Journal of Chromatography, 488 (1989) 53–72
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4557

RACEMATES VERSUS ENANTIOMERICALLY PURE DRUGS: PUTTING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO WORK IN THE SELECTION PROCESS

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SUMMARY

In view of the current negative attitude towards pharmaceutical preparations containing two or more drug substances, it is not surprising that the stereoisomeric composition of drugs has become a key issue in their development, regulatory approval and marketing. As the requirements placed on the purity of drugs become more and more stringent, the question arises of whether a racemic mixture should be automatically considered as being 50% impure. The answer is complex and requires careful comparative evaluations of the activities, toxicities and pharmacokinetics of the two enantiomers. In addition to the risk–benefit factors, the cost–benefit factors should also be carefully weighed in order to reach a decision which will guarantee drug safety. Excessive development costs and an unnecessary regulatory burden resulting from systematic demands for enantiomerically homogeneous drugs can be avoided with very potent drugs administered at low dosages and low-potency drugs for which the "inactive" isomer is not considerably more toxic than the active isomer. Regardless of the final decision taken, during the preclinical and clinical drug development it is important to have analytical methods suitable for conducting pertinent studies. This paper discusses the importance of stereochemistry in drug development and the role of high-performance liquid chromatography as a pre-eminent tool for analytical and preparative enantioselective separations.

INTRODUCTION

The fundamental concepts of stereochemistry and optical activity were set forth by Jean-Baptiste Biot in 1815 and Louis Pasteur in 1848 and were subsequently included in the general theory of organic structure in three dimensions by Van 't Hoff and Le Bel at the time when the general principles of atomic structure now taught in freshman chemistry courses were treated as metaphysical concepts. In one of his lectures before the Council of the Société Chimique de Paris reporting on what is now considered an epochal discovery, Pasteur said, "Most natural organic products, the essential products of life, are asymmetric and possess such asymmetry that they are not superimposable on their images... Thus we find introduced into physiological principles and investigations the idea of this influ-

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ence of the molecular asymmetry of natural organic products of great character which establishes perhaps the only well marked line of demarcation that can at present be drawn between the chemistry of dead matter and the chemistry of living matter" [1].

Indeed, life processes are governed by highly stereoselective processes of inter- and intracellular communication: the discriminatory capacity of enzymes, receptors, carrier molecules, etc., is based on molecular asymmetry. This stereospecificity also applies to the endogenous messenger molecules and substrates whose action is also based on complementary chemistry. This high stereoselectivity of biological systems is reflected not only in the biosynthesis, but also in metabolism, storage and transport processes. It is, therefore, not surprising that the same principles of stereochemistry hold true in the action of pharmaceuticals and pesticides. This paper is concerned only with pharmacologically active substances.

THE STEREOCHEMISTRY OF PHARMACEUTICALS

The stereoisomeric composition of pharmaceuticals is becoming an important issue not only in the course of drug development but also drug approval and clinical use. At present, the pharmaceutical industry is rapidly moving towards precisely targeted, zero-risk drug therapies. However, a rapid survey of the 700 most frequently prescribed drugs shows that half of them contain a chiral centre and approximately half of them are marketed as racemic mixtures [2].

It has been said that "plasma protein binding can function as a biological machine that performs what pharmaceutical companies often neglect: the resolution of drugs into enantiomers" [3]. However, today no formal government regulations exist to define industry requirements in the development of enantiomerically pure drugs. Two diametrically opposed views exist. According to Ariëns [4], "inactive isomers should be regarded as impurities, isomeric ballast, not contributing to the effect aimed at". The situation is aggravated if the drug contains more than one chiral centre because, for example, with a molecule with two asymmetric centres 25:25:25:25 mixtures of stereoisomers are applied. Before advocating one or the other point of view, it is important to discuss briefly the main phases in the biological action of drugs and the possible actions of one isomer of a racemic mixture.

The induction of a desired pharmacological effect is a complex sequence of several chemical and physico-chemical processes, as outlined in Fig. 1. The active

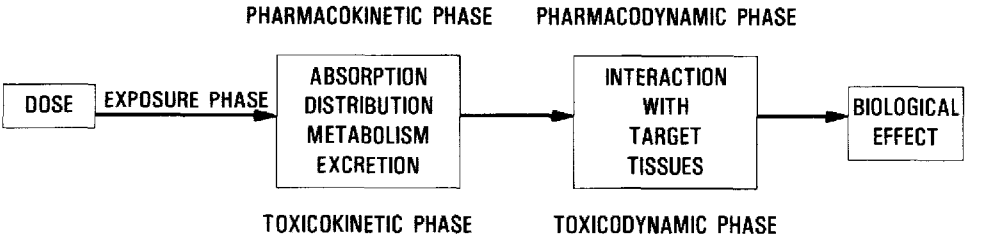

Fig. 1. The main phases in the biological action of drugs.

isomer responsible for the desired action will be referred to as the eutomer and the inactive isomer as the distomer. The exposure phase determines the relationship between the dose applied and the fraction available for subsequent absorption and biological effect. During the pharmacokinetic phase the drug is absorbed, distributed, metabolized and excreted. The exposure phase and the kinetic phase determine the biological activity of the molecule. In the pharmacodynamic phase, the fraction of the molecule available for the pharmacological effect interacts with the molecular sites of action in target tissues such as receptors, carrier molecules, enzymes, antibodies, etc.

