Stereoisomeric separations

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Use of chiral stationary phases to resolve molecules of pharmacological interest

Recent advances in the resolution of stereoisomers by high performance liquid chromatography on chiral stationary phases (HPLC-CSPs) provide the capabilities for rapid analysis of the enantiomeric composition of pharmaceutical preparations. With the commercially available HPLC-CSPs developed by W.H. Pirkle (Department of Chemistry, University of Illinois at Urbana-Champaign), it is possible to measure rapidly and accurately the stereoisomeric composition of a number of pharmacologically active substances, both before and after their administration.

Studies concerned with the isomeric composition of pharmaceuticals are important for enantiomers that differ in potency, pharmacological actions, or plasma disposition kinetics, or in cases in which one isomer is converted in vivo to the other. Examples of drugs with enantiomers having these differences include propranolol — the second most prescribed drug in the United States (1) — whose (*S*)-enantiomer is 100 times as potent as the (*R*)-isomer, which is, in turn, metabolized faster than the (*S*)-form (2,3). Nonsteroidal anti-inflammatory agents such as ibuprofen and naproxen (numbers 8 and 23 in the U.S. market, respectively) (1) also show differences in potency and metabolism between their isomeric forms. In these cases, the (*S*)-isomers are the active forms; the inactive (*R*)-enantiomers are activated in the body by their conversion into (*S*)-enantiomers (4,5).

The magnitude of the analytical problem involved is reflected by the report that in the United States in 1982, 12 of the 20 most prescribed drugs and 114 of the top 200 drugs prescribed contained at least one asymmetric center (1). Each molecule presents the chemist and the pharmacologist with a separate question of how to determine its enantiomeric composition.

Traditionally, one of the major analytical approaches to this question has involved the measurement of the specific rotation of a solution of the enantiomer or the racemic mixture. These procedures are often lengthy and are most often inexact. Consider, for example, the official method for the determination of the stereochemical purity of dextroamphetamine as presented in *The United States Pharmacopeia* (*USP XX*) (6). The current USP monograph requires preparation of an analytical solution of the drug containing 400 mg/10 ml for the determination of specific rotation, yet the analysis affords results accurate to only ±12%. In this procedure, the equivalent of more than 25 tablets must be ground and triturated with 1 ml of methanol during sample preparation; the entire sample must then be taken through a cumbersome procedure prior to the final determination.

By contrast, with the Pirkle-type HPLC-CSP, the stereochemical purity of dextroamphetamine can be determined in 1 hr by using just one 10-mg tablet. As little as 0.5% levoamphetamine can be detected in the presence of 99.5% dextroamphetamine (7) (Figure 1).

THE RESOLUTION OF ENANTIOMERS AS DIASTEREOMERS

The resolution of enantiomers on HPLC-CSPs is an extension of, as well as a significant departure from, previous work on the separation of enantiomers as diastereomers. The physical properties of *d*- and *l*-enantiomers are identical, but chemical reactions that add another chiral center create a diastereomeric pair, each of which has distinct physical properties. (See Figure 2.) Therefore, although an enantiomeric pair cannot be separated by ordinary chromatographic means, the diastereomeric pair can often be separated easily.

For example, *d*- and *l*-mandelic acids can be readily resolved by first converting them to their 2-octyl esters with *d*-2-octylbromide. The resulting diastereomeric esters (*d*,*d*- and *d*,*l*-esters) can be separated on silica gel by using a hexane/methylene chloride mobile phase (8).

The applicability of diastereomeric methods to routine assays is hindered by enantiomeric contamination of the derivatizing agent, which leads to false determinations. One of the standard methods for the determination of the enantiomeric composition of propranolol, for example, involves the synthesis of amides using (−)-*N*-trifluoroacetyl-l-prolyl chloride (TPC) (9–11). Silber and Riegelman (9) found that commercial TPC was contaminated with from 4% to 15% of the (+)-enantiomer and that the reagent rapidly racemized during storage. Efforts to synthesize optically pure TPC were unsuccessful.

