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Chiral Separation by Chromatographic and Electromigration Techniques. A Review

Gerald Gübitz* and Martin G. Schmid
Institute of Pharmaceutical Chemistry and Pharmaceutical Technology, Karl-Franzens University, Universitätsplatz 1, A-8010 Graz, Austria

Dedicated to Prof. W. Fleischhacker on occasion of his 70th birthday

ABSTRACT: This review gives a survey of different chiral separation principles and their use in high-performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC), thin-layer chromatography (TLC), capillary electrophoresis (CE) and capillary electrochromatography (CEC) highlighting new developments and innovative techniques. The mechanisms of the different separation principles are briefly discussed and some selected applications are shown. Copyright © 2001 John Wiley & Sons, Ltd.

Key words: chromatography; capillary electrophoresis; capillary electrochromatography; enantiomer separation

Introduction

Almost half of the drugs in use are chiral. It is well known that the pharmacological effect is restricted in most of the cases to one of the enantiomers (eutomer) [1]. Nonetheless, only about 25% of drugs are administered as pure enantiomers. There can be qualitative and quantitative differences in the activity of the enantiomers. The pharmacologically inactive enantiomer (distomer) can show unwanted side effects; in some cases antagonistic and even toxic effects are observed. The enantiomers can differ in absorption, distribution, protein binding and affinity to the receptor [2]. Furthermore, the metabolic pathways can differ.

The guidelines for the development of new drugs issued by regulating authorities require efficient methods for enantiomeric purity control. For enantiomer separation on analytical scale a great variety of methods based on chromatographic techniques such as HPLC, GC, SFC, TLC have been developed during the past three decades. More recently, CE and CEC have also been shown to be useful techniques for this purpose.

The ideal way to obtain pure drug enantiomers would be enantioselective synthesis. This is, however, not always practicable and usually complicated and expensive. Therefore, the separation of racemic mixtures of intermediate or final products is often required. In addition to classical methods, as there are formation of diastereomeric pairs using chiral reagents followed by repeated recrystallization or the use of stereoselective enzymes, chromatographic techniques, especially LC, have become increasingly relevant also on preparative scale.

This article will present an overview of chromatographic and electroseparation techniques for chiral separation. Since thousands of papers on applications of chiral separation methods have already appeared, a comprehensive review of all the publications in this field would fill several books. Therefore, this review

*Correspondence to: Institute of Pharmaceutical Chemistry and Pharmaceutical Technology, Karl-Franzens University, Universitätsplatz 1, A-8010 Graz, Austria. E-mail: guebitz @uni-graz.at

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will focus on the description of chiral separation principles, mentioning several milestones and highlighting new approaches and some selected examples for applications. The reader will be referred to specialized review articles for details. Several recent comprehensive reviews and book articles summarize chromatographic [3-9] and electrophoretic methods [10-17] for chiral separation in general.

High performance liquid chromatography (HPLC)

HPLC can be used to separate enantiomers either indirectly with chiral derivatization reagents or directly with chiral stationary phases or chiral mobile phase additives. Each of these techniques has advantages and disadvantages. Indirect separation is based on the use of chiral derivatization reagents to form diastereomeric derivatives which differ in their chemical and physical behavior and therefore can be separated on achiral stationary phases. This approach circumvents the need for expensive columns with chiral stationary phases and is more flexible; however, derivatization represents an additional step which can involve undesirable side reactions, formation of decomposition products and racemization. Furthermore, the chiral derivatization reagent has to be of high enantiomeric purity and the presence of derivatizable groups in the analyte is a prerequisite. The direct approach using columns with chiral stationary phases is more convenient and also applicable for separations on preparative scale, but requires a collection of expensive columns to solve a variety of problems, is required. The chiral mobile phase approach represents a simple and flexible alternative, which is, however, not always applicable. Since the mobile phase containing the chiral selector cannot be reused, this technique cannot be applied with expensive reagents.

Indirect separation

Specialized reviews report on the use of chiral derivatization reagents for amino acids [18], drug enantiomers bearing hydroxy groups [19], neurotransmitters [20], amphetamine analogues [21] and for biomedical chromatography in general [22]. An excellent overview of fluorescent chiral derivatization reagents has been given by Toyo'oka [23].

The most frequently used chiral derivatization reagents are 1-(9-fluorenyl)ethylchloroformate and *o*-phtaldialdehyde in combination with chiral thiols [23]. (*O*,*O*′-*R*,*R*)-diacylated tartaric acid anhydrides were used for derivatization of β blockers [24]. Kleidernigg *et al*. [25] introduced a new chiral derivatization reagent, (1*R*,2*R*)- or (1*S*,2*S*)-*N*-[(2-isothiocyanato)cyclohexyl]-3,5 dinitrobenzoylamide (DDITC) for the derivatization of primary and secondary amines and amino alcohols. 1-(6-Methoxy-2-naphthyl)ethyl isothiocyanate (NAP-IT) and 2-(6-methoxy-2-naphthyl)-1-propylchloroformate (NAP-C) were used by Büschges *et al*. [26] for the derivatization of adrenoceptor antagonists and antiarrythmic drugs. Brückner and Wachsmann [27] synthesized *N*-[4-[(*S*)-1-carbamoyl-2-methyl-propylamino] - 6 - chloro - [1, 3, 5]triazin - 2-yl] - L -phenylalanine starting from cyanuric chloride and used this reagent for the indirect enantioseparation of amino acids. A new chiral fluorescent tagging reagent, (1,*R*,2*R*)-*N*-[(2-isothiocyanato)cyclohexyl]-6-methoxy-4-quinolinylamide was prepared by Kleidernigg and Lindner [28] and applied to amino acids and amines. A series of new fluorescent chiral benzoxadiazole-amino acid derivatives for amines were synthesized by Al-Kindy *et al*. [29]. Yasaka *et al*. [30] reported the preparation of (*S*)-(+)-1-methyl-2-(6,7-dimethoxy-2,3-naphthyalimido)ethyl trifluoromethansulfonate as a chiral fluorescent derivatization reagent for carboxylic acids. More recently, Inoue *et al*. [31] introduced 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride as a chiral fluorescent labeling reagent for prolyl dipeptides.

Direct methods

Overviews of different chiral separation principles and various applications to different compound classes are given in References [3–7].

Specialized reviews report on the application of different HPLC approaches to the chiral separation of various drug classes [32], NSAIDs [33], drugs possessing carboxyl groups [34], β-

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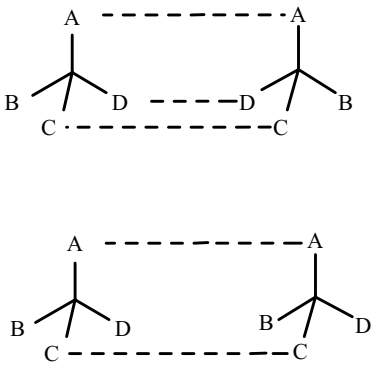

Figure 1. Three-point interaction model

adrenoceptor blocking drugs [35], the chiral drug analysis in biological fluids [36] and the application of column switching methods in chiral drug analysis in biological samples [37] .

Lipkowitz discusses theoretical aspects of different separation principles based on atomic-level molecular modeling [38]. Several models for the requirements to obtain chiral recognition have been discussed. The most reliable model is the three-point interaction model which postulates that three interactions have to take effect and at least one of them has to be stereoselective (Figure 1) [39]. Some modifications of this model have been discussed, but in general this model can be applied to most of the chiral separation principles.

The different chiral separation principles will be discussed in the following sections.

Phases based on multiple hydrogen bonds. This principle was first reported by Dobashi and Hara [40,41] using amino acid amides as chiral selectors chemically bonded to silica. The authors applied this principle to the chiral separation of amino acid and hydroxy acid derivatives using non-aqueous mobile phases. On a tartaric acid amide phase hydroxy carbonyl, β-amino alcohol derivatives, diols, barbiturates and hydantoines were resolved [42]. Since hydrogen bonds as exclusive forces are not very strong, the application of such phases is limited.

Chiral π-donor and π-acceptor phases. The principle was introduced by Pirkle [43,44]. The first phase developed in this group contained (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine (*R*)-*N*-DNBPG as a chiral selector with π-acceptor properties. Subsequently, a considerable number of chiral brush-type π-acceptor and π-donor phases have been developed by this group including several types of dinitrobenzoyl amino acid based chiral stationary phases (CSPs), hydantoin-, 2-arylamidoalkane-, *N*-aryl amino acid-, phthalide- and naproxen-derived CSPs and found application to a broad spectrum of compounds including several drugs. An overview of the immense work done in this lab is given by Welch [45]. Pirkle proposed a chiral recognition model for the *N*-DNBPG phase based on π–π interactions, dipole stacking and hydrogen bondings [46]. Several specially designed CSPs were developed making use of the reciprocal nature of chiral recognition by immobilizing the target analyte and checking several compounds as potential chiral selectors [47]. Thereby, special phases for the chiral separation of β-blockers [48] or NSAIDs [49–51], for example, were developed. The Whelk-O 1 CSP, prepared in the Pirkle lab and commercialized by Regis Technologies (Morton Grove, IL, USA), was shown to be applicable to a broad range of compounds [52].

2,4,6-Trichloro-1,3,5-triazine was used to bind different π-basic chiral selectors to silica gel [53, 54]. These phases showed enantioselectivity for amino acid derivatives containing π-acid groups. CSPs containing a naphthylethylamine moiety were prepared via (*R*,*R*) tartaric acid [55] or urea derivatives of amino acids [56]. Machida *et al*. [57] synthesized a tartaric diamide phase bearing a *p*-chlorophenyl residue and applied it to the resolution of 1,2-diols, 2,2′-dihydroxy-1,1′-binaphthyl and some β-blockers. Cholic and deoxycholic acid derivative phases found applications in the chiral separation of derivatized amino acids, amines, alcohols, hydantoins [58] and 3-hydroxy-benzodiazepin-2-ones [59].

Hyun and Min [60] prepared a CSP starting from (*R*)-4-hydroxyphenylglycine and grafting (*R*)-*N*-butanoyl-4-allyloxyphenylglycine *N*-propylamide to silica gel and studied the resolution behaviors of *N*-Pr-amides, *N*,*N*-di-Et amides and Et esters of *N*-(3,5-dinitrobenzoyl) amino acids. 3,5-dinitrobenzoyl groups carrying selectors derived from trans-1,2-diaminocyclohexane [61]

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and 1,2-diphenylethane-1,2-diamine [62,63] were used as the basis for CSPs which resolved aromatic secondary alcohols and some carboxylic acids.

A new CSP, in which the 1,2-diphenylethane-1,2-diamine moiety was substituted by 1,2-diphenyl-1-amino ethane, showed enantioselectivity for various amides, ureas, carbamates and esters [64]. A CSP containing an ergot alkaloid as chiral selector was found to show enantioselectivity for 2-aryloxypropionic acids, analogs of chrysanthemic acid and NDAIDs [65]. Quinine and quinidine carbamate and hydrazide based ion-exchange CSPs have been recently prepared and applied to the chiral separation of amino acid derivatives and profens [66,67]. In addition to ionic interactions, hydrogen bondings and π–π interaction were proposed as forces for chiral recognition.

Combinatorial chemistry was used to design new selectors. Following the chiral reciprocity principle, Lewandowski *et al*. [68] found among a library of 140 synthesized 4-aryl-1,4-dihydropyrimidines a candidate which was used for the preparation of a polymeric CSP for the chiral separation of *N*-3,5-dinitrobenzoyl leucine. To find a suitable selector for *N*-2,5-dinitrobenzoylated amino acid derivatives, the same group attached a library of 36 different amino acid anilides to polymer beads which were used as an HPLC CSP [69]. After observing some enantioselectivity for the target analyte, packings with a reduced number of selectors were prepared ending up with several proline-based CSPs showing selectivities for *N*-3,5-dinitrobenzoyl leucine diallylamide up to 23. Welch *et al*. prepared a CSP based on the (*S*)-Glu-(*S*)-Leu fragment of a dipeptide library obtained by combinatorial chemistry, which was able to resolve *N*-(2-naphthyl)alanine diethylamide, the target analyte, on preparative scale [70].

A comprehensive discussion about the mechanism on π-acceptor and π-donor phases is given by Lipkowitz [38].

Cyclodextrin phases. Inclusion into the chiral cavity of CDs is a very frequently used approach for chiral separation. CDs are cyclic oligosaccharides; they consist of six (α-CD), seven (β-CD) or eight (γ-CD) glucopyranose units (Figure 2). CDs

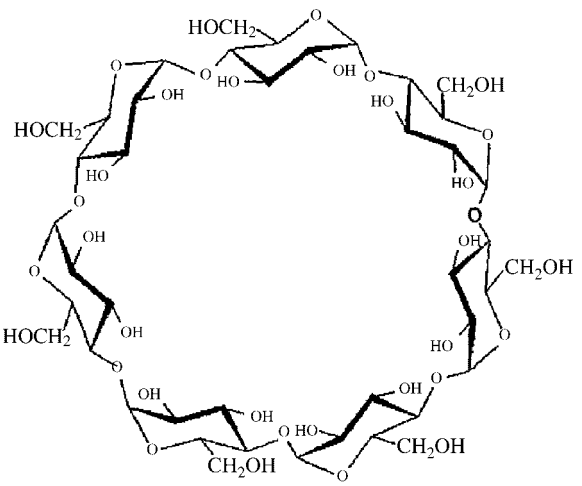

Figure 2. Formula of β-cyclodextrin

possess a hydrophilic surface and a truncated cone with a hydrophobic cavity. The depth of the cavity and the solubility can be modified by derivatization. The hydroxy groups in positions 2, 3 and 6 are available for derivatization. Chiral recognition is based on inclusion of the bulky hydrophobic group of the analyte into the hydrophobic cavity of the CD. Additionally, lateral interactions of the hydroxyl groups at the C-2 and C-3 at the upper rim of the CD, such as hydrogen bonds and dipole–dipole interactions with the analyte are to be taken into account.

