A Strategy for Developing HPLC Methods for Chiral Drugs

Molecules that relate to each other where an object and its mirror image are not superimposable are called chiral (from Greek word *cheiro*, meaning “hand”); that is, they are like a pair of hands. These molecules also are called enantiomers. Major differences in biological activity have been observed in chiral molecules. The difference in spatial arrangements of atoms in a molecule (that is, its stereochemistry) can influence its pharmacological, metabolic, or toxicologic activity. This is why regulatory requirements in the pharmaceutical industry demand detailed investigations of chiral molecules. Before initiating method development, it is important to develop a basic understanding of stereochemistry. Basic information on stereochemistry is provided in this article to help readers develop better understanding of the separation mechanisms that come into play in various separation methods used for chiral compounds. This knowledge can allow readers to select a desirable chiral separation method, based upon the molecular structure of the chiral compound of interest. Logical reasons for the selection process are discussed later in this article.

The most difficult problem for an investigator in chiral separations is to determine where to start. Cost considerations, availability of equipment, and know-how play important roles in the selection process. The scientific literature suggests that chromatographic methods are generally favored over nonchromatographic methods. Of the various chromatographic methods (such as thin-layer chromatography, gas chromatography [GC], high performance liquid chromatography [HPLC], supercritical fluid chromatography [SFC], or capillary electrochromatography), HPLC methods are likely to be found most useful (exceptions can be found in references 1 and 3–5). The next step is to select an appropriate column, based upon various considerations (see short discussion in the following section). The primary concern for any investigator in this area, notwithstanding the high cost of the columns, is which column will work best in a particular situation. This article attempts to answer these questions, and it provides examples that will help readers make intelligent decisions in this complex field.

Determine the Chirality of the Molecule

First the investigator must take a close look at the molecule that is to be resolved and then answer the following question: Is there a stereogenic center? The simplest example of this is an asymmetric carbon with four different substituents. As simple as it might sound, this process appears cumbersome when you are looking at many carbon atoms in a molecular structure. The simplest way to counter this problem is to number all carbon atoms in the structure and look at each of them in turn to see if they are asymmetric or not.

If no asymmetric carbon is found, look at the plane of symmetry of the whole molecule and other atoms, such as sulfur and nitrogen, which also can confer chirality.

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If one asymmetric carbon or stereogenic center is found, the investigator can expect two enantiomers. For two stereogenic centers, the number of enantiomers is four. It should be clear that this number grows rapidly as the number of asymmetric centers increases; the $2^n$ rule applies, except for fused rings, where $n$ is the number of asymmetric centers.

**Stereoisomerism:** Molecules that are isomeric but have a different spatial arrangement are called stereoisomers. Symmetry classifies stereoisomers as either enantiomers, as defined previously, or diastereomers. Stereoisomerism can result from a variety of sources besides the single chiral carbon (stereogenic or chiral center) mentioned previously — that is, a chiral atom that is a tetrahedral atom with four different substituents. Detailed discussion on these topics can be found in several books and review articles (1–8); a short summary is provided here. It is not necessary for a molecule to have a chiral carbon to exist in enantiomeric forms, but it is necessary that the molecule, as a whole, be chiral. There are two simple molecular sources of chirality: molecules having a stereogenic center and those having a stereogenic axis. Stereoisomerism is possible in molecules that have one or more centers of chirality, helicity, planar–axial–torsional chirality, or topological asymmetry.

The amounts of energy necessary to convert given stereoisomers into their isomeric forms can be used for their classification. Stereoisomers with low-energy barriers to this conversion are termed conformational isomers, whereas high-energy-barrier conversions are described as configurational isomers. Diastereomers differ in energy content and, thus, in every physical and chemical property; however, the differences can be so minute as to be nearly indistinguishable.

**Stereocchemistry and biological activity:** The importance of determining the stereoisomeric composition of chemical compounds, especially those of pharmacological importance, has been well recognized for some time now (9–12). However, a fairly large number are still used in racemic or diastereomeric forms. It is important to remember that some enantiomers might exhibit potentially different pharmacologic activities, and the patient might be taking a useless or even undesirable enantiomer when ingesting a racemic mixture. To ensure the safety and effect of currently used and newly developing drugs, it is necessary to isolate and examine both enantiomers separately. Furthermore, it is necessary, in at least three situations, to measure and control the stereochemical composition of drugs. Each situation presents a specific technical problem during manufacture, where problems of preparative scale separations might be involved; quality control (or regulatory analysis), where analytical questions of purity and stability predominate; and metabolic and pharmacologic studies of plasma disposition and drug efficacy, where ultratrace methods can be required (1).