An additional complication is that enantiomers can have quite different rates of reaction and/or equilibrium constants when they react with another chiral molecule. As a result, the generation of two diastereomeric products in proportions different from the starting enantiomeric composition may occur (12).

ADVANTAGES OF RESOLVING ENANTIOMERS AS ENANTIOMERS

Both of these problems can be avoided by resolving the enantiomeric pairs as enantiomers. Consider the cases of enantiomeric amines and carboxylic acids. Both classes

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of compounds exhibit poor chromatographic properties, whereas the amide derivatives, which can be made from either class, are in general more suitable for chromatographic separations.

If the derivatizing reagent contains an asymmetric center, with the consequent production of diastereomers the procedure is subject to all the shortcomings described above. By contrast, if the reagent is achiral (symmetric), the problems of stereochemical contamination and different reaction rates are avoided.

This direct enantiomeric approach has added benefits. There are a large number of readily available achiral reagents that may be chosen to enhance detectability or to otherwise improve chromatographic properties. In addition, because the difference in relative reaction rates is not a factor, the chosen reaction does not need to be quantitative. And, of course, in some cases the enantiomeric substances of interest can be resolved per se; that is, without derivatization.

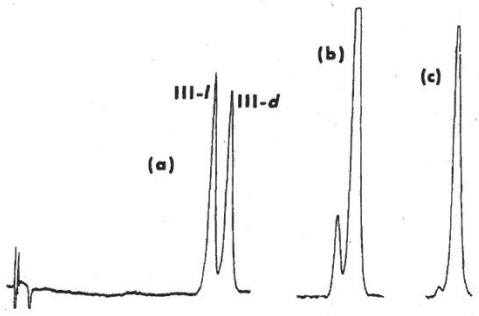

FIGURE 1: HPLC chromatograms of the naphthoylamide derivatives of *l*-amphetamine (III-*l*) and *d*-amphetamine (III-*d*) mixtures: (a) 50:50 III-*l*/III-*d*, (b) 10:90 III-*l*/III-*d*, and (c) 1:99 III-*l*/III-*d*.

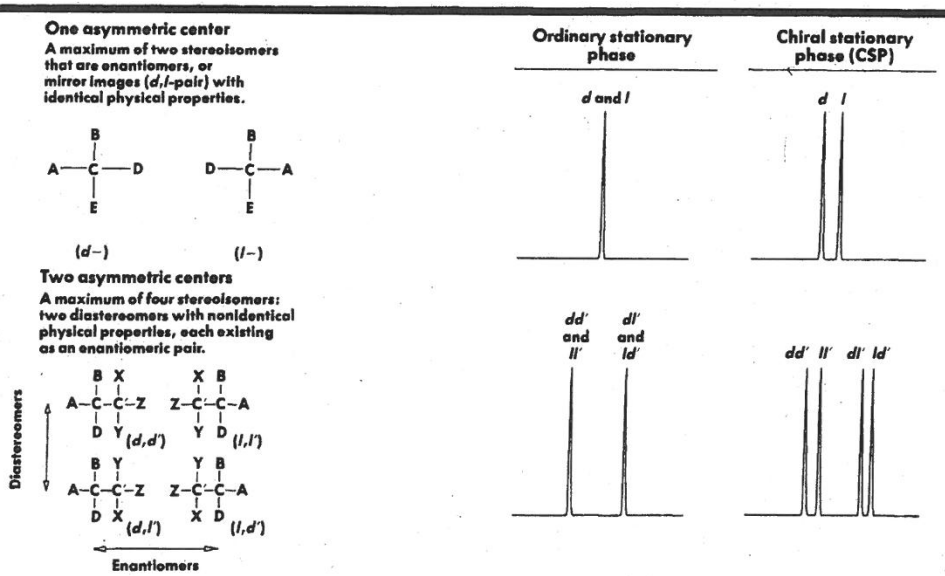

FIGURE 2: Stereochemical properties and chromatographic behavior of enantiomers and diastereomers.