CDs have been used in HPLC both in form of chiral mobile phase additives (CMPAs) or CSPs. A overview of the use of CDs in HPLC and CE has been given by Bressolle *et al*. [71].

CD-CSPs can be used in normal-phase, polar-organic and reversed phase mode. The first HPLC-CSPs containing CDs chemically bonded to silica gel were developed by Armstrong *et al*. [72] and applied to a broad spectrum of compounds [73]. Chang *et al*. [74] studied the effects of mobile phase and ring size of the CDs using several drug classes. Armstrong and De Mond [75] observed that β-blockers showed improved resolution using the organic polar mode on native β- or γ-CD phases. A comparison of the chiral recognition ability of a β-CD-phase and heptakis-2,3-*O*-dimethyl-β-CD in polar or-

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ganic and reversed phase mode for a series of compounds is given by Armstrong *et al*. [76].

In addition to chemically bonded CD-CSPs, HPLC phases were prepared by adsorbing β-CD-polymers to silica [77]. Several CD derivatives have been developed showing improved enantioselectivity in many cases. The most commonly used derivatives are methylated, acetylated, carboxymethylated and hydroxypropylated CDs. The preparation and application of phases containing permethylated CDs was reported by Ciucanu and König [78]. Riering and Sieber [79] evaluated different permethylated CD-CSPs by means of several drugs. The use of perphenylated cyclodextrins was described by Ciucianu [80]. The preparation and study of the separation behavior of a selectively methylated β-CD phase has recently been reported by Araki *et al*. [81]. Stalcup and Gahm [82] showed that CSPs containing sulfated β- CD can be applied to the chiral resolution of various drug classes. Naphthylethylcarbamate derivatized β-CD phases show increased selectivity due to the formation of additional π-π- interactions [83,84]. The chiral recognition mechanism, however, is postulated to depend greatly on the mobile phase mode. Hargitai et al. [85] prepared a series of 3,5-dimethylphenylcarbamate derivatized β-CD phases and studied the influence of degree of substitution of carbamate groups on β-CD on resolution. Li and Purdy [86] developed some other multi-interaction CD-based phases, among them methylbenzylamine- and naphthylethylamine- modified CD phases, which showed enantioselectivity for a great variety of compounds. In addition to inclusion complexation, hydrogen-bondings, π–π interactions, hydrophobic interactions and steric repulsion are assumed to be responsible for chiral recognition. Feng *et al*. [87] described the preparation of silica-based phases containing β-CD derivatized with 8-quinolinol as a chiral selector. Several CD CSPs are commercially available (Astec, Whippany, NJ, USA).

Several authors report the use of CDs as CMPAs [88–92]. Szemán and Ganzler [92] observed that the degree of substitution of CM-CD has a strong influence on the resolution of an aminomethyl benzodioxane derivative, ephedrine and oxprenolol. Rousell and Favrou [93] studied CM-β-CD and a cationic β-CD whereby the latter showed improved enantioselectivity for phenylhydantoin amino acids and barbiturates.

CSPs based on polysaccharides. Polysaccharide-based phases showed a very broad applicability to different compound classes. Several specialized reviews report the development and applications of polysaccharide-based phases [94–98]. Native cellulose showed only weak chiral recognition ability [99,100]. Hesse and Hagel [101] discovered that microcrystalline cellulose triacetate (CTA-I) produces a tertiary structure upon swelling and forms chiral cavities which are able to include stereoselectively compounds with aromatic residues. Several compounds were resolved on CTA-I [102,103]. Okamoto's group [104,105] prepared cellulose triacetate in a different way and coated macroporous aminopropylsilanized silica gel with the biopolymer. It was observed that the enantioselectivity on this phase, called CTA-II, was completely different from that of CTA-I. Similarly, cellulose tribenzoate and other cellulose ester phases were prepared [105,106]. Contrary to the inclusion mechanism proposed for CTA-I, the main interactions were assumed to be hydrogen bondings and dipole–dipole interactions on this type of phases [107]. Francotte and Wolf [108] prepared spherical beads of cellulose tribenzoate derivatives for preparative LC. Okamoto's group developed a series of CSPs based on cellulose trisphenylcarbamate derivatives (CTPCs) adsorbed to macroporous silica [109]. The chiral recognition mechanism on CTPC phases was elucidated by X-ray analysis, NMR studies [110, 111] and computer simulations [38]. CTPC has a left-handed 3/2 helical conformation and the glucose residues are regularly arranged along the helical axis. A chiral helical groove exists with polar carbamate groups inside the groove and hydrophobic aromatic groups outside the groove. Polar enantiomers may insert in the groove to interact with the carbamate residues via hydrogen-bond formation. In addition to these polar interactions, π–π interactions between the phenyl group of the CTPC and the aromatic groups of an analyte are assumed to be an essential contribution to chiral recognition. The

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substituents on the phenyl ring play also an important role in distinct enantioselectivity [97].

Substitution of cellulose by amylose was found to result in different enantioselectivity [112]. Contrary to cellulose, left-handed 4/1 helical structure is postulated for amylose [113]. 3-Fluoro-, 3-chloro and 3-bromo-5-methylphenylcarbamated of cellulose and amylose as CSPs were investigated by Chankvetadze *et al*. [114] and were found to show better chiral recognition ability than the corresponding 3,5-difluoro- and 3,5-dimethylphenylcarbamates.

Recently, new cyclohexylcarbamates of cellulose and amylose prepared by Kubota *et al*. [115] showed resolving abilities comparable to those of tris(3,5-dimethylphenylcarbamates) of cellulose and amylose. A drawback of coated-type phases is the solubility of the cellulose derivatives in some solvents. To improve stability of CTPC- and ATPC-based phases, regioselective chemical bonding of the selector to aminopropyl-silanized silica gel via a diisocyanate spacer was carried out [116–118]. A slightly lower chiral recognition compared to the coated-type phases was observed with these CSPs. Oliveros *et al*. [119] published a procedure for covalent binding by polymerization of mixed polysaccharide derivatives containing a 3,5-dimethylphenylcarbamate group and a 10-undecenoate spacer. Enomoto *et al*. [117] described the immobilization of amylose to silica by reducing the terminal residue of each molecule involving an enzymatic polymerization of α-D-glucose-1-phosphate. Recently, approaches for fixation based on photochemical and thermal treatment have been patented [120]. An overview of different approaches for the preparation of covalently bonded polysaccharide phases was recently published by Franco *et al*. [121].

Besides cellulose and amylose other polysaccharides such as, chitosan [109,122], chitin [123] and amylopectin [124] were used for the preparation of CSPs.

To enhance enantioselectivity, regioselectively substituted polysaccharides were synthesized [113,125]. A survey of different CSPs based on regioselectively modified cellulose, amylose and amylopectin derivatives was published by Felix [126].

There is a broad range of commercially available polysaccharide phases from Daicel (Tokyo, Japan) based on cellulose or amylose esters and carbamates. Several of them can be used in normal-phase, polar organic and reversed-phase mode [127,128]. Tachibana and Ohnishi [129] give an overview of numerous applications of polysaccharide based phases using reversed-phase conditions. Recently, Safni *et al*. [130] reported on the use of an anion exchanger modified with heparin as CSP for the separation of chloroquine enantiomers.

Macrocyclic antibiotics. Macrocyclic antibiotics have been shown to be very effective chiral selectors both for HPLC and CE. The glycopeptides vancomycin [131], teicoplanin [132], ristocetin A [133] and avoparcin [134] as well as the polypeptide thiostrepton [131] and the ansamycin rifamycin B [131] have been used for the preparation of chiral HPLC phases. A comprehensive review on the application of macrocyclic antibiotics as chiral selectors for both HPLC and CE was recently given by Ward and Fattis [135]. Macrocyclic antibiotics have several stereogenic centers and functional groups allowing multiple interactions with chiral analytes. The glycopeptides consist of an aglycon portion of fused macrocyclic rings that form a characteristic basket shape and a carbohydrate moiety. Hydrophobic parts of the analyte may be included into the hydrophobic basket and hydrogen bonds with the pendant arms as well as dipole stacking, ionic-, π–π interactions and steric repulsions are assumed to be the main interactions responsible for chiral recognition. Vancomycin [131] found application among others to the chiral separation of barbiturates, hydantoins, piperidine-2,6-dione and cyclic amides [136], dihydropyrimidinones [137], pyridone derivatives [138] and semi-synthetic ergot alkaloids [139]. A CSP based on vancomycin derivatized with 3,5-dimethylphenylisocyanate [131] showed different chiral recognition ability and resolved for example hydroxyzine and althiazide. Teicoplanin [132] was used for the separation of amino acids, dipeptides [140,141] and unusual aromatic β-alkyl amino acids [141]. Ristocetin A was shown to be applicable to a broad spectrum of compounds [133] using either normal-phase, polar

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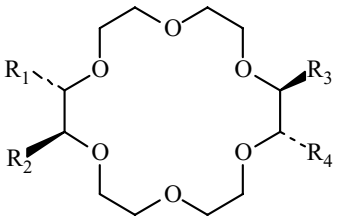

Figure 3. General structure of a chiral crown ether

organic or reversed-phase mode. Avoparcin, has recently been introduced as a chiral selector and was demonstrated to have chiral recognition properties complementary to the other macrocyclic antibiotics [134]. Recently, a new CSP containing the macrocyclic glycopeptide A-40 926 was introduced and compared regarding its chiral recognition ability with the teicoplanin phase [142,143]. Acquarica studied different immobilization procedures for teicoplanin comparing different silica gel supports [144]. Macrocyclic antibiotic phases have been commercialized by Astec (Whippey, NJ, USA).

Synthetic chiral macrocycles

Crown ethers. Crown ethers are macrocyclic polyethers which are known to form host–guest complexes with alkali- and earth-metal ions as well as primary ammonium cations. If a chiral crown ether is used, the inclusion of primary amines is stereoselective (Figure 3). This principle was introduced by Cram and coworkers [145]. This group prepared crown ether phases based on polystyrene or silica for classical LC and demonstrated the applicability of these phases to the chiral separation of amino acids [146].

The formation of hydrogen bonds between the 3 hydrogens attached to the nitrogen of the analyte and the dipoles of the oxygens of the macrocyclic ether are assumed to be the main interactions. Additionally, substituents of the crown ether are perpendicular to the plane of the macrocyclic ring, forming a chiral barrier, which divides the space available for the substituents at the chiral centre of the analyte into two domains. Thus, two different diastereomeric inclusion complexes are formed.

An HPLC phase using a polymeric crown ether derivative adsorbed on silica was developed by Shinbo *et al*. [147] and applied to the chiral separation of amino acids. This type of phase has been commercialized by Daicel (Tokyo, Japan) under the name Chirapack CR(+) and has found application to the chiral separation of amino acids and various primary amines [148,149] and unusual β-alkyl-amino acids [150,151]. One problem with this coated phase is stability. Recently, new crown ether phases using 18-crown-6-tetracarboxylic acid chemically bonded to aminopropylsilanized silica gel were prepared in different ways [152–154]. More recently, Hyun *et al*. [155] prepared a crown ether phase derived from a (diphenyl-subsituted 1,1′-binaphthyl) crown ether. These phases were applied to the chiral separation of amino acids and other compounds with primary amino groups. Three novel chiral pyridino-18-crown-6 derivatives attached to a Merrifield resin were synthesized by Horvath and Huszthy [156] and their chiral recognition ability was checked using α-(1-naphthyl)ethylammonium perchlorate.

Other synthetic macrocycles. A C3 symmetric, cup-shaped macrocyclic chiral selector, grafted to 3-mercaptopropyl silica gel, was described by Gasparrini *et al*. [157]. This receptor-like CSP showed enantioselectivity for amino acid derivatives and small peptides. Two C2 symmetric two-armed receptor miming selectors containing tetra-amide subunits, which were prepared from (*R*,*R*)-1,2-diaminocyclohexane and phtalic or trimesic acid connected by a *N*-(4-allyloxy benzoylated)-(*R*,*R*)-2,3-diaminopyrrolidine, were grafted to 3-mercaptopropyl silica [158]. Pieters *et al*. [159] sythesized cage-like C3 symmetric receptors containing two 1,3,5-triaryl benzene moieties linked by 3 amino acid spacers through peptide bonds. The selectors were immobilized to 3-mercaptopropyl silica through allyloxy groups (Figure 4). The applicability of these CSPs was checked by the chiral separation of ($\pm$)-1,1′ binaphtyl 2,2′ diol derivatives. Hu *et al*. [160] described the synthesis of a macrocyclic dibenzodicyclohexanotetramide containing CSP and evaluated this CSP by means of α-amino butyric acid methylester and α-methylbenzylamine.

