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Review

Enantiomeric derivatization for biomedical chromatography

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Abstract

Derivatization reactions aimed at creating the basis for the chromatographic resolution of biologically and pharmaceutically important enantiomers are reviewed, with emphasis on the literature published in the last 10 years. Three main aspects of chiral derivatization are discussed. (a) Enantiomers containing suitable functional groups (amino, carboxyl, hydroxyl, epoxy, *etc.*) are transformed into covalently bonded diastereomeric derivatives using homochiral derivatizing agents. The diastereomers formed (esters, amides, urethanes, urea and thiourea, etc., derivatives) can be separated on achiral stationary phases. The derivatization reactions often afford further advantages, such as the improvement of chromatographic properties and the detectability of the solutes using UV and fluorimetric detectors. (b) Covalent but achiral derivatization is often necessary even with the use of chiral stationary phases enabling in principle direct enantioseparations (Pirkle-type columns, cyclodextrin-bonded phases, glycoprotein column and functionalized cellulose columns). The main goals of these derivatization reactions (which are analogous to those discussed above), are to introduce functional groups into the molecule of the enantiomers that improve the possibilities for chiral interactions or block functional groups to avoid non-specific interactions. (c) In the broader sense, the dynamic formation of diastereomers using chiral mobile phase additives (cyclodextrins, various reagents to form diastereomeric ion pairs, adducts, mixed metal complexes) can also be considered to be chiral derivatization reactions and is therefore briefly discussed also.

Contents

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List of abbreviations

AGP	Acid glycoprotein
Aib	Aminoisobutyric acid
APMB	(−)-2-[4-(1-Aminoethyl)phenyl]-6-methoxybenzoxazole
CD	Cyclodextrin
DCC	Dicyclohexylcarbodiimide
EDC	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide
Fl	Fluorescence
FMOC	Fluorenylmethyloxycarbonyl
GC	Gas chromatography
GITC	2,3,4,6-Tetra-O-acetyl-β-D-glycopyranosyl isothiocyanate
Gly	Glycine
HPLC	High-performance liquid chromatography
Leu	Luecine
NP	Normal-phase
NMR	Nuclear magnetic resonance
OPA	*o*-Phthalaldehyde
ORD–CD	Optical rotatory dispersion–circular dichroism
Phe	Phenylalanine
RP	Reversed-phase
SFC	Supercritical fluid chromatography
Su	Succinyl
S/N	Signal-to-noise ratio
TLC	Thin-layer chromatography
UV	Ultraviolet
Val	Valine

1. Introduction

1.1. The necessity for enantiomeric separations in pharmaceutical and biomedical chromatography

As can be seen in Fig. 1, about 56% of the drug materials currently used in therapy are chiral compounds. Whereas most natural and semi-synthetic drugs are administered as the pure enantiomers, almost the opposite situation exists in the field of synthetic drugs, where about 88% of the chiral compounds are used as the racemates in therapy [1]. A considerable decrease in the latter figure can be expected in forthcoming years, at least in the field of new entities in drug therapy. The reason for this is that on the basis of the continuously increasing knowledge related to enantioselective drug actions and side-effects [2–4] and the recent achievements in the industrial-scale preparation of pure enantiomers by synthetic or biotech-

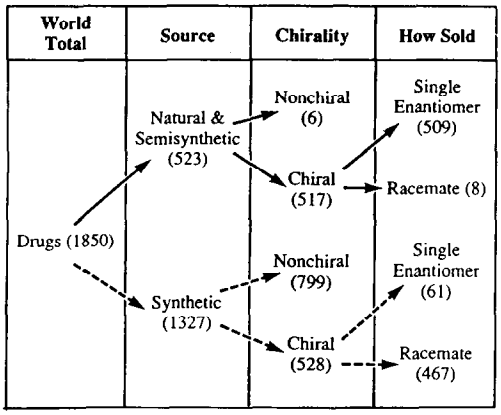


Fig. 1. Sales distribution of single enantiomer and racemate drugs currently on the market worldwide (with permission from ref. 1).

nological methods, the drug registration authorities already prefer enantiometrically pure new drugs to the corresponding racemates and this tendency will certainly increase in the near future.

The fact that chirality problems are among the main issues in drug research and development raises several problems to be solved by analysts working in the pharmaceutical and biomedical areas [5] and chromatography plays a predominant role in solving these problems. The most typical analytical tasks are as follows.

Chromatographic separation and determination of enantiomers with the aim of developing methods for their determination in biological samples in order that the possible differences in their pharmacokinetics, pharmacodynamics and metabolism can be monitored [6].

If on the basis of pharmacological results and of the studies outlined above it is decided that the pure enantiomer will be introduced and marketed, analytical methods are necessary to check the enantiomeric purity of the drug substance, *i.e.*, the detection and quantification of the undesired antidope down to the 0.1% level. Although other methods (NMR, ORD–CD spectroscopic and thermal) are also available for this purpose, the role of chiral chromatography is predominant in this field also. These methods have to be used routinely if the drug is introduced into therapy.

Similar methods have to be used to check the enantiomeric stability of the drug material, especially during prolonged storage of its pharmaceutical formulations.

In the course of the industrial-scale synthesis or separation of the enantiomerically pure material, the steps and intermediates involved have to be checked by enantioselective (mainly chromatographic) methods.

1.2. Separation strategies in chiral chromatography

Although no separation of the enantiomers can be achieved if conventional chromatographic separation units (columns, capillaries, TLC plates) are used, their diastereomeric derivatives prepared by means of enantiomerically pure derivatizing agents can be separated with the aid of these units containing achiral selectors. The formation of the diastereomeric derivative can be achieved in two general ways. First, the enantiomers react with the chiral reagent in a reaction vessel prior to the chromatographic run to form a pair of covalently bonded diastereomeric derivatives. This possibility can be used with all important branches of chromatography, *i.e.*, TLC, HPLC, GC and SFC. Second, the chiral reagent is dissolved in the chromatographic solvent to form diastereomeric adducts, ion pairs, metal complexes, *etc.*, with the enantiomers to be separated. In these cases (where the use of GC is naturally excluded), the formation of the diastereomeric adducts, *etc.*, does not involve the formation of covalent bonds. Although even this dynamic formation of the diastereomeric adducts can be regarded as a derivatization procedure prior to the separation on the achiral column, *etc.*, this branch of chiral chromatography is often mentioned among the "direct" enantioseparations because of the lack of a *preliminary* derivatization reaction.

A third possibility for the separation of enantiomers is the use of real direct separation procedures involving the use of separation units containing immobilized chiral selectors: either natural chiral macromolecules (proteins, carbohydrates or their derivatives), cyclodextrins or

small chiral molecules covalently or ionically bonded to the chromatographic support. In this case the basis of the separation is the difference between the stability constants of the diastereomeric adducts of the two enantiomers formed with the immobilized chiral molecules. This kind of chiral separation does not necessarily require preliminary derivatization but achiral derivatization often improves the resolution of the enantiomers.

1.3. Scope and limitations

In accordance with the general aims of this special volume, this main article deals with all the three kinds of derivatization outlined in the preceding section: formation of covalently bonded diastereomers, thus creating the basis for the separation using achiral columns; achiral covalent derivatization aiming at improving the separation on chiral columns; and the dynamic formation of adducts, ion pairs and metal complexes for the separation to be performed on an achiral column.

Only HPLC derivatization will be discussed, omitting often analogous procedures for chiral GC, TLC, SFC and capillary electrophoretic separations. Another restriction is that only pharmaceutical and biomedical aspects of chiral HPLC derivatization will be dealt with.

The limitations of this section do not permit us to cover the extremely large number of publications from the beginning even in this restricted field. A previous review on covalent chiral derivatization [7] covered the literature up to 1986. In this article only a few of the most important papers published before this date will be discussed and selection will be made mainly of the papers published afterwards.

The importance of chiral chromatography is reflected by the large number of books devoted to this topic [4,8–14]. These and some important reviews are good sources for the literature on chiral HPLC derivatization [4a,7,15–17].

Most of the reagents and reactions discussed in Section 2.3 and summarized in Tables 1–3 have already been described before 1986. References to these original versions of most of the methods in Section 2.3 can be found in the above-mentioned reviews.

2. Formation of covalently bonded diastereomeric derivatives and separation on achiral columns

2.1. Introductory remarks

The derivatization of enantiomers using homochiral reagents to form their diastereomeric derivatives followed by the separation of the latter on achiral chromatographic columns was the first, widely used method for the enantiomeric separation of chiral drugs and related materials (see references to reviews in Section 1.3).

After the introduction of newer techniques, namely separation of the underivatized enantiomers on achiral columns by using chiral mobile phase additives and especially after the appearance of highly effective chiral HPLC stationary phases, it was often predicted that the importance of covalent derivatization would rapidly decrease. The large number of publications that appeared until recently indicates that in contrast to these predictions, the popularity of covalent chiral derivatization does not seem to have decreased significantly.

This general method is still a method of choice in addition to the other two branches of chiral separations. The reasons for this are as follows: the large variety of reagents and reactions permitting diastereomeric derivatives with excellent separation and detection possibilities to be formed; the possibility of tailor-made separations, *i.e.*, being in possession of the *R*- and *S*-forms of the reagent, it is achievable that the minor peak (enantiomeric impurity) elutes before the main peak; and the possibility of using relatively inexpensive achiral columns.

2.2. The most important features of chiral derivatization reactions and reagents

2.2.1. Good chromatographic properties of the derivatives

The retention time and the peak shape of the derivatives and their separability from the other components of the complex sample can be optimized in the usual ways used in achiral HPLC: proper column selection (usually reversed-phase but in many instances normal-phase systems), solvent composition and pH of the eluent, *etc.*

2.2.2. Sufficient separation of the diastereomers formed

This, of course, is also greatly influenced by the above-mentioned solvent composition and pH, but in this instance the most important factor is the proper selection of the derivatizing reagent, enabling diastereomers with different molecular fine structures suitable for their chromatographic separation to be formed.

One of the most important structural features of the diastereomeric derivatives is the distance between the two chiral centres. In most instances the optimum distance is two to four atoms. This is valid for the most of the derivatives in Tables 1–3 but in some instances it is five [18,19] and in one instance eight [20] (see Fig. 1).

Another important point is the conformational rigidity of the diastereomeric derivatives, which creates the basis for the separation. The bulkiness of the groups in the vicinity of the chiral centres in the enantiomeric compound or the derivatizing agent favourably influences the conformational rigidity and hence the extent of the resolution. It is especially advantageous if in the analyte and/or the reagent the chiral centre is incorporated into a ring system. For example, the successful separations with the 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate reagent (see Table 1) can be attributed to the presence of these structural features. In accordance with this rule, the exchange of the four acetyl groups in the reagent to bulkier benzoyl groups greatly improves the resolution of the enantiomers of oxirane-derived α-amino alcohols. It is interesting, however, that using the even more bulky tetrapivaloyl reagent poor separation of the enantiomers is obtained [21].

The role of the bulkiness of remote groups in the molecule of the enantiomers to be separated is more difficult to explain. For example, the neomenthylthiourea derivatives of amino acid enantiomers could only be resolved if their carboxyl groups were esterified with the bulky *tert.*-butyldimethylsilyl group; the methyl esters were not separated [22]. In contrast to this, the *tert.*-butyloxycarbonyl (BOC) protecting group in BOC-L-Leu-O-Su and other related reagents has to be removed from the diastereomeric derivative after the derivatization of amino acids to achieve good resolution [23]. In another method, however, amino acid enantiomers with bulky protecting groups were successfully separated without the removal of the protecting group when their carboxyl groups were derivatized with L- or D-O-(4-nitrobenzyl)tyrosine methyl ester reagent [24].

The formation of hydrogen bonds also plays a predominant role in establishing the conformational differences between the diastereomeric derivatives, thus creating the basis for the separation. The classical example is the enantiomeric separation of propranolol and related derivatives as their urea or thiourea derivatives. Here the basis of the separation is the hydrogen bond between the free hydroxyl group and the carbonyl or thiocarbonyl group in the urea or thiourea moieties. The etherfication of the hydroxyl group greatly reduces the separability of the diastereomeric derivatives [25].

