Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 349–355

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Chiral interconversion monitoring of a drug candidate by supercritical fluid chromatography (SFC)

Partha S. Mukherjee*

Analytical Development, Pharmaceutical and Analytical R&D, AstraZeneca Pharmaceuticals LP, Wilmington, DE 19803, USA

ARTICLE INFO

Article history:
Received 23 April 2009
Received in revised form 4 May 2009
Accepted 5 May 2009
Available online 15 May 2009

Keywords:
Supercritical fluid chromatography
Chiral
Enantiomerization
Racemization
Interconversion
Enantiomeric excess

ABSTRACT

Stereoisomer interconversion of chiral drug substances is of significant importance if it occurs within pharmacological and pharmaceutical time scales and under physiological and shelf life conditions. Several analytical techniques exist for the determination of first order rate constants and enantiomerization energy barriers by dynamic and stopped flow chromatography, mathematical models and functions, and computer programs. The focus of this work is to utilize a simple supercritical fluid chromatography (SFC) chiral assay to determine the possibility of interconversion of the desired R and less active S isomers of a drug candidate. The rate constants of racemization and enantiomerization, the half life of racemization, and enantiomerization energy barriers were determined for the R → S (or, forward) and S → R (or, reverse) conversions. The method was selective and sensitive enough to detect less than 1% interconversion occurring under the conditions studied. The method also demonstrated that R ⇌ S racemization was possible only under extreme conditions of prolonged heating (80 °C) and highly basic pH (9.5).

1. Introduction

Chiral separation has gained significant attention in pharmaceutical analysis over the past few years with an increasing trend in synthesis of chiral compounds and exploration of chiral drug switches. Configurational stability of chiral drug substances is of pharmaceutical and pharmacological concern, as most biochemical processes are stereochemically regulated and enantiomers can have different interactions in terms of pharmacokinetics, pharmacodynamics, and toxicological properties. The monitoring of configurational and conformational lability in terms of chiral interconversion is particularly important in the following cases:

- The distomer has pharmacological activity markedly different from the intended isomer (eutomer). Associated with this phenomenon, an inverse relationship exists between the effective dose of chiral drugs and their enantiomeric potency ratios, also known as Pfeiffer's rule [1–3].
- The distomer is pharmacologically toxic or causes detrimental side effects. A well-known pharmaceutical example of this is the case of thalidomide.

Since the late 1980s, the U.S. Food and Drug Administration (FDA) has recognized the importance of drug stereomers and has set forth guidelines requiring configurational characterization of chiral drugs during pharmaceutical development [4,5]. These dictate that drug manufacturers assess the stereochemical integrity of enantiomers as well as examine the potential of interconversion of individual isomers. Further impetus for characterizing chiral interconversion is supported by observations described by Testa et al. [6] and Reist et al. [7] that no stereoisomer is configurationally stable under all combinations of temperature and pH. Also, two time scales and related conditions are relevant to stability of drugs: the pharmaceutical time scale, in the range of days to years to include manufacturing and shelf life, and the pharmacological time scale, in the range of minutes to days to cover physiological conditions (37 °C, pH 7.4) during exposure. Enzymatic inversion of chiral molecules is also possible and has been reported [6]. Physiologic enantiomeric ratio can also change as a result of metabolism and excretion rate differences. These, however, are considered to be in vivo enrichment of a particular enantiomer and not a stereochemical interconversion [6].

It is important to point out the differences between enantiomerization and racemization, which are both associated with compounds possessing one chiral center. Enantiomerization refers to the reversible first order interconversion of one enantiomer to the other [7]. Typically this passes through a transition state (e.g., Ar–CO bond rotation) [8–11], or an intermediate (e.g., base catalyzed pyramidal carbanion or acid catalyzed acyl carbonium ion [12–14], a fast keto ⇌ enol tautomerism [15,16], etc.).

In contrast, racemization refers to the irreversible conversion of an enantiomer (be it optically pure or impure) into a racemic

* Tel.: +1 302 885 6992; fax: +1 302 886 7470.
E-mail address: partha.mukherjee@astrazeneca.com.

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.05.005

mixture (50:50 molar ratio of original and the converted enantiomer) [7,17,18]. This is also associated with a loss in optical activity of the chiral substance over time. A racemic mixture is, therefore, the endpoint of the interconversion of two enantiomers. The rate constant of racemization k_{rac} refers to this first order kinetic process. In an achiral environment, the two antipodal enantiomers have identical free energies, and their k_{rac} should be the same. The relationship between the rate constant for enantiomerization (k_{enant}) and the rate constant of racemization (k_{rac}) is: $k_{rac} = 2 \times k_{enant}$, under a given set of conditions [7,19].

The half life of racemization ($t_{1/2rac}$) is the time during which the enantiomeric purity of a chiral compound reduces to 50% of its original enantiomeric excess (e.e). The e.e is calculated as the % difference in the mole fractions (or, peak area% in chromatography) of the two enantiomers. Thus, a racemate has an e.e = 0.

An experimental approach to k_{enant} determination is to measure the rate during very early phase of conversion, when essentially most of the enantiorich form is still unchanged. During this period, the other isomer has not yet accumulated to a significant amount and the reverse conversion rate is negligible. The overall observed (or, apparent) rate constant of the kinetics during this period is a fairly accurate estimate of the true k_{enant} for the relevant process.

