and B.J. Clark, J. Chromatogr., 434 (1988) 377.
[91] A. Valiente Barderas and F. Duprat, J. Liq. Chromatogr., 17 (1994) 1709.
[92] G.B. Cox and R.W. Stout, J. Chromatogr., 384 (1987) 315.
[93] B.A. Bidlingmeyer, J.K. Del Rios and J. Korpl, Anal. Chem., 54 (1982) 442.
[94] B.J. Clark and J.E. Mama, J. Pharm. Biomed. Anal., 7 (1989) 1883.
[95] M. Sueyasu, T. Ikeda, K. Otsubo, T. Taniyama, T. Aoyama and R. Oishi, J. Chromatogr. B, 665 (1995) 133.
[96] N. Husain and I.M. Warner, Am. Lab., 11 (1993) 80.
[97] D. Sybilska, Ordered Media in Chemical Separations (ACS Symposium series, No. 326), American Chemical Society, Washington, DC, 1987, p. 218.
[98] M. Katagi, H. Nishioka, K. Nakajima, H. Tsuchihashi, H. Fujima, H. Wada, K. Nakamura and K. Makino, J. Chromatogr. B, 676 (1996) 35.
[99] G. Geisslinger, K. Dietzel, D. Loew, O. Schuster, G. Rau, G. Lachmann and K. Brune, J. Chromatogr. B, 491 (1991) 139.
[100] J. He, A. Shibukawa, T. Nakagawa, H. Wada, H. Fujima, F. Imai and Y. Go-oh, Chem. Pharm. Bull., 41 (1993) 544.
[101] W. Naidong and J.W. Lee, J. Pharm. Biomed. Anal., 11 (1993) 785.
[102] W. Naidong and J.W. Lee, J. Pharm. Biomed. Anal., 12 (1994) 551.
[103] S.S. Baum and H. Rommelspacher, J. Chromatogr. B, 660 (1994) 235.
[104] D. Castoldi, A. Oggioni, M.I. Renoldi, E. Ratti, S. Di Giovine and A. Bernareggi, J. Chromatogr. B, 655 (1994) 243.
[105] A. Shibukawa, M. Kadohara, J. He, M. Nishimura, S. Naito and T. Nakagawa, J. Chromatogr. A, 694 (1995) 81.
[106] B. Rochat, M. Amey and P. Baumann, Ther. Drug Monit., 17 (1995) 273.
[107] C.B. Eap, L. Koeb, K. Powell and P. Baumann, J. Chromatogr. B, 669 (1995) 271.
[108] Y. Deng, W. Maruyama, P. Dostert, T. Takahashi, M. Kawai and M. Naoi, J. Chromatogr B., 670 (1995) 47.
[109] H. Kim and C.C. Lin, J. Pharm. Biomed. Anal., 13 (1995) 1415.
[110] B. Ba,G. Eckart and J. Leube, J. Chromatogr. B, 572 (1991) 277.
[111] S. Eto, H. Noda, M. Minemoto, A. Noda and Y. Mizukami, Chem. Pharm. Bull., 39 (1991) 2742.
[112] K. Röna and I. Szabo, J. Chromatogr., 573 (1992) 173.
[113] S. Eto, H. Noda and A. Noda, J. Chromatogr. B, 658 (1994) 385.
[114] K. Sakamoto and Y. Nakamura, Xenobiotica, 24 (1994) 329.
[115] R.H. Pullen, J.J. Brennan, R. Lammers and G. Patonay, Anal. Chem., 67 (1995) 1903.
[116] S. Fanali, in A. Chrambach, M.J. Dunn and B.J. Radola (Editors), Advances in Electrophoresis, V.C.H., Vol. 7, 1994.
[117] R. Vespalee and P. Böeek, Electrophoresis, 15 (1994) 755.
[118] S.A.C. Wren, R.C. Rowe and R.S. Payne, Electrophoresis, 15 (1994) 774.
[119] S. Terabe, K. Otsuka and H. Nishi, J. Chromatogr. A, 666 (1994) 295.
[120] H. Nish and S. Terabe, J. Chromatogr. A, 694 (1995) 245.
[121] S.A.C. Wren and R.C. Rowe, J. Chromatogr., 603 (1992) 235.
[122] Y.Y. Rawjee, D.U. Staerk and G. Vigh, J. Chromatogr., 633 (1993) 291.
[123] Y.Y. Rawjee, R.L. Williams and G. Vigh, J. Chromatogr. A, 652 (1993) 233.
[124] W. Lindner, B. Böhs and V. Seidel, J. Chromatogr. A, 697 (1995) 549.
[125] C. Vogt, A. Georgi and G. Werner, Chromatographia, 40 (1995) 287.
[126] S. Fanali and P. Bocek, Electrophoresis, 11 (1990) 757.