A newer example is the resolution of D- and L-phenylalanine methyl esters as the (*Z*)-L-Val-Aib-Gly derivative [20] (see Table 1). The capacity factors of (*Z*)-L-Val-Aib-Gly-L-Phe-OCH_3 and of the LD diastereomer are 10.79 and 6.09, respectively (α = 1.77). The conformation of the LL isomer with two intramolecular hydrogen bonds is shown in Fig. 2. NMR and circular dichroism studies revealed that the LD isomer adopts another β-turn conformation in which there is only one hydrogen bond between the valine C=O and the phenylalanine. NH. This is

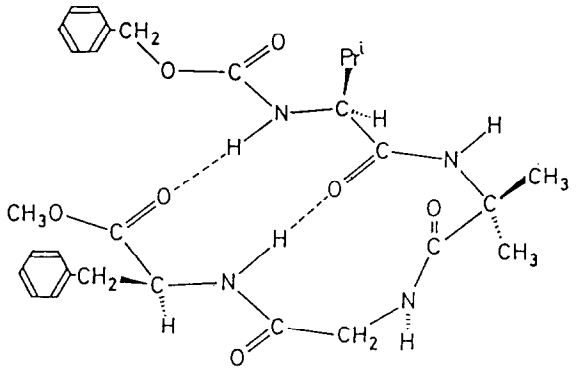


Fig. 2. Conformation of the LL isomer of (Z)-L-Val-Aib-Gly-L-Phe-OMe (with permission from ref. 20).

why the LL isomer is more hydrophobic and for this reason can interact more strongly with the octadecylsilica layer of the stationary phase than the LD isomer, leading to longer retention times and good separation in the RP system.

2.2.3. Unidirectional derivatization reaction taking place possibly under mild conditions

The most widespread reactions are completed at room temperature within 1 h without the formation of side-products and do not require an extraction step to be used. These are, however, not prerequisites: in many instances prolonged reaction time and/or heating of the reaction mixture are necessary. The occurrence of side-reactions does not preclude reliable data from being obtained if their chemistry is known, the side-products do not disturb the monitoring of the peaks of the diastereomeric derivatives and the side-reactions do not show stereospecificity [24].

2.2.4. Absence of kinetic resolution and racemization

When introducing a new reagent, new reaction conditions or the application of the reaction to new problems, it has to be carefully checked if the ratio of the peak areas of the diastereomeric derivatives is close to unity when samples are taken from the reaction mixture of the racemate with the reagent during and after the reaction. It is also an important task during the validation of the method to check the enantiomeric stability of the reaction product during the conditions of the reaction by checking for the absence of the peak of the other diastereomeric derivative when derivatizing a pure enantiomer.

2.2.5. Chiral purity and stability of the reagent

The at least 99% enantiomeric purity of the reagent and its chiral stability are naturally among the most important factors determining the success of the indirect chromatographic resolution. Various reviews [4a,7,12a,16,26] usually mention the case of N-trifluoroacetyl-(*S*)-(−)-prolyl chloride [27] or anhydride [28] as deterrent examples: these reagents are claimed to racemize during storage and are therefore not sufficiently pure for enantiomeric derivatization. Reagents with such properties are no longer used: the reagents in Tables 1–3 fulfil the above-mentioned requirements.

Of course, checking of the enantiomeric purity of the reagents belongs to the validation protocol of chiral chromatographic methods based on chiral derivatization. If the enantiomers (or at least one of them) is available in enantiomerically pure form, then this can be done by running the derivatization reaction with the pure enantiomer(s); the relative peak area of the diastereomeric derivative will be characteristic of the enantiomeric impurity of the reagent. If this is a small, constant value, it can be taken into correction.

The prerequisites regarding the enantiomeric purity of the reagent depend on the type of problem to be solved. If the aim of the study is to determine the enantiomeric purity of an enantiomeric drug, the purity of the reagent should be at least 99.9%. Using an enantiomerically pure reagent, 0.1–0.2% or even lower concentrations of enantiomeric impurity in enantiomeric drugs and related materials can easily be determined, provided that the separation and the detectability are good. As an example, the detection of 0.1% of (*S*)-3-aminoquinuclidine in the *R*-enantiomer and *vice versa* after derivatization with (*R*,*R*)-O,O-dibenzoyltartaric anhydride is demonstrated in Fig. 3 [29].

If the goal is the determination of commensurable amounts of the enantiomers in biological

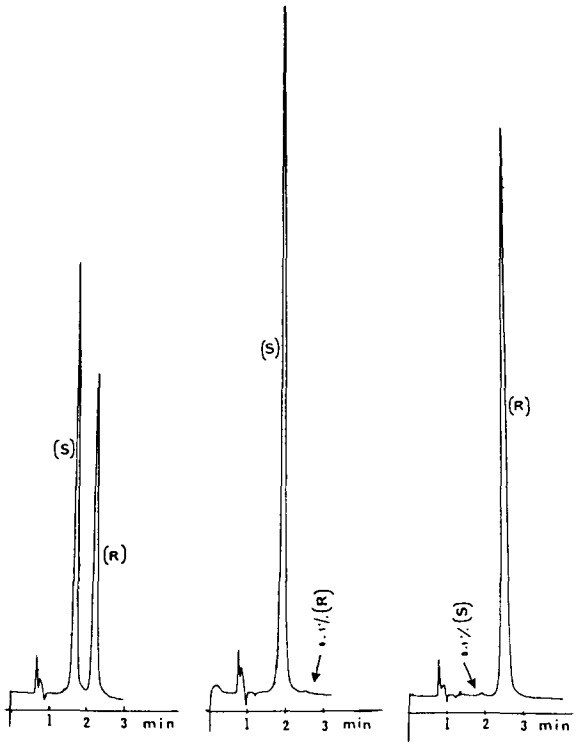


Fig. 3. HPLC separation of (*R*)- and (*S*)-3-aminoquinuclidine mixtures derivatized with (*R*,*R*)-O,O-dibenzoyltartaric acid anhydride. Column, 150 × 4.6 mm I.D., Zorbax C_8; mobile phase, 0.15% acetic acid in water (adjusted with triethylamine to pH 4.2)–methanol–acetonitrile (60:25:15); flow-rate, 2 ml/min; detection, UV 254 nm (with permission from ref. 29).

samples during the pharmacokinetic examination of the racemic drug, 1–2% of the enantiomeric impurity in the reagent is tolerable (or can be corrected for).

2.2.6. *Good chromophoric or fluorophoric properties of the reagent*

Although the primary aim with the derivatization reagent in indirect chiral chromatography is to ensure the formation of well separable diastereomeric derivatives, in many instances it is very advantageous if at the same time the reagent improves the detectability of the separated enantiomers by intorducing chromophoric or fluorophoric groups into their molecules. Low detection limits are very important in both the detection and the determination of traces of enantiomeric impurities and the measurement of very low concentrations of the enantiomers of the racemate drugs in body fluids, especially if the enantiomers themselves are spectrophotometrically or fluorimetrically only poorly active.

The question of detection limits using various types of reagents is discussed by Krull *et al.* in this volume. The limit of UV detection is in the range 1–100 ng per peak depending on the chromophoric properties of the compound and/or the derivatizing agent. Of course, much lower detection limits are obtainable with fluorimetric derivatization. For example, using (*S*)-flunoxaprofene (a *p*-fluorophenylbenzoxazole derivative) for the derivatization of amino acids, a detection limit of 0.1–0.5 ng per peak was obtainable [30], and as little as 10 fmol (1.5 pg) of (*S*)-(+)-2-phenylpropionic acid could be detected (signal-to-noise ratio = 3) after derivatization with (−)-2-[4-(1-aminoethyl)phenyl]-6-methoxybenzoxazole [31].

It is important to note that the UV or fluorescence characteristics of the diastereomers formed are not necessarily equal and therefore have to be checked in the course of the validation of a new method. For example, in the investigation of a wide range of drugs after derivatization with (−)-(*S*)-flunoxaprofen isocyanate reagent (see eq. 3 and using a fluorimeric detector, the *R*/*S* peak-area ratios ranged between 0.97 and 1.03 in most instances (including propranolol and metoprolol), but the ratio was 1.203 for mexiletine and 1.09 for tranylcypromine [32]. In another study [33], the *R*,*R*,*R*/*S*,*R*,*R* peak-area ratio of the diacetyl tartrate derivative of propranolol was 1.08 when using a UV detector at 290 and 1.25 when a fluorimetric detector was used (290/335 nm).

2.2.7. *Possibility of the proper selection of the elution order*

In the detection and determination of enantiomeric impurity, it is an important point greatly influencing the success of the investigation that the impurity peak elutes before the main peak. This can be achieved by changing from normal- to reversed-phase separation or *vice versa* (see Fig. 4) [31].

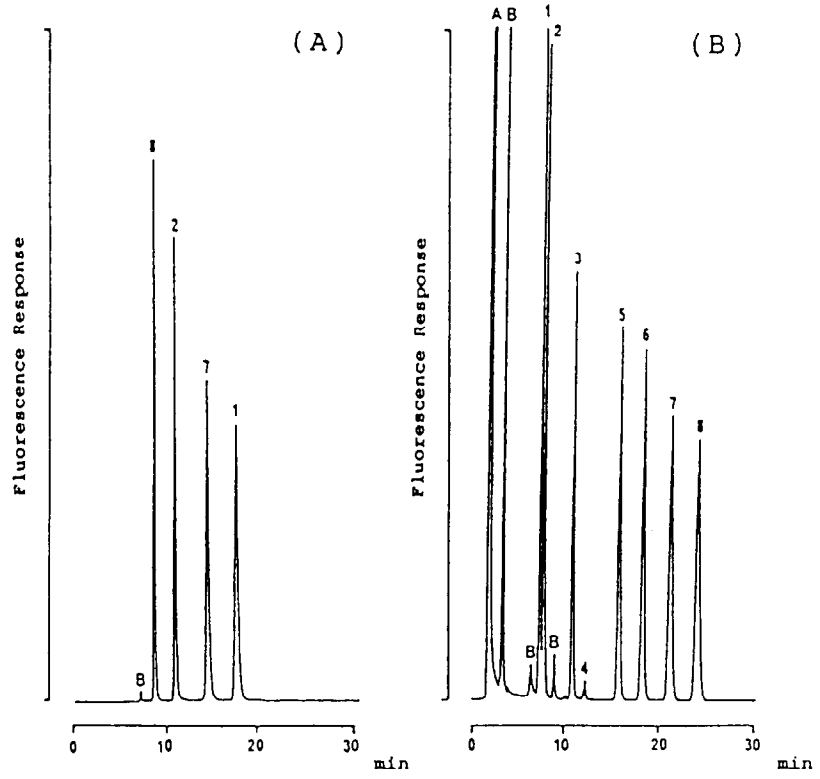


Fig. 4. HPLC separation of APMB {(−)-2-[4-(1-aminoethyl)phenyl]-6-methoxybenzoxazole} derivatives of 2-arylpropionic acids. (A) Normal-phase HPLC. Column, 250 × 4.6 mm I.D., TSK gel silica-60, 5 μm; mobile phase, *n*-hexane–ethyl acetate–2-propanol–acetic acid (900:50:50:1); flow-rate, 1 ml/min; detection, fluorescence (320 nm excitation, 380 nm emission). (B) Reversed-phase HPLC. Column, 150 × 4.6 mm I.D., TSK gel ODS-80TM, 5 μm; mobile phase, acetonitrile–water–acetic acid (600:400:1); flow-rate and detection as in (A). Peaks: 1, 2 = (*S*)- and (*R*)-2-phenylpropionic acid; 3, 4 = (*S*)- and (*R*)-naproxen; 5, 6 = (*S*)- and (*R*)-flurbiprofen; 7, 8 = (*S*)- and (*R*)-ibuprofen; A = (−)-APMB; B = degradation peak of the reagent (with permission from ref. 29).

Another possibility is to purchase or synthesize both enantiomers of the derivatizing reagent. In possession of this pair of reagents, it is easy to assure the proper elution order [24,34–38]; see, *e.g.*, Fig. 5.

2.3. Covalent enantiomeric derivatization of some important functional groups

2.3.1. Derivatization of amines

Of the reagents and reactions in the section "Activated carboxylic acids" in Table 1, some merit special attention. The carboxylic chloride derivatives of the chiral drugs naproxen, flunoxaprofen and benoxaprofen used as reagents [30,43–47] can result in diastereomeric derivatives with excellent chromatographic properties and, owing to their strong fluorescence, low detection limits.

A solid-phase derivatization reagent [41,42] is prepared by reacting polymer-bonded (copolymer of 96% styrene–4% divinylbenzene) 4-hydroxy-3-nitrobenzophenone with FMOC-L-proline using DCC as the coupling agent. As shown in reaction 1, the active ester formed reacts with amines (*e.g.* amphetamine) to form the FMOC-L-prolyl derivative with good chromatographic and fluorescence properties. This enabled the derivatization reaction to proceed in a reaction column at 60°C within 5 min in the mixture of acetonitrile and water which is the eluent for the C_{18} column positioned after the reaction column, thus creating the basis for the on-line derivatization–resolution of enantiomeric amine drugs in physiological fluids.

