ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis 27 (2002) 357–372

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

www.elsevier.com/locate/jpba

Review

Pharmaceutical and biomedical applications of enantioseparations using liquid chromatographic techniques

Jun Haginaka *

Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 11-68, Koshien Kyuban-cho, Nishinomiya, Hyogo 663-8179, Japan

Received 9 July 2001; received in revised form 24 July 2001; accepted 24 July 2001

Dedicated to Professor Dr Gottfried Blaschke on the occasion of his 65th birthday

Abstract

The chiral separation methods using liquid chromatographic techniques can be divided into two categories: one is a direct method, which is based on a diastereomer formation on stationary phase or in mobile phase. The other is an indirect method, which is based on a diasteromer formation by reaction with a homochiral reagent. The enantiomer separation on a chiral stationary phases followed by derivatization with an achiral reagent is also dealt with this review article as the indirect method. The pharmaceutical and biomedical applications of enantioseparations using the direct and indirect methods have been considered in this review. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Stationary phases, liquid chromatography; Chiral stationary phases, liquid chromatography; Chiral separation; Chiral derivatizing reagent

Nomenclature

AGP	α_1-acid glycoprotein
2-APAs	2-arypropionic acid derivatives
AP-OTf	2-(anthracene-2,3-dicarboximido)-1-propyltrifluoromethanesulfonate
β-CD	β-cyclodextrin
CBH	cellobiohydrolase I
CD	circular dichroism

* Corresponding author. Tel.: +81-798-45-9949; fax: +81-798-41-2792.
E-mail address: haginaka@mwu.mukogawa-u.ac.jp (J. Haginaka).

0731-7085/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0731-7085(01)00652-5

CDITC	*N*-[(2-isothiocyanato)cyclohexyl]-6-methoxy-4-quinolinylamide
Chiralcel OD	*tris*(3,5-dimthylphenyl carbamate)cellulose
Chiralpak AD	*tris*(3,5-dimetylphenylcarbamate)amylose
CSP	chiral stationary phase
DBD-Apy	4-(*N*,*N*-dimethylaminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole
DBD-CO-Hz	(*N*-hydrazinoformyl)methyl]-*N*-methyl]amino-7-(*N*,*N*-dimethylamino)sulfonyl-2,1,3-benzoxadiazole
DBD-F	(*N*,*N*-dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole
DBD-*N*-Me-Ala	4-(*N*-1-carboxyethyl-*N*-methyl)amino-7-(*N*,*N*-dimethyl)amino-2,1,3-benzoxadiazole
DBD-Pro	4-(2-carboxypyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulphonyl)-2,1,3-benzoxadiazole
DBD-PyNCS	4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole
DBD-Pz	4-(*N*,*N*-dimethylamino)sufonyl-7-piperazine-2,1,3-benzoxadiazole
DIB-Cl	4-(4,5-diphenyl-lH-imidazol-2-yl)benzoyl chloride
ELSD	evaporative light scattering detection
FLEC	fluorenylethyl chloroformate
FMOC-L-Pro	9-fluorenylmethyl chloroformate-L-proline
GITC	2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate
LC	liquid chromatography
3-MPA	3-mercaptopropionic acid
MS	mass spectrometry
NBD-Apy	4-nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole
NBD-CO-Hz	*N*-(4-nitro-2,1,3-benzoxadiazoyl-7-yl)-*N*-methyl-2-aminoacetohydrazide
NBD-F	4-fluoro-7-nitro-2,1,3-benzoxadiazole
NBD-N-Me-Ala	4-(*N*-1-carboxyethyl-*N*-methyl)amino-7-nitro-2,1,3-benzoxadiazole
NBD-Pro	4-(2-carboxypyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole
NEA	1-(1-naphthyl)ethylamine
NEIC	naphthylethylisocyanate
OPA	*o*-phthalaldehyde
PITC	phenyl isocyanate
PTC-AAs	phenylthiocarbamoyl derivatives of amino acids
RAM	restricted access media
SPE	solid-phase extraction

1. Introduction

Two enantiomeric forms of a drug can have different properties, such as different potencies and different toxicities [1]. Thus, in the preparation and analysis of chiral drugs, it is of vital importance to separate their enantiomeric forms. In the 1970s, racemic thalidomide was chromatographically separated on optically active polyamide by Blaschke's group [2]. This study prompted the development and progress of chiral stationary phases (CSPs), and many research groups have sought to suitable CSPs for chromatographic resolution of enanitomeric forms

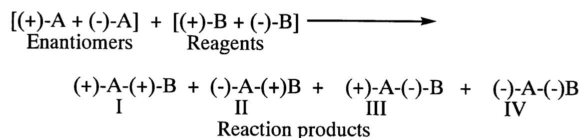
over the last three dacades [3,4]. Those include natural chiral selectors such as proteins, oligosaccharides, macrocyclic antibiotics and alkaloids, semisynthetic chiral selectors such as modified oligosaccharides and polysaccharides, and synthetic chiral selectors such as Pirkle type selectors, crown ethers, proline derivatives and helical synthetic polymers.

The chiral separation methods can be divided into two categories: one is a direct method, which is based on a diastereomer formation on a stationary phase or in a mobile phase [5,6]. The other is an indirect method, which is based on a diasteromer formation by reaction with a homochiral reagent. The enantiomer separation on a CSP followed by derivatization with an achiral reagent is also dealt with in this review article as the indirect method. The direct method, which can separate enantiomeric forms based on formation of a diastereomeric complex on a stationary phase, is suitable for the analysis of enantiomers in a standard sample and pharmaceutical preparations, where the low amount of the antipode level should be determined. It is suitable for preparative purposes [6]. However, for trace analysis of the enantiomers of a drug and its metabolite(s) in complex matrices such as biological samples, liquid–liquid extraction, solid-phase extraction (SPE) or column switching technique was followed by the enantiomer separation on a CSP. Further, for biomedical and pharmaceutical applications of enantioseparations, mass spectrometry (MS) combined with liquid chromatography (LC) separations, which includes LC–MS–MS, has been utilized for the sensitive and specific detection of a target drug [7]. On the other hand, the indirect method, which involves the reaction with a homochiral reagent and the separation of the diastereomeric derivatives on an achiral stationary phase, is an efficient technique for separation of many enantiomers having functional groups. It is essential that the chiral derivatization reaction proceeds completely in both enantiomers, and that the racemization reaction does not occur [5,6]. Further, if the optical purity of the derivatization reagent is not known, or is not taken into consideration, the optical purity of the target compound will not be determined precisely. The enantiomers of A are to be separated and determined as their diastereomeric derivatives (I and II), which can be resolved as respective peaks on achiral stationary phases (Scheme 1). However, the peaks of the products III and IV produced with (−)-B overlap with the peaks of II and I, respectively, on achiral stationary phases, because II and III, and I and IV are enantiomeric pairs. The indirect method was unsuitable for the analysis of enantiomers in a standard sample and pharmaceutical preparations, and were unsuitable for preparative purposes. However, they were suitable for trace analysis of enantiomers in complex matrices such as biological samples because of the introduction of a highly sensitive tag(s). Those include ultraviolet-visible, fluorescence and electrochemical tags [6,10]. Among them, the fluorescence derivatization is the most effective for determinations in complex matrices, in terms of sensitivity and/or selectivity.

Many book chapters and review articles dealt with the separations of drug enantiomers using LC techniques [1,3,4,8–13]. This review article focuses on recent developments in the biomedical and pharmaceutical applications of enantioseparations using LC techniques.

2. Direct methods

Though direct methods using chiral mobile phase additives can separate many enantiomers by addition of chiral selectors to a mobile phase on an achiral stationary phase, the applicability of the methods in the biomedical and pharmaceutical areas is limited because of purity of chiral selectors and detection problem. This review article only deals with the enatioseparations based on CSPs.



Scheme 1. Derivatization of enantiomers with a chiral reagent with less than 100% optical purity.

