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Analytical Survey

Chiral drugs: an industrial analytical perspective

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Abstract: In the pharmaceutical industry, chiral drug candidates introduce a unique set of challenges to all disciplines involved in the drug development process. For the analytical chemist in particular, the generation of relevant information about a variety of stereoisomeric issues is necessary. Chiral drug candidates, whether a single isomer or a mixture of isomers, require more analytical information than achiral drug candidates. This information can be derived from enantioselective spectroscopic and chromatographic techniques. Chiral analytical methods require proper development and validation to ensure accurate results. Issues related to method development and validation for complete stereochemical characterization are discussed, with primary emphasis on the generation of analytical data required for the registration of a chiral drug candidate. The presentation of pertinent analytical data depends on an awareness of the problems encountered during the development process and the appropriate use of methodology for the determination of stereoisomeric purity.

Keywords: *Chiral; enantiomers; quantitation; method validation.*

Introduction

The growth in chiral drug development is reflected in a 1986 survey which estimated that approximately one-half of the 700 most frequently prescribed drugs contain at least one stereogenic centre [1]. The refinement and implementation of asymmetric synthetic techniques in the pharmaceutical industry has dramatically increased the ability to produce therapeutic agents which are enantiomerically pure [2, 3]. As the ability to manufacture stereochemically pure bulk drugs on a large-scale becomes more feasible, the need for chiral analytical support becomes critical. Although several authors have addressed the issues of chiral drug development from an industrial perspective, they have focused primarily on the drug discovery process, large-scale asymmetric synthesis, and preparative chiral purification techniques [4, 5]. There has been little mention of the rôle of the analytical chemist in the endeavour to produce and market stereochemically pure drug products. In this paper, the impact of producing chiral drug products in the field of analytical chemistry in the pharmaceutical industry is discussed.

Background

The major question the analytical chemist faces in chiral drug development is: What additional analytical information is required for the development of chiral drug products? In answering this question, the analytical chemist must consider the following questions: Will the initial development and clinical testing involve the use of an enriched/pure enantiomer or a racemate? What structure confirmation techniques are necessary for the complete stereochemical characterization (i.e. determination of absolute configuration) of a chiral drug candidate? Which analytical techniques are best suited for determining the enantiomeric purity and stability of the chiral drug candidate? What problems will be encountered in developing and validating robust chiral methods? Do the undesired isomers present in a chiral drug candidate need to be determined accurately, or is an acceptance limit test appropriate? Which method of quantification and reporting of results should be used in conjunction with regulatory specifications?

To put these questions into perspective, the three approaches for chiral drug development must be addressed. They are: (1) development, clinical testing and registration of the racemate; (2) early stage development and clinical testing of the racemate, followed by further clinical evaluation and registration of the pure enantiomer once efficacy is established; or (3) development, clinical testing and registration

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of the pure enantiomer. The decision to develop either a single enantiomer or racemate should be based on the efficacy of each enantiomer, their relative rates of metabolism, pharmacological differences, and/or potential toxicological differences. All of these factors need to be carefully evaluated for each drug candidate before deciding whether the pure enantiomer or the racemate is the best candidate for early clinical development. A more detailed discussion of these considerations has been recently outlined in a Pharmaceutical Manufacturers Association (PMA) position paper [6].

Although analytical chemists may not decide the development program for a chiral drug candidate, this decision has a significant impact on their rôle in the development process. The first approach, the development and registration of a racemate, has similar analytical resource requirements to those of an achiral drug candidate. For this reason the requirements to develop a racemate will not be discussed. The other approaches would be required if preliminary development data indicated that one enantiomer were preferred. For example, warfarin is administered clinically as the racemate. Chiral analysis of physiological fluids showed that the (*S*)-enantioner is four times more potent than the (*R*)-enantiomer and is eliminated more rapidly [7]. Such preliminary pharmacokinetic and activity data may indicate a significant advantage for the development of a specific enantiomer. If the sponsor chooses to register a pure/enriched enantiomer, early development programs and clinical trials can progress using either the pure enantiomer or the racemate. Following confirmation of clinical safety and efficacy with either the racemate or enantiomer (Phase I and II studies), the pure enantiomer is carried forward for a new drug application (NDA) submission. The following discussion will focus on the analytical implications of these two approaches (development of either a pure enantiomer or the racemate in early clinical trials) when registering a pure enantiomer for NDA submission.

If all drug candidates were successful, the decision whether to develop a single enantiomer or a racemate for early clinical testing would not be critical in terms of the chiral analytical support for a NDA submission. In this situation, both cases require identical chiral support over the duration of the project. However, since all candidates do not result in a NDA submission, the differences in chiral analytical support for each case (through early clinical trial evaluation) are significant. The largest portion of analytical chiral support for development of a pure enantiomer occurs prior to an investigative new drug (IND) submission because of the need for complete stereochemical characterization. Since most drug candidates progress to this stage of development, considerable analytical resources are required for all projects in this category. In contrast, development of the racemate through early clinical evaluation does not require significant analytical chiral support until safety and efficacy are established. Once established, further development involves the pure enantiomer and requisite analytical chiral support. The differences and resulting analytical resource requirements for each case are discussed in the following paragraphs.

When progressing through early clinical trials with the racemate, the first stage involves screening for pharmacological activity in animal or other activity tests (Fig. 1, solid lines). If sufficient activity is observed, the candidate is developed with the intent of filing an IND for the racemate. To this point, only achiral analytical support is necessary. Following the IND submission, the racemate is investigated in clinical trials to determine efficacy in humans. To obtain meaningful pharmacokinetic data, chiral analytical methodology to assess potential differences in the metabolic rates of the enantiomers will be required. If acceptable clinical efficacy for the racemate is established, then resolution of the enantiomers is undertaken (Fig. 1, dotted lines). The individual enantiomers are then retested for pharmacological activity in either animal or other activity tests to determine if one enantiomer is more active than the other, or if they are equivalent. Assuming that one enantiomer has an advantage over the other enantiomer or the racemate, then the decision should be made to proceed with the more active enantiomer. This enantiomer would be clinically evaluated under an IND amendment and, if efficacious, a NDA submission would be made for the pure enantiomeric product. Following successful clinical evaluation of the racemate, the need for chiral analytical support becomes significant and will equal that of any achiral analytical support necessary for a NDA submission.

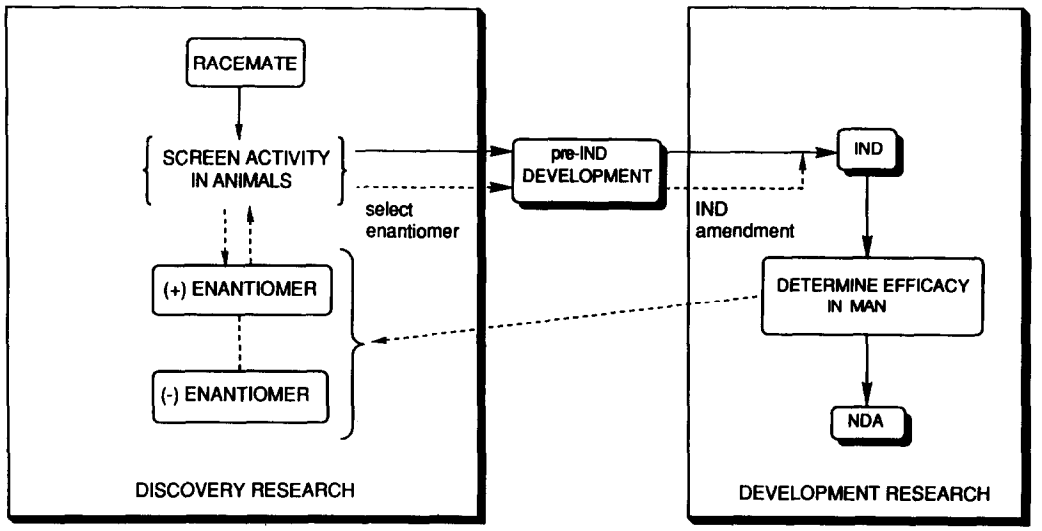

Figure 1
Registration of a pure enantiomer through the initial development of the racemate (dotted lines indicate additional work if clinical efficacy and safety are established).

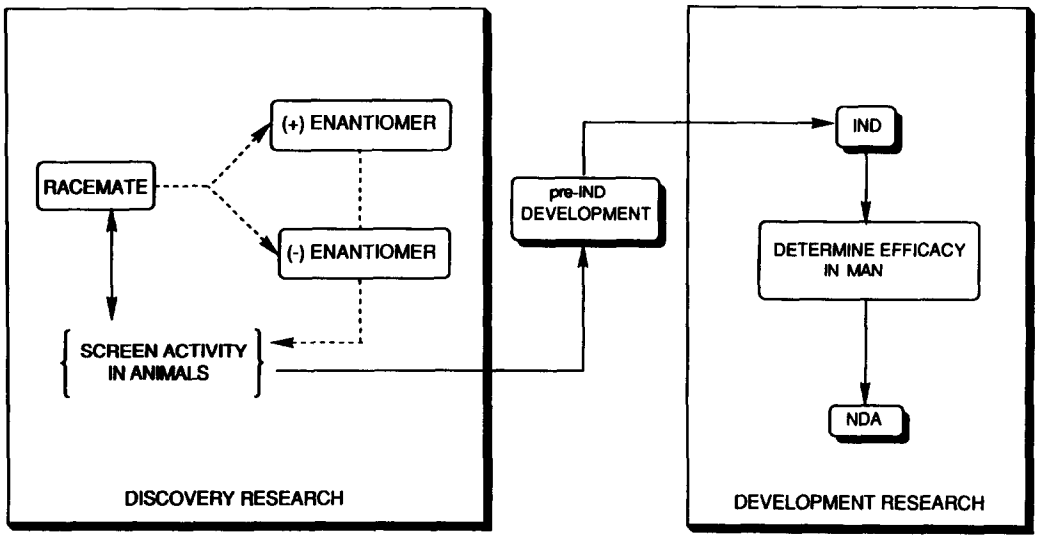

Figure 2
Registration of a pure enantiomer through the initial development of the pure enantiomer (dotted lines indicate preliminary screening efforts for racemate and each enantiomer).