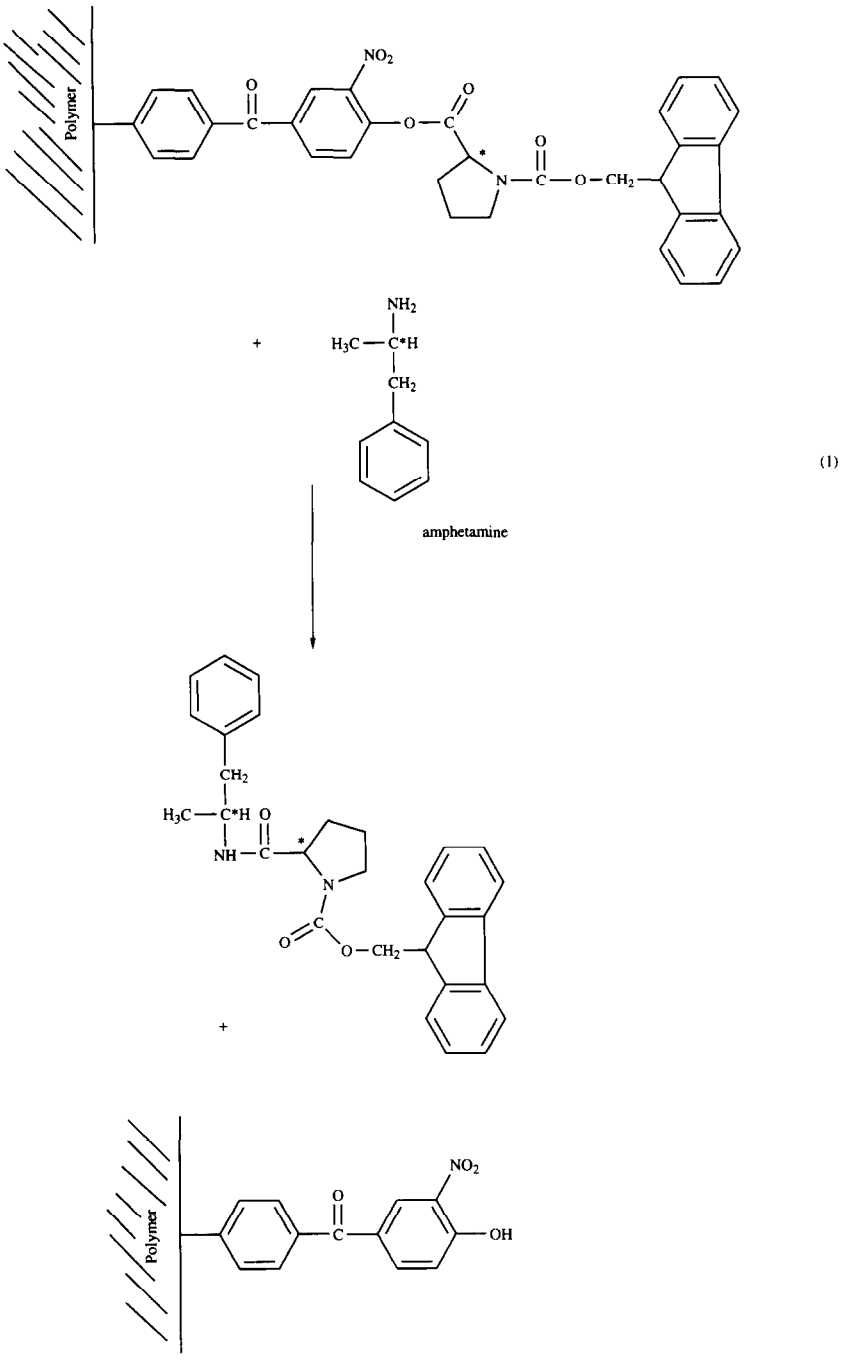
Table 1
Chiral derivatization of amines

Reagent(s)	Enantiomers separated	HPLC	Detection (λ, nm)	Biological sample	Ref.
Activated carboxylic acids					
1-(4-Nitrophenylsulphonyl)-L-prolyl chloride	Flecainide	RP	UV (280)	+	39
Dansyl-L-proline–triethylamine–diethyl phosphorocyanidate	Amino acids, amino alcohols	RP	Fl (345/515)	+	40
(*R*,*R*)-O,O-Dibenzoyltartaric acid anhydride	3-Aminoquinuclidine	RP	UV (254)	–	29
(*Z*)-L-Val-α-aminoisobutyryl-Gly-O-N-oxysuccinimide	α- and β-amino acid methyl esters	RP	UV (254)	–	20
Fluorenylmethyloxycarbonyl-L-proline (FMOC-L-proline) solid-state reagent	Amphetamine	RP	Fl (254/313)	+	41, 42
(*S*)-(+)-Naproxen chloride	Tocainide, tranylcypromine	NP	Fl (313/365)	+	43
	Carvedilol	NP	Fl (285/355)	+	44
	Baclofen butyl ester	NP	Fl (335/365)	+	45
(*S*)-(+)-Flunoxaprofen chloride	Tranylcypromine and other amines	NP	Fl (305/355)	+	46
	Amino acids	NP	Fl (305/355)	–	30
	Propranolol	RP	Fl (305/355)	+	47
(*S*)-(+)-Benoxaprofen chloride	Baclofen butyl ester	NP	Fl (313/365)	+	45
Chloroformates					
(–)-Menthyl chloroformate	Encainide and metabolites	NP	UV (261)	+	48
	Promethazine	RP	UV (258)	–	49
	Atenolol	RP	Fl (230/305)	+	50
tert.-Butyl 3-chloroformoxy-butyrate	Metoprolol	RP	Fl (228/306 or 272/306)	+	51
5-Chlorocarbonyl-L-isomannide 2-mononitrate and other chloroformates	Tyrosine	RP	UV (240)	–	51
(+)-1-(9-Fluorenyl)ethyl chloroformate	Propranolol	RP	Fl (260/340)	+	52
	Amino acids	RP			53
	Atenolol	RP	Fl (227/310)	+	54
	N-6-(*endo*-2-Norbornyl)-9-methyladenine	RP	UV (270)	–	55
Isocyanates					
(*R*)- or (*S*)-1-(1-naphthyl)ethyl isocyanate	Salsolinol	RP	Electrochemical	+	38
	Propafenone	NP	UV (220)	+	56
	Acebutolol	NP	Fl (220/389)	+	57
	3-Aminoquinuclidine	NP	UV (254)	–	29
(*R*)-α-Methylbenzyl isocyanate	3-Aminoquinuclidine	NP	UV (254)	–	29
	Propranolol and other β-blockers	RP	Fl (295/345)	+	47
		NP	UV (265–320)	–	58
(*R*)-α-Methylbenzyl isocyanate (after demethylation with vinyl chloroformate)	Promethazine, methixene, thioridazine, etc.	NP	UV (254)	–	59
(*S*)-α-Methoxybenzyl isocyanate	Amphetamine, mexiletine	NP	UV (254)	–	60
(–)-(*S*)-Flunoxaprofen isocyanate	Propranolol, metoprolol,	RP, NP	Fl (296/356	+	32
(–)-(*S*)-Naproxen isocyanate	mexiletine, cibenzoline, tranylcypromine		276/356)		

(Continued on p. 60)

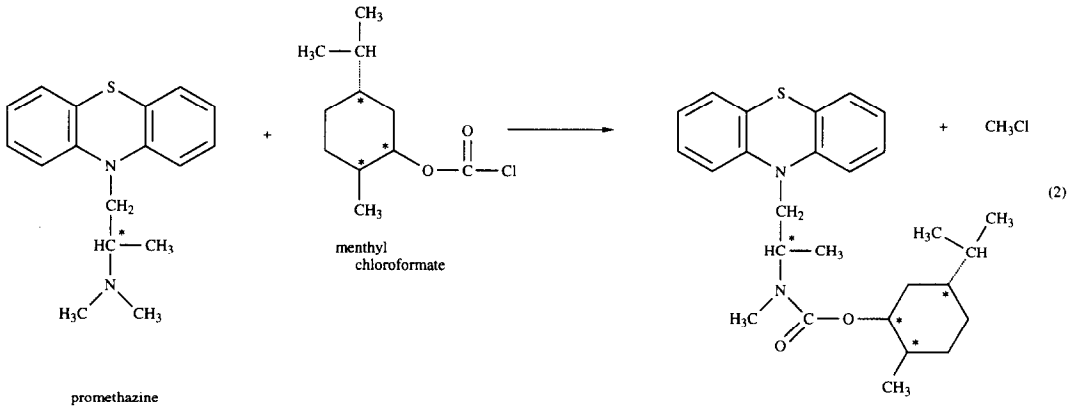
Table 1 *(continued)*

Reagent(s)	Enantiomers separated	HPLC	Detection (λ, nm)	Biological sample	Ref.
Isothiocyanates					
2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC)	Metoprolol	RP	UV (222)	+	61
	Chloramphenicol	RP	UV (254)	–	63
	α-Methylamino acids	RP	UV (250)	–	63
	Epinephrine	RP	UV (250)	–	64
	Trimethoquinol	RP	UV (200–300)	–	65
	Norephedrine, ψ-norephedrine	RP	UV (254, 380)	–	66
	3-Aminoquinuclidine	RP	UV (254)	–	29
	β-Blockers	NP	UV (265–320)	–	58
GITC or (*R*)-α-methylbenzyl isothiocyanate, (*S*)-1-(1-naphthyl)-ethyl isothiocyanate, (*R*)-1-(2-naphthyl)ethyl isothiocyanate	Mexiletine, tocainide, flecainide, propafenone	RP	UV (254)	+	67
(*R*)-α-Methylbenzyl isothiocyanate	Thiamphenicol	RP	UV (254)	–	62
(4*S-cis*)-2,2-dimethyl-5-isothiocyanate	Labetalol	RP	UV (254)	–	68
2,3,4,6-Tetra-O-benzoyl-β-D-glucopyranosyl isothiocyanate	Amino acids, β-blockers	RP	UV (231)	–	21
N-Haloarylamino acid derivatives					
1-Fluoro-2,4-dinitrophenyl-5-L-alaninamide (Marfey's reagent)	Amino acids, hydrolyzed amino acid derivatives and peptides	RP	UV (340)	–	69, 70
	Diaminopimelic acid	RP	UV (220, 254, 325, 400)	+	71
	α-Alkylamino acids	RP	UV (340)	–	72
Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-valinamide, etc.	Amino acids	RP	UV (340)	–	73, 74
N^2-[(4-chloro-6-(alkyl- or aryloxy)-1,3,5-triazinyl)-L-alaninamides (6-methoxy; 6-(2-naphthoxy); 6-(4-methoxy-1-naphthyl); 6-(4-phenylazoanilino)]	Amino acids	RP	UV (232, 365) Fl (340/415)	–	18
o-Phthalaldehyde (OPA) + chiral thiols					
OPA + N-acetyl-L-cysteine	Amino acids, α-alkylamino acids and their amides	RP	Fl (338/415)	–	75
OPA + N-acyl-L-cysteines (acyl = acetyl, *tert.*-butoxycarbonyl, *n*-butyryl, iso-butyryl, pivaloyl, benzoyl)	Amino acids	RP	UV (338)	+	76, 78
OPA + N-acetyl-L-cysteine or N-*tert.*-butoxycarbonyl-L-cysteine	Amino acids, α-alkylamino acids	RP	Fl (344/443)	–	72
OPA + N-isobutyryl-L (or D)-cysteine	Amino acids, peptide hydrolysates	RP	Fl (230/445)	+	34, 77, 79
OPA + N-acyl-L-cysteines (acyl = acetyl, propionyl, butyryl, isobutyryl, valeroyl, isovaleroyl, trimethylacetyl, *tert.*-butoxycarbonyl)	Amino acids	RP	Fl (344/443)	–	80
OPA + 1-thio-β-D-glucose (or galactose or mannose)	Leucines in protein hydrolysates	RP	Fl (360/420)	+	81
	Amino alcohols	RP	Fl (360/420)	–	82
OPA + N-acetyl-L-cysteine (or 1-thio-β-D-glucose, N-acetyl-D-penicillamine, 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside)	Amphetamine, mexiletine, tocainide, tranylcypromine, rimantidine, amino acids, etc.	RP	UV (254) Fl (338/425) Electrochemical	–	83, 84

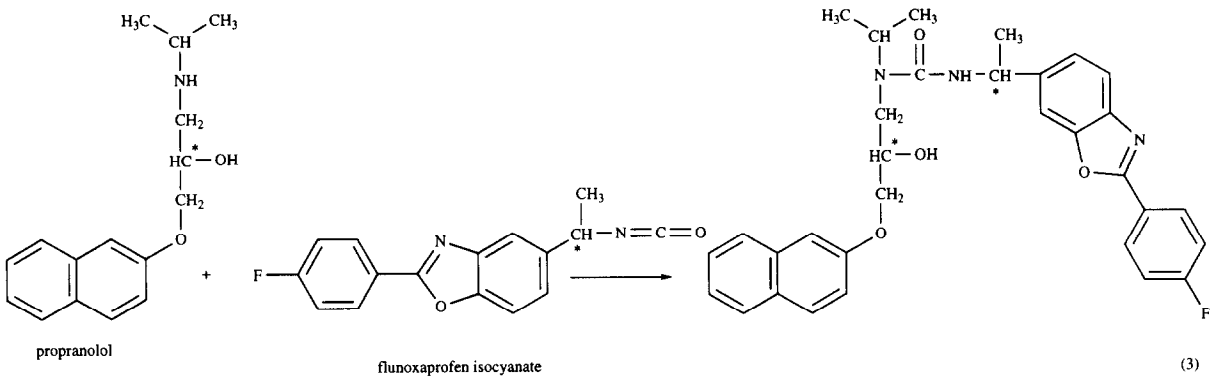


Reaction 1.