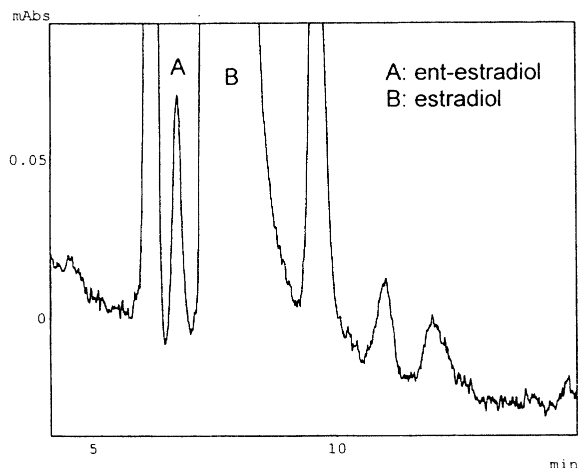


Fig. 1. Chromatogram of a batch of estradiol drug substance spiked with 0.05% *ent*-estradiol. Reproduced from Ref. [17] with permission. LC conditions: Chiralpak AD-R column (250 × 4.6 mm i.d.); mobile phase, acetonitrile-water (50:50, v/v); flow-rate, 1.0 ml/min; column temperature, 40 °C; detection, 278 nm.

2.1. Pharmaceutical analysis

In pharmaceutical applications, the quantitation of the antipode in pharmaceuticals or drug substances can be attained with the direct separation of drug enantiomers using CSPs. The quantitation of the opposite enantiomer of tiagabine hydrochloride was attained using a *tris*(3,5-dimthylphenyl carbamate)cellulose CSP (Chiralcel OD) [14]. Similarly, crown ether dynamically coated CSP was used for the enatiomer separation of hydrophobic amino compounds [15]. The method could determine as little as 0.05% of the D-form in L-alanine-β-naphthlamide. Ovomucoid and Chiralcel OD columns were used in reversed-phase and normal phase modes, respectively, for the quantitation of fluoxetine hydrochloride enantiomers [16]. The chromatographic conditions provided a much more discriminating test, compared to an optical rotation method proposed for pharmacopeial use [16]. Chiral resolution of enantiomeric steroids was attained using *tris*(3,5-dimetylphenyl carbamate)amylose (Chiralpak AD-R, R means a column for reversed-phase mode) under reversed-phase mode [17]. Fig. 1 shows the chromatogram of an estradiol sample spiked with 0.05% of the unnatural enantiomer of estradiol (*ent*-estradiol), whose concentration level is the quantitation limits. The method was successfully validated for the quantitation of *ent*-estradiol in drug substances.

For the optimization of enantioselective analytical methods, chemometrics, factorial design [18–20] or neural network [21–23], was used to examine the influence of the descriptor variables on chromatographic responses. Two CSPs based on α_1-acid glycoprotein (AGP) and cellobiohydrolase I (CBH) were tested for the enantioseparations of twelve closely structurally related amino alcohols using factorial design [20]. The changes in mobile phase buffer pH and column temperature affected the enantioselective retentions for the two CSPs. Further, the ionic strength and 2-propanol concentration affected them for the AGP and CBH columns, respectively [20].

For the detection of enantiomeric compounds which have no chromophore, evaporative light scattering detection (ELSD) method was effective [24,25]. Carnitine and *O*-acetylcarnitine [24], and underivatized amino acids [25] enantioseparated using teicoplanin CSPs were easily detected by ELSD.

2.2. Biomedical applications

With regard to the assays of enantiomeric drugs in biological fluids, liquid-liquid extraction methods were generally utilized for extraction and concentration of the target drugs [26–37]. The simultaneous determinations of disopyramide and its active metabolite, mono-*N*-dealkyldisopyramide, in plasma and urine were attained with a Chiralpak AD column using hexane–ethanol plus diethylamine as an eluent, following the liquid–liquid extraction with dichloromethane after protein precipitation with trichloroacetic acid [37]. Typical chromatograms of drug-free human plasma, human plasma spiked with disopyramide and mono-*N*-dealkyldisopyramide, and treated subject plasma are shown in Fig. 2. As an alternative approach to liquid-liquid extraction, the disposable SPE columns such as C18, nonpolar/strong cation mixed phase and phenylboronic acid were used for the assays of ketopro-

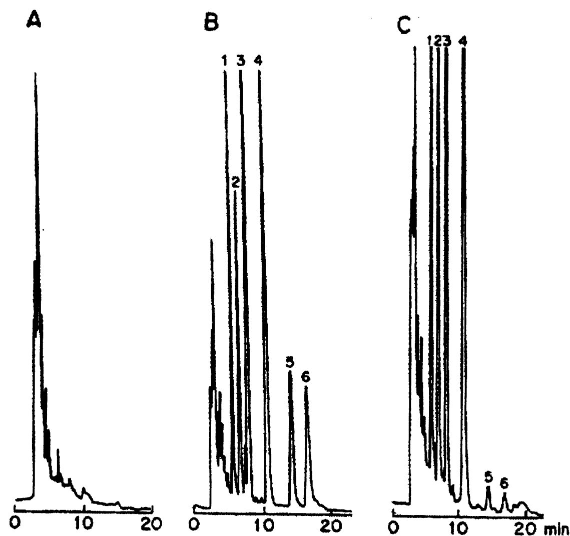
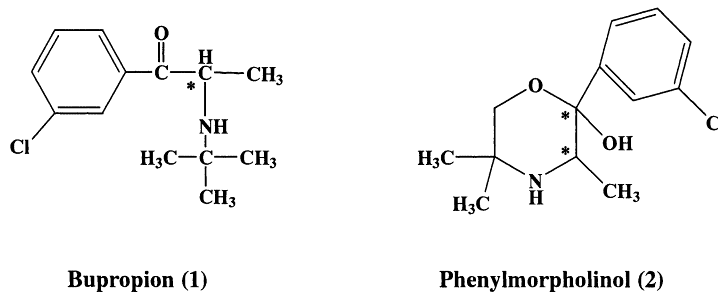


Fig. 2. Chromatograms referring to the analysis of disopyramide and mono-N-dealkyldisopyramide enantiomers in plasma. Reproduced from Ref. [37] with permission. (A) Blank plasma; (B) plasma spiked with 625 ng/ml of disopyramide and mono-N-dealkyldisopyramide enantiomers; (C) plasma sample from a healthy volunteer collected 6 h after the administration of 100 mg of Dicorantil (racemic disopyramide). Peak assignments: (+)-(S)-disopyramide (1); (−)-(R)- disopyramide (2); metoprolol (3 and 4); (+)-(S)-mono-N-dealkyldisopyramide (5); (−)-(R)-mono-N-dealkyldisopyramide (6). LC conditions: Chiralpak AD column (250 × 4.6 mm i.d.); mobile phase, hexane–ethanol (91:9, v/v) plus 0.1% diethylamine; flow-rate, 1.2 ml/min; detection, 270 nm.

fen [38], reboxetine [39] and albuterol [40] enantiomers, respectively, in biological samples.

For the enantiomeric determinations of drugs and their metabolites in serum or plasma, tedious and time-consuming pretreatment procedures such as liquid–liquid extraction, SPE or membrane-based extraction have been required [41]. Among those pretreatment procedures, SPE is most widely used for the extraction of the target compounds in biological fluids. However, direct injection of serum or plasma samples onto LC or SPE materials causes protein denaturation at the partitioning phase and accumulation on the particles, resulting in undesired loss in the capacity and selectivity of the packing materials [42,43]. Thus, it is inevitable to remove serum or plasma proteins before loading the samples onto the LC or SPE materials. Recently, restricted access media (RAM) were introduced for direct injection of proteinaceous samples onto the LC or SPE materials [42,43]. Direct serum injection assays of pindolol enantiomers were attained using Pinkerton GFF2 RAM system [44]. The method involves on-line sample clean-up using Pinkerton GFF2 RAM column separation and heart-cut of the drug on a C18 column, and the chiral separation of the drug enantiomers on an AGP column. The alkyl-diol RAM columns were used in a column-switching system for the determination of ketoprofen enantiomers in plasma [45] and atenolol enantiomers in urine [46], in conjunction with human serum albumin and teicoplanin columns, respectively.