Accurate assessment of the isomeric purity of substances is critical since isomeric impurities can have unwanted toxicologic, pharmacologic, or other effects. Such impurities can be carried through the synthesis, preferentially react at one or more steps, and yield an undesirable level of another impurity. Frequently, one isomer of a series can produce a desired effect, while another can be inactive or even produce some undesired effect. Some examples of activity differences are given in Table I. Large differences in activity between stereoisomers point out the need to accurately assess isomeric purity of pharmaceuticals.

**Regulatory requirements:** the Food and Drug Administration (FDA) of the United States issued a set of initial guidelines in 1987 on the submission of new drug applications, where the questions relating to stereochemistry were approached directly in the guidelines on the manufacture of drug substances (13,14). The finalized guidelines require a full description of the methods used in the manufacture of the drug, including testing to demonstrate its identity, strength, quality, and purity. Therefore, the submissions to the FDA should show the applicant’s knowledge of the molecular structure of the drug substance. For chiral compounds, this includes identification of all chiral centers. The enantiomer ratio, although 50:50 by the definition for a racemic, should be defined for any other admixture of stereoisomers. The proof of structure should consider stereochemistry and provide appropriate descriptions of the molecular structure. An enantiomeric form is considered an impurity, and therefore, it is desirable to explore potential in vivo differences between these forms.

**Separation Methods**

Approximately 40 years ago, systematic research was initiated for the design of chiral stationary phases functioning to separate enantiomers by GC. This led to molecular design and preparation of chiral phase systems for LC. Lately, these efforts have
been directed toward finding new types of chiral stationary and mobile phases on the basis of the stereochemical viewpoint. Many factors can be responsible for the extent of interactions of stereoisomeric molecules in any environment such as dipole–dipole interactions, electrostatic forces, hydrogen bonding, hydrophobic bonding, inductive effects, ion–dipole interactions, ligand formation, partition coefficient differences, pK values, resonance interactions—stabilization, solubilities, steric interference (size, orientation, and spacing of groups), structural rigidity–conformational flexibility, temperature, and van der Waals forces. The nature and effects of some of these factors can influence the chromatography of stereoisomers; therefore, they must be carefully reviewed before developing a separation method (see further discussion below).

It is well recognized now that HPLC methods offer distinct advantages over classical techniques in the separation and analysis of stereoisomers, especially for enantiomers that are generally much more difficult to separate. These methods show promise for moderate-scale separations of synthetic intermediates as well as for final products (15–19). For large-scale separations and in consideration of the cost of plant-scale resolution processes, the separation methods offer substantial increases in efficiency over recrystallization techniques.

There are basically two approaches to the separation of an enantiomer pair by HPLC. In the indirect approach used rarely, the enantiomers can be converted into covalent, diastereomeric compounds by a reaction with a chiral reagent, and these diastereomers are separated on a routine, achiral stationary phase. In the direct approach which is often called chiral HPLC, the enantiomers or their derivatives are passed through a column containing a chiral stationary phase (CSP). Chiral HPLC and SFC are ideally suited for large-scale preparation of optical isomers.

**Separation of Chiral Compounds by HPLC**

As mentioned previously, the chromatographic separation of enantiomers can be achieved by various methods; however, it is generally desirable to use some kind of chiral discriminator or selector (20,21). Two different types of selectors can be distinguished: a chiral additive in the mobile phase or a chiral stationary phase. Of these approaches, chiral stationary phases are more commonly used for separations of enantiomers, and they are discussed at some length in the following sections.