Although almost all the steps involved between the exposure and the pharmacological effect are stereoselective, this is particularly true for drug interactions with enzymes (pharmacokinetic phase) and receptors (pharmacodynamic phase). The potential differences between the two enantiomers manifest themselves in an asymmetric environment. The distomer may then exert different actions which are case dependent. The following cases are theoretically possible. (1) Both enantiomers may have similar actions but different affinities for the active sites and, therefore, different intrinsic activities. (2) The two enantiomers may act as competitive antagonists. (3) The two enantiomers may have opposite effects (functional antagonism) not related to competitive antagonism; for example, with certain barbiturates the (−)-isomer acts as a depressant and the (+)-isomer as a convulsant. The distomer can also act as a non-competitive antagonist of the eutomer. (4) The distomer can also inhibit the side-effects of the eutomer; for example, in certain diuretics, the (+)-isomer is responsible for the diuretic action and uric acid retention (undesired effect) while the (−)-isomer acts as a uricosuric. However, in these instances the racemic 1:1 ratio is insufficient for proper therapeutic action of the drug, more favourable ratios being 1:4 or 1:8 [4]. This example demonstrates that even in cases where both enantiomers are needed, their optical ratio is different from that naturally obtained by non-asymmetric synthesis. (5) The unwanted effect may reside only in one isomer while the other isomer is predominantly responsible for the desired therapeutic effect; for example, in ketamine, the (+)-isomer has a hypnotic and analgesic effect whereas the (−)-isomer exerts the unwanted CNS-stimulatory action [4]. (6) Although both enantiomers exert the desired action, the side-effects may reside predominantly in one isomer; for example, timolol is prepared as the optically pure (*S*)-form and officially indicated for the treatment of hypertension and reduction of mortality and reinfarction after heart attack. However, in glaucoma, the (*R*)-isomer is a more potent receptor antagonist in the eye than in pulmonary and cardiac tissues, which makes it preferable as a more ocular-selective antiglaucoma agent [4]. (7) The two enantiomers may exert different pharmacological actions, both of which may be of potential therapeutic interest; for example, in hydroxy-N-methylmorphinan, the (+)-(*d*)-isomer is a potent analgesic whereas the (−)-(*l*)-isomer is an antitussive [4]. (8) One enantiomer may undergo in vivo stereospecific inversion. This phenomenon is encountered with certain non-steroidal anti-inflammatory agents, such as ibuprofen in which the inactive (*R*)-enantiomer undergoes bioactivation into the (*S*)-enantiomer [5]. Further, there is often considerable inter-individual variation in the ratio of the

two enantiomers.

In view of the above, even if the ultimate decision is to market a racemic mixture rather than the enantiomerically pure drug, studies of the pharmacokinetics and clinical pharmacology of a drug should be carried out on separate isomers to justify the decision taken. This leads us to the problem of sometimes tedious and always more costly preparation of pure enantiomers. This problem can be generally solved in four ways: (1) resolution; (2) synthesis by the use of optically pure intermediates (chiral carbon pool); (3) chemically controlled induction of chirality or asymmetric synthesis; (4) chromatography.

Resolution is a classical process whereby a chiral racemic molecule is reacted with an enantiomerically pure agent. The resulting mixture of diastereomers is separated by crystallization and cleaved to recover the agent and individual enantiomers. This largely empirical method is possible only with certain types of functional groups on the molecule to be separated for which suitable enantiomerically pure derivatization reagents exist. In spite of significant recent advances, resolution by crystallization remains an inefficient process: the maximum theoretical yield is 50% and, owing to losses during the process, the actual yield is often significantly lower.

By means of second-order asymmetric transformations, a considerably higher yield can be achieved. By careful choice of reaction conditions, one diastereomer can be induced to crystallize under the equilibrium conditions. After its precipitation, the solution equilibrium is re-established by racemization of the remaining salt. Kinetic resolution employs either a chemical or enzymatic reaction of one member of a racemic pair. Amino acids, alcohols and acids of high enantiomeric purity have been obtained by means of enzymatic conversions.

The second major source of chiral pharmaceuticals is the chiral carbon pool, which uses naturally occurring chiral molecules such as carbohydrates, amino acids and microbiologically derived compounds such as tartaric acid or lactic acid, in addition to the standard building blocks derived from natural substances. This approach relies on the enantiomeric purity of the starting materials.

Asymmetric synthesis is an exciting and continuously growing field of scientific exploration [6,7] which involves chemical or biochemical conversion of a prochiral substrate to a chiral product. The reaction takes place at an unsaturated site having prochiral faces such as C=C, C=N or C=O. This approach has already been used for the synthesis of L-DOPA, used in the treatment of Parkinson's disease, and cilastatin, a broad-spectrum antibiotic. However, although a very valuable tool, it is not applicable in all instances. Further, a 100% chiral excess is rarely obtained.

Finally, thin-layer and liquid chromatographic (LC) preparative resolutions of racemic mixtures are rapidly becoming an alternative to the above-mentioned methods. However, it is LC that in recent years has demonstrated the greatest power, versatility and convenience in preparative applications.

CHROMATOGRAPHIC RESOLUTION OF RACEMATES

All approaches are based on the formation of diastereomeric complexes which are either transient or covalent diastereomeric adducts.

When two chiral molecules, A and B, undergo a reaction that does not affect the asymmetric centre(s), four products can be formed:

$$(R,S)\text{-A} + (R,S)\text{-}B \rightarrow \underset{\mathbf{1}}{(R)\text{-A}\text{-}(R)\text{-B}} + \underset{\mathbf{2}}{(R)\text{-A}\text{-}(S)\text{-B}} + \underset{\mathbf{3}}{(S)\text{-A}\text{-}(R)\text{-B}} + \underset{\mathbf{4}}{(S)\text{-}A\text{-}(S)\text{-}B} \quad (1)$$

The adducts **1** and **4** and **2** and **3** are enantiomers of each other; the adducts **1** and **3** and **2** and **4** are diastereomers. If the corresponding mixture is analysed on a non-chiral stationary phase, two peaks will be obtained as the adducts **1** and **4** and **2** and **3** will have identical distribution properties. If, however, the same racemic molecule is reacted with an enantiomerically pure agent, (*R*)-B, only two products will be obtained:

$$(R,S)\text{-A} + (R)\text{-B} \rightarrow (R)\text{-A}\text{-}(R)\text{-B} + (S)\text{-A}\text{-}(R)\text{-B} \quad (2)$$

Reactions 1 and 2 demonstrate the importance of the enantiomeric purity of chiral selectors if the resulting mixtures are to be separated on a non-chiral phase. In this instance, the use of racemic chiral selectors, partially impure or racemized during storage or chemical derivatization, will lead to erroneous results for the enantiomeric composition of the selectand. For indirect separation methods it is advisable, therefore, to determine the actual extent of enantiomeric contamination of the derivatization agent prior to the reaction. It may be argued that even an enantiomerically impure reagent can be used if the appropriate correction to diastereomeric peak ratios is made. However, this is contingent upon identical reaction rates of the derivatization reaction for the two enantiomers. An alternative is the analysis of the reaction mixture on an enantioselective column, provided that chromatographic conditions are available that permit the separation of all enantiomers.

