Ion-pair Chromatography; Ligand Exchange Chromatography; Liquid Chromatography; Molecular Imprints as Stationary Phases; Protein Stationary Phases; Supercritical Fluid Chromatography; Synthetic Multiple Interaction ('Pirkle') Stationary Phases; Thin-Layer (Planar) Chromatography.

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Chiral Derivatization

S. Görög, Chemical Works of Gedeon, Richter Ltd, Budapest, Hungary

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Introduction

The Importance of Enantiomeric Separations

The separation of enantiomers of chiral compounds by chromatographic methods and related techniques is one of the important tasks in modern analytical chemistry, especially in the analysis of compounds of biological and pharmaceutical interest. However, analysis of this kind is also required in food analysis and the analysis of pesticides, flavours and fragrances. The reasons for this are as follows:

- As a consequence of the existing or potential differences between the biological–pharmacological activities of the antipodes of racemic drugs, analytical methods are required for their simultaneous determination in biological samples, thus enabling one to follow the fate of the enantiomers of the administered drug (candidate) in the animal or human organism.
- Asymmetrical syntheses are in the focus of interest in various fields of organic chemistry especially in the synthesis of drugs administered as the pure enantiomer. In these cases and also if the preparation of the enantiomers is carried out by classical resolution techniques, analytical methods are necessary for the determination of their enantiomeric purity.

Possibilities for Enantiomeric Separations

Since in achiral environment the physicochemical properties of the antipodes of racemates are identical, their separation is not possible if the generally used, achiral separation systems – ordinary high-performance liquid chromatography (HPLC) and gas chromatography (GC) columns, thin-layer chromatography (TLC) plates, capillary electrophoresis (CE) capillaries, etc., with ordinary mobile phases – are used. The main possibilities for the separation of enantiomers are:

- Transformation of the enantiomers to covalently bonded diastereomeric derivatives by reacting them with homochiral derivatizing reagents prior to their chromatographic separation using achiral stationary phases or and mobile phases. The detailed description of this general method, which is often referred to as an indirect method, is described here.
- Incorporation of the chiral reagent in the mobile phase for the dynamic formation of diastereomeric adducts, ion pairs or complexes with the enantiomers to be separated during the chromatographic run. In this case also, achiral stationary phases are used.
- Separation of the enantiomers on chiral stationary phases (HPLC, GC and TLC). Although in principle this general method does not require derivatization, the separation can be improved in many cases by modifying the enantiomers using precolumn achiral derivatization. These aspects are also briefly discussed here.

Covalent Chiral Derivatization of Enantiomers and Separation of the Diastereomeric Derivatives on Achiral Columns

Introductory Remarks: the Role of Covalent Chiral Derivatization in Enantiomeric Separations

The derivatization of enantiomers using homochiral reagents to form their diastereomeric derivatives

separable on achiral GC or HPLC columns or TLC plates was the first, widely used general method in the chiral analysis of drugs and related materials. After the introduction of newer chromatographic and capillary electrophoretic techniques briefly mentioned above, the importance of enantioseparations based on covalent chiral derivatization has naturally decreased to some extent. However, this general method is still a method of choice widely used especially in HPLC. The reasons for this are the large number of commercially available homochiral reagents and well-established reactions enabling diastereomeric derivatives with excellent separation and detection possibilities to be formed and the possibility of tailor-made separations using inexpensive, achiral columns, i.e. being in possession of the *R*- and *S*-forms of the reagent, it is possible to have the peak of the enantiomeric impurity eluting before the main peak.

The importance of covalent chiral derivatization can be characterized by the large number of publications (above 300) from the early 1970s up to the present time. References to the most important publications from this field can be found in chapters in books and review papers listed in Further Reading which cover the literature until 1993. The relatively large number of papers published after 1993 describing new applications of previously described derivatization reagents, moreover the introduction of new reagents is an indication that this branch of chiral analysis has retained some of its importance to the present day (see figure legends).

Important Features of Chiral Derivatization Reactions and Reagents

The presence of a suitable functional group in the molecule of the analyte The prerequisite of the use of any kind of enantioseparation based on covalent, chiral derivatization is the presence of at least one functional group (amino, hydroxyl, carboxyl, epoxy, thiol, etc.) in the molecule of the chiral compound which is capable of reaction with the reactive group of the derivatizing reagent.

Good chromatographic properties of the derivatives The optimization of the chromatographic parameters of retention time, peak shape and separability of the enantiomers from other components of the complex mixture by proper selection of the column (usually reversed-phase but in some cases normal phase columns), and the composition and pH of the eluent is done in the usual ways for achiral HPLC.

Sufficient separation of the diastereomers formed This is also influenced by the above-mentioned solvent composition and column selection, but in this case it is even more important to select a proper derivatizating reagent enabling diastereomers to be formed with sufficiently different molecular fine structures for their chromatographic separation. If the number of the chiral centres in the analyte is more than one, the reagent should enable the separation of all stereoisomers. An example for this is the separation of (*S*,*R*), (*R*,*S*), (*S*,*S*) and (*R*,*R*) nadolol in **Figure 1**.

The structural features which influence the separation of the diastereomers are:

- The distance between the two chiral centres. In the majority of cases this is two to four atoms but there are many exceptions to this rule.
- Conformational rigidity of the diastereomers favours resolution. Bulky groups in the vicinity of one of the chiral centres or the incorporation of one of them into a ring system are especially advantageous. For example, in the case of one of the most widely used chiral derivatizing reagent, GITC (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyrasonyl isothiocyanate, see later) the chiral centres of the reagent are in the pyrane ring. The exchange of the acetyl groups to the bulkier benzoyl groups improves the resolution. The position and bulkiness of the remote functional groups also influences the separation of the diastereomers. In these instances, however, the direction of the influence is difficult to predict. As an example, the investigation leading to the highly efficient derivatizing agent for the amino group (+)-2-methyl-2β-naphthyl-1,3-benzodioxole-4-carboxylic acid chloride is mentioned. It was found that the difference between the bulkiness of the two substituents at C-2 favourably influences the separation and with the isomeric 5-carboxylic acids only poor separation is achievable.
- The formation of hydrogen bonds. For example, in the case of propranolol the secondary amino group of the molecule is transformed by chiral isocyanates or isothiocyanates to diastereomeric urea or thiourea derivatives. The formation of a hydrogen bond between the carbonyl or thiocarbonyl group of the latter and the free hydroxyl group of propranolol plays an important role in the separation: the resolution deteriorates with etherification of the hydroxyl group.

