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Enantioselective Bioanalysis of Beta-Blocking Agents: Focus on Atenolol, Betaxolol, Carvedilol, Metoprolol, Pindolol, Propranolol and Sotalol

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The recent developments in enantioselective HPLC-separation techniques are impressive and are driven by industrial and academic interests; thus there is for instance a high demand for developing stereoselective assays for chiral drugs in biological fluids. The beta-blocking agents, which possess an amino- propanol- or -ethanol side chain with at least one chiral centre, represent one of the most intensively investigated groups of more than 40 drugs introduced world wide. Seven of the most popular beta-blockers were chosen as representatives: atenolol; betaxolol; carvedilol; metoprolol; pindolol; propranolol; and sotalol, these span the whole range of lipophilicity to hydrophilicity (polarity). Enantioselective HPLC bioassays for these β-blockers published so far, including techniques based on chiral derivatizing agents (CDAs), chiral stationary phases (CSPs) and chiral mobile phase additives (CMPAs) have been reviewed and documented in the light of general aspects together with pharmacokinetic and pharmacodynamic considerations.

INTRODUCTION

The differences between the single enantiomers of a racemic mixture in their biological activity as a sum of enantioselective pharmacodynamic, pharmacokinetic and toxicologic activities have to be viewed as a result of stereoselective and/or stereospecific interactions of chiral compounds within a chiral biological environment, viz. the human body. The neglect of stereoselectivity in action leads to 'highly sophisticated scientific nonsense' and to misinterpretations of drug disposition data as pointed out by Ariens (1984) and Evans *et al.*, (1988), respectively.

In order to allow investigations on biological activities of chiral drugs various enantioselective techniques for resolution of the (*R*)- and the (*S*)-enantiomer (or (+)- and (−)-enantiomers if stereochemically less defined) in biological fluids have been developed in the last decade. In the course of the successful development of chromatographic separation techniques for enantiomers (Allenmark, 1988; Krstulovic, 1989; König, 1987), the major part of the methods applied to bioanalysis is based on high-performance liquid chromatography followed by gas-chromatography (Dyas, 1992). The rapidly growing field of chiral separation techniques also influenced significantly the understanding of 'chiral recognition mechanisms' of biological systems as well as of chromatographic separation techniques.

The knowledge of pharmacodynamic and pharmacokinetic behaviours of chiral compounds (Williams and Lee, 1985; Tucker and Lennard, 1990; Eichelbaum and Gross, 1990) is no longer of academic interest only. New recommendations of the Food and Drug Administration in the United States (FDA) were released in summer 1992 concerning new developments of chiral drugs or redevelopments ('racemic switches') of drugs now marketed as racemates. Although pharmaceutical companies still have the choice a priori to develop chiral drugs either as racemates or as single enantiomers, justifications for developing the racemate are now requested by the FDA (Stinson, 1992). One group of candidates for 'racemic switches' is the group of currently marketed beta-blocking agents, with more than 40 different structures; and (*S*)-atenolol seems to be a prime candidate.

BETA-BLOCKING AGENTS

Beta-adrenergic antagonists, commonly termed beta-blockers, belong to one of the most widely used and therefore extensively studied group of drugs. The clinical use ranges from cardiovascular disorders, such as angina pectoris and hypertension, to the therapy of migraine, glaucoma and hyperthyreosis, to mention only a few. All beta-blockers have an amino-alcohol structure with at least one chiral center in their side chain and most of them are marketed as racemic mixtures, except (*S*)-timolol and (*S*)-penbutolol. Beta-blockers can be classified according to their pharmacodynamic effects, β_1-selectivity, intrinsic activity and membrane stabilising properties, to their pharmacokinetic aspects, and to their lipophilicity and hydrophilicity. The compounds described in this review are

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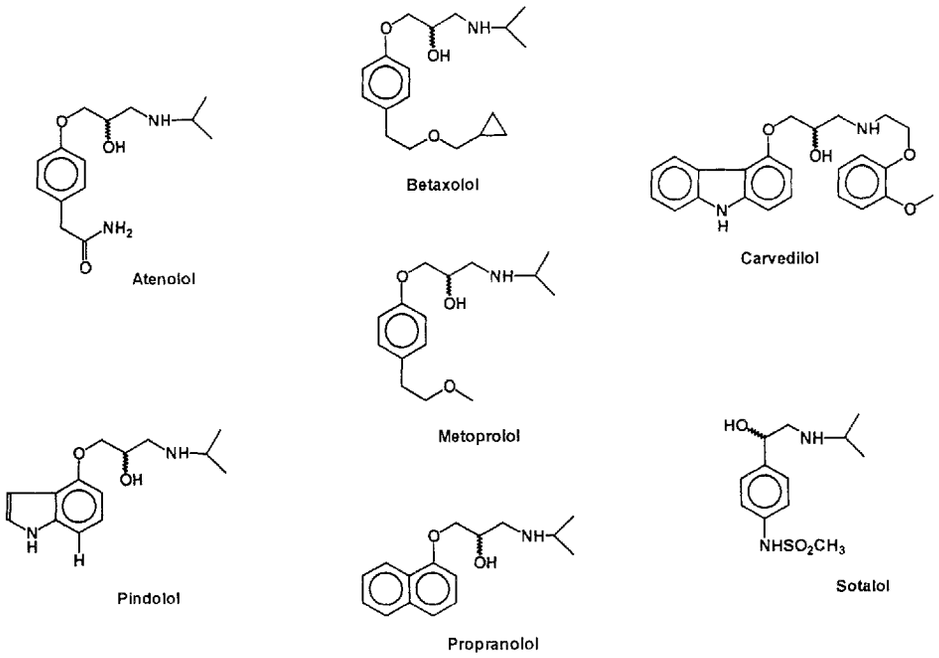


Figure 1. Structures of the described beta-blocking agents.

chosen with respect to their widespread clinical use, and because they cover the whole range of the classifications described above (see also Fig. 1). However, the literature on these compounds is massive and only selected papers could be considered for citation.

It has been well documented that the single enantiomers of beta-blocking agents, as well as of several other drugs, differ largely in their pharmacodynamic and pharmacokinetic profiles. This review does not discuss the differences between enantiomers of beta-blockers in their biological activity in detail, the interested reader is referred to the review of Walle *et al.*, (1988) (and literature cited therein); only a few examples of stereoselective pharmacodynamics and pharmacokinetics of the investigated beta-blockers will be mentioned, just to point out the necessity of enantioselective pharmacodynamic and pharmacokinetic investigations of chiral drugs. The main focus of this paper is on enantioselective bioanalytical methods.

Propranolol is one of the most prominent and most intensively investigated beta-blocking drugs. The compound is an excellent example for demonstrating possible different pharmacodynamic and pharmacokinetic profiles of (*R*)- and (*S*)-enantiomers. Already in 1966 Howe and Shanks could show by animal studies that (*S*)-propranolol is more than 100 times more potent in blocking beta-receptors than the corresponding (*R*)-enantiomer (Howe and Shanks, 1966). In contrast to the beta-blocking activity, the thyreostatic activity, inhibition of the conversion of thyroxin to triiodothyronine lies only in (*R*)-propranolol (Buchinger *et al.*, 1988). The antiarrhythmic class 1-activity (Stark *et al.*, 1989) and the decrease of intraocular pressure (Alkondon *et al.*, 1986) are effected by both enantiomers of propranolol. Corresponding results can be prognosticated for structure-related beta-blockers. Recently, a comparative pharmacodynamic study on (*R*,*S*)-atenolol (Stoschitzky *et al.*, 1993) demonstrated that half dosed (*S*)-atenolol decreased heart rate and blood pressure to the same extent as the racemic drug. It was also shown that (*R*)- and (*S*)-sotalol have similar class III antiarrhythmic activities (Singh *et al.*, 1987) but only the (*R*)-enantiomer exhibits the beta-blocking activity (Kato *et al.*, 1986). For carvedilol, a newly launched beta-blocking drug with additional α-blocking activities, it could be demonstrated that only the (*S*)-enantiomer exerts beta-blocking activity, whereas no difference between the two enantiomers was found with respect to their α-blocking effect (Bartsch *et al.*, 1990).

Lipophilic drugs such as propranolol and metoprolol undergo an extensive hepatic metabolism (Nelson and Shetty, 1986), presumably mediated by different cytochrome P-450 isoenzymes (Murray, 1992) which exhibit a genetic polymorphism within the population. The metabolism of (*R*,*S*)-metoprolol strongly cosegregates with the 'debrisoquine oxidation phenotype', resulting in 1.5 times greater bioavailabilities of the (*S*)-enantiomer than of the (*R*)-enantiomer in 'extensive metabolizers' (Lennard, 1989; Lennard *et al.* 1983). Hydrophilic beta-blockers such as atenolol (Boyd *et al.* 1989; Mehvar *et al.*, 1989) and sotalol (Sallustio *et al.*, 1992), which are hardly metabolized, show slightly but significantly higher plasma levels of the (*R*)- than of the (*S*)- enantiomer. Possible explanations could be related to the findings of Lindner and coworkers (Stoschitzky *et al.*, 1992) who demonstrated that (*S*)-atenolol is stereoselectively taken up by and released from adrenergic nerve endings by membrane depolarization. However, no differences between the pharmacokinetics of betaxolol enantiomers occurred, after oral or intravenous administration of the racemic drug, despite the close structure relationship to the extensively and stereoselectively metabolized drug metoprolol (Stagni *et al.*, 1990). Pindolol is an example of stereoselective renal clearance as described by Hsyu and Giacomini (1985). Recent investigations of carvedilol enantiomers demonstrated that AUCs of (*S*)-carvedilol were significantly lower than those of (*R*)-carvedilol after intravenous and oral administration (Neugebauer *et al.*, 1990), which is in contrast to the findings for propranolol and metoprolol for example, compounds which are also hepatically metabolized.

CHIRAL HPLC-SEPARATION TECHNIQUES

In the last ten years the development of chiral liquid chromatographic techniques became very popular, as did attempts to summarize the latest results by editing books (Lough 1989; Zief and Crane, 1988; Allenmark, 1988; Krstulovic, 1989). However, only less than one third of the published work touches the field of bioanalysis (Dyas, 1992). Generally speaking, the chiral HPLC-separation techniques can be divided into three groups: (1) the 'indirect techniques' using a chiral derivatizing agent (CDA) forming diastereomers; (2) the 'direct methods' using a chiral stationary phase (CSP); and (3) methods based on chiral additives to the mobile phase (CMPA). The latter technique is also quite popular for enantioseparations by capillary electrophoresis and electrokinetic chromatography.

Recently liquid chromatographic methods for enantioselective separation of beta blockers were reviewed by Vandenbosch *et al.* (1993), the application to biological matrix including radioimmuno-assays, stable isotope techniques and radioreceptor-assays by Walle and Walle (1989) and by Dyas (1992) including GC and thin-layer chromatography. The object of this paper was to give an overview of the current status of stereoselective HPLC-separation techniques applied to quantitate simultaneously the single enantiomers of atenolol, betaxolol, carvedilol, metoprolol, pindolol, propranolol and sotalol but in a biological matrix, thus having input for 'stereoselective investigations' on clinical, biological and pharmaceutical topics.