In addition to the requirements of optical purity, the derivatization agent must also react with the target functional group readily and selectively. The derivatives formed should afford similar detector responses and, preferably, their detectability should be increased with respect to the parent compound. This requirement

TABLE I

TYPICAL DERIVATIZATION REACTIONS

Functional group	Product	Reaction
Amino	Amides	RR′NH + XCOR → RR′NCOR
	Carbamates	RR′NH + ClCOOR″ → RR′NCOOR″
	Ureas, thioureas	RR′ + O=C=NR″ → RR′NCONHR″
Hydroxy	Esters	ROH + NCCOR′ → ROOCR′
	Carbonates	ROH + ClCOOR′ → ROCOOR′
	Carbamate	ROH + R′NCO → ROCONHR′
Carboxy	Esters	RCOOH + R′OH → RCOOR′
	Amides	RCOOH + RNH_2 → RCONHR′

is of utmost importance in drug metabolism and pharmacokinetic studies where usually low drug levels are often encountered.

The most typical chemical reactions involved are outlined in Table I.

THE CHOICE BETWEEN AN ENANTIOMERICALLY PURE DRUG AND A RACEMIC MIXTURE

In reality, the concept of 50% impurity [4] in a racemic mixture is entirely valid only in a limited number of instances as there are relatively few examples of enantiomers having totally different pharmacological activities and/or toxicities. This line of thought is often rationalized with the examples of the difference in excitatory and depressant effects of the enantiomers of barbiturates and the antiarrhythmic potencies and anticholinergic side-effects of the enantiomers of disopyramide [8,9].

In everyday life, more pronounced differences are observed in the pharmacokinetics and potencies of the two enantiomers. However, with very potent drugs with highly stereoselective receptor interaction, the use of a single enantiomer would offer little advantage over the racemate as the dose administered will be small and hence the unwanted pharmacological side-effect would be less pronounced. Conversely, for low-potency drugs, the unwanted side-effect of the "inactive" enantiomer would have to be considered. The final choice between the enantiomerically pure compound and the racemate will have to be made on the basis of the risk–benefit and cost–benefit factors; the risk–benefit factors include toxicity, potency, pharmacology and pharmacokinetics. If the toxicity of one enantiomer is considerably higher, a stereochemically pure drug should be envisaged. However, the situation is rarely clear-cut and several factors may be intertwined. Theoretically, the potency of the eutomer can at best be twice that of the racemate unless the distomer antagonizes the desired action. The pharmacokinetic profile should include parameters such as half-life, the area under the curve, the ability to cross the blood–brain barrier and first-pass metabolism, etc.

Regardless of the approach used in preparing an enantiomerically pure substance, the production costs are always more elevated than the costs of producing a racemic mixture. If a stereoselective synthesis is used, for example, the starting materials will be more costly than for an ordinary synthesis and the overall yield will be lower. It has been estimated [10] that the actual production costs of an enantiomer are at least twice as high as those for a racemate. Hence it is very important also to estimate the cost–benefit factors when deciding on one stereoisomer versus a racemate. These include the development costs, manufacturing costs and willingness of the customer to pay for a more expensive product. It is important to dispel the widespread belief that the resources in industry are limitless owing to high benefits. The extremely elevated development costs, often unjustifiably increased, the regulatory burden and the inevitable modifications to social security programmes have resulted in dramatic changes in the pharmaceutical industry. Hence all factors should be carefully weighed before the final decision is taken.

Nevertheless, as stated before, regardless of the final choice, during the drug

development it is extremely important to have analytical methods suitable for conducting pharmacological studies. This paper highlights the more recent developments in the field of chiral high-performance liquid chromatographic (HPLC) separations with special reference to compounds of pharmacological interest.

CHIRAL HPLC SEPARATIONS

Principle of chiral recognition

The first model for chiral recognition in chromatography, originally developed by Dalgliesh in 1952 [11] and subsequently re-examined by Pirkle and Finn [12] and Davankov and Kurganov [13], is based on a "three-point interaction" between the solute and the chiral stationary phase (CSP). This simplified model of chiral recognition originates from the Cahn–Ingold–Prelog convention according to which at least three of the four bonds of the chiral carbon atom are needed to determine the configuration. According to this model, at least three interactions between the solute and CSP must be involved in the chiral recognition model. The interactions between the chiral selector and selectand, which may be either attractive or repulsive, may involve hydrogen bonding, dipole–dipole, π–π, electrostatic, hydrophobic or steric interactions. However, if one interaction defines an axis rather than a point (e.g., interactions between two aromatic rings), only two interactions may be sufficient. The highest enantioselectivity will be obtained when the three interaction points (one of which must be stereochemical) are on the asymmetric centre or in close proximity. The degree of enantioselectivity decreases with increasing distance between the chiral centre and the interaction groups because of the smaller difference between the average conformations of the transient diastereomeric adducts.

More sophisticated models based on NMR studies [14], X-ray crystallography [15] and molecular mechanics have been used to investigate the mechanism of chiral recognition. For example, by means of computer graphics of X-ray crystallographic data, Armstrong et al. [15] predicted and rationalized the separation of the enantiomers of propranolol on β-cyclodextrin. However, the success of these predictions and their validity is as yet uncertain because of the complexity of the interactions at the molecular level.

Methods of separation

Chiral HPLC separations can be achieved by indirect analysis or by direct analysis. The indirect separations are based on diastereomeric derivatization prior to chromatography. As the diastereomers formed have different physical and chemical properties, they are separable on conventional columns. This method eliminates the need for the expensive chiral HPLC columns but suffers from a certain number of limitations which have been discussed above.

Most chiral separations are performed by the direct methods which rely on the temporary formation of short-lived diastereomeric adsorbates. There are two different approaches to the direct separation of chiral compounds: (a) the use of achiral stationary phases and chiral mobile phase additives and (b) the use of chiral stationary phases and achiral mobile phases.

The former stereoselective separations involve the addition of a chiral complexing agent to the mobile phase and proceed via one or more of the following mechanisms: (i) stereoselective complexation in the mobile phase, (ii) stereoselective binding of the chiral selector to the stationary phase with subsequent "dynamic" complexation and (iii) formation of diastereomeric adducts with different distribution properties between the mobile and stationary phases.

This separation mode circumvents the need for a chiral column and retention can be regulated by varying the concentration of the chiral additive. The elution order can be controlled by using either the (*S*)- or the (*R*)-enantiomer of the selector. Chiral complexation is possible even with enantiomerically pure selectors. However, the optical impurity affects the separation factor adversely [16]. Fig. 2 illustrates the separation of the enantiomers of propranolol with D- and L-N-benzoxycarbonylglycylproline of varying enantiomeric purity. Most of these separations are carried out in the normal-phase mode as aprotic solvents promote the formation of chiral adducts. Usually, the mobile phases reported contain dichloromethane; alcohols and water, in contrast, decrease the stereoselectivity [17].