For a chiral compound, any possible on-column interconversion can be measured by a variety of dynamic chromatographic methods, as noted by Krupcik et al. [20], if the process occurs within the time frame of separation. These different dynamic approaches are used for the determination of rate constants and interconversion energy barriers of enantiomers. On-column stopped flow techniques [21], computer simulation (e.g., discontinuous plate, stochastic, and continuous flow models) [21,22], programs (e.g., SIMUL, Mimesis, ChromWin) [21,23], approximation functions, and deconvolution methods [24,25] have also been employed for the determination of rate constants and enantiomerization energy barriers.

Combination of chiral separation with classical kinetic methods has also been reported for the determination of interconversion rate constants [20]. In this approach, the interconversion of an isomer (pure or enantiorich) in solution is performed outside the separation system (off-line) by controlling temperature for a required time. Aliquots of converting enantiomer solution are sampled at regular intervals and analyzed by enantioselective chromatographic methods. The chiral column is maintained at conditions (e.g., temperature, mobile phase, etc.) which quench any possible on-column interconversion. Achiral chromatographic detectors are typically used and give equal responses for both enantiomers. The apparent rate constants may be determined directly from peak areas, in this case.

In general, an assay to monitor the chiral interconversion should have the following attributes: (a) be stereospecific for the chiral center of interest, (b) provides baseline or better resolution of the intended and the other isomer, and (c) sensitive enough to detect the interconversion occurring at a low concentration, particularly if the minor isomer elutes immediately following a tailing major isomer peak [26].

SFC has gained particular attention in monitoring both off-column (classic) and on-column (dynamic) interconversion of stereoisomers due to the advantages of faster separation speed, higher efficiency, less mobile phase consumption, and significantly less organic solvent waste generation offered by this technique, as compared to traditional HPLC [27]. In this work, the interconversion possibility of a therapeutically important chiral drug candidate was investigated by SFC. The R isomer of this compound was found to possess different potency compared to the S (minor) isomer. It was, therefore, necessary to investigate if the R isomer would racemize to the less active S isomer during typical pharmacological and pharmaceutical time scales, and thus lose its therapeutic activity. It would potentially also provide the synthetic chemist with useful information to optimize conditions and maximize yield of the desired isomer, if significant interconversion is occurring. A sensitive and selective chiral SFC method was employed to accurately demonstrate any interconversion from R to S and vice versa, and preliminary studies have been conducted to measure the interconversion rates in co-solvent containing media at a range of pH from 3 to 9.5.

2. Experimental

2.1. Materials and reagents

The basic drug candidate (initial optical purity 99%) and its S enantiomer (initial optical purity 99%) consisted of one chiral center (of the configuration of $R_1R_2R_3C$–H adjacent to a carbonyl group), and were obtained from AstraZeneca compound management (Wilmington, DE). This is identical with AZM in a previously reported work [28]. Among the various lots of the compound studied, the S (minor) enantiomer was present at a minimal 1% level. HPLC grade methanol and USP grade 200 proof ethanol were obtained from J.T. Baker (Phillipsburg, NJ) and Pharmco (Brookefield, CT), respectively. Dimethylethylamine (DMEA) was used in the SFC method development as additive, and purchased from Acros Organics (NJ). The drug compound is a diprotic base and it was necessary to include basic additives in the mobile phase to reduce the possibility of peak tailing. Lactic acid (pH adjusted to 3.0) was prepared in-house. Potassium phosphate monobasic buffer and sodium borate buffer solutions were products of Fisher Scientific (Fairlawn, NJ) and LabChem (Pittsburgh, PA), respectively. The pH of the three buffer solutions were checked prior to use by a Beckman pH meter model Φ40. SFC grade carbon dioxide was supplied by MC Industries (Malvern, PA).

2.2. Preparation of method development reference solution

An in-house, fit-for-purpose SFC method optimized for this drug candidate was used during the interconversion evaluation study. The details of the method development and validation have been published earlier [28,29] and therefore beyond the scope of this paper. For feasibility evaluation of interconversion, the following solution was prepared:

Approximately 5 mg of each enantiomer were weighed out in a 5 ml volumetric flask and brought to volume with 0.1 M lactic acid (pH 3.0) as vehicle. The solution was sonicated for approximately 5 min using a sonication bath (Branson model 2200) and also vortexed briefly to ensure complete dissolution, using a Thermolyne Maxi Mix II model 37600 mixer. This produced a stock solution (2 mg/ml) of racemic mixture. This stock solution was further diluted 50:50 with mobile phase modifier and used as the reference, to ensure baseline resolution of the enantiomers by SFC.

2.3. Preparation of solutions of R isomer for interconversion study

For evaluation of the effect of pH on the interconversion of R to S, the following solutions were prepared:

(1) Approximately 1 mg/ml of R isomer in 1 ml of 0.1 M lactic acid (pH 3.0).
(2) Approximately 1 mg/ml of R isomer in 1 ml of 0.05 M potassium phosphate monobasic buffer (pH 7.0).
(3) Approximately 1 mg/ml of R isomer in 1 ml of 40 g/l sodium borate buffer (pH 9.5).