Chiral derivatizing agents (CDA)

The so called 'indirect separation technique' involves a chiral derivatization step of the chiral analyte (*R*)-A and (*S*)-A with an chiral derivatizing agent (e.g. (*R*)-CDA) leading to 2 diastereomeric derivatives: (*R*)-A-(*R*)-CDA and (*S*)-A-(*R*)-CDA. These differ in their physico-chemical properties and can thus be separated using conventional achiral chromatography, reversed phase or normal phase chromatography. However, this technique puts high demands on the optical purity and stability of the CDA which has to be carefully validated as described by Lindner and Petersson (1985), and Allenmark (1988). If the reagent (*R*)-CDA contains the correspondent antipode (*S*)-CDA as an optical impurity, two additional products are formed: (*R*)-A-(*S*)-CDA and (*S*)-A-(*S*)-CDA. The four derivatives (two enantiomeric pairs of diastereomers) will appear only as two peaks on, for example, a non-chiral reversed phase type chromatogram, because (*R*)-A-(*R*)-CDA and (*S*)-A-(*S*)-CDA, as well as (*R*)-A-(*S*)-CDA and (*S*)-A-(*R*)-CDA, are two pairs of enantiomers which cannot be separated by achiral chromatography. The optical purity of the reagent has therefore to be determined using a 'direct method' or by derivatizing a chiral reference compound with known optical purity. The optical stability of a reagent owning at least two chiral centres should be expected to be better compared with those with only one centre, because it becomes less likely that both chiral centres racemize equally well. The intermediate would be a diastereomer which can probably be eliminated by purification via a crystallization step. Good examples for this strategy are CDAs such as diacetyl tartaric acid anhydrides (Lindner *et al.*, 1984) or GITC (Nimura *et al.*, 1980).

The derivatization procedure in the course of bioanalysis can, however, be time-consuming and sometimes difficult owing to severe drawbacks. However, chiral derivatizing agents have proved their usefulness in enantioseparation of beta-blocking agents in a biological matrix. Table 1 shows the CDAs utilized in bioanalysis so far.

One of the first CDAs used in bioanalysis of beta-blockers (Hermansson and von Bahr, 1980; Silber and Riegelman, 1980) was *N*-trifluoroacetyl-*L*-prolyl chloride, first described by Weygand *et al.*, (1957). Einarsson *et al.* (1987) developed an additional acid chloride derivative, (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC), for separation of amino acid enantiomers and chiral amines. (*R*,*S*)-metoprolol was used a model substance to demonstrate the applicability of this reagent to enantioseparation of beta-blockers. The use of (−)-menthyl chloroformate (MCF) had already been reported from GC-separations of chiral amines and hydroxyl compounds by Westley and Halpern (1968). The application for separation of beta-blockers was reported by Schmitthenner *et al.*, (1989) and Mehvar (1989a). Hydrophilic as well lipophilic beta-blockers have been derivatized and the diastereomers separated under reversed phase conditions. Maximal derivatization efficiency was obtained in a two-phase system of acetonitrile and a saturated solution of sodium carbonate within 30 s. Since menthylchloroformate precipitates in the presence of water it is necessary to remove the excess of reagent, e.g. with 4-hydroxy-L-proline, prior to injection into the reversed phase system, or to extract the derivatives into an organic solvent. Chloroformates react with amines and alcohols producing carbamates and carbonates. However, under mild alkaline conditions selective reaction with primary and secondary amines can be achieved as described by Ahnhoff *et al.*, (1990), who investigated 11 chloroformate derivatives in view of their reactivity and chromatographic properties.

The symmetric anhydride of *tert.*butoxycarbonyl-L-leucine (BOC-L-Leu) was developed by Hermansson (1982) as CDA. L-leucine was taken as a basis for this CDA instead of the first developed L-proline homologue owing to its tendency to racemize to a certain extent. (*R*,*R*)-*O*,*O*′-diacetyltartaric acid anhydride (DATAAN) introduced by Lindner *et al.*, (1984), was shown to separate 15 different beta-blockers and other compounds owning an amino-alcohol structure. The CDA reacts in an aprotic solvent with the hydroxy group of the beta-blocker side chain in about 4 hours, while the amino group is protected by ion pair formation with a strong acid e.g. trifluoro acetic acid. The diastereomeric ester derivatives form an intermolecular zwitterionic ring of the negatively charged carbonyl group and the positively charged amino group, thus enantioseparation is related to the pH of the mobile phase in a reversed phase system.

Thompson *et al.* (1982) investigated the reaction of (*R*,*S*)-propranolol with (*R*)-(+)-phenylethyl isocyanate (PEIC), also referred to as α-methyl benzyl isocyanate. Isocyanates react with primary and secondary amines as well as with hydroxyl groups forming urea derivatives and carbamates, respectively. At room tem-

Table 1. Chiral derivatizing agents (CDAs) applied to bioanalysis

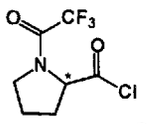
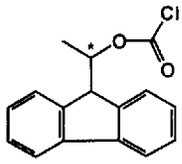
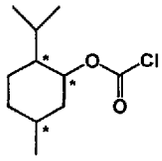
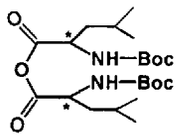
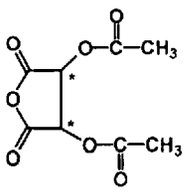
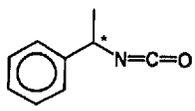
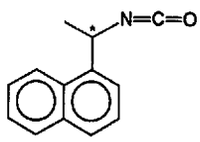
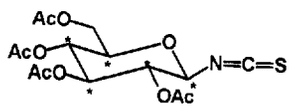
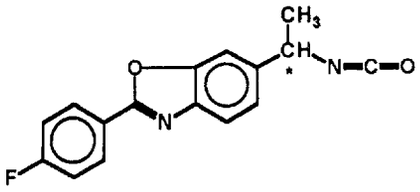
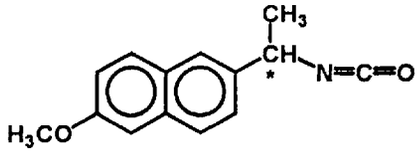
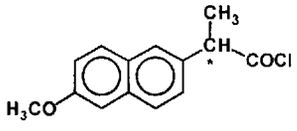
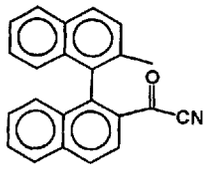
Reagent	Formula	Resolved drugs[a]	References
N-trifluoroacetyl-L-prolyl chloride		P	Hermansson and von Bahr (1980) Silber and Riegelman (1980)
(+)-1-(9-fluorenyl)ethyl chloroformate (Flec)		A P	Rosseel *et al.* (1989) Roux *et al.* (1991)
(−)-menthyl chloroformate (MCF)		A P S	Mehvar (1989) Prakash *et al.* (1989) Fiset *et al.* (1993)
Anhydride of *tert.* butoxy carbonyl-L-leucine		P P P	Hermansson (1982) Hermansson and von Bahr (1982) Guttendorf *et al.* (1989)
(*R,R*)-*O,O'*-diacetyl tartaric acid anhydride		P	Lindner *et al.* (1989)
(+)-(*R*)-phenyl ethyl isocyanate		C P P, 4OHPSO4 M	Stahl *et al.* (1993) Tsuru *et al.* (1987) Laganiere *et al.* (1989) Wilson and Walle (1984) Schäfer *et al.* (1990) Spahn *et al.* (1989)
(−)-(*S*)-phenyl ethyl isocyanate		A M Pin S	Chin *et al.* (1989) Plugman *et al.* (1987) Hsyu and Giacomini (1986) Sallustio *et al.*, (1992)
(+)-(*S*)-1-(1naphtyl)ethyl isocyanate		S M	Carr *et al.* (1991) Bhatti and Foster (1992)
(−)-(*R*)-1-(1naphtyl)ethyl isocyanate		B	Darman and Thenot (1986)
2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate		A C M P, 4OHP P, 4OHPSO4 Pin	Webb *et al.* (1988) Eisenberg *et al.* (1989) Neugebauer *et al.* (1990) Schuster *et al.* (1988) Walle *et al.* (1988) Miller *et al.* (1992) Eller *et al.* (1993) Hasegawa *et al.* (1989)
(−)-(*S*)-flunoxaprofen isocyanate		P	Martin *et al.* (1989)
(−)-(*S*)-naproxen isocyanate		P	Martin *et al.* (1989)
(−)-(*S*)-naproxen chloride		C	Spahn *et al.* (1990)

Table 1. *(Continued)*

Reagent	Formula	Resolved drugs[a]	References
(+)-2-methyl-1,1'-binaphtalene-2'carbonyl cyanide		P	Shao *et al.*, (1991)

[a] A, atenolol; B, betaxolol; C. carvedilol; M, metoprolol; Pin, pindolol; P, propranolol, 4OHP, 4-hydroxypropranolol, 4OHPSO4, sulphate conjugate of 4-hydroxypropranolol, S, sotalol.

perature in the presence of inert solvents urea formation is the preferred mechanism, as validated by GC-MS experiments. The reaction is complete after some minutes and the derivatives of (*R*,*S*)-propranolol were resolved in reversed phase mode within 20 min. Further GC experiments with trimethylsilylated-propranolol-methylbenzyl carbamate derivatives showed a loss of stereoselectivity compared with the underivatized diastereomers. This results led to the presumption that an intramolecular hydrogen-bonding between the carbonyl group and the hydroxyl group enhanced the physicochemical differentiation of the diastereomers. (+)-(*S*)-1-(naphtyl)ethyl isocyanate (NEIC) shows an improved stereoselectivity paired with fluorescence sensitivity for detection compared with the phenyl homologue. Owing to the high lipophilicity of the resulting derivatives normal phase HPLC is generally preferred, in order to shorten retention times. Gal and Sedman (1984) introduced (*R*)-1-phenylethyl isothiocyanate (PEITC) as a CDA owing to a better chemical stability than the cyanate derivatives. A further CDA of this type is the 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) developed by Nimura *et al.*, (1980), initially introduced for resolution of amino acids and later applied to the resolution of beta-blockers by Sedman and Gal (1983). The stereoselective mechanism responsible for the good separation of diastereomers is presumably the formation of rigid conforms via intramolecular hydrogen bondings between the acetyl groups and the hydroxy group in the β-position of the derivatized amino function (Kinoshita *et al.*, 1981). The reagent can be applied in organic solvents but also in aqueous solutions up to a water content of 30% (beyond that it precipitates). It should be noticed that the commercially available reagent implies chemical impurities of unknown structures sometimes causing co-eluting peaks on the chromatogram. Ahnhoff *et al.*, (1992) investigated the formation of these products as well as their elimination by pre-treatment of the reagent solution with another amine.

(−)-(*S*)-Flunoxaprofen and (−)-(*S*)-naproxen were introduced as their isocyanate derivatives as CDA for resolution of beta-blockers by Martin *et al.*, (1989). The applicability to serum samples was demonstrated by analysing plasma samples obtained from 2 volunteers after administration of racemic propranolol.HCl. (−)-(*S*)-naproxen chloride found application for resolution of carvedilol enantiomers in the course of enantioselective pharmacokinetic investigations in humans (Spahn *et al.*, 1990).

A different type of CDA is (+)-2-methyl-1,1'binaphtalene-2'-carbonyl cyanide developed by Goto *et al.*, (1990), which owns a chiral axis as chiral element instead of a chiral carbon atom.