Alternatively, the enriched/pure enantiomer is evaluated in early clinical trials. Initially, the racemate is screened for activity in animal or other activity tests. If the racemate has desirable pharmacological activity, the enantiomers are separated and screened to determine which isomer is more active (Fig. 2, dotted lines). If sufficient activity is observed for one of the enantiomers, the candidate undergoes further development, with the intent of filing an IND for the pure enantiomer. This requires complete stereochemical characterization, including structure confirmation and method development. The pure enantiomer then proceeds to clinical trials and the efficacy in humans is assessed (Fig. 2, solid lines). Contrary to the case in which the racemate is initially developed, analytical support for the development of an enriched/pure enantiomer requires both achiral and chiral test methods to support an IND submission. As with the racemate, analytical methodology is necessary to assess potential differences in the rates of metabolism for each enantiomer. If a sponsor has a policy to develop and clinically test only pure enantiomers, then the need for pre-IND analytical

support increases substantially over that required for initial clinical evaluation of the racemate.

The developmental and scientific risks involved in both of these cases have to be assessed. Since the majority of drug candidates do not proceed beyond early clinical trials, initial development of the racemate appears to be advantageous for the analytical chemist. With the exception of chiral methodology to address potential differences in the rates of metabolism for each enantiomer, this approach minimizes chiral method requirements until clinical efficacy and safety are established. While this route appears to be desirable, non-analytical problems encountered during initial evaluation of the racemate could delay registration. If these delays require characterization of each enantiomer, the analytical resource requirements become significant. Problems which could necessitate the testing of each enantiomer are: differing rates of metabolism during clinical trials, undesired side-effects in clinical trials or adverse events during long-term toxicology studies. Since the cause of these problems cannot be assigned to an individual enantiomer, a decision would be required to terminate the candidate or re-evaluate each enantiomer separately. In addition to losing research time, the testing of each enantiomer requires the isolation, screening and analytical characterization of each enantiomer. If the source of the problem is resolved, clinical evaluations could be resumed for the desired enantiomer. In the case of the development of a pure enantiomer, all toxicological and clinical effects can be attributed to a single enantiomer. Since these results are not known *a priori*, the implications of each scenario must be considered prior to implementation of a clinical plan.

The analytical requirements to develop a pure enantiomer, for early clinical testing, can be established by assessing the analytical support required by each customer. For example, synthetic process chemists require analytical methodology to evaluate both achiral and chiral purity of key intermediates and the chiral drug candidate. Formulation development scientists and toxicologists need to determine the potency and stability (achiral and chiral) of the drug candidate in their formulations. Assays to determine drug concentration in physiological fluids are needed by clinical physicians. In short, all disciplines requiring achiral analytical support will also require chiral assay support. Thus, the analytical support required for the development of a chiral drug candidate is substantial when compared with that of an achiral drug candidate.

Analytical Techniques

Recent advances in analytical technology have provided the analytical chemist with additional techniques to address the stereochemical characterization of drug candidates. In many cases, an individual technique is insufficient to characterize the chiral properties of a drug candidate, and therefore several complementary techniques must be utilized. Several of the more common spectroscopic and chromatographic techniques, including their ability to distinguish between individual enantiomers or a racemate, are listed in Table 1.

Table 1
Analytical methods to characterize chiral drug candidates

Method	Can distinguish
Spectroscopic	
Optical rotation	(+) from (−) and (±)
NMR	(+) from (−) and (±)
IR	(+) or (−) from (±)
XRD	(+) or (−) from (±)
Chromatographic	
GC	(+) from (−) and (±)
HPLC	(+) from (−) and (±)
TLC	(+) from (−) and (±)
Other	
Melting range	(+) or (−) from (±)

Spectroscopy

Spectroscopic techniques are the oldest and most frequently used methodologies for the analysis of chiral drug candidates. They include optical rotation, circular dichroism (CD), nuclear magnetic resonance spectroscopy (NMR), infrared spectroscopy (IR) and powder X-ray diffraction (XRD). Whilst these techniques are powerful tools for stereochemical evaluation, difficulties occur during the analysis of a pure enantiomeric drug candidate when the analytical chemist is attempting to measure both the desired enantiomer and trace levels (0.05–1.0%) of an enantiomeric impurity. These problems are magnified when performing these analyses on the drug product because of matrix effects and low analyte levels. Another significant limitation of spectroscopic techniques is their frequent inability

to resolve all isomers of compounds with multiple chiral centres.

Optical rotation is comparatively simple and can be used to distinguish between a racemate and its enantiomers as well as between one enantiomer and its antipode [8]. In order to quantify trace enantiomeric impurities, the drug must have sufficient rotation to provide precise measurements of rotational differences. The main problem with these determinations is the requirement of pure or well characterized reference standards of each enantiomer. Several additional precautions must be taken to ensure that a method is properly validated and provides accurate results. These include the effects of temperature, solvent and wavelength on rotation. The contributions from any potential chiral impurities, pharmaceutical excipients or counterions present in the sample must be individually determined. If any of these components are present at concentrations which can alter the optical rotation of the desired analyte significantly, the accuracy of the measurement can be distorted. In many cases, the judicious selection of the solvent or wavelength can minimize the effects of undesired species on the rotation of the enantiomers [9]. The analyst must fully investigate these properties prior to implementation of this technique as a quantitative test procedure.

NMR spectroscopy using chiral shift reagents or chiral solvating agents can be used as an identity test or for the quantification of enantiomers [10–13]. It can be used to distinguish between enantiomers as well as the racemate. Quantification and method validation require the assessment of potential interferences from other chiral impurities, excipients or counterions present in the sample. In addition, resolution of the enantiomers is dependent on the concentration of the chiral shift reagent or solvating agent, therefore these effects must be determined. For optimum detection limits and accurate quantification, the signals correlating to the enantiomers of interest must have baseline separation and be singlets. For example, Maple has determined a limit of detection of 0.2% for L-diisopropyl tartrate relative to D-diisopropyl tartrate using ultrahigh resolution ^{13}C NMR [14].

Infrared spectroscopy and powder XRD can distinguish between a racemate and its enantiomers, but not between one enantiomer and its antipode [8]. Quantification and method validation require the assessment of potential interferences from other chiral impurities, excipients or counterions present in the sample. Limitations for these techniques are similar to those of NMR, with the primary difficulty being the selection of an appropriate signal region and an acceptable limit of detection. In addition, accurate quantification by XRD requires that the enantiomers be crystalline. Any amorphous components can severely limit the ability to monitor the signal of interest.

Chromatography

Recent advances in chiral chromatographic technology make this the preferred technique if satisfacory separation between enantioners can be achieved. Chiral separations have been demonstrated using gas chromatography (GC), high-performance liquid chromatography (HPLC), and thin-layer chromatography (TLC) [15–23]. Chromatographic methods can be used to distinguish between individual enantiomers and also the racemate, while providing excellent detection limits for quantification of trace chiral impurities. Perry *et al.* have reported enantiomeric purities of 99.9967% when the enantiomeric impurity elutes prior to the desired enantiomer [24, 25]. Chromatographic techniques are generally more rugged, specific and inexpensive compared with many of the spectroscopic techniques. This combination of features provides the analyst with a reliable, routine technique to assess the chiral purity of a drug candidate.

Chiral separations can be performed in either a direct or indirect mode. The direct separation of enantiomers using chiral stationary phases is preferred to the formation of diastereomers using chiral derivatizing reagents, followed by separation on achiral columns (indirect separation). There are advantages and disadvantages to both modes of separation, especially with respect to peak shape, elution order and quantification. A complete review of these issues will not be presented, as they have previously been described [20, 24–30].

Other

Melting range data can distinguish between an enantiomer and the racemate, but not one enantiomer from its antipode [8]. The accuracy of the methodology is limited and the ability to determine trace enantiomeric impurities is generally not feasible.

Analytical Characterization

Generally, more than one analytical technique is required to characterize the chiral properties of a drug candidate. One example of each technique, for the same drug candidate, will be discussed.

