CHIRAL SEPARATION METHODS FOR PHARMACEUTICAL AND BIOTECHNOLOGICAL PRODUCTS

Edited by

Satinder Ahuja
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Calabash, North Carolina

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CONTENTS

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PREFACE

Enantiomers or *chiral* molecules (from the Greek word *cheiro*, meaning "hand"; that is, they are like a pair of hands) relate to each other as an object and its mirror image. This "handedness" of small and large molecules has created a lot of interest in the pharmaceutical and biotechnology industries because they can have different pharmacologic, metabolic, and/or toxicologic activities.

The handedness of the molecules relates to the difference in spatial arrangements of atoms in a molecule. *Stereoisomers* are molecules that are isomeric but have a different spatial arrangement. Symmetry classifies stereoisomers as either enantiomers or *diastereomers*. There are two molecular sources of chirality: molecules that have a stereogenic center and those that have a stereogenic axis. Stereoisomerism is also possible in molecules that have one or more centers of chirality, helicity, planar/axial/torsional chirality, or topologic asymmetry.

The 1960s public health catastrophe brought about by the use of thalidomide reinforced our thinking on the need for regulatory controls, since one isomer can produce a desired effect whereas the other may produce an undesired effect. In 1992, the U.S. Food and Drug Administration issued a policy statement for the development of new stereoisomeric drugs, where the question of stereochemistry was approached directly. To ensure that similar problems are not encountered in the future, the guidelines emphasized the importance of separating and isolating the isomers so that appropriate pharmacologic, metabolic, and/or toxicologic studies could be conducted. Chiral separations entail the most intriguing, and at times 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. The molecules with (+) rotation are called *dextrorotatory* and those with (−) rotation are called *levorotatory*. An accurate evaluation of the isomeric purity of active drug substances is critical because the impurities may be carried through the synthesis, preferentially react at one or more steps, and produce an undesirable level of another impurity.

This book provides valuable information on chiral separations of pharmaceuticals and biotechnology products by:

- Covering a variety of modern methods, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), and capillary electrophoresis (CE).
- Dealing with the impact of chirality on biological activity of small and large molecules.

- Providing detailed information on useful chiral stationary phases (CSPs) for HPLC.
- Including handy information on selection of an appropriate CSP based on mechanistic studies.
- Offering strategies for fast method development with GC, HPLC, SFC, and CE.
- Discussing preparatory methods utilized in the pharmaceutical industry.

The first three chapters in this book provide a broad overview of chiral separations, regulatory considerations in drug product development, and basic considerations in method development.

Chapters 4 to 6 discuss the development of popular polysaccharide columns, various techniques that can be used for separations on them, and mechanistic studies on chiral separations to help us understand how these columns operate so that we can develop better methods with them. Chapters 7 and 8 provide comparisons of chiral columns and chiral separation screens for pharmaceutical analysis and purification.

Separations of chiral compounds by GC, SFC, and CE are discussed at length in Chapters 9 to 11. These methods come in handy in various situations. A strategy of method development for HPLC, SFC, and CE is covered in great detail in Chapter 12. The reader may find significant advantages in this integrated approach to method development with these techniques commonly used in chiral separations today.

Chapter 13 covers preparatory separations in the industrial environment. Chemical sensors provide an interesting promise for chiral separations in the future (Chapter 14). Preliminary studies indicate a vast potential for a variety of chiral applications. The current status of what is being done or not done in terms of chiral separations of biotechnology products is covered in Chapter 15. Some useful suggestions have been made to assist future developments in this field, including the control of biogenerics.

I would like to thank all the authors for their valuable contributions, which make this book a useful resource for laboratory investigators, managers, and regulators who are involved in chiral separations in the pharmaceutical industry.