Chiral ion-pairing agents such as 10-camphorsulphonic acid and modified di-

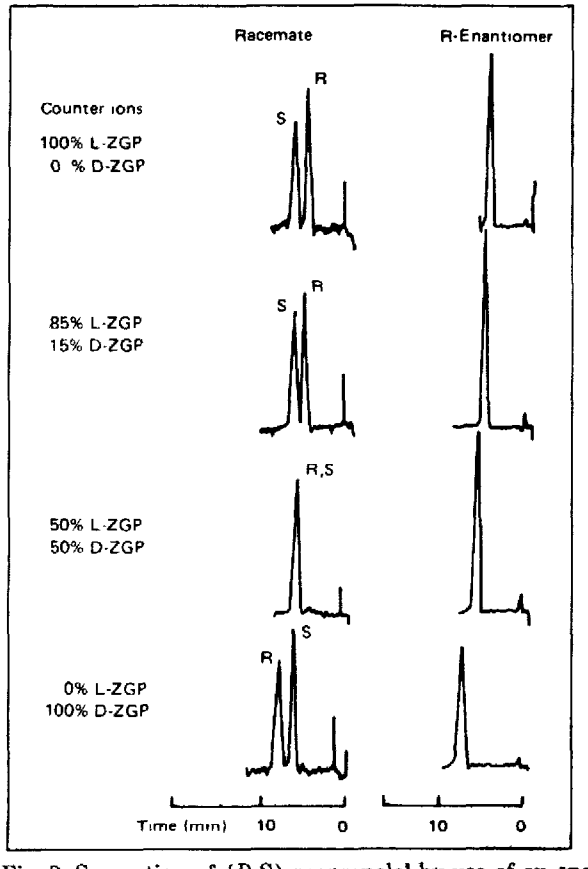

Fig. 2. Separation of (*R,S*)-propranolol by use of an enantiomerically impure chiral counter ion. Stationary phase, LiChrosorb DIOL; mobile phase, 2.25 m*M* mixture of D- and L-N-benzoxycarbonylglycylproline (ZGP) and 0.25 m*M* triethylamine in dichloromethane (containing 80 ppm of water). Reproduced from ref. 17 with permission.

peptides have been used for the resolution of β-amino alcohols; amines such as quinine and quinidine have been used for the separation of acids with hydrogen-bonding functions [18]; tartaric acid mono-*n*-octylamide was used for the resolution of amino acid enantiomers and some amino alcohols [19]; cyclodextrins [20], proteins [21], chiral bidentate ligands, such as L-proline, and L-histidine, L-phenylalanine or L-valine and transition metals such as Cu^{II}, Zn^{II}, Ni^{II} or Cd^{II}, have been used for the separation of α- and β-amino acids [22,23], α-hydroxy acids [24], thyroid hormones [24], dipeptides [24], etc. The technique, pioneered by Davankov and co-workers [23,25] and known as chiral ligand-exchange chromatography, will not be discussed further, because of its limited use for the separation of pharmaceutical products.

Chiral stationary phases

At present, there is no universal CSP equivalent to reversed-phase packings. Therefore, analytical chemists are often faced with the problem of choosing the most suitable stationary phase for a particular application among approximately 33 chiral columns commercially available at present. As almost all chiral columns are still expensive, it is important to select the most suitable phase in order to avoid unnecessary expense. With the growing body of knowledge about the different mechanisms of chiral recognition and the increasing number of applications, the classification of phases according to the types of interactions involved and the establishment of general guidelines for the choice of a column based on molecular structure are of great importance. This subject has recently been treated by Wainer [26] and Krstulović [24].

CSPs can be classified into four categories: Pirkle-type phases (type I), chiral polymers (type II), affinity phases (type III) and chiral ligand-exchange phases (type IV). The distinction between type I and II phases is based on the difference in the location of the interactive sites. Only the most commonly employed phases will be discussed.

Type I phases. These phases were developed by Pirkle and Finn [12]. The most commonly used is the (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine phase prepared by reacting the α-amino group of (*R*)-phenylglycine with 3,5-dinitrobenzoyl chloride.

The latter reagent is responsible for π–π interactions and the amide bond for hydrogen bonding and dipole stacking interactions. The product of this reaction is then ionically or covalently bonded to the aminopropyl functionalized silica. They are commonly used with non-aqueous mobile phases composed of hexane and an alcohol as a polar modifier. The active forces are hydrogen bonds, π–π interactions and dipole–dipole interactions. Often, derivatization of the solute is required in order to introduce functional groups that are essential for chiral recognition; thus amines must be converted to amides [27] or carbamates [28] and carboxylic acids to esters [29] or amides [27]. Compounds with α,β-amino alcohol functionalities are often separated after conversion to the corresponding oxazolidone [30] and oxazolidine derivatives [31].

In order to maximize the π–π interactions, the most commonly used derivatization reagents contain a naphthyl moiety (when 3,5-dinitrobenzoyl CSPs are used) or a 3,5-dinitrobenzoyl moiety (when naphthylalanine CSPs are used).

Relatively few examples of separations of drugs on these columns are available; α-methylarylacetic acid-based anti-inflammatory agents [5], barbiturates, hydantoins and glutarimides [30] have been separated. Strict structural requirements imposed on the chiral solute in order to achieve structural complementarity with the stationary phase, needed for chiral recognition, seriously reduce the number of applications. The general attitude among chromatographers is that if a derivatization step is needed, it is more convenient to form covalent diastereomeric compounds which can then be separated on a conventional column.

Recently, these phases have been used in supercritical fluid chromatography (SFC) for the separation of amino acid derivatives [32]. However, the selectivity was found to be lower than in conventional chiral HPLC, probably owing to the adsorption of carbon dioxide molecules on the stationary phase. However, the use of SFC for chiral separations is a promising field as it operates under more favourable kinetic conditions owing to decreased solvent viscosity and thus higher solute diffusivity. Therefore, increased column efficiencies are to be expected. This has recently been confirmed by Steuer et al. [33], who used SFC for the separation of amino alcohols as diastereomeric ion pairs.