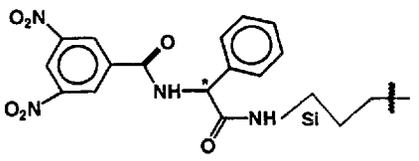
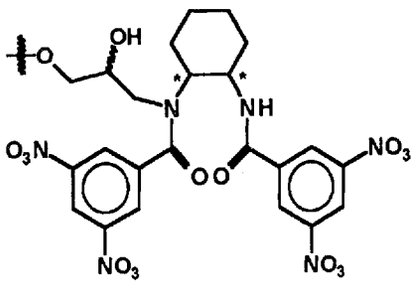
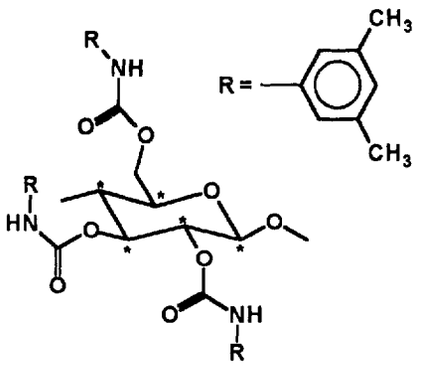
Chiral stationary phases

The 'direct' enantioseparation technique relies on the formation of reversible diastereomeric complexes of the chiral analyte (selectant, SA) and a chiral host molecule (selector, SO) representing the chiral stationary phase (CSP). The chemical nature of the CSPs spans from small molecules which are ionically physisorbed or covalently bound onto a silica surface (the so called brush-type CSPs), to chiral biopolymers such as cellulose or proteins also strongly adsorbed or bound onto a surface.

The separation of stereomers by a chiral host molecule requires at least 3 different interactions according to the three point interaction model of Dalgliesh (1952). However, the chiral stationary phase represents a layer or cluster of particular chiral host molecules which may be oriented to each other in a certain way so that the multipoint attachment rule cannot be defined so strictly. Thus one could state that a 'chiral cavity' or a 'chiral bay area' gets created or exists which is spatially selective for one enantiomer but not for the antipode. If a chiral analyte fulfills this SO–SA binding the compound can be separated directly, otherwise one might also consider an achiral derivatization to introduce further structural and binding elements necessary for chiral recognition. A derivatization step may also be needed to mask polar groups in order to reduce non-stereoselective interactions of the analyte and the stationary phase. Methods for direct enantioseparations can be roughly classified into two groups depending on whether or not achiral derivatization of the analyte is required for chiral separation. A concept characterizing the various CSPs was initially made by Wainer (1987), who divided them into 5 groups, based on the type of chiral selector and the intermoleculars SO–SAs interactions which are probably responsible for chiral recognition and retention. Some prominent CSPs used for beta-blockers in the course of bioanalysis are listed in Table 2.

CSPs based on small molecules including 'Pirkle type' CSPs. Attractive or repulsive SO–SA interactions by hydrogen bondings, π-π- and/or dipole–dipole interactions between the analyte (SA) and the CSP (SO) are the principle mechanism of chiral recognition of such CSPs.

Table 2. Chiral stationary phases (CSPs) applied to bioanalysis

Reagent (resolved derivatives)	Chiral selector	Resolved drugs[a]	References
(3,5)-dinitrobenzoyl-α-phenyl glycine (oxazolidine-2-one)		P	Wainer *et al.* (1984)
(*R,R*)-3,5-dinitrobenzoyldiamino cyclohexane (oxazolidine-2-one)		A P	Egginger *et al.* (1993a) Egginger *et al.* (1993b)
tris (3,5 dimethylphenyl) carbamate of cellulose (underivatized)		B M M, αOHM P P, 4OHP	Krstulovic *et al.* (1989) Rutledge and Garrick (1989) Ching *et al.* (1989) Straka *et al.* (1990) Herring *et al.* (1991) Balmer *et al.* (1991) Straka *et al.* (1988) Takahashi *et al.* (1988) Herring and Johnson (1993)
α_1 acid glycoprotein (A: N, O, bisacetyl) (M: underivatized)		A M	Enquist and Hermansson (1990) Walhagen and Edholm (1989) Walhagen *et al.* (1989) Persson *et al.* (1990)
ovomuvoid (underivatized)		P	Tamai *et al.* (1990)

[a] αOVM, α-Hydroxy-metoprolol. For rest of key see table 1.

The first commercially available CSP of this type was developed by Pirkle *et al.*, (1981) using 3,5-dinitrobenzoyl-α-phenylglycine as chiral selector. Focused on β-blockers, it was found necessary to convert the amino function to amide-derivatives (Pirkle *et al.*, 1981), urea derivatives (Yang *et al.* 1988) or oxazolidine-2-one derivatives (Wainer *et al.*, 1984) to fulfill the binding requirements. Based on Pirkle's Reciprocal Principle (Pirkle and Däppen, 1987) a large number of investigations dealing with structural requirements of the chiral selector (Pirkle *et al.*, 1984) and of the analyte (e.g. propranolol by Dyas *et al.*, 1990) have been performed, and many efforts in designing new chiral stationary phases have been undertaken in the recent past.

Consequently, and adapting the original Pirkle concept a new CSP was designed by Misiti and coworkers (Gasparrini *et al.*, 1991) based on (*R,R*)-(3,5-nitrobenzoyl)diaminocyclohexane ((*R,R*)-DNB-DACH). This CSP has proved to separate several beta-blockers as their oxazolidine-2-one derivatives with α-values of 1.14–1.55 and resolution factors (R_s) from 1.2–3.3. The formation of a rigid structure via the derivatization of the analyte with phosgene or 1,1-carbonyldiimidazole was shown to be essential to produce stereo-selectivity; e.g. *N*- or *O*-acetylated (*R,S*)-propranolol was not resolved on this CSP. Because of the availability of the (*R,R*)- and (*S,S*)-DNB-DACH CSP the application of this CSPs for determination of enantiomeric excess more than 99.9% was found possible in the course of bulk drug analysis; its application to bioanalysis will be shown later.

CSPs based on large molecules including cellulose type CSPs. As pointed out earlier chiral cavities within the chiral selector layer (macro-molecules) may play a major role as the chiral discrimination mechanisms, together with hydrogen-, π-π- and/or dipole interactions. Okamoto *et al.*, (1986) described the separation of several underivatized beta-blockers on a CSP based on cellulose or amylose derivatives adsorbed on macroporous silica gel. In particular, the cellulose *tris* (3,5-dimethyl)phenyl carbamate derivative, commercially available as Chiralcel OD® (Daicel Chemical Industries), found widespread application for bioanalysis of beta-blockers and their metabolites. The first generation of this CSP was applied in normal phase mode and exhibits a chemical instability towards

certain organic solvents such as CH_2Cl_2, THF and maybe large amounts of alcohols, whereas the new generation, the so called OD-R, can be used in reversed phase mode with almost equal efficiency.

CSPs based on inclusion complexation. Biopolymers (cellulose triacetate, α, β, γ-cyclodextrine) or synthetic polymers may form chiral cavities or chiral bay areas into which the solute molecule may enter, completely or in part, respectively, thus leading to different diastereomeric inclusion complexes with different association and/or dissociation constants. Prominent representatives of such chiral selectors are cyclodextrines (α- β- γ-CD) and derivatives thereof also bound onto silica. Armstrong and Demond (1984) introduced the first such a chiral stationary phase and later reported the resolution of several beta-blockers (Armstrong *et al.*, 1992). Nakagawa *et al.*, (1992) demonstrated the separation of underivatized atenolol enantiomers on phenylcarbamate derivatives of β-cyclodextrine and furthermore the applicability of this technique to serum samples.

Imprinted phases, produced by polymerization of e.g. metacrylic or itaconic acid in the presence of a 'print molecule' (template) should also be mentioned as being a concept with high potential (Sellegren *et al.*, 1988). Fischer *et al.*, (1991) reported the development of an imprinted CSP for β-blockers using (*S*)-timolol as template. However, this technique appears to be highly stereoselective and therefore more suitable for preparative separations of enantiomers of beta-blockers; the efficiency of these CSPs are usually rather poor and their analytical application therefore limited.

Ligand exchange type CSPs. Amino acid derivatives have been the ideal SO model compounds for chiral ligand-exchange separations of amino acids and derivatives. This technique was first described in the 1960s by Davankov *et al.*, (1988). The resolution of the enantiomers is based on the formation of diastereomeric SO–Me–SA chelate complexes between the enantiomers of the analyte (SA), and the chiral SO molecule (such as e.g. L-proline covalently bound) and a transition metal ion (Me, usually Cu^{2+}). The selector ligand molecule can be introduced via the mobile phase as additive but can also be covalently bound to the stationary phase (see later).

CSPs based on immobilized proteins. CSPs based on proteins were developed with regard to the stereoselective binding properties of drugs to certain proteins found by *in vitro* measurements. Based on Allenmark's early work (Allenmark, 1982) Hermansson (1983) introduced the first protein type CSP based on α_1-acid glycoprotein, AGP (Enantiopac®). Separations of several cationic drugs were described by Hermansson (1985): including neutral but moderately polar compounds; beta-blockers as such or as their oxazolidine-2-one derivatives; and amines as such or as acetyl derivatives. By changing the linking procedure of the AGP to the silica support, a second generation of this CSP, the CHIRAL-AGP®, was introduced owning higher stability than the Enantiopac-columns. Enquist and Hermansson (1990) demonstrated the separation of β-blockers and other drugs on CHIRAL-AGP without any achiral derivatization.

Several other CSPs based on proteins such as bovine serum albumin (BSA) introduced by Allenmark *et al.*, (1983), ovomucoid (OVM) by Haginaka *et al.*, (1990) avidin by Miva *et al.*, (1987) and Oda *et al.*, (1991), cellulase by Erlandsson *et al.*, (1990), immobilized enzymes as α-chymotrypsin by Wainer *et al.*, (1988) and trypsin by Thelohan *et al.*, (1989) have been developed in the last few years. As a general trend and because of the basic properties of, for example, BSA, these CSPs express stereoselectivity for acidic drugs and acidic proteins such as AGP, OVM etc. and show good enantioselectivity not only for basic drugs such as β-blockers but also for neutral compounds.

However, several investigators have attempted to use protein CSPs as models for the calculation and prediction of protein binding of drugs: first for AGP (Jewell *et al.*, 1989) and later for BSA (Wainer, 1991). It could be demonstrated that the BSA–CSP, but not the AGP–CSP seem to reflect the protein binding of drugs *in vitro*. In order to circumvent the differences between bovine and human proteins Domenici *et al.*, (1990) developed a further protein type CSP by immobilizing human serum albumin (HSA) rather than BSA onto silica gel.

A CSP based on the di-peptide glycyl-L-proline was developed by Ohwa *et al.*, (1990) capable of separating a series of beta-blockers including atenolol, metoprolol, pindolol and propranolol but at normal phase conditions.

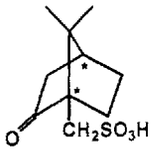
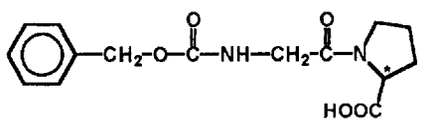
Chiral mobile phase additives (CMPAs)

Enantioseparation by the mean of chiral mobile phase additives is mainly relying on the formation of reversible diastereomeric ion pairs using a chiral counter ion, and on diastereomeric inclusion complexes using cyclodextrines, respectively. Some CMPAs applied to bioanalysis so far are summarized in Table 3.

Petersson and Schill (1981) reported the use of (+)-10-camphor sulfonic acid (CSA) as a mobile phase additive for resolution of the enantiomers of several beta-blockers, e.g. metoprolol and propranolol. This type of ion pair chromatography is usually performed under straight phase conditions and controlled water content of the mobile phase (Duncan *et al.*, 1990). Higher stereoselectivity compared with CSA (Leeman and Dayer, 1988) was achieved with benzoxycarbonyl-glycyl-L-proline (L-ZGP) which was introduced by Petersson and Josefsson (1986). Petersson *et al.*, (1987), in a fundamental and basic study, investigated the influence of the optical purity of a chiral selector, in this case a chiral counter-ion using L-ZGP, and (*R*,*S*)-propranolol as model substances. They demonstrated that complete determination and separation of propranolol enantiomers were possible even when the chiral selector was contaminated to a certain degree (e.g. 20%) with its antipode owing to the reversibility of the diastereomeric ion pairs formed. This is in contrast to the chiral derivatizing agents where a precise knowledge of the optical purity of the reagent is required for correct determination of the single enantiomers, as pointed out above.