Figure 3 shows the powder XRD patterns for a crystalline racemate and its individual enantiomers. These data demonstrate that this technique is unable to distinguish between the (*S*)-and (*R*)-enantiomers, but that the (*S*)- and (*R*)-patterns are significantly different from the racemic crystal pattern. In this example, the

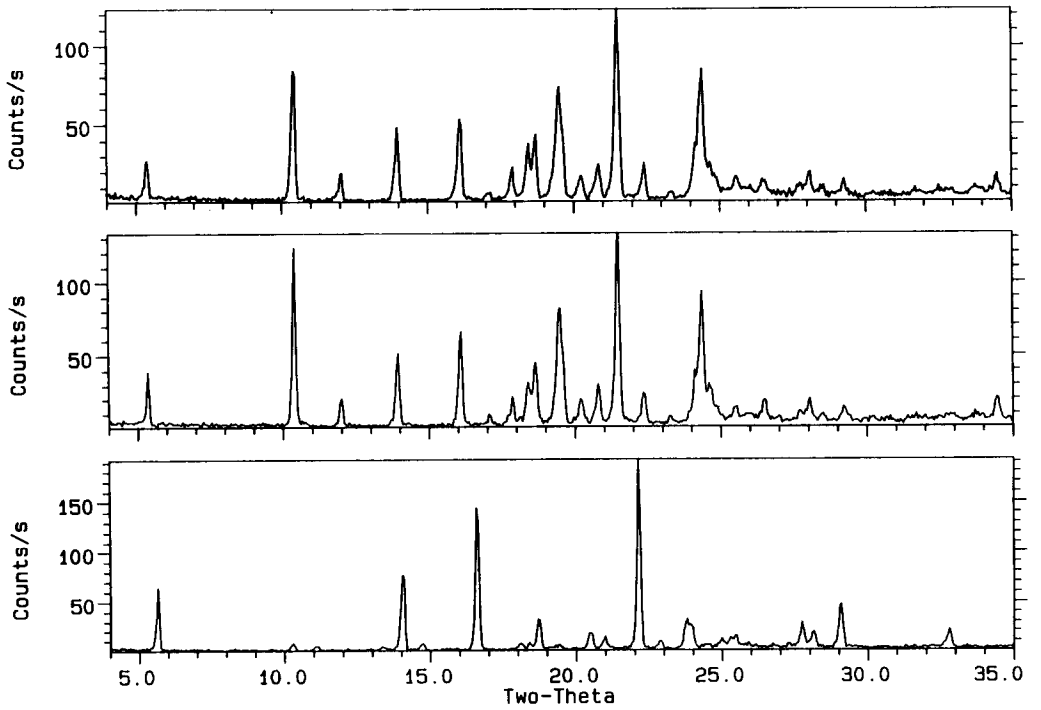

Figure 3
Powder XRD patterns for a chiral drug candidate (top trace, (*S*)-enantiomer; middle trace, (*R*)-enantiomer; bottom trace, racemate).

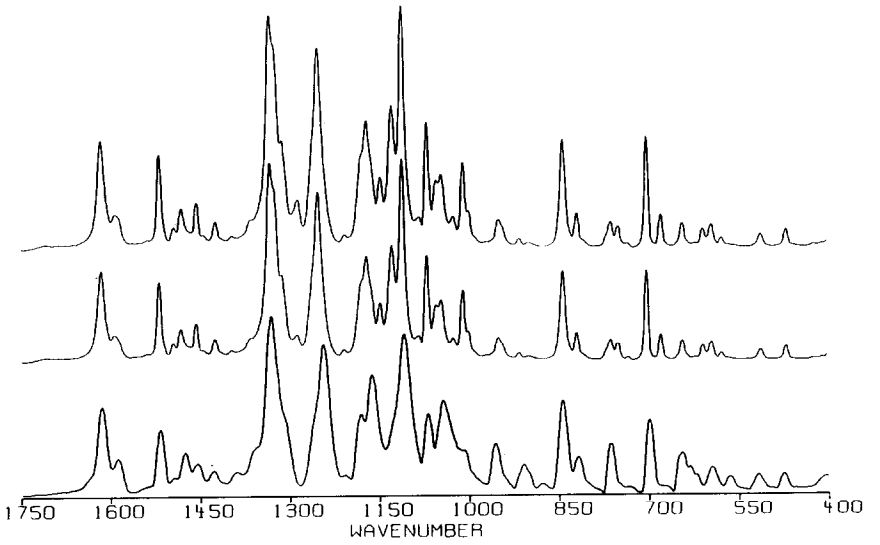

Figure 4
Infrared spectra for a chiral drug candidate (top trace, (*S*)-enantiomer; middle trace, (*R*)-enantiomer; bottom trace, racemate).

powder XRD technique could be used as an identity method to confirm the presence of the racemate or an enantiomer (*R* or *S*).

Infrared spectra for the same racemate and its enantiomers are shown in Fig. 4. In the expanded region of the spectra (1750 to 400 wavenumbers) one observes no difference between the spectra for the (*S*)- and (*R*)-enantiomers, but that a moderate difference exists for the racemate. These small differences between the racemate and enantiomer spectra would allow for adequate differentiation of the species, but not quantification.

Optical rotation for the same enantiomers is ±39.9° in methanol. These data would provide confirmation of identity for either enantiomer or the racemate. Because of the minimal rotation of the compound, this technique would not provide accurate quantification of trace levels of an enantiomeric impurity.

Separation of the enantiomers by LC using a chiral column proved to be the most successful solution. The method separated both enantiomers, which allowed quantification of either enantiomer or the racemate (Fig. 5). Achiral impurities present in the bulk drug substances were also separated from the enantiomer(s) of interest. Specificity for both achiral and chiral components provided the ability to determine both chiral and achiral degradation products. Chromatographic methods frequently provide better accuracy, precision and limit of detection when compared with their spectroscopic counterparts.

Chiral Method Development and Validation

Introduction

In the pharmaceutical industry the analyst is faced with two problems when developing a method. First, satisfactory resolution of the enantiomers must be obtained. Second, a validated method for the quantitative determination of chiral purity must be developed. While achieving chiral resolution is not trivial, it is only a small portion of the task facing the analytical chemist. The second problem, that of validation of a quantitative method for chiral purity determination, is much more time consuming and in many cases much more difficult. The major points that must be addressed in validating a chiral method are the same as those for achiral method validation. These criteria include sensitivity, selectivity, reproducibility, repeatability and stability-indication.

For chiral pharmaceutical products, the major effort is directed toward validation of methods to quantify a trace level enantiomeric impurity in a highly purified chiral drug. Since the principles of method development and validation are similar for most techniques, the

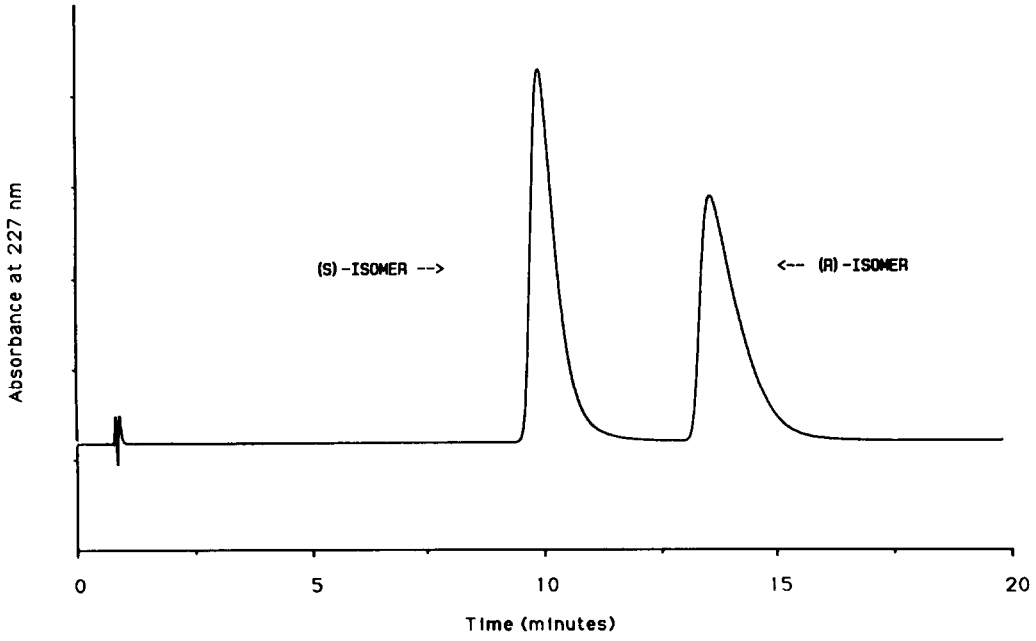

Figure 5
Chromatographic determination of enantiomers for a chiral drug candidate.

following discussion will focus on method development and validation processes for chromatographic techniques.

Enantiomeric resolution

Achieving enantiomeric resolution is the preliminary step in chiral method validation, and it can be accomplished by either the direct or indirect mode of separation. The advantage of using the direct mode (chiral mobile phase additives, charge-transfer, cellulose-based and protein columns) is that in most cases no derivatization of the analyte is required. Another benefit arises as the column begins to racemize. The quantitative results are not significantly affected since the peak areas do not change as resolution decreases. Quantitative capabilities are not lost until the enantiomer peaks lose baseline resolution or an achiral impurity coelutes with the enantiomeric impurity peak. Disadvantages of the direct mode include: (1) higher limit of detection due to poor peak asymmetry; (2) poor selectivity between achiral impurities and the enantiomers; (3) sample solubility problems due to limited mobile phase choices and limited organic modifier concentrations; and (4) limited ability to control elution order, which can result in loss of the enantiomeric impurity peak if it elutes on the tailing edge of the desired enantiomer peak.

The advantages of using the indirect mode of separation (derivatization to form diastereomers) include improved peak symmetry and resolution since the separation occurs on achiral columns. Disadvantages may include: (1) the need for a derivatizable functional group which allows for diastereomer formation; (2) knowledge of the chiral purity and stability of the derivatizing agent; (3) mild and reproducible reaction conditions; (4) quantitative derivatization conditions; and (5) sample preparation complexity.

Sensitivity

When the compound of interest is a racemate or a near racemate, baseline resolution and peak symmetry are not as critical for determining the ratio of the two enantiomers. An example of this situation is illustrated in Fig. 6, where both enantiomers are adequately resolved using a chiral column. This separation was suitable for the intended purpose, the analysis of the racemate, even though peak symmetry was not optimum.