The fluorenyl fluorophoric group [as the (+)-1-(9-fluorenyl)ethyl derivative] is also widely used in the homogeneous phase [52,54,55]. This reagent and other chloroformates, including (−)-menthyl chloroformate, are classical chiral reagents for transforming amines into the corresponding carbamates. It is interesting that the latter reagent has also been successfully used for the chiral derivatization of tertiary amines, such as promethazine [49]. The reaction (see reaction 2) involves the demethylation of the tertiary amine moiety ($\alpha_{+/-} = 1.15$).



Reaction 2.

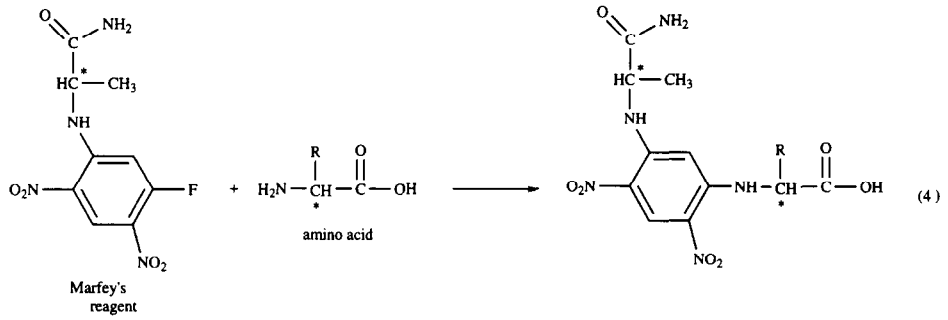


Reaction 3.

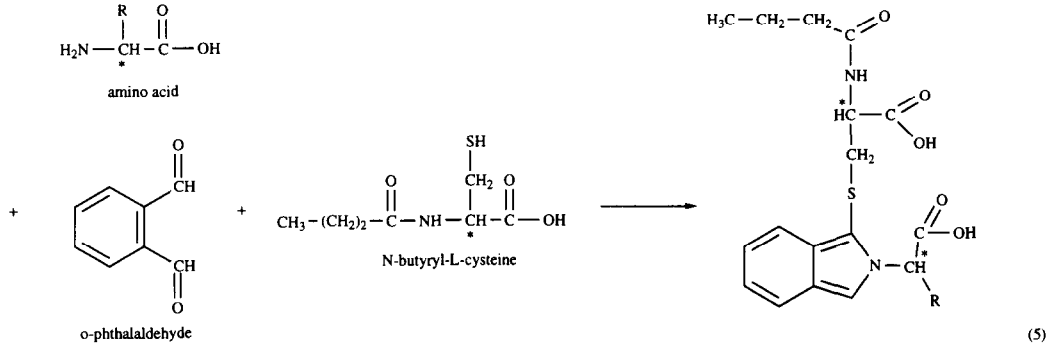
Isocyanates and isothiocyanates form the corresponding urea or thiourea derivatives with primary and secondary amines. Under the reaction conditions adopted for the derivatization reactions, drugs containing both amino and hydroxyl groups (*e.g.* β-blockers) react at their amino moieties only. Enantiomeric 1-(1-naphthyl)ethyl [29,38,56,57] and α-methylbenzyl [29,47,58,59] isocyanates are among the classical chiral derivatizing agents. Drug-related derivatizing agents, already mentioned among the carboxylic chloride reagents, have also found application here [32]. As an example, the derivatization of propranolol with (−)-(*S*)-flunoxaprofen reagent is shown in reaction 3 ($\alpha_{-/+} = 1.14$).

Of the isothiocyanate reagents, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate is most widely used [29,58,61–67]. Its recently introduced tetrabenzoyl analogue [21] retains the good resolution capabilities but greatly improves the detectability by the UV detector.

Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alaninamide) is one of the most widely used reagents for the resolution of amino acid enantiomers. It is especially useful in controlling peptide reactions for possible racemization during certain reaction steps. In this application the peptides have to be hydrolysed first either by acidic reagents such as 6 *M* hydrochloric acid or enzymatically followed by reaction with the reagent (see reaction 4) [69,70]).



Reaction 4.



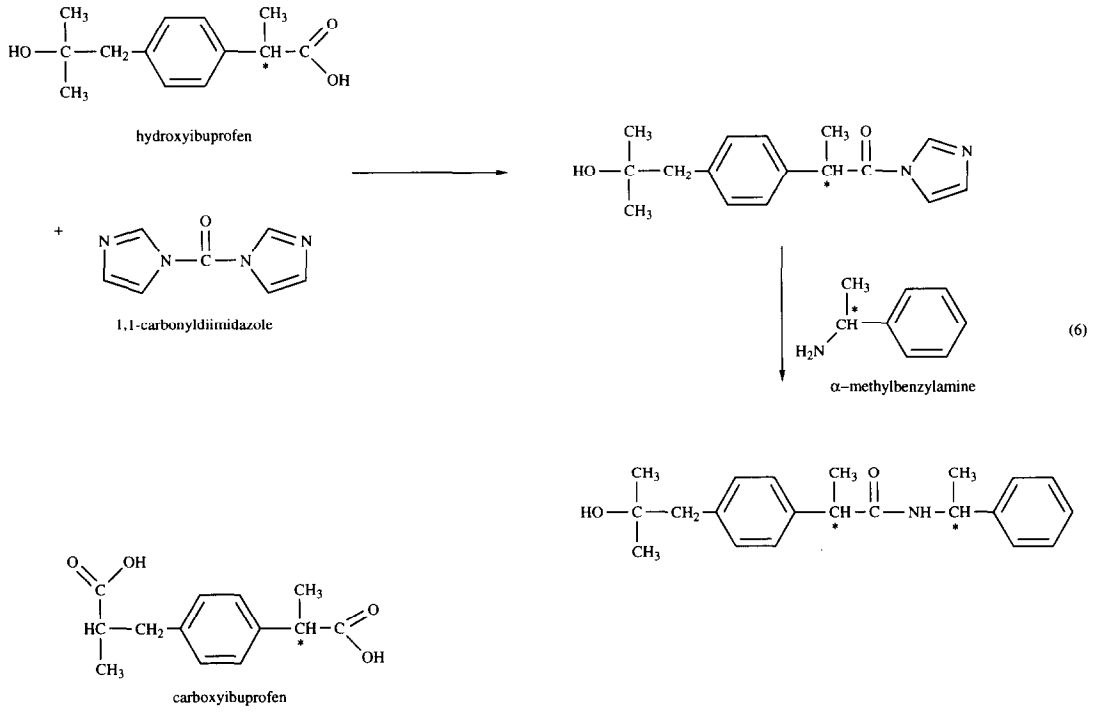
Reaction 5.

In a recent study, it was found that the 5-L-valinamide analogue of the reagent gives better resolution [73,74]. Good separation and sensitive fluorimetric monitoring were possible with the monochloro-1,3,5-triazinyl-L-alaninamide derivatives, where the derivatization reaction is (as with the Marfey's reagent) the nucleophilic replacement of the aromatic halogen atom by the amino group of the amino acid [18].

The other general reaction for the chiral derivatization of amino acids is their reaction with *o*-phthalaldehyde and chiral thiols to form fluorimetrically highly active isoindole derivatives. As the thiol reagent, among others 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside [83, 84] and N-acetyl-L-cysteine [75–77] have been used. The replacement of the acetyl group in the latter reagent by an isobutyryl group improved the separation [34,77,79,80]. The reaction equation with this reagent and the chromatograms obtained after derivatization with the L- and D-forms of the reagent are shown as reaction 5 and Fig. 5, respectively.

2.3.2. Derivatization of carboxyl group

Chiral carboxylic acids are transformed into their diastereomeric derivatives via esterification with chiral alcohols or amidation with chiral amines. Several examples for the derivatization with chiral alcohols such as (+)-2-octanol, (−)-menthol, (+)-1-phenylethanol and (−)-2-butanol can be found in earlier reviews [4a,7–10, 12a,13,15,16]. Inspection of the literature of the last few years shows that interest has shifted towards chiral amines as the derivatizing agents. As is seen in Table 2, the general method to convert carboxylic acids into their amide derivatives begins with the activation of the carboxyl group to enable it to react with the chiral amines. The activators include carbodiimides to transform the carboxyl group into reactive N-acylurea derivatives, thionyl chloride to form carboxylic chlorides, chloroformates to form mixed anhydrides and 1,1-carbonyldiimidazole to form the reactive N-acylimidazole derivatives. An application of the last activator followed by the classical chiral amine reagent, (*S*)-(−)-α-



(6)

Reaction 6.

methylbenzylamine reagent, for the derivatization of the carboxyl groups of ibuprofen metabolites is shown in reaction 6 and Fig. 6 [85].

One of the metabolites, carboxyibuprofen, has two chiral centres and is therefore present in four stereoisomeric forms. After the derivatization with (*S*)-(−)-α-methylbenzylamine reagent, all four diastereomeric derivatives together with the diastereomeric pair of the derivatives of the other metabolite (hydroxyibuprofen) have been well resolved [85].

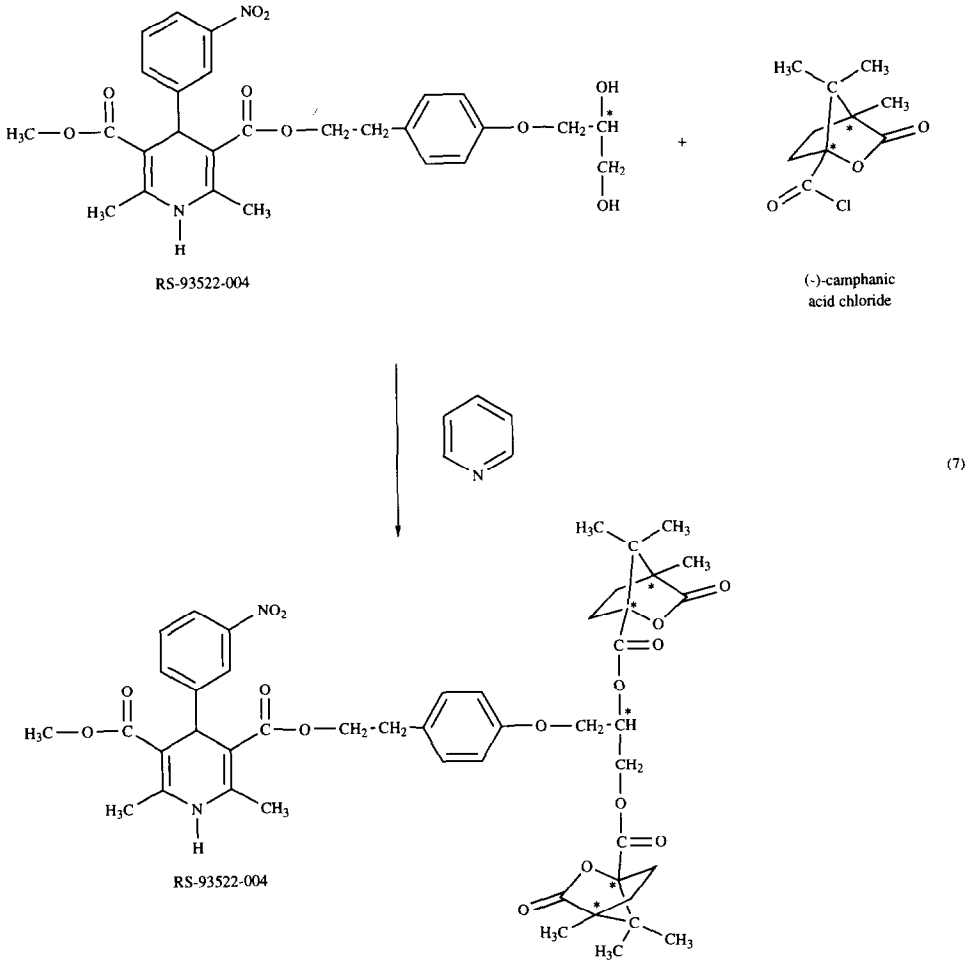
Of the newer chiral amine reagents, the amine derivatives of flunoxaprofen, benoxaprofen and naproxen [86–88] and (−)-2-4-(1-aminoethyl)-phenyl-6-methoxybenzoxazole [86], permitting very sensitive fluorimetric monitoring of the chromatogram, and 1-ferrocenylethyl (or propyl)amine [92], a reagent for application with an electrochemical detector, are worth mentioning.