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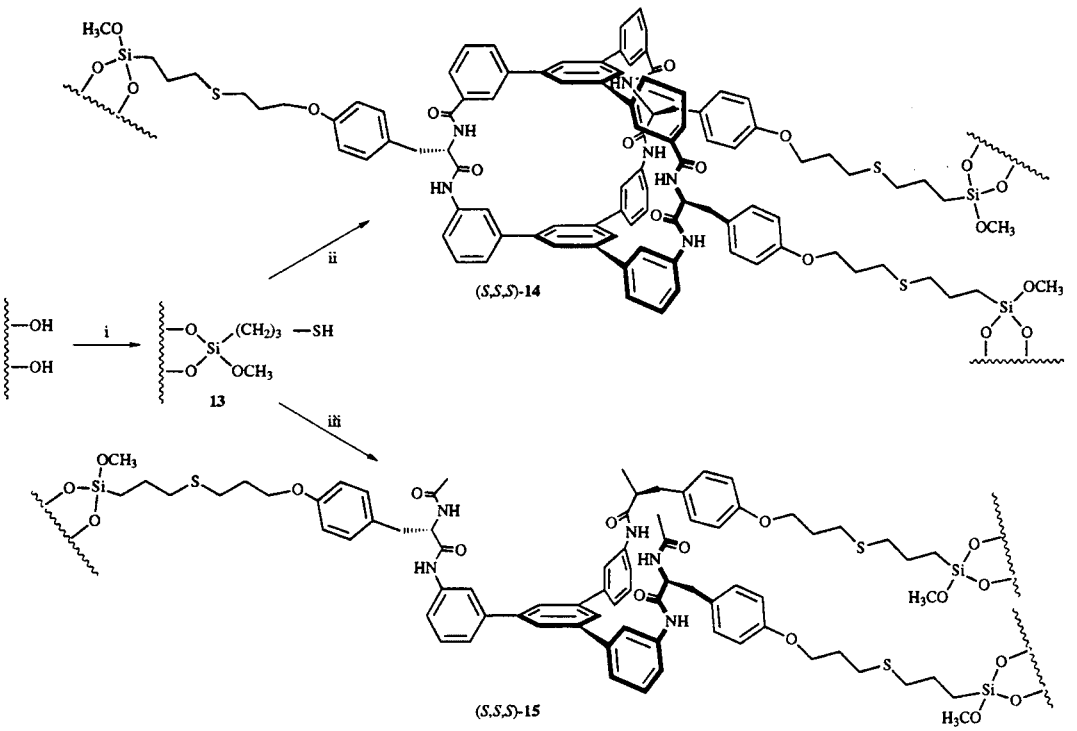

Figure 4. Synthesis of CSP (*S,S,S*)-14 and (*S,S,S*)-15 (From Reference [159] with permission.)

Chiral synthetic polymers as CSPs. A comprehensive review on the synthesis and application of chiral synthetic polymeric CSPs was given recently by Nakano [161]. In this chapter only the most important development in this field will be discussed.

Blaschke and coworkers [162] designed polyacrylamides and polymethacrylamides with chiral side chains. These phases were used among others for the chiral separation of benzodiazepines, barbiturates and hydantoins. Chemically bonding of the polymers to silica gel resulted in pressure-stable phases suitable for HPLC [163]. Phases of this type have been commercialized by Merck (Darmstadt). Hosoya *et al*. [164] described the preparation of a CSP based on copolymerization of phenylethylamine and methacryloyl chloride.

Helical isotactic polymethacrylates have been synthesized by Okamoto's group [165]. Triphenylmethyl methacrylate (TrMA) showed enantioselectivity to a broad variety of compounds. Both the durability and the separation ability were improved when the polymer was supported on macroporous silica gel [166]. More than 200 compounds were resolved on this CSP, which has been commercialized by Daicel. Polydiphenyl-2-pyridyl methacrylate (D2PyMA) was a further CSP developed by Okamoto's group [167]. Recently, Buchmeiser's group prepared polymers by ring-opening metathesis polymerization using norbornene derivatives of L-valine, L-phenylalanine [168] or β-CD [169] as monomers. These polymers were grafted to norbor-2-ene-5-yl-methylsilanized silica.

The development of polymeric monolithic phases is a new approach introduced by Hjerten's group [170]. Monolithic phases are obtained by *in situ* copolymerization of a monomer, a crosslinker and a selector. The applicability of this new technique for enantiomer separation by HPLC was demonstrated with a series of β-blockers using allylated CBH-I as a chiral monomer for the preparation of the CSP [171]. A new approach for synthesizing monolithic polymers has recently been presented by

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Sinner and Buchmeiser [172]. The authors made use of the principle of ring-opening metathesis polymerization using a norbornene derivative of β-CD as chiral monomer component.

Chiral imprinted polymers. This approach is based on polymerizing a monomer with a cross-linking agent in the presence of a chiral template molecule. After removing the template molecule, a chiral imprinted cavity remains, which shows high stereoselectivity to the template molecule or closely related molecules. This principle was introduced by Wulff's group [173,174]. Whereas Wulff used a covalent attachment technique based on the formation of a template–monomer complex through reversible covalent bonding, Mosbach's group [175] developed a non-covalent route making use of hydrogen bonding, electrostatic interactions, hydrophobic interactions, etc. to bind the template to the monomer.

Molecular imprinted polymers (MIPs) were developed among others for the chiral separation of amino acids [176], amino acid derivatives [175,177], peptides [178], carboxylic acids [177], β-blockers [179,180], cinchona alkaloids [181,182], NSAIDs [183,184] and benzodiazepines [185].

A ligand exchange molecularly imprinted polymer was prepared by Vidyasankar *et al*. [186] based on methacrylate-derivatized silica using Cu(II)-*N*-(4-vinylbenzyl) iminodiacetic acid as an achiral monomer and L-Phe as template. This imprinted polymer showed enantioselectivity for Phe and Tyr but not for other amino acids.

Recently, methods for combinatorial synthesis and screening of a series of MIPs have been described [187,188]. Monolithic MIPs were prepared by *in situ* polymerization in the column [189–191]. This approach is advantageous with respect of circumventing packing of the columns. Tan and Remcho [192] reported the preparation of a capillary coated with a polymer MIP using Dns-L-Phe as template for chiral open-tubular LC and CEC of Dns-Phe. A multi-step swelling polymerization technique using water as suspension medium was developed by Haginaka *et al*. [184]. A polymer imprinted with (*S*)-naproxen with a hydrophilic polymer layer at the outer surface was prepared which was suitable for the direct injection of plasma samples of NSAIDs.

For further details and applications the reader is referred to specialized reviews [193–195].

The advantage of chiral phases based on MIPs is the high, antibody-like selectivity; disadvantages are the relatively low efficiency and the restricted range of applicability, since one special phase shows chiral recognition only for the same molecule used as template or very closely related compounds.

Protein-based CSPs. The ability of proteins to bind drugs stereoselectively has been utilized for the chromatographic and capillary electrophoretic separation of drug enantiomers. Proteins consist of chiral amino acid building blocks and glycoproteins additionally contain sugar moieties. Proteins form a three-dimensional structure, whereby hydrophobic, electrostatic interactions and hydrogen bonds are assumed to be the interactions responsible for chiral recognition.

Proteins have been used as mobile phase additives and for CSPs. A recent comprehensive review reports on the development of protein-based CSPs and their application [196]. The first protein-based CSP was a bovine serum albumin (BSA) phase [197]. Subsequently, several BSA phases have been developed. In addition to silica gel, agarose and polymers have been used for the immobilization of BSA [198,199]. Nakamura *et al*. [198,200] prepared a BSA-multilayer-adsorbed porous hollow fiber membrane as a CSP. Haginaka and Kanasugi [201] isolated a BSA fragment of molecular mass 35236 and resolved 2-arylpropionic acid derivatives, benzodiazepines, warfarin and benzoin on a CSP prepared from this fragment.

Human serum albumin (HSA)-based CSPs were introduced by Domenici *et al*. [202] and found application among others to the resolution of NSAIDs and benzodiazepines. An improvement in enantioselectivity was observed, when Cys 34 in HSA was derivatized by ethacrylic acid [203]. α_1-Acid glycoprotein (AAP) CSPs, introduced by Hermansson [204], found application to a broad spectrum of drugs [204,205]. The addition of 2-propanol to the mobile phase showed an increase in resolution in many cases. Phases based on ovomucoid from chicken egg white were developed by Miwa *et al*. [206] and applied

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to the chiral separation of acidic, basic and neutral drug enantiomers [207]. Pinkerton *et al*. [208] showed that only one of three domains in chicken and turkey ovomucoids is responsible for chiral recognition. Phases of this type showed enantioselectivity for benzodiazepines and 2-arylpropionic acid derivatives. Haginaka *et al*. [209] isolated a new ovoglycoprotein, OGCHI from chicken egg white and bonded it to aminopropyl-silica gel [210]. While this CSP showed enantioselectivity preferably for basic compounds, a newly developed CSP prepared from Japanese quail egg white was found to be more suitable for acidic compounds [211]. Miwa *et al*. [212] immobilized avidin to DSC-activated aminopropyl-silica gel and resolved on this CSP acidic compounds such as 2-arylpropionic acid derivatives. Oda *et al*. used a more hydrophobic spacer for immobilization of avidin and observed improved chiral recognition ability on this CSP [213]. This phase found application to the chiral separation of a variety of acidic, basic and neutral drugs [213,214] and was shown to be applicable to direct injection of serum samples of drugs [215]. CSPs based on riboflavin binding protein were prepared from chicken egg white [216], chicken egg yolk [217] and quail egg white [218]. Several enzymes have been used for the preparation of CSPs. CSPs containing trypsin [219] and α-chymotrypsin [220] as chiral selectors showed enantioselectivity for amino acids and derivatives, dipeptides and aryloxypropionic acids [219,221]. Two cellobiohydrolases, CBH I and CBH II [222] and more recently CBH 58 [223], found application as chiral selectors for the preparation of silica-based CSPs which showed remarkable enantioselectivity for a broad spectrum of drugs, among them *β*-blockers [224]. Lysozyme [225]- and pepsin [226]-based CSPs showed enantioselectivity for basic and neutral enantiomers but not for acidic enantiomers. Recently, the preparation of amyloglucosidase-based CSPs and its application to amino-alcohols was reported [227]. Several protein-based CSPs are available from ChromTech AB, Sweden.

Ligand-exchange chromatography. Ligand-exchange chromatography (LEC) introduced by Davankov and Rogozhim [228] in the early seventies, was one of the first successful separation principles in chiral chromatography.

Chiral recognition on chiral stationary phases is based on the formation of ternary mixed metal complexes between the selector and analyte ligand.

Mobile phase (m) A_m

$\Updownarrow$

Stationary phase (s) $A_s + MS_S \rightleftharpoons AMS_s$

A: Analyte
M: Metal
S: Selector

The original phases prepared by Davankov for classical column chromatography were based on polystyrene–divinylbenzene polymers containing amino acid residues complexed with metal ions. These phases showed remarkable enantioselectivity for amino acids. For adapting this principle to HPLC, Gübitz *et al*. [229–232] prepared chiral LEC phases based on amino acids chemically bonded to silica gel via 3-glycidoxypropyltrimethoxysilane. Phases of this type have been commercialized by Daicel, Tokyo, Japan (ChiralPak WH). These phases have been shown to be suitable for the chiral separation of underivatized amino acids [229–232], α-alkyl and *N*-alkyl amino acids [232,233], amino acid derivatives [232], dipeptides [232], hydroxy acids [234] and thyroid hormones [235]. Subsequently, a large number of chiral LEC phases and their applications have been published [236–238]. Recent developments are the preparation of a chiral stationary phase by covalent attachment of *S*- and *R*-phenylalaninamide [239] and (*S*,*R*)- and (S,S)-N^2-(2-hydroxypropyl)-phenylalaninamide [240] to silica. Gübitz *et al*. [241] prepared two new chiral ligand-exchange chromatography (CLEC-) phases by binding L-proline via a (2-hydroxycyclohexyl)ethylene and a 6-hydroxy-4-oxa-8-aza-*n*-decene spacer to silica. The phases showed improved enantioselectivity for amino acids, amino acid derivatives, dipeptides and hydroxy acids. The latter phase was also found to be applicable for the chiral resolution of barbiturates. Wachsmann and Brückner [242] reported the synthesis of a new CLEC phase by binding L-proline or L-lysine to aminopropylsilanized silica through a triazine spacer. While the first phase

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was found to be suitable to resolve underivatized amino acids and *N*-(2,4-dinitrophenyl) amino acids, the second phase resolved Dns-amino acids.

An alternative to chemically bonded phases are chiral-coated phases. Davankov *et al*. [243] used a reversed-phase column coated with *N*-*n*-alkyl-L-hydroxyproline (L-Hypro) derivatives for the direct enantiomeric separation of amino acids. Similarly, *N*-*n*-decyl-L-histidine coated phases were used [244]. Yamazaki *et al*. [245] showed that C_{12}-L-Hypro coated phases can be used for the chiral separation of sympathomimetics. Ôi *et al*. [246] adsorbed Schiff bases of amino alcohols on reversed-phase columns and resolved racemic amino acids, hydroxy acids, amines and amino alcohols. The same group [247] used *N*,*S*-dioctyl-D-penicillamine and *N*,*S*-dioctyl-N-methyl-D-penicillamine as coatings on reversed-phase columns and applied these columns to the chiral separation of amino acids, *N*-acetylamino acids, glycyldi- and tripeptides, and amino alcohols. Wan *et al*. [248] synthesized chiral selectors derived from L-proline and L-phenylalanine by alkylation and arylation. The selectors containing C_7, C_9, C_{12}-chains or methoxybenzyl, naphthylmethyl or anthrylmethyl groups were adsorbed onto the surface of porous graphitic carbon. The authors resolved 36 racemic amino acids on these phases.