The R isomer was completely soluble in pH 3.0 solution following brief sonication. The compound was only sparingly soluble at pH

Table 1
Analytical SFC system.

Component	Description
SFC system	Berger analytical
Autosampler	Berger ALS Model 719
Sample injection volume	10 μl, full loop
Detector	Variable wavelength HP 1050
Detection wavelength	244 nm
Analytical column	Chiralpak AD-H, 4.6 × 250 mm, 5 μm
Pumps	Berger fluid control module FCM 1100/1200
Inlet pressure	160 bar
Outlet pressure	100 bar
Mobile phase	28% ethanol with 0.3% DMEA/72% CO_2
Modifier selection valve	6 position valco
Thermal control module	Berger TCM 2000
Flow rate	2.2 ml/min
TCM temperature	40 °C
Nozzle temperature	60 °C
Wash solvent	Methanol

7.0 and 9.5, following an extended sonication (15 min), with lower solubility at higher pH. The saturated solutions of (2) and (3) were filtered through MillexHV® 0.45 μm filters (Millipore, Carigwahill, Ireland). The exact concentrations of these filtered solutions at pH 7.0 and 9.5 were, therefore, less than 1 mg/ml. All three above solutions were further diluted 50/50 with the ethanolic mobile phase modifier to prevent precipitation.

Each of the three diluted solutions were analyzed to obtain day 0 value of % e.e of R isomer at room temperature, prior to initiation of study. The values of % e.e determined on day 0 were almost identical to the previously reported % e.e of this isomer in the manufactured lot, which indicated that no racemization occurred due to sonication. One milliliter aliquot of each of the solutions were transferred to individual 2 ml HPLC autosampler vials, crimp-sealed, and placed in an oven (Precision Scientific model 18) preset at 80 °C. Initial investigation indicated that no detectable interconversion occurred at lower temperatures. Sample vials at each pH were removed from the oven at regular intervals over a 2-week period, cooled to room temperature, and a 10 μl aliquot injected onto the chiral column for SFC assay.

2.4. Preparation of solutions of S isomer for interconversion study

The reverse process of S → R was also investigated in a separate study under the same storage and separation conditions mentioned in Section 2.3. It was appropriate to investigate the conversion possibility at high pH only, based on the results observed with R → S. Approximately 1 mg/ml solution of the S enantiomer was prepared in pH 9.5 sodium borate buffer with sonication. The solution was filtered, and further diluted 50/50 with the ethanolic mobile phase modifier to prevent precipitation. Approximately 1 ml of this solution was transferred to individual 2 ml HPLC autosampler vials, crimp-sealed, and placed in the same oven (preset at 80 °C) previously utilized for R → S conversion study. Samples were removed at regular intervals over the next 6 weeks and analyzed by the same SFC system used for the forward conversion.

2.5. Analytical chromatographic system

Details of the SFC instrument components and method conditions are provided in Table 1.

3. Results and discussion

3.1. Results from method development for chiral interconversion

3.1.1. Chromatographic parameters

The method was enantioselective, as demonstrated by the lack of interferences across the elution windows of the R and S enantiomers (Fig. 1) and therefore suitable for monitoring chiral interconversion.

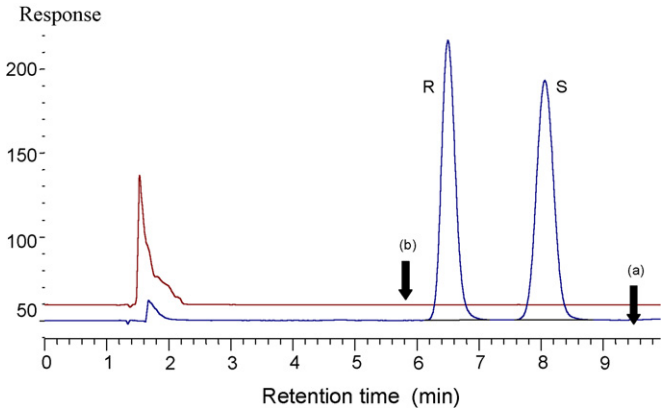

Fig. 1. A representative chromatogram from SFC method development for the chiral drug candidate. Chromatogram (a) represents the racemic mixture in the same solvent at 0.5 mg/ml of each enantiomer. Chromatogram (b) represents blank solvent vehicle (0.1 M lactic acid, pH adjusted to 3.0). This figure is identical to Fig. 1 in [28].

3.1.2. Sensitivity of the interconversion assay

Based on an external standardization procedure, the concentration of the R isomer in pH 9.5 solution on day 0 was determined to be 0.25 mg/ml. 0.9% of S impurity in this manufactured lot could be accurately quantified in the same chromatogram. This suggested that the method is sensitive enough to detect at least a 0.9% conversion of original R isomer in solution by this method. It should also be noted that the sensitivity of the method could be further enhanced by optimizing the currently employed separation conditions (e.g., to elute the peaks earlier while maintaining baseline resolution), or injecting larger volume of the sample. For the purpose of this study (i.e., to explore the possibility of interconversion), this method was however considered to be fit-for-purpose.