2.3.3. Derivatization of hydroxyl group

The chiral derivatization of enantiomers with hydroxyl groups is carried out via esterification with various chiral acids or acid derivatives (Table 3). Direct coupling with the carboxylic acid reagent can be done by using dicyclohexylcarbodiimide as the coupling agent [36], but enzymatic glucuronidation is also worth mentioning [93].

More widespread is the use of acyl chlorides [37,94–97)], of which flunoxaprofen chloride, enabling highly sensitive fluorimetric detection to be carried out, merits special attention [97]. Reaction 7 shows the reaction of RS-93522-004, a calcium channel antagonist, with (−)-camphanic acid chloride. The chromatogram in Fig. 7 illustrates the separation of the four enantiomers of the two diastereomeric drug molecules [94].

Further chiral reagents for the derivatization of hydroxyl groups are acyl cyanides [98,99] and isocyanates [100], transforming the hydroxyl compounds into the corresponding esters and carbamates, respectively.



(7)

Reaction 7.

2.3.4. Miscellaneous derivatization reactions

The derivatization of chiral epoxides (oxiranes) usually requires two derivatization steps. In a recently published method [101], the first step is the opening of the oxirane ring by sodium sulphide at pH 10 to form a vicinal hydroxythiol derivative. In the second step, this reaction product serves as the chiral thiol reactant in the *o*-phthalaldehyde–amino acid–thiol reaction leading to diastereomeric isoindole derivatives, which is described in Section 2.3.1, and eq. 5 as a derivatization reaction for the resolution of amino acid enantiomers.

In another method, the oxirane ring is opened with 2-propylamine to give the corresponding chiral 1,2-amino alcohol. The amino group of the latter is derivatized with the 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate reagent (see Section 2.3.1) [102] or its tetrabenzoyl analogue [21] to form the diastereomeric thiourea derivatives.

The derivatizing agent for the separation of the enantiomers of gossypol, containing two aldehyde groups, is (*R*)-(−)-2-amino-1-propanol. The diastereomeric Schiff's bases formed are separated in an RP system ($\alpha_{-/+} = 2.2$) [103].

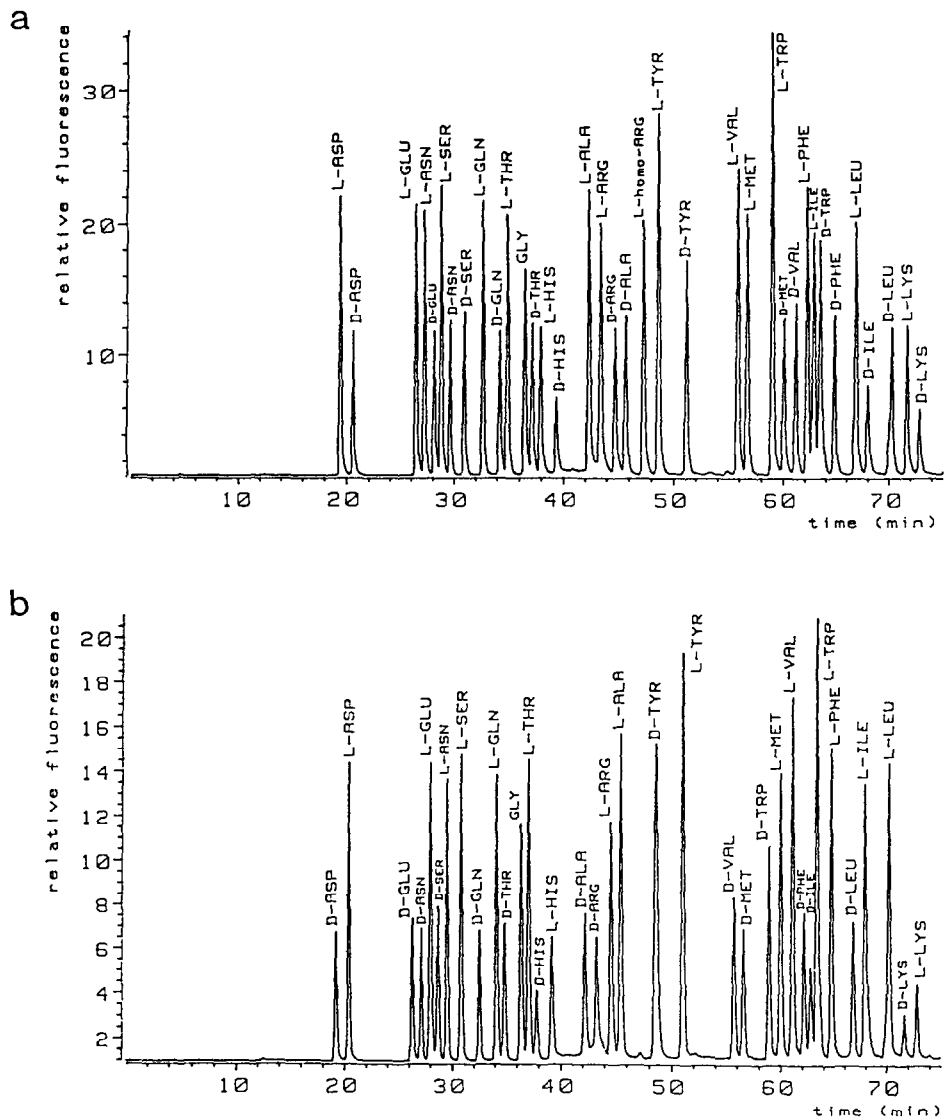


Fig. 5. HPLC elution profiles of a standard of DL-amino acids (L:D molar ratio 2:1), Gly and L-*homo*-arginine (internal standards) derivatized with (a) *o*-phthalaldehyde–N-isobutyryl-L-cysteine and (b) *o*-phthalaldehyde–N-isobutyryl-D-cysteine. Column, 250 × 4 mm I.D., Hypersil ODS, 5 μm; mobile phase, gradient elution, A = 23 m*M* sodium acetate (pH 6), B = methanol–acetonitrile (600:50), linear gradient from 0% B to 53.5% B in 75 min; flow-rate, 1 ml/min; detection, fluorescence (230 nm excitation, 445 nm emission) (with permission from ref. 34).

The aim of some enzymatic derivatization methods is to improve the confidence of the identification of the *R*- and *S*-enantiomers separated by chiral HPLC. This is especially important when complex biological samples containing several D- and L-amino acids are investigated, where retention matching with standards is not a sufficiently reliable means of identification. Selective oxidative deamination of D-amino acids by using the enzymes D-amino acid oxidase and catalase results in a decrease in or the disappearance of the peaks of D-amino acids in

Table 2
Chiral derivatization of carboxylic acids

Reagent(s) (carboxyl group activator + chiral amine)	Enantiomers separated	HPLC	Detection	Biological sample	Ref.
Dicyclohexylcarbodiimide + (1*R*,2*R*)-(−)- or (1*S*,2*S*)-(+)-2-amino-(4-nitrophenyl)-1,3-propanediol	α-Chloropropionic acid, (*Z*)-phenylalanine, *cis*-permethrinic acid, etc.	RP	UV (220)	−	35
Dicyclohexylcarbodiimide + L- or D-O-(4-nitrobenzyl)tyrosine methyl ester	N-Protected amino acids	NP	UV (270)	−	24
1,1-Carbonyldiimidazole + (*S*)-(−)-α-methylbenzylamine	Ibuprofen metabolites	RP	UV (232)	+	85
Thionyl chloride or N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide + flunoxaprofen amine or benoxaprofen amine or naproxen amine	Ibuprofen, becloboric acid, ciclotropium metabolite	RP, NP	Fl (305/355)	+	86–88
Ethyl chloroformate + L-leucinamide	Ketoprofen	RP	UV (275)	+	89
Diphenylphosphinyl chloride + L-leucinamide	Ofloxacin	RP	Fl (298/458)	+	19
Di-*tert*.-butyl carbonate + ethyl chloroformate + (*S*)-α-methylbenzylamine	Baclofen (preparative separation)	NP	UV (260)	−	90
(−)-1-(1-Anthryl)ethylamine	Ramipril	NP	UV (254)	−	91
1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide + 1-ferrocenylethylamine or 1-ferrocenylpropylamine	Ibuprofen, naproxen	RP	Electrochemical	−	92
2,2′-Dipyridyl disulphide–triphenylphosphine + (−)-2-[4-(1-aminoethyl)-phenyl]-6-methoxybenzoxazole	Naproxen, ibuprofen, flurbiprofen	NP, RP	Fl (320/375, 380)	−	31

the chromatograms run before and after the enzymatic reaction, thus facilitating their identification [104]. This principle has been used in the form of a postcolumn solid-phase reactor system where the amino acids in the HPLC effluent are first deaminated alternatively in reactors containing immobilized L- and D-amino acid oxidase enzymes. The basis of monitoring the chromatogram at 514 nm is the reaction of the hydrogen peroxide (formed in the course of the oxidative deamination) to form a quinoneimine dye with 2,4-dichlorophenol-6-sulphonyl chloride–4-

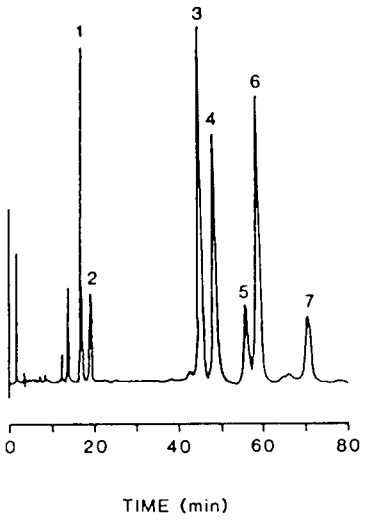


Fig. 6. HPLC separation of the distereomeric (*S*)-α-methylbenzylamide derivatives of ibuprofen metabolites extracted from human urine from a healthy volunteer dosed with the racemate. Column, 250 × 4.6 mm I.D., Ultrasphere C_8, 5 μm; mobile phase, water–methanol–*n*-butanol (62:30:8); flow-rate, 1 ml/min; detection, UV at 232 nm. Peaks: 1, 2 = (*S*)- and (*R*)-hydroxyibuprofen; 3 = internal standard (β-naphthoic acid); 4, 5, 6, 7 = (*SS*)-, (*RS*)-, (*SR*)- and (*RR*)-carboxyibuprofen (with permission from ref. 85).

aminoantipyrine reagent in a second reactor containing immobilized peroxidase enzyme [105].

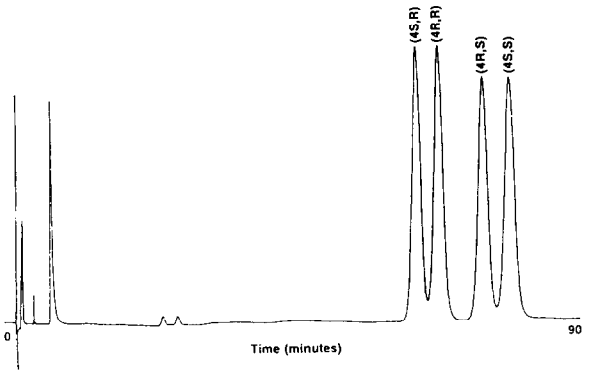


Fig. 7. HPLC separation of the calcium channel antagonist RS-93522-004 biscamphanate diastereomers. Column , 250 × 4.6 mm I.D., Nucleosil silica, 5 μm; mobile phase, 0.1% acetonitrile and 4% 2-propanol in isooctane; flow-rate, 2 ml/min; detection, UV at 229 nm (with permission from ref. 94).