However, for a chiral drug candidate that is essentially a pure enantiomer (>99% chiral purity), baseline resolution and optimum peak symmetry are necessary for adequate limit of detection for the enantiomeric impurity. Although the enantiomer separation in Fig. 6 appeared to be adequate for the racemate, the

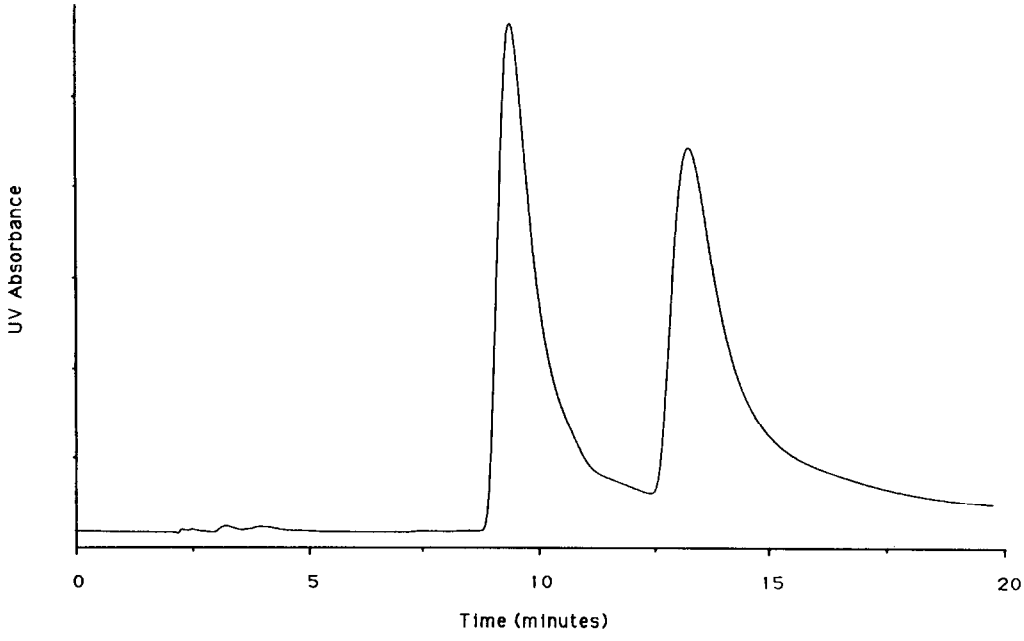

Figure 6
Direct chiral separation of a racemate.

same chromatographic conditions were insufficient for determining trace levels of the enantiomeric impurity. Figure 7 shows the difficulties involved when the enantiomeric impurity elutes on the tail of the drug peak. In this example, the tail of the drug candidate peak masks the enantiomeric impurity until it reaches the 2% level. By addition of a small amount of an amine modifier to the mobile phase, the method was able to detect the chiral impurity down to the 0.1% level (Fig. 7).

Unfortunately, with most chiral columns there are no systematic means to control the elution order. The exceptions are the charge-transfer columns, which are frequently available with either the (+) or (−) chiral stationary phase. Since the undesired enantiomer may be present at an order of magnitude less than the drug candidate, elution of the enantiomeric impurity prior to the major enantiomer peak is desired. Selection of the appropriate charge-transfer stationary phase can provide the analytical chemist with this ability [21]. This obviates problems concerning elution of the enantiomer impurity on the tail of the drug peak (i.e. limit of quantification). Selection of mode of separation (direct or indirect) and the elution order of the enantiomers is critical,

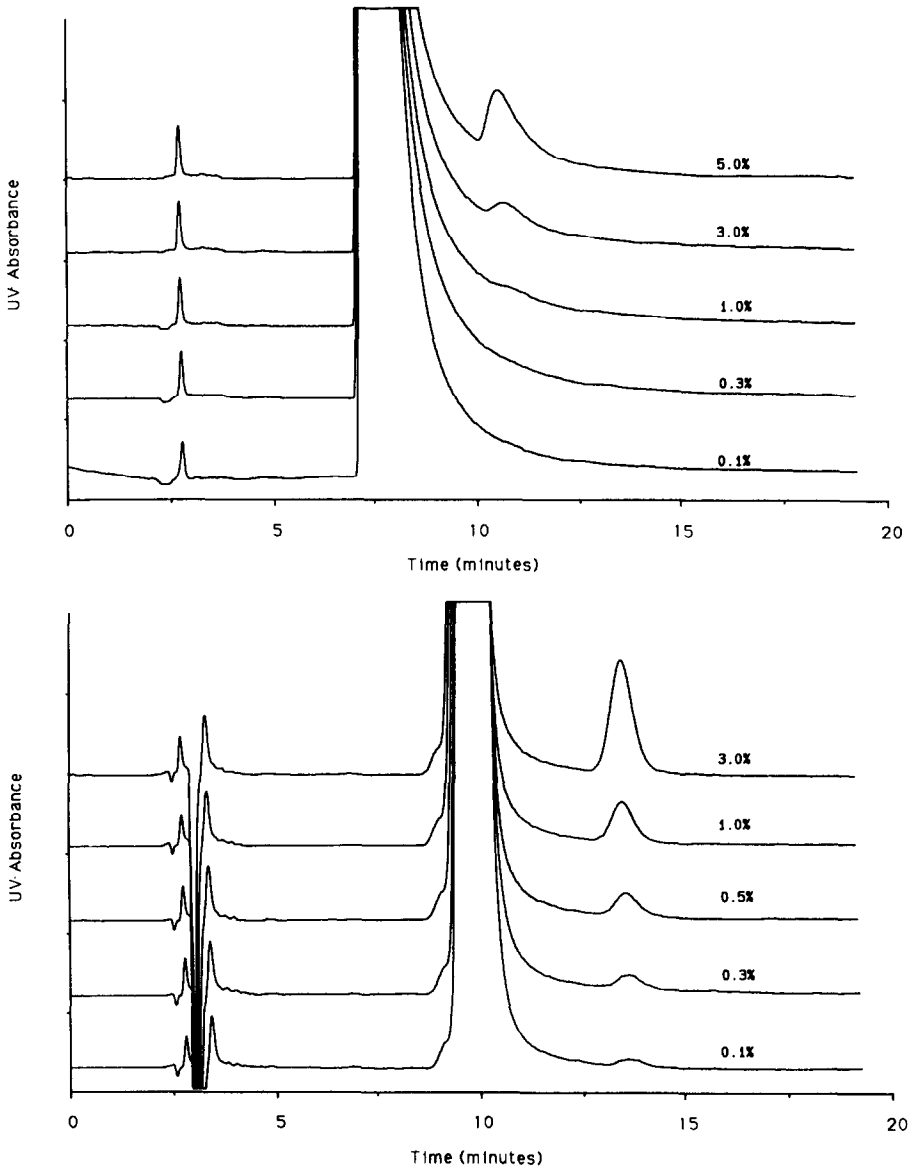

Figure 7
Quantification problems associated with the elution of the enantiomeric impurity on the tail of the desired enantiomer (top chromatogram, no amine modifier; bottom chromatogram, 0.2% amine modifier).

particularly if the resolution between the enantiomers is minimal.

Selectivity

The use of chromatographic methodology in the pharmaceutical analysis of chiral drugs requires not only chiral separation, but also the assurance that any other process related substances, degradation products, excipients or physiological interferences present in the sample matrix are separated from the enantiomers. Figure 8 shows a chromatogram from a method which separates the starting material, a process intermediate, a synthetic precursor and the enantiomers of interest. To assure adequate selectivity, the drug candidate should be stressed to determine if any degradation products will interfere with the enantiomers of interest. While chiral columns are extremely efficient for chiral separations, it has been observed that achiral efficiencies on the same column are significantly less. This is illustrated by the lack of separation between the process intermediate and the starting material (Fig. 8). The combination of lower efficiencies for achiral components, the lack of column robustness, and restrictions in mobile phase composition for chiral columns often require the development of a separate assay for achiral impurities. In some cases, the use of the indirect mode of separation will provide adequate separation of all achiral impurities and enantiomers. This is the result of fewer restrictions on mobile phase composition and better overall column efficiencies for achiral columns.