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CHAPTER 1

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

Chiral Separation Methods for Pharmaceutical and Biotechnological Products, Edited by Satinder Ahuja

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 toxocologic 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

observed for various chiral separations; this can result in poor method transference. 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 chromatograpy 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 intoduced into our armamentarium of modern medicine are based on proteins, which are complex organic macromolecules whose structures are coded in an organisim'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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CHAPTER 2

Regulatory and Development Considerations of Chiral Compounds

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1 OVERVIEW

The importance of chirality in biological systems has been well recognized for over 50 years, due largely to its observed pervasiveness in nature. Although it may be self-evident that chirality plays a critical role in biological activity, it was not until the 1980s that its importance in drug development was recognized. With the advent of improved assay sensitivity and separation techniques of the day, Ariens et al. correctly identified racemic mixtures as fixed-dose combinations of a *eutomer*, or desired enantiomer, and *distomer*, or biologically inactive and/or toxic enantiomer [1]. Since then, the universe of chiral science has been expanding at an astonishing rate, with significant developments in analytical methods, the discovery of new stereoselective catalyzing agents, improved manufacturing and separation process controls, and in vitro characterization as evidenced by a larger pool of patents and intellectual property litigation in this area.

Although regulatory policies for chiral drug development were put in place nearly 20 years ago, the timing for when to implement these tests in the development plan has not been well defined. Regulatory and development considerations have grown significantly more complicated and layered since then and appear to have resulted in an ad hoc approach instead of a highly integrated program. The focus of this chapter is to provide a more holistic project development plan based on the combined current regulatory guidance and industry experience for the development of racemic drugs and selective enantiomers.

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2 HISTORY OF REGULATORY GUIDANCE

Several regulatory agencies and competent authorities (CAs) have issued guidance documents on chiral development, beginning in the mid-1980s. A summary of the major guidance documents from the U.S. Food and Drug Administration (FDA), European Medicines Evaluation Agency (EMEA), Japanese Ministry of Health, Labor, and Welfare, and Health Canada are summarized below for ease of review and comparison.

2.1 U.S. Food and Drug Administration

In 1987, the FDA published a series of guidelines on the content and format of information to be submitted in new drug applications (NDAs). The guideline on chemistry and manufacturing data noted for the first time that the structural identity of a compound must be verified and all chiral centers identified. For enantiomeric structures that had ratios different from a racemic mixture, those had to be identified, as well as providing a diagram of the chemical structure and all known isomers.

However, it was not until 1992 that the agency issued a policy statement specifically regarding development of new stereoisomeric drugs* as either individual enantiomers or racemates. The rationale for the 1992 policy stemmed from a convergence of two major aspects. First, a growing body of historical data demonstrated different characteristics for enantiomers in biological systems as well as pharmacokinetic differences in absorption, distribution, metabolism, and excretion (ADME); however, these differences in enantiomeric profiles were not studied routinely, due to the difficulty in separation. The second aspect driving the 1992 policy statement was that the technological advances now allowed large-scale commercial separation of chiral compounds and production of single enantiomers. Thus, the agency's perspective shifted to address acceptable manufacturing controls of synthesis, impurities, pharmacological and toxicological assessment, characterization of metabolism and distribution, as well as clinical evaluation of stereoisomeric drugs.

The 1992 policy cited examples of enantiomers in which both had similar qualities, albeit some enantiomers had different concentration–response relationships (or receptor affinity/receptor activation kinetic) profiles compared to each other, such as:

- Dobutamine enantiomers are both positive ionotropes.

*The FDA Policy Statement for the Development of New Stereoisomeric Drugs, issued May 1, 1992. The policy noted that the term *stereoisomers* included enantiomers, geometric isomers (e.g., *cis/trans*), and diastereomers (isomers with more than one chiral center that are not mirror images of one another). Since the agency considered diastereomers and geometric isomers as chemically and pharmacologically distinct, and which can be separated without chiral techniques, these are generally treated as separate drugs, with some very specific exceptions (e.g., in vivo interconversion). There is no reason to develop a mixture of diastereomers or geometric isomers unless they fortuitously present a reasonable fixed-dose combination.

