

Overview of Chiral Separations

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1 INTRODUCTION

Enantiomers of a molecule relate to each other as an object and its mirror image that are not superimposable. They are also called *chiral* (from the Greek word *cheiro*, meaning “hand”); that is, they are like a pair of hands. The “handedness” of small and large molecules has sparked great interest in pharmaceutical and biotechnology industries [1–10]. This difference in spatial arrangements of atoms in a molecule (i.e., the molecule’s stereochemistry) can influence its pharmacologic, metabolic, and toxicologic activity. Molecules that are isomeric but have a different spatial arrangement are called *stereoisomers*. Symmetry classifies stereoisomers as either *enantiomers*, as defined above, or *diastereomers*. Stereoisomerism results from a variety of sources besides the single chiral carbon. There are two simple molecular sources of chirality: molecules that have a stereogenic center and those that have a stereogenic axis. Stereoisomerism is possible in molecules that have one or more centers of chirality, helicity, planar/axial/torsional chirality, or topologic asymmetry.

The amounts of energy necessary to convert given stereoisomers into their isomeric forms may be used for further classification. Stereoisomers with low-energy barriers to this conversion are termed *conformational isomers* (e.g., proteins in the case of biotechnology products), whereas high-energy-barrier conversions are described as *configurational isomers* (e.g., small molecules). Diastereomers differ in energy content, and thus in every physical and chemical property; however, the differences may be so minute as to be nearly indistinguishable.

Very often, one isomer of a series may produce a desired effect, while another may be inactive or even produce an undesired effect. Chiral separations represent

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the most intriguing and, by some measures, most difficult separations of chemical compounds in that the molecules to be separated have the same molecular weight and physical and chemical properties, except for the rotation of polarized light. As mentioned above, isomeric impurities may have unwanted toxicologic, pharmacologic, or toxicologic effects. Therefore, an accurate assessment of the isomeric purity of substances is essential. Such impurities may be carried through the synthesis, preferentially react at one or more steps, and yield an undesirable level of an additional impurity.

2 REGULATORY CONSIDERATIONS

Regulatory guidance for development of chiral compounds is generally consistent among regulatory bodies in the United States, the European Union, Canada, and Japan (Chapter 2). The focus is to develop specific enantiomeric methods early in the program:

- To determine the relative pharmacological contribution, compared to that of the racemate, of each enantiomer in animals and in humans
- To compare the toxicology profile of the racemate to the individual enantiomers to confirm their relative activity

Based on these data, the sponsor may make a logical choice to proceed with development of the racemate or a single enantiomer.

Although regulatory guidance documents do not specify biologics or biotech-derived products, one can assume that for the generation of a single, purified active pharmaceutical ingredient (API), many of these same concepts apply. A major caveat to the category of products approved under the Public Health Service Act vs. the Food, Drug, and Cosmetic Act is that several approved biologics consist of a pool of heterologous proteins, such as polyclonal antibodies (e.g., intravenous gamma globulin, vaccine antigens, and some isozyme preparations). Given the rigor or orthogonal analytical methods used in biologics development and process validation, it is assumed that issues relating to chiral activity will not be lost in the program.

3 BASIC CONSIDERATIONS IN METHOD DEVELOPMENT FOR CHIRAL COMPOUNDS

Cost considerations, availability of equipment, and know-how play important roles in the selection process for an appropriate method (Chapter 3). Paper chromatography (PC) and thin-layer chromatography (TLC) have been used where cost considerations outweigh other factors. PC is used very rarely these days; however, TLC can be a very useful qualitative technique that entails minimal costs. It can also provide good indications as to which HPLC method would be

most suitable for resolving enantiomers. Of course, it can also be used as an independent technique with limitations of resolution and low precision. A significant amount of coverage was provided in earlier texts [3–6] to enable the reader to try TLC; those texts include a number of reference sources for TLC aficionados. Commonly used methods for separation of enantiomers today can be classified broadly into the following four categories:

- Gas chromatography (GC)
- High-performance liquid chromatography (HPLC)
- Supercritical fluid chromatography (SFC)
- Capillary electrophoresis (CE)

Detailed discussion of these methods is provided in this book. Since HPLC methods are generally favored for a variety of reasons, some basic information on selecting a suitable method for HPLC has been included in this chapter. A basic understanding of chiral discrimination by various chiral stationary phases (CSPs) has been provided to help with method development. A strategy for fast method development is also provided in this chapter.

4 SEPARATION OF CHIRAL COMPOUNDS ON POLYSACCHARIDE COLUMNS

The popularity of polysaccharide-based chiral stationary phases has been well documented (Chapter 4). Based on published information, it appears that derivatized polysaccharides are by far the most widely used CSPs in the separation of enantiomers. An incredible number of chiral separations have been and continue to be made with just four commercial chiral stationary phases: Chiralpak AD and AS and Chiralcel OD and OJ. Now these same problems can usually be solved with just three immobilized columns: Chiralpak IA, IB, and IC. In various studies, either of these sets of columns offers resolution for more than 85% of the compounds that have been investigated. Mechanisms of separation and method development are also discussed in this chapter.