Heldin *et al.*, (1991) described the separation of enantiomeric amino alcohols using tartaric acid derivatives as chiral complexing agents in organic stationary

Table 3. Chiral mobile phase additives (CMPAs) applied in bioanalysis of beta blockers

Reagent	Formula	Resolved drugs[a]	References
(+)-10-camphor sulphonic acid		M	Leeman and Dayer, 1984
N-benzoxycarbonyl-glycyl-L-proline		M P	Leeman and Dayer, 1988 Petersson and Joseffson 1986

[a] For key see table 1.

or mobile phases. Nucleosil CN as well as porous graphitic carbon were used as solid support whereas the latter exhibits higher stereoselectivity.

It should be mentioned that Cyclodextrines can also be used successfully as a chiral mobile phase additive leading to diastereomeric inclusion complexes of the analyte and the chiral cavity of the host polysaccharide (Armstrong, 1984). However, no applications in the field of HPLC bioanalysis have been reported so far.

ENANTIOSELECTIVE HPLC-BIOANALYSIS OF BETA-BLOCKERS

Propranolol. The first 'indirect' enantioseparations of a beta-blocking drug by HPLC were reported by Hermansson and von Bahr (1980), quickly followed by Silber and Riegelman (1980), after chiral derivatization of propranolol with *N*-trifluoroacetyl-L-prolylchloride as the CDA. The first research group performed the derivatization in chloroform at room temperature for 15 min in the presence of a basic catalyst. The method was applied to plasma samples obtained from two subjects after administration of racemic propranolol. However, the results had to be corrected mathematically because the commercially available reagent was contaminated with the D-isomer. The optical stability of this CDA during storage was problematic (Silber and Riegelman, 1980) and a new CDA was developed by Hermansson (1982). The symmetric anhydride of *tert.*-butoxycarbonyl-L-leucine (BOC-L-Leu) L-leucine was taken as the basis for this reagent instead of L-proline owing to racemization phenomena of the L-proline-derivatives. The diastereomers of propranolol and metoprolol were separated by reversed phase HPLC and *N*,*N*-dimethyloctylamine was used to improve the peak symmetries. The method was adapted by Guttendorf *et al.*, (1989) for quantifying the enantiomers of propranolol in 100 µL of rat whole blood microsamples. Cyclopentyldesisopropyl-propranolol was incorporated as internal standard into this intensively validated assay. The use of freshly prepared CDA and the performance of the cleavage of the BOC-group at strictly controlled reaction conditions, utilizing trifluoroacetic acid at 0 °C for 7 min., was important to enable good diastereoseparation and for the reproducibility of this assay. The derivatives were separated within a run time of 60 min, whereby the (*R*)-propranolol-derivative eluted on the down slope of an unidentified large peak. Two concentration time profiles after an intravenous dose of racemic propranolol to rats were shown, exhibiting a three-times higher terminal half-life for (*S*)- than for (*R*)-propranolol.

(+)-(*R*)-phenylethyl isocyanate (PEIC), first applied by Thompson *et al.*, (1982) as CDA for propranolol, subsequently found wide application for the separation of several β-blockers and their metabolites by HPLC. Wilson and Walle (1984) described the simultaneous enantioselective determination of propranolol and 4-hydroxypropranolol in urine. The free bases of (*R*,*S*)-4-hydroxy-propranolol and (*R*,*S*)-propranolol were extracted with ether at pH 9.6, the conjugates were cleaved by pre-treatment with glusulase prior to extraction.

The highly hydrophilic sulfate conjugates of (*R*,*S*)-4-hydroxypropranolol found in human urine were extracted via an ion-pair procedure as described by Wingstrand and Walle (1984). The extracts were purified by reversed phase chromatography, and pretreated with glusulase prior to derivatization with PEIC. The reaction was performed in chloroform at room temperature. Under these derivatization conditions the formation of a late eluting pair of peaks on the chromatogram (R_t ca 50 min) (assumed to be the di-derivatives of the amino- and hydroxyl-group of 4-hydroxypropranolol) was observed. The carbamates but not the ureides could be selectively cleaved by shaking the reaction mixture with 0.1 M HCl. The structure of the diastereomeric mono-derivatives was verified via electron-impact MS. In order to improve the insufficient resolution of the diastereomers obtained in the reversed phase mode, normal phase conditions were examined, resulting in shorter retention times, higher degree of resolution and improved peak symmetries. This assay was slightly modified by Schaefer *et al.*, (1990) who used liquid/liquid extraction at pH 10.5 in the presence of the antioxidant ascorbic acid for the extraction of the free bases of propranolol and its 4-OH-metabolite (suspected to be easily decomposed by oxidation). Triethylamine was used as a basic catalyst to prevent the formation of the carbamate derivative; no reaction of the phenolic OH group was observed under these conditions. In contrast to Wilson and Walle (1984) this group used reversed phase conditions and achieved separation of propranolol and 4-hydroxypropranolol derivatives within 40 min. A plasma concentration profile of one subject was given, showing preferred glucoronidation for the (*S*)-isomer of propranolol and 4-

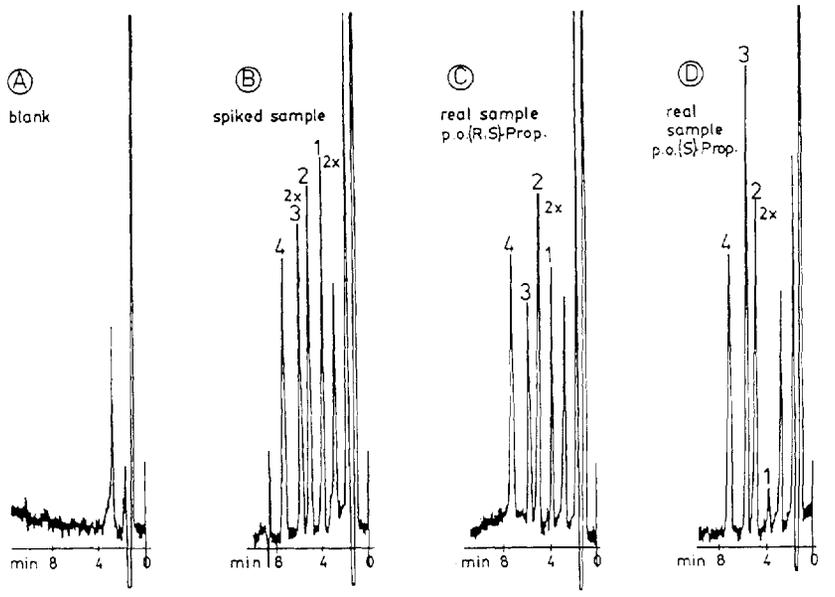


Figure 2. Typical chromatograms of (A) blank human plasma (B) human plasma spiked with 30 ng of the racemic drugs (*R,S*)-propranolol and (*R,S*)-internal standard (C) human plasma sample 2 h after oral administration of 80 mg of (*R,S*)-propranolol and (D) human plasma sample after oral administration of 40 mg of (*S*)-propranolol.

hydroxypropranolol, whereas the sulphatation favoured the (*R*)-isomer of (4)-hydroxy-propranolol.

Laganiere *et al.*, (1989) established a bioassay for the determination of propranolol in plasma microsamples using PEIC as CDA and cyclopentyldesisopropylpropranolol as internal standard. The derivatization was carried out at 4 °C for 30 min and the derivatives were separated under reversed phase conditions using methanol/isopropanol/methylene chloride/water (67/7.5/1/25.5, by volume) as mobile phase, within 20 min. The detection limit of a 100 µL sample was given with 2–3 ng/mL.

The determination of conjugates of 4-hydroxypropranolol via the unconjugated compound after enzymatic hydrolysis is sometimes critical owing to the chemical instability of this compound. Walle *et al.*, (1985) developed a method for direct determination of the intact sulphate conjugate after extraction via an ion-pair procedure (Wingstrand and Walle, 1984) and derivatization of the metabolite with GITC. The derivatization was performed in acetonitrile/water with 0.2% triethylamine within 5 min. The limit of quantitation was 40 ng/enantiomer. Recently, this reagent was combined with an immunoaffinity isolation of the sulphate conjugate of 4-hydroxypropranolol from plasma described by Eller *et al.*, (1993). A 0.5 mL aliquot of plasma was diluted with phosphate buffer, applied onto the top of the extraction column, incubated for 30 min, and eluted with 20 mL acetonitrile/water (95/5, v/v). The immunoaffinity gel was reported to be reusable for at least 100 applications. The correlation coefficient in the range of 1–200 ng/mL was 0.992. The recovery over the whole range was only about 40%. The assay was applied to plasma samples obtained from two subjects after single oral doses of 80 mg (*R,S*)-propranolol. However, no stereoselective plasma concentration profiles of 4-hydroxypropranolol suphate conjugate were shown. The (*R*)/(*S*)-ratio of the 2 hour sample is given with 3.2 and 4.3 for the male and the female subject, respectively. Miller (1991) used also GITC for the determination of propranolol and 4-hydroxypropranolol in plasma. The compounds were extracted from alkalinized plasma into hexane and derivatized in acetonitrile at room temperature for 40 min. The derivatives were reported to be stable for at least 36 hours at room temperature. The resolution values of the enantiomers of propranolol and 4-hydroxypropranolol were 1.5 and 2.5, respectively, whereby the (*S*)- elutes before the (*R*)-isomer.

(*R,R*)-*O,O'*-diacetyl tartaric acid anhydride introduced by Lindner *et al.* (1984) was applied for bioanalysis of propranolol (Lindner *et al.*, 1989) using liquid/liquid extraction via laboratory made Extrelut® extraction columns and N-*tert.*butylpropranolol as internal standard. The extraction yield was ca. 70% and the limit of determination was 0.6 and 1.2 ng for the (*R*)- and (*S*)-isomer, using fluorimetric detection. Despite a derivatization of >97% (4 hours, at 40 °C) the signals of the first eluting (*R,R,R*)-derivative of propranolol as well of the internal standard had double the intensity of those of the (*S,R,R*)-derivatives, probably due to the different fluorescent characteristics of the diastereomers. Using a mobile phase of methanol 0.2% acetate buffer (55/45, v/v) pH 4.8 the resolution factors were 4.0 and 3.8 for propranolol and internal standard, respectively. Typical chromatograms are shown in Fig. 2. Owing to the availability of the (*S,S*)-enantiomer of the CDA and to the high resolution values of the diastereomeric derivatives this method can be used for the determination of trace amounts of one enantiomer beside a more than 200-fold excess of the other enantiomer. The method was applied to a comparative human pharmacokinetic study administering (*R,S*)-propranolol and half-dosed optically pure (*S*)-propranolol to eight volunteers (Lindner *et al.*, 1989).

(−)-menthyl chloroformate was used as CDA by Prakash *et al.* (1989). Propranolol enantiomers and (+)-flecainide as internal standard were extracted via liquid/liquid extraction. After a reaction time of 15 min, aliquots of the reaction mixture were injected

onto the HPLC-system. The retention times for (*S*)- and (*R*)-propranolol derivatives were 12 and 13 min, respectively. The structure of the derivatives was verified by MS. The assay was applied to enantioselective pharmacokinetic investigations in humans and dogs, showing higher plasma concentrations of the (*S*)-enantiomers in humans but higher levels of the (*R*)-enantiomer in dogs.