Another alternative technique is the use of chiral metal complexes as additives to the mobile phase in combination with achiral stationary phases. In this case a monodendate (MS) or bidendate selector metal complex (SMS) is present in the mobile phase and forms a mixed selector–analyte complex (AMS). Partition between the mobile ($_m$) and the stationary phase ($_s$) takes place according the following equilibria:

Mobile phase	$A_m + MS_m$	$\rightleftharpoons$	AMS_m
	$\Downarrow \quad \Downarrow$		$\Downarrow$
Stationary phase	$A_s + MS_s$	$\rightleftharpoons$	AMS_s

A: Analyte
M: Metal
S: Selector

The use of bis (L-prolinato)copper complexes was first described by Gil-Av's group [249]. This simple approach, however, is not applicable to underivatized amino acids because of detection problems. Numerous publications deal with the application of this basic technique using different metal complexes [236–238]. Selected examples of recent developments in this field are the use of (*S*)-phenylalaninamide [239], (*S*,*S*)-N^2-(2-hydroxypropyl)-phenylalaninamide [240] and (*S*,*S*)-*N*,*N*′-bis(phenylalanyl)ethanediamine as chiral additives to the mobile phase.

Chiral ion-pairing chromatography. This principle is based on the formation of diastereomeric ion-pairs between a chiral counter-ion and the analyte enantiomers which can be resolved on adsorption-based columns.

In 1981 Pettersson and Schill [250] first reported the use of (+)-10-camphor sulfonic acid (CSA) as mobile phase additive in combination with a diol-silica-based stationary phase for the chiral separation of *β*-blockers. CSA also found application as counter-ion for the chiral separation of methylphenidate [251] and several alkaloids [252–255]. *N*-benzoxycarbonyl-glycyl-L-proline (ZGP) was found to show higher enantioselectivity for *β*-blockers compared to CSA due to the presence of several polar functions allowing more points of interactions [256]. Quinine and other cinchona alkaloids have been used as counter ions to resolve enantiomeric acids on surface modified silica supports using indirect detection [257,258]. Karlsson and Pettersson have shown that porous graphite carbon is a useful support for chiral ion-pairing chromatography [259] using ZGP, *N*-benzyloxycarbonylglycylglycyl-L-proline (L-ZGGP) and captopril as chiral counter-ion for the resolution of various bases and quinine for the chiral separation of acids. Knox and Jurand [260] reported the chiral separation of Trp and glycylphenylalanine using L-Leu–L-Leu–L-Leu as a zwitterionic counter ion. (+)-Tartaric acid was used as a counter-ion by Gaskell and Crooks [261] for the chiral separation of *β*-blockers. Pettersson and Gioeli [262] introduced (−)2,3,4,6-di-*O*-isopropylidene-2-keto-L-gulonic acid as a chiral counter-ion dissolved in polar mobile phases for chiral ion-pair chromatography of several basic drugs.

Separation on preparative scale. The use of chiral chromatography on a preparative scale has

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attracted increasing interest for the production of enantiomerically pure drugs in industry [263]. Several chiral phases are available for preparative separation, among others cellulose derivative-based phases (CTA I, Chiralcel OD, OJ, OB, Chiralpack AD, AS, Daicel), a polymeric phase (Chiraspher, Merck), crosslinked L-diallyltartramide derivatives (Kromasil CHI-DMB, CHI-TTB) and a Pirkle type phase (DNBPG). A recent review [264] summarizes the applications of preparative LC.

One technique, which allows separation of enantiomers at a 100 g scale is the batch chromatographic mode [265]. This technique uses columns with an internal diameter between 5 and 30 cm. To increase sample throughput, techniques such as overlapping injections, peak shaving and recycling have been applied [265].

A special technique for preparative LC is simulated moving-bed (SMB) chromatography [266]. A typical SMB system consists of an array of columns connected in series and several pumps and valves. One recycling pump is needed for delivering the mobile phase flow through all columns. Further pumps are required to inject the feed and fresh eluent and withdraw the raffinate and extract flows. The valve system controls opening and closing of the inlet and outlet stream of each column at definite intervals. A countercurrent movement of stationary and mobile phase is simulated by controlled switching of the recycle fluid stream and the external and internal fluid flow streams on the different columns. A detailed description and applications of this technique are given in a recent review [267].

Counter-current chromatography (CCC) and centrifugal partition chromatography (CPC)

These techniques are based on multiple partition of compounds between two immiscible liquids [268]. A recent review deals with the detailed description and application of these techniques to chiral separation [269]. Only a few papers report chiral separations using these techniques. The first use of CCC in chiral separations was reported by Prelog's group [270]. The authors used (*R*,*R*)-di-5-nonyltartrate as a chiral selector and achieved a partial resolution of racemic norephedrine. The most efficient chiral selectors used in CCC and CDC are *N*-dodecyanoyl-L-proline-3,5-dimethylanilide, sulfated β-CD, albumin and vancomycin [269].

Gaschromatography (GC)

Indirect separation

A survey of chiral derivatization reagents for GC and their application to various compound classes including applications to pharmacokinetic studies is given by Srinivas *et al*. [271].

Most frequently used chiral derivatization reagents in GC are *S*(−) heptafluorobutyryl prolyl chloride, (−) menthyl chloroformate, *S*-α-methoxy-α-trifluoromethylphenyl acetyl chloride, *S*(−)-trifluoroacetyl prolyl chloride and *R*-(−)-2,2,2-trifluoro-1-(9-anthryl) ethanol [271]. More recently applied derivatization reagents are isopinocampheylamine [272] and *O*,*O*′-(*R*,*R*)-diacylated tartaric acid anhydrides [273].

Direct separation

CSPs based on amino acids and diamides. Pioneering work in the field of chiral separation by chromatography in general was done by Gil-Av's group. They developed the first chiral GC phases based on an amino acid derivative, *N*-trifluoroacetyl-L-isoleucine lauryl ester [274] and *N*-trifluoroacetyl-L-valyl-L-valine cyclohexylester [275] and resolved *N*-trifluoroacetyl amino acids on these columns. Chiral recognition on such phases is based on the formation of multiple hydrogen bonds. A valine diamide was linked to polysiloxanes yielding a phase called Chirasil-Val [276] which found broad application for chiral separation of amino acids and other compounds after transformation into volatile derivatives. This basic type of CSP has subsequently been modified [277].

CSPs based on metal complexes. Schurig [278] introduced the principle of complexation gas chromatography using a dicarbonyl rhodium(I)-

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3-trifluoroacetyl-(1*R*)-camphorate dissolved in squalene as CSP and resolved 3-methylcyclopentene on this phase. Later a series of 1,3-diketonate bis chelates of manganese(II), cobalt(II) and nickel(II) derived from perfluoroacylated terpene-ketones were investigated as CSPs [279]. To increase the thermostability, an immobilized polysiloxane-based phase (Chirasil-Nickel) was developed [280]. Applications of complexation GC included the chiral separation of pheromones, flavors and oxiranes [281].

CSPs based on cyclodextrins. Different approaches for the preparation of CSPs based on CDs were investigated. Schurig and Novotny [282] used permethylated β-cyclodextrin dissolved in a moderately polar polysiloxane as CSP. Later this selector was chemically bonded to a polysiloxane backbone creating the Chirasil-Dex CSP [280]. König and coworkers [283,284] used per-*n*-pentylated CD derivatives, which are liquid at room temperature as CSPs (Lipodex). Phases of this type showed enantioselectivity for a broad spectrum of compounds [285]. Armstrong and coworkers [286] developed a series of more polar CD phases with different selectivity. Chiral recognition of CD phases is based in several cases on inclusion into the chiral cavity, but hydrogen bonds, dipole–dipole interactions, electrostatic interactions and hydrophobic interactions are assumed to be the main binding forces.

A specialized review deals with the use of CDs as chiral selectors for the gas chromatographic separation of chiral components in essential oils, aromas and flavors [287].

CSPs based on cyclocholates. Recently, Bucaille *et al*. [288] prepared two chiral cyclocholates and checked their chiral recognition ability in mixtures with polysiloxanes in capillary GC.

CSPs based on calixarenes. Calixarenes represent a new class of chiral selectors applied in chiral chromatography. A chiral calixarene phase was recently prepared by Pfeiffer and Schurig [289] by linking L-valine tert butylamide to the eight hydroxy groups of a resorc[4]arene. This selector was bonded to a dimethylpolysiloxane (Chirasil-Calix). Another group [290] synthesized thiacalix[4]arenes containing pendant chiral amines. These phases were applied to the chiral separation of derivatives of amino acids, alcohols and amines. Chiral recognition might be partially based on inclusion into the basket shaped cavity and on hydrogen bondings.

For more detailed information on chiral separations by GC the reader is referred to an excellent recent review [291].

Supercritical, sub-critical fluid chromatography (SFC) and enhanced-fluidity liquid chromatography (EFLC)

Many chiral phases successfully applied in HPLC or GC chromatography have been also investigated in super- and sub-critical fluid chromatography [292–294]. Usually carbon dioxide is used as mobile phase which can be modified to a certain extent with organic additives such as alcohols or acetonitrile and acids or bases. Separations have been carried out either in packed columns (or capillaries) or open tubular columns (or capillaries).

Advantages over HPLC reported are shorter equilibrium times and faster chromatographic separations. Carbon dioxide has a viscosity that is about one order of magnitude less than that of water and is environmentally friendly. Drawbacks are the restricted applicability and the limited polarity regarding the mobile phase.

Brush-type π-acceptor and donator phases

Mourir *et al*. [295] reported the first application of SFC to chiral separations using the Pirkle phase containing (*R*)-*N*-(3,5-dinitrobenzoyl)-phenylglycine as chiral selector. Various brush-type CSPs have been applied to the chiral separation of a broad spectrum of compounds, among them amino acid derivatives, antimalarials, pyrethroids, β blockers and β-agonists [295]. The Whelk-O-1 phase was applied for the chiral separation of drugs on analytical and preparative scales [296]. Terfloth *et al*. [297] incorporated the same selector into polysiloxanes and immobilized the polymer thermally to silica gel. This phase was used for the chiral separation of NSAIDs. Blum *et al*. [296] compared a Whelk -O-1

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and a Chiracel column for the chiral separation of verapamil, warfarin and other compounds on preparative scale.

CSPs based on metal complexes

Schurig and coworkers [280] also extended the use of CSPs based on nickel camphorate complexes bonded to siloxanes, successfully used in complexation GC, also to SFC.

Cyclodextrin-based CSPs

The first SFC-separation on a β-CD-CSP was reported by Macaudiere *et al*. [298]. Williams *et al*. [299] compared the LC and SFS separation of ancymidol enantiomers on a naphthylethylcarbamoylated β-CD phase. Jung and Schurig [300] showed that an immobilized polysiloxane anchored permethyl-β-CD (Chiralsil-Dex) open-tubular capillary, originally prepared for GC, can also be used also for SFC and demonstrated its applicability by separating NSAIDs, norgestrel and hexobarbital. Lee and coworkers [301] compared the performance of packed and open-tubular SFC columns containing the same cyclodextrin-modified polymer. A polymeric CSP based on permethylated β-cyclodextrin was developed by Bradshaw *et al*. [302] and applied to the chiral separation of NSAIDs, anticoagulants hexobarbital, dihydrodiazepam and norgestrel. Armstrong *et al*. [303] reported the preparation of a CSP based on methylated β-CD having short methylsiloxane polymers as substituents. Shen and coworkers [304] prepared two different encapsulated particles for packed capillary column SFC.

Polysaccharide-based phases

Juvancz *et al*. [305] prepared an open-tubular column coated with benzoyl derivatives of cellulose and resolved some polar aromatic compounds on this column. Cellulose tris (3,5-dimethylphenylcarbamate) (Chiracel OD) found application to the chiral separation of β-blockers, benzodiazepines, calcium channel blockers and imidazole derivativatives [292]. NSAIDs [293], benzodiazepines and β-blocker [292] were resolved on amylose tris (3,5-dimethylphenylcarbamate) (Chiracel AD). Whatley [306] reported the separation of glibenclamide on cellulose- and amylose-based phases on a preparative scale. Yaku and Morishita [307] compared different cellulose-based CSPs for their ability to resolve dilthiazem enantiomers.

CSPs based on macrocyclic antibiotics

Vancomycin and teicoplanin phases were applied to the resolution of aryloxypropionic acids, arylpropionic acids, benzodiazepines, local anesthetics and β-blockers [292]. Recently, Sun and Olesik [308] reported the application of enhanced-fluidity liquid chromatography (EFLC) to chiral separations using a vancomycin CSP and CO_2 or CHF_3 in combination with organic modifiers in normal- and reversed-phase mode.

Polymeric phases

Macaudiere *et al*. [309] separated the enantiomers of bi-β-naphtol and α-methylene-γ-lactone on a (+)-polytriphenylmethyl methacrylate coated on macroporous silica particles under subcritical fluid chromatographic conditions. Petersson *et al*. [310] synthesized CSPs for open-tubular columns by copolymerization of cyclohexylidenebisbenzamide and methylsiloxane and applied these phases in among others to the chiral resolution of diols, mephenytoin and *trans*-stilbene oxide.

Thin Layer Chromatography (TLC)

Compared to other chromatographic techniques, TLC has been used less frequently for chiral separations. TLC might not be able to compete with HPLC or GC regarding separation efficiency; however, it shows several advantages. TLC is a very simple, inexpensive, rapid and flexible technique; many samples can be processed parallel on one plate and very selective detection can be carried out by using spray reagents. For chiral separation, in principal chiral stationary phases or chiral mobile phases can be used. However, only one type of TLC plate containing a chiral stationary phase, which is based on the LEC-principle, is commercially available. Details about applications of chiral

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TLC to different compound classes can be found in specialized reviews [9,311,312].