**Mechanism for chiral separations**: To develop an optimum method, it is important to understand the mechanism of chiral separation. Our understanding of chiral separations with some of the systems is quite good, while it remains poor for protein and cellulose stationary phases. The separation basis with various chiral stationary phases is discussed below in their respective group; some general comments are included here. A number of chiral recognition models have been proposed to account for optical resolutions by HPLC; these are often based upon the three-point interaction rule advanced by Dalglish (22) in 1952. He arrived at this conclusion from paper chromatographic studies of certain aromatic amino acids. He assumed that the hydroxyl groups of the cellulose were hydrogen-bonded to the amino carboxyl groups of the amino acid. A third interaction was caused, according to these views, by the aromatic ring substituents. It led to the postulation that three simultaneously operating interactions between an enantiomer and the stationary phase are needed for chiral discrimination. However, this is not always necessary as steric discrimination also could result from steric interactions.

Chiral separations also are possible through reversible diastereomeric association between an enantiomeric solute and a chiral environment that is introduced into the column. Because chromatographic resolutions are possible under a variety of conditions, it might be concluded that the necessary difference in association can be obtained by many types of molecular inter-

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**Figure 1: Flow diagram to select an appropriate CSP (1).**
actions. The association, which may be expressed quantitatively as an equilibrium constant, will be a function of the magnitudes of the binding as well as the repulsive interactions involved. The latter are usually steric, although dipole–dipole repulsions also could occur, whereas various kinds of binding interactions can operate. These include hydrogen bonding, electrostatic and dipole–dipole attractions, charge–transfer interaction, and hydrophobic interaction (in aqueous systems).

CSPs, where steric fit is of primary importance, include those based upon inclusion phenomena, such as cyclodextrin and crown ether phases. It is possible to construct chiral cavities for the preferential inclusion of only one enantiomer. Molecular imprinting techniques are very interesting in this respect (1,23). The idea is to create rigid chiral cavities in a polymer network in such a way that only one of two enantiomers will find the environment acceptable.

A Primer for Selecting a Suitable CSP

As mentioned earlier, it is important to study the solute to determine what kind of interactions it can bring about. This information is very helpful in the selection of the desired CSP. The chromatographic analysis of entantiomeric compounds can be based upon whether the normal-phase or reversed-phase mode is desirable (1). However, the preferred approach is to select a CSP based upon various considerations discussed in the following. A conventional classification of common chiral stationary phases is provided in Table II. The table also provides a quick review of various interactions on CSPs. Alternatively, the chromatographic separations of chiral compounds can be viewed in terms of normal or reversed phase; the terms used more commonly by the chromatographers. A flow diagram (Figure 1) is provided to help readers select an appropriate CSP.

Brush-type columns (Type 1): The CSP in brush-type or Pirkle columns is composed of various selectors capable of ionic or covalent bonding. These CSPs are generally composed of an optically pure amino acid bonded to γ-aminopropylsilanized silica gel. An amide or urea linking of a π-electron group to the asymmetric center of amino acid provides for π-electron interactions and one point of chiral recognition. It has been proposed that at least three points of interaction are necessary between the chiral molecule and the CSP. One of these interactions must be stereochemically dependent. When three points of interaction occur, a transient diastereomeric complex is formed between the solute and the CSP. The compounds that can be separated on these columns are as follows: amines, amino acids, carboxylic acids, esters, ethers, hydroxy acids, ketones, and lactones.
Two types of columns can be used. For the older brush-type columns, derivatiza-
tion was frequently required. This was cum-
bensome, and it complicated separations
unnecessarily. Columns such as α-Burke
columns and Whelk-O 1 that obviate the
need for derivatization have evolved.

**Underivatized separations:** An α-
Burke II column (Regis Technologies,
Morton Grove, Illinois) is useful for reso-
lution of metoprolol and related com-
ounds. A mobile phase composed of
85:10:5 methylene chloride–ethanol–
methanol containing 10 mM ammonium
acetate is used at a flow rate of 1 mL/min
for a 25 cm × 4.6 mm i column. The α
value of the enantiomers is 1.28. Related
compounds such as alprenolol, atenolol,
betaxolol, bufarol, bupronolol,
oxprenolol, practolol, pindolol,
pronethalol, and propanolol can be
resolved with slight modifications of
mobile phases.