The element of symmetry of a chiral axis is the basis of the CDA (+)-2-methyl-1,1′-binaphtalene-2′-carbonyl cyanide which was developed by Goto *et al.*, (1990) and applied by Shao *et al.*, (1991) for the determination of propranolol in plasma. After extraction via C_{18} cartridges (*R*,*S*)-propranolol and (+)-bufuralol as internal standard were derivatized within 20 min at 60 °C. After decomposing the excess of reagent with methanol, the derivatives were purified via an ion exchange column (18 mm × 6 mm ID) and the dried eluate was redissolved in the mobile phase and injected into the HPLC-system using a mobile phase consisting of hexane/ethylacetate (15/1, v/v) with 0.3% methanol as modifier. The symmetric peaks of Internal standard (I.St.-)- and of (*R*)-(*S*)-propranolol-derivative eluted at ca. 5 min and at 9 and 11 min, respectively. The recovery was reported to be ca. 90%. and the detection limit was 100 pg/enantiomer/mL. Silanized glass ware was used throughout the assay.

An enantioselective bioassay using (+)-(9-fluorenyl)ethyl chloroformate as CDA was developed by Roux *et al.*, (1991) and applied to a bioavailability study comparing an immediate formulation and a sustained release formulation of racemic propranolol. After liquid/liquid extraction of (*R*,*S*)-propranolol with (*R*,*S*)-4-methyl-propranolol as internal standard and derivatization, the excess of the reagent was removed via C_{18} Bond-Elut columns using dichloromethane as eluent. The detection limit was 0.5 ng/mL. The α- and R_s values were 1.07 and 2.74 for (*S*)- and (*R*)-propranolol and 1.08 and 2.62 for (*S*)- and (*R*)-I.St.

Wainer *et al.*, (1984) presented the first 'direct resolution method' of bioanalysis of (*R*)- and (*S*)-propranolol in serum using a Pirkle-type CSP (*R*)-*N*-(3,5)-dinitrobenzoyl)phenylglycine. (*R*,*S*)-propranolol and (*R*,*S*)-pronethalol as internal standard were extracted with ether, derivatized with phosgene and the resulted oxazolidine-2-ones were separated on the CSP using hexane/isopropanol/acetonitrile (97/3/1, by volume) as mobile phase and fluorimetric detection. The capacity factors of the enantiomeric derivatives were 57 and 62, $\alpha = 1.09$ and $R_s = 1.4$. No separation could be achieved for the internal standard ($k' = 16$). The extraction yield was reported to be more than 99%. Plasma concentration time curves for 1 volunteer were shown, exhibiting higher values for (*S*)- than for (*R*)-propranolol.

Another bioassay based on the direct separation of underivatized propranolol was described by Straka *et al.* (1988). (*R*,*S*)-propranolol and (*R*,*S*)-verapamil as internal standard were resolved on a Chiralcel OD CSP after ether extraction, using hexane/2-propanol/*N*,*N*-dimethyloctamine (92/8/0.01, by volume) as mobile phase and fluorimetric detection. Under these conditions the k' values were 2.9 and 6.5 for (*R*)- and (*S*)-propranolol, respectively, α-value = 2.2 and $R_s = 3.7$. This method was applied to a pharmacokinetic study dealing with the stereoselective accumulation of (*R*,*S*)propranolol in steady state conditions by LaLondne *et al.* (1988). It was observed during these studies that the (*S*)/(*R*) ratio decreased during steady state compared to single dose regimens.

Recently, the determination of the enantiomers of propranolol and 4-hydroxypropranolol in urine using Chiralcel OD as CSP was presented by Herring and Johnson (1993). (*R*,*S*)-propranolol, (*R*,*S*)-4-hydroxypropranolol and (*R*,*S*)-verapamil as internal standard were extracted at pH 10 after enzymatic hydrolysis of the conjugates. Using hexane/ethanol/diethylamine (99/9/0.1, by volume) as mobile phase. R_s-values were 3.53 and 3.76, respectively, with a run time of 30 min. The verapamil enantiomers were not resolved under these conditions. The extraction efficiency was 77% for propranolol- and 66% for 4-hydroxypropranolol enantiomers, over a range of 1–12.5 μg/mL.

Recently, another direct method was developed by Lindner and co-workers (Egginger *et al.*, 1993b) relying on liquid/liquid extraction of the enantiomers of propranolol and '*n*-pentylpropranolol' as internal standard followed by achiral derivatization of the drugs with phosgene and separation of the corresponding oxazolidine-2-one derivatives on a (*R*,*R*)-DACH-DNB CSP. The reaction was performed in dichloromethane at 40 °C for 3 hours, and the oxazolidine-2-one derivatives were separated using dichloromethane/methanol (99.75/0.25, v/v) as mobile phase. The retention times of the (*R*)- and (*S*)-propranolol derivatives were 8.8 and 10.8 min, $\alpha = 1.27$, and those of (*R*)- and (*S*)-internal standard. 7.8 and 9.8 min, $\alpha = 1.31$. The study was applied in the course of extensive comparative human pharmacokinetic and pharmacodynamic investigations (Stoschitzky *et al.*, 1992a), dealing with the haemodynamic effects, plasma concentrations and influence on thyroid hormones of (*R*,*S*)-propranolol compared to optically half-dosed (*S*)-propranolol after single dose and at steady state conditions. Mean plasma concentration time curves of (*R*)- and (*S*)-propranolol at steady state are shown in Fig. 3.

The protein type CSP based on ovomucoid commercially available as Ultron ES-OVM® has also been used for the separation of propranolol enantiomers in rat or mouse blood and several tissues by Tamai *et al.* (1990). Therefore a column switching device was used, coupling the OVM-CSP with a precolumn (particle size 44–88 μm) on which plasma, whole blood and tissue homogenates were directly injected. The calibration curve ranged from 0.5–100 pmol/enantiomer and the detection limit was 0.2 ng/mL using fluorimetric detection. 50 mM sodium dihydrogene phosphate pH 4.6 containing 12% ethanol was used as mobile phase. The influence of pH, salt concentration, column temperature and flow rate on α, k' were investigated. Under the chosen conditions the retention times were 14.4 min for (*S*)- and 16.8 min for (*R*)-propranolol, $\alpha = 1.15$.

Petersson and Josefsson (1986) investigated the use of the chiral counter ion *N*-benzoxycarbonyl-glycyl-L-proline (ZGP) for determination of propranolol enantiomers in biological fluids. For the separations a DIOL-stationary phase together with 2.5 mM ZGP in dichloromethane containing 1 mM triethylamine and 500 p.p.m. H_2O as modifier was used.

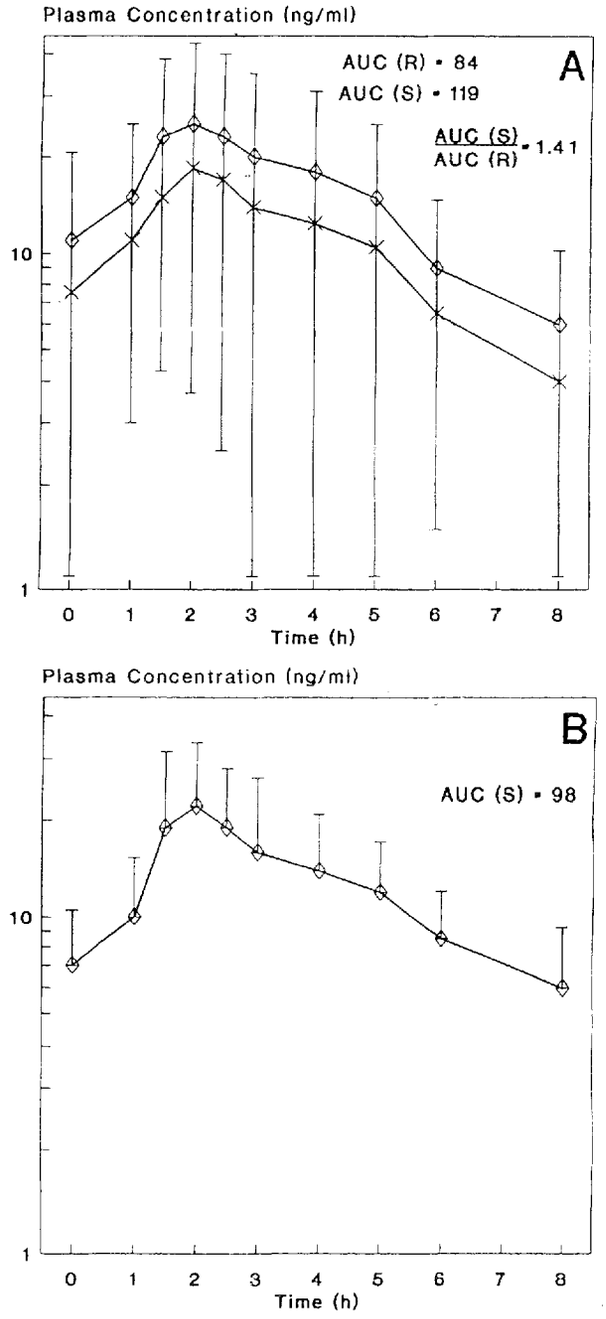


Figure 3. Mean plasma concentration time curves of (*R*)- and (*S*)-propranolol of 10 healthy volunteers at steady state after the 22nd oral application (three times daily over 1 week) of (A) 40 mg (*R,S*)-propranolol.HCl and (B) 20 mg (*S*)-propranolol.HCl (8th day of the study).

Metoprolol. The first 'indirect' enantioseparation for metoprolol in plasma was described by Hermansson and von Bahr (1982) using the symmetric anhydride of *tert.*butoxy-L-leucine (BOC-L-Leu) as CDA in the presence of a basic catalyst. The *tert.*-butoxycarbonyl-group had to be cleaved with trifluoroacetic acid prior to chromatography in order to get sufficient stereodifferentiation of the diastereomers. The *k'* values for (*S*)- and (*R*)-metoprolol-L-leucine derivatives were 4.03 and 5.86 min. Using fluorescence detection the detection limit was given with <1ng/enantiomer/mL plasma. This assay was applied by Lennard *et al.* (1983) in the course of a pharmacogenetic study in extensive and poor metabolisers.

(−)-(*S*)-phenylethyl isocyanate (PEIC) was used by Pflugman *et al.* (1987) as CDA. The derivatization procedure was stopped after 45 min and the excess of PEIC was quenched with ethanol amine. The diastereomeric derivatives were separated in reversed phase mode within a run time of 20 min. Owing to late eluting peaks the total run time was more than 30 min. Two commercially available metabolites (a propanoic acid derivative and a phenyl acetic acid derivative) were shown not to interfere with the metoprolol derivatives. The limit of detection was 2 ng/enantiomer/mL plasma using fluorimetric detection. This assay was further validated via a radio receptor assay by Spahn *et al.* (1989) demonstrating a close agreement between the HPLC- and receptor-assay.

The naphtyl homologue of PEIC, (+)-(*S*)-1-(1-naphtyl)ethyl isocyanate was used by Bhatti and Foster (1992) as CDA and (*R,S*)-propranolol as internal standard. The reaction time was reported to be only 30 s. The derivatives were resolved in normal phase mode ((*R*)- and (*S*)-metoprolol 14.1 and 16.3 min, $R_s > 1.5$; (*R*)- and (*S*)-internal standard 8.5 and 10.5 min) and fluorimetrically detected.

GITC was used by Schuster *et al.* (1988) as CDA for the enantioseparation of metoprolol and α-hydroxy-metoprolol. The enantiomers were extracted via liquid/liquid extraction, derivatized within 30 min at room temperature and UV-detected at 222 nm. The R_s- and α-values were 1.72 and 1.43 for metoprolol, and 0.95 and 1.28 for α-hydroxy-metoprolol.