- Ibuprofen enantiomers are both anti-inflammatory agents.
- Warfarin and phenprocoumon enantiomers are both anticoagulants.
- Bupivicaine enantiomers both produce local anesthesia, but the incidence of severe arrhythmias in isolated rabbit heart is much less with the *S*(−)-enantiomer than the *R*(+)- isomer or the racemate.
- Quinolones and β-lactam antibiotic enantiomers are all antibacterial.

Conversely, there are numerous examples of enantiomers with strikingly different qualities, such as:

- The *R*(+)-enantiomer of thalidomide has sedative action but the *S*(−)-enantiomer is a teratogen.*
- The *l*-propranolol enantiomer is a β-adrenergic receptor antagonist (β-blocker), but *d*-propranolol is not.
- The *d*-sotalol enantiomer is a type 3 antiarrhythmic, whereas *l*-sotalol is a β-blocker.
- The *d*-levodopa isomer is associated with granulocytopenia.
- The *d*-levamisole isomer is associated with vomiting.
- The *d*-carnitine isomer is associated with myasthenia gravis symptoms.

In addition to the pharmacological differences, the agency also noted that there were numerous cases of enantiomers having different pharmacokinetic (PK) profiles. Although these differences might not have a clinical impact, they could make nonchiral blood level assays difficult to interpret with accuracy or confuse the interpretation of nonclinical data if the animal disposition of the isomers differed significantly from those in humans.

Despite the differences in enantiomer activity described above, the agency's 1992 policy statement noted that development of racemates historically resulted in only a few recognized adverse profiles. Even in the light of feasibly preparing purified enantiomers, the agency recognized that development of racemates was still appropriate but that the following data should be considered:

- Appropriate manufacturing and control procedures should be used to assure enantiomeric composition with respect to identity, strength, quality, and purity. Manufacturers should notify compendial authorities of these specifications and tests.

*Thalidomide was widely marketed in over 46 countries as a sleep aid. Its use during pregnancy was responsible for over 10,000 children born with profound birth defects. Thalidomide would have been approved in the United States were it not for the vigilance of a medical officer, Frances O. Kelsey. The public health catastrophe prompted stronger regulation (Kefauver–Harris Amendment of 1962) for demonstrating the safety of a product prior to marketing. Although it was commonly thought the toxicity resided solely with the *S*(−) form of thalidomide, several in vitro studies showed that the drug could racemize quickly in various aqueous media. Metabolic inversion has been observed with other compounds, such as 2-arylpropionic acid (2-APA) and mandelic acid.

- PK evaluations that do not use a chiral assay will be misleading if the disposition of the enantiomers is different. Therefore, techniques to quantify individual stereoisomers should be available. However, if the PK of the enantiomers is demonstrated to be the same or to exist as a fixed ratio in the target population, a nonchiral selective assay may be used.

Based on the synopsis above, the agency's 1992 policy statement was meant as an outline of the particular studies expected during the development of enantiomeric drugs. In general, the enantiomeric composition of a drug should be known for the material used in pharmacology, toxicology, and clinical studies. The finished product specifications should assure identity, strength, quality, and purity with respect to enantiomeric purity.

With respect to PK profiles of enantiomers, quantitative assays should be used for individual enantiomers early in the drug development program, which will allow assessment of any potential for interconversion and the ADME profile. If the drug is a racemate and the PK profiles of the isomers are different, sponsors should monitor the enantiomers individually to determine properties such as dose linearity and the effects of altered metabolic or excretory function as well as drug–drug interactions. If the PK profile is the same for both isomers or a fixed ratio between the plasma levels of enantiomers is demonstrated in the target population, an achiral assay or assay that monitors the most relevant enantiomer is acceptable for later evaluation.

Unless it proves particularly difficult, the main pharmacologic activity of the isomers should be compared in in vitro systems, animals, and/or in humans. A relatively mild toxicology profile of the racemate would usually support further development without separate toxicology assessments of each enantiomer. Conversely, if there are findings other than those associated with a natural extension of the pharmacological activity, unusual toxicity, or toxicity approaching the effective dose in animals or near the planned dose in humans, a toxicology evaluation of the individual enantiomers is warranted.