Reproducibility

Another concern in the development and validation of chiral drug assays is the reproducibility of the separation. Direct chiral separations are generally more susceptible to resolution changes, than their achiral counterparts, arising from minor mobile phase composition variation. Therefore, the analyst must investigate the dependence of resolution on mobile phase composition. With certain types of chiral stationary phases, lot to lot variability can also significantly alter the separation. Figure 9 illustrates the difference in a chiral separation for two cyclodextrin columns from the same manufacturer. While the peak symmetry and resolution for the "good" column were not ideal, the enantiomers of interest were resolved. The use of an "identical" cyclodextrin column, which was derived from a different manufacturing lot of stationary phase, exhibited unacceptable resolution. Minor modification to the mobile phase did not restore adequate resolution for the second column to be considered useful. This lot to lot variability was observed for several columns from the same manufacturer and prevented the

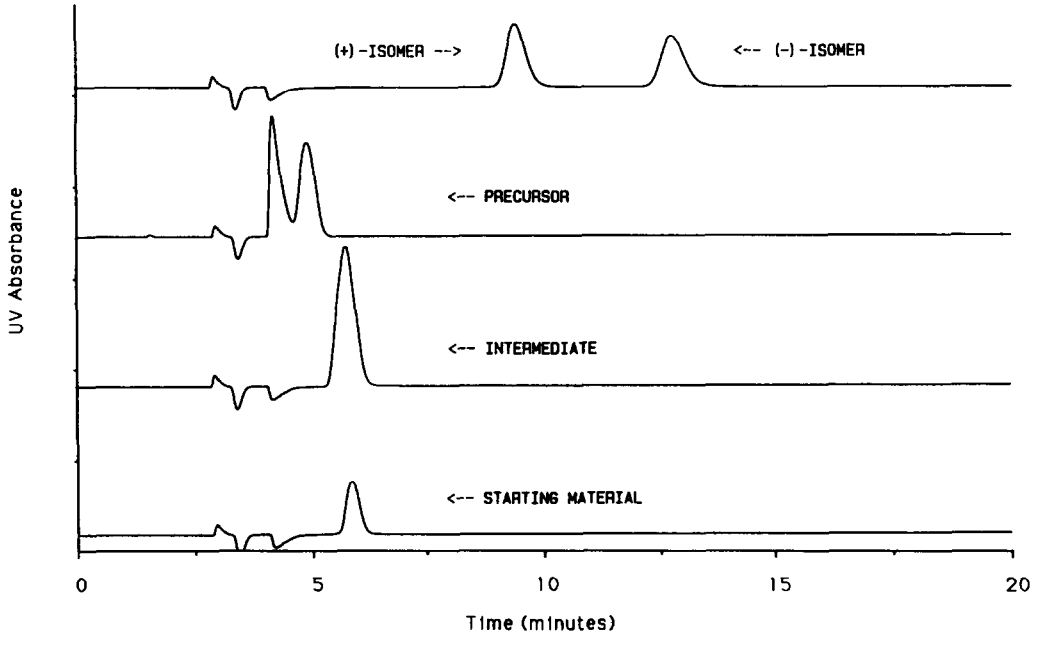

Figure 8
Confirmation of the resolution of achiral impurities from the enantiomers of interest.

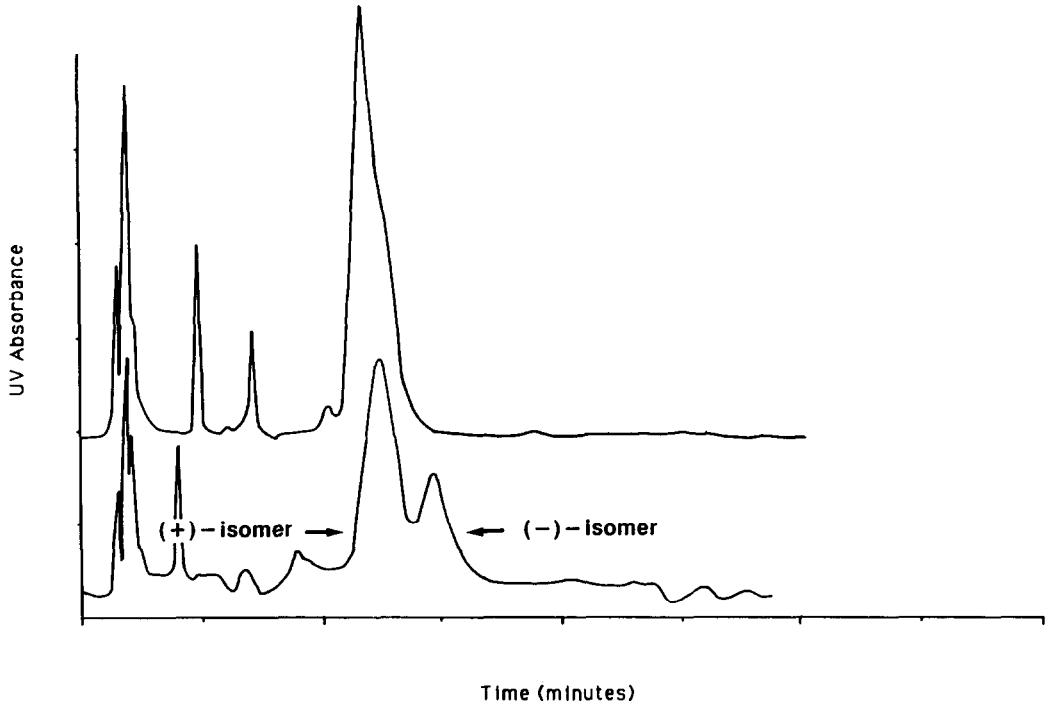

Figure 9
Resolution differences resulting from manufacturing lot variation for a cyclodextrin HPLC column.

development of a reproducible method. In this case, the method ruggedness was improved by using an achiral column with cyclodextrin as a mobile phase additive. This change of procedure provided a method which was rugged and transferable to several different laboratories.

Stability indication

Many of the achiral chromatographic methods developed for stability-indicating assays need a chiral counterpart. In addition to determining the achiral stability of the chiral drug substance, the analytical chemist must be concerned with chiral stability (i.e. racemization). Frequently, the chromatographic conditions developed for the bulk drug substance can be directly translated into chiral assays for the drug product, test article preparations for toxicology, and physiological fluids. While the analyst can generally use these conditions, validation data for other matrices must confirm that no additional interferences or recovery problems occur.

While achiral assays are performed at most stability protocol time points, this is not feasible for chiral assays. In many cases, chiral columns are not able to withstand the rigour of long-term stability studies because of limited chiral column lifetimes. Therefore, the goal of the chiral assay in stability studies is to confirm that the enantiomer composition does not change with time. This can be accomplished by assaying the initial and final samples for enantiomeric composition. These chiral stability studies would be required for the drug substance, drug product, test article formulations for toxicology and physiological fluids. In various matrices these measurements can be hampered by low enantiomer levels (e.g. microgram dosage formulations, trace levels in toxicology test article formulations or trace levels in physiological fluids). While the achiral assay of a drug candidate at low levels is not trivial, the analyst is generally measuring a single component. In the case of chiral drug candidates, it is necessary to measure the enantiomeric ratio as a function of time. These measurements are further complicated by the poor peak symmetry exhibited during chiral separations, which contribute to poorer detection limits. This requires optimization of peak shape so sensitivity will be improved.

Figure 10 shows chromatograms obtained during a stability study for a toxicology test article formulation. The study was designed to address the chiral stability of the drug candidate in rodent feed. Standard practice is to

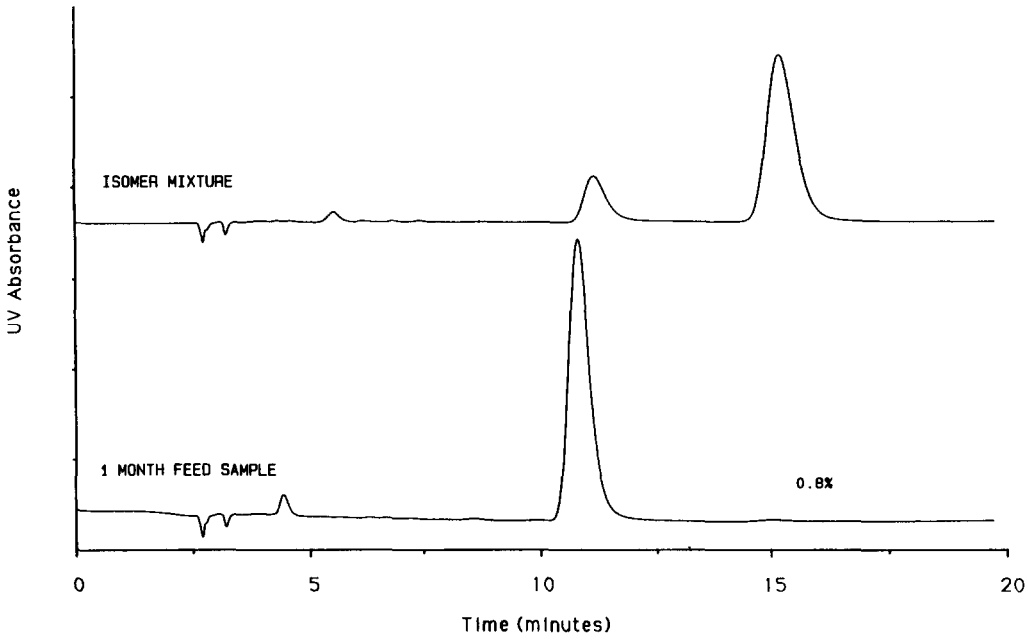

Figure 10
Chiral stability assay for toxicology test article preparation. Enantiomeric impurity present in bulk drug substance at the 0.9% level (top, standard of both enantiomers; bottom, 1 month old feed preparation).

determine the level of drug in the feed, homogeneity of the preparation, and achiral stability of the drug in the rodent feed over a 1-month period. In order to assess whether racemization occurred in the rodent feed, fresh and aged samples were assayed to determine changes in enantiomeric ratio. The fresh and aged samples gave equivalent enantiomeric ratios within experimental error, while also matching the enantiomeric ratio for the drug substance lot mixed into the feed during preparation of the test article mixture. These data, in conjunction with the achiral stability data for the test article mixture, assure that the toxicology preparation is stable for the desired storage period. In studies of this type, the chiral methodology only has to prove that the ratio of the enantiomers does not change since the achiral assay determines potency decreases not associated with racemization.