Ligand exchange

Ligand exchange represents the most frequently used chiral separation principle in TLC.

Weinstein [313] impregnated reversed phase silica plates with *N*,*N*-di-n-propyl-L-alanine/copper(II) complexes. Alak and Armstrong [314] and Günther [315] used the copper(II)complex of (2*S*, 4*R*, 2′*RS*) - 4-hydroxy - 1-(2′-hydroxydodecyl)-proline as chiral selector coated on C18 bonded silica gel plates. On plates of this type, commercialized first by Macherey-Nagel under the Name 'chiral plate' and later by Merck (Darmstadt, Germany) under the name 'CHIR' plate, a broad spectrum of chelate-complex forming compounds, such as amino acids, α-methylamino acids, *N*-alkyl amino acids, dipeptides, α-hydroxy acids, halogenated carboxylic acids and some heterocyclic compounds were resolved [311,312,316]. Remelli *et al*. [317] reported on the use of a histidine-based stationary phase for the separation of amino acids.

Cyclodextrins

Armstrong [318] described the resolution of amino acid derivatives, metallocenes, sulfonates and a crown ether using reversed phase TLC in combination with β-CD as additive to the mobile phase. Several groups resolved amino acids on TLC-plates containing different sorbents using α-CD, β-CD, 2-O-[*R*-2-hydroxypropyl)]-β-CD or a soluble cyclodextrin polymer as mobile phase additives [311, 312]. A new CSP for TLC containing a 3,5-dinitrobenzoyl substituted β-CD was synthesized by Zhu *et al*. [319] and evaluated with the chiral separation of Dns-amino acids using both normal phase and reversed phase mode. Bieganowska *et al*. [320] resolved 2-amino-1-butanol derivatives using β-CD as a mobile phase additive. The effect of the addition of bis(2-ethylhexyl)orthophosphoric acid on retention and resolution was studied.

Polysaccharides

The use of native crystalline cellulose as a chiral stationary phase for TLC and the application to some aromatic amino acids was described by Bach and Haas [321], Yuasa *et al*. [322] and Lederer [323]. Lepri [324] resolved 21 different racemates on microcrystalline cellulose triacetate plates. Cellulose phenyl carbamate phases were successfully applied by Suedee and Heard [325] to the resolution of β-blocker enantiomers. Malinowska [326] reported the use of chitin and chitosan as chiral stationary phases in TLC.

Macrocyclic antibiotics

Armstrong and Zhou [327] introduced vancomycin as chiral selector for enantiomeric separation by TLC. Bhushan and Parshad [328] reported the use of macrocyclic antibiotics for the resolution of enantiomeric Dns-amino acids by TLC.

Proteins

Lepri's group reported the use of BSA as mobile phase additive in chiral TLC [329,330]. The same group studied the mechanism of the separation of the enantiomers of warfarin, amino acids and several other chiral compounds on reversed-phase plates using BSA as mobile phase additive [329,330].

Molecularly imprinted polymers

Kriz *et al*. [331] prepared a polymer using L-phenylalanine anilide as a print molecule and resolved L- and D-phenylalanine anilide on plates containing this polymer. Suedee *et al*. [332] used quinine as a print molecule and studied its enantioselectivity using quinine, quinidine, cinchonine, cinchonidine and ephedrine analogues. Recently, the same group prepared imprinted CSPs containing (−)-pseudoephedrine and (−)-norephedrine as a print molecule, whereby the latter showed chiral recognition ability for a series of adrenergic drugs.

Ion-pair chromatography

Li *et al*. [333] recently reported the use of a chiral ion-pairing reagent, ammonium-D-10-camphorsulfonate as mobile phase additive for the resolution of aromatic amino alcohols using silica gel plates.

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Capillary Electrophoresis (CE)

Chiral separation by CE can be performed either indirectly using a chiral derivatization agent forming diastereomeric pairs, which can be resolved under achiral conditions or directly using chiral selectors as additives to the electrolyte. The techniques in CE are capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), electrokinetic chromatography (EKC), isotachophoresis (ITP) and isoelectric focusing (IEF). In capillary electrochromatography (CEC), which represents a recent technique, similarly to HPLC chiral stationary phases or chiral mobile phase additives can be applied. Several comprehensive reviews give a survey of chiral separation principles used in CE and applications to various compound classes [10–17].

Indirect separation

Several reagents used in HPLC for chiral derivatization have also been applied in CE.

Some recently developed chiral derivatization reagents for CE are (+) and (−) 1-(9-anthryl)-2-propyl chloroformate (APOC) [334], (1*R*,2*R*)- and (1S, 2S) -*N*-[(2-isothiocyanato)cyclohexyl]-6-methoxy-4-quinolinylamide) [335] and *R*-(−)- or *S*-(+)-4 - (3 -isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole [336].

Direct separations

Cyclodextrins. CDs are the most frequently used chiral selectors in CE.

The first application of CDs for chiral separations was reported by Snopek *et al*. [337] using the principle of ITP. Guttman *et al*. [338] used *β*-CD incorporated in a gel and Fanali [339] published the first paper dealing with the use of CDs in CZE. Subsequently, several hundreds of papers appeared on the use of CDs as chiral selectors in CE and about 800 compounds were resolved. CDs found application among others to the chiral separation of *β*-blockers, sympathomimetics, antipsychotics, antidepressants, hypnotics, barbiturates, local anesthetics, antiasthmatics, anticoagulants, anti-epileptics, antihypertensives, calcium-channel blockers, antimalarials, antibacterials, antivirals, antifungals, etc. The use of CDs as chiral selectors is subject of several selective reviews [13,15,16,340].

The depth of the cavity and the solubility of native CDs can be increased by derivatization. The hydroxy groups in positions 2, 3 and 6 are available for this process. Several neutral and charged CD derivatives have been synthesized. To date, various derivatives are commercially available and the enantioselectivity has been shown to vary drastically among them.

Neutral CD derivatives. A great variety of neutral derivatives of CDs such as heptakis-*O*-methyl-CD (M-CD), heptakis (2,6-di-*O*-methyl) CD (DM-CD), heptakis (2,3,6-tri-*O*-methyl) CD (TM-CD), hydroxyethyl-CD (HE-CD) and hydroxypropyl-CD (HP-CD) have been synthesized and applied to a great variety of compounds [341].

Since most of the CD-derivatives represent mixtures of different products showing different substitution patterns, separations are often difficult to reproduce. Therefore, some groups isolate the single isomers or synthesize selectively substituted derivatives. Selectively methylated and acetylated CDs were synthesized by Miura *et al*. [342] and applied to the chiral separation of amino acids derivatives. A new uncharged CD-derivative, cyanoethylated *β*-CD has recently been introduced by Aturki *et al*. [343]. Acidic drugs were found to be more strongly complexed than basic analytes, requiring lower CD-concentrations. Zerbinati *et al*. [344] introduced ethylcarbonate-*β* and *γ*-CDs and applied them to the resolution of racemic dichlorprop herbicides. Chiari *et al*. [345] synthesized a new vinylpyrrolidine-*β*-CD copolymer by radical copolymerization of vinylpyrrolidone and methacroyl-*β*-CD and evaluated this selector by means of sympathomimetic drugs. Li *et al*. [346] described the use of mono-3-*O*-phenylcarbamoyl-*β*-CD as a new chiral selector and evaluated this selector using verapamil and propafenone. Recently, the synthesis of L-Ala-Crown(3)-L-Ala capped *β*-CD was reported [347]; it showed chiral recognition ability for Dns-amino acids.

A systematic screening of drugs comparing *α*-CD [348], *β*-CD [349], *γ*-CD [350] and neutral CD-derivatives [341] was done by Koppenhoefer *et al*.

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Negatively charged CDs. Negatively charged CDs are suitable chiral selectors for the separation of basic and neutral drugs. The improved selectivity compared to neutral CDs is mainly attributed to the counter-current mobility. Sulfated CDs, sulfobutyl-and sulfoethyl ether-β-CD are the most frequently used charged CDs [13,15,16].

A series of single isomer β- and γ-CD derivatives was introduced by Vigh's group [351–354]. These authors prepared derivatives completely sulfated in 6-position and completely substituted on their larger rims with hydrophylic groups, moderately hydrophobic groups or hydrophobic methyl functional groups. The authors have shown that neutral, basic, zwitterionic and even acidic enantiomers can be separated. Carboxyl functional CDs such as carboxymethyl-β-CD (CM-β-CD) [355] carboxyethyl-β-CD (CE-β-CD) [355] and succinyl-β-CD [355,356] found application to a broad spectrum of neutral and basic compounds [13,15,16]. Phosphated CDs [357,358] are another group of negatively charged CDs successfully applied to the separation of some drug enantiomers.

Positively charged CDs. Cationic CDs such as 6-[(3-aminoethyl)amino]-6-deoxy-β-CD, 6^{A}-methylamino-β-CD, 6^{A},6^{D}-dimethylamino-β-CD, a hepta-substituted methylamino-β-CD, mono (6-amino-6-deoxy)-β-CD were the first cationic CDs described that found application to the chiral separation of various acidic and neutral compounds [13,15,16]. A polycationic CD derivative, heptakis(6-hydroxyethylamino-6-deoxy-β-CD) (beta-CD-EA) was developed by O'Keeffe *et al*. [359] and applied to acidic compounds such as NSAIDs, Dns-amino acids, and phenoxypropionic acid herbicides.

Haynes *et al*. prepared a new hepta-substituted single isomer cationic β-CD (heptakis (6-methoxyethylamine-6-deoxy)-β-CD [360] and checked its separation behavior towards NSAIDs and phenoxypropionic acid herbicides. Galaverna *et al*. recently introduced histamine-modified cationic β-cyclodextrins as chiral selectors and demonstrated their applicability to chiral separation by means of Dns-amino acids, carboxylic acids and hydroxy acids [361].

CDs containing quaternary ammonium groups show some advantages, because they are strong bases and therefore the electrophoretic mobility is pH independent. Furthermore, only very low selector concentrations are required to resolve acidic enantiomers because of the strong ionic interactions. 2-Hydroxy-3-trimethylammoniopropyl-β-CD was investigated by several groups [362–364] and applied to the chiral separation of basic, neural and acidic compounds. A reversal of the EOF was observed with this selector [364,365]. Another quaternary ammonium-β-CD (QA-β-CD) of undefined structure, which is commercially available (Cerestar-USA, Hammond, IN, USA) was applied to the chiral separation of various acidic analytes [366,367].

Amphoteric CDs. Two new amphoteric CD derivatives, mono-(6-glutamylamino-6-deoxy)- β-CD (Glu-β-CD) and AM-β-CD of undefined structure have recently been introduced and shown to be applicable to neutral, acidic and basic compounds [368,366].

CDs and non-chiral additives. The combination of CDs with a chiral micelle forming surfactants such as sodium dodecyl sulfate (SDS) is utilized in the principle of CD-mediated micellar electrokinetic chromatography (CD-MEKC) introduced by Terabe *et al*. [369]. While uncharged CDs migrate with the same velocity as the EOF, the negatively charged micelles migrate in the direction opposite to the EOF. Partition of hydrophobic analytes between the bulk solution, the CD and the micelle phase takes place, causing retention of the analyte, which enables separation of uncharged analytes with neutral CDs. Several authors report the change from CD-CZE to CD-MEKC as a means of reversing the enantiomer migration order [370,371]. A method for the chiral separation of diols is based on the use of a mixture of CDs and borate. [372–374]. Chiral resolution is assumed to be based on the formation of mixed CD-borate diol complexes.

Carbohydrates. A variety of linear neutral and charged carbohydrates were also found to be applicable as selectors for chiral separations. Specialized reviews summarize the applications

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of different carbohydrates to various compound classes [375,376].

Neutral Mono-, oligo- and polysaccharides. Low and high molecular mass maltodextrins and dextrans were successfully applied as chiral selectors, mainly to acidic compounds [375,376].

Chankvetadze *et al*. [377] showed that water soluble, native polysaccharides such as amyloses of different molecular mass, laminaran, pullulan as well as derivatized polysaccharides, methylcellulose and hydroxypropylcellulose and carboxymethyl amylose can be used as chiral selectors. The same author [378] studied the influence of the kind of linkage of different malto- and oligosaccharides on the enantioselectivity. Nakamura et al. [379] showed that even monosaccharides such as D-glucose, D-mannose and some of their derivatives can exhibit some limited chiral recognition ability.

The chiral recognition mechanism for polysaccharide based selectors is still not completely clear. In the case of dextrins the formation of a helical structure with hydrophobic character is assumed to be responsible for binding of hydrophobic molecules. Lateral binding forces such as hydrogen bonds and dipole–dipole interactions with hydroxy groups of the sugar molecules are to be taken into account [375,380].

Charged polysaccharides. Negatively charged polysaccharides such as heparin, chondroitin sulfate C, chondroitin sulfate A, dextran sulfate and λ-carrageenan have been used for the chiral separation of bases [375]. More recently investigated selectors on the basis of sulfated glycosaminoglycan are dermatan sulfate DS (chondroitin sulfate B) [381], a fucose containing glycosaminoglycan (FGAG) and a depolymerized holothurian glycosaminoglycan (DHG) [382] and pentosan polysulfate [383].

Phinney *et al*. [384] investigated citrus pectins (polygalacturonic acid sodium and potassium salts and esterified pectins) as chiral selectors for the separation of basic drugs including antihistamines, antimalarials and broncho- and vasodilators.