The Whelk-O 1 (Regis Technologies)
column originally was developed for sepa-
rating the enantiomers of naproxen
(Figure 2). Naproxen can be resolved by
using a normal-phase method with
80:20:0.5 hexane–isopropanol–acetic
acid on a 25 cm × 4.6 mm Whelk-O 1
column at a flow rate of 1 mL/min in 16
min (5). Alternatively, 60:40
methanol–0.1% phosphate can be used at
the same flow rate with the same run
time. The α value is 2.1 in the normal-
phase mode and 1.7 in the reversed-
phase mode. Other arypropionic acids
that have been resolved in the normal-
phase mode are ibuprofen, ketoprofen,
and flubiprofen. Various pharmaceuti-
cals that have been resolved on this column
are cyclohexizide, bendroflumethiazide,
oxazepam, mephenytoin, bupivacaine,
and p-chloro-warfarin.

An improved version of this column,
Whelk-O 2, has recently been designed to
improve the resistance of stationary phase
to hydrolysis, while using strong organic
modifiers such as trifluoroacetic acid.

**Polysaccharide columns (Type 2):** As
mentioned previously, the resolving
capacity of polysaccharides such as cellul-
lose was first realized by Dalgliesh with the
observation that a racemic amino
acid could occasionally give two spots in
paper chromatography. Optical resolu-
tion of amino acids is possible on cellu-
llose by TLC as well. This has led to the
use of cellulose and cellulose derivatives,
as well as amylose, for chiral separations
by liquid chromatography. Cellulose, a
linear polymer, has the chemical constitu-
tion of a linear poly-β-d-1,4-glucoside
(see Figure 3).

Cellulose forms long chains of at least
1500 (+)-d-glucose units per molecule. The
molecular weight of cellulose ranges from
2.5 × 10^5 to 1 × 10^6 or more. Each of
the (+)-d-glucose repetitive unit
contains five chiral centers and three
hydroxyl groups. All the ring substituents
are equatorial.

Broadly substituted cellulose columns
can be divided into two major categories:
cellulose esters and cellulose carbamates.
The popular phases (Chiralpak IA, Chira-
lpak IB, and Chiralpak IC) were created to
be more stable to a broad range of
mobile phase diluents, as well as to ele-
vated temperatures.

**Inclusion CSPs (Type 3):** Chiral dis-
crimination on a stationary phase can be
achieved by creation of chiral cavities, in
which stereoselective guest–host interac-
tions influence the resolution. Included in
this group are cyclodextrins, crown
ethers, polycrylates, and polycycla-
lamides. The applicability of this
approach has been extended by utilizing
macrocyclic antibiotics as chiral station-
ary phases instead of cyclodextrins by
Armstrong and colleagues (8–10).

**Cyclodextrin Columns**
Cyclodextrins are produced by the par-
tial degradation of starch and the enzy-
matic coupling of cleaved glucose units
into crystalline, homogeneous toroidal
structures of different molecular size.
Alpha-, β-, and γ-cyclodextrins have been most widely characterized; they

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### Table 1: Activities of some chiral compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity</th>
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<tbody>
<tr>
<td>Amphetamine</td>
<td>d-isomer is a potent CNS stimulant; l-isomer has little effect</td>
</tr>
<tr>
<td>Propoxyphene</td>
<td>α-l is antitussive; α-d is analgesic</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>l-isomer is 10 times more active as a vasoconstrictor than d-isomer</td>
</tr>
<tr>
<td>Synephrine</td>
<td>l-isomer has 60 times the pressor activity of d-isomer</td>
</tr>
<tr>
<td>Propranolol</td>
<td>S–(−) isomer has only β-adrenergic blocking activity</td>
</tr>
<tr>
<td>Warfarin</td>
<td>S–(−) isomer is 5 times more potent anticoagulant than R–(+ isomer</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>d-isomer is anti-ascorbic; l-isomer is not active</td>
</tr>
</tbody>
</table>
contain 6, 7, and 8 glucose units, respectively, and are chiral. For example, β-cyclodextrin has 35 stereogenic centers, and the toroidal structure has a hydrophilic surface resulting from the 2-, 3-, and 6-position hydroxyl groups making them water-soluble.