3.2. Possibility of on-column interconversion

Typically, a peak cluster consisting of a peak of the unchanged enantiomer and a plateau formed by the inter converting species are observed during a dynamic chiral chromatography process [30–33]. The retention time, area, and width of the enantiomer peak and the plateau height depend on the analyte interconversion kinetics (dictated by separation temperature) and the chromatographic conditions (type of chiral column and the mobile phase flow rate). Different kinetic models have been proposed for the dynamic equilibrium occurring between stationary and mobile phases during on-column interconversion.

It should be noted that no on-column racemization was observed in this study, under the temperature and conditions of separation. The peaks were well resolved and no plateau formation or coalescence of peaks (typical of on-column interconversion) was noted. The extent of interconversion observed in the chromatograms were solely due to that occurring under the storage conditions of temperature, pH, solvent, and sampling time.

3.3. Conversion of R enantiomer to S enantiomer at different pH

3.3.1. Conversion at pH 3.0, 80 °C

It was observed that the conversion process of R → S isomer was extremely slow, if at all, over 2 weeks at 80 °C and pH 3.0 (Fig. 2). This also demonstrates the selectivity of the method under this condition. The original e.e (~98.2%, as determined on day 0) of R was maintained throughout the period of study. The % e.e of R was determined as (area% of R – area% of S). The area% was calculated

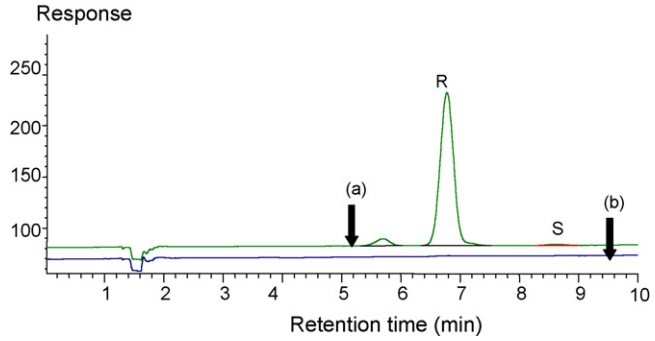

Fig. 2. (a) Typical chromatogram of the R enantiomer on day 14 at pH 3.0, 80 °C during the R → S conversion. Chromatogram (b) represents solvent vehicle (0.1 M lactic acid, pH adjusted to 3.0) stored simultaneously under same conditions for 14 days.

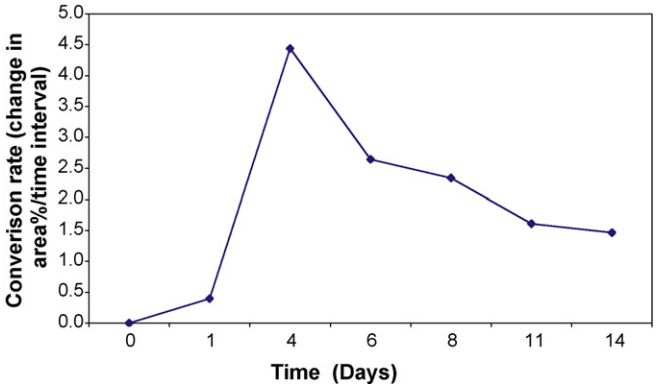

Fig. 4. Plot of conversion rate (change in area%/time interval) of R → S vs. time, as monitored for R enantiomer at pH 9.5, 80 °C over a period of 2 weeks.

as the ratio of the area of R isomer to the total area of (R + S) isomers, as monitored in chiral separation. The underlying assumptions were that the interconversion proceeded without any interference from concurrent degradation pathways, and the rates of degradation were the same during both forward and reverse conversions. The results also suggested that this particular condition probably had the enantiomerization energy barrier high enough to retard the interconversion proceeding via an intermediate. Since the method detected the presence of <1% of S in R isomer in the original manufactured lot, it was concluded that an interconversion occurring at this low level could be accurately monitored by this method.

3.3.2. Conversion at pH 7.0, 80 °C

The conversion rate of R → S was slightly higher at pH 7.0 as compared to pH 3.0, when stored at 80 °C for 2 weeks. Monitoring chiral area% vs. time indicated that there was only a 0.6% increase in the peak area of S over the 2 weeks monitored at 80 °C at pH 7.0.

This result provides evidence that under physiological non-enzymatic conditions (pH 7.4, 37 °C), the possibility of interconversion will be insignificant for this particular chiral drug candidate.

3.3.3. Conversion at pH 9.5, 80 °C

The conversion was the highest at this pH, as monitored over 2 weeks (Fig. 3). 34% of the R enantiomer was converted to S over this time period when heated at 80 °C.

The maximum extent of conversion was observed between day 1 and day 4. The conversion rate was determined by the change in area% of R divided by the interval between the two corresponding analysis time points. The rate slowed beyond day 4, as observed in Fig. 4. This was probably because by this time, significant S isomer had been generated in the solution, which allowed the reverse conversion process of S → R to increasingly contribute to the overall observed rate of change in total R content in the solution. In other words, a certain % of R isomer content depleted by R → S process was being replenished by the S → R contribution occurring simultaneously.