The 1992 policy statement outlined enantiomer development for chemistry and manufacturing as well as nonclinical and clinical studies. A brief synopsis is provided below.

Chemistry, Manufacturing, and Controls Data

- Analytical methods for racemates and enantiomers should include a stereochemically specific identity test and/or selective assay. This identity and assay method should be in place for drug substance and drug product. With respect to stability testing, a selective method should also be in place, but once it has been demonstrated that stereochemical conversion does not occur, the stereoselective tests might not be required.
- Impurity limits for each isomer should include all isomeric components, impurities, and contaminants. The maximum allowable level of impurity in product used for clinical trials should not exceed the level that was assessed in nonclinical toxicity studies.

- Labeling should include a name with the appropriate stereochemical description.

Nonclinical Studies

- Pharmacologic activity of the individual enantiomers should be characterized for the principal mechanism of action and any other important effects with respect to potency, specificity, maximum effect, and so on.
- Pharmacokinetic profiles should include an in vivo assessment of interconversion and disposition for each enantiomer and be compared later to the clinical PK profile in phase I and phase II.
- Toxicology on the racemate should be sufficient; however, if toxicity other than that predicted by its known pharmacologic properties should occur at low multiples of exposure, toxicity of the individual enantiomers is warranted to ascertain the relative contribution of each for the toxicity observed. If the toxicity of significant concern can be eliminated by development of a single enantiomer, that would be the preferred route of development.
- To study a single stereoisomer from a mixture that has already been studied nonclinically, an abbreviated pharmacology/toxicology evaluation would be required. This program would include a repeated-dose toxicity study up to three months and a reproductive toxicity segment II study in the most sensitive species. The positive control would probably include the racemate. If there is no difference between the single enantiomer and the racemate, no further studies would be required. If the single enantiomer demonstrated a different or more toxic profile than that racemate, further studies would be necessary as well as an evaluation of its implications for human dosing.

Clinical and Biopharmaceutical Studies

- As noted above, a racemate may be considered suitable for development barring no significant differences in the activity and disposition of the enantiomers. Individual enantiomers should be evaluated in case there is toxicity of the racemate at clinical doses that is either unexpected or suggests an unrelated pharmacologic effect. It should be noted that some enantiomers may not have different toxicity profiles, but their metabolites may. Thus, it is also relevant to consider individual programs when the activity of one is clearly superior to that of the other.
- If a racemate is studied, the PK of the two isomers should be evaluated in phase I to confirm a lack of in vivo interconversion. Based on phase I and phase II PK data in the target population, it should then be possible to determine if an achiral assay or monitoring of just one enantiomer is sufficient to evaluate both.
- If a racemate has been the subject of an approved market application and a sponsor wishes to develop the single enantiomer, the FDA requires a new NDA. The evaluation must include a determination of any significant conversion to the other isomer and whether the PK profile of the single

isomer is comparable to that of the racemate. However, bridging with data of a racemate already on the market is not possible.

The combined effect of the FDA policy, refinements in chiral separation, and improved analytical methods over the last 20 years has been a greater effort required from the pharmaceutical industry to define the pharmacology and toxicology of individual enantiomers from racemic drugs. By increasing the scrutiny of a racemate vs. single-enantiomer profiles, the regulatory authorities influenced the industry's decision to develop more single enantiomers in lieu of racemic drug mixtures.

2.2 European Union European Medicines Evaluation Agency

The most specific guidance by either the European Union (EU) or the European Medicines Evaluation Agency (EMEA) was the *Investigation of Chiral Active Substances* issued in October 1993 [2]. This guidance was principally revised from the *Clinical Investigation of Chiral Active Substances/III/3501/91* to include quality and nonclinical development considerations. It mirrored many of the same points as the FDA 1992 *Policy Statement for the Development of New Stereoisomeric Drugs* but provided more specific guidance for chemistry and nonclinical studies.