The cavity in cyclodextrins is composed of the glucoside oxygen and methylene hydrogen, giving it an apolar character. As a result, cyclodextrins can include polar molecules of appropriate dimensions in their cavities and bind them through dipole–dipole interactions, hydrogen bonding, or London dispersion forces. In general, cyclodextrins are stable from pH 3 to 14. Listed in the following section is a general plan that can be utilized for making initial choices. β-Cyclodextrin has been used in the largest number of applications because it has been found useful for low-molecular weight analytes in the pharmaceutical and environmental areas. These columns can be operated in the following modes: reversed phase or normal phase.

To achieve a chiral separation in the reversed-phase mode, it is essential that the analyte have at least one aromatic ring. Exceptions are heterocyclic analytes and t-boc amino acids. For cyclodextrin inclusion, the molecular weight of the polyaromatic ring structure is not as critical as its bulk. The most important consideration is proper fit of the molecule in the cyclodextrin cavity. This fit is a function of both size and shape of the analyte relative to the cavity. For example, an analyte like norgestrel, a five-ring steroid structure, is better separated on a β-cyclodextrin column, while the enantiomer of a naphthalene-like structure or single substituted aromatic ring would fit better on a β-cyclodextrin column. For chiral recognition, solvent strength is independent in most cases because it affects only displacement of the analyte from the cavity (1).

Normal-phase separations on cyclodextrin columns have generally been carried out with mobile phases such as hexane–isopropanol, acetonitrile–methanol, methanol, or ethanol. π–π hydrogen bonding forces primarily influence enantiomeric separations. It is possible to override inclusion complexation in favor of interacting directly with secondary hydroxyl groups across the larger opening of a cyclodextrin toroid or the appendant carbamate, acetate, or hydroxypropyl functional groups. β-Blockers such as propanolol, timolol, and atenolol, and compounds like warfarin can be separated.

**Bonded Derivatized Cyclodextrins**

A number of bonded cyclodextrins are currently available. The carbamate coupling of the π bases, 1-naphthylethyl to a bonded cyclodextrin, creates a complex environment that has demonstrated diverse chiral separations. It has been labeled as multimodal chiral stationary phase because it can be used in a normal- as well as a reversed-phase mode.
with appropriate modifiers. Analyte structure, solubility, and stability dictate the proper selection of the mobile phase. For example, if the analyte is $\pi$ acidic, normal-phase solvents can be used. If the analyte is not $\pi$ acidic but contains two hydrogen bonding groups, one on or near the stereogenic center, polar modifiers must be used. Of the three carbamates available, the $\delta$-naphthylethyl carbamate has shown the greatest selectivity and versatility. Because the naphthylethyl carbamate does play a role in enantioselectivity, the $R$ form can be useful if the separation does not occur on the $S$ form. A variety of compounds such as catelol, labetolol, nadolol, metoprolol, pinدولol, propanolol, timolol, oxazepam, suprofen, comuachlor, and warfarin have been resolved on cyclodextrin columns (1).

**Macrocyclic Antibiotics**

The chiral selectors in this group include vancomycin, ristocetin A, teicoplanin, avoparcin, rifamycin B, and thiostrepton. Chiroteic V is based upon covalently bonding the amphoteric glycopeptide vancomycin to 5-mm silica gel. These ligands are linked to assure their stability while retaining essential components for chiral interactions. For example, vancomycin contains 18 chiral centers surrounding three pockets or cavities. Five aromatic ring structures bridge these cavities. Hydrogen donor and acceptor sites are readily available close to the ring structures. It has been claimed that the selectivity of this column is similar to $\alpha$-1 acid glycoprotein (AGP), and it is stable when 0–100% organic modifier is used.

Broad selectivity has been demonstrated by vancomycin in both reversed-phase and normal-phase solvents, and limited selectivity with polar organic mobile phases. Since vancomycin contains peptide, carbohydrate, and other ionizable groups, it would be expected to offer different selectivity in these modes. The structure of vancomycin indicates that all the typical interactions outlined for protein phases and other cellulosic polymer type phases are possible with this phase. The potential interactions and their relative strengths are as follows: $\pi-\pi$ interactions — very strong; inclusion — weak because of shallow pockets; dipole stacking — medium to strong; steric interactions — weak; and anionic or cationic binding — strong.