Selection of the elution order This is especially important if the aim of the analysis is the determination of trace level enantiomeric impurity in a drug which is administered as the pure enantiomer. In order to have the impurity peak appear before the main peak the

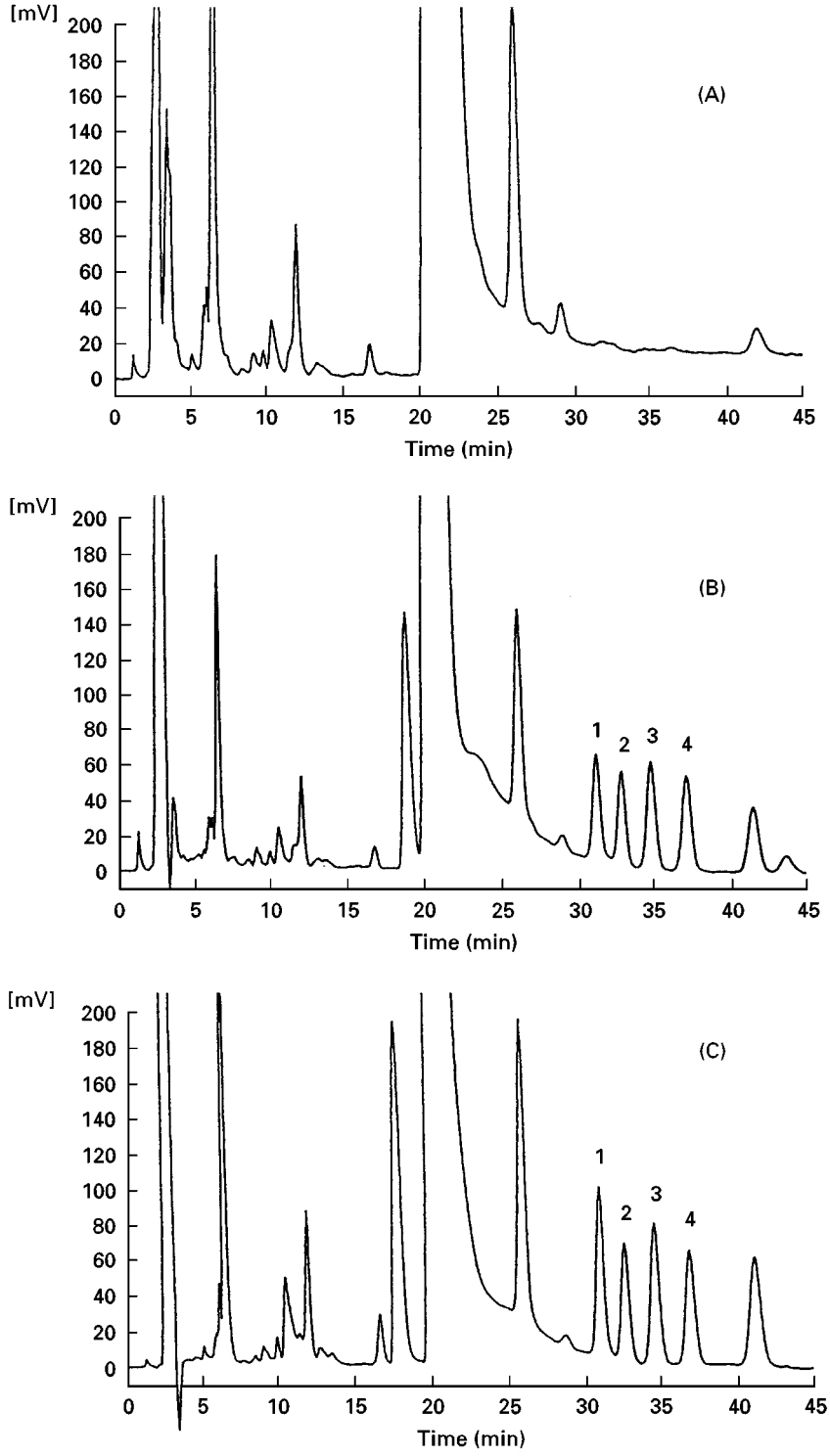


Figure 1 Resolution of the four diastereomers of the two racemates of nadolol as urea derivatives after reaction with (*R*)-(−)-1-(naphthyl)ethyl isocyanate and their determination in dog plasma extracts. Column YMC-AM-303 ODS(250 × 4.6 mm, 5-μm); mobile phase, water–acetonitrile (60:40, v/v); flow rate, 1 mL min^{-1}; temperature, 40°C; UV detection at 285 nm. (A) Blank control dog plasma; (B) plasma spiked with 50 ng ml^{-1} of each diastereomer; (C) plasma obtained 2 h after oral administration of 1 mg kg^{-1} of racemic nadolol. Peaks: 1, (*S*,*R*)-nadolol; 2, (*R*,*S*)-nadolol; 3, (*R*,*R*)-nadolol; 4 (*S*,*S*)-nadolol. Reproduced with permission from Hoshino M, Yajima K, Suzuki Y and Okahira A (1994) *Journal of Chromatography B* 661: 281, copyright Elsevier.

proper chromatographic system (normal- or reversed-phase) has to be selected; it is even more advantageous to select a derivatizing agent that is available in both the *R* and *S* forms. Curves a and b in **Figure 2** demonstrate this: the elution order of amino acids changes upon changing from *R* to *S* reagent in their derivatization with the *o*-phthalaldehyde-*N*-butyryl-cysteine reagent.

Unidirectional derivatization reaction taking place under mild conditions The most widely used reactions are completed at room temperature within 1 h and the reaction mixture can be injected directly into the chromatograph. The necessity of heating or extraction of the reaction mixture does not preclude a reaction being used and nor does the occurrence of side reactions, provided that their products do not interfere with the detection and quantification of the peaks of the main products and the side reactions do not show stereospecificity.

Enantiomeric purity and stability of the derivatizing reagent The enantiomeric purity of the reagent is one of the most important factors determining the success of the determination of the enantiomeric purity of the analyte. It is evident that when using a homochiral reagent containing its antipode as an impurity, the latter also reacts with the main component of the analyte. This results in a diastereomeric derivative which has the same retention time as that originating from the reaction of the main component of the reagent and the impurity of the analyte. It is therefore difficult to estimate if the satellite peak originates from the impurity of the reagent or from that of the analyte. If the aim of the study is the determination of the enantiomeric purity of a drug administered as pure enantiomer and the test limit for the antipode is 0.5%, the enantiomeric purity of the reagent should be at least 99.9%. If the requirement for the enantiomeric purity of the drug is higher, the purity of the reagent should be even higher. If the aim is the determination of commensurable amounts of enantiomers (e.g. in biological samples), 1–2% of the enantiomeric impurity in the reagent is tolerable.

The enantiomeric purity of the reagent can be checked if the enantiomers of the analyte (or at least one of them) are available in enantiomerically pure form. The relative peak area of the diastereomeric impurity after the reaction with the reagent will be characteristic of enantiomeric impurity of the reagent.