However, the earliest guidance regarding stereochemistry was published in 1989 by the European Commission (EC) Committee for Proprietary Medicinal Products (CPMP) in Volume II, *Notice to Applicants for Marketing Authorizations for Medicinal Products for Human Use in the Member States of the EC*, which specifically required the Expert Report to include an evaluation of chiral data.

In addition, the CPMP guideline on analytical validation was adopted in July 1989 and published in the July 1990 addendum to Volume III of the *Rules Governing Medicinal Products in the European Community*, which notes that "methods or procedures of analysis proposed must take account of technical and scientific progress and enable the starting material, intermediate, and finished product to be checked by means of generally accepted methods." Although the reference in Volume III does not specifically mention chiral assays or analysis of single enantiomers, it is implied that the most advanced analytical methods available at the time should be used.

With respect to the chemistry aspects, the EU guidance noted that the drug substance (DS) may be a racemate (equimolar), a nonequimolar mixture of enantiomers, a single enantiomer, or a mixture of enantiomers and/or diastereomers. In the case of a single enantiomer, the other enantiomers will be considered an impurity. The analytical methods used [e.g., optical rotation, chiral high-performance liquid chromatography (HPLC), optical rotary dispersion, circular dichroism, nuclear magnetic resonance (NMR)] should be validated to demonstrate the limits of detection and quantitation for both the DS and impurities. Note that for NMR, the method cannot distinguish enantiomers from racemic mixtures

without proper shift reagents or latently installed chiral auxiliary agents. Solid-phase methods include polymorph analysis by x-ray particle diffraction (XRPD) and differential scanning calorimetry (DSC).

The EU guidance also provided more detail than the U.S. guidance for nonclinical testing for a single enantiomer vs. a racemate as well as a single enantiomer from an approved racemate. The guidance also noted nonclinical considerations for developing a new racemate from an approved single enantiomer and a non-racemic mixture from an approved racemate or single enantiomer. Similar to what was noted in the U.S. guidance for development of a single enantiomer, the 1993 EU guidance recommended an analysis of animal in vivo ADME data for comparison to human ADME data to ascertain any conversion of one enantiomer into another (e.g., metabolic inversion) or into a racemic metabolic pool. Based on those findings, a chiral bioanalytical assay might be required for continued clinical development.

If the sponsor elected to develop a racemate DS instead of a single enantiomer, justification and support by animal PK/PD and ADME studies were required. Two potential scenarios might occur: rapid conversion of the enantiomer to the racemate in vivo, or no/slow conversion in vivo that allowed for separate enantiomer effects and ADME data pools. The pharmacodynamic (PD) response should be compared for the racemate vs. the single enantiomer. The pharmacokinetic (PK) response should be measured by enantiospecific analytical methods. Both the PK and PD animal data should be compared to human phase I data. With respect to toxicology, it is acceptable to perform toxicology studies using the racemate, especially if sufficient quantities of the preferred enantiomer are unavailable and/or the animal in vivo studies indicate conversion to the racemate. However, if toxicity other than that predicted by an extension of the pharmacological properties occurs at relatively low multiples of the exposure intended for clinical study, relevant toxicity studies should be repeated with the racemate and purified enantiomers to help discern the activity and toxicity of each component.

It is also critical to have proper process controls when dealing with an enantiospecific drug substance. If the API synthetic scheme uses a chiral starting material, regulatory agencies expect strict specification controls on the starting material source and acceptance criteria. The sponsor who procures the chiral starting material or raw material with enantiospecific consistencies should expect, and implement, release testing in-house and batch analysis to establish trending data. On the other hand, if the sponsor chooses to optimize an enantiospecific process by which the desired chirality is induced during a specific step in the process scheme, adequate in-process test controls for enantiospecificity are expected. It is also critical that the enantiospecific process be planned thoroughly and carefully to avoid an early induced chiral center on any intermediates during the process scheme and/or so as not to undergo racemization, due to severe reaction parameters of any step.