Positively charged polysaccharides such as diethylaminoethyl dextran (DEAE-dextran), and the aminoglycoside antibiotics streptomycin sulfate, kanamycin sulfate and fradiomycin sulfate were introduced by Nishi *et al*. [385] for the resolution of some acidic analytes. Since then there have been no further reports on the use of positively charged polysaccharides.

Chiral crown ethers. The only one chiral crown ether used up to now in CE is 18-crown-6-tetracarboxylic acid (18C6H4), introduced by Kuhn *et al*. [386] for the chiral separation of amino acids.

Besides the formation of inclusion complexes, ionic-, dipole–dipole interactions or hydrogen bonds between the carboxylic groups of the selector and polar groups of the analytes may act as additional supporting interactions.

In addition to amino acids, 18C6H4 found application in the chiral separation of sympathomimetics [387], dipeptides [388,389] various amino acid derivatives [390] and different drugs containing primary amino groups [391]. Mori *et al*. described the chiral separation of various drugs using 18C6H4 in non-aqueous medium [392].

Tanaka *et al*. [393] described a partial filling technique in a CE-MS system. The partial filling of the capillary with 18C6H4 should prevent entrance of the non-volatile selector into the CE-MS interface.

Surveys of applications of chiral crown ethers to various compounds are given in specialized reviews [16,394].

Calixarenes. Calixarenes are macrocyclic compounds consisting of benzene rings linked by methylene groups forming a hydrophobic cavity which is able to form host–guests complexes. Peña *et al*. synthesized a water-soluble (*N*-L-alaninoacyl)calix[4]arene and (*N*-L-valinoacyl)calix[4]arene [395]. The authors resolved with these chiral calixarenes 1, 1′-binaphtyl-2,2′-diyl hydrogen phosphat, 1,1′ bi-2-naphtol and 1, 1′-binaphtyl-2,2′-diamine as model compounds. Grady *et al*. [396] prepared a (*S*)-di-naphthylprolinol calix[4]arene which was coated on the wall of the capillary. Racemic 2-phenylglycinol was used as a model analyte.

Macrocyclic antibiotics. Macrocyclic antibiotics as chiral selectors were introduced by Armstrong

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[397]. Three classes of antibiotics have been introduced as chiral selectors: Ansamycins such as rifamycin B, rifamycin SV; the glycopeptides vancomycin, ristocetin and teicoplanin introduced by Armstrong [397] and the aminoglucoside antibiotics streptomycin, fradiomycin and kanamycin investigated by Nishi *et al*. [385]. Macrocyclic antibiotics possess several asymmetric centres and many functional groups allowing multiple interactions with the analytes. The semirigid basket-shaped aglycan, which has hydrophobic properties, enables the formation of host–guest inclusion complexes and there are pendant polar arms, which can form hydrogen bonds. Ionic, dipole–dipole, π–π, hydrophobic interactions and steric repulsion are assumed to take effect [398]. A comprehensive description of the properties of these selectors and their applications to the chiral separation of a broad spectrum of compounds is given in several specialized reviews [399–401]. While rifamycin B showed enantioselectivity for basic compounds, rifamycin SV and the glycopeptide antibiotics were found to be suitable for the chiral separation of acidic compounds. To overcome detection problems arising from the strong UV-absorption of the selectors, partial filling methods [402] and counter-current processes [403] have been applied. In the latter approach, coated capillaries are used to suppress the EOF and a suitable pH to provide the selector (vancomycin or ristocetin A) and the analytes with opposite charges. Thus, the positively charged selector moves to the cathode, clearing the detection window, and the analytes can be detected without interferences at the anodic side. Further macrocyclic antibiotics of the glycopeptide type investigated as chiral selectors are A 82846B [404], LY307599 [405], Actaplanin A [406], Avoparcin [407], Hepta-tyr [408] and A 35512B [409].

Proteins. Proteins can be positively or negatively charged depending on the pH applied. Their charges give them electrophoretic mobility and they can be used for the separation of basic and acidic analytes. The tertiary structure of proteins is assumed to be an important factor for the chiral recognition ability of proteins. The main interactions are hydrogen bondings, dipole–dipole, and hydrophobic interactions.

A great variety of proteins, such as BSA, HSA, AAG, avidin, conalbumin, ovomucoid, ovoglycoprotein and casein were used as chiral selectors in CE for a broad spectrum of compounds. For detailed information the reader is referred to specialized reviews [410–412].

Further proteins investigated as chiral selectors are quail egg white riboflavin binding protein [413], applied to the chiral resolution of basic drugs such as oxazepam, oxprenolol, prilocaine, bupivacaine, etc. and native flavoprotein isolated from chicken egg white and chemically modified flavoproteins [414] which found application among others to NSAIDs, proglumide and aminoglutethimide. Ovotransferrin was applied to the chiral separation of trimetoquinol [415] and iron-free human transferrin was shown to exhibit chiral recognition for tryptophan esters [416] and several drugs [417].

Several enzymes were found to be useful chiral selectors. Fungal cellulase [418] and cellobiohydrolase I (CBH I) [64,419–421] showed remarkable chiral recognition ability for *β*-blockers.

Pepsin [422] found application to several basic drugs and lysozyme [423] was used for the chiral separation of tryptophan, PTH- and Dns-amino acids.

Two groups [424,425] reported the use of cyclohexapeptides prepared by combinatorial synthesis for chiral separations. Several cyclic peptide libraries were prepared and checked for their ability to resolve DNP-amino acids.

Ligand exchange CE (LECE). The principle of ligand exchange successfully applied in chiral HPLC was transferred to CE by Zare's group using a L-histidine [426]- or aspartame-Cu(II) [427] complexes for the chiral separation of Dns-amino acids. Desiderio *et al*. [428] resolved hydroxy acids using L-Pro-, L-4-hydroxyproline (L-Hypro)- or aspartame-Cu(II) complexes. The first approach for direct separation of underivatized amino acids using L-Pro or L-Hypro-Cu(II) complexes as chiral selectors was published by Schmid and Gübitz [429].

N-alkyl-hydroxy-proline such as *N*-(2-hydroxyoctyl)- and *N*-2-hydroxypropyl)-L-Hypro derivatives have recently been synthesized and

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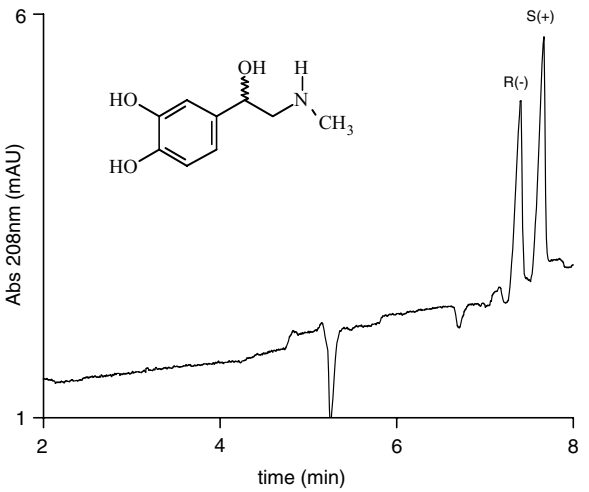

Figure 5. Chiral separation of epinephrine by ligand exchange CE: Conditions: 10 mM HO-L-Hypro, 5 mM Cu(II) at pH 12, *U*=15 kV. (From Reference [432] with permission.)

applied as Cu(II) complexes to the chiral separation of underivatized aliphatic and aromatic amino acids and dipeptides [430,431]. Compared to L-Hypro, these selectors showed improved resolution with significant lower selector concentrations. *N*-(2-hydroxyoctyl)-L-Hypro was also shown to be applicable for the chiral resolution of sympathomimetic drugs [432] (Figure 5) as well as hydroxy acids and β-blockers [433].

A comprehensive survey of applications of LECE is given in a recent review [434].

Chiral surfactants. Terabe *et al*. [435] introduced the principle of micellar electrokinetic chromatography (MEKC) using chiral surfactants as chiral selectors. Surfactants are amphiphilic molecules composed of a polar head group and a hydrophobic tail. Above the critical micelle concentration (CMC) micelles are formed. Since these micelles act as pseudostationary phases, this technique has been called MEKC. More recently, the term micellar capillary electrophoresis has also been used [436,441]. The chiral separation of analytes is based on their ability to form aggregates with the micelles and their partition coefficients between the chiral micelle phase and the electrolyte bulk phase.

A variety of different classes of surfactants found application as chiral selectors: bile salts, saponines, long chain *N*-alkyl-L-amino acids and *N*-alkanoyl-L-amino acids, *N*-dodecoxycarbonyl amino acids, alkylglycoside surfactants and polymeric amino acid based surfactants. Detailed descriptions and surveys of the applications of these approaches to various compounds are presented in recent reviews [437–439].

Ding and Fritz synthesized new carbamate type surfactants from amino acids (L-Leu, L-Val, L-Isoleu, L-Ser) and alkylchloroformates with chain lengths of C4 to C11 [440], and checked their enantioselectivity by means of propranolol, atenolol, ketamine, laudanosine, nefopam, benzoin and hydrobenzoin. Polymeric amino acid and dipeptide based surfactants have been introduced by Warner's group [441,442]. The monomers were synthesized by coupling the *N*-hydroxysuccinimide ester of 10-undecylenic acid to amino acids or dipeptides. Recent reviews deal with the development and application of polymeric surfactants [436,442,443].

Several chiral glycosidic surfactants have been prepared and used as chiral selectors in CE [444]. Alkyl-glucopyranoside-based surfactants were synthesized by Tickle *et al*. [445] and by El Rassi's group [446]. Recently, Ju and El Rassi presented new chiral glycoside surfactants, cyclohexyl–alkyl-D-maltosides [447]. These cyclohexyl–alkyl-β-D-maltosides have a chiral maltose polar head group and a cyclohexyl–alkyl hydrophobic tail. Steroidal-glycosidic surfactants were prepared by Mechref and El Rassi [448]. These surfactants can be charged *in situ* by complexation of the polyolic moiety with borate.

More recently, two new amphiphilic aminosaccharide derivatives were prepared by Horimai *et al*. [449]. These negatively charged selectors were applied above their CMC in borate buffer pH 9.5 for electrokinetic separation of Dns-amino acids and new quinolon antibacterial agents.

Non-aqueous CE (NACE). Non-aqueous solvents show several advantages regarding solubility of chiral selectors or samples and reduce unwanted interactions with the capillary wall. Often an increase in selectivity can be observed in non-aqueous solvents. Different forms of chemical equilibria in aqueous and non-aqueous systems can lead to different selectivities. Weak interac-

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tions which are disrupted by water can become effective in non-aqueous systems. A lower Joule heating is produced and since higher voltage can be applied, shorter retention times result. Furthermore, non-aqueous solvents are better compatible with CE-MS coupling than aqueous BGEs. The use of non-aqueous CE for chiral separations has been reviewed recently [450].

Karbaum and Jira [451] investigated a series of solvents for their suitability for non-aqueous CE and discussed the advantages of NACE.

First applications of non-aqueous solvents in chiral CE-separations were reported by Valkò [452] and Wang and Khaledi [453] using CDs in formamide (FA), dimethylformamide (DMF) and *N*-methylformamide (NMF) as solvents. Neutral and charged CDs were used in NACE for several applications. Comparative studies on the separation of basic compounds in aqueous and non-aqueous systems using sulfated-*β*-CD (S-*β*-CD) in formamide showed that a significant reduction of band broadening is observed in non-aqueous medium [454]. Vincent and Vigh demonstrated the advantages of the use of the single isomer heptakis (2,3-diacetyl-6-sulfato)-*β*-CD (HDAS-*β*-CD) in pure methanol for the chiral separation of basic drugs [455]. The use of a quaternary ammonium *β*-CD (QA-*β*-CD) in pure organic solvents for the chiral separation of amino acids derivatives and some profens was reported by Wang and Khaledi [456].

Mori *et al*. applied a chiral crown ether (18C6H4) in FA to the chiral separation of several compounds including some drugs with primary amino groups [392].

The use of chiral ion-pairing reagents in CE was not successful in aqueous medium. In non-aqueous medium, however, several chiral ion-pairing reagents were successfully applied for chiral CE separations. The interfering effect of water on the inter-molecular interactions, such as hydrogen bonds, responsible for chiral recognition is thereby eliminated.

(+)-*S*-Camphor-10-sulfonic acid was the first ion-pairing reagent reported; it was applied for the chiral separation of *β*-blockers [457]. As electrolyte, acetic acid in acetonitrile containing Tween 20 was used. Stalcup and Gahm [458] used quinine as a ion-pairing reagent in non-aqueous medium for the chiral resolution for acidic compounds using acetic acid–ammonium acetate–methanol as BGE. Piette *et al*. [459] compared native cinchona alkaloids and carbamoylated derivatives for their applicability as chiral ion-pairing reagents for the enantioseparation of *N*-protected amino acids using ammonium acetate in methanol and ammonia-octanoic acid in an ethanol–methanol mixture as non-aqueous BGEs. In addition to the primary ionic interactions, hydrogen bonding, dipole–dipole, π–π, hydrophobic and steric interactions are to be taken into account.

More recently, Karbaum and Jira [460] have shown that LECE is also possible in non-aqueous solvents. Using L-proline–Cu(II) in 25 mM ammonium acetate/1 M acetic acid in methanol the authors resolved several aromatic amino acids.