The enantiomeric stability of the reagent is also an important prerequisite to obtain reliable results. For example, *N*-trifluoroacetyl-(*S*)-(−)-prolyl chloride or anhydride, were found to racemize upon storage. Reagents which are available commercially at the present time fulfil this requirement.

The absence of kinetic resolution and racemization The absence of kinetic resolution, e.g. difference between the reaction rates of the two enantiomers with the reagent and the enantiomeric stability of the analyte and its diastereomeric derivative are important prerequisites of the applicability of a reagent to a given purpose. These can be checked by comparing the peak areas of the diastereomers during and after the reaction with a racemate. The peak area should be close to unity.

Good chromophoric or fluorophoric properties of the reagent Although the primary aim of derivatization in chiral chromatography is the formation of easy to separate diastereomeric derivatives it is advantageous if, at the same time, the reagent improves the detectability of the separated enantiomers by introducing chromophoric or fluorophoric groups into their molecules. A typical example for such 'dual-purpose' fluorophoric reagents is (−)-2-[4-(1-aminoethyl)phenyl]-6-methoxybenzoxazole. The detection limit for the enantiomers of 2-phenylpropionic acid after derivatization with this reagent is as low as 10 fmol (1.5 pg). Many more examples of this type are presented in the next section.

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 during the validation of a new method by comparing the spectra and band intensities of the diastereomers.

Covalent Enantiomeric Derivatization of Some Important Functional Groups

Derivatization of amines The reagents suitable for the chiral derivatization of amines can be categorized as follows:

Activated carboxylic acids These are usually carboxylic chlorides and the reaction with primary and secondary amines leads to diastereomeric carboxamides. The classical reagent *R*(+)-α-methoxy-α(trifluoromethyl)phenylacetyl chloride is still in use for the preparation of gas chromatographically separable diastereomers. Some others ('dual-purpose' reagents with strong UV absorption or fluorescence) include 1-(4-nitrophenylsulfonyl)-L-prolyl chloride, dansyl-L-proline activated by triethylamine/diethyl phos-

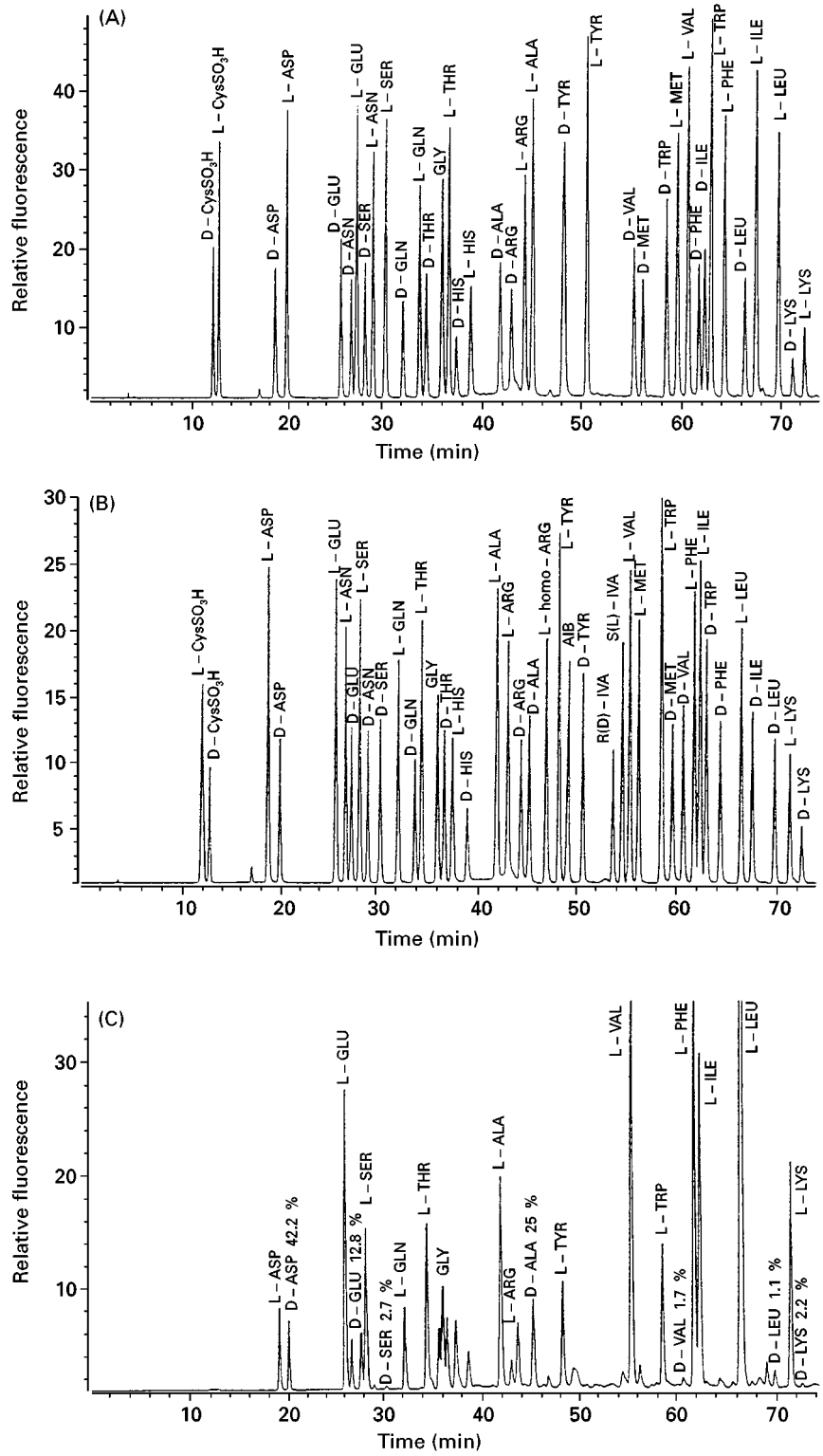


Figure 2 High-performance liquid chromatographic (HPLC) elution profile of the mixture of L and D-amino acids (L : D = 2 : 1), glycine and L-*homo*-arginine (internal standard) derivatized as the isoindoles with (A) *o*-phthalaldehyde-*N*-isobutyryl-D-cysteine and (B) *o*-phthalaldehyde-*N*-isobutyryl-L-cysteine; (C) amino acids from an ethanolic extract of *Lactobacillus acidophilus*, derivatization with *o*-phthalaldehyde-*N*-isobutyryl-L-cysteine. Column, Hypersil ODS (250 × 4 mm, 5 μm); mobile phase, gradient elution; A = 23 mM sodium acetate (pH 5.95); B = methanol–acetonitrile (600 : 50 v/v), linear gradient from 0% B to 53.5% B in 75 min; flow rate 1 mL min^{-1}; fluorescence detection, 230 nm excitation, 445 nm emission. Reproduced with permission from Brückner H, Haasmann S, Langer M, Westhauser T and Godel H (1994) *Journal of Chromatography A* 666: 259, copyright Elsevier.