Direct injection of the biological samples after liquid-liquid extraction or solid-phase extraction resulted in interferences by biological matrix components and drastically diminished the column life. Thus, the on-line achiral–chiral system using a trimethylsilyl-silica gel column and an AGP column was developed for the enantiomeric determination of the phenylmorpholinol metabolite (**2**) of bupropion (**1**) (their structures, see Scheme 2)



Scheme 2. Structures of bupropion (**1**) and phenylmorpholinal (**2**).

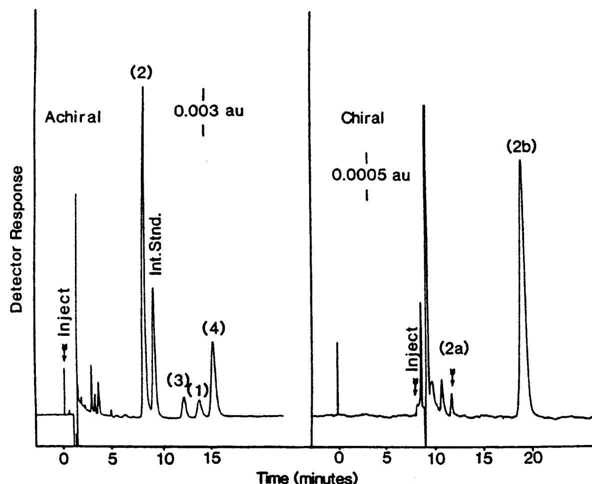
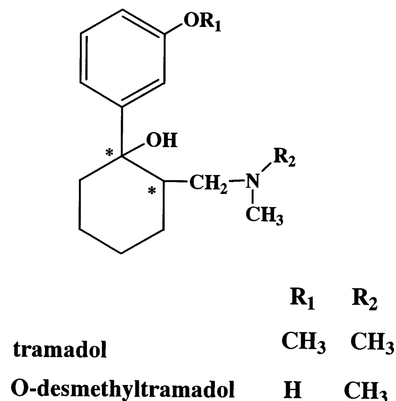


Fig. 3. Chromatograms of a 100 μl injection of a human steady-state plasma sample extract. Reproduced from Ref. [47] with permission. LC conditions: achiral column, Supelcosil LC-1 (150 × 4.6 mm i.d.); chiral column, Chiral-AGP (100 × 4 mm i.d.). Peak assignments: bupropion (1); phenylmorphorinol (2); *erythro*-amino alcohol (3); *threo*-amino alcohol (4); (+)-phenylmorpholinol (2a); (−)-phenylmorpholinol (2b). Concentration of phenylmorpholinol (2) is estimated to be 2167 ng/ml (equivalent to 2071 ng/ml of (−)-phenylmorpholinol (2b)).

in human plasma after liquid–liquid extraction [47]. It was found that **2** is present in human steady-state plasma in enantiomeric (−)-form, which corresponds to peak 2b in the chiral chromatogram in Fig. 3. Since a C18 column has been known to provide very strong retention of a target drug, it is very difficult to match the eluents between achiral and chiral systems. For the stereoselective determination of montelukast, a potent and selective leukotriene D_4 receptor antagonist, in plasma after protein precipitation [48], Biomatrix RAM column was used as an achiral column. Enantiomer separations of chlorpheniramine and its main monodesmethyl metabolite in urine were attained using a coupled achiral–chiral LC system after liquid–liquid extraction [49]. The two compounds were separated from the biological matrix on a cyanopropyl column and reinjected into a Chiralpak AD-R column in a reversed-phase mode. For the enantiomeric separations of tramadol and its metabolite [50], and varapamil and its metabolites [51], an off-line achiral–chiral LC system was used. For the simultaneous separation of verapamil and its seven metabolites, a rapid and simple achiral system using Hisep C18 and Novapak C18 columns was used. The achiral preseparation steps in off-line mode have been recommended as effective clean-up and preconcetration procedures. An AGP column was used for successful chiral separations of verapamil and its seven metabolites [51].

LC–MS, especially LC–MS–MS, was effectively used for etection of a drug and its metabolites in biological fluids [52–56]. On-line LC–MS–MS system using atmospheric pressure chemical ionization was developed for the enantiomeric determinations of tramadol (**3**) and its active metabolite, *O*-desmethyltramadol (**4**), in human plasma (their structures, see Scheme 3). After off-line solid-phase extraction on a disposable extraction cartridges, the enantiomeric separations of tramadol and *O*-desmethyltramadol were achieved on a Chiralpak AD column [55]. The MS–MS ion traditions monitored were 264 → 58 for tramadol, 264 → 58 for *O*-desmethyltramadol and 264 → 58 for ethyltramadol, used as an internal standard. Fig. 4 shows a typical chromatogram of a plasma extract spiked with racemic tramadol and racemic *O*-desmethyltramadol. These two drugs were enantioseparated and the absence of interfering endogenous components at retention times of tramadol and *O*-desmethyltramadol was demonstrated.



Scheme 3. Structures of tramadol (**3**) and *O*-desmethyltramadol (**4**).

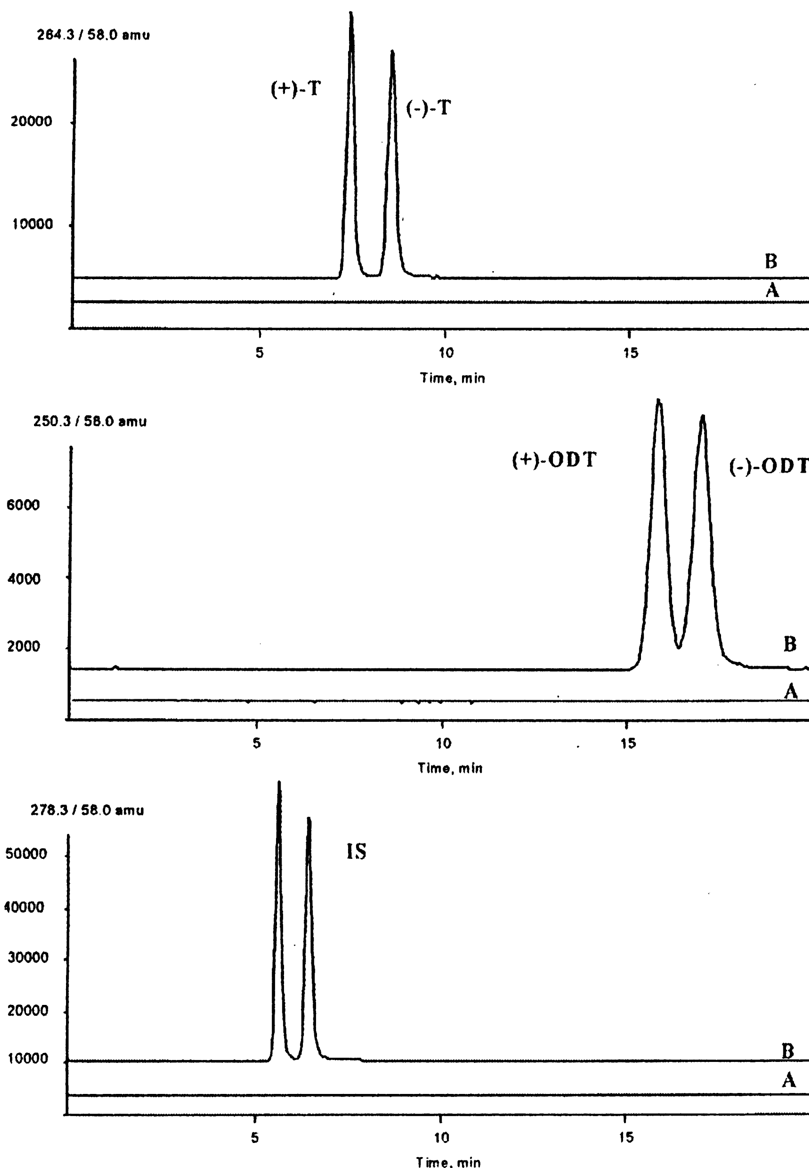


Fig. 4. Selective reaction monitoring ion chromatogram of blank plasma (A) and spiked plasma (B) samples with 25 ng/ml for tramadol (T) and 10 ng/ml for *O*-desmethyltramadol (ODT) racemates. Reproduced from Ref. [55] with permission. LC conditions: column, Chiralpak AD (250 × 4.6 mm i.d.); mobile phase, isohexane–ethanol–diethylamine (97:3:0.1, v/v); flow-rate, 1.0 ml/min; column temperature, 25 °C.