A similar approach was applied to the determination of the enantiomeric ratio for a chiral drug in plasma samples. Due to desired pharmacological effects (offsetting agonist/antagonist properties which minimized addictive potential), this drug was administered to animals as a racemate. The goal of the study was to determine if the individual enantiomers were metabolized at different rates. This was monitored by determining the enantiomers ratio as a function of time. Achiral determination of plasma concentrations was achieved using GC with electron capture detection. Potential changes in the enantiomeric ratio in plasma were monitored using a chiral HPLC column and fluorescence detection. The enantiomer determinations were complicated by plasma drug level concentrations of nanograms per millilitre. Once adequate sensitivity and linearity had been established, accuracy and recovery were validated by spiking the plasma with the enantiomer mixtures at varying ratios (4:1, 2:1, 1:1, 1:2) and determining if the peak area ratios were consistent with theoretical values (Fig. 11). Following validation of the method, authentic plasma samples were assayed.

Quantification — direct

Using the direct separation mode, the chiral purity of a drug can be determined by three methods: (1) comparison of the enantiomeric impurity peak area to the total peak areas for both enantiomers; (2) comparison of the enantiomeric impurity peak area to a standard curve prepared from a reference standard of the desired enantiomer; or (3) comparison of the enantiomeric impurity peak area to a standard curve prepared from a reference standard of the enantiomeric impurity.

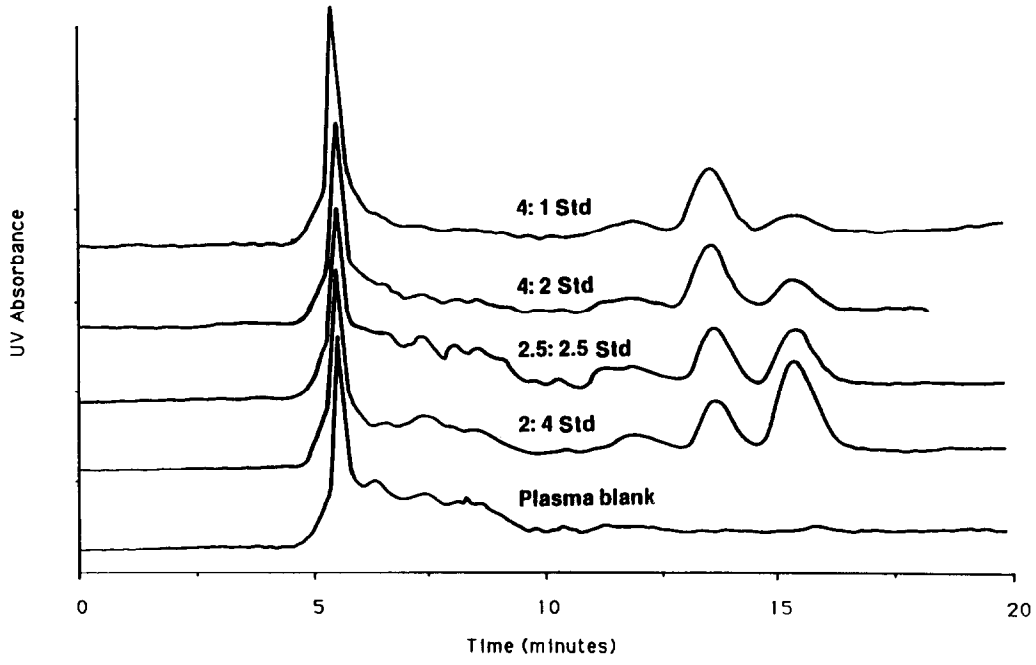

Figure 11
Study of enantiomer ratios for chiral drug candidate spiked into plasma.

Through validation studies, the analytical chemist must determine which of these approaches provides accurate and precise results.

The comparison of relative enantiomer peak areas is preferable since only one chromatographic injection is required for the quantification of the enantiomer impurity. The analyst must demonstrate linearity of response by spiking known amounts of the enantiomer impurity into the desired enantiomer over the range of quantification (e.g. if the analyst wanted to measure chiral impurities from 0.1 to 5%, then the enantiomer impurity should be spiked over this range). The relative peak areas for these spiked samples should agree with theoretical values for confirmation of method validation. If this criterion is not met then the analytical chemist must use the "high–low" approach, where two injections per sample are necessary [31]. Either of these area per cent approaches eliminate the multiple injections required for generating standard curves and more importantly, the need for a characterized enantiomer reference standard. As a minimum, the analytical chemist should evaluate both the area per cent and standard curve approaches to ensure accurate results. Results obtained for the validation of a chiral impurity method, using spiked samples to investigate the accuracy of the area per cent and standard curve approaches are presented in Table 2. For this particular case, both approaches provided excellent precision and agreed with theoretical values. Assuming that one of these approaches will work cannot be

Table 2
Comparison of accuracy (peak area versus standard curve method)

Per cent of minor isomer (area of minor peak versus total peak area)	Per cent of minor isomer (from standard curve of isomer)
2.27	2.16
2.22	2.11
2.24	2.13
2.25	2.13
2.22	2.15
Average = 2.24%	Average = 2.14%
RSD = 0.95%	RSD = 0.91%

made *a priori* and must be confirmed experimentally in all cases.

In some situations, the area per cent method for quantification is inaccurate because of nonlinearity of response or other chromatographic anomalies. The observed peak ratios for spiked samples have a high or low bias when compared with theoretical values. If this phenomenon is observed during validation studies, the analytical chemist must quantify the level of enantiomer impurity versus a standard curve. Disadvantages of using standard curves include multiple chromatographic injections for the standards and samples, and the need for a reference standard of the enantiomer impurity. While one would assume that a standard curve of the enantiomer impurity is necessary for quantification, another possibility exists that does not require a characterized reference standard of the enantiomeric impurity. Since the UV chromophore of the enantiomers is identical, the detector response for all enantiomers is equal. Therefore, standard curves can be generated from a reference standard of the desired enantiomer or enantiomer impurity. The benefit of using the desired enantiomer as the standard derives from the existence of a fully characterized reference standard, which is available from the synthesis of the bulk drug substance. Once again, the analytical chemist cannot assume that this procedure can be validated and must confirm this by careful experimental evaluation. If this approach fails, then a reference standard of the enantiomeric impurity must be synthesized, fully characterized and experiments performed to determine the validity of this approach. If quantification against a standard curve of the enantiomer impurity is necessary, this would significantly impact analytical resources because two reference standards would need to be maintained.

While it has been infrequent that a chiral impurity method could not be validated using either the area per cent or a standard curve of the desired enantiomer, these situations occur. Figure 12 illustrates an assay in which both the area per cent and standard curve (of the desired enantiomer) approaches provided inaccurate results when compared with the theoretical response. Initial validation attempts using area per cent values for samples spiked with the enantiomeric impurity were inaccurate when compared with theoretical values. Therefore, standard curves of reference standards for both the desired enantiomer and enantiomeric impurity were evaluated during further validation studies. The experimental results shown in Fig. 12 illustrate the divergence of the two standard curves. Only the standard curve for the enantiomeric impur-

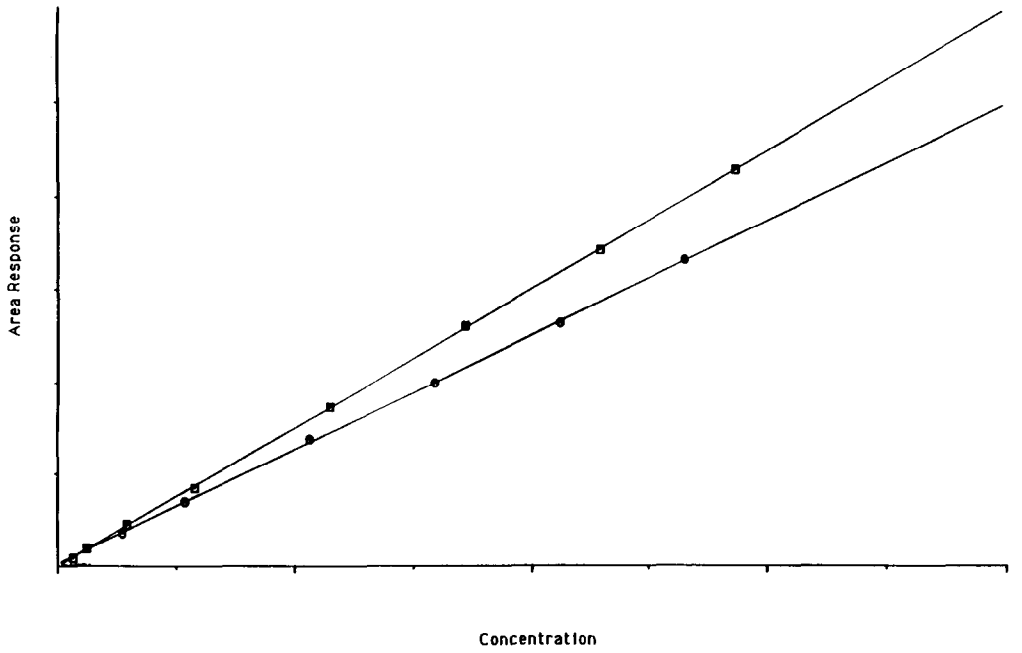

Figure 12
Validation of standard curve response. Standards curves were generated using known quantities of the desired enantiomer and enantiomeric impurity (top curve, desired enantiomer; bottom curve, enantiomeric impurity and theory).

ity gave the results which corresponded to theoretical values. Since the UV responses for both enantiomers are equivalent and the reference standards both exceed 99.8% purity (achiral and chiral) this was an unexpected result. The reason for the observed difference for the standard curve of the desired enantiomer versus theory has not been determined.

When choosing one of these approaches to quantification, the analytical chemist cannot assume that a particular approach will provide accurate results without careful investigation. Assuming that one of the methods is correct, without proper supporting data, can lead to significant errors in the quantification of enantiomeric impurities.