In principle, development of a single enantiomer from an approved racemate can utilize existing toxicity data of the racemate to help bridge the toxicity data program of the enantiomer; however, use of the racemate data is decided on a case-by-case basis. Some key considerations include:

- *Pharmacodynamics*. The profile of the enantiomer should be compared to that of the racemate.
- *Pharmacokinetics*. The profile of the enantiomer should be compared to that of the racemate.
- *Toxicology*. A suitable bridging program may be comprised of (1) an acute toxicity study of the enantiomer compared to the racemate; (2) a repeated-dose study up to three months in a single most appropriate species, and (3) a study of pre- and postnatal development, including maternal function with the modification of starting treatment at conception using the racemate as a positive control. Results for the single enantiomer should be compared to those for the racemate.

The EU guidance also outlined clinical considerations, which largely mirrored the U.S. guidance and repeated many of the same points brought out for nonclinical testing (e.g., comparison of PK/PD profiles). In the situation regarding development of a nonracemic mixture from an approved racemate or single enantiomers, the EU guidance noted that this approach was largely similar to a fixed combination product that has been developed to optimize the molar ratio of compounds for therapeutic benefit. Thus, the sponsor developing this type of product should also refer to the EU guidance *Fixed-Combination Medicinal Products*.

In addition to the EU guidance *Investigation of Chiral Active Substances*, points to consider for development of chiral compounds are noted in several other guidances, such as *Investigation of Chiral Active Substances*, Committee for Veterinary Medicinal Products [3].

2.3 Japan (Ministry of Health, Labour, and Welfare)

While the Japanese regulatory authorities have not issued specific guidance on the development of chiral compounds, they appear to be using an approach similar to that adopted by the United States and the EU. A retrospective analysis of drug approvals in Japan from 1988 through 2007 assessed the number of enantiomers, racemates, and achiral drugs that were approved, which showed a definite trend of increased development of single isomers rather than racemates [4].

Shimizawa et al. [4] analyzed 76 drug approvals in Japan from January 2001 through 2003, which were comprised of 29 achiral drugs (39%), 23 single enantiomers with multiple chiral centers (30%), 14 single enantiomers with one chiral center (18%), and 10 racemic drugs (13%). The total number of single enantiomeric drugs was 37. Twenty-nine (29) of the 37 (78%) single enantiomeric drugs were synthesized from an enantiomeric starting material; three products (8%) were derived by asymmetric synthesis, and the remaining five products (14%) were derived by asymmetric resolution (e.g., crystallization and/or chromatographic separation).

Confirmation of stereochemistry was confirmed by XRPD for 22 products (59%); 11 products (30%) did not specify a method; and four products (11%) were confirmed by identification through the authentic samples. Specifications for

assuring chirality appeared largely to be those using optical rotation (21 products, or 57%), optical rotation and optical purity by HPLC (11 products, or 30%), optical purity by HPLC only (two products, or 5%), and not set (three products, or 8%). With respect to pharmacokinetic assessment, a PK evaluation was performed for all the products, but only 12 products were associated with any reported assessment of chirality on PK; 10 products were reported with chiral inversion.

An analysis by Shimizawa et al. showed that Japanese government approval of single-enantiomer drug products rose from 39% (of all drugs approved in Japan) in 1988–1990 to 56% in 2000–2002. Conversely, the number of racemic drug products approved in the same period dropped from 32% to 11%. The Japanese approvals were similar to approvals observed worldwide, with single enantiomers accounting for 27% in 1996, up to 39% in 2002 [5,6].