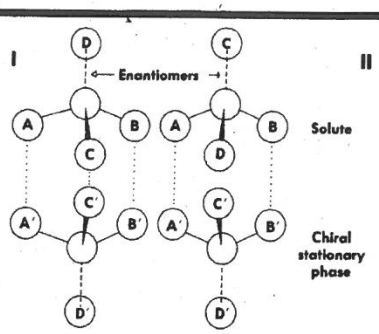

FIGURE 3: Three-point chiral interaction model.

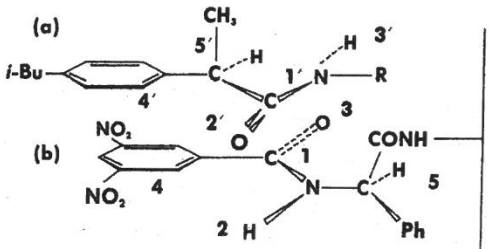

FIGURE 4: Proposed interaction model between the 1-naphthalenemethylamide of (*R*)-ibuprofen and the covalently bonded Pirkle-type HPLC-CSP.

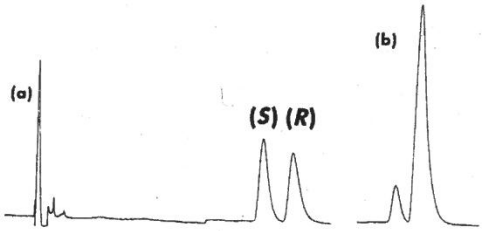

FIGURE 5: Resolution of enantiomeric mixtures of ibuprofen 1-naphthalenemethylamide; (a) racemic mixture, (b) 9:91 mixture of the (*S*)- and (*R*)-enantiomers.

THEORY OF HPLC RESOLUTION OF ENANTIOMERS AS ENANTIOMERS

One approach to this type of direct enantiomeric resolution in HPLC uses the formation of temporary diastereomeric complexes between the solute enantiomers and a specially constructed enantiomeric stationary phase bonded to silica (the CSP). The difference in stability between the diastereomeric complexes leads to a difference in retention time between the two enantiomers; that is, the solute enantiomer that forms the less stable diastereomeric complex with the CSP will elute first.

The CSPs are designed on the basis of the three-point chiral recognition model proposed by Dalgliesh (13), who postulated that chiral recognition requires a minimum of three simultaneous interactions between the CSP and solute. At least one of these interactions must be stereochemically controlled and may be either attractive or repulsive. The relative strengths of the resulting diastereomeric complexes determine the resolution and elution order of the two enantiomers. This situation is depicted in Figure 3. In this case, enantiomer (I) interacts with the CSP at sites A · · · A′, B · · · B′, and C · · · C′, whereas its mirror image, enantiomer (II), lacks the C · · · C′ interaction. If the C · · · C′ interaction is an attractive interaction, enantiomer (I) will be retained on the column longer than (II). If the C · · · C′ interaction is repulsive, however, then the diastereomeric complex involving enantiomer (II) is more stable and (I) will be eluted first. If C and C′ interact minimally or not at all, then no separation will be observed; this phenomenon is the key concept of the three-point model.

PIRKLE'S HPLC-CSP

This general approach is incorporated into the CSPs designed by Pirkle and co-workers (14,15). One of the CSPs that they have developed uses (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine, which is bound to a γ-aminopropyl packing through either ionic or covalent bonds. Figure 4b depicts the covalently bound modified CSP. This CSP has a number of possible sites for interaction (Figure 4b):

- the dipole formed by the amide linkage between the 3,5-dinitrobenzoyl (DNB) moiety and the phenylglycine amine (site 1)
- the amide hydrogen, which is available for hydrogen bonding (site 2)
- the amide carbonyl, which is available for hydrogen bonding (site 3)
- the 3,5-DNB ring, which is electron-poor and available for π-π bonding with other aromatic rings (site 4)
- the carbonyl and phenyl groups on the phenylglycine moiety, which can interact with the solute either attractively (through mechanisms similar to those above) or repulsively (through their relative steric bulk) (site 5).