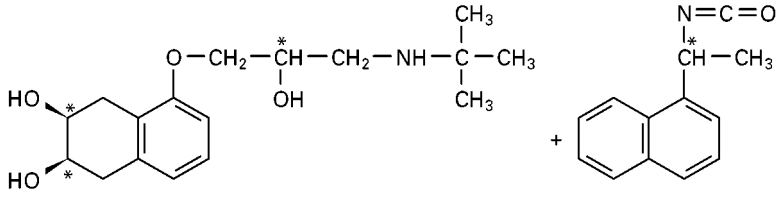
phorocyanidate, *N*-[4-(6-methoxy-2-benzoxazolyl)] benzoyl-L-phenylalanine or -proline, activated by 2,2′-dipyridyl disulfide/triethylphosphine, *N*-benzyloxycarbonyl-L-phenylalanine, activated by acetic anhydride(+)-2-methyl-2β-naphthyl-1,3-benzodioxole-4-carboxylic acid chloride, (*S*)-(+)-naproxen chloride, (*S*)-(+)-flunoxaprofen chloride, (*S*)-(+)-benoxaprofen chloride, etc. Their strong fluorescence enables the enantiomers of chiral drugs such as tranylcypromine, tocainide, carvedilol, baclofen and propranolol to be determined. (The isocyanate, isothiocyanate and chloroformate derivative of these compounds have also been introduced as chiral derivatizing agents as shown in the subsequent sections.)

An on-line solid-phase derivatization reagent is fluorenylmethyloxycarbonyl-L-proline (FMOC-L-proline), bonded to beads of a styrene-divinylbenzene copolymer as the active ester of a 4-hydroxy-3-nitrobenzophenone moiety. By positioning the HPLC column after the reaction column, the transformation of chiral amines (e.g. amphetamine) to their highly fluorescent diastereomeric FMOC-L-prolyl derivatives and their separation was achieved.

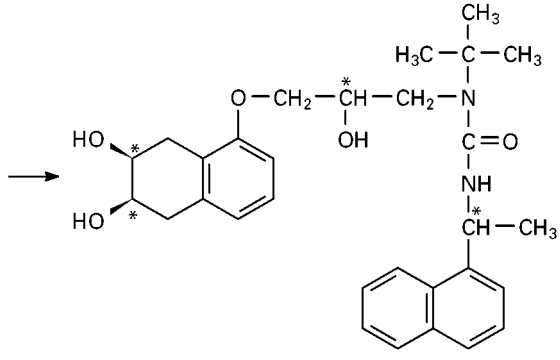
Chloroformates The above-mentioned FMOC group has been incorporated into another types of chiral reagents: (+)-1-(9-fluorenyl)ethyl chloroformate is one of the most widely used derivatization reagents for the chiral HPLC of amino acids, β-blockers, to form the corresponding carbamate derivatives.

An interesting feature of another widely used reagent of the chloroformate type, (−)-menthyl chloroformate is that it is suitable for the derivatization of the tertiary amine promethazine via demethylation of the dimethylamino moiety.

Isocyanates These reagents form diastereomeric urea derivatives with chiral primary and secondary amines. (*R*)-(−)- and (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate and (*R*)-α-methylbenzyl isocyanate are among the classical chiral derivatizing reagents. Isocyanate derivatives of the drugs mentioned in one of the previous sections as the carboxylic chlorides and (*R*)-*N*-3,5-dinitrobenzoyl)phenyl glycine have also been used for the derivatization of β-blockers and other amines. Eqn [1] shows the reaction between nadolol and (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate, while the separation of the four diastereomers of the two racemates is depicted in Figure 1. It is remarkable that by selecting a suitable reagent and proper chromatographic conditions not only can the four diastereomers be separated but the interference from dog plasma can also be eliminated.

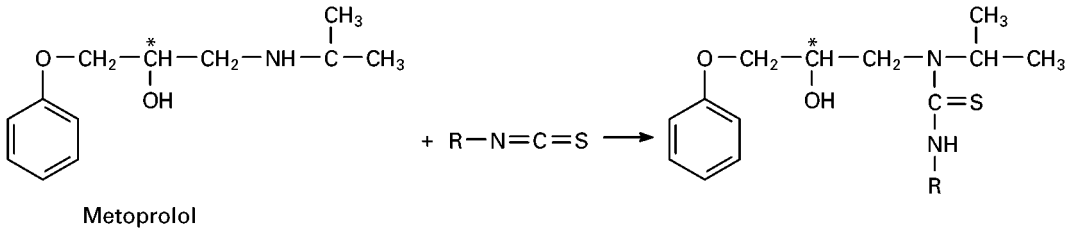


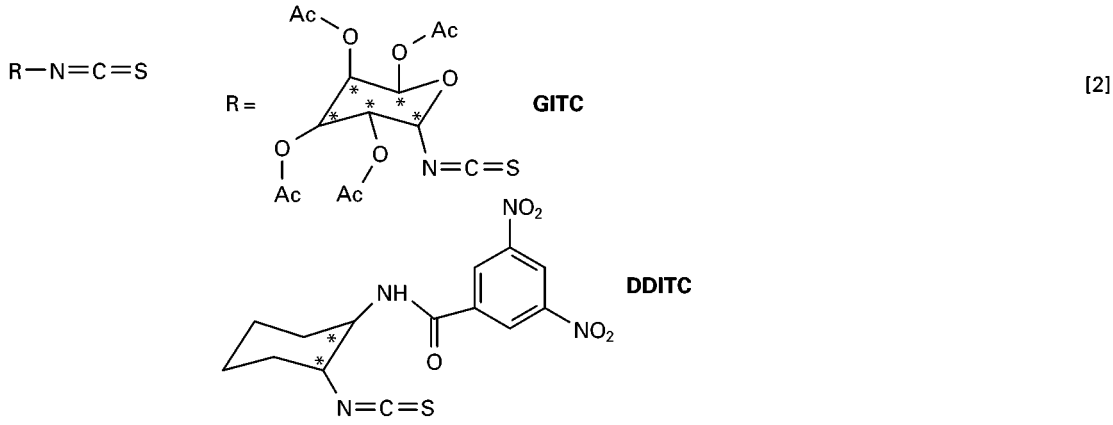
[1]



Isothiocyanates Of the isothiocyanates forming thiourea derivatives with primary and secondary amines, GITC is most widely used. If the four acetyl groups are replaced by benzoyl groups the sensitivity of the detection is greatly improved. Other reagents of this type leading to highly fluorescent derivatives include (*R*)-(−)- and (*S*)-(+)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole and its 7-(*N*,*N*-dimethylaminosulfonyl) analogue as well as DDITC ((1*R*,2*R*)- and (1*S*,2*S*)-*N*-[(2-isothio-

cyanato)cyclohexyl]-3,5-dinitrobenzoylamide, which excels with the high chemical stability, high UV activity and the excellent separability of the diastereomeric derivatives. The equation of the reaction of GITC and DDITC with the β-blocker metoprolol and the separation of the derivatives are depicted in eqn [2] and **Figure 3**, respectively.