Type II phases. In 1973, Hesse and Hagel [34] used triacetylcellulose for the semi-preparative and preparative chromatographic separation of enantiomers. However, these polysaccharide derivatives did not have sufficient mechanical stability for their use in HPLC. Recently, several very promising polysaccharide phases have been developed by Okamoto's group [35,36] by impregnating silica with the various polysaccharide derivatives (Table II). Relatively little is known about the exact mechanism of chiral recognition, although it is generally admitted that the formation of diastereomeric complexes involves hydrogen bonding, π–π and dipole interactions. These interactive sites are located within the cavities, rather than on the surface as in type I phases. These phases can be used with eluents composed of a non-polar solvent (hexane) modified with an alcohol or with polar eluents (e.g., ethanol); chlorinated solvents should be avoided.

The morphology and the type of ccellulose used were shown to be important for chiral recognition [37]; however, the molecular mass of the cellulose derivative, the solvent used for depositing the phase on the support and the nature of the support were found to have an effect. Most compounds succesfully separated on these phases have a rigid structure and bulky groups close to the chiral centre. Compounds with polar groups such as carboxylic acids and primary amines are usually derivatized prior to chromatography. Among examples of drug separations, the most important are those of β-adrenergic blocking agents which can be separated underivatized on tris(3,5-dimethylphenylcarbamate)-cellulose (Chiralcel OD; Daicel Chemical Industries) [36,38–40]. β-Adrenergic blocking agents used in the treatment of hypertension contain in their general formula **5** a chiral centre in position 2 (*):

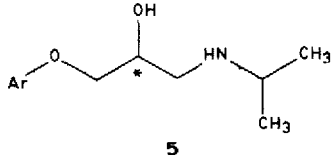

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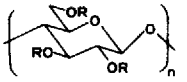
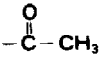
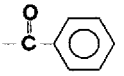
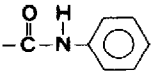
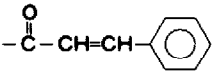
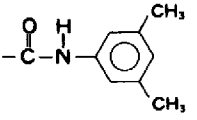
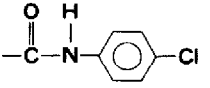
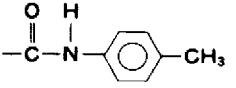
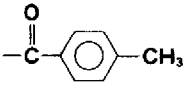
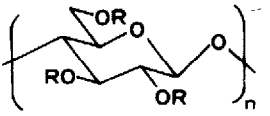
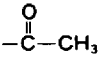
TABLE II

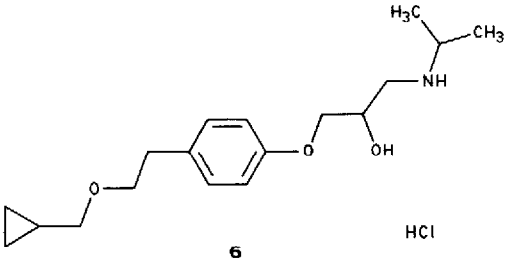
POLYSACCHARIDE STATIONARY PHASES: STRUCTURES, CHROMATOGRAPHIC CONDITIONS AND SELECTED APPLICATIONS

Courtesy of Daicel Chemical Industries.

Column	Adsorbent	R	Separation	Mobile phase
				
	Coated on silica gel			
Chiralcel OA			Compounds containing aromatic, carbonyl, nitro, sulphinyl, cyano, hydroxy group, etc.	Hexane–2-propanol or ethanol
Chiralcel OB				
Chiralcel OC				
Chiralcel OK				
Chiralcel OD				
Chiralcel OF			Aromatic drugs and agrochemicals	Hexane–2-propanol
Chiralcel OG				Hexane–2-propanol or hexane–ethanol
Chiralcel OJ				Hexane–2-propanol or ethanol
				
Chiralcel CA-1				Ethanol (+water)

Although many of these drug substances are developed and marketed as racemic mixtures of both enantiomers, for some of them only one enantiomer, usually the (*S*)-isomer, is preferred as it has been demonstrated that the principal pharmacological effect was due to the (*S*)-enantiomer. Further, the hepatic oxidation of drugs such as propranolol, metoprolol, alprenolol and bufuralol is highly stereospecific [39]. It is, therefore, important to have a method for the precise and accurate determination of the enantiomeric excess of bulk drugs obtained through resolution or enantiospecific synthesis and the determination of the metabolic disposition of racemic drugs.

Betaxolol, (±)-1-{4-[2-(cyclopropylmethoxy)ethyl]phenoxy}-3-isopropylamino-2-propanol hydrochloride (**6**), is a cardioselective β-adrenergic blocking



6

agent characterized by a high biovailability (90%) and a long half-life of about 15 h in man [41]. The drug, marketed as a racemic mixture under the name Kerlone, is highly efficacious for the treatment of hypertension. The same drug substance, used for the treatment of glaucoma, is marketed under the name Betoptic. The only previously reported method for the resolution of enantiomers of betaxolol required a lengthy derivatization with (*R*)(−)-1-(1-naphthyl) ethylisocyanate.

We therefore developed a direct method for the routine control of the bulk drug and the analysis of enantiomers of betaxolol in rat hepatocyte suspensions. The best results in terms of resolution, peak symmetry and analysis time were obtained with the mobile phase hexane–2-propanol–diethylamine (87:13:0.05, v/v/v), with $R_s > 2$ and asymmetry factors for the two peaks equal to 1 (Fig. 3a). The addition of diethylamine markedly improved the efficiency of the separation and peak symmetries (Fig. 3b). In all instances the (*R*)-enantiomer was eluted before the (*S*)-enantiomer, thus resulting in a high precision of the determination of the enantiomeric excess of (*S*)-betaxolol (Fig. 3c).

This separation method was also applied to the determination of betaxolol in rat hepatocytes. Fig. 4 shows the chromatograms of a control rat hepatocyte suspension and a suspension, at time zero, containing equal amounts of both enantiomers (2.5 μg/ml betaxolol). No interfering endogenous peaks were detected. This chromatographic technique therefore permits the relative and absolute concentrations of the enantiomers to be followed over the incubation time (<2 h) necessary to metabolize the compounds completely. The resulting concentration–time data allow a comparison of the relative rates of hepatic metabolism of the two enantiomers.

Fig. 5 shows the highly efficient separation of cicloprolol on a Chiralcel OD column. Other major β-blockers have been resolved on the same stationary phase [24].