3. Derivatization with achiral reagents prior to separation on chiral columns

3.1. Introductory remarks

As has already been mentioned in Section 1.2, precolumn derivatization can be necessary in certain instances even if direct resolution of the enantiomers is carried out using chiral stationary phases. The aim of this derivatization is to improve the chromatographic properties of the enantiomers for their separation on the chiral column.

Although the necessity for derivatization makes the term "direct separation" at least questionable, there are some advantages of this kind of derivatization as compared with the chiral derivatization described in Section 2: achiral reagents are used, which are less expensive than the chiral reagents; the enantiomeric purity and stability of the reagent do not have to be taken into account; the problem of kinetic resolution does not come into question; and there is no possibility of forming diastereomeric derivatives with different UV and fluorescence properties.

3.2. Derivatization prior to the use of Pirkle-type chiral columns

In the Pirkle-type and related chiral stationary phases, the chiral selectors are small, synthetic, chiral molecules attached to the silanol groups of the silica support by means of achiral spacers. The chiral selectors contain polar groups and aromatic moieties (phenyl, dinitrophenyl, naphthyl, *etc.*), enabling the "three-point interaction" to take place between the chiral selector and the analyte. In addition to hydrogen bonding and dipole–dipole interactions, π–π interactions between the aromatic moieties in the selector and analyte play a predominant role. Too strong interactions between the highly polar groups of the analyte and the chiral selector have to be avoided because this would lead to the loss of resolution.

On the basis of this, the primary aim of derivatization is the introduction of aromatic

Table 3
Chiral derivatization of alcohols and phenols

Esterification reagent	Enantiomers separated	HPLC	Detection	Biological sample	Ref.
Carboxylic acids					
Uridine 5′-diphosphoglucuronic acid + 5′-diphosphoglucuronyltransferase enzyme [D-(+)-glucuronidation]	2-(N-Propyl-N-2-thienyl-amino)-5-hydroxytetralin	RP	UV (225)	–	93
(−)-(1*S*,2*R*,4*R*)-*endo*-1,4,5,6,7,7-hexachlorobicyclo[2.2.1]-hept-5-ene-2-carboxylic acid [(−)]-HCA] + dicyclo-hexylcarbodiimide + NaOH (postcolumn)	Warfarin	RP	Fl (313/370)	+	36
Acyl chlorides and anhydrides					
(+)-HCA (as above) + thionyl chloride	Hydroxylated derivatives of 7-methylbenz[*c*]acridine and dibenz[*a*,*j*]acridine	NP	UV (254, 280)	–	37
(−)-Camphanic acid chloride	2-[4-(2,3-Dihydroxypropoxy)-phenyl]ethyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate and β-blockers	NP	UV (229)	–	58, 94
(*R*)-(+)-α-Methoxy-α-trifluoromethyl-phenylacetyl chloride (Mosher's reagent)	(*E*)- and (*Z*)-10-hydroxyami-triptyline	RP	UV (254)	+	95
β-Naphthylsulphonyl-L-prolyl chloride	Enprosil (prostaglandin)	RP	UV (270)	–	96
Flunoxaprofen chloride	(8*R*)-3α-hydroxy-8-iso-propyl-1α*H*,5α*H*-tropanium bromide (metabolite of ciclotropium)	RP	Fl (310/365)	+	97
(*R*,*R*)-O,O-Diacetyltartaric anhydride	Propranolol	RP	Fl (290/335)	+	33
Acyl cyanides and isocyanates					
(−)-2-Methyl-1,1′-binaphthalene-2′-carbonyl cyanide	Propranolol	NP	Fl (318/408)	+	98
(−)-2-Methoxy-1,1′-binaphthalene-2′-carbonyl cyanide	β-Blockers	NP	Fl (330/420)	–	99
(*R*)-1-(1-Naphthyl)ethyl isocyanate + 4-pyrrolidinopyridine	Diacylglycerol derivatives	NP	UV (280)	+	100

moieties into the molecule of the analyte. As is seen in the examples in Table 4, phenyl, naphthyl and 3,5-dinitrophenyl groups are introduced by means of the general reactions described in detail in Section 2 and in Tables 1–3, in such a way that the above-mentioned $\pi-\pi$ interaction can take place and in addition one of the above-mentioned highly polar functional groups (amine, hydroxyl or carboxyl) is blocked. Another advantage of the introduction of these groups is the increase in the sensitivity of UV or fluorescence detection. It can be seen in Table 4 that for compounds with two polar functional groups, the introduction of an aryl group is not always satisfactory: amino acids, hydroxy fatty acids, *etc.*, have to be transformed into the corresponding ester or amide derivative prior to the second derivatization reaction. Other examples not included in Table 4 are the separation of enantiomeric dihydrodiols of polycyclic aromatic hydrocarbons, where the prerequisite for obtaining good resolution is the transformation of the

Table 4
Derivatization of chiral amino, hydroxyl and carboxyl compounds with achiral reagents for their enantiomeric separation on Pirkle-type and related chiral stationary phases

Reagent	Derivative	Enantiomers separated	HPLC	Biological sample	Ref.
		Amino derivatives			
3,5-Dinitrobenzoyl chloride	3,5-Dinitro-benzamide	Propranolol, pindolol, etc.	NP	–	106
		Tocainide, mexiletine, etc.	NP	–	107
		Amino acids	RP	–	108
		Amino acid methyl esters	NP	–	109
		Amino acid esters	NP	–	110
		Amino acid 2-propyl esters or *n*-propylamides, dimethylamides	NP	–	111
		Amino acid alkylamides	NP	–	112
		Di- and tripeptide methyl esters	NP	–	113, 114
		1-(4-Methoxybenzyl)-10-hydro-xydecahydroisoquinoline, etc.	NP	–	115
Solid-phase polymer-bonded 3-nitro-4-hydroxybenzo-phenone 3,5-dinitrobenzoate or hydroxybenztriazole 3,5-dinitrobenzoate		Amino acids, amphetamine, ψ-ephedrine, norephedrine	NP	+	116, 117
4-Nitrobenzoyl chloride	4-Nitrobenzamide	Amino acid propyl esters	NP	–	118
3,5-Dinitrophenyl-, methyl-1-naphthyl isocyanates	Urea derivatives	Tocainide, mexiletine, etc.	NP	–	107
3,5-Dinitrophenyl isocyanate	3,5-Dinitro-phenylurea	1-(4-Methoxybenzyl-10-hydro-xydecahydroisoquinoline	NP	–	115
1-Naphthyl isocyanate	1-Naphthylurea	β-Blockers, etc.	NP	–	119
Ethyl-, cyclohexyl-, phenyl-, 4-methoxyphenyl-, 4-fluoro-phenyl-, 1-naphthyl-isocyanates and isothio-cyanates	Urea and thiourea derivatives	Propranolol	NP	–	120, 121
		Carboxyl derivatives			
Aniline, 3,5-dimethylaniline–dicyclohexylcarbodiimide	Carboxanilides	N-Protected amino acids	NP	–	122

Aniline, 4-alkylanilines, etc.–thionyl chloride or 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline		Ibuprofen, α-phenylbutanoic 2-isopropoxyphenylacetic acids, etc.	NP, RP	–	123
3,5-Dimethylaniline–1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC)		Arylpropanoic acids	NP, RP	–	124
3,5-Dinitroanaline–thionyl chloride		Naproxen	NP	–	125
1-Naphthylmethylamine–EDC + 1-hydroxybenztriazole	Carboxamide	N-[4,4-Di(3-methylthien-2-yl)-but-3-enyl]nipecotic acid	RP	–	126
		Hydroxyl derivatives			
Benzoyl chloride	Benzoate	β,β-Caroten-2-ol, -2,2′-diol, 2-hydroxyechinenone	NP	+	127
3,5-Dinitrobenzoyl chloride	3,5-Dinitro-benzoates	Glycerol ethers	NP	–	128
		Pantolactone	RP	–	129
1-Naphthoyl chloride	1-Naphthoates	Hydroxylated polyenoic fatty acid methyl esters	NP	–	130
		18- and 19-Hydroxyeicosatetranoates (as methyl esters)	NP	–	131, 132
1-Naphthyl isocyanate	1-Naphthyl-carbamates	Secondary alcohols	NP, RP	–	133
		Ibutilide	RP	–	134
3,5-Dinitrophenyl isocyanate	3,5-Dinitro-phenyl-carbamates	Secondary alcohols	NP	–	135
		Lactic acid ethyl ester, etc.	NP	–	136
		Ketones (as ketoximes)	NP	–	137
		2-Hydroxy fatty acid methyl esters	NP	–	138
		Mono- and diacylglycerols and derivatives	NP	+	139–142
		Pantothenic acid methyl ester	RP	–	129

hydroxyl groups into their methyl ethers [143] and the formation of O-acetyl or O-methyl derivatives of lorazepam to avoid racemization in the mobile phase [144]. It is worth mentioning that, as it is seen in Table 4, in most instances (unlike in Tables 1–3), there is no indication of the use of the separation method for the purpose of investigating biological samples. The interest of analysts working in this field seems to have been concentrated on the very interesting theoretical questions related to the structural basis of the resolution.

3.3. Derivatization for the use of cyclodextrin-bonded phases

Although cyclodextrins or their derivatives covalently bonded to silica are extremely effective selectors for a wide variety of enantiomers even without derivatization, in many instances it is necessary or at least useful to derivatize the chiral compounds, especially if they do not contain aromatic groups [145,146]. For example, amino acids are usually investigated as the dansyl [147], 3,4-dinitrobenzoyl [148] or *o*-phthalaldehyde [149] derivatives, but recently other derivatization reactions such as reaction with naphthalene-2,3-dicarboxaldehyde–cyanide reagent to form highly fluorescent, fairly well separable derivatives [150] have also been described. Further reagents for the derivatization of amino acids prior to separation on bonded cyclodextrin phases are the highly fluorescent 9-fluorenylmethyl chloroformate and 9-fluorenylmethoxycarbonyl glycine chloride [151–153] and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate [154]. Of the several other examples, the derivatization of penicillamine with the *o*-phthalaldehyde–2-mercaptoethanol reagent [155] can be mentioned.

3.4. Derivatization for the use of α_1-acid glycoprotein column

α_1-Acid glycoprotein, immobilized on silica (Chiral AGP), is one of the most widely used chiral stationary phase for the enantiomeric separation of a wide variety of chiral drugs with several applications in the biological–clinical field [14,156]. Neutral drugs not containing ionizable groups and many of the basic drugs where there are bulky substituents on the basic nitrogen can be resolved without derivatization using phosphate buffer in the pH range 5–7 with relatively low concentrations (up to 15%) of organic modifiers (mainly 2-propanol or acetonitrile).

The enantiomeric resolution of drugs containing carboxyl groups such as naproxen [157] and ibuprofen [158] can greatly be improved by the dynamic formation of ion pairs with N,N-dimethyloctylamine dissolved in the eluent. Anionic additives (*e.g.* octanoic or phenylbutyric acid) improve the resolution, column efficiency and peak symmetry for some basic drugs such as ephedrine, atropine and homatropine [156,159,160].

Covalent achiral derivatization is also carried out in some instances. In the course of the determination of atenolol enantiomers in human plasma, hydroxyl and secondary amino groups of the extracted enantiomers are acetylated prior to the chromatographic separation. The aim of this is partly to improve the resolution of the enantiomers and partly to separate their peaks from endogenous peaks [161]. Another example of the effect of acylation of amino groups on the separation is demonstrated in Fig. 8. Here the resolution of the enantiomers of alanine benzyl ester is shown with and without formylation of the primary amino group using mixed acetic–formic anhydride [162]. Esterification of the carboxyl group,, *e.g.* in the case of mandelic acid, has a dramatic effect on the separation; the separation factor of the free acid at pH 7 in the presence of 2% of 2-propanol is 1.00, whereas 1.27 and 1.93 were found for the methyl and ethyl ester, respectively [163]. Another interesting achiral derivatization improving the resolution of the enantiomers of several β-blockers is their reaction with phosgene, leading to oxazolidone derivatives [163].