Compared with cyclodextrins, the shallow pockets for inclusion yield weaker energies. This leads to faster kinetics, which can in turn lead to faster separations. Reversed-phase conditions favor inclusion and hydrogen bonding. Under these conditions, changes in pH can produce cationic or anionic interactions. Dipole stacking and $\pi-\pi$ complexation are favored in normal-phase solvents. Polar organic mobile phases enhance the potential for all of the previous interactions. Analytes such as acids, amides, esters, and neutral compounds can be resolved.

Chiroteic T (Astec, Whippany, New Jersey) is based upon bonding the amphoteric glycoside teicoplanin to a 5-mm silica gel through covalent linkage. Teicoplanin contains 20 chiral centers surrounding four pockets or cavities. Hydrogen donor and acceptor sites are readily available close to seven aromatic rings.

Separations of warfarin enantiomers on Chiroteic T and V columns is shown in Figure 5 with the same mobile phase (10:90 acetonitrile–1% triethylammonium acetate, pH 4.1). Much better resolution is observed with Chiroteic V.

Recently Chiroteic V2 and T2 have been created to produce higher selectivity and higher capacity.

**Selection of mobile phases:** The mobile phase functions equally in reversed-phase or normal-phase solvents because of the complex structure of the macrolide and ionizable groups. In the reversed-phase mode, optimization is accomplished by controlling the type and amount of organic modifier, type of buffer, and pH. Efficiency and selectivity are affected by ionic strength, buffer type, flow rate, and temperature. Of the various organic modifiers, tetrahydrofuran gives greater selectivity and efficiency. Typical composition of organic modifier–buffer (pH 4.0 to 7.0) is 10:90. Alcohols as modifiers generally require higher concentration, for example, 20% for comparable retention to acetonitrile or tetrahydrofuran. Ammonium nitrate and triethylamine acetate buffers have been found useful. In general, analytes act more favorably at a pH where they are not ionized. Lower column temperatures are favored because of enhancement of weaker bonding forces.

In normal-phase separations, peak efficiency and resolution can be improved with ethanol as the polar modifier of hexane. A good starting point might be 20% ethanol. In most cases, ethanol works better than isopropanol.

**Applications:** A wide range of amino acid derivatives have been resolved on these columns. Analytes such as neutral molecules, amides, acids, esters, and cyclic amines show considerable enantioselectivity. Other amines have been separated with varying degrees of success. Benoxaprofen, ibuprofen, fenoterol, mephobarbital, naproxen, warfarin, albuterol, citulline,
DOPA (3,4-dihydroxy-phenylalanine), and phenylalanine have been resolved on chirobiotic columns (1).

**Ligand exchange (Type 4):** Davankov and Kurganov (16) were the first to indicate that cross-linked resins with fixed ligands, \((R)\)-N,N’-dibenzyl-1,2-propane-diamine in the form of copper(II) complexes, display high enantioselectivity for alanine, serine, and leucine. Various amino acids, including baclofen (1), can be resolved on a reversed-phase C18 column with a chiral mobile phase of aqueous cupric acetate and N,N-di-\(n\)-propyl-l-alanine (DPA) containing 15% acetonitrile (17,18). Cation exchange chromatography can then be used to break the Cu-DPA-baclofen complex on a Dowex-50W column (Dow Chemical, Midland, Michigan) to yield small quantities of the optical isomers for mechanistic studies. A number of ligand exchangers are sold by Daicel (Chiral Technologies), including Chiralpak WH, Chiralpak WM, and Chiralpak WE. These columns are useful for resolution of amino acids and their derivatives (15). The mobile phase is simply aqueous copersulfate. Chiralpak Ma (+) is another ligand-exchange column that is useful for hydroxy-carboxylic acids.

Investigations have revealed that a ligand-exchange column can be a simpler and more useful approach for separating enantiomers of baclofen (1), a drug similar to phenylalanine. The selection of Chiralpak WH column, a column designed to serve as a ligand exchanger (19), considerably shortened the method development time. It is fairly well known that the separation of amino acids is significantly affected by the molarity of CuSO₄ used for the separation. However, the effect of organic solvents such as methanol on the retention of free amino acids is not well known, except for valine, which shows a decrease in retention time with an increase in methanol concentration up to 20%. Temperature has a significant effect on retention — for example, the retention time of phenylalanine decreases significantly with increasing temperature. These considerations led to an optimal mobile phase (containing 0.25 mM copper sulfate, run at a flow rate of 1.5 mL/min at 50 °C) for the resolution of d- and l-forms of baclofen from the racemate (1).