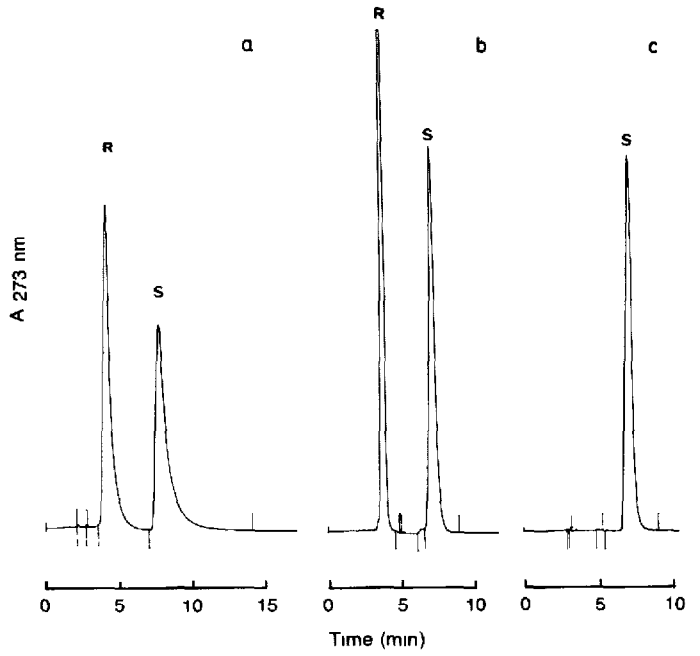


Fig. 3. Enantiomeric separation of betaxolol β-adrenergic blocking agent. (a) and (b) Racemic mixture; (c) (*S*)-betaxolol bulk drug. Chromatographic conditions: (a) mobile phase, hexane–2-propanol (87:13, v/v); column, Chiralcel OD (250×4.6 mm I.D.; 10 μm average particle size); (b) and (c) mobile phase, hexane–2-propanol–diethylamine (87·13:0.05, v/v/v); flow-rate, 1.5 ml/min; detection, 273 nm. Concentration: 2 mg/ml in mobile phase.

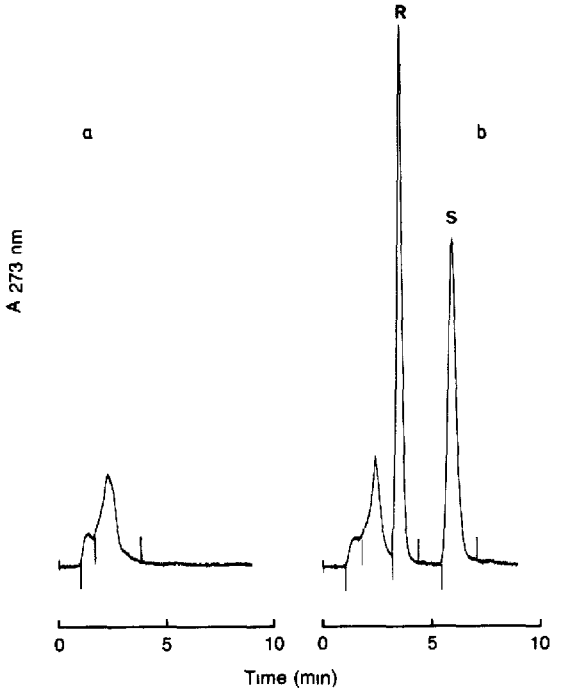


Fig. 4. Analysis of rat hepatocytes: (a) control rat hepatocytes suspension; (b) suspension containing 2.5 μg/ml betaxolol. Chromatographic conditions as in Fig. 3a. Reproduced from ref. 40 with permission.

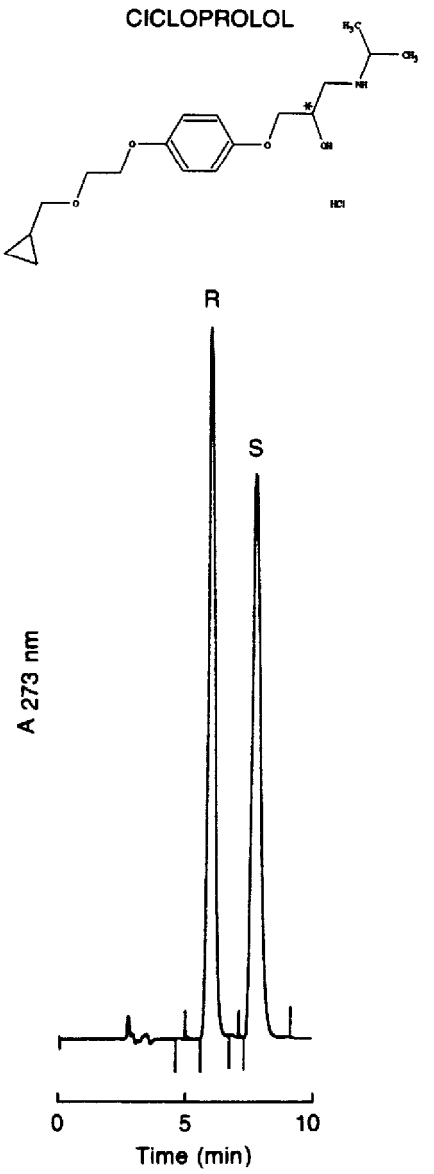


Fig. 5. Enantioselective HPLC separation of cicloprolol, an anti-angina pectoris drug. Chromatographic conditions: column, Chiralcel OD (250×4.6 mm I.D.; 10 μm average particle size); mobile phase, hexane–2-propanol–diethylamine (80:15:0.05, v/v/v); flow-rate, 1.0 ml/min; detection, 280 nm.

The α-, β- and γ-cyclodextrins (CDs) are cyclic oligosaccharides composed of 6–8 D-glucose units linked together through α-(1,4) linkages. The molecule has the shape of a hollow truncated cone and contains 30–40 chiral centres. Whereas the openings of the cone are rimmed with secondary and primary hydroxy groups, the interior is relatively hydrophobic. The chiral recognition afforded by these phases is based on the inclusion of the solute molecules in the chiral cavity of the stationary phase. Hydrophobic interactions, hydrogen bonding and steric interactions are operative on these phases, which are used with buffered aqueous–