The enantioseparations of warfarin and 7-hydroxywarfarin in human plasma and urine, respectively, were attained using Chiralcel OD column with ultraviolet or fluorescent detection, and their absolute configuration was determined simultaneously by circular dichroism (CD) detection [57]. Similarly, enantiomers of lorazepam in human plasma were determined using a β-cyclodextrin (β-CD) column based on CD detection [58]. Chiroptical detection was useful for the pharmacokinetic study of chiral drugs.

Recently, SPE on disposable cartridges, dialysis or column switching used for sample preparation techniques, which can be fully automated and applied to enantioselective analysis in biological fluids, are compared, with regard to selectivity, detectability, elution of the analytes from the extraction sorbent, sample volume and analyte stability (Table 1)[41]. The three sample preparation techniques had advantages and disadvantages: in column-switching systems, stability problems were often observed with the precolumn but the recent introduction of RAM gives some interesting perspectives. The SPE procedure with disposable extraction cartridges is an 'open' system that can sometimes cause problems if the analytes are particularly sensitive to light or to oxygen, However, the wide range of extraction sorbents commercially available combined to the high flexibility in the choice of the eluting solvent are two important advantages. The principal limitation of the dialysis approach lies in the recovery of the analytes, which is usually somewhat lower than those observed with the other two approaches.

3. Indirect method

The indirect methods were divided into two categories: one is to derivatize the enantiomers using a homochiral derivatizing reagent (structures, see Scheme 4) and to separate the derivatives using an achiral stationary phases. The other is to derivatize the enantiomers using an achiral derivatizing reagent (structures, see Scheme 5) and to separate the derivatives using a CSP.

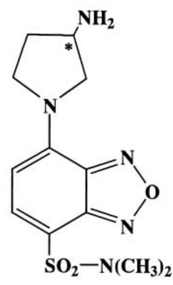
3.1. Derivatization with chiral reagent

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) as a chiral derivatization reagent was used for the assays of S-(−) and R-(+) enantiomers of propranolol and 4-hydroxypropranolol in human plasma [59]. The method involved liquid–liquid extraction for sample clean-up, derivatization with GITC and separation of the derivatized products on a C18 column. The proposed assay was used to quantify the enantiomers of propranolol and 4-hydroxypropranolol, respectively, in human plasma. Similarly, GITC was used for the assays of the enantiomers of atenolol [60] and unusual secondary aromatic amino acids [61]. (+)-(S)-Naphthylethylisocyanate ((S)-NEIC) was used for the determination of mefloquine enantiomers in plasma, urine and whole blood [62]. The method involved liquid–liquid extraction of mefloquine from biological fluids and derivatization of the residue by (S)-NEIC, and separation of the resulting diastereomers on a silica gel column. Further, enantiomers of acebutolol and its active metabolite, diacetolol, in human serum were determined after dervatization with (S)-NEIC following liquid–liquid extraction [63]. Enantiomerically pure

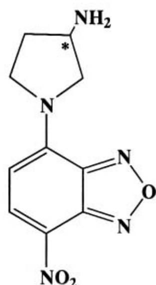
Table 1
Comparison of different sample preparation methods coupled to enantioselective LC analysis

	SPE on DEC	Dialysis	Column-switching
Selectivity	Wide range of sorbents available for DEC	Dialysis: non selective process, wide range of sorbents available for TEC	Limited number of RAM sorbents available so far
Detectability	High recoveries, possibility of reconcentration of the extract	Recoveries lower than 100%	High recoveries
Elution	Free choice of the eluting solvent	By the LC mobile phase	By the LC mobile phase
Sample volume (μl)	100–1000	100–370	10–1000
Stability	Open system	Totally closed system	Totally closed system

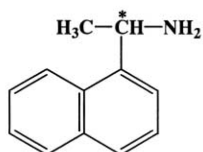
Reproduced from Ref. [41] with permission. SPE, solid-phase extraction; DEC, disposable extraction cartridge; TEC, trace enrichment cartridge; RAM, restricted access media.