**Protein phases (Type 5):** Chiral AGP (ChromTech, AB, Norsborg, Sweden) is a second-generation chiral selector that is based upon the \(\alpha\)-1-acid glycoprotein as the chiral stationary phase. The process of immobilizing AGP on porous spherical 5-mm silica has been patented. This CSP has been found useful for resolving a broad range of compounds such as racemic amines, acids, and nonprotonic compounds without derivatization. A number of examples of drugs such as alprenolol, atenolol, bupivacaine, chlorhalidone, disopyramide, ephedrine, ethotoin, felodipine, fenoprofen, hexobarbital, metoprolol, pheniramine, and verapamil have been resolved on Chiral AGP (1). The resolution ability of this column is due to the unique nature of the chiral stationary phase and the fact that enantioselectivity is generally recognized that the greatest selectivity can be induced by choosing proper mobile phase composition. For bioanalytical work, this CSP has been recommended highly. The typical mobile phase for this column is phosphate buffer with an organic modifier. Enantioselectivity and retention can be regulated by changing the mobile phase composition with respect to any of the following parameters: pH, organic modifier, modifier concentration. For the types of various modifiers that have been used and their respective concentrations, see Table 10.6 in reference 1. Typical operating conditions entail the use of 10 mM buffer at pH 7.0, with or without an organic modifier. Modifier concentrations as high as 15% iso-propanol or 10% acetonitrile have been used. The pH can affect the resolution of different analytes. A number of other protein-based columns are also available for certain applications. It should be noted that the protein columns tend to be less stable and have low capacity.

Table III can offer some help in selecting a suitable CSP on the basis of resolution, efficiency, capacity, column stability, mobile phase compatibility, and analysis time.

### A Fast Approach to CSP Selection

A significant savings in time can be made by use of a chiral database searchable that is based upon molecular structures. Christian Roussel and others (24) have provided CHIRBASE, a molecular-oriented factual database that includes tens of thousands of entries, and the entire system can be searched by ISIS software.

<table>
<thead>
<tr>
<th>Table III: Comparison of various CSPs (1)</th>
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<tr>
<td><strong>Brush-type</strong></td>
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<tr>
<td>Resolves enantiomers</td>
</tr>
<tr>
<td>High efficiency</td>
</tr>
<tr>
<td>Column stability</td>
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<tr>
<td>Mobile phase compatibility</td>
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<tr>
<td>Inversion of elution order</td>
</tr>
<tr>
<td>High capacity</td>
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<td>Analysis time</td>
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(ISIS Software, Inc., Richmond, Virginia). The list provides comprehensive structural, experimental, and bibliographic information on successful and unsuccessful chiral separations (25). Assistance can be sought from the major column manufacturers (for example, Astec/Supelco, Chiral Technologies, Regis Technologies, and ChromTech AB, Norsborg, Sweden) who offer practical background information on their columns as well as technical assistance in developing analytical and/or preparative enantiomeric separations.

With over 170 CSPs commercially available, reliable and rapid enantioselectivity prediction of a new chiral molecule with any CSP still remains elusive. Of the commercially available CSPs, the polysaccharides and macrocyclic glycopeptides have been favored by various groups for the screening strategies in HPLC. Two screening strategies have proposed by Perrin and colleagues (26,27) for the enantiomeric separation of drugs using polysaccharide columns in the isocratic normal- and reversed-phase modes. In both modes, three complementary columns are used that have broad enantiorecognition abilities for a wide range of pharmaceutical compounds. These columns are the Chiralcel OD-H, Chiralpak AD and Chiralcel OJ for the normal phase. The compounds are screened on each column using two mobile phases containing 90:10 (v/v) of n-hexane–2-propanol or n-hexane–ethanol at a flow rate of 1 mL/min. Diethyl amine (0.1% v/v) is added to the mobile phase for the analysis of basic compounds; however, for the acidic compounds 0.1% (v/v) trifluoroacetic acid is used instead. This strategy was applied to a set of 36 drugs. The study also has shown that for basic compounds, the screening on the Chiralcel OD-H and the Chiralpak AD columns is usually sufficient to achieve the separation. Resolution of the enantiomers was observed for 32 compounds on at least one column. Short analysis times (that is, 20 min or less) were usually achieved.