[127] S. Palmarsdottir, J. Chromatogr. A, 666 (1994) 337.
[128] C.L. Copper, J.B. Davis, R.O. Cole and M.S. Sepaniak, Electrophoresis, 15 (1994) 785.
[129] T.E. Peterson, J. Chromatogr., 630 (1993) 353.
[130] B. Chankvetadze, G. Endresz and G. Blaschke, J. Chromatogr. A, 700 (1995) 43.
[131] H. Nishi, K. Nakamura, H. Nakai and T. Sato, J. Chromatogr. A, 678 (1994) 333.
[132] A.W. Garrison, P. Schmitt and A. Kettrup, J. Chromatogr. A, 688 (1994) 317.
[133] A. Aumatell, R.J. Wells and D.K.Y. Wong, J. Chromatogr A., 686 (1994) 293.
[134] I.E. Valko, H.A.H. Billiet, J. Frank and K.C.A.M. Luyben, J. Chromatogr. A, 678 (1994) 139.
[135] L.A. St Pierre and K.B. Sentell, J. Chromatogr. B, 657 (1994) 291.
[136] S.K. Branch, U. Holzgrabe, T.M. Jefferies, H. Mallwitz and N.W. Matchett, J. Pharm. Biomed. Anal., 12 (1994) 1507.
[137] S. Fanali and Z. Aturki, J. Chromatogr. A, 694 (1995) 297.
[138] S. Piperaki, S.G. Penn and D.M. Goodall, J. Chromatogr. A, 700 (1995) 59.
[139] M.W. Matchett, S.K. Branch and T.M. Jefferies, J. Chromatogr. A, 705 (1995) 351.
[140] M.W.F. Nielen, Anal. Chem., 65 (1993) 885.
[141] S.A.C. Wren and R.C. Rowe, J. Chromatogr., 635 (1993) 113.
[142] T. Schmitt and H. Engelhardt, Chromatographia, 37 (1993) 475.
[143] B. Chankvetadze, G. Endresz and G. Blaschke, J. Chromatogr. A, 704 (1995) 234.
[144] P. Gareil, J.P. Gramond and F. Guyon, J. Chromatogr. B, 615 (1993) 317.
[145] A. Guttman, A. Paulus, A.S. Cohen, N. Grinberg and B.L. Karger, J. Chromatogr., 438 (1988) 211.

[146] S. Fanali, J. Chromatogr., 603 (1989) 298.
[147] J. Snopeck, H. Soini, M. Novotny, E. Smolkova-Keulemansova and I. Jelinek, J. Chromatogr., 559 (1991) 215.
[148] K.D. Altria, D.M. Goodall and M.M. Rogan, Chromatographia, 34 (1992) 19.
[149] P. Baumy, P. Morin, M. Dreux, M.C. Viaud, S. Boye and G. Guillaumet, J. Chromatogr. A, 707 (1995) 311.
[150] I. Bechet, P. Paques, M. Fillet, P. Hubert and J. Crommen, Electrophoresis, 15 (1994) 818.
[151] M. Heuermann and G. Blaschke, J. Chromatogr., 648 (1993) 267.
[152] I.E. Valko, H.A.H. Billiet, J. Frank and K.C.A.M. Luyben, Chromatographia, 38 (1994) 730.
[153] E.C. Rickard and R.J. Bopp, J. Chromatogr. A, 680 (1994) 609.
[154] B.A. Ingelse, F.M. Everaerts C. Desiderio and S. Fanali, J. Chromatogr. A, 709 (1995) 89.
[155] S. Fanali, J. Chromatogr., 545 (1991) 437.
[156] A. Guttman and N.Cooke, J. Chromatogr. A, 680 (1994) 157.
[157] F. Thunecke, H. Hartenstein, C. Sicker and C. Vogt, Chromatographia, 38 (1994) 470.
[158] M.M. Rogan, K.D. Altria and D.M. Goodall, Electrophoresis, 15 (1994) 808.
[159] H. Nishi, H. Nakamura, H. Nakai, T. Sato and S. Terabe, Chromatographia, 40 (1995) 638.
[160] J.Szjtli, Inclusion Complexes, Akademia Kiado, Budapest, 1982.
[161] S. Terabe, Trends Anal. Chem., 8 (1989) 129.
[162] S. Terabe, K. Otsuka and H. Nishi, J. Chromatogr. A, 666 (1994) 295.
[163] H. Nishi and S. Terabe, J. Chromatogr. A, 694 (1995) 245.
[164] S. Terabe, H. Ozaki, K. Otsuka and T. Ando, J. Chromatogr., 332 (1985) 211.
[165] T. Schmitt and H. Engelhardt, J. High Resolut. Chromatogr., 16 (1993) 525.
[166] T. Schmitt and H. Engelhardt, Chromatographia, 37 (1993) 475.