Dual selector systems. Dual selector systems containing either two chiral selectors or one chiral selector and a separation-supporting agent have been found to improve or even enable separation in several cases. It was shown that the combination of neutral native with synthetic neutral or charged CD derivatives often significantly enhances resolution . Since specialized reviews deal with the use of dual CD systems, this topic will not be discussed here in detail [461,462]. Another approach reported is the combination of CDs with chiral surfactants such as bile salts [463–465], decanoyl-*N*-methyl glucanoid [466] and poly (sodium-*N*-undecenyl-D-valinate) [467].

18C6H4 was used in combination with CDs [468,469]. It was shown that not only chiral but also non-chiral crown ethers can support chiral recognition of CDs [470]. Armstrong *et al*. [471] discussed the mechanism postulating the formation of 'three body' complexes between an amine, a cyclodextrin and 18-crown-6 .

Ion-pairing reagents were found to have a supporting effect on the chiral separation using CDs [472,473]. It was found out that a significant improvement in resolution is observed independent if the counter ion is chiral or not. The influence of (+)- or (−)-camphorsulfonic acid, alkylsulfonic acids and alkanoic acids of different chain lengths as well as sodium cyclamate on the

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chiral resolution of basic compounds using different neutral CDs was investigated. Basic counter ions such as quinine and (*S*)-hyoscyamine were found to support the resolution of acid compounds using CDs.

Horimai *et al*. [474] used a dual selector system consisting γ of -CD and Zn(II)-D-phenylalanine and applied this system to the chiral separation of drugs with quinolone structure.

Diverse selectors. Inglese *et al*. [475] introduced (+)-(5R,8S,10R)-1-allylterguride, a semisynthetic product, derived from ergot alkaloids as chiral selector and demonstrated its applicability for chiral separations using hydroxy acids and herbicides. Nair *et al*. [476] reported on the ability of *d*(+)-tubocurarine chloride, a macrocyclic bis(benzylisoquinoline) alkaloid, to resolve carboxylic acids.

Isotachophoresis (ITP) and isoelectric focusing (IEF). ITP was the first of the electroseparation techniques to be applied for chiral separations [337]. Only few special applications of this technique to chiral separations have appeared in the past years.

Kaniansky *et al*. [477] and Hoffmann *et al*. [478] described ITP – systems for preparative isolation and purification of enantiomers. Coupled ITP-CZE systems for sample pretreatment and chiral separation were developed by Dankova *et al*. [479], Fanali *et al*. [480] and Tousaint *et al*. [481].

Glukhovsky and Vigh [482] used preparative IEF for the separation of Dns-phenylalanine enantiomers on mg/h scale.

New techniques. Zhao and Jorgenson [483] recently introduced the approach of cyclic capillary electrophoresis and give examples of the application of this new technique to chiral separation. The authors report the achievement of 100 million plates with this technique.

Liu and Fang [484] described the possibility of combining flow injection (FI) with CE and demonstrated its applicability by means of the chiral separation of intermediate enantiomers in chloramphenicol synthesis.

The use of microfabricated devices for CE is a recent trend. There are already a few examples of the application of microchip-CE for chiral separations [485,486].

Capillary Electrochromatography (CEC)

CEC can be regarded as a hybrid method between CE and HPLC combining the efficiency of CE and the selectivity of stationary phases. While in HPLC a conical flow profile is produced by the hydrodynamic flow, in CEC a rather plug-like flow profile is generated by electroosmotic flow, resulting in higher efficiency. In chiral open tubular capillary electrochromatography (OT-CEC), the chiral selector is covalently attached or coated on the inner surface of a capillary. Packed CEC (P-CEC) uses either an achiral stationary phase in combination with a chiral mobile phase or a chiral stationary phase. A new alternative to silica based packed capillaries is the use of monolithic chiral stationary phases prepared by *in-situ* polymerization. Different CEC techniques and applications are summarized in specialized reviews [487–490].

Open tubular capillaries

Pioneering work in CEC has been done by Schurig's group [491,492]. Permethylated β-cyclodextrin was attached via an octamethylene spacer to a dimethylpolysiloxane and coated on the capillary wall (Chirasil-Dex). These phases were applied to the chiral separation of several drugs. Schurig *et al*. [493] demonstrated the concept of unified chromatography using the same capillary coated with Chirasil-Dex for GC, capillary HPLC, SFC and CEC. Francotte and Jung [494] used capillaries coated with 3,5-dimethylphenylcarbamoyl cellulose and *p*-methylbenzoyl cellulose and applied the same capillaries for CEC and open tubular HPLC. The immobilization of proteins such as BSA [495] or AAG [496] via silanes directly onto the capillary wall represents a simple way of preparing open tubular capillaries. Liu *et al*. [497] simply adsorbed proteins, peptides and amino acids on the capillary wall by rinsing the capillary with a buffer containing the selector. These phases were evaluated by means of amino acids and some other compounds.

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Chiral imprinted polymers as CSPs also found application in CEC. Several reviews report on recent developments and applications in this field [498–502].

Open tubular capillaries containing chiral imprinted polymers coated as a thin films to the capillary wall were prepared by Tan and Remcho [499] used Dns-L-Phe as print molecule with methacrylic acid and 2-vinylpyridine as functional monomers and ethylene dimethacrylate as crosslinker in an *in situ* polymerization technique. To obtain a thin film at the capillary wall, the capillary was evacuated to effect shrinking of the polymer. Brüggemann *et al*. [503] reported the preparation of very thin coatings using *S*(+)-2-phenylpropionic acid as a print molecule.

Packed capillaries

Achiral stationary phases with chiral mobile phases. Several authors described the use of cyclodextrins as additives to the electrolyte in combination with achiral stationary phases, such as C18 [504], diol silica [505] or bare silica phases [506]. Lämmerhofer and Lindner [507] resolved amino acid derivatives on an ODS-stationary phase using a quinine derivative as an ion-pairing reagent in the mobile phase.

Chiral stationary phases. Since most of these packings are silica based frits have to be prepared by sintering a zone at the end of the packing. Li and Lloyd [508] used an HPLC-grade material containing native β-CD as packing. These packings, however, showed relatively low efficiency.

Wistuba *et al*. [509] prepared a packing material based on permethylated β-CD immobilized to 5 μm (mercaptopropyl) methyl-silica gel (Chiralsil-Dex-silica 2). The authors showed that the same capillary can be used for CEC, capillary-HPLC and pressure supported CEC. Recently, the same group [510] reported the preparation of a polysiloxane-linked permethyl-β-CD thermally immobilized on silica (Chirasil-Dex silica 1). Recent studies by the same authors [511] deal with the comparison of open tubular CD coated capillaries and packed capillaries with CD-CSPs using both CEC and capillary-LC. Zhang and El-Rassi [512] prepared a CSP containing a hydrophylic sulfonated sublayer to provide a strong EOF, to which a top layer of hydroxypropyl-β-CD was immobilized. This approach produced fast separations of anionic analytes with negative mobility such as Dns-amino acids and phenoxy acid herbicides.

A comprehensive overview of the applications of cyclodextrins in chiral electrochromatography was recently published by Schurig and Wistuba [511].

The use of proteins, such as HSA and AGP immobilized on HPLC-grade silica gel was described in early publications [513]. The relatively low efficiency obtained on these phases would certainly be improved by using smaller silica gel particles. There are no recent papers on this subject.

Several groups recently investigated polysaccharide derivative-based CSPs for chiral CEC separations in aqueous [514–517] or non-aqueous medium [518]. Several drug enantiomers were resolved on these phases. Efficiency was found to be higher than with HPLC.

Poly-*N*-acryloyl-L-phenylalanine ethylester covalently bound to silica (Chiraspher) [514] or a helically chiral poly(diphenyl-2-pyridylmethyl methacrylate) [519], which was coated to wide-pore aminopropyl-silanized silica, was used by Krause *et al*. for chiral separations by capillary-HPLC and pressure-supported CEC.

Recently, new CSPs containing macrocyclic antibiotics have been prepared. Dermaux *et al*. [520] and Wikström *et al*. [521] immobilized vancomycin to silica gel. These phases were applied to the chiral separation of neutral, basic and acidic compounds in both reversed phase and polar organic mode with remarkable efficiency. Carter-Finch and Smith [522] and Karlsson *et al*. [523] investigated capillaries packed with a commercial teicoplanin CSP (Chirobiotic T). It was shown that this phase can be used for the chiral separation of a broad spectrum of compounds using reversed phase and non-aqueous conditions.

Wolf *et al*. [524] reported the use of two brush-type CSPs, an (S) naproxen-derived CSP and of a (3*R*,4*S*) Whelk-O-CSP for chiral CEC. Efficiencies of up to 200 000 plates/m were observed on these phases. Screening studies were performed with the latter phase investigating 41 neutral analytes [525].

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Lämmerhofer and Lindner [526] developed an anion exchange CSP based on tert.-butyl quinine carbamate immobilized on silica. This CSP was applied both for the HPLC and CEC separation of amino acid derivatives and was also found to be applicable to non-aqueous CEC [527].

Lin *et al*. [528] prepared molecularly imprinted polymers by copolymerization of methacrylic acid or 2-vinylpyridine as functional monomers and ethylene glycol dimethacrylate as crosslinker in the presence of initiator 2,2′-azobis isobytyronitrile (AIBN) using Dns-L-leucine or L-phenylanilide as print molecules. After sieving, the particles were packed into the capillary.

Monolithic CSPs

Monolithic phases are a novel alternative to capillaries containing packings on silica gel basis.

Packing of capillaries and the preparation of frits by sintering a packing zone are rather complicated procedures. Furthermore, both the silica particles and the frits can be sources of air-bubble formation.

The technique of preparation of monolithic phases by in-situ polymerization in the column was introduced by Hjertén *et al*. [529].

The capillary is first treated with *y*-methacryloxy propyltrimethoxysilane to provide functional groups for immobilizing the polymer at the capillary wall The monomers including an allylated selector, a crosslinker and a charge-providing agent are copolymerized *in situ* in the capillary.

Koide and Ueno [530] prepared monolithic CSPs by incorporating *β*-CD polymers such as poly *β*-CD and CM-*β*-CD polymer in a polyacrylamide gel or by *in situ* polymerization of allyl carbamoylated *β*-CD (AC-*β*-CD), acrylamide, *N*,*N*′-methylenebisacrylamide and *N*-(2-acrylamidoethyl) triethylammonium iodide, in the presence of *N*,*N*,*N*′,*N*′-tetramethylethylendiamine and ammonium peroxodisulfate in the capillary [531]. Recently, the same group developed a monolithic CSP containing a chiral crown ether, which was applied to the chiral separation of compounds containing primary amino groups [532].

Végvári *et al*. [533] prepared completely homogeneous polyacrylamide based gels by copolymerization of 2-hydroxy-3-allyloxy-propyl-*β*-CD, acrylamide *N*,*N*′-methylenebisacrylamide and 2-acrylamido-2-methylpropane sulfonic acid for the preparation of negatively charged gels and dimethyl–diallyl ammonium chloride for positively charged gels, respectively. The applicability of these phases was demonstrated by means of neutral, basic and acidic drugs.

Peters *et al*. [534] reported the preparation of a 'moulded' monolithic CSP by copolymerization of the chiral monomer 2-hydroxyethyl methacrylate (N-L-valine-3,5-dimethylanilide) carbamate with ethylene dimethylacrylate, 2-acrylamido-2-methyl-1-propanesulfonic acid and butyl or glycidyl methacrylate in the presence of a porogenic solvent. The applicability of this phase to chiral CEC separation was tested using *N*-(3,5-dinitrobenzoyl)leucine diallylamide as a model compound. Recently, Lämmerhofer *et al*. [535,536] prepared monolithic quinidine CSPs by copolymerization of *O*-[2-(methacryloyloxy)ethylcarbamoyl]-10,11-dihydroquinidine, ethylene dimethylacrylate and glycidyl methacrylate or 2-hydroxyethyl methacrylate (Figure 6). The phase was evaluated by means of the chiral separation of several amino acid derivatives, whereby efficiencies up to 250 000 plates/m were achieved. Figure 7 shows the application of these phases to the chiral separation of DMB-Leu.

Schmid *et al*. [537] synthesized a monolithic ligand-exchange CSP by in-situ copolymerization of methacrylamide and *N*-(2-hydroxy-3-allyloxypropyl)-L-4-hydroxyproline as a chiral selector in the presence of piperazine diacrylamide as a crosslinker and vinylsulfonic acid as a charge providing agent. This CSP was applied to the chiral separation of underivatized amino acids [537] and hydroxy acids [538] using phosphate buffer pH 4.4/copper(II)sulfate as mobile phase . The same capillary was used for CEC, capillary LC and pressure supported CEC. To speed up separation, 'short-end injection' using a bed of 8.5 cm in length, was applied. Under these conditions the enantiomers of Phe were baseline resolved within 4 min (Figure 8).

Schweitz *et al*. [539,540] developed monolithic imprinted polymers for chiral CEC separation using propranolol, metoprolol or ropivacaine as print molecules for the chiral separation of *β*-blockers and local anesthetics, respectively. The polymers were prepared by filling the capillary

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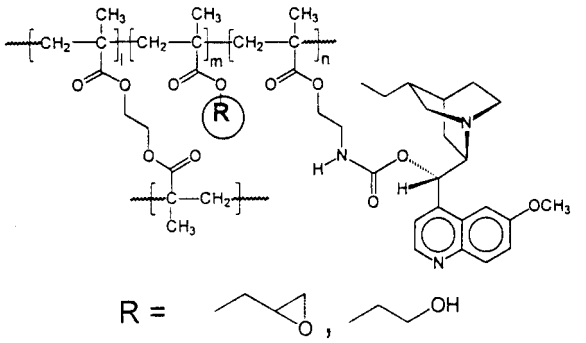

Figure 6. Simplified chemical structure of the chiral monolithic polymer prepared by copolymerization of quinine-functionalized chiral monomer, ethylene dimethylacrylate, and glycidyl methacrylate or 2-hydroxyethyl methacrylate (From Reference [535] with permission.)