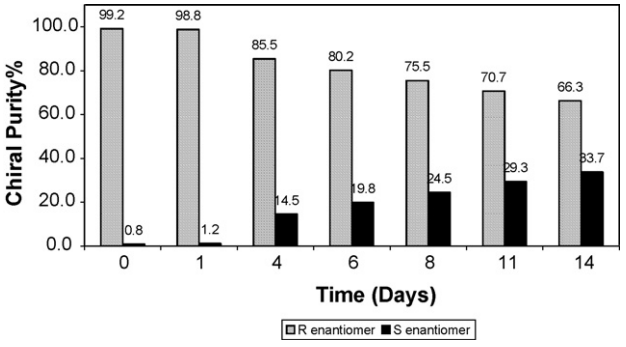

Fig. 3. Graphical representation of change in chiral purity % (or, area%) of R and S enantiomers vs. time, as monitored at pH 9.5, 80 °C over a period of 2 weeks for the R → S conversion.

3.4. Conversion of S to R enantiomer

As the rate of racemization and enantiomerization were detectable only under conditions of high temperature and pH for the R → S process, the reverse conversion study was limited only to these conditions to generate relevant data. The possibility of racemization of S → R was, however, investigated over a much prolonged period of time (compared to R → S) of 43 days, to ensure that a complete or full racemization was observed from the chromatographic data. The conditions of storage and separation were maintained the same for both processes. It was observed that the S isomer almost completely racemized (~50:50 S:R) following storage under the conditions for 43 days.

As can be seen from Fig. 5, the rate of conversion slowed down as it approached the racemization ratio of 50:50. This was also evident from the rate (change in area% of the S isomer divided by the interval between two corresponding analysis time points) vs. time. The area% was calculated as a ratio of the area of S isomer to the total areas of R + S. This was similar to the trend observed during the monitoring of the enantiomerization of the R to S isomer, within the

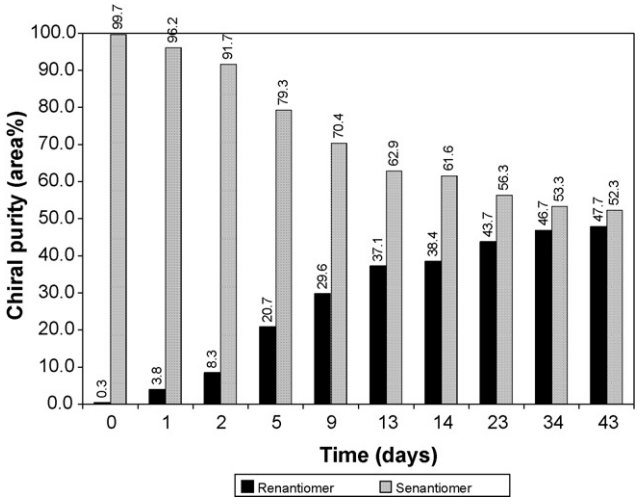

Fig. 5. Graphical representation of change in chiral purity % of S and R enantiomers vs. time, as monitored at pH 9.5, 80 °C over a period of 43 days for the S → R conversion.

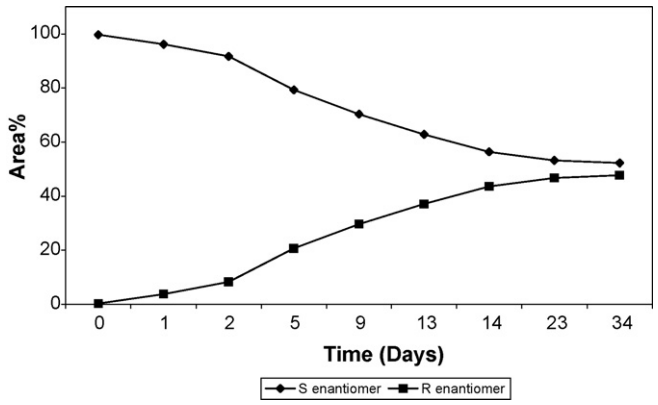

Fig. 6. First order kinetic plot for R and S enantiomers, as monitored at pH 9.5, 80 °C during the S → R conversion.

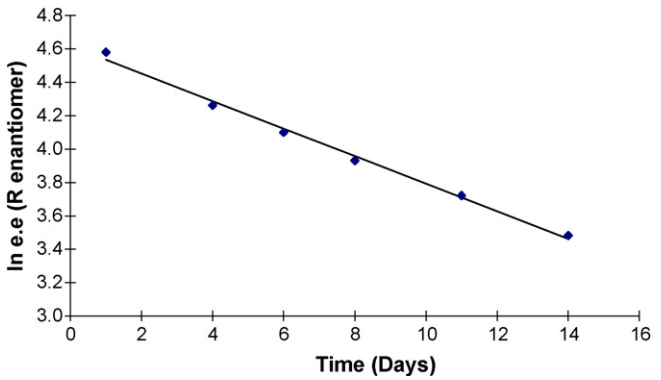

Fig. 7. Linear plot of logarithm of % e.e of R enantiomer vs. time, as monitored at pH 9.5, 80 °C over a period of 2 weeks for the R → S conversion.

same separation time scale.