Quantification — indirect

Using the indirect separation mode, the analyst derivatizes the sample to produce diastereomers and then determines the levels of the corresponding diastereomer. The same arguments for quantification using direct chiral separation apply to indirect separation. These options include quantification by: (1) area per cent ratios of the diastereomers; (2) comparison with a standard curve of the derivatized desired enantiomer; (3) comparison with a standard curve of the derivatized enantiomeric impurity; or (4) comparison with a standard curve of the appropriate diastereomer. Again, the analytical chemist cannot assume that one of these approaches will provide accurate results without supporting validation data. Depending on the method of quantification, reference standards would be required for either the desired enantiomer, the enantiomer impurity or the appropriate diastereomer. In the worst case, the analyst would need to maintain four reference standards (all enantiomers and diastereomers).

When utilizing indirect chiral separation, an understanding of the derivatization chemistry and the stability of the derivatization product is mandatory. For quantitative determinations, the chiral and achiral purity of the derivatizing reagent and its chiral stability is necessary. The analyst must also confirm that the molar absorbances for the diastereomers are equivalent. Figure 13 illustrates a problem which can occur when using an indirect separation. In this case a chiral drug containing a secondary amine is reacted with Mosher acid as the chiral derivatizing reagent. Several situations, which can affect the accuracy of quantification, are possible depending on the purity of the derivatizing reagent. For example, if the derivatizing reagent is 100% chiral purity and the compound is 98% chiral purity, allowing these two compounds to react with each other produces two diastereomers in the ratio of 98:2. Separation of these diastereomers on an achiral column would result in a correct assignment of chiral purity. Reversing the purity of the two components (i.e. if the derivatizing reagent were 98% pure and the compound were 100% pure) gives identical results (98:2), even though the drug has 100% chiral purity. The assumption that the derivatizing reagent has 100% chiral purity (98% actual) leads to a significant quantification error. For the examples given, the worst case would be that in which both the reagent and drug have 98% chiral purity. This would result in an incorrect chiral purity assignment of 96% for the drug; therefore, the analytical chemist must also develop chiral methods to assess the purity and chiral stability of the derivatizing reagent.

In some instances, the analytical chemist is only interested in determining whether the chiral drug candidate racemizes (e.g. formulations, toxicology test article preparations). These types of chiral assays only need to determine changes in relative enantiomer ratios as a function of time. Therefore, the absolute enantiomeric purity of the chiral derivatizing reagent is unnecessary.

Quantification — multiple chiral centres

Quantification of isomers in chiral drugs having more than one asymmetric centre need not be more difficult than those containing only one asymmetric centre. For example, while a compound with three stereogenic centres can have eight stereoisomers (i.e. four diastereomers and the four corresponding enantiomers), the analytical chemist is generally only interested in quantifying the desired isomer, i.e. all eight isomers need not be separated from one another, as long as no single isomer or combination of isomers co-elute with the desired isomer. In this respect, these separations are not significantly different than those for compounds with a single centre of asymmetry. Several approaches which can be applied to this problem include the use of a chiral assay, or the combination of achiral and chiral assays. This choice is dependent on whether the analytical chemist is trying to quantify only one enantiomer, or one enantio-

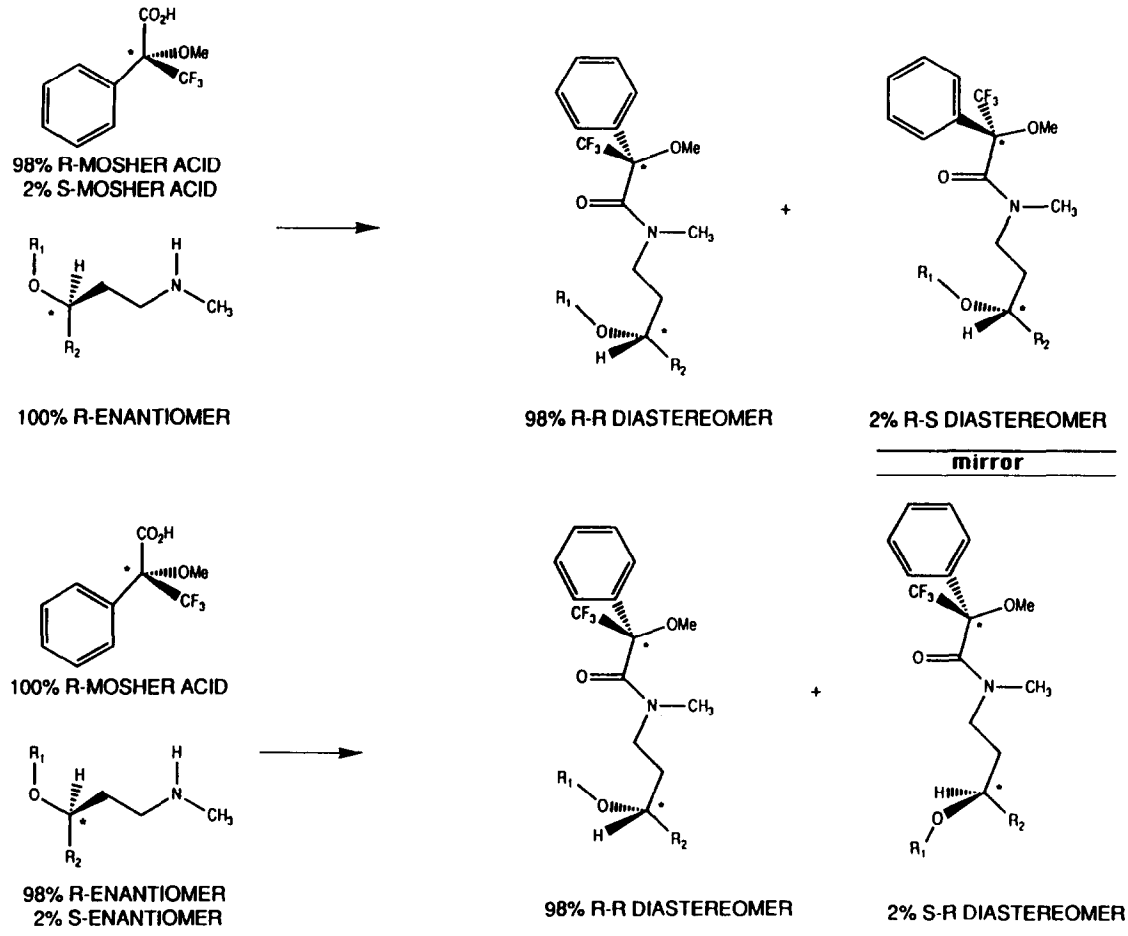

Figure 13
Potential inaccuracies for indirect separations caused by derivatizating agent purity.

mer and all diastereomer impurities which may be present.

In the second case, the probability of resolving all seven undesired isomers from each other and the desired isomer with a single chiral assay is minimal. Therefore, the analytical chemist would probably require a combination of achiral and chiral assays. In the example shown in Fig. 14 (compound with three stereogenic centres), two achiral assays were necessary to obtain adequate separation of the four diastereomers. In the achiral reversed-phase separation shown in the upper pair of chromatograms, diastereomer pairs 1 and 4 were easily separated from the diastereomer pair of interest (diastereomer pair 2) while diastereomer pair 3 was not. In the lower chromatogram, diastereomer pairs 1, 3 and 4 were separated from the diastereomer pair of interest (diastereomer pair 2) using a normal phase separation. But in this chromatographic system, diastereomer pairs 3 and 4 were not completely resolved from one another. If the only goal were to determine the levels of the enantiomers present in diastereomer pair 2, the normal phase separation provided adequate quantification of diastereomer pair 2. The individual levels of each isomer could then be determined using a chiral assay (Fig. 15). If the analyst wanted to individually quantify each diastereomer pair, then combining the results of the two achiral separations provided information on individual diastereomer pairs. Once the amount of diastereomer pair 2 was known, the desired isomer level was determined using a separate chiral assay (Fig. 15). For this specific sample, accurate quantification of the desired isomer was possible even though all of the potential isomers could not be separated from one another.

In drugs that contain more than one stereogenic centre, determining chiral stability is simplified when compared with the compound with a single asymmetric centre. If a compound