2.4 Canada (Health Canada)

In July 1998, Health Canada published a Therapeutic Products Programme (TPP) guidance document entitled *Stereochemical Issues in Chiral Drug Development*; it became effective May 1, 2000. The overall content followed much of what the U.S. and EMEA guidance documents noted with respect to development considerations for single enantiomers, racemates, and nonracemic mixtures. The key distinction is that since it is one of the most recent guidance documents on this topic, there is more detail in the development plan regarding when certain analytical methods or assessments should be performed.

Of particular note is the preamble, which states that the sponsor should develop and validate an enantiomer-specific assay as early as possible in drug development and use it until the combined data clearly demonstrate that the same results are achieved with a nonselective assay. A list of chiral methods to be considered include NMR, infrared (IR), gas chromatography (GC), chiral HPLC, chiral GC, x-ray crystallography, melting point, circular dichroism (CD), optical rotation, optical rotary dispersion (ORD), and others.

3 CHIRAL SWITCHES AND BRIDGING RACEMIC DATA TO ENANTIOMER PROGRAMS

Although the guidelines detailed in Section 2 touched on chiral switches and bridging data from racemic to enantiomer programs, this was actually an early and intense area of interest by both industry and regulators. Before the FDA issued the 1992 policy statement, the agency formed a stereoisomer committee in 1989 to study and formulate key areas of stereoisomer development. In response, the Pharmaceutical Manufacturers Association (PMA) formed its own committee to articulate industry viewpoints on the development of chiral compounds. The PMA issued a position paper in 1990 which stated that a manufacturer's decision to market an enantiomer or racemate should be case by case after considering all available data and providing the regulatory authorities with data that prove

the safety and efficacy of the drug proposed. For economical and practical reasons, it is preferred to make that decision (racemic vs. enantiomer) as early as possible in the development, but the fact remains that the information required is gathered gradually during development. Thus, the industry should not be disposed prematurely against development of racemic drugs and cited numerous examples: antihypertensive agents such as verapamil, atenolol, metoprolol, and captopril, or the bronchodilators salbutamol and antihistamine terfenadine. While the PMA recognized that, more data might be required to justify the selection and development of a racemate over an enantiomer, the agency's requirements should not be interpreted as a discouragement of racemic drug development. The most important point was that if a development team decided to switch from a racemate to a single enantiomer, there should be some conservation of effort.

Thus, a key consideration by the PMA was how and when bridging of racemic development data could be applied to a chiral switch, particularly with respect to avoiding a complete repeat of the racemate nonclinical program with the enantiomer. The industry position was that it might not be necessary to repeat acute and subchronic toxicology studies with the single enantiomer if they have been conducted properly with the racemate; the PMA conceded that chronic toxicity studies should be done with the single enantiomer. The agency concurred (up to a point), and in the FDA 1992 policy, it noted:

> Unless it proves particularly difficult, the main pharmacology activities of the isomers should be compared in in vitro systems, in animals and/or in humans. A relatively benign toxicologic profile using the racemate would ordinarily support further development without separate toxicologic evaluation of the individual enantiomers. If, however, there are toxic findings other than those that are natural extensions of the pharmacologic effects of the drugs, and especially if they are unusual or occur near the effective dose in animals or near the planned human exposures, toxicologic evaluation of the individual isomers in the study where the toxicity was detected should be undertaken.

The 1992 policy specifically noted that an abbreviated pharmacology/toxicology evaluation could be conducted to allow the existing knowledge of the racemate to be applied to the enantiomer. The longest repeated-dose toxicity study (up to three months) and reproductive toxicity segment II study in the most sensitive species would need to be performed with the enantiomer. The positive control group should include the racemate. If there is no difference between the toxicology profiles of the enantiomer and the racemate, no further studies would be needed. However, if the enantiomer were more toxic than the racemate, further studies would be warranted.

The thrust of the agency policy was that toxicity data of the racemate could be used to bridge the nonclinical program of an enantiomer only if they were tested side by side or as controls in these studies. There did not appear to be any allowance for historical comparisons of the racemic toxicology profile to a standalone enantiomer profile performed separately. That is an important point to note, especially when a development team may, or may not, know if an enantiomer has