The fact that temperature accelerated the racemization process was obvious from a separate experiment where by heating the S isomer solution (pH 9.5) at 105 °C yielded a 50:50 ratio of R and S within 2 days. This was also accompanied by significant degradation, as expected.

3.5. Evaluation of kinetic profile of $R \rightleftharpoons S$ interconversion

It was assumed that the interconversion proceeded through a first order mechanism, according to Eq. (1):

$$C_t = C_o \cdot e^{-kt} \quad \text{or,} \quad A_t = A_0 \cdot e^{-kt} \tag{1}$$

where C_o is the concentration of compound at time 0, C_t is the concentration of compound at time t, k is the first order rate constant, A_0 is the peak area of enantiomer R at time 0, and A_t is the peak area of enantiomer R at interconversion sampling time t.

A plot of C_t vs. time theoretically provides a convex curve typical of first order reaction. The observed peak area is directly proportional to the concentration in the linear range of detector response. In this case, the area% of S and R isomer obtained from S → R were plotted vs. time, and profiles corresponding to typical first order kinetics were observed for both isomers (Fig. 6). Logarithmic plots on the same data also confirmed a first order kinetic process, based on linearity of plots. The calculated area% values based on Eq. (1) were in close agreement with the corresponding chromatographically observed area%s. For example, the mean ($n = 5$) differences in observed and calculated area% for the R isomer was ~2%.

3.6. Rate constants for conversion from R to S enantiomer

3.6.1. Rate constant of racemization

To determine the rate constant for racemization (k_{rac}) of the R to S interconversion, ln logarithmic values of % e.e for R were plotted vs. corresponding time (Fig. 7). This is an accepted procedure for the determination of rate constant for racemization in solutions, as reported in literature [9].

An unweighted univariant linear regression analysis performed on the data indicated the k_{rac}, at pH 9.5 and 80 °C, as determined from the slope, was 0.082 day^{-1} (Table 2). The day 0 e.e for the R isomer determined from the regression intercept (98.3%) correlated well with the observed e.e of 98.4%.

3.6.2. Rate constant of enantiomerization

In classical first order kinetic studies, the interconversion is performed in a stationary system, and the forward rate constant (k_1) is calculated from the equation [20,32]:

$$k_1 = \frac{1}{2t} \ln \frac{C_{R,0}}{2C_R - C_{R,0}} = \frac{1}{2t} \ln \frac{A_{R,0}}{2A_R - A_{R,0}} \tag{2}$$

where $C_{R,0}$ is the concentration of enantiomer R at time 0, C_R is the concentration of enantiomer R at interconversion sampling time t, and $A_{R,0}$ and A_R are the corresponding peak areas, and should be directly proportional to the respective concentrations.

It is a valid assumption that k_1 measured very early in the interconversion process (close to time $t = 0$), will provide a fairly accurate estimate of the enantiomerization rate constant $k_{enant}^{R \to S}$ for the forward reaction. This is true when there is a predominantly large e.e of the R isomer, and not enough S isomer has yet accumulated in the same reaction vessel. The reverse reaction (which is dependent on initial concentration of S) is, therefore, still negligible. The overall rate of solution kinetics under this condition would measure the $k_{enant}^{R \to S}$.

The forward rate constant (or, $k_{enant}^{R \to S}$) was measured using Eq. (2) in this manner for R → S using relevant time points ($n = 3$) early in the process, and determined to be 0.039 day^{-1} (Table 2). This was in close agreement (within 95%) with the value (0.041 day^{-1}) calculated from 1/2 the rate constant of racemization (Section 3.6.1).

Table 2
Regression analysis parameters for determination of racemization and enantiomerization rate constants.

Parameter	Rate constant	Correlation coefficient (r)	Intercept	Standard error	Text reference
$k_{rac}^{(R \rightleftharpoons S)}$ measured	0.082 day^{-1}	0.997	4.618	0.033	Section 3.6.1
$k_{enant}^{R \to S}$ calculated	0.041 day^{-1}				Section 3.6.1
$k_{enant}^{R \to S}$ measured	0.039 day^{-1}				Section 3.6.2
$k_{rac}^{(S \rightleftharpoons R)}$ measured	0.105 day^{-1}	0.999	4.628	0.021	Section 3.7.1(a)
$k_{rac}^{(S \rightleftharpoons R)}$ measured	0.113 day^{-1}	0.999	−0.018	0.062	Section 3.7.1(b)
$k_{enant}^{S \to R}$ measured	0.05 day^{-1}				Section 3.7.2
$k_{enant}^{S \to R}$ calculated	0.05 day^{-1}				Section 3.7.2

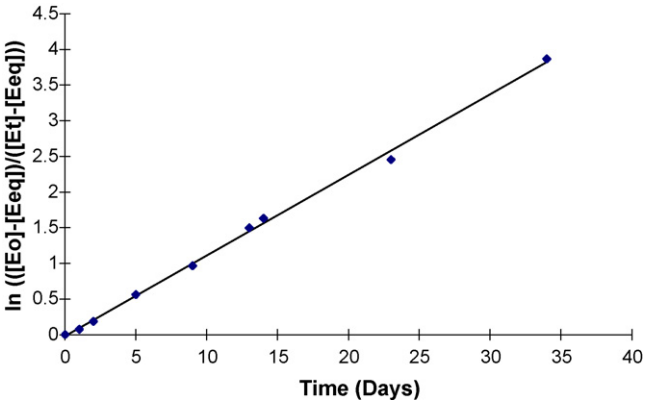

Fig. 8. Logarithm plot of equilibrium area% of S enantiomer vs. time (using Eq. (3), see text for details) for the determination of rate constant of racemization from slope.