5 CHIRAL SEPARATIONS BY VARIOUS TECHNIQUES

Three cases of chiral separations based on phase conversion of a popular Chiralpak AD column are presented in Chapter 5. Examples of successful chiral separation by converting this column from the normal phase to the reversed phase are demonstrated. By phase conversion, some of the compounds changed enantiomeric elution order, whereas others did not. Advantages of phase conversion in chiral separations are also discussed. It should be noted that improper preparation of a normal mobile phase could cause loss of chiral resolution previously

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observed for various chiral separations; this can result in poor method transfer-ence. Finally, a very interesting case of achieving chiral resolution on rotamers with achiral columns is shown that makes one wonder whether the separation is chiral.

6 CHIRAL DISCRIMINATION STUDIES BY NUCLEAR MAGNETIC RESONANCE

Although polysaccharide-based CSPs have been commercialized for more than two decades, the chiral discrimination mechanisms are still unclear at the molecular level (Chapter 6). Chiral recognition exhibited by polysaccharide-based CSPs depends on the higher-ordered structures of the polymers, which makes it difficult to understand the chiral recognition mechanism. Problems often arise with regard to the selection of appropriate systems, with fitting mobile phases, from the polysaccharide-based CSPs available. Unfortunately, no selector–selectand combinations or reliable chiral recognition models have been developed to allow for predictions with respect to separability, magnitude of enantioselectivity, elution order, and suitable chromatographic conditions.

Insight into chiral discrimination at the molecular level for polysaccharide-based CSPs is hindered by the complexities of the polymer, such as the exact stereochemical structure, the geometry of the interaction, the accessible binding sites, and the multiplicity of sites with different affinities for enantiomers. Numerous techniques, such as x-ray crystallography, nuclear magnetic resonance, calorimetric studies, infrared, and computational methods have been used to provide insight into chiral recognition mechanisms for other CSPs. These studies can help improve our understanding of the chiral stationary-phase structures, chiral cavities, and surface properties.

7 COMPARISON OF CHIRAL CHROMATOGRAPHY COLUMNS

Analytical laboratories must be ready continually to address the changing nature of molecules in developments in the pharmaceutical industry (Chapter 7). A majority of compounds screened for chiral method development have been adequately resolved on polysaccharide-based stationary phases, including Chiralpak WH, Chiralpak WM, and Chiralpak WE, AD, OD, AS, and OJ in many laboratories. However, as new phases become available, it is important to characterize their capabilities as well. After optimizing the analysis parameters for several chiral columns produced by different manufacturers, the column series was challenged by chemical entities representative of those developed for commercial use as pharmaceuticals. The chromatographic results were assessed vs. polysaccharide-based phases to gauge how successful various chiral columns are in developing efficient stereoselective methods for resolving chemical entities progressing to market.

8 CHIRAL SEPARATION SCREENS FOR ANALYSIS AND PURIFICATION

The pharmaceutical industry strives to produce effective, safe, and high-quality medicines. Analysts play a critical role in the chiral discovery process because each enantiomer has the potential to produce different therapeutic effects or adverse effects, and may even be metabolized differently (see Chapter 8). Chiral chromatography, analytical and preparative, is now considered an integral part of pharmaceutical analysis and drug discovery. A series of chiral HPLC (normal, polar, and reversed phases), and chiral SFC screens have been developed and implemented. These allow scouting many conditions and columns rapidly and effectively. Parallel chiral HPLC systems and chiral SFC have been found to be very useful. Several examples illustrating the performance of the screens are discussed in detail.

9 SEPARATIONS OF ENANTIOMERS BY GAS CHROMATOGRAPHY

High efficiency, sensitivity, and speed of separation are important advantages of enantioseparation by high-resolution capillary gas chromatography (HRC-GC). Because of the high separation power of HRC-GC (Chapter 9), contaminants and impurities can be separated from the chiral analytes; the simultaneous analysis of multicomponent mixtures of enantiomers (e.g., derivatized proteinogenic α -amino acids). Ancillary techniques such as multidimensional GC (i.e., in series-coupled column operation), interfacing, and coupling methods such as gas chromatography–mass spectrometry (GC-MS) are important tools in chiral analysis. Employing the ion-monitoring mode selected, trace amounts of enantiomers can be detected by GC-MS. The universal flame-ionization detector (FID) is linear over five orders of magnitude, and detection sensitivity can be increased further to the picogram level by electron-capture detection (ECD) and element-specific detection, usually aided by special derivatization strategies. In contrast to liquid chromatography or electromigration methods, the delicate choice of solvents (buffers), modifiers, and gradient elution systems is not necessary in GC. However, the prerequisites for the use of GC are volatility, thermal stability, and resolvability of the chiral analyte; these restrict the exclusive use of enantioselective GC.