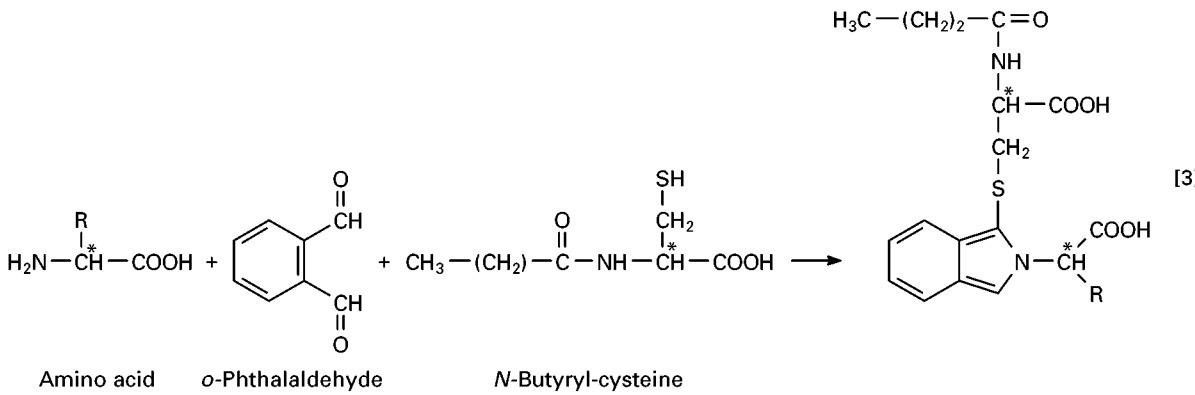




[2]

N-Haloarylamino acid derivatives Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alaninamide) is one of the most generally used reagents in the analytical control of racemization during peptide synthesis. The peptides are split either by hydrochloric acid or enzymatically. The nucleophyllic attack of the α-amino group of the amino acids on the C–F bond activated by the two nitro groups on the aromatic ring results in a smooth reaction to form diastereomeric aniline derivatives with good UV detectability (see eqn [4]). The valinamide analogue of the Marfey reagent gives even better resolution.

o-Phthalaldehyde + chiral thiols This dual derivatization reaction leading to fluorimetrically highly active isoindole derivatives is another generally used method in the chiral analysis of amino acids (see eqn [3]). As the chiral thiol *N*-acetyl-L-cysteine is most widely used but the use of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside and *N*-isobutyryl-L-cysteine (and D-cysteine) have been found to be advantageous in the resolution of the diastereomers. The reaction of amino acids with *o*-phthalaldehyde and the latter reagent is shown in eqn [3], while in **Figure 1** the separation of as many as 36 enantiomers of 18 amino acids and amino acid analogues as well as glycine is depicted together with a practical applicaion.



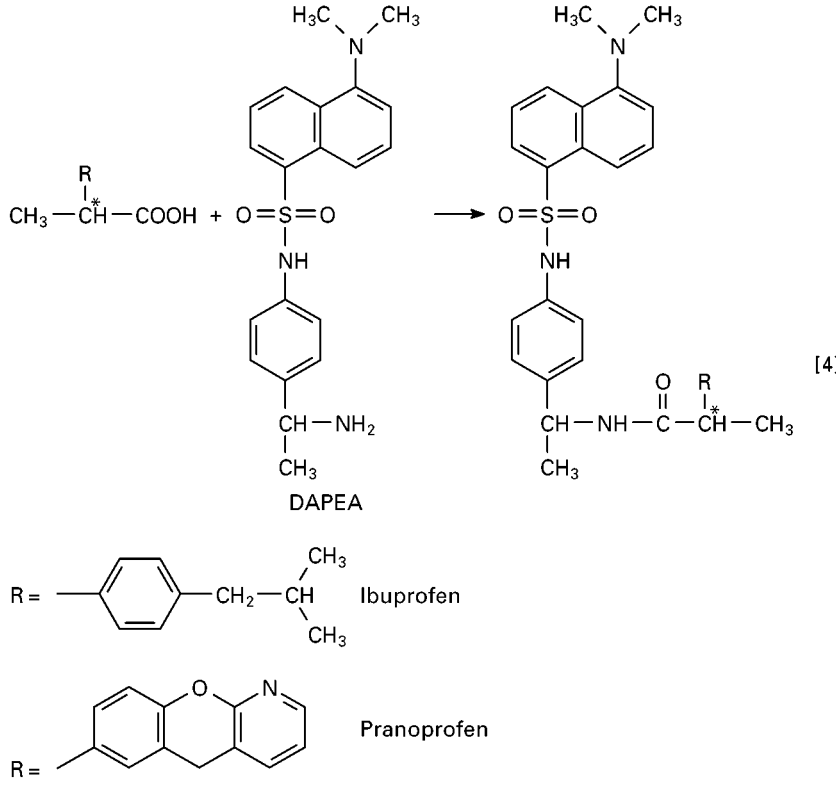
[3]

Enzymatic deamination Chiral HPLC of a mixture of amino acid enantiomers with and without selective oxidative deamination of D-amino acids with the aid of the enzymes D-amino acid oxidase and catalase enables D-amino acids to be identified in the mixture.

Derivatization of the carboxyl group

Esterification with chiral alcohols (+)- or (−)-2-octanol, (+)-1-phenylethanol, (−)-menthol, (+)- or (−)-2-butanol are the classical reagents for the chiral analysis of carboxylic acids. The reactions usually require harsh conditions and for this reason the danger of racemization should be taken into consideration.