3.7. Rate constants for conversion of S to R enantiomer

3.7.1. Rate constant of racemization

The rate constant of racemization (k_{rac}) of the S to R isomer was measured by the following two procedures:

(a) By plotting the logarithm values of e.e (expressed as area%) of S monitored with time, in same manner as in Fig. 7. The slope of the unweighted linear regression analysis (Table 2) indicated a rate constant of racemization (k_{rac}) of 0.105 day^{-1}. The day 0 % e.e for the S isomer determined from the regression intercept (98.3%) correlated well with the observed e.e of 99.4%.

(b) By plotting the data according to the following equation [7,16]:

$$\left(\frac{\ln([E_o]-[E_{eq}])}{([E_t]-[E_{eq}])}\right) = k_{rac}\cdot t \quad (3)$$

where $[E_o]$ is the concentration of decreasing stereoisomer (S) at time 0. This is also equivalent to the area% of the isomer, as the area% is directly proportional to the concentration of the isomer in the solution, $[E_{eq}]$ is the concentration of decreasing stereoisomer (S) at equilibrium (or, following racemization), and $[E_t]$ is the concentration of decreasing isomer (S) at time t.

The slope of the above plot (Fig. 8) equals k_{rac} with a zero intercept. The slope of the unweighted linear regression analysis (Table 2) indicated a k_{rac} for S → R of 0.113 day^{-1}. This was in close agreement (within 93%) with the rate of racemization obtained from the plot by procedure outlined in (a). Also, as seen in Table 2, the calculated intercept value was close to the predicted value of 0. It should be noted that Eq. (3) could not be used for determination of k_{rac} from R → S conversion, as the process was not monitored to the point of racemization.

3.7.2. Rate constant for enantiomerization

In a manner similar to that described for R → S conversion (based on Eq. (2)), the reverse rate constant of enantiomerization $k_{enant}^{S\rightarrow R}$ (n = 3) was measured to be 0.05 day^{-1}. This was in excellent agreement with the rate constant of enantiomerization (0.05 day^{-1}) calculated as 1/2 the rate constant of racemization [determined in Section 3.7.1(a)]. The value of 0.05 day^{-1} was also in close agreement (0.06 day^{-1}) with the calculated $k_{enant}^{S\rightarrow R}$ from Section 3.7.1(b), as expected.

Theoretically, the rate constants of racemization are expected to be the same for the R → S and S → R conversion processes. The observed difference in the rate constants of racemization for the forward (R → S) and reverse (S → R) processes in this study was because the two studies were done separately, and the forward reaction was not followed through the racemization procedure. An ideal situation would be where the samples of individual pure R and pure S isomers are placed in a more precisely controlled temperature oven at the same time, and then followed through the interconversion procedure at same sampling times, till racemization is noted on both pathways. This would also require preparation of the pure R and pure S solutions in identical concentrations, in same solvent buffer strength, and monitored under identical separation conditions. For this fit-for-purpose study, the observed difference was acceptable within experimental error margin. It was assumed that the k_{rac} value determined from the S → R conversion would be more accurate, as this process was followed through the true racemization endpoint, and should be considered as the k_{rac} for this drug candidate.

3.8. Half life of racemization based on chromatographic data

The half life for first order racemization was calculated from the equation:

$$t_{1/2rac} = \frac{\ln 2}{k_{rac}} = \frac{0.693}{k_{rac}} \quad (4)$$

The half life of racemization was determined to be 6.6 days at 80 °C and pH 9.5, based on k_{rac} obtained from the S → R pathway. The $t_{1/2rac}$ was also calculated using k_{rac} as 2 times the value of $k_{enant}^{S\rightarrow R}$ obtained from Eq. (2), and determined to be 6.2 days. A mean calculated value of 6.4 days was assigned the half life of racemization for this particular drug candidate, at 80 °C and pH 9.5. This value corroborated closely with the experimental data (6.8 days) obtained from a plot of e.e of S isomer vs. time.

3.9. Enantiomerization energy barrier for the interconversion process

It was assumed that the enantiomerization energy barriers (ΔG) would be very similar for both R → S and S → R processes, under the solvent and temperature studied. The following equation was used to determine the enantiomerization energy barrier [19,32]:

$$\Delta G_{R\rightarrow S} = -RT\ \ln[(k_{1R\rightarrow S}^{app}/k)\cdot(h/k_B T)] \quad (5)$$

$\Delta G_{R\rightarrow S}$ is the energy barrier to enantiomerization from R to S isomer in kJ mol^{-1}, R is the gas constant = 8.31441 J K^{-1} mol^{-1}, T is absolute temperature in K (80 °C = 353 K), $k_{1R\rightarrow S}^{app}$ is the apparent first order rate constant for conversion of R to S isomer in s^{-1} (or, k_{enan}, as calculated from the R → S process), k is the transmission coefficient = 0.5 (as the interconversion is postulated to be a two-step process via an intermediate, as described in Section 3.10), k_B is Boltzmann constant = 1.380662 × 10^{-23} J K^{-1}, and h is the Planck's constant = 6.626176 × 10^{-34} J s.