10 SEPARATIONS OF CHIRAL COMPOUNDS BY SFC

SFC has been used successfully for chiral separations at the analytical, semipreparative, and preparative scales (Chapter 10). Commercial systems have demonstrated excellent performance, robustness, and cost-effectiveness. For industrial purposes, SFC at a simulated moving bed (SMB) on a production scale has been demonstrated on a prototype in the lab. The production capacity

can be obtained at the metric tons level. Excellent economic advantages have been demonstrated compared to liquid-based SMB operations.

11 CHIRAL SEPARATIONS BY CAPILLARY ELECTROPHORESIS

Cyclodextrins (CDs) are most frequently used as a selector in chiral CE (Chapter 11). The numerous applications reported over the past several years indicate their potential and popularity. The development of anionic derivatives has boosted their popularity. Some derivatives, such as the highly sulfated CDs, show broad enantioselectivity toward a large number of structurally diverse compounds. They are suitable for developing screening approaches or separation strategies for industries (e.g., in drug development and in quality control). This explains the 18% market share of the applications described from the pharmaceutical industry and the continuous growth predicted in this field. For crown ethers, only small molecules bearing an amino group, such as amino acids, can be separated, although occasionally, separation of a small drug molecule has also been reported. The same applies for ligand-exchange CE, where the analytes must have free-electron pairs and where applications are also limited primarily to amino acids. For macrocyclic antibiotics, the number of applications reported has decreased notably in recent years. This can be attributed to their limited enantioselectivity in CE and the fact that they absorb ultraviolet light at wavelengths below 250 nm. Adsorption onto the capillary wall and limited enantioselectivity may also be reasons that proteins are not used as frequently.

12 HIGH-THROUGHPUT SCREENING AND METHOD-DEVELOPMENT STRATEGIES

Since chiral recognition mechanisms are not fully understood, making the prediction of enantioseparation rather difficult. Some generic screening and method-development strategies have been developed to avoid time-consuming trial-and-error approaches (Chapter 12). These include normal-phase liquid chromatography (NPLC), reversed-phase liquid chromatography (RPLC), polar organic solvent chromatography (POSC), super- and subcritical fluid chromatography, and capillary electrophoresis. When one technique fails to separate certain compounds, it is possible that another technique will succeed in obtaining a baseline resolution. The fact that these techniques complement each other enlarges the spectrum of chiral compounds that can be separated with one of the defined strategies.

13 PREPARATORY SEPARATIONS

Preparatory separations have been employed successfully in a challenging preparation of an enantiopure single diastereomer of a pharmaceutical intermediate

from a mixture of four different stereoisomers (Chapter 13). Column screening, modeling, and optimization have led to the identification of an HPLC method employing a step gradient to enhance separation productivity and to reduce solvent consumption. The separation was carried out on a fairly large scale that afforded a substantial amount of the enantiopure single diastereomer.

14 CHIRAL ANALYSIS WITH SENSOR TECHNOLOGY

It is abundantly clear that verification of enantiomeric purity is an important analytical requirement in the pharmaceutical industry. Chiral purity assays are often performed via chromatographic techniques, and performance is controlled by “adsorption” of the analyte onto the coating. Since it is not always known which CSP would provide optimal specificity for a given enantiomeric pair, chromatographic method development can be a time-consuming and expensive process (Chapter 14). Chemical sensors are being investigated to improve the efficiency of column method development. The leading platform for the sensor application is the quartz-crystal microbalance (QCM) because of its ability to make real-time condensed-phase measurements. QCM sensors are coated with stereospecific coatings; the coated sensor readily produces unique responses upon exposure to enantiomeric isomers. Preliminary studies that assess the nature of the analyte–coating interaction indicate vast potential for future chiral applications. Research from various groups is promoting the potential for stereospecific applications for chemical sensors. The research activities are progressing to achieve two important applications: to establish whether sensor technology can be used for direct enantiomeric impurity determinations for pharmaceutical applications, and to determine if sensors make the selection of chiral LC columns more efficient for preparative and analytical needs.

15 CHIRALITY OF BIOMOLECULES AND BIOTECHNOLOGY PRODUCTS

A large number of successful biotechnology products that have been introduced into our armamentarium of modern medicine are based on proteins, which are complex organic macromolecules whose structures are coded in an organism’s DNA. Each protein has a unique genetically defined amino acid sequence that determines its specific shape and functions. It is well known that proteins are composed of chiral amino acids. Unfortunately, chiral studies are largely ignored on biomacromolecules such as proteins, as they are not monitored to assure that they indeed correspond in terms of all chiral components to the original macromolecules produced biologically. This may stem from the fact that monitoring biological activity is considered adequate in many cases. Alternatively, it is assumed that their unique structure assures appropriate chirality of its components; that is, appropriate folding would not occur if an alternative enantiomer were to be incorporated in the molecule.

Chapter 15 reviews what is being done to monitor biomolecules such as proteins and biotechnology products based on proteins and their building blocks (i.e., amino acids and peptides). With the upcoming advent of biogenerics, it is desirable that all chiral components in new products correspond to the original macromolecules. Furthermore, extensive physicochemical testing needs to be performed to assure that denaturation of proteins has not occurred and that they are refolded properly in case any unfolding occurred during processing.

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