[167] N.W. Smith, J. Chromatogr. A, 652 (1993) 259.
[168] A. Nardi, A. Eliseev, P. Bocek and S. Fanali, J. Chromatogr., 638 (1993) 247.
[169] K. Otsuka and S. Terabe, Trends Anal. Chem., 12 (1993) 125.
[170] H. Nishi and T. Fukuyama, J. Chromatogr., 553 (1991) 503.
[171] S. Terabe, Y. Miyashita, Y. Ishihama and O. Shibata, J. Chromatogr., 636 (1993) 47.
[172] V.C. Anigbogu, C.L. Copper and M.J. Sepaniak, J. Chromatogr. A, 705 (1995) 343.
[173] S. Terabe, J. Pharm. Biomed. Anal., 10 (1992) 705.
[174] S. Terabe, Y. Miyashita, O. Shibata, E.R. Barnhart, L.R. Alexander, D.G. Patterson, B.L. Karger, K. Hosoya and N. Tanaka, J. Chromatogr., 516 (1990) 23.
[175] R. Furuta and T. Doi, J. Chromatogr. A, 676 (1994) 431.
[176] J.E. Noroski, D.J. Mayo and M. Moran, J. Pharm. Biomed. Anal., 13 (1995) 45.
[177] J. Wang and I.M. Warner, J. Chromatogr. A, 711 (1995) 297.
[178] S.A.C. Wren and R.C. Rowe, J. Chromatogr., 635 (1993) 113.
[179] R. Furuta and T. Doi, Electrophoresis, 15 (1994) 1322.
[180] T. Okubo, H. Kitano and N. Ise, J. Phys. Chem., 80 (1976) 2661.
[181] I. Satake, T. Iketanoue, T. Tafeshita, K. Hayakawa and T. Maeda, Bull. Chem. Soc. Jpn., 58 (1985) 2746.
[182] J. Snopek, I. Jelinek and E. Smolkova-Keulemansova, J. Chromatogr., 438 (1988) 211.
[183] I. Jelinek, J. Snopek and E. Smolkova-Keulemansova, J. Chromatogr., 439 (1988) 386.
[184] I. Jelinek, J. Dohnal, J. Snopek and E. Smolkova-Keulemansova, J. Chromatogr., 464 (1989) 139.
[185] I. Jelinek, J. Snopek, J. Dian and E. Smolkova-Keulemansova, J. Chromatogr., 472 (1989) 308.
[186] J. Snopek, I. Jelinek and E. Smolkova-Keulemansova, J. Chromatogr., 609 (1992) 1.
[187] E. Dubrovcakova, B. Gas, J. Vacik and E. Smolkova-Keulemansova, J. Chromatogr., 623 (1992) 337.
[188] S. Mayer and V. Schuring, J. High Resolut. Chromatogr., 15 (1992) 129.
[189] S. Mayer and V. Schuring, J. Liq. Chromatogr., 16 (1993) 915.
[190] S. Mayer, M. Schleimer and V. Schuring, J. Microcol. Sep., 6 (1994) 43.
[191] D.W. Armstrong, Y. Tang, T. Ward and M. Nichols, Anal. Chem., 65 (1993) 1114.
[192] A. Guttman, A. Paulus, A.S. Cohen, N. Grinberg and B.L. Karger, J. Chromatogr., 448 (1988) 41.
[193] I.D. Cruzado and G. Vigh, J. Chromatogr., 608 (1992) 421.
[194] H. Soini, M.G. Riekkola and M.V. Novotny, J. Chromatogr., 608 (1992) 265.
[195] J. Prunonosa and R. Obach, J. Chromatogr. B, 574 (1992) 127.
[196] E. Francotte, S. Cherkaoui and M. Faupel, Chirality, 5 (1993) 516.
[197] M. Heuermann and G. Blaschke, J. Pharm. Biomed. Anal., 12 (1994) 753.
[198] J.M. Dethy, S. De Broux, M. Lesne, J. Longstreth and P. Gilbert, J. Chromatogr. B, 654 (1994) 121.
[199] C. Desiderio, S. Fanali, A. Küpfer and W. Thormann, Electrophoresis, 15 (1994) 87.
[200] T. Ohara, A. Shibukawa and T. Nakagawa, Anal. Chem., 67 (1995) 3520.
[201] S. Palmarsdottir and L.E. Edholm, J. Chromatogr., 693 (1995) 131.
[202] R.L. Sheppard, X.C. Tong, J.Y. Cai and J.D. Henion, Anal. Chem., 67 (1995) 2054.
[203] G. Hempel and G. Blaschke, J. Chromatogr. B, 675 (1996) 139.
[204] A.M. Abushoffa and B.J. Clark, J. Chromatogr. A, 700 (1995) 51.