Various combinations of these sites provide a large number of ways for the CSP to interact with the solute as well as the possibility of interaction with a wide range of molecules. In fact, the commercially available ionic and covalent forms of this column are now finding widespread application (Table I). Both analytical and preparative prepacked columns containing the Pirkle-type HPLC-CSP discussed in this article are commercially available in ionically and covalently bonded forms[1].

The preparative columns raise the exciting possibility of rapid and facile accumulation of pure enantiomers for synthesis and pharmacological use.

APPLICATIONS OF THE PIRKLE-TYPE HPLC-CSP

One class of compounds that typifies the use of this CSP are enantiomeric amides. Pirkle and co-workers (15–17), and Wainer and Doyle (7, 18–21) have reported the resolution of a large number of enantiomeric amides that were synthesized by starting with either enantiomeric amines or enantiomeric carboxylic acids. A good example of how this CSP works is found in the resolution of the enantiomers of the widely used nonsteroidal anti-inflammatory agent ibuprofen (21).

To be chromatographed and resolved on the Pirkle-type HPLC-CSP, ibuprofen, 2-(*p*-isobutylphenyl)propionic acid, must first be converted to an amide (Figure 4a). A number of amides of ibuprofen have been resolved: the separation factor ranges from $\alpha = 1.04$ for the cyclohexylamide to $\alpha = 1.12$ for the 1-naphthalenemethylamide (Figure 5). For each amide that was resolved, the (*S*)-enantiomer was eluted first, suggesting

that the diastereomeric complex formed between the (*S*)-enantiomer and the CSP is less stable than that formed between the (*R*)-enantiomer and the CSP. This conclusion leads to the proposed interaction model (Figure 4), in which there is a dipole-dipole stacking interaction between the amide dipoles of the CSP and solute (sites 1 and 1′). This interaction takes place first and is the driving force behind the formation of the CSP-solute complex.

Once the initial interaction takes place, a number of other interactions take place between the CSP and the solute. The attractive interactions include:

- a hydrogen bond formed between the amide hydrogen of the CSP and the amide carbonyl of the solute (sites 2 and 2′)
- a hydrogen bond formed between the amide carbonyl of the CSP and the amide hydrogen of the solute (sites 3 and 3′)
- a π-π attractive interaction between the DNB ring of the CSP and the phenyl ring of the solute (sites 4 and 4′).

All of these interactions are important for the formation of the CSP-solute complex, but they are not solely responsible for the chiral discrimination between the two enantiomers. The stereochemically controlled interaction is a steric interaction involving the α-methyl group (site 5′). Figure 4 depicts the complex formed between the (*R*)-isomer, which is eluted last, and the CSP. For this isomer, the α-methyl group points away from the CSP and does not interfere with the complex. When its mirror image, the (*S*)-enantiomer, is considered, the α-methyl group points toward the complex, destabilizing it through a steric interaction. The difference in the stability of the two diastereomeric CSP-solute complexes results in a lower capacity factor (k') for the (*S*)-enantiomer; therefore the stereoisomers are resolved.

As stated above, this type of interaction is just one of many that are available to the CSP. The large number of possible interactions gives the Pirkle-type HPLC-CSP its broad applicability. A survey of the types of compounds that have been resolved by the ionic and covalent forms of this CSP is presented in Table I.