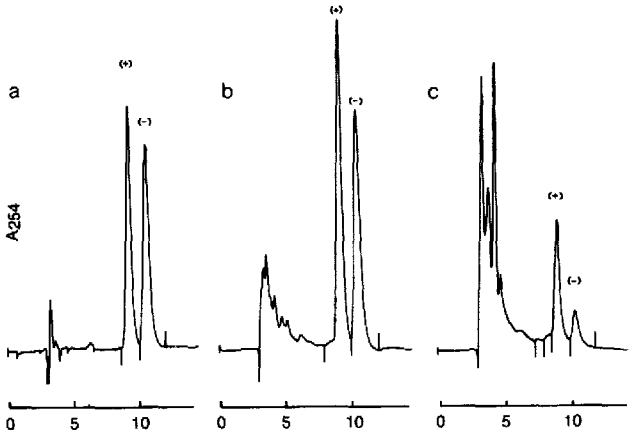


Fig. 6. Enantioselective HPLC separation of an anti-inflammatory agent. (a) Racemic mixture; dichloromethane extract of rat plasma (b) 3 h and (c) 6 h after administration of the drug. Chromatographic conditions: column, Cyclobond I (β-cyclodextrin) (250×4.6 mm I.D.; 10 μm average particle size); mobile phase, 0.05 *M* KH_2PO_4 (pH 5.3)-methanol (35:65, v/v); flow-rate, 1.0 ml/min; detection, 254 nm.

organic eluents containing methanol, ethanol or acetonitrile as the organic modifier. Retention is controlled by means of pH, type of buffer and organic modifier used and the temperature.

These phases are compatible with other reversed-phase systems. This is advantageous for the analysis of biological matrices where the separation selectivity needed is often achieved through coupled-column systems [42]. Preparative separations are also possible. When used with non-polar eluents, these phases are achiral and give selectivities similar to those of diol phases, owing to the solvent competition for the hydrophobic interior of the cavity.

The use of this type of column is illustrated with the analysis of the rat plasma levels of the enantiomers of an anti-inflammatory agent. Fig. 6a shows the separation of a reference racemic mixture and Fig. 6b and c the enantiomers in a dichloromethane extract of rat plasma 3 and 6 h after administration of the drug. It is evident that the (−)-enantiomer is metabolized faster than the (+)-enantiomer. Other polymers which have been immobilized on silica and used as chiral stationary phases are poly(triphenylmethyl methacrylate) and acryloyl-(*S*)-phenylalanine.

Type III phases. These phases utilize the stereo-differentiating properties of proteins. Currently, the two most useful chiral protein columns are human α-acid glycoprotein (EnantioPac, chiral AGP), originally developed by Hermansson [43], and bovine serum albumin (Resolvosil), developed by Allenmark et al. [44]. As a number of carboxylic acid moieties are tied up in the binding to the silica, the stereospecificity of the protein is modified with respect to the native protein.

The enantioselective properties based on principles of bioaffinity of the two proteins involve a combination of hydrogen bonding, electrostatic interactions

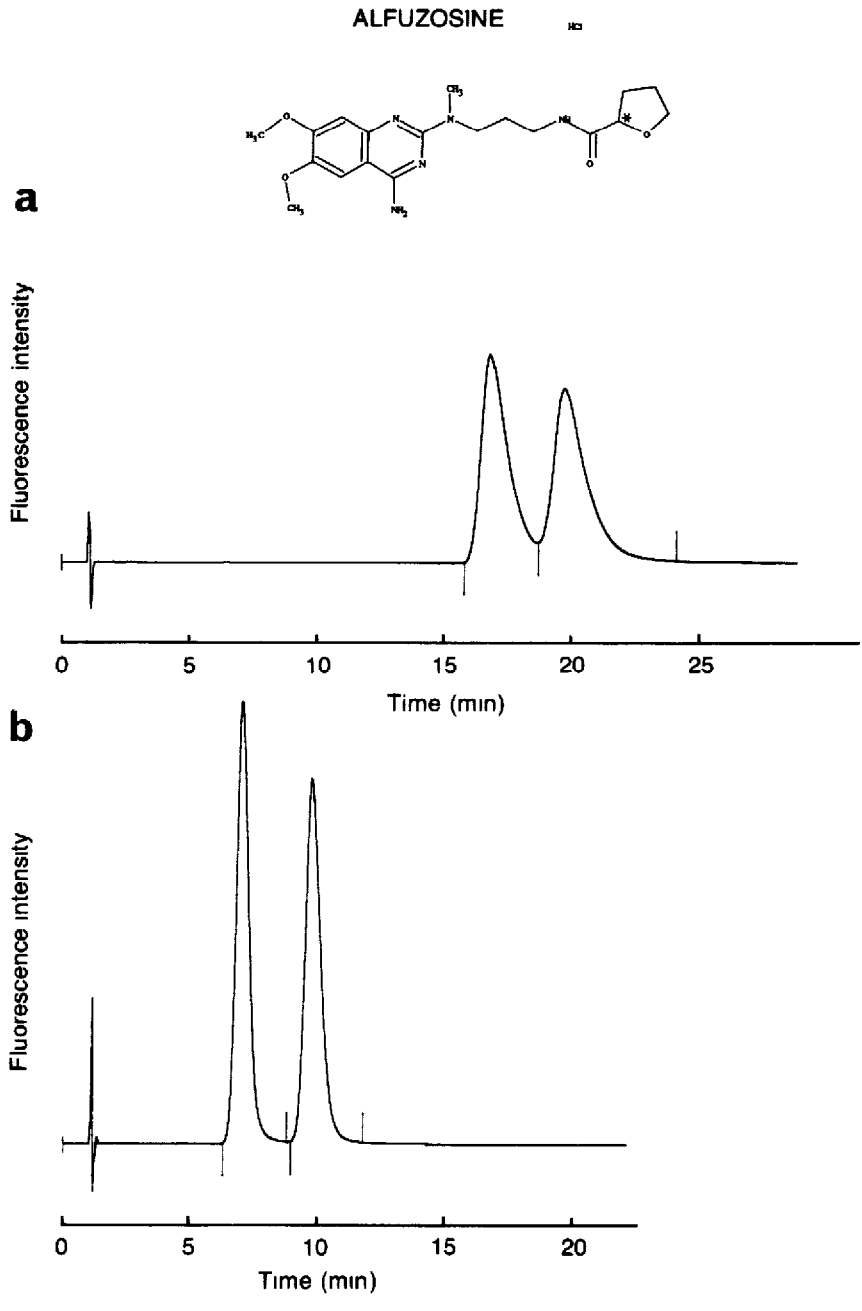

ALFUZOSINE
a
Fluorescence intensity
0
5
10
15
20
25
Time (min)
b
Fluorescence intensity
0
5
10
15
20
Time (min)

Fig. 7.