Amidation with chiral amines Prior to their reactions with chiral amines the carboxyl group should be activated. Possibilities for this are, e.g. reaction with thionyl chloride to form carboxylic chlorides, with chloroformates to form mixed anhydrides, with 1,1-carbonyldiimidazole to form reactive *N*-acylimidazoles and with the classical coupling agent dicyclohexylcarbodiimide to form the reactive *N*-acylurea derivatives. The classical but still widely used amine reagent is (*S*)-(−)-α-methylbenzylamine, but several others with excellent separation power, UV or fluorometric properties are also in use, e.g. (*R*)-α-methyl-4-nitrobenzylamine, (*R*)-(+)-1-(1-naphthyl)ethylamine, (−)-1-(1-anthryl)ethylamine, *R*-(−)- and (*S*)-(+)-amphetamine, (1*R*,2*R*)-(−)- or (1*S*,2*S*)-(+)-2-amino-(4-nitrophenyl)-1,3-propanediol, L-leucinamide, L-alanine-β-naphthylamide, L- or D-*O*-(4-nitrobenzyl)tyrosine methyl ester, (−)-2-[4-(1-aminoethyl)-phenyl]-6-methoxybenzoxazole and other related derivatives where the 1-aminoethyl group is replaced by L-leucyl or D-phenylglycyl groups, drug-related amines (flunoxaprofen amine, benoxaprofen amine and naproxen amine), (*R*)- and (*S*)-1-(4-dansylaminophenyl)ethylamine, etc. The reaction of ibuprofen and pranoprofen with the last reagent and the separation of the diastereomeric carboxamide derivatives are shown in eqn [4] and **Figure 4**, respectively.



[4]

Derivatization of the alcoholic and phenolic hydroxyl groups The most frequently used general method for the derivatization of the hydroxyl group of chiral alcohols and phenols is esterification. A great variety of chiral carboxylic acids have been used for this purpose such as *R*(+)- and *S*(−)-α-methoxy-α(trifluoromethyl) phenylacetic acid (Mosher's acid), *R*(+)-*trans*-chrysanthemic acid, (−)-menthenyloxyacetic acid for the gas chromatographic or HPLC

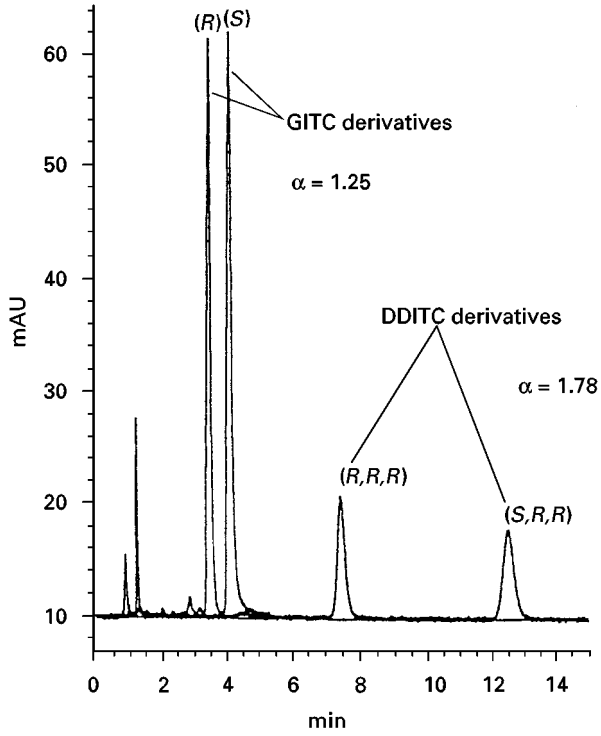


Figure 3 Resolution of (*R*,*S*)-metoprolol derivatized as (*R*,*R*)-DDITC- and GITC-thioureas. Column, Hypersil ODS (125 × 4 mm, 5 μm); mobile phase, acetonitrile-20 mM ammonium acetate 55:45, v/v; flow rate 1 mL min^{-1} detection at 254 nm. Reproduced with permission from Kleidernigg OP, Posch K and Lindner W (1996) *Journal of Chromatography A* 729: 33, copyright Elsevier.

separation with UV detection and (−)-(1*S*,2*R*,4*R*)-*endo*-1,4, 6,7,7-hexachlorobicyclo[2.2.1]-hept-5-ene-2-carboxylic acid and (*S*)-(+)-2-tert-butyl-2-methyl-1,3-benzodioxolo-4-carboxylic acid for HPLC separation with fluorimetric detection, etc. Dicyclohexylcarbodiimide can be used as the coupling agent, but the use of *in situ* transformation of the acids to their chlorides by the addition of thionyl chloride is more widespread. The direct enzymatic D-(+)-glucuronidation reaction of phenols has also been described.

Acyl chlorides, anhydrides and acyl cyanides can be used directly. For example (−)-camphanic acid, *R*(+)-α-methoxy-α(trifluoromethyl)phenylacetyl chloride, β-naphthylsulfonyl-L-prolyl chloride, flunoxaprofen chloride, (*R*,*R*)-*O*,*O*-diacetyl- (or di-*p*-toluoyl-) tartaric anhydride and (−)-2-methyl (or methoxy)-1,1′-binaphthalene-2′-carbonyl cyanide lead to well-separable, UV or fluorimetrically active diastereomeric ester derivatives with chiral alcohols.

As an illustration, the reaction of delmopinol and its analogue used as the internal standard in the determination of the drug in plasma with the reagent (*R*,*R*)-*O*,*O*-di-*p*-toluoyl-tartaric anhydride is shown in eqn [5]. The separation is depicted in **Figure 5**.