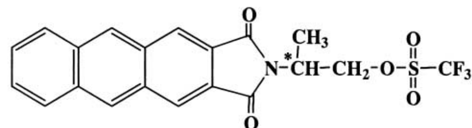
DBD-Apy



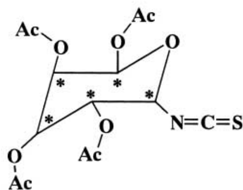
NBD-Apy



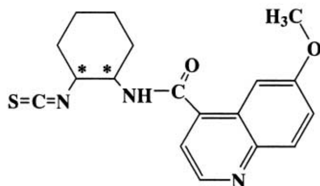
NEA



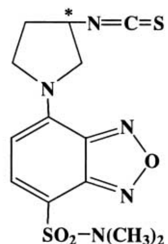
AP-OTf



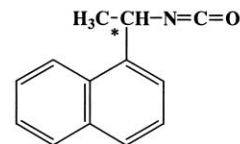
GITC



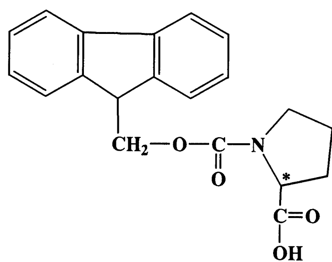
CDITC



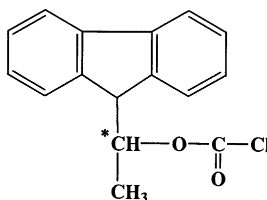
DBD-PyNCS



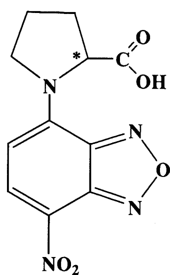
NEIC



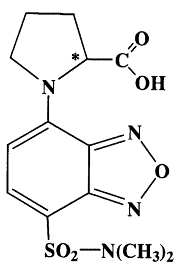
FMOC-L-Pro



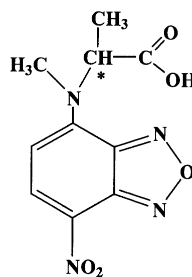
FLEC



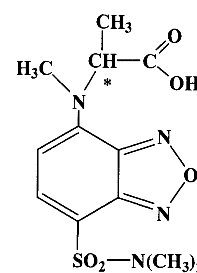
NBD-Pro



DBD-Pro

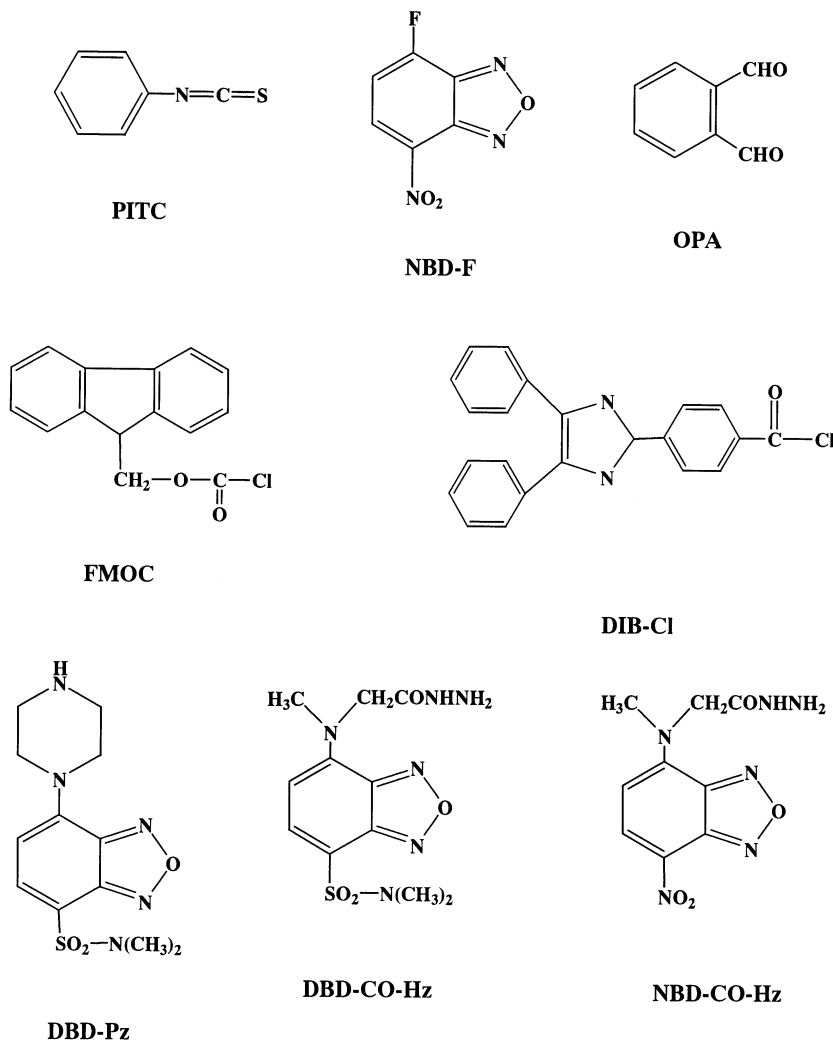


NBD-N-Me-Ala



DBD-N-Me-Ala

Scheme 4. Structures of a homochiral derivatizing reagent.



Scheme 5. Structures of an achiral derivatizing reagent.

trans-1,2-diaminocyclohexane based (1R,2R)- and (lS,2S)-*N*-[(2-isothiocyanato)cyclohexyl]-6-methoxy-4-quinolinylamide ((R,R)- and (S,S)-CDITC) was designed as a new fluorescence tagging chiral derivatizing agent for the enantiomeric separation of amino acids and amines [64]. These derivatives were separated on a C18 column. A fluorescent chiral tagging reagent, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [R-(−)-DBD-PyNCS], has been used for the derivatization of amino acids [65] and thiols [66]. The enantiomeric separations of propranolol in rat plasma and saliva, and thiols were attained based on diastereomer formation with R-(−)-DBD-PyNCS [67]. Further, R-(−)-DBD-PyNCS was applied to chiral resolution of 17 amino acids and discrimination of D/L-amino acids in peptide sequences [68]. 9-Fluorenylmethyl chloroformate-L-proline (FMOC-L-Pro)[69] or (−)-fluorenylethyl chloroformate (FLEC)[70] were employed for the enantiomeric determination of amphetamine and various amphetamine-type compounds by LC. FLEC was applied for the separation of enantiomers of α-hydroxy acids

[71]. Further, 4-(2-carboxypyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole (NBD-Pro), 4-(2-carboxypyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulphonyl)-2,1,3-benzoxadiazole (DBD-Pro), 4-(*N*-1-carboxyethyl-*N*-methyl)amino-7-nitro-2,1,3-benzoxadiazole (NBD-*N*-Me-Ala), 4-(*N*-1-carboxyethyl-*N*-methyl)amino-7-(*N*,*N*-dimethyl)amino-2,1,3-benzoxadiazole (DBD-*N*-Me-Ala) have been synthesized for the resolution of enantiomers of amines [72].

For enantiomeric determination of 2-arypropionic acid derivatives (2-APAs), a few chiral derivatizing reagents were reported [73–75]. The fluorescent reagents with a benzofurazan structure, (S)-(+)-4-(*N*,*N*-dimethylaminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole ((S)-DBD-Apy), (R)-(−)-4-nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole ((R)-NBD-Apy) by LC-MS were utilized for the enantiomeric separation and the detection of 2-APAs. The separation on a C18 column after the diastercomeric derivatization with (S)-DBD-Apy or (R)-NBD-Apy and detection by electrospray ionization MS afforded high sensitivity [73]. Methylated *N*ε-dansyl-L-lysine, which has a free amino group and an asymmetric carbon atom, was examined as a chiral derivatizing reagent for ibuprofen [74]. The method was successfully applied for the determination of ibuprofen in urine. (R)-1-(1-Naphthyl)ethylamine ((R)-NEA) was used for the derivatization of the enantiomers of flurbiprofen and ibuprofen in human serum, serum dialysate and urine samples [75]. Chromatograms of drug free urine and urine obtained from a volunteer, 2–4 h post drug administration, both prior to and following treatment with NaOH are shown in Fig. 5. No interfering peaks were observed in the chromatograms of blank and blank alkali treated urine samples. (S)-(+)-2-(Anthracene-2,3-dicarboximido)-1-propyl trifluoromethanesulfonate (AP-OTf) was synthesized as a chiral fluorescence derivatization reagent for carboxylic acids [76,77].

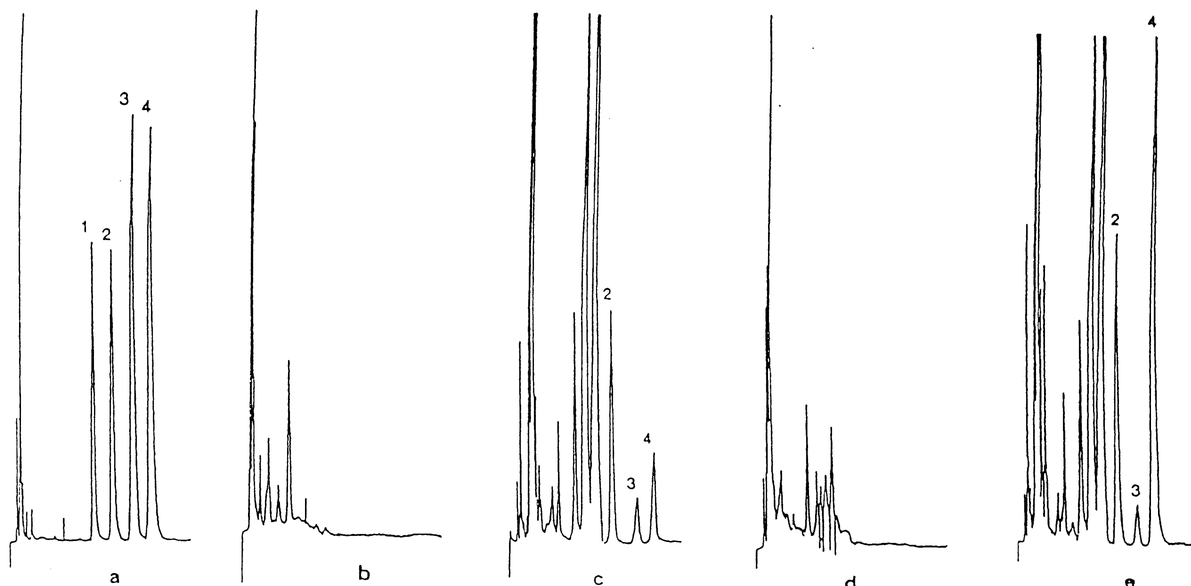


Fig. 5. Chromatograms of (a) standard solutions of racemic ibuprofen (5 ng/ml) derivatized with (R)-NEA, (b) extract of a blank urine sample, (c) extract of a urine sample of a volunteer 2–4 h following oral administration of 400 mg of the racemic drug, (d) extract of alkali treated blank urine and (e) extract of volunteer urine sample as in (c) following treatment with alkali. Reproduced from Ref. [75] with permission. LC conditions: column, Resolve C18 (150 × 2.1 mm i.d.); mobile phase, 10 mM phosphate buffer (pH 3.5)–acetonitrile = 50:50 (v/v); detection, excitation wavelength at 290 nm and emission wavelength at 330 nm. Peak assignments: 1, (R)-flurbiprofen; 2, (S)-flurbiprofen; 3, (R)-ibuprofen; 4, (S)-ibuprofen.