The $\Delta G_{R\rightarrow S}$ and $\Delta G_{S\rightarrow R}$ were determined to be 94.3 kJ mol^{-1} (or, 22.5 kcal mol^{-1}) and 93.7 kJ mol^{-1} (or, 22.3 kcal mol^{-1}) at 80 °C, respectively.The values of ΔG obtained for the chiral compound at 80 °C and in the solvent investigated were similar to the ones obtained for other compounds [22,33]. Also, the enantiomerization energy barriers of this drug candidate were in the region amenable to evaluation by Dynamic HPLC (70–100 kJ mol^{-1}) and also by Dynamic SFC (70–150 kJ mol^{-1}) [32–34]. The compound may, therefore, also be evaluated by these two dynamic chromatography methods for on-column racemization possibilities. This would only be possible, however, by manipulation of currently employed separation conditions (for example, by raising the column temperature much above 40 °C, which may cause undesirable on-column thermal degradation, or by lowering the flow rate below 2.2 ml/min, which would adversely affect the chromatography in this case).

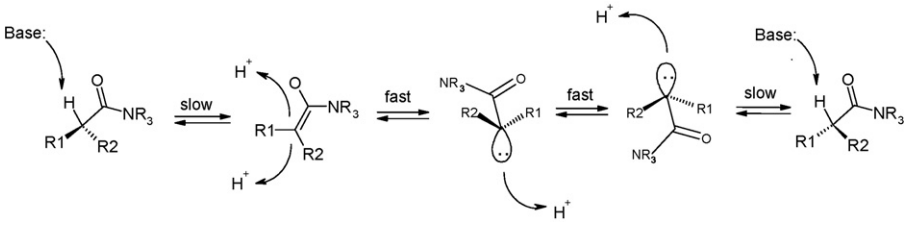

Fig. 9. Postulated mechanism of the R ⇌ S interconversion for the drug candidate as monitored at pH 9.5 and 80 °C. Racemization of a chiral center α to an amide through an intermediate is represented as proceeding through either the planar O-enolate or through the tautomeric rapidly interconverting pyramidyl C-enolate.

3.10. Postulation of interconversion pathway for the drug candidate

For interconversion of R ⇌ S, the drug candidate possessed a carbonyl group adjacent to the $R_1R_2R_3C$–H single chiral center. Typically the interconversion in such compounds has been postulated to proceed through the formation of a base catalyzed carbanion ion $R_1R_2R_3C^-$ (deprotonated intermediate) [6,12]. It may also be possible that the transition state passes through a fast keto ⇌ enol tautomeric equilibrium, which has been reported for other compounds [15,16]. Fig. 9 indicates the postulated interconversion pathway for structures with such generic attributes. At equilibrium, both the desired isomer and the minor isomer remain in dynamic exchange process with the intermediate, leading to the maintenance of the racemic ratio, as observed during the reverse process.

4. Conclusions

A chiral SFC method has been employed to monitor the interconversion of the therapeutically relevant R isomer to the less active S isomer of a potential drug candidate. This interconversion study was performed in a classical off-line stationary system, and monitored by an enantioselective method. The rate constants of enantiomerization and racemization, half life of racemization, and enantiomerization energy barriers were calculated from relevant chromatographic data and based on first order kinetics. The relatively high energy barrier of enantiomerization at lower temperature ensured no on-column interconversion occurred during the enantioselective separation. The method was sensitive enough to detect the interconversion occurring at less than 1% level, as observed in pH 7.0. The conversion proceeded to racemization only under prolonged heating (~40 days) at 80 °C and in highly basic (pH 9.5) solution. The data suggests that interconversion may not be of any concern in the pharmaceutical or non-enzymatic physiologic conditions/time scale. However, because the samples examined contained a co-solvent (ethanol), further study may be required to confirm this conclusion in true formulations and physiologic media.

Acknowledgments

The author would like to thank Dr. John P. McCauley for providing insight on interconversion mechanism and the schematic of the postulated pathway (Fig. 9), and Dr. Steve Cook for providing useful feedback on this article.

References

[1] B. Testa, W. Trager, Chirality 2 (1990) 129–133.
[2] P. Lehmann, Trends Pharmacol. Sci. 7 (1986) 281–285.
[3] C. Pfeiffer, Science 124 (1956) 29–31.
[4] W. DeCamp, Chirality 1 (1989) 2–6.
[5] FDA's policy statement, Chirality 4 (1992) 338–340.
[6] B. Testa, P. Carrupt, J. Gal, Chirality 5 (1993) 105–111.
[7] M. Reist, B. Testa, P. Carrupt, M. Jung, V. Schurig, Chirality 7 (1995) 396–400.
[8] D. Cassarini, L. Lunazzi, S. Alcaro, F. Gasparrini, C. Villani, J. Org. Chem. 60 (1995) 5515–5519.
[9] A