Several bioassays in the 'direct mode' have been published using a cellulose tris(3,5-dimethylcarbamate) (Chiralcel OD) as CSP. Enantioselective bioanalysis of metoprolol was reported by Rutledge and Garrick (1989). The given capacity factors for (*R*)- and (*S*)-metoprolol were 2.4 and 5.3, $\alpha = 2.2$ and $R_s = 5.5$ using a mobile phase of hexane/2-propanol-/diethylamine (91/8/1, by volume) and the limit of detection was 2 ng/enantiomer/mL plasma. Ching *et al.* (1989) described a method including liquid/liquid extraction of (*R,S*)-metoprolol (alprenolol as internal standard) out of plasma into dichloromethane, followed by further purification of the extract via CN-Bond-Elut® cartridges to minimize co-extracted compounds and to reduce the water content of the extracts in view of the chemical instability of this CSP to polar solvents. The retention times of (*R*)- and (*S*)-metoprolol were 7.8 amd 16.2 min ($\alpha = 3.1$ and $R_s = 3.2$) using hexane/isopropanol/diethylamine (90/10/0.01, by volume) as mobile phase. The limit of detection was 3 ng for (*R*)- and 6 ng/mL for (*S*)-metoprolol. Straka *et al.*, (1990) used racemic verapamil as internal standard and hexane/2-propanol (90/10, v/v) as mobile phase. Under given conditions the retention times for the (*R*)- and (*S*)-metoprolol were 6 and 14 min, $\alpha = 3.8$, no enantioseparation could be achieved for internal standard ($R_t = 9$ min). The limit of detection was 5 ng/mL, and comparable to the methods described above. Some endogenous compounds were removed first via ether extraction of the acidified serum prior to the ether extraction of the drug of the alkalinized serum. The serum concentration time curves given in all three papers exhibits large interindividual differences in the stereoselectivity and the plasma concentrations, related to the genetically determined phenotype of the subjects. Balmer *et al.*, (1991) presented a method for enantioselective determination of metoprolol and its α-hydroxymetabolite in plasma and

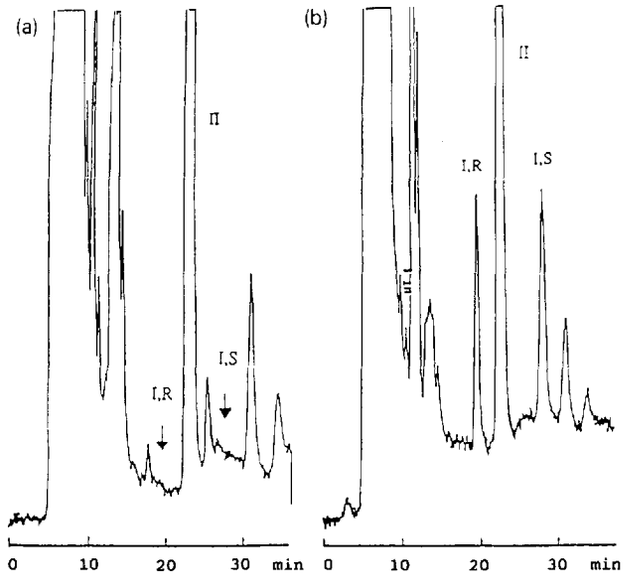


Figure 4. Separation of metoprolol enantiomers in human plama: (a) blank plasma; (b) patient plama. Peaks I,*R* and I,*S* correspond to (*R*)- (37 nmol/L) and (*S*)-metoprolol (47 nmol/L), respectively, Peak II corresponds to the internal standard (S)-alprenolol.

urine. The same group also investigated the influence of the water content of the mobile phase on chiral separations. They achieved separation of the enantiomers of the parent drug, its metabolite and of internal standard (alprenolol) within 30 min using hexane containing 4% 1-propanol, 0.1% diethylamine and 1.5 g water/L as mobile phase, as shown in Fig. 4. An extraction yield of more than 95% for metoprolol and its metabolite and a detection limit down to 10 nmol/L was reached. The analysis of dog plasma showed the presence of a further metabolite, a side chain desaminated and hydroxylated diol-product.

Chiral-AGP was used by Walhagen and Edholm (1989) in combination with a column switching system. The enantiomers were separated first on the chiral protein column, and each eluent fraction was trapped and compressed on two separate achiral columns (4×5 mm ID) and finally separated on a C_{18} column. Plasma samples were pre-treated with solid phase extraction prior to injection and the absolute recovery was estimated to be 85%. This method was modified and also coupled to a thermospray mass spectrometer by Walhagen *et al.*, (1989). Deuterium labelled metoprolol was used as internal standard. A schematic diagram of such a column switching set-up and chromatograms obtained from plasma extracts are shown in Fig. 5.

Persson *et al.* (1990) described a bioassay relying on liquid/liquid extraction in alkaline conditions followed by a back extraction step into phosphoric acid and a second re-extraction step into hexane in alkaline conditions employing 2-dehydroxymetoprolol as internal standard. The enantiomers were resolved on Chiral-AGP and EnantioPac, showing lower α-values (1.4 versus 1.7) but much higher R_s values (1.7 versus 3.3) for the enantiomers of metoprolol with the new Chiral-AGP than with the EnantioPac CSP. No separation was achieved for the internal standard. Using a gradient system the detection limit was found to be 2 nmol/L.

Leeman and Dayer (1988) investigated the enantioseparation of metoprolol and its metabolites by the chiral additive camphor sulphonic acid to the mobile phase which consisted of dichloromethane and propanol or methanol as modifier. Using fluorimetric detection the limit of detection was 5 ng/ml plasma.

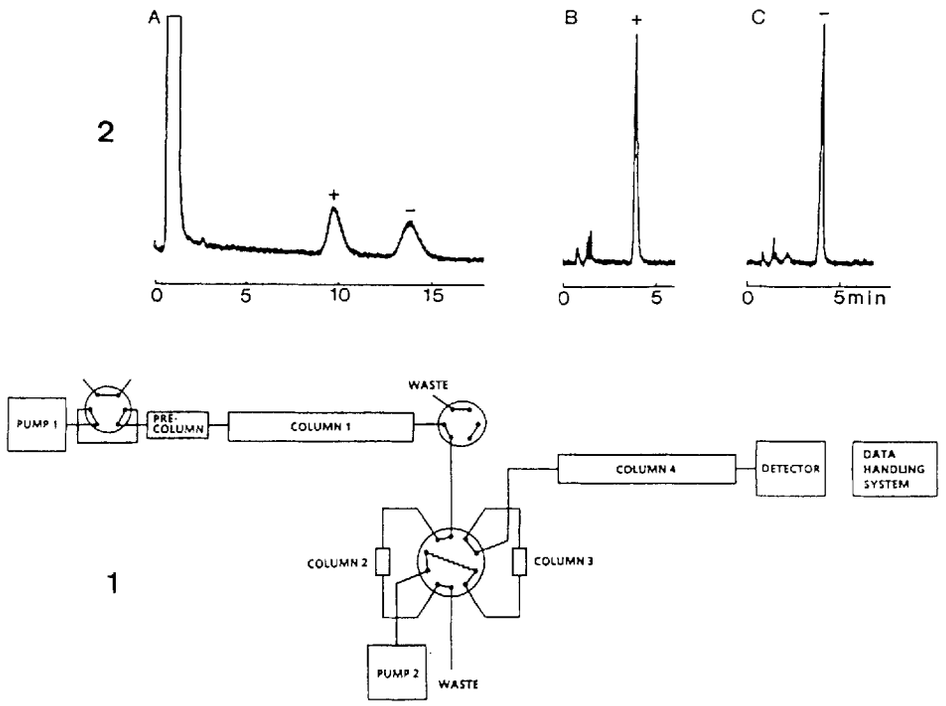


Figure 5. (1) Schematic diagram of the coupled-column chromatography system (CCC): column 1, chiral protein type CSPs; columns 2 and 3 = C_{18} achiral for trapping; column 4, C_{18} achiral. (2) Chromatogrms (A) after direct injection of 53 pmol of a standard solution onto the AGP-column, (B) spiked plasma containing 70 nmol/L of racemic metoprolol after separation by CCC, (+)-metoprolol first eluted enantiomer, (C) as in (B) (−)-metoprolol, second eluted enantiomer.

Betaxolol. The first 'indirect' enantioselective determination of betaxolol enantiomers in bioanalysis was reported by Darmon and Thenot (1986). They used (−)-(*R*)-naphtyl ethyl isocyanate as CDA and (*S*)-cycloprolol as internal standard. This reagent was chosen to improve the fluorescent activity and a detection limit of 0.5 ng/enantiomer/mL plasma could be obtained. The reaction was complete within 15 min and the derivatives were separated within 10 min under reversed phase conditions. To shorten the retention times and to improve the chromatographic behavior tetramethylethylene diamine (0.4%) and tetrahydrofuran (14%) were added to the mobile phase. This method was applied to single dose pharmacokinetic investigations in three human subjects after p.o. application of 20 mg of the racemic drug. Slightly higher blood concentrations for (*S*)- than (*R*)-betaxolol were observed in two subjects, whereas the third subject exhibited the opposite case which was rather surprising. NEIC was used in a recent pharmacokinetic investigation on betaxolol enantiomers in humans after application of the racemic drug (Stagni *et al.*, 1990).

Krstulovic *et al.* (1988) used a Chiralcel OD-CSP for the first 'direct' enantioselective determination of underivatized betaxolol in hepatocyte suspensions. The mobile phase was hexane/2-propanol/diethylamine (87/13/0.05, by volume) and a R_s factor >2 could be obtained. Furthermore, this method was reported to be suitable for determination of an enantiomeric excess of $>0.05\%$ in the bulk drug.

Carvedilol. The first 'indirect' enantioselective bioassay for carvedilol and its active metabolite *o*-desmethylcarvedilol was described by Eisenberg *et al.*, (1989) utilizing GITC as CDA combined with a solid phase extraction procedure. Plasma samples were diluted with a solution of guanidine. HCl and the enantiomers of carvedilol and *N,N*-bis-carvedilol as internal standard were extracted via C_{18} extraction columns and derivatized in acetonitrile for 30 min at room temperature in the presence of triethylamine as basic catalyst. The acidified samples were injected on an ODS 75×4.6 mm ID analytical column with a mobile phase consisting of acetonitrile/methanol/water/triethylamine (29/29/41.5/0.5, by volume) at pH 2.5. The k'-, α-, and R_s-values for the enantiomers of carvedilol and the *o*-desmethylmetabolite were 10.0 min (*S*), 12.2 min (*R*), $\alpha = 1.22$, $R_s = 3.32$ and 5.9 min (*S*), 7.2 min (*R*), $\alpha = 1.22$ and $R_s = 2.26$, respectively. The recoveries of the enantiomers of analytes and internal standard were about 90% in this extensively validated assay.

In another study on the stereoselective disposition of carvedilol enantiomers after intravenous and oral administration GITC was also used as CDA and liquid/liquid extraction followed by a re-extraction step utilized for sample pretreatment (Neugebauer *et al.*, 1990). The derivatives were resolved within 26 min, no k'- or α-values were given.

Spahn *et al.*, (1990) described a method for the measurements of carvedilol enantiometers in plasma and urine using (*S*)-naproxen chloride as CDA. Carvedilol enantiomers were extracted at pH 9.8 from plasma or urine into diethyl ether, derivatized with naproxen chloride and resolved under normal phase conditions using fluorimetric detection as carvedilol and also naproxen show native fluorescent properties.