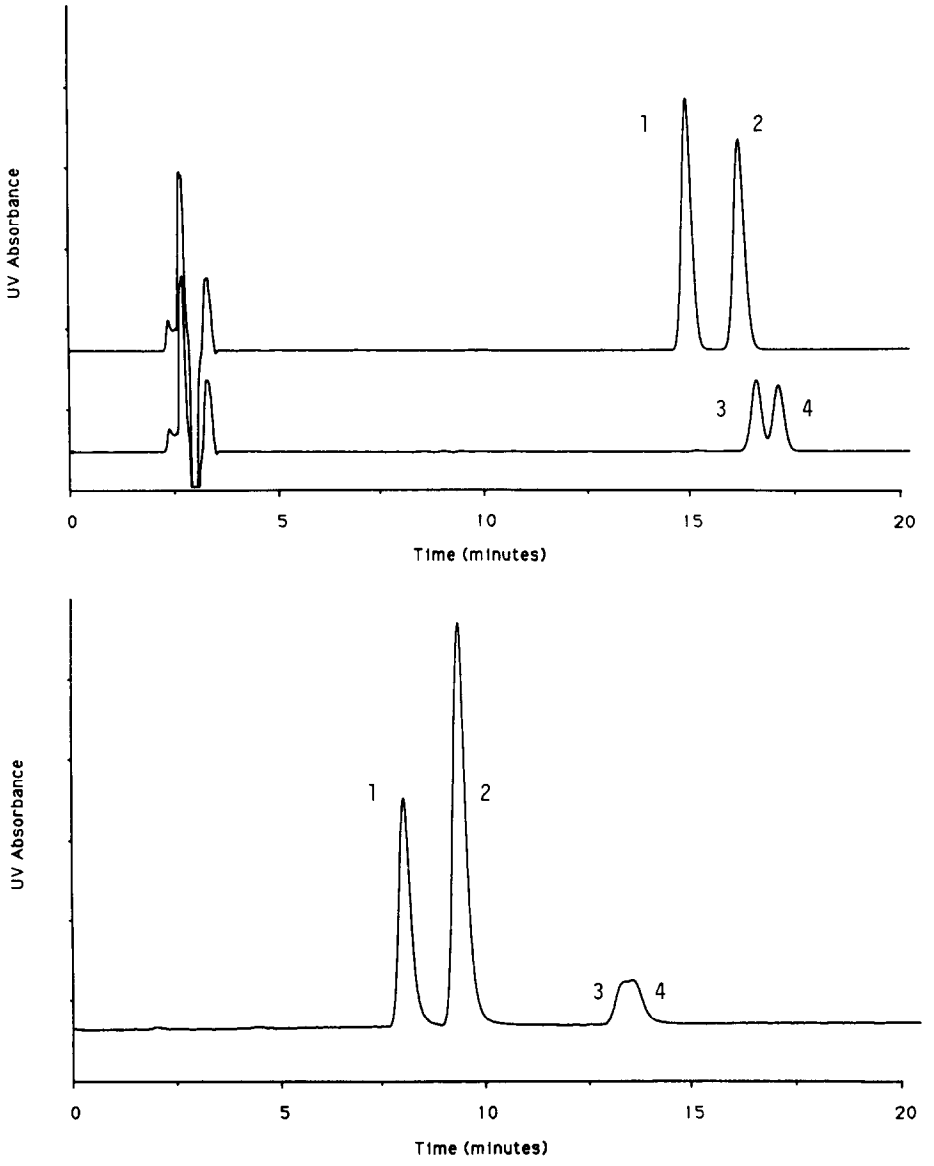

Figure 14
Achiral separation of diastereomer pairs (top, reversed-phase mode, bottom, normal phase mode; diastereomer pair 2 contains the enantiomer of interest).

with multiple chiral centres were to racemize, one or more of the stereogenic centres would undergo stereochemical inversion and one or more diastereomers would be formed (rather than the enantiomer). Unlike compounds with a single asymmetric centre (which form an enantiomer), diastereomer formation can be monitored using an achiral separation. Therefore, following the initial characterization of a compound with multiple chiral centres using chiral and/or achiral assays, the chiral assay is unnecessary. The analytical chemist only needs to monitor diastereomer formation, since for an enantiomer to form all stereogenic centres in the drug molecule would have to undergo simultaneous inversion. The probability of simultaneous stereochemical inversion for a compound with more than one asymmetric centre is minimal.

Summary of method development and validation

Analytical method development and validation for a chiral drug candidate is significantly more difficult than that for an achiral drug candidate. In addition to the achiral assay requirements, the analytical chemist must develop their chiral assay counterparts (Table

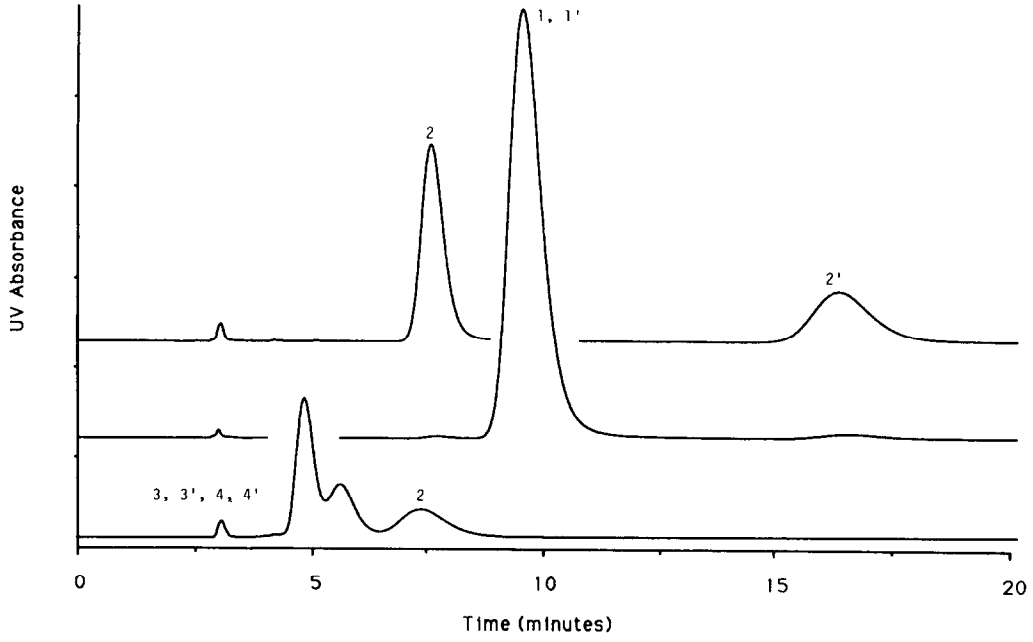

Figure 15
Direct chiral assay for enantiomeric impurity in diastereomer pair 2 (peak 2' is the enantiomeric impurity to be quantified).

Table 3
Requirements for drug substance characterization

Achiral	Chiral
Bulk drug identity test	Enantiomer specific identity
Bulk drug stability-indicating assay	Enantiomer stability
Bulk drug impurities assay	Enantiomer impurities
Stress testing of bulk drug	Racemization of bulk drug
Reference standard characterization	Chiral potency assignment
Structure proof	Absolute stereochemical assignment
Toxicology test article assay	Racemization of test article

3). Although the requirements listed in Table 3 are specific to the drug substance, additional chiral assays are required for the drug product, toxicology test article preparations and physiological fluids. Likewise, the chemist must also examine the chiral stability of the bulk drug substance and any formulations. Another significant requirement for the chiral drug candidate is the assignment of absolute stereochemical configuration. The resources and methodology required for this task are greater than those for achiral structure confirmation.

Assignment of Chiral Purity and Potency

The reporting of chiral purity and potency data for reference standards, drug substance, drug product and stability studies is an important rôle for the analytical chemist. Therefore, one must be aware of the final use of these data and assure the recipient that the reported values are appropriate for their end use. For example, the analyst can report achiral purity (corrected for counterions, volatiles, residues and other impurities), total isomer purity (all diastereomers and enantiomers) or enantiomeric specific purity (desired enantiomer only). Since purity values for chiral compounds can be reported in a variety of formats, one needs to define both the achiral and chiral purity of the reference standard to ensure proper assignments. If an assay does not distinguish chirality, the achiral potency value must be used to report analytical results. For example, the determination of drug substance potency during a stability study uses an achiral assay and therefore, the achiral potency value should be used. The use of the chiral potency

value would lead to incorrect assignment since the achiral method is unable to distinguish enantiomers. Conversely, when an assay for enantiomeric purity is performed the chiral purity value is required since a distinction is made between the enantiomers.

Potency can be assigned for chiral compounds either as an optical purity, enantiomeric excess or chiral chromatographic purity. It is important that the analyst understand the differences in potency assignment, since the results are not interchangeable in all cases (e.g. optical purity is equivalent to enantiomeric excess, but not equal to chiral chromatographic purity). Improper assignment of potency could lead to errors or incorrect conclusions on the part of the person using these data. Per cent optical of a sample is equal to the observed rotation divided by the rotation obtained from a 100% chirally pure standard, times 100 as indicated in equation (1):

$$\text{optical purity} = [(\alpha)/(\alpha)_{max}] \times 100. \quad (1)$$

The per cent enantiomeric excess (EE) or enantiomeric purity (EP) of a sample is equal to the amount of the major enantiomer minus the amount of the minor enantiomer, divided by the sum of the two enantiomers, times 100 as indicated in equation (2):

$$\text{enantiomeric excess} = [(R - S)/(R + S)] \times 100. \quad (2)$$

Chiral chromatographic purity is defined as the area of the enantiomeric impurity divided by the sum of the peak areas of the enantiomers:

$$\text{chiral chromatographic purity} = [R/(R + S)] \times 100. \quad (3)$$

Each assignment of potency has its purpose during the development of a chiral drug candidate and the analytical chemist must confirm the intent of use of these data with the submitter.

Summary

The development of chiral drug candidates has a significant impact on the industrial analytical chemist. This has not been fully recognized by the scientific community. Although analytical chemists have little control over the selection of a racemate or pure enantiomer as the drug candidate during clinical evaluations, the development of the pure enantiomer requires significantly more analytical development work for an IND submission. Selection of the racemate for preliminary testing can minimize analytical involvement unless problems are encountered in formulation stability, toxicology or pharmacokinetic studies which could be attributed to one of the enantiomers.

Adequate analytical tools exist to verify the chiral integrity of most drug candidates and properly validate analytical methodologies. Caution must be exercised when determining the proper method for quantitative assignment of chromatographic chiral purity. These concerns must be coupled with the proper assignment of chiral purity and/or potency for the end user of the data. The availability of inexpensive, rugged chiral chromatographic stationary phases which mimic the separation characteristics of achiral stationary phases would eliminate much of the need for dual assays (achiral and chiral). In turn, this would significantly reduce requirements for the industrial analytical chemist.

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