The classes of compounds are diverse, but they appear to have a number of common features in their mode of interaction with the CSP. This interaction appears to involve at least two attractive interactions: for example, a hydrogen-bonding interaction and a π-π interaction, and one or more additional interactions, one of which is steric in nature. Pirkle and co-workers have reviewed a number of these interaction models (16).

APPLICATION TO BIOAVAILABILITY STUDIES

The broad applicability of the Pirkle-type HPLC-CSP opens up a number of possibilities for pharmacological studies that were previously impossible or extremely difficult. An example of this type of application is the recent report by Wainer et al. of a method for the determination of *d*- and *l*-propranolol levels in blood (22). Using this method, these isomers can be resolved as enantiomeric oxazolidones using the ionically bound Pirkle-type HPLC-CSP, and propranolol levels as low as 0.5 ng/ml in whole blood can be detected (Figure 6).

This assay, unlike the ones described previously (9–11), does not utilize the formation of diastereomers and, therefore, avoids errors caused by contamination in the derivatizing agent. This analytical procedure and others like it can be adapted to pharmacological studies or to clinical situations in which the monitoring of the levels of a particular isomer in blood is of interest.

LIMITATIONS OF THE PIRKLE-TYPE HPLC-CSP

Although there have been a number of successes using the Pirkle-type HPLC-CSP, there have also been failures.

TABLE I: ENANTIOMERIC SEPARATIONS USING THE PIRKLE-TYPE HPLC-CSP (*R*)-*N*-(3,5-DINITROBENZOYL)PHENYLGLYCINE

Type of Compound	Reference
Alkyl carbinols	15,16,23
Propranolol analogs	15,16,25
Cyclic alcohols	15,16,22,23
Aryl acetamides	15,16
α-Methylarylacetic acids	21
Tropic acid derivatives	20
Oxazolidones	15,16,24,25
Amino alcohols as oxazolidines	26
Phenethylamines	7,18,19
Aryl-substituted: hydantoins, hydroxyphosphonates, lactams, and succinimides	15,16
Phthalides	15,16
Bi-β-naphthols	15,16
Hydroxy sulfides	15,16
Alkyl-aryl, diaryl, cyclic, and β-hydroxy sulfoxides	15,16
Spiro-2,2-dithiolane-1-oxides	15,16
1-Aryl-1-alkyl-2,2-dithiolane-1-oxides	15,16
Diels-Alder adducts of acrylamides and anthracenes	15

The inability of the CSP to resolve a molecule tells us a great deal about the column and how it works. For example, the 1-naphthalenemethylamide of ibuprofen is resolved on the CSP, whereas the 1-naphthalenemethyl ester is not (21). This difference suggests the importance of the amide dipole-dipole interaction between the CSP and solute. The dipole moment of an ester, which is approximately one-half that of an amide, is apparently not strong enough for the initial attractive interaction that is essential for the formation of the CSP-solute complex. On the basis of this consideration, it could be assumed that amide derivatives of tropic acid would be resolved on the Pirkle-type HPLC-CSP, but that ester derivatives of tropic acid, such as atropine, would not be resolved. This is indeed the case (20).

Weems and Yang (23) and Kasai et al. (24) also have reported the inability of the Pirkle-type HPLC-CSP to resolve some enantiomeric pairs. Weems and Yang worked with a series of dihydrodiols and tetrahydrodiols of benzo[*a*]pyrene and benz[*a*]anthracene. Of the 16 enantiomeric pairs chromatographed, six were not resolved (23). Kasai et al. chromatographed 80 enantiomeric alkyl carbinols. The CSP was able to resolve 33 of 42 members of a series of acyclic alkyl carbinols and their acetates, but only 10 of 38 substituted 1-indanols, 1-tetralols, and their acetates (24).

Application of the Pirkle-type HPLC-CSP obviously is not a completely straightforward process. The choice of whether or not to derivatize the solute and what type of derivative to use plays a large part in the chromatographic approach. Additional complications arise from differences between the ionically bound CSP and the co-

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