Recently, Stahl *et al.* (1993) applied (+)-(*R*)-phenyl ethyl isocyanate as CDA in the course of investigations on the stereopharmacokinetics of carvedilol in models for reduced liver functions. Aliquots of plasma, urine and bile were extracted with ether. The evaporated extracts were derivatized in the presence of methanolic triethylamine and the derivatives were separated under normal phase conditions using diisopropyl ether/dichloromethane/methanol (95/5/2, by volume) as mobile phase. The conjugates were cleaved with β-glucuronidase prior to extraction after pre-extraction of the parent compound. The coefficients of variation were $<10\%$ and the correlation coefficient was 0.999 from 1 ng to 1.5 μg/mL.

Pindolol. (−)-(*S*)-PEIC was used as CDA by Hsyu and Giacomini (1986) for the determination of the enantiomers of pindolol in plasma and urine. The method relied on a three step solvent extraction (ether-HCl-ether), a derivatization step within 30 s and separation of the diastereomers under reversed phase conditions with baseline separation within 20 min. At a concentration of 8 ng/enantiomer/mL plasma the recovery was 75% and the corresponding value at 238 ng was 35%. Calibration was performed via an external standard protocol.

Hasegawa *et al* (1989) used GITC as CDA for determination of pindolol enantiomers in plasma and tissue. The extraction procedure was similar to that described above; 1-Nitronaphtaline was incorporated as internal standard into this assay. The diastereomeric derivatives were separated within a run time of 30 min. The method was applied for enantioselective pharmacokinetic studies on the effect of endotoxine on pharmacokinetic parameters of **(R,S)**-pindolol in rats.

Atenolol. In order to investigate the stereoselective uptake and release of atenolol enantiomers of and from models of adrenergic nerve endings, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate was used as CDA to set up a bioassay by Webb *et al.* (1988). It was shown that the uptake into and release of atenolol from adrenergic cells are stereoselective favouring the (*S*)-enantiomer (Bagwell *et al.*, 1989). Similar results have been found by Lindner and co-workers (Stoschitzky *et al.* 1992b) judging the serum levels of (*R*)-and (*S*)-atenolol of patients who were treated chronically with (*R,S*)-atenolol.

(-)-Phenylethyl isocyanate was used by Chin *et al.*, (1989) with (*R,S*)-4-methylpropranolol as internal standard for determination of (*R,S*)-atenolol in plasma and urine after liquid/liquid extraction with ethyl acetate. The retention times (R_t) of the (*R*)- and (*S*)-atenolol derivatives were 16 and 18 min. Only the first eluting peak of the internal standard could be used for quantitation because the second eluting peak co-eluted with an interfering peak present in the plasma. However, the reproducibilities ranged from 2–6% for plasma and from 2–9% for urine. The recovery was ca. 65% and the limit of determination was 50 ng/mL utilizing fluorimetric detection (native fluorescence of atenolol).

Mehvar (1989) used (−)-menthyl chloroformate as CDA and methoxamine.HCl as internal standard. Baseline separation was achieved only for the first

eluting pair of diastereomers of (*R*)- and (*S*)-atenolol ($R_s = 1.94$) but not for (*R*)- and (*S*)-internal standard ($R_s = 0.96$). The total run time was 25 min. In this study liquid/liquid extraction using ethyl acetate and fluorimetric detection was also used. Both methods were applied to pharmacokinetic studies of (*R,S*)-atenolol in humans and rats and it was shown that the AUC of (*R*)-atenolol was slightly but significantly higher than that of (*S*)-atenolol (Boyd *et al.* 1989; Mehvar *et al.* 1989).

A further indirect method was described by Rosseel *et al.* (1991) using (+)-1-(9-fluorenyl)ethyl chloroformate as CDA and (*R,S*)-practolol as internal standard. The reaction was complete after 30 min and the diastereomeric derivatives were separated under reversed phase conditions (R_t (*S*)- 5.9 min and R_t (*R*)-atenolol-derivative 6.5 min; 7.8 and 8.6 min for practolol-derivatives, used as internal standard) and fluorimetrically detected.

Enquist *et al.* (1991) described the first direct method for the bioanalysis of (*R,S*)-atenolol in plasma and urine; it is based on achiral derivatization of the drug with acetic anhydride and resolution of the bis-acetylated enantiomers on a α_1-acid glycoprotein (AGP) chiral stationary phase. The degree of extraction was reported to be approximately 95% using dichloromethane containing 3% heptafluorobutanol. Acetylation of atenolol prior to chromatography was performed to circumvent chromatographic interferences of endogenous compounds and to reduce the capacity factors of the enantiomers in order to increase the sensitivity. Furthermore, an improvement in enantioselectivity of the acetylated atenolol enantiomers ($\alpha = 1.71$, $R_s = 2.6$) compared with the underivatized isomers was observed. The detection limit was 6 ng/enantiomer/mL plasma. The column was reported to be stable for more than 1000 injections of plasma and urine samples whereas a washing procedure had to be performed after every tenth injection.

Recently, another direct method, applied for a human comparative pharmacokinetic and pharmacodynamic study, was developed in Lindner and co-workers (Egginger *et al.*, 1993a) using (*R,R*)-DACH-DNB (see Gasparrini *et al.*, 1991) as chiral stationary phase. An achiral derivatization of atenolol with phosgene leading to oxazolidine-2-one derivatives was undertaken to form rigid analytes preferentially resolved on this CSP.

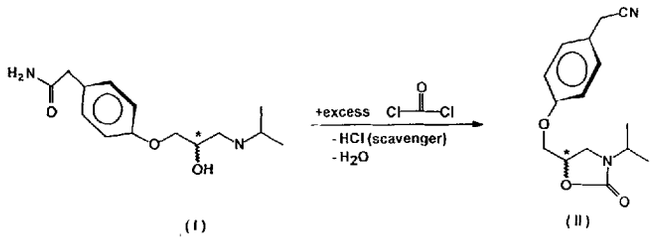


Figure 6. Structures and reaction scheme of (*R,S*)-atenolol (I) with phosgene leading to the corresponding chiral oxazolidine-2-one derivatives (II).

Under these conditions a simultaneous quantitative conversion of the acetamid group to the stable corresponding nitrile-group was performed as depicted in Fig. 6. The mobile phase was dichloromethane/methanol (98/2, v/v) and k' values were 3.4 and 4.2 for (*R*)- and (*S*)-atenolol derivatives, α-values were 1.22 and $R_s > 1.5$. Chromatograms of plasma extracts are shown in Fig. 7. Using the native fluorescence for detection and liquid/liquid extraction for sample preparation (recovery $\approx 80\%$) the limit of detection was 0.5 ng/enantiomer/mL plasma. Calibration was performed via an external standard protocol. The results of the pharmacokinetic study showed slightly but significantly higher AUCs for (*R*)- than for (*S*)-atenolol as shown in Fig. 8.

Sotalol. Three 'indirect' methods for the determination of the single enantiomers of the rather hydrophilic compound sotalol have been published so far. Carr *et al.*, (1991) used (+)-(*S*)-1-(1-naphtyl)ethyl isocyanate as CDA and racemic atenolol as internal standard. The resulting diastereoisomers were resolved in normal phase mode with fluorimetric detection.

Salustio *et al.* (1992) used (−)-(*S*)-phenylethyl isocyanate (PEIC) and (*R,S*)-atenolol as internal standard. The drugs were extracted from plasma using solid-phase extraction cartridges, the extracts were treated with PEIC in chloroform overnight at 4° to minimize evaporation of the reacting solvent, and the resulting diastereomeric derivatives were separated on a reversed phase C_{18} column within 30 min and detected fluorimetrically. Fiset *et al.* (1993) used (−)-menthyl chloroformate as CDA and (*S*)-atenolol as internal standard. The drug was extracted by liquid/liquid

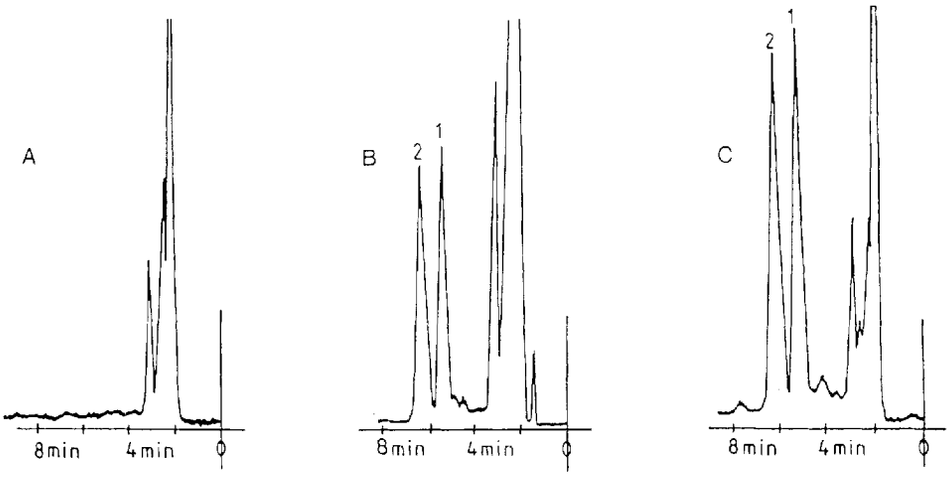


Figure 7. Typical chromatograms of plasma extracts of (A) blank plasma, (B) spiked plasma containing 100 ng of each atenolol enantiomer; (C) patient sample. Peak 1 corresponds to (*R*)- and peak 2 to (*S*)-atenolol-derivative.

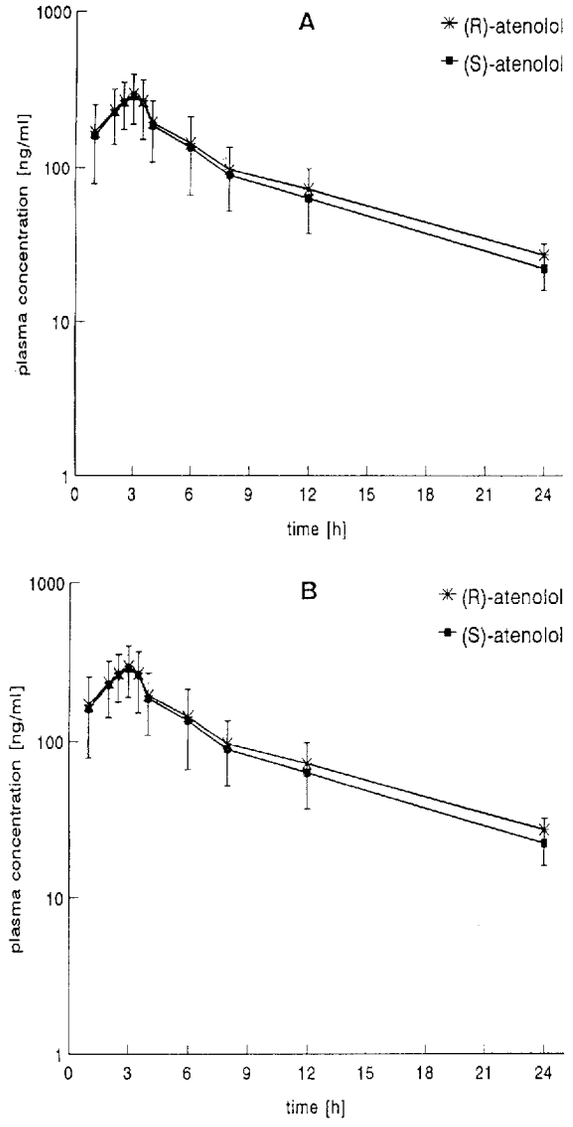


Figure 8. Mean plasma concentration time curves of 12 human subjects after a single oral dose of 100 mg (*R,S*)-atenolol (A) and of 50 mg (*R*)- or 50 mg (*S*)-atenolol (B).

extraction at pH 9 with a recovery of ca. 90%, and the derivatization was completed within 1 min. The diastereomeric derivatives were resolved on a C_8 reversed phase column with a run time of 16 min and fluorimetrically detected. The limit of quantitation was given with 20 ng/enantiomer/mL plasma or urine. The plasma concentration time curves shown documented slightly higher plasma concentrations for (*R*)- than for (*S*)-sotalol. The results were similar to those obtained for (*R,S*)-atenolol, although it shoudl be pointed out that sterically (*R*)-atenolol and (*S*)-atenolol are equivalent considering the Cahn–Ingold–Prelog rule (see also Fig. 1).