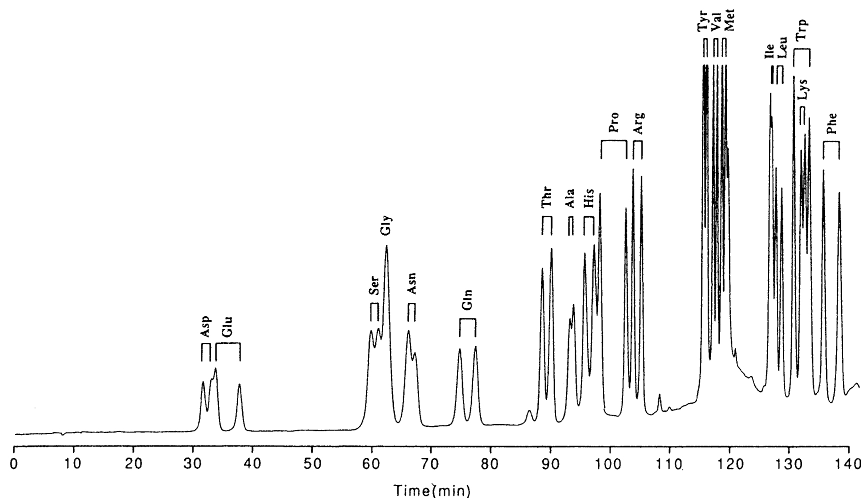


Fig. 6. Separation of a mixture of 37 PTC amino acids. Reproduced from Ref. [79] with permission. Amount of each enantiomer was 1 nmol, and Gly was 2 nmol. The elution order of enantiomers was D-form and then L-form. Ile was a mixture of D-allo-Ile and L-Ile. Column and temperature: Octyl-80Ts (at 30 °C) + phenylcarbamoylated β-CD (at 20 °C). Mobile phase: A, 100 mM ammonium acetate (pH 6.5) containing 1 mM sodium butanesulfonate; B, 100 mM ammonium acetate (pH 6.5)– methanol = 50:50 (v/v) containing 1 mM sodium butanesulfonate. Gradient: isocratic elution until 50 min (B, 0%), linear gradient elution from 50 to 80 min (B, 0–20%) then from 80 to 110 min (B, 20–80%), isocratic elution from 110 to 150 min (B, 80%). Flow-rate: 0.7 ml/min. Detection, 254 nm.

Beraprost, which is composed of four optical isomers, was derivatized with this reagent, and all four isomeric derivatives were separated using a silica gel column. The method was successfully applied to determine beraprost in human plasma [77]. 4-(*N*,*N*-Dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) and R-(−)-DBD-APy, respectively, were used for chiral separation of racemic metabolite, M1, from erdosteine, a thiol and two carboxyl groups in the M1 structure [78]. The derivatives were completely separated on a C18 column. Erdosteine with a carboxyl group was also labelled with R-(−)-DBD-APy and separated together with M1 derivatives. The simultaneous determinations of racemic M1 and erdosteine in rat plasma after administration of erdosteine were attained using the proposed derivatization and separation methods.

3.2. Derivatization with achiral reagent

Phenyl isocyanate (PITC), which is used for N-terminal sequencing of peptides or proteins, was used for the enatiomeric separations of the derivatized amino acids [79]. The enantiomeric separation of phenylthiocarbamoyl derivatives of amino acids (PTC-AAs) was carried on a series of a C8 column coupled to a phenylcarbamoylated β-CD column by optimizing the column temperature, ion-pairing reagent and content of methanol. Under the best conditions, all the individual PTC-AAs were well separated within 150 min, as shown in Fig. 6. Further, the applicability of the method was demonstrated by the sequence/configuration analysis of a peptide containing a D-amino acid ([D-Thr^2]-leucine enkephalin-Thr). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was employed for the determination of minute amounts of D-Leu in biological samples [80] using coupled achiral–chiral columns, micro C18 and *N*-(3,5-dinitrobenzoyl)-(S)-1-naphthylglycine columns. Without the use of the column-switching system, many interfering peaks due to the endogenous substances in the rat hippocampus were observed. While using the column-switching system, most of the interfering compounds were re-

moved, and selective determination of D- and L-Leu in the rat hippocampus could be attained. The precolumn derivatization using *o*-phthalaldehyde (OPA) and 2-mercaptoethanol was used for the chiral resolution of racemic phenylalanine at trace levels in urine [81]. The method involved the retention of OPA-derivatized phenylalanine on a C18 column, the zone transfer of the retained band of the amino acid from the column and the separation on an analytical β-CD column. This allowed the chiral resolution and quantification of the enantiomers of the amino acid using reversed-phase HPLC and fluorescence detection. Derivatization in situ with OPA-3-mercaptopropionic acid (3-MPA) and FMOC afforded the enantioselective determination of selfotel in human urine [82]. Chromatographic separations of the FMOC derivatives of selfotel enantiomers were achieved using a column switching system consisting of a C18 column and a Chiralcel OD column. Both reagents have previously been described for the analysis of primary and secondary amino acids [10]. With a combination of these reagents, secondary amino acids like selfotel can selectively be determined after formation of FMOC derivatives. Possible interfering primary amino acids were first derivatized with the OPA-3-MPA reagent. The method was successfully utilized to determine selfotel stereospecific pharrnacokinetics. For the determination of the enantiomers of methamphetamine and its major metabolites, amphetamine and *p*-hydroxymethamphetamine, in urine samples, 4-(4,5-diphenyl-lH-imidazol-2-yl)benzoyl chloride (DIB-Cl) was used [83,84]. The derivatives of six enantiomers were separated isocratically on a Chiralcel OD-R column following a preseparation on a C18 column. The method was successfully applied to discriminate between (S)-(+)-methamphetamine and its corresponding metabolites found in abusers' urine and their antipodes in a sample taken from a Parkinsonian patient on selegiline [(R)-(−)-*N*-methyl-*N*-(1-phenyl-2-propyl)-2-propinylamine; Deprenyl] therapy as shown in Fig. 7 [83].

2-APAs were derivatized with the fluorogenic reagents, 4-(*N*,*N*-dimethylamino)sufonyl-7-piperazine-2,1,3-benzoxadiazole (DBD-Pz) and (*N*-hydrazinoformyl)methyl] - *N* - methyl]amino - 7 - (*N*, *N* - dimethylamino)sulfonyl - 2,1,3 - benzoxadiazole (DBD-CO-Hz), and their enantiomeric separation by a Chiralcel OD-R in a reversed-phase mode [85]. The derivatives with DBD-Pz were enantiomerically separated well under the elution con-

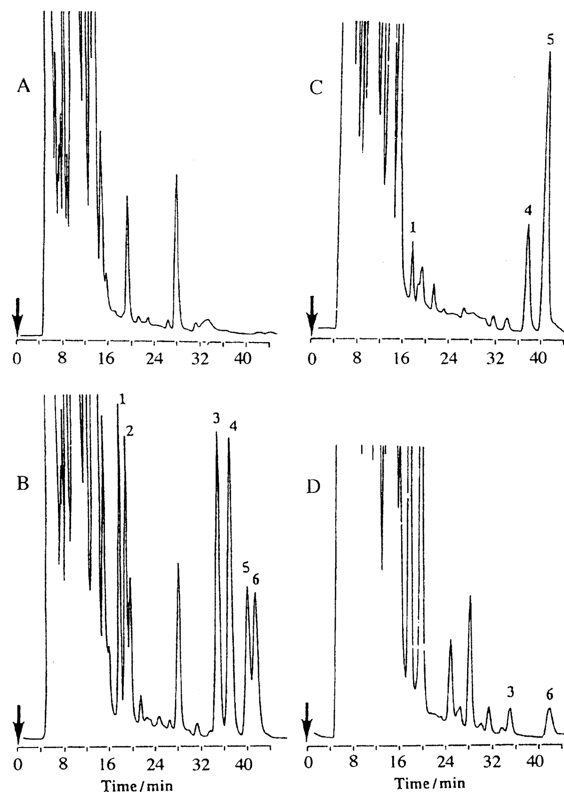


Fig. 7. Typical chromatograms of DIB-Cl derivatives with fluorescence detection of (A) 10-fold diluted control human urine, (B) 10-fold diluted human urine spiked with methanphetamines at a concentration of 10 μmol/l per enantiomer, with 2.5 pmol of each compound injected on-column, (C) 10-fold diluted methanphethamine abuser's urine sample and (D) Parkinsonian's urine after selegiline intake. Reproduced from Ref. [83] with permission. LC conditions: columns, Disopak SP-120-5-ODS (250 × 4.6 mm i.d.) and Chiralcel OD-R (250 × 4.6 mm i.d.); mobile phase, 2-propanol-acetonitrile–phosphate–citrate buffer (pH 4.5) = 1:59:40 (v/v) containing 50 mM sodium hexafluorophosphate; flow-rate, 0.6 ml/min; detection, excitation wavelength at 330 nm and emission wavelength at 440 nm. Peak assignments: l, (S)-(+)-*p*-hydroxymethamphetamine; 2. (R)-(−)-*p*-hydroxymethamphetamine; 3, (R)-(−)-amphetamine; 4, (S)-(+)-amphetamine; 5, (S)-(+)-methamphetamine; 6, (R)-(−)-methamphetamine.