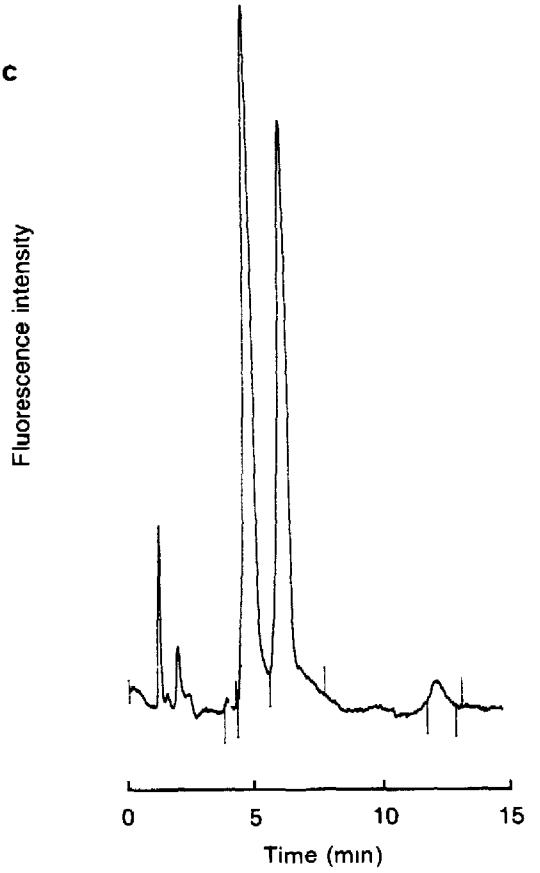


Fig. 7. Enantioselective HPLC separation of alfuzosine antihypertensive agent. (a) and (b) Reference racemic mixture; (c) dichloromethane extract of rat plasma 30 min after administration of the drug. Chromatographic conditions: column, Chiral AGP (100×4.0 mm I.D.; 5 μm average particle size; ChromTech, Sweden); mobile phase, (a) 0.1 *M* ammonium acetate (pH 7.5)–2-propanol–acetonitrile (96·2·2, v/v/v); (b) 0.1 *M* ammonium acetate (pH 7.5) (containing 30 mmol/l tetrabutylammonium bromide)–2-propanol–acetonitrile (96:2·2, v/v/v); (c) 0.1 *M* ammonium acetate (pH 7.5) (Containing 30 mmol/l tetrabutylammonium bromide)–2-propanol–acetonitrile (93:2:5, v/v/v); fluorimetric detection, excitation 265 nm, emission 380 nm; flow-rate, 0.9 ml/min.

and hydrophobic interactions. In addition, charge-transfer interactions may also be operative on BSA and ion-pairing on AGP. Hence it is difficult to derive a detailed model of chiral recognition. Nevertheless, a prerequisite for optical resolution on BSA columns is the presence of aromatic and relatively polar groups in the racemic compound. Steric effects also seem to be highly important and account for the largely unpredictable results from substituent variation.

Both columns are normally used with buffered aqueous–organic eluents. Retention can be controlled by means of pH, ionic strength of the buffer, polarity of the organic modifier and cationic (amines, quaternary ammonium salts) or anionic (aliphatic organic acid) additives. These phases have been used for the separation of a variety of underivatized anionic and cationic solutes, mostly drugs.

Both columns exhibit excellent selectivities but limited efficiencies and sample capacities. Preparative separations are difficult.

The second-generation AGP columns have considerably better mechanical stability, which makes it possible to use higher flow-rates (up to 1 ml/min vs. 0.5 ml/min), and better chemical stability, which allows the use of solvents such as acetonitrile, methanol, ethanol, 2-propanol, 1-propanol and aqueous buffers in the pH range 3.5–8. The column efficiencies are considerably higher and resolution factors of at least 2 are commonly encountered.

The use of the newly developed [45] chiral AGP column is illustrated with the separation of enantiomers of alfuzosine, N-{3-[(4-amino-6,7-dimethoxy-2-quinazolinyl)methylamino]propyl}tetrahydro-2-furancarboxyamide hydrochloride (Fig. 7a), an antihypertensive agent. Fig. 7a and b show the dramatic effect of tetrabutylammonium bromide (TBA) on the separation of the enantiomers of alfuzosine; at pH 7.4, the protein is negatively charged, resulting in strong interactions with the quinazoline ring and, therefore, excessively long retention times. On addition of TBA these interactions are diminished while a satisfactory resolution is maintained.

Fig. 7c shows the enantiomers of alfuzosine in a sample of rat plasma. Owing to the presence of an excellent fluorophore (quinazoline), fluorimetric detection is ideally suited to the determination of alfuzosine in biological samples.

CONCLUSIONS

The remarkable enantioselectivity exhibited by biological systems, which is reflected in the biosynthesis, metabolism, storage and transport processes, is well documented in the literature. It is, therefore, not surprising that the stereochemistry of drugs has become the focus of intensive research in many laboratories. The concept of 50% contamination has been advocated for drug substances containing one chiral centre and it is expected that regulatory agencies will continue to focus their attention on this issue. However, this view should be considered as extreme, because with the development of highly potent drugs the doses administered decrease and hence the probability of an unwanted side-effect also decreases. Exceptions include drugs with poorly defined pharmacological effects or those for which only the adverse effect would be stereoselective.

Nevertheless, during the development of a new drug containing a chiral centre it is important to evaluate critically the potencies, toxicities and pharmacokinetic properties of individual enantiomers in order to guarantee the safety of the pharmaceutical preparation.

Enantiomeric separations by HPLC are rapidly becoming an efficient tool for both analytical and preparative separations needed in preclinical and clinical investigations. At present, a universal chromatographic system does not exist. However, a large selection of commercially available columns permit the separation of all the major classes of organic compounds. In spite of the recent advances in this field, two major problems exist: (1) most chiral columns offer limited efficiency, which results in peak dispersion and lower detection sensitivities; and (2) there is an urgent need for more specific detection devices to avoid false

enantiomeric peaks in biological extracts; chiroptic detectors such as polarimeters with laser sources or circular dichroism detectors have not yet given satisfactory results. The problem of sometimes inadequate column selectivities can be circumvented by using highly selective extractions or by column-switching techniques.

The major future development should be in the field of detection and scale-up of separations on less expensive packing material.

ACKNOWLEDGEMENTS

The author thanks Dr. Alexander Wick, Director of the Chemistry Department at Synthelabo Recherche (L.E.R.S.), for his timely advice, the Analytical Group for technical support and Ms. Claire Delavault for the preparation of the manuscript.

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