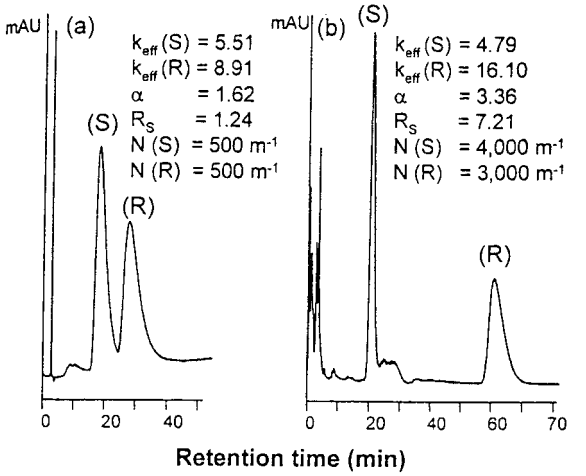

Figure 7. Electrochromatographic performance of monoliths with glycidyl methacrylate (a) and 2-hydroxyethyl methacrylate (b) as comonomers. Conditions: capillary column 335 nm (250 nm active length) $\times$ 0.1 mm ID, pore size 993 (a) and 1163 nm (b); analyte DNB-(*R,S*)-leucine; mobile phase, 44 mM acetic acid and 4 mM triethylamine in acetonitrile/methanol (80:20,v/v); voltage, -25 kV; temperature, 30°C. (From Reference [535] with permission.)

pretreated with γ-methacryoxypropyltrimethoxysilane with a pre-polymerization mixture of consisting of print molecule, functional and crosslinking monomers (methacrylic acid and trimethylolpropane trimethacrylate), radical initiator (AIBN) and solvent (toluene). Polymerization was performed by placing the capillary under a UV source. After removing the print molecule by flushing the capillary with

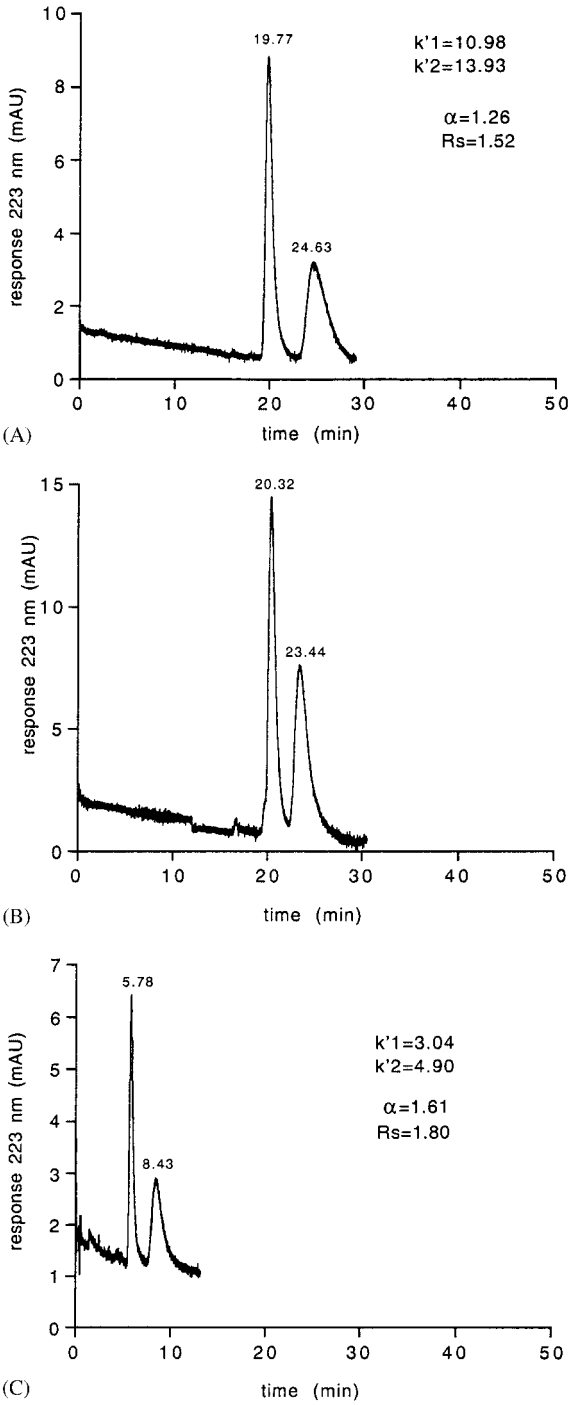

Figure 8. Chiral separation of DL-Phe by ligand exchange electrochromatography comparing (A) CEC, (B) pressure-driven nano-HPLC and (C) pressure-supported CEC. Conditions: Mobile phase, 50 mM sodium dihydrogenphosphate/0.1 mM Cu(II), pH 4.6; stationary phase: ligand exchange continuous bed (26 cm $\times$ 0.75 mm); (A) 30 kV, (B) 12 bar, (C) 30 kV and 12 bar.(From Reference [537] with permission.)

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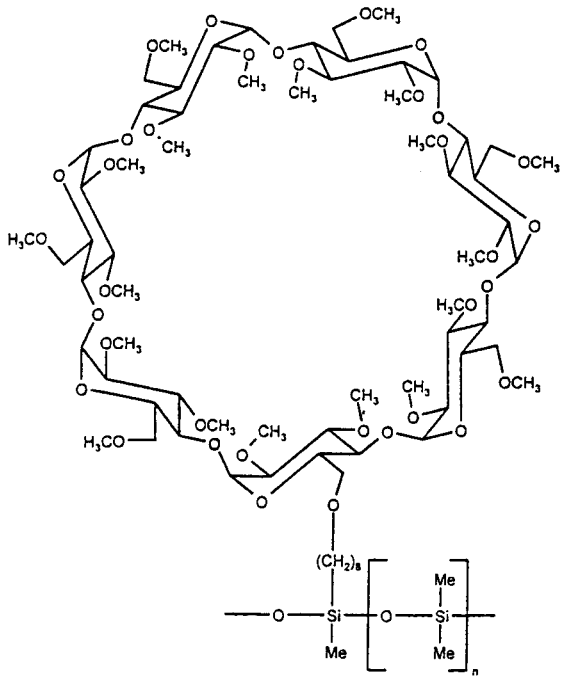

Figure 9. Chemical structure of the Chirasil-Dex CSP (From Reference [510] with permission.)

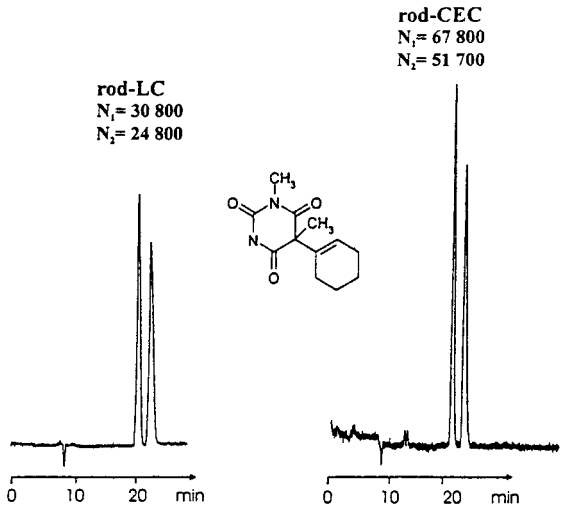

Figure 10. Enantiomer separation of hexobarbital on a Chirasil-Dex monolith by CEC an LC. Conditions: 20 cm (overall length 40 cm) × 0.1 mm ID capillary; 20 mM MES, pH 6.0/MeOH 7/3 v/v. UV detection, 230 nm. CEC: 25 kV,12 bar; LC: 50 bar. (From Reference [510] with permission.)

acetonitrile, an imprint remains, which shows high enantioselectivity for the same or very similar molecules.

Lin *et al*. [541] applied a thermally induced *in situ* polymerization procedure for the preparation of an imprinted polymer using D-phenylalanine as a print molecule. This phase showed high chiral recognition ability for phenylalanine, but was weak for tyrosine and phenylglycine. Chirica and Remcho [542] prepared a monolithic column by flushing a capillary packed with a polymer, containing L-Dns-phenylalanine as an imprint molecule, with an aqueous potassium silicate solution followed by heating from 40 to 160°C for several days.

Recently, Wistuba and Schurig [543] prepared a monolithic phase by sintering the silica bed of a packed capillary at 380°C and subsequently coating it with permethylated β-CD covalently linked via an octamethylene spacer to dimethylpolysiloxane (Chirasil-Dex, Figure 9). The phase was evaluated with several compounds, among others barbiturates, benzoin and some profens. Remarkable efficiencies were obtained. Figure 10 shows the comparison of the chiral separation of hexobarbital by LC and CEC using the Chirasil-Dex monolith.

Kato *et al*. [544] prepared a monolithic column by injecting a suspension of 5 µm silica particles modified with (*S*)-*N*-3,5-dinitrobenzoyl-1-naphthylglycine or (*S*)-*N*-3,5-dinitophenylaminocarbonylvaline in tetraethylorthosilicate, ethanol and aqueous hydrochloric acid in to a capillary and heating the capillary for 1 h at 120°C. The columns were tested using NBD-amino acids.

Miscellaneous

Enantiomerization studies

Some chiral compounds with stereo-labile units can invert their configuration. If this interconversion takes place within the time of a chromatographic run, it can be followed by chromatography. Techniques such as dynamic gas chromatography (DGC) [545,546], dynamic liquid chromatography (DHPLC) (5,547–550] and SFC [551] have been created to investigate such phenomena. Another approach is to use stopped-flow chromatography [550].

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Reversal of enantiomeric elution order (EMO)

Reversal of the EMO in chromatographic and electromigration techniques is required, for example, in connection with enantiomeric purity checks for drugs. The distomer present in traces in a sample of the eutomer should always be the first peak, otherwise it could be covered by the tailing of the eutomer. One way would be to change to a selector possessing opposite chirality. This is, however, only rarely possible in HPLC using chiral stationary phases. In CE reversal of the EMO can sometimes be obtained by changing from a neutral to a charged selector, by changing the mobility of the analyte or the selector by varying the pH or by reversing the direction of the EOF. A survey of different possibilities for reversing the EMO in CE has recently been given by Chankvetadze *et al*. [370].

Chiral analysis of drugs in biological fluids

The chiral separation and quantification of drugs in biological fluids is of relevance in connection with pharmacodynamic investigations, drug disposition studies, metabolism studies and gained recently in interest also in toxicological and forensic analysis as well as in analysis of drugs of abuse. The analysis of drug enantiomers in biological fluids requires the development of very selective methods to separate the analytes from the biological matrix in addition to chiral separation. Furthermore, often the chiral separation and quantification of the metabolites is also desired. A crucial point in bioanalysis is detection sensitivity, which can be a problem with CE with conventional UV-detection. Laser-induced fluorescence detection (LIF) or the coupling of CE with MS might overcome this drawback.

A general discussion of the problems arising with chiral drug analysis in biological fluids by HPLC is given by Ducharme *et al*. [36]. Since direct injection of biological samples reduces the life-time of the columns, sample pretreatment by conventional liquid–liquid extraction or solid-phase extraction is mostly included. Column coupling and column switching methods are elegant methods to solve such problems [37,552,553]. Görög and Gazdag [22] reported the use of chiral dervivatization reagents for biomedical chromatography. Bressolle *et al*. gave an specialized overview of the use of cyclodextrins for chiral analysis in HPLC and CE including applications to biological fluids [71]. Comprehensive reviews dealing with the chiral analysis of drug enantiomers and their metabolites by CE and applications to pharmacokinetic studies have recently been published by Bojarski and Aboul-Enein [554] and by Zaugg and Thorman [555]. Several recent examples for the determination of drug enantiomers and their metabolites in biological fluids have been listed in a review by Blaschke and Chankvetadze [556].

Examples for the use of GC for chiral separation of drugs following pre-column derivatization for pharmacokinetic studies were given by Srinivas *et al*. [271].

Abbreviations

18C6H4	18-crown-6-tetracarboxylic acid
AGP	α_1-acid glycoprotein
AIBN	2,2′-azobis(isobutyronitrile)
ATPC	amylose trisphenyl carbamate
BGE	background electrolyte
BSA	bovine serum albumine
CD	cyclodextrin
CD-MEKC	CD-mediated micellar electrokinetic chromatography
CEC	capillary electrochromatography
CLEC	chiral ligand-exchange chromatography
CSP	chiral stationary phase
CTPC	cellulose trisphenyl carbamate
CZE	capillary zone electrophoresis
Dns	dansylated
EKC	electrokinetic chromatography
EMO	enantiomer migration order
EOF	electroosmotic flow
L-Hypro	L-4-hydroxyproline
IEF	isoelectric focusing
ITP	isotachophoresis
LECE	ligand-exchange capillary electrophoresis
MEKC	micellar electrokinetic chromatography
MES	2-(*N*-Morpholino)ethanesulfonic acid
NA	non-aqueous

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NBD	7-Nitrobenz-2-oxa-1,3-diazol
NMF	N-methylformamide
NSAID	non-steoridal antiinflammatory drug
OT-CEC	open tubular capillary electrochromatography
P-CEC	packed capillary electrochromatography
SDS	sodium dodecyl sulfate

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