GENERAL ASPECTS OF ENANTIOSELECTIVE BIOANALYSIS; FOCUS ON BETA-BLOCKERS

Enantioselective bioanalysis is essential for investigations on stereoselective pharmacokinetics and its application to clinical pharmacology and *vice versa*: The growing demands on pharmacokinetics and clinical studies of each xenobiotic and thus also of each stereoisomer of a drug administered as a combination of the stereoisomers (for instance as a 1:1 mixture in the case of a racemic drug), have stimulated the development of enantioselective assays in the last decade.

Enantioselective bioanalysis can be seen as the combination of two disciplines: chiral separation techniques and analysis of drugs in biological samples. However, the introduction of a chiral separation technique into a bioassay results usually in a more complex total analysis method, with its own problems and requirements concerning extraction, chiral derivatization or enantioseparation on a CSP, detection and validation. In addition to the demands of a non-chiral bioassay extensively reviewed by Peng and Chiou (1990) the criterion of enantioselectivity needs further efforts in setting up and validating the total method. This aspect is of special importance for the pharmaceutical industry, when deciding the question whether a new drug which contains centers of chirality will be developed as single stereoisomer or as mixture thereof (Wozniak *et al.*, 1991). The following considerations will stress the specific problems connected with bioanalysis in general and in view of the combination with a chiral HPLC-assay.

To study the pharmacokinetic behavior of a compound in the course of the development or redevelopment of a drug several hundreds to thousands of samples have to be analysed. Therefore, the method has to be rugged, fast, easy to perform, reproducible and automatisable. Often serum/plasma levels of drugs and metabolites in the low ng- or even pg-range/mL have to be analysed, which require particularly high sensitivity of the detection-systems. In order to distinguish between endogenous compounds, metabolites, the parent drugs and their enantiomers, a particular emphasis on selectivity is required of the total analytical system. Chemical selectivity in extraction, derivatization, chromatography and detection as well as enantioselectivity or diastereoselectivity in chromatography are crucial points. In this context reference should be made to a recent book on 'selective sample-handling' edited by Frei and Zech (1988).

Extraction. An isolation of the analyte and/or of the metabolites together with the internal standard out of the biomatrix blood, serum, urine etc. is usually performed prior to chromatography via extraction. Isolation of the analytes by means of precipitation of plasma proteins can be critical in view of extensive bindings of lipophilic drugs to these proteins accompanied by co-precipitation of the analyte, eventually leading to losses, expressed as low recoveries. In view of the possible different binding ratios of enantiomers to proteins false results due to discrimination phenomena become obvious. This could be circumvented by direct injection of biological samples onto an HPLC column, however all sorts of problems can arise as can be seen from the review by Shihabi (1988). The use of column switching systems might minimize the loss of compounds during sample preparation, shorten the process of sample preparation and enable automation. However, the establishment of such assays is time consuming, exhaustive and needs expertise, particularly, when it comes to 'trouble shooting'. Extraction methods for plasma or urine samples based on chroma-

tographic retention in dedicated columns often termed 'solid phase extraction columns', should be expected to provide higher chemical selectivity than conventional liquid/liquid methods based only on different solubility or distribution rates. Furthermore, the solid phase extraction procedures are generally more suitable for automation. In any case the goal of any sample preparation procedure is to obtain 'clean extracts' containing only the analytes of interest; this has an additional advantage if one has to integrate an additional derivatization reaction in the course of sample preparation: the risk of co-reacting and co-chromatographed compounds diminishes to a certain extent. However, the appearance of very large peaks, caused by the large excess of reagent needed to guarantee quantitative results, is quite often a very annoying complication. The peak integration routine can easily become disturbed and the problems involved are well known. The use of a reliable software package seems essential and it should also contain peak height calculations.

To return to sample preparation, an attempt should be made to obtain extracts which are as pure as possible to prolong the HPLC-column life time, particularly with regard to the usually quite expensive CSPs.

Chiral derivatization. The precision of 'indirect methods' is strongly related to the optical purity of the CDA as described above. The optical stability of a CDA as well as of the chiral analyte during storage and derivatization has to be controlled. The reaction may often be time consuming and frequently require strict control of temperature and time. During the reaction of a mixture of chiral analytes and a chiral reagent different reaction and degradation kinetics may occur because of the diastereomeric character of the reaction products. Therefore, it is recommended to use a chiral internal standard with a similar structure to that of the analyte and thus expecting similar reaction kinetics of the enantiomers. By this strategy one could also pay attention to the possibility of different detector responses of diastereomeric molecules, as observed for the (*R*)-propranolol- and (*S*)-propranolol-(*R*,*R*)-*O*,*O*′-diacetyltartaric ester derivatives (Lindner *et al.* 1984). At this point, the advantage of a CDA which is transparent to the chosen type of detection should be mentioned, but in many cases reliance is placed on a sensitive detection of the analyte *per se*.

Chiral stationary phases. These exhibit in many cases good enantioselectivity resulting in large α values, but often with modest resolution capacities owing to broad peaks. The CSPs quite often also lack the chemical selectivity needed to separate the analytes from interfering compounds. Large α- and resolution values are of 'scientific' and 'academic' interest, but in practice one wants only base-line separation in a minimum of time. The very late eluting enantiomer becomes broad leading to a higher limit of detection. The combination of a chiral stationary phase together with an achiral conventional stationary phase can be a helpful tool to circumvent these problems to a certain extent, as described by Walhagen and Edholm (1989) for the bioanalysis of metoprolol (see also Fig. 5). The optimization of chromatographic parameters such as retention and resolution is on the one hand limited because of the 'chemical limits' of CSPs e.g. Chiralcel-OD or the protein based CSPs, where the selection of mobile phase conditions is crucial but also time consuming owing to the manifold parameters to be investigated, e.g. for the protein type CSPs. The type of solvent, type and strength of buffer, type and concentration of modifiers and temperature are parameters to be optimized. Recent developments in mathematical operations (chemometrics) are expected to have an impact setting
up optimized (usually set for a minimum analysis time at maximum resolution values) HPLC-bioassays. Furthermore, it should be mentioned that column-to-column variations of a particular CSP (Straka *et al.*, 1990) might be a problem when several columns have to be used during long-term investigations, in routine analysis, or when the bioassay has to be transferred to another laboratory.

A special analytical problem is the determination of the optical purity and/or of the optical stability of chiral compounds by HPLC. Trace amounts of one enantiomer have to be analysed beside the bulk drug. As a consequence it would be appropriate that both antipodes of a chiral reagent or a chiral stationary phase are available in order to chose the optimized conditions freely.

Detection. In addition to the chromatographic system the method of detection should also contribute to the assay specificity. The commonly used detection methods in HPLC are UV-absorbance, fluorescence and electrochemical detection. The relatively selective detection mode of UV-diodarray detection are often lacking very high sensitivity and one would therefore prefer at least fluorescence detection. Mass spectrometry interfaced with GC or LC would provide both sufficient specificity and sensitivity in bioanalysis; the complexity and high costs of these systems have limited their widespread use, but they are certainly the methods of choice in the near future. LC(GC)–MS can also be useful or necessary in order to check the peak-purity of the analyte-peaks, and thus to check the selectivity of the chromatographic system or of the whole method, when setting up an HPLC-bioassay incorporating an optical or electrochemical detector.

The attempt to improve the limit of detection and of determination of an analyte by introducing via derivatization an additional chromophor- or fluorescence tag into the molecule often leads to an enormous increase in 'co-detected' compounds on the chromatogram to be quantified. This requires a more extensive sample clean-up to deal with this inherent problem.

Detection principles based on immunoaffinity type reactions (immunoassays) should have high potential in this context and will be introduced more widely than is currently the case.

Validation. Bioanalysis together with pharmacokinetics play an important role in the development of new substances concerning preclinical and clinical studies, developments of new formulations, and investigations in bioavailability and metabolism. Furthermore, it has become more and more the basis of therapeutic drug monitoring and drug managements in patients. In the recent past increasing importance has been attached to the validation of bioanalytical methods which had become an essential part of a method. Common recom-

mendations of method validation of bioanalytical methods also taking account of enantioselective methods were established in order to prove the results on which several far-reaching decisions may be based. A detailed discussion of this point was not within the scope of this review. Some recommendations for validation in bioanalysis were described by Karnes *et al.* (1991). Shah *et al.* (1992) gave a summary report, published in several journals, of the conference on *Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies*, December 1990, Washington DC.

SUMMARY AND FUTURE TRENDS

Within the last few years a large number of investigations on the development of new enantioselective HPLC-methods or the adaptation and improvement of already existing ones have been carried out in the field of bioanalysis of beta-blockers, obviously to improve the visibility and convenience and to be faster and more efficient. Some of the methods appeared to be suitable for enantioselective bioanalysis, thus providing more stereochemical information on the biological activity of various drugs. The examples were selected with respect to their reliability, sensitivity, uniqueness and trend setting.

Besides the improvement of conventional HPLC-systems the development of microsystems in HPLC promises shorter run times, and high resolution capacities paired with lower consumption of solvents. However, this move is still slow to come. Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) which have already been utilized for some biomedical and clinical applications as reviewed by Deyl and Struzinsky (1991) gain very rapidly in popularity and advances are being made to improve the total method sensitivity.

Supercritical or subcritical fluid chromatography has been applied for enantioseparation on type 1 CSP (Pirkle type CSP) by Macaudiere *et al.* (1989) and on Chiralcel OD by Lee *et al.* (1991), providing shorter analysis times and often higher resolution capacities compared to normal phase liquid chromatography, and of course only very low consumption of organic solvents. However, the applicability to bioanalysis has not yet been demonstrated and has still to be examined.

Direct introduction of biological samples into the HPLC-system in combination with CSPs as described by Tamai *et al.* (1990) for (*R*,*S*)-propranolol seems to be an interesting and elegant method which deserves further investigations and developments. In particular 'tailor made CSPs' in combination with a fully automated column switching system for direct injection of biological samples (this also includes solid phase extraction) could be of great interest, particularly if one considers that in the course of modern drug developments thousands of biosamples have to be analysed.

CONCLUSION

The enormous number of publications dealing with different, stereoselective characteristics of chiral drugs seems more and more to prove that stereoselectivity in biological activity is rather the rule than the exception. Enantioselective bioanalysis is a necessity to produce correct pharmacokinetic data on chiral compounds and furthermore to deal with various possible pharmacokinetic phenomena such as e.g. enantiomer/enantiomer interactions or *in vivo* racemizations. In the recent past increasing attention has also been attached to the individual disposition of drug metabolism—the 'poor and extensive metabolisers' (Eichelbaum and Gross, 1990)—with regard to the consequences in clinical pharmacology.

Enantioselective analytical methods as well as preparative enantioseparation techniques and enantioselective synthesis, respectively, are the basis of 'stereoselective drug development'. The huge advances in these fields should contribute to bringing about the call for optically pure drugs, rather than racemic mixtures (but founded exceptions should be excluded) as already recommended by the FDA (see above).

The well-aimed administration of stereoisomers with regard to the individual disposition of metabolism should greatly contribute to drug safety in the future.

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