In the reversed-phase strategy, the three columns (Chiralcel OD-RH, Chiralpak AD-R and Chiralcel OJ-R) are used with two mobile phases. The compounds are first analyzed with a mobile phase consisting of an aqueous phosphate buffer, pH 2.0, containing 100 mM potassium hexafluorophosphate, mixed with acetonitrile (60:40 v/v). The chaotropic agent hexafluorophosphate is added to the mobile phase to achieve the separation of basic analytes at low pH. According to Perrin and colleagues (27), most enantiomers can be separated with this approach. If no or very little enantioselectivity is achieved for some components, all three columns are investigated with a basic mobile phase consisting of an aqueous 20 mM borate buffer, pH 9.0, mixed with acetonitrile (60:40 v/v) at a flow rate of 0.5 mL/min. Enantioselective separation is achieved on at least one of the columns for 89% (33 out of 37) of the drugs analyzed. Analysis times are usually less than 30 min.

Another screening strategy using the same type of columns but with normal-phase gradient elution has been proposed (28). Each compound is analyzed on four columns: Chiralcel OD-H, Chiralpak AD, Chiralcel AS, and Chiralcel OJ. n-Hexane–2-propanol and an n-hexane–ethanol gradient elution system are used to screen the compounds except for the Chiralpak AD column, where only the former system is used. To speed-up the analysis, a column-switching device is used. The gradient is run from 20% to 70% alcohol in 20 min with a flow rate of 0.75 mL/min. With the baseline separation is not achieved, isocratic optimization of the separations is necessary.

Four polysaccharide-based CSPs and

![Figure 5: Comparison of separations of warfarin on Chirobiotic T and V (1).](Image)
three macrocyclic glycopeptide based CSPs have been evaluated for rapid screening for of over 55 chiral compounds of pharmaceutical interest (29). The polysaccharide columns are employed in the normal phase and polar organic modes and showed overall enantioselectivity for 87% of the compounds tested. The macrocyclic glycopeptide columns are employed in the reversed-phase and polar organic mode and showed enantioselectivity for 65% of the analytes. When the results from both the polysaccharide and the macrocyclic glycopeptide screen are combined, they show enantioselectivity for 53 out of 55 enantiomeric pairs (96%). This shows that the two screens are complementary—i.e., that, both types of columns should be included to achieve the highest probability of success. The screens can be automated using a column switcher that allows for different combinations of CSP and mobile phase to be tested overnight. It is claimed that the method development can be completed within 24 h for a given compound. The column coupling approach has been applied on macrocyclic glycopeptide CSPs. It allows evaluation of this entire class of chiral selectors with a single coupled column for the ability to separate a molecule. Even if a partial separation is obtained on the coupled column, a baseline separation is potentially possible with one of the columns in this class (30).

A parallel multicolumn screening approach has been published by Zhang and colleagues (31). The modified HPLC system allows simultaneous screening of five CSPs in parallel using a regular HPLC autosampler and a pump with five UV detectors.

Future chiral screening program will focus on various separation techniques besides HPLC (32). These include SFC, capillary electrophoresis (CE), and GC. This could lead to a unified strategy for chiral method development screening.

Summary and Conclusions
The selection of an appropriate column (CSP) is the most important step in method development of chiral compounds. Selectivity, mode of operation, compatibility, robustness, efficiency, loadability, and reproducibility also need to be considered. The trial-and-error approach generally used in chiral separations can be extremely time-consuming. Some of the previously mentioned books can be helpful in column selection. Most chiral separations can be achieved on two types of CSPs: polysaccharide-based and macrocyclic glycopeptides. These columns can be operated in the reversed-phase, normal-phase, and polar organic modes with complementary enantioselectivities. The chiral databases as well as the column screening approach discussed previously can shorten the selection process. Improved columns need to be designed based upon careful evaluations of molecular architecture of CSP as it can provide clues relating to which portion of their structure offers the desired selectivity.

References
5. S. Ahuja, Chiral Separations: Applications and Technology (American Chemical Society, Washington, DC, 1997).