dition of H_2O/MeOH, based on the π–π interaction between the derivatives and the stationary phase. The rigid and bulky stucture of DBD-Pz was demonstrated to be more effective as compared to the less rigid ones. The derivatives with DBD-CO-Hz were more efficiently separated into each enantiomer with H_2O/CH_3CN as the eluent. The effective separation was based on hydrogen-bonding interaction between the acid hydrazide of the derivatives and the carbamoyl moiety of the stationary phase. There was a reversal in the elution order of the enantiomers between the two fluorescent derivatives. The method was demonstrated to be useful for the enantiomeric quantification of flurbiprofen in rat plasma. Further, *N*-(4-nitro-2,1,3-benzoxadiazoyl-7-yl)-*N*-methyl-2-aminoacetohydrazide (NBD-CO-Hz) was used for the enantiomeric assays of 2-APAs [86].

DBD-Pz was used for the determination of D- and L-lactic acid. HPLC separation of the lactic acid derivative was achieved using a C18 column followed by enantiomeric separation on a phenylcarbamoylated β-CD chiral column in off-line [87] and on-line [88] modes. Further, DBD-Pz was used for the enantiomeric assay of a natriuretic hormon, 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxy chroman, in rat plasma, urine and bile using C18 and Chiralcel OD-R columns [89].

4. Conclusions

For the separations of drug enantiomers in pharmaceuticals and in biological fluids using LC techniques, the direct and indirect methods have been developed. The direct method, which can separate enantiomeric forms based on a diastereomeric complex formation on a stationary phase, are suitable for the analysis of enantiomers in a standard sample and pharmaceutical preparations. However, for trace analysis of the enantiomers of a drug and its metabolite(s) in complex matrices such as biological samples, liquid–liquid extraction, SPE or column switching technique was followed by the enantiomer separation on a CSP. Further, for biomedical applications of enantioseparations, LC–MS–MS has been utilized for the sensitive and specific detection of a target drug. On the other hand, the indirect methods, which involves the reaction with a homochiral reagent and the separation of the diastereomeric derivatives on an achiral stationary phase, or the reaction with an achiral reagent and the enantiomer separation on a CSP, is an efficient technique for separation of many enantiomers having functional groups. It is essential that the derivatization reaction proceeds completely in both enantiomers, and that the racemization reaction does not occur. They were suitable for trace analysis of enantiomers in complex matrices such as biological samples because of the introduction of highly sensitive tags. In the future, these direct and indirect methods could be complementarily used for pharmaceutical and biomedical applications of enatioseparations using LC techniques.

References

[1] W.I. Wainer (Ed.), Drug Stereochemistry: Analytical Methods and Pharmacology, second ed., Marcel Dekker, New York, 1993.

[2] G. Blaschke, H.P. Kraft, K. Fickentscher, F. Köhler, Arzneim.-Forsch. 29 (1979) 1640–1642.

[3] G. Blaschke, Angew. Chem. Int. Ed. Engl. 19 (1980) 13–24.

[4] B. Chankvetadze (Ed.), Chiral Separations, Amsterdam, Elsevier, 2001.

[5] M. Zief, L.J. Crane (Eds.), Chromatographic Chiral Separations, Marcel Dekker, New York, 1988.

[6] J. Haginaka, in: I.D. Wilson, E.R. Adlard, M. Cooke, C.F. Poole (Eds.), Encyclopedia of Separation Science, Academic Press, New York, 2000, pp. 2381–2387.

[7] J. Ermer, M. Vogel, Biomed. Chromatogr. 14 (2000) 373–383.

[8] A.M. Krstulovic (Ed.), Chiral Separations by HPLC: Applications to Pharmaceutical Compounds, Ellis Horwood, Chichester, 1989.

[9] S. Allenmark, Chromatographic Enantioseparation. Methods and Applications, second ed., Ellis Horwood, New York, 1991.

[10] T. Toyo'oka (Ed.), Modern Derivatization Methods for Separation Sciences, Wiley, Chichester, 1999.

[11] W. Lindner, Chromatographia 24 (1987) 97–107.

[12] D.R. Taylor, K. Maher, J. Chromatogr. Sci. 30 (1992) 67–85.

[13] T. Toyo'oka, Biomed. Chromatogr. 10 (1996) 265–277.
[14] A.M. Rustum, V. Estrada, J. Chromatogr. B 705 (1998) 111–117.
[15] Y. Machida, H. Nishi, K. Nakamura, J. Chromatogr. A 830 (1999) 311–320.
[16] B.A. Olsen, D.D. Wirth, J.S. Larew, J. Pharm. Biomed. Anal. 17 (1998) 623–630.
[17] M. Kummer, G. Werner, J. Chromatogr. A 825 (1998) 107–114.
[18] S.A. Wood, J.M. Long, R.J. Simmonds, J.W. Bridges, D. Stevenson, J. Pharm. Biomed. Anal. 16 (1997) 231–237.
[19] V. Wsól, A.F. Fell, E. Kvasnicková, I.M. Hais, J. Chromatogr. B 689 (1997) 205–214.
[20] A. Karlsson, A. Aspegren, J. Chromatogr. A 866 (2000) 15–23.
[21] D. Bylund, A. Bergens, S.P. Jacobsson, Chromatographia 44 (1997) 74–80.
[22] T.D. Booth, K. Azzaoui, I.W. Wainer, Anal. Chem. 69 (1997) 3879–3883.
[23] V. Dohnal, M. Farková, J. Havel, Chirality 11 (1999) 616–621.
[24] I. D'Acquarica, F. Gasparrini, D. Misiti, C. Villani, A. Carotti, S. Cellamare, S. Muck, J Chromatogr. A 857 (1999) 145–155.
[25] D.S. Risely, M.A. Strege, Anal. Chem. 72 (2000) 1736–1739.
[26] T. Wikberg, T. Korkolainen, M. Karlsson, Chirality 8 (1996) 511–517.
[27] M.A. Campanero, A. L.-Ocáriz, E. G.-Quetglas, Chromatographia 48 (1998) 203–208.
[28] E. Gorichon, C. Martin, K.N. Bangchang, J. Karbwang, A. Thuillier, R. Farinotti, F. Gimenez, J Chromatogr. B 712 (1998) 259–262.
[29] M. Kosel, C.B. Eap, A.P. Baumann, J. Chromatogr. B 719 (1998) 234–238.
[30] S.H. Gorman, J. Chromatogr. B 730 (1999) 1–7.
[31] M. Sueyasu, K. Fujito, K. Makino, H. Suto, Y. Kataoka, R. Oishi, J. Chromatogr. B 723 (1999) 307–311.
[32] L.R.P. De Abreu, V.L. Lanchote, C. Bertucci, E.J. Cesarino, P.S. Bonato, J. Pharm. Biomed. Anal. 20 (1999) 209–216.
[33] H.Y. A.-Enein, V. Serignese, Biomed Chromatogr. 13 (1999) 520–524.
[34] T. Nishikawa, H. Kanazawa, Y. Kamijo, Y. Ohtani, R. Kondo, H. Takeuchi, T. Okuda, Biomed. Chromatogr. 14 (2000) 243–248.
[35] D.W. Boulton, C.L. Devane, Chirality 12 (2000) 681–687.
[36] Z. Desta, N.V. Soukhova, A. Morocho, J. Park, S.K. Mahal, D.A. Flockhart, J. Chromatogr. B744 (2000) 263–272.
[37] R. Bortocan, V.L. Lanchote, E.J. Cesarino, P.S. Bonato, J. Chromatogr. B 744 (2000) 299–306.
[38] J. Boisvert, G. Caillé, I.J. McGilveray, S.A. Qureshi, J. Chromatogr. B 690 (1997) 189–193.
[39] R.R. Walters, S.C. Buist, J. Chromatogr. A 828 (1998) 167–176.
[40] K.M. Fried, P. Koch, I.W. Wainer, Chirality 10 (1998) 484–491.
[41] A. Ceccato, B. Toussaint, P. Chiap, P. Hubert, J. Crommen, Enantiomer 4 (1999) 305–315.
[42] K.-S. Boos, C.-H. Grimm, Trends Anal. Chem. 18 (1999) 175–180.
[43] J. Haginaka, in: I.D. Wilson, E.R. Adlard, M. Cooke, C.F. Poole (Eds.), Encyclopedia of Separation Science, Academic Press, New York, 2000, pp. 4087–4091.
[44] F. Mangani, G. Luck, C. Fraudeau, E. Vérette, J. Chromatogr. A 762 (1997) 235–241.
[45] W.R.G. Baeyens, G. Van der Weken, J. Haustraete, H.Y. A.-Enein, S. Corveleyn, J.P. Remon, A.M. G.-Campana, P. Deprez, J Chromatogr. A 871 (2000) 153–161.
[46] G. Lamprecht, T. Kraushofer, K. Stoschitzky, W. Lindner, J. Chromatogr. B 740 (2000) 219–226.
[47] R.F. Sucknow, M.F. Zhang, T.B. Cooper, Biomed. Chromatogr. 11 (1997) 174–179.
[48] L. Liu, H. Cheng, J.J. Zhao, J.D. Rogers, J. Pharm. Biomed. Anal. 15 (1997) 631–638.
[49] B.T. Hiep, V. Khanh, N.K. Hung, A. Thuillier, F. Gimenez, J. Chromatogr. B 707 (1998) 235–240.
[50] M.A. Campanero, B. Calahorra, M. Valle, I.F. Troconiz, J. Honorato, Chirality 11 (1999) 272–279.
[51] E. Brandsteterová, I.W. Wainer, J. Chromatogr. B 732 (1999) 395–404.
[52] T.A. Kolbah, A.P. Zavitsanos, J Chromatogr. A 759 (1997) 65–77.
[53] H. Kanazawa, Y. Konishi, Y. Matsushima, T. Takahashi, J. Chromatogr. A 797 (1998) 227–236.
[54] H. Stenhoff, A. Blomqvist, P.-O. Lagerström, J. Chromatogr. B 743 (1999) 191–201.
[55] A. Ceccato, F. Vandebist, J.-Y. Pabst, B. Streel, J. Chromatogr. B 748 (2000) 65–76.
[56] A.P. Watt, L. Hitzel, D. Morrison, K.L. Locker, J. Chromatogr. A 896 (2000) 217–227.
[57] H. Takahashi, T. kashima, S. Kimura, N. Muramoto, H. Nakahata, S. Kubo, Y. Shinoyama, M. Kajiwara, H. Echizen, J. Chromatogr. B 701 (1997) 71–80.
[58] H. Kanazawa, Y. Kunito, Y. matsushima, S. Ohkubo, F. Mashige, J. Chromatogr. A 871 (2000) 181–188.
[59] S.T. Wu, Y.P. Chang, W.L. Gee, L.Z. Benet, E.T. Lin, J. Chromatogr. B 692 (1997) 133–140.
[60] X. Lin, T.-W. Yao, S. Zeng, J. Chromatogr. B 742 (2000) 433–439.
[61] A. Péter, G. Török, G. Toth, W. Van den Nest, G. Laus, D. Touwé, D.W. Armstrong, Chromatographia 48 (1998) 53–58.
[62] E. Souri, H. Farsam, F. Jamali, J. Chromatogr. B 700 (1997) 215–222.
[63] J.S. Oleksiak, M. Walczak, J. Bojarski, H.Y.A. Enein, Chirality 11 (1999) 267–271.
[64] O.P. Kleidernigg, W. Lindner, J. Chromatogr. A 795 (1998) 251–261.
[65] T. Suzuki, T. Watanabe, T. Toyo'oka, Anal. Chim Acta 352 (1997) 357–363.

[66] D. Jin, K. Takehana, T. Toyo'oka, Anal. Sci. 13 (1997) 113–115.
[67] T. Toyo'oka, M. Toriumi, Y. Ishii, J. Pharm. Biomed. Anal. 15 (1997) 1467–1476.
[68] D. Jin, K. Nakamura, S. Murofushi, T. Miyahara, T. Toyo'oka, J. Chromatogr. A 822 (1998) 215–224.
[69] R. H.-Hernández, P. C.-Falcó, L.A.T. Genaro, Analyst 123 (1998) 2131–2137.
[70] C. Gunaratna, P.T. Kissinger, J. Chromatogr. A 828 (1998) 95–103.
[71] B. Fransson, U. Ragnarsson, J. Chromatogr. A 827 (1998) 31–36.
[72] S. Al-kindy, T. Santa, T. Fukushima, H. Homma, K. Imai, Biomed. Chromatogr. 12 (1998) 276–280.
[73] T. Santa, J. Luo, C.-K. Lim, K. Imai, Biomed. Chromatogr. 12 (1998) 73–77.
[74] T. Hayamizu, S. Kudoh, H. Nakamura, J. Chromatogr. B 710 (1998) 211–218.
[75] S.C. Tan, S.H.D. Jackson, C.G. Swift, A.J. Hutt, Chromatographia 46 (1997) 23–32.
[76] K. Akasaka, H. Meguro, H Ohrui, Tetrahedron Lett. 39 (1997) 6853–6856.
[77] K. Akasaka, H Ohrui, H. Meguro, T. Umestu, Anal. Sci. 13 (1997) 461–466.
[78] N. Muramatsu, T. Toyo'oka, K. Yamaguchi, S. Kobayashi, J. Chromatogr. B 719 (1998) 177–189.
[79] T. Iida, H. Matsunaga, T. Fukushima, T. Santa, H. Homma, K. Imai, Anal. Chem. 69 (1997) 4463–4468.
[80] T. Inoue, K. Hamase, A. Morikawa, K. Zaitsu, J. Chromatogr. B 744 (2000) 213–219.
[81] P. Khan, P.R. Fielden, Anal. Commun. 35 (1998) 37–40.
[82] B. Knoche, S. Milosavljev, S. Gropper, L.A. Brunner, M.L. Powell, J. Chromatogr. B 695 (1997) 355–363.
[83] C. Al-dirbashi, N. Kuroda, F. Menichini, S. Noda, M. Minemoto, K. Nakashima, Analyst 123 (1998) 2333–2337.
[84] O.Y. Al-dirbashi, M. Wada, N. Kuroda, M. Takahashi, K. Nakashima, J. Forensic Sci. 45 (2000) 708–714.
[85] T. Fukushima, T. Santa, H. Homma, S.M. Al-kindy, K. Imai, Anal. Chem. 69 (1997) 1793–1799.
[86] T. Santa, A. Takeda, S. Uchiyama, T. Fukushima, H. Homma, S. Suzuki, H. Yokosu, C.K. Lim, K. Imai, J. Pharm. Biomed. Anal. 17 (1998) 1065–1070.
[87] T. Fukushima, S. Adachi, H. Ichihara, S. Al-kindy, K. Imai, Bomed. Chromatogr. 13 (1999) 418–424.
[88] H. Ichihara, T. Fukushima, K. Imai, Anal. Biochem. 269 (1999) 379–385.
[89] A. Hattori, T. Fukushima, K. Imai, Anal. Biochem. 281 (2000) 209–215.