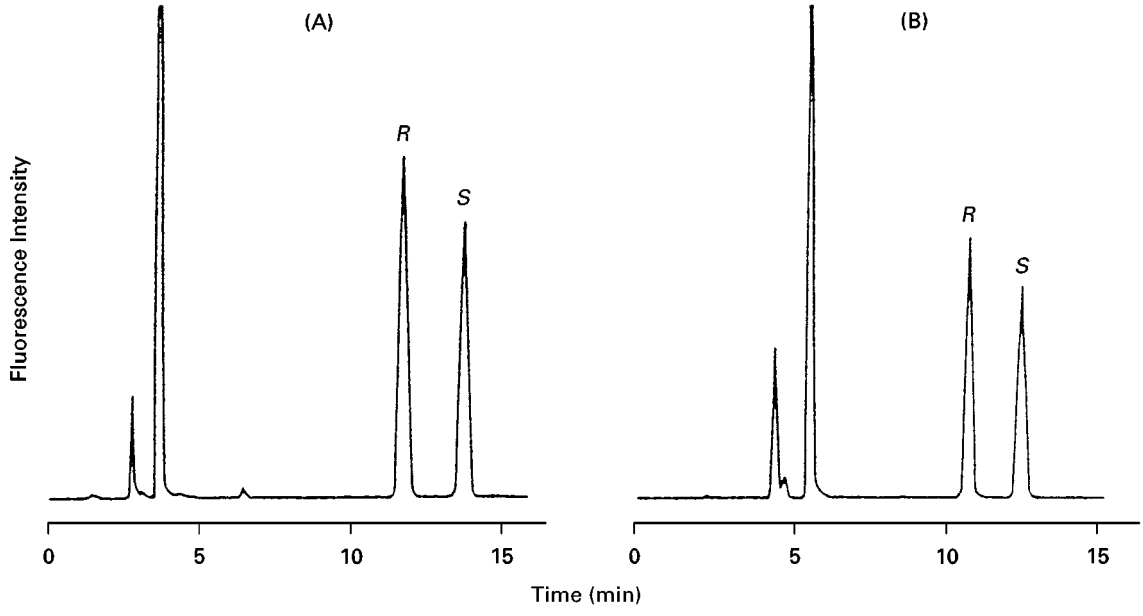
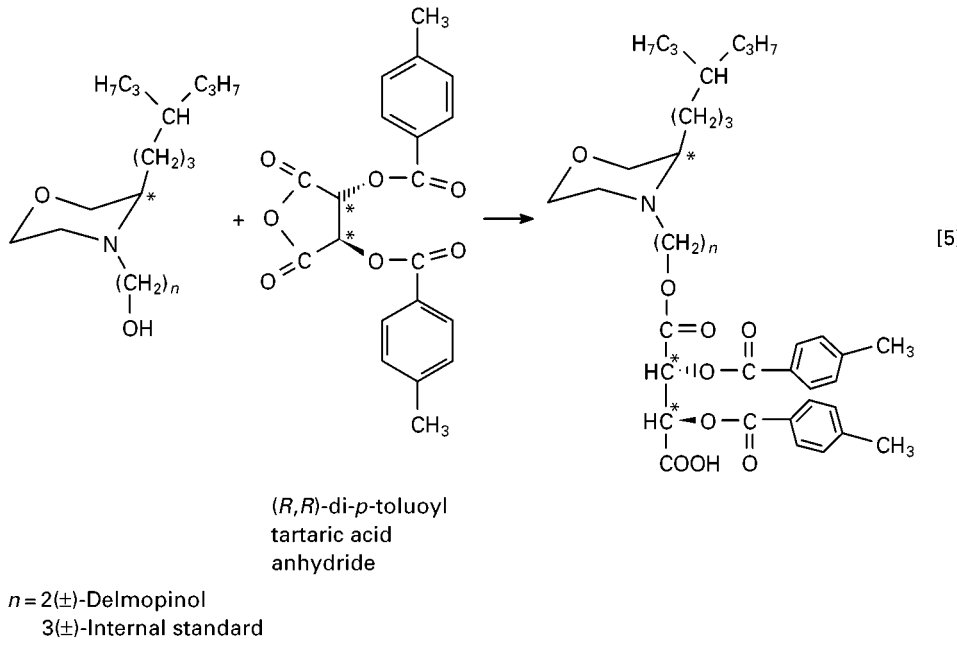


Figure 4 Resolution of (A) (*R*,*S*)-ibuprofen and (B) (*R*,*S*)-pranoprofen as the carboxamides formed with (*S*)-1-(4-dansylaminophenyl)ethylamine. Column ODS-80^{TM} (150 × 4.6 mm, 5 μm); mobile phase: 50 mM sodium acetate (pH 6.5)–acetonitrile (A) 30 : 70, v/v and (B) 45 : 55, v/v, and flow rate 1 mL min^{-1}; fluorescence detection, 338 nm excitation, 535 nm emission. Reproduced with permission from Iwaki K, Bunrin T, Kameda Y and Yamazaki M (1994) *Journal of Chromatography A* 662: 87, copyright Elsevier.



[5]

Further derivatization reactions include the use of (*R*)-1-(1-naphthyl)ethyl isocyanate for the derivatization of, e.g. diacylglycerol derivatives to form the corresponding carbamates. The lower reactivity of the hydroxyl group compared with the amino group can be overcome by using a suitable catalyst (4-pyrrolidinopyridine). (−)-Menthyl chloroformate, which has already been mentioned, has also found application here as a reagent for amines; the reaction products with chiral alcohols are carbonates.

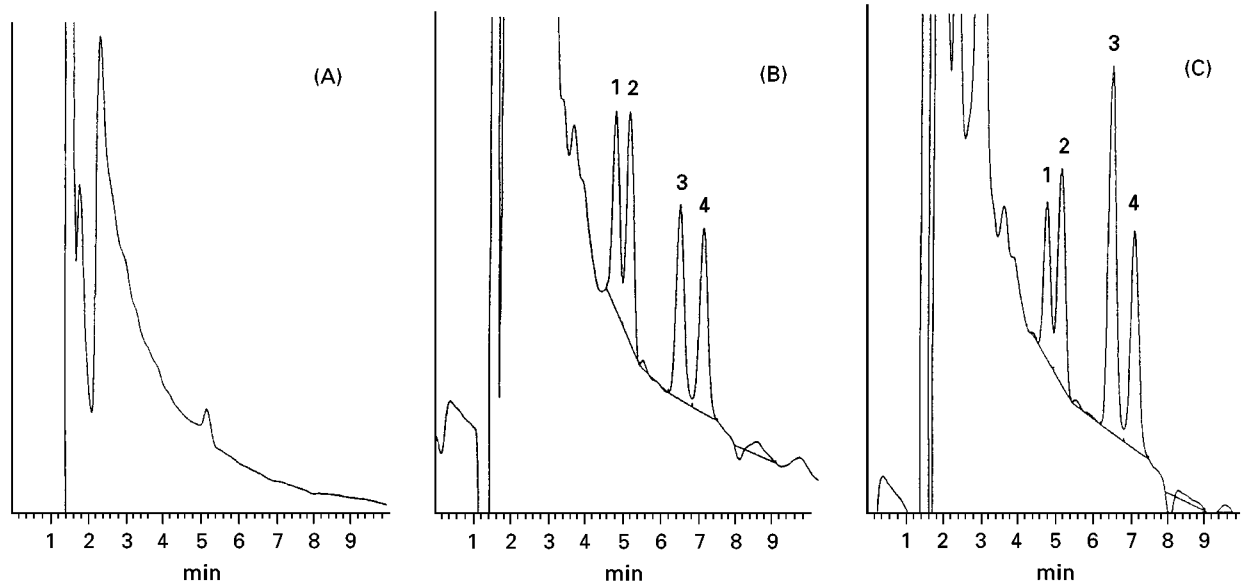


Figure 5 Resolution of (−)- and (+)-delmopinol (peaks 1 and 2) and (−)- and (+)-internal standard (peaks 3 and 4) and their determination in human plasma extracts. Derivatization with (*R,R*)-O,O′-di-*p*-toluoyl tartaric acid anhydride to form the esters. (A) Blank plasma; (B) plasma spiked with 6 ng of each enantiomer; (C) authentic plasma sample. Column, Hypersil ODS (125 × 4 mm, 5 μm); mobile phase, 100 mM ammonium acetate–acetonitrile 35 : 65, v/v, pH 5.7; flow rate, 0.8 mL min^{-1}; electrochemical detection. Reproduced with permission from Egginger G, Blaschke E, Lindner W and Olsson A-M (1994) *Journal of Chromatography A* 666: 275, copyright Elsevier.