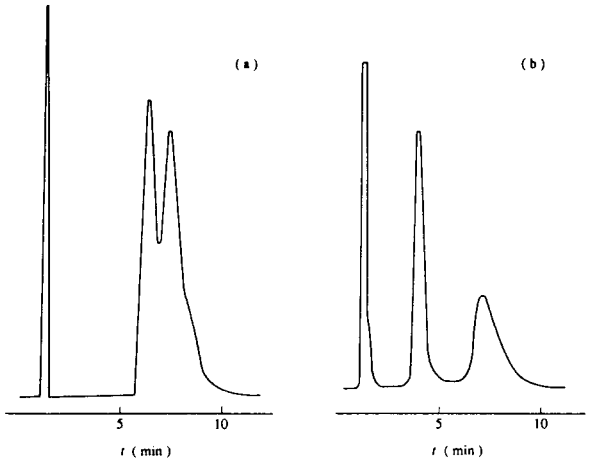


Fig. 8. HPLC separation of the enantiomers of alanine benzyl ester. (a) Separation without derivatization; (b) separation after formylation. Column, 100 × 4.6 mm I.D., Chiral AGP; mobile phase, 0.01 *M* phosphate buffer (pH 8) containing 1% 2-propanol; flow-rate, 0.8 ml/min; detection, UV at 210 nm (with permission from ref. 162).

3.5. Derivatization for the use of functionalized cellulose phases

Columns with functionalized cellulose bonded to silica are at present the most popular chiral stationary phases for the resolution of enantiomeric drugs both in the biological–clinical field and in checking their enantiomeric purity. The functional groups attached to the hydroxyl groups of the carbohydrate units in the bonded cellulose macromolecules are mainly substituted benzoyl or phenylcarbamoyl groups to create the basis for the $\pi-\pi$ interaction between the stationary phase and the enantiomers, which plays a predominant role in the separation mechanism. In most instances no derivatization of the analytes is necessary. In some instances, however, derivatization of the carboxyl group (*e.g.* formation of the methyl ester of flurbiprofen using diazomethane [164]) improves the resolution. The introduction of an aromatic group, *e.g.* by the benzoylation of the hydroxyl group in carotinoids [165], is also advantageous with compounds not containing an aromatic moiety for the above-mentioned $\pi-\pi$ interaction.

4. Dynamic formation of diastereomeric adducts, ion pairs and metal complexes and their separation on achiral columns

4.1. Introductory remarks

As already described in Section 1.2, direct chiral separation methods are based on the formation of stable molecular associates between the chiral stationary phase and the enantiomers to be separated. In these instances the basis of the separation is the difference between the stability constants of the acceptably stable diastereomeric associates formed. The separation on chiral columns after derivatization with achiral reagents prior to the separation is outlined in Section 3.

In this section, the separation on achiral stationary phases using chiral mobile additives, including the dynamically coated stationary phases, will be discussed.

4.2. Separation based on cyclodextrin inclusion complex formation

The concept of separation methods based on inclusion complex formation is simple: the solute molecule can penetrate into the toroidal cavity in the host molecule, where the interaction with the surface of the cavity leads to a significant change in the chromatographic properties of these solutes. The strength of the interaction controls the retention of the solute: stronger interaction results in a greater change in the chromatographic properties.

Several groups have studied the separation mechanism and the chromatographic behaviour of the inclusion complexes applying α-, β- and γ-cyclodextrins (CDs) as chiral mobile phase additives in reversed-phase systems, of which the pioneering work of Sybilska and co-workers [166–169] merits special mention. Several reviews have been published on the comparison of the different CDs and their application in chiral separations [11a,12d,170, *etc.*]. In this section, only the application of CDs as mobile phase additives will be discussed (chemical derivatiza-

tion aspects of the use of chemically bonded CD stationary phases was briefly discussed in Section 3.3).

The influence of different parameters on the retention of solutes capable of forming inclusion complexes can be summarized as follows:

The α-, β- and γ-CDs and their different derivatives differ in the number of glucose units, which is six, seven and eight, respectively. Accordingly, different cavity diameters may be selected to suit the sizes and structure of the solute molecule.

The following are some limitations that have great importance in the formation of inclusion complexes. An appropriate size and a lipophilic portion of the guest molecule are necessary to the interaction with the host CD cavity. Weak reaction or no complex formation is observed with solutes having a smaller or larger size than the CDs. In these cases the presence of CDs does not influence the retention parameters of the solutes. Chiral separations require the solute molecule to enter the hydrophobic cavity in such a way as to place the chiral centre in association with the polar groups at the edge of the cavity. If there is no interaction between these (hydroxy) groups and the substituents attached or near to the chiral centre of the solute, the separation is minimal or nil. Generally, it is not the degree or nature of the penetration into the chiral cavity that is the main criterion for resolution to occur, but the existence of interaction between the secondary hydroxyls and the guest molecules. Very polar and apolar compounds cannot be separated owing to their small retention in reversed-phase systems and to the high organic solvent concentration in the eluent to obtain a reasonable analysis time.

Attempts have been made to choose the proper column and mobile phase composition for the resolution of chiral solutes with different molecular structures. It has been found that CDs are not adsorbed on the surface of either polar or apolar stationary phases [171,172].

The polarity and the type of stationary phases play important roles in the retention, separation selectivity and efficiency. The same resolving power was found on various octadecylsilica columns at constant eluent composition with entirely different retardations [171]. The suitable concentration of the organic modifier depends on the polarity of the stationary phase. This was investigated by Gazdag *et al.* [172] by using γ-CD as the chiral reagent in the mobile phase and also by Takeuchi and Nagae [173] using microcolumn LC. It can be concluded that stronger inclusion complex formation and more effective enantioselective separation can be achieved by decreasing the organic solvent content in the eluent.

The influence of the type and concentration of CDs in the eluent has also been studied in detail [171,172,174]. It was found that α-CD may be primarily applied for the separation of small molecules (one-ring systems), whereas β- and γ-CDs are suitable for the resolution of compounds with larger molecular size (2–4-ring systems). β-CD and its derivatives and γ-CD (forming the most polar complexes) have been most widely and successfully used in practice as mobile phase additives for the enantiomeric separation of various drugs and biologically active compounds. Some of the recent applications are summarized in Table 5.

Using different organic solvents, it was found [175] that the elution order of the compounds to be separated is not altered; the selectivity of the separation is not significantly affected by the nature of the organic solvent in the eluent. Figs. 9 and 10 show the capacity factors, selectivity and the resolution as a function of the concentration of the organic solvent in the eluent with methanol (Fig. 9) and acetonitrile (Fig. 10) as organic modifiers. The molecules of the solutes and the organic solvents compete for the preferred location in the hydrophobic cavity, which results in various degrees of interaction of the compounds to be tested with CDs. On increasing the concentration of the organic modifier, the capacity factors of the solute complexes decrease similarly to those with ordinary reversed-phase systems. The two above-described opposing effects may result in unchanged or increased retentions of the compounds.

The effect of elevated column temperature [175,180] on the separation characteristics (capacity factors, selectivity and resolution) is unpredictable. In the resolution of the enantio-

Table 5
Some enantiomeric separations based on complexation with cyclodextrins (CDs) as mobile phase additives

Mobile phase additive	Enantiomers separated	Stationary phase[a]	Biological sample	Ref.
γ-CD	Norgestrel	C_{18}	–	171, 175, 176
γ-CD	Budesonide, etc.	Cyanopropylsilica	–	172, 176
β-CD, methyl-β-CD	Propranolol, Gly-Phe	Porous graphitic carbon	–	177
γ-CD	Dansylphenylalanine	C_{18}, C_8, trimethylsilica	–	173
β-CD	Renin inhibitor RO-42-5892/001 after dabsylation	C_8	+	178
β-CD	Dansylamino acids	C_{18}, C_8	–	179
β-CD	Tipredane, phenylthioproline	Trimethylsilica	–	180
γ-CD	1,1'-Binaphthyl-2,2'-diyl hydrogenphosphate	C_{18}	–	181
β-CD	Oxazepam	Porous graphitic carbon	–	182
β-CD	Terbutalin, chlorthalidone, oxazepam	Phenylpropylsilica, cyanopropylsilica	+	183
β-CD	Thromboxane antagonists	C_8	–	184
β-CD	Brompheniramine (semi-preparative separation)	Cyanopropylsilica	–	185

[a] C_{18} = dimethylocadecylsilica; C_8 = dimethyloctylsilica.

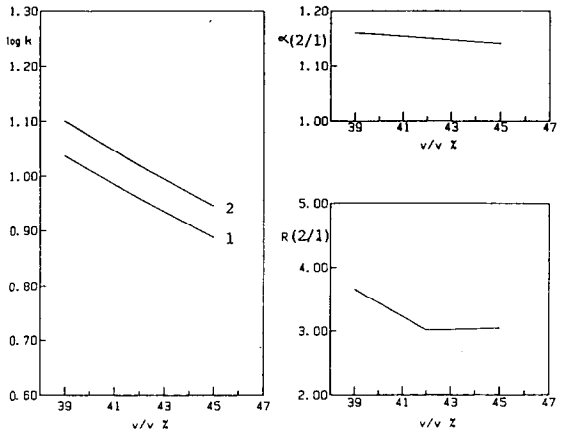


Fig. 9. Dependence of the capacity factors (k'), separation factor (α) and resolution (R_s) of (1) D-norgestrel and (2) L-norgestrel on methanol concentration in an eluent containing 10 mM γ-cyclodextrin; flow-rate, 1 ml/min; column, 250 × 4.6 mm I.D., Ultrasphere ODS, 5 μm; detection, UV at 244 nm; temperature, 27°C (with permission from ref. 175).

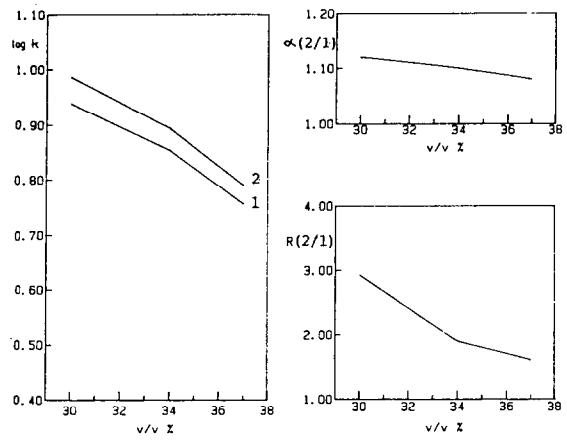


Fig. 10. Dependence of the capacity factors (k'), separation factor (α) and resolution (R_s) of (1) D-norgestrel and (2) L-norgestrel on acetonitrile concentration in an eluent containing 10 mM γ-cyclodextrin. For other conditions and reference, see Fig. 9.

mers of norgestrel [175], an elevated column temperature results in a decrease in retention.

If the guest compound contains ionizable functional groups(s), the inclusion complex formation is affected by the pH and the ionic strength. In contrast, the selectivity and efficiency of the separation are independent of the salt concentration and pH when guest molecules without ionizable groups are examined [174].

Chiral separations with porous graphitic carbon as the achiral stationary phase in conjunction with β-CD as the mobile phase additive can afford significant advantages by enabling poorly ionizable drugs to be investigated at the extreme ends of the pH range [177].

Method development and systematic optimization of the methods involving the use of CDs as mobile phase additives can be found in the literature with the aim of finding the best available separation system [174,175,182,186].

An interesting possibility for enhancing the utility of enantiomeric separations with chiral mobile phase additives has been described by Szepesi and Gazdag [176], who combined CD inclusion complex formation with ion-pair chromatography.

The usefulness of coupled-column chromatography for the direct separation of drug enantiomers in biological fluids has been demonstrated by Walhagen and Edholm [183] by using the first achiral column for the separation of the enantiomers from endogenous materials followed by enantiomeric separation on another achiral column using CD in the mobile phase.

4.3. Separation with formation of diastereomeric ion pairs (complexes)

Organic cations and anions can interact with ion-pairing reagents in HPLC eluents at proper pH, *etc.*, conditions to form less polar, undissociated ion pairs. This technique is frequently used for the separation of polar, ionizable compounds in reversed-phase systems. Using chiral ion-pairing reagents the ionizable enantiomers can be transformed into their diastereomeric ion pairs, thus creating the basis for their resolution on achiral columns.

Some general features and advantages of the use of chiral counter-ions dissolved in the mobile phase are as follows. The most important point is to select the appropriate counter ion fulfilling the requirements of the formation of stable diastereomeric complexes ensuring the optimum elution order. The reagents should be optically pure and soluble in the eluent and it is often advantageous if the reagent improves the detectability of the solute. The basis for the resolution is the formation of diastereomeric complexes (ion pairs) with different stabilities or distribution properties between the mobile and stationary phases. Commercially available achiral stationary phases can easily be selected for the separation of the diastereomeric complexes. The variability of mobile phase composition over a wide range affords excellent possibilities for method development and optimization.

The chiral counter ions are generally used in mobile phases of low polarity (normal-phase systems) to promote a high degree of ion-pair formation. In some instances reversed-phase systems have also been applied using optically active zwitterions for the stereoselective separation of, *e.g.* tryptophan and its analogues [187,188]. For the separation of optically active octahedral metal complexes in ion-exchange chromatography, (+)-tartrate was used [189].

The fundamentals (equations, different retention models describing the interactions) can be found in the monographs cited earlier [8,11b,12c,15,170].

In this section mainly normal-phase ion-pair chromatographic aspects will be discussed.

4.3.1. Selection of the chiral counter ion

This point has great importance because the success of the enantioselective separation is highly dependent on the counter ion chosen.

The primary interaction between the enantiomeric solute and the counter ion to form a stable diastereomeric complex is of ionic character and for this reason in most instances a relatively strong acid–base character of the reagent is important.

To form a stable and rigid diastereomeric complex, a hydrophobic interaction is needed. This means that a lipophilic nature of the reagent is also necessary to permit this secondary inter-

action. The structure of the counter ion not only has a great effect on the retention but also influences the stereoselectivity of the separation. Improved stereoselectivity can be achieved by the introduction of bulky and rigid groups in the vicinity of the chiral centre of the counter ion [190]. According to Pettersson and Schill [191], the presence of a polar functional group in the chiral reagent is also necessary for chiral recognition (tertiary interaction).

The enantiomeric purity is one of the most important properties of chiral ion-pairing reagents as the amount of the enantiomeric impurity in the reagent determines the lowest detectable enantiomeric impurity content in the solute. In this respect, the situation is similar to that in enantiomeric derivatization (see Section 2.2.5). In certain instances, however, making use of the equilibrium nature of the formation of the diastereomeric complexes enantiomerically impure reagents can also be used for the determination of enantiomeric ratios [192].

Other important features of the ion-pairing reagents to be considered are low UV absorbance in the spectral range used for monitoring the chromatograms and good solubility in the mobile phase.

4.3.2. Optimization of the separation (resolution, selectivity, efficiency)

Most papers report on the use of very simple mobile phase compositions for normal-phase ion-pair separation: an apolar eluent (*e.g.* dichloromethane) modified with small amounts of polar components (alcohols, water, etc.). Much higher variability and excellent resolutions have been achieved by Szepesi *et al.* [193] using three-component solvent mixtures as eluents in the separation of various stereoisomeric eburnane alkaloids using 10-camphorsulphonic acid as the chiral ion-pairing reagent.

On the basis of the results of this study, it can be stated that the nature of the apolar solvent does not have a significant influence on the enantioselectivity. The retention of the solutes is dependent on the nature of the hydrophobic constituent. The selection of the most suitable "moderator" (medium-polarity) solvent is of great importance from the point of view of the stereoselective separation. For the separation of the enantiomers of apovincaminic acid ethyl ester, dioxane and tetrahydrofuran can be advantageously applied, but in the separation of other enantiomeric eburnane alkaloids, in accordance with the results of Pettersson and co-workers [190,191,194], chloroform or dichloromethane is more appropriate. The retention of the compounds can be easily controlled by changing the ratio of hydrophobic and moderator solvents without a decrease in selectivity and efficiency (see Fig. 11). A linear correlation has been found between the capacity factors and the ratio of hexane and dioxane in the mobile phase.

The efficiency of the column and the stereoselectivity of the separation are greatly

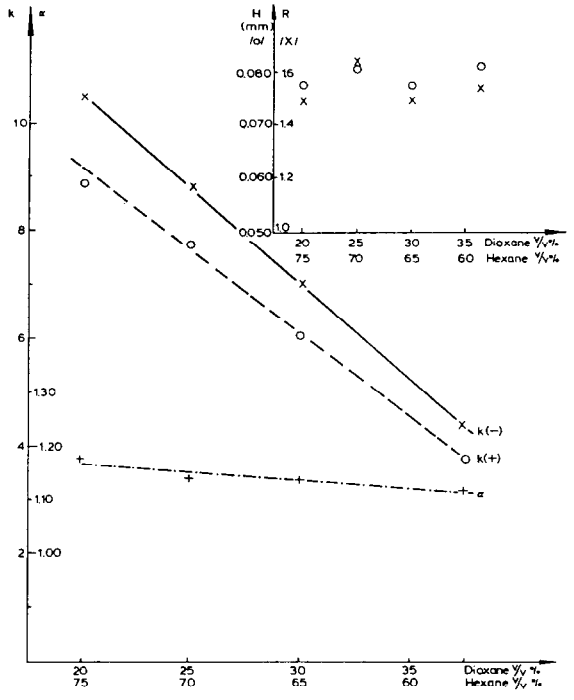


Fig. 11. Dependence of capacity factors (k'), separation factor (α), resolution (R_s) and height equivalent to a theoretical plate (H) of (+)- and (−)-apovincaminic acid ethyl ester (vinpocetine) on the ratio of hydrophobic and moderator solvents in the mobile phase. Column, 250 × 4.6 mm I.D., Nucleosil 10 CN; eluent, (hexane–dioxane)–1-butanol (95:5) containing 2 m*M* (+)-10-camphorsulphonic acid and 1 m*M* diethylamine; flow-rate, 1.5 ml/min; detection, UV at 280 nm (with permission from ref. 193).

affected by the selection and concentration of the polar component in the eluent. The effect of water merits special attention. Pettersson and co-workers [190,196,197] have found that mobile phases containing 80–500 ppm of water often give good stereoselectivity with an acceptable time requirement for the equilibration of the column. However, using a cyanopropylsilica stationary phase, a water concentration of up to 1% did not affect the separation when the above-mentioned ternary solvent mixtures [193] were used as the mobile phases. Similar results have been reported by Ladányi *et al.* [195].

The functional groups at the surface of the stationary phase control the retention of the diastereomeric ion pairs and for this reason the proper selection of the stationary phase (see Table 6) is an important point in the optimization of the separation.

The achiral additives (see Table 6) as competitors for ion-pair formation with the counter ion and for adsorption on the stationary phase also play an important role in regulating the retention and improving the peak shape and separation efficiency without a decrease in stereoselectivity.

The dependence of the capacity factor of an enantiomeric solute on the concentration of the counter ion is very complex [190], as the latter can also adsorb on the stationary phase and compete with the diastereomeric ion pairs for the limited adsorption capacity of the stationary phases. By changing the chiral ion-pairing reagent from (+) to (−) or *vice versa*, the elution order of the diastereomeric ion pairs can be changed, which is of great importance especially in optical purity testing of solutes [192,193] (see Section 2.2.7).

Some applications or enantioseparations with chiral ion-pairing and complexing agents are summarized in Table 6.

4.4. Separation by chiral ligand-exchange chromatography

Ligand-exchange chromatography is based on separation effected by the preferential mixed chelate complexation of the solute ligand with a metal ion and a second (system) ligand introduced into the chromatographic system. Stereoselective resolution of isomeric ligands can be achieved if the system ligand is optically active and forms a chiral complex with the metal ion.

Of the immense literature on this method, only a few introductory papers [204–206] and some important reviews [11c,12b,207,208] are listed here.

The ligand-exchange equilibrium can be accomplished by two general approaches, as follows. Enantiomeric amino acid ligands are bonded to or coated on the stationary phase (polymeric resins, silica, modified silica), on which copper(II) ions are subsequently loaded. The ligand-exchange equilibrium on which the resolution is based takes place on the surface of the stationary phase. Alternatively, very efficient separation of enantiomeric solutes with complex-forming ability can be achieved using conventional achiral stationary phases such as reversed-phase and ion-exchange packings if a metal ion and a chiral ligand are added to the mobile phase. The mechanism of the resolution is based on the difference in the stabilities and therefore the equilibrium concentrations of the two diastereomeric ternary complexes formed and the difference in their partitioning and/or adsorption behaviour (dynamic ligand-exchange chromatography).

In both instances the resolution may also be influenced by other interactions, such as hydrophobic or steric interactions between the chiral additive and the solute molecule and between the ternary complex of various structures and the characteristics of the column packing surface [209]. Further, the success of the ligand exchange depends on the concentration of the chiral additive, the molar ratio of the chiral ligand and the metal ion, the pH and the composition of the mobile phase.

Chiral ligands applied in this technique are designed on the basis of the following criteria: two or more functional groups are necessary in the vicinity of its asymmetric centre for chelate formation; they should have a bulky moiety to induce steric repulsion; and their optical purity is important.

Some chiral ligands used in the mobile phase

Table 6
Some enantiomeric separations based on the formation of diastereomeric ion pairs

Chiral ion-pairing reagent	Achiral additive	Enantiomers separated	Stationary phase	Biological sample	Detection	Ref.
(+) or (−)-10-Camphorsulphonic acid	Diethylamine	Vincamine (8 stereoisomers) and derivatives	Cyanopropyl-silica	−	UV (280)	183
	Diethylamine	(*E*)-2,4-Dimethoxy-8-aza-D-homo-9β,13α-gona-1,3,5(10)-trien-12-one oxime	Silica	−	UV (281)	195
	Triethylamine-*tert.*-butylamine, etc.	Propranolol and derivatives	Cyanopropyl silica	−	UV (280)	199
	−	Azelastine	Cyanopropyl-silica	−	UV (286)	200
	−	Nomifensine (N-trifluoroacetylated)	Silica	−	UV (254)	201
N-Benzyloxycarbonyl-glycyl-D-proline	Triethylamine	Propranolol	Silica-diol	−	UV	192
	Triethylamine	Propranolol	Silica-diol	+	Fl (292/340)	196
	Triethylamine	β-Blockers, promethazine, etc.	Silica-diol, porous graphitic carbon	−	UV	197
	Triethylamine	Propafenone	Cyanopropyl-silica	+	UV (300)	202
N-Acetyl-L-valine-*tert.*butylamide	−	Amino acid N-(4-nitrobenzoyl)isopropyl esters	Silica	−	UV (265)	203
L-Leu-L-Leu-L-Leu	Phosphate (pH 6.3)	Tryptophan	Octadecyl-silica	−	UV (254)	188
(2*R*,3*R*)-Dicyclohexyl tartrate	Phosphate pH (2.8)	Ephedrine, atropine, homatropine, etc.	Porous graphitic carbon	−	UV (254)	198

in some recent studies are N,N-di-*n*-propyl-L-alanine [210], N-*n*-dodecyl-L-hydroxyproline [211], L-phenylalanine [212], phenylalanyl, valyl, *etc.*, amides [213], (*S*,*S*)-N,N′-bis-(amino-acyl)ethane or propane derivatives (acyl = alanine, phenlalanine, valine) [214], *etc.* Some new ligand-exchange stationary phases prepared by binding or coating the chiral ligand to the solid support are RP-18–octyl-5′-AMP [215], Chiralpak WH, Chiral Hypro-Cu-Daltosil [216], RP-18–cyanocobalamine [217] and RP-18–N-salicilidene-(*R*)-2-amino-1,1-bis(2-butoxy-5-*tert.*-butylphenyl)-3-phenyl-1-propanol [218].

In most instances copper(II) salts are used in the mobile phase to form the ternary complexes [210–218], but in earlier reviews the use of Ni(II), Fe(II), Fe(III), Zn(II) and Co(II) was also described. The enantiomers to be separated should also have two or more chelating functions. Amino acids are therefore eminently suitable for this type of enantiomeric separation: most papers published on the application of chiral ligand-exchange chromatography deal with the analysis of amino acids and derivatives. For example, also among the selectands considered above, the enantiomeric separation of free α-amino acids [210,215,217,218], β-amino acids [211], dansylamino acids [213,214] and amino acid amides [210] represent the great majority. Hydroxy acids such as lactic, glyceric, 2- and 3-hydroxybutyric [218] and 3- and 4-hydroxy-mandelic [212] acid, amino alcohols such as β-blockers [218], norephedrine, *etc.* [218], nor-adrenaline, phenylethanolamine, *etc.* [211], and pirmenol [216] represent other groups of chiral drugs resolvable by ligand-exchange chromatography.

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