Derivatization of the aldehyde group The importance of this kind of chiral derivatization reactions is much smaller than those described for amines, carboxylic acids and alcohols. For example, aldoses can be transformed with L-cysteine methyl ester to diastereomeric thiazolidine derivatives which can be separated by GC after trimethylsilylation. The two aldehyde groups of gossypol were transformed with (*R*)-(−)-2-amino-1-propanol to the diastereomeric Schiff's bases separable by HPLC.

Derivatization of epoxides The oxirane ring of chiral epoxides is opened by sodium sulfide to form a vicinal hydroxythiol derivative. In the second step of the derivatization reaction the thiol group reacts with *o*-phthalaldehyde and a chiral amino acid to form the diastereomeric isoindole derivative mentioned in the section dealing with the derivatization of the amino group. Another ring-opening reagent is 2-propylamine. The secondary amino group of the 1,2-amino alcohol is then reacted with 2,3,4,6-tetra-*O*-acetyl (or benzoyl)-*β*-D-glucopyranosyl isothiocyanate to form the diastereomeric thiourea derivatives.

Derivatization of the thiol group The formation of diastereomeric isoindole derivatives from chiral amino acids, chiral thiols and *o*-phthalaldehyde (already mentioned in the section dealing with the derivatization of the amino group; see eqn [4]) can also be used for the enantioseparation of thiols. Reagents of other types are (*R*,*R*)-dinitrobenzoyldiaminocyclohexyl isothiocyanate and *N*-[(2-isothiocyanato)-cyclohexyl]-pivalinoyl amide which transform the thiol compound to their diastereomeric dithiocarbamate derivatives.

Derivatization of Enantiomers With Achiral Reagents to Improve Their Chromatographic Properties and Their Separation on Chiral Columns

Introductory Remarks

Chiral columns enable the direct separation of enantiomers. In many cases the chromatographic properties of the enantiomers can be improved by transforming them to suitable derivatives using achiral reagents.

Pirkle-Type Chiral Columns

The purposes of using pre-column derivatization are:

- Blocking of the polar groups of the analyte (amino, carboxyl, hydroxyl groups) to avoid strong interactions with the polar groups of the chiral selector thus improving the separation.
- The introduction of aromatic rings into the analyte to enable π–π interactions to take place between the aromatic moieties of the chiral selector and the analyte which play a dominant role in the resolution of the enantiomers.
- Improve UV detectability.

To fulfil these requirements amino groups in the analyte are usually transformed to aromatic acyl derivatives by 4-nitro- or 3,5-dinitrobenzoyl chloride or to aromatic urea or thiourea derivatives by means of phenyl-, 4-fluorophenyl-, 4-methoxyphenyl-, 3,5-dinitrophenyl- or 1-naphthyl-isocyanates and isothiocyanates. Carboxyl derivatives are derivatized after activation with thionyl chloride or carbodiimides to form anilides, 3,5-dimethylanilides, 3,5-dinitroanilides, naphthylmethylamides, etc. The derivatizing agents for the hydroxyl group are benzoyl chloride, 3,5-dinitrobenzoyl chloride, 1-naphthyl chloride to form ester derivatives and 1-naphthyl- or 3,5-dinitrophenyl isocyanates to form the corresponding carbamates.

Cyclodextrin-Bonded Phases

In the case of enantiomeric HPLC separations on cyclodextrin-bonded stationary phases the necessity of pre-column achiral derivatization is not as general as with the Pirkle-type phases. The introduction of the aromatic ring into analytes often improves their separation. Amino acids are usually derivatized with dansyl chloride, 3,4-dinitrobenzoyl chloride, *o*-phthalaldehyde/2-mercaptoethanol, etc., or with highly fluorescent reagents such as 9-fluorenylmethyl chloroformate, 9-fluorenylmethoxycarbonylglycine chloride, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. etc.

α_1-Acid Glycoprotein Column

In most instances no derivatization is necessary to obtain good enantioseparation. In the case of carboxylic acids, separation is improved by the addition of ion pairing agents to the eluent. For some basic drugs anionic reagents in the eluent also improves the column efficiency, peak symmetry and resolution.

Covalent derivatization is only seldom used. Acetylation or formylation of the amino and hydroxyl groups, esterification of the carboxyl group

with 2-propanol have been found in some instances to improve the separation.

Functionalized Cellulose Phases

In the overwhelming majority of cases enantiomers can be separated on these columns without derivatization. In a few instances esterification of the carboxyl group and the introduction of a benzoyl group into the molecule have been found advantageous.

Conclusions and Future Trends

It is indisputable that the direct chromatographic enantioseparations based on chiral stationary phases has greatly surpassed the importance of indirect separations based on covalent chiral derivatization. The fact that the overwhelming majority of papers reporting on the development and application of chiral separations are from these fields does not necessarily reflect the real of the contribution of covalent chiral derivatization in the solution of practical problems in pharmaceutical and biomedical analysis. In some areas (e.g. the determination of small concentrations of D-amino acids in various biological samples, etc.) the well-established classical methods are still in use. It is also remarkable that the combination of chiral derivatization with the use of chiral stationary phases has proved to be suitable for the solution of very delicate problems (separation of several enantiomers and diastereomers of drugs with more than one chiral centre). It should also be noted that the combination of chiral chromatography with fluorescence derivatization enables the limit of detection of enantiomeric impurities to be decreased to the 10 ppm. level. These tendencies are expected to continue in the near future.

See also: **II/Chromatography: Liquid:** Derivatization. **III/Chiral Separations:** Amino Acids and Derivatives; Capillary Electrophoresis; Cellulose and Cellulose Derived Phases; Chiral Derivatization; Countercurrent Chromatography; Cyclodextrins and Other Inclusion Complexation Approaches; Gas Chromatography; Ion-Pair Chromatography; Ligand Exchange Chromatography; Liquid Chromatography; Molecular Imprints as Stationary Phases; Protein Stationary Phases; Supercritical Fluid Chromatography; Synthetic Multiple Interaction ('Pirkle') Stationary Phases; Thin-Layer (Planar) Chromatography.

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Countercurrent Chromatography

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Introduction

Countercurrent chromatography (CCC) is a generic term for support-free liquid/liquid partition chromatography, which can be used for the separation of a variety of enantiomers by dissolving a chiral selector (CS) in the liquid stationary phase. The method eliminates the time-consuming procedure which involves chemically bonding the chiral selector to a solid support for chiral chromatography. It also provides an important advantage over the conventional methods in that the same column can be used for the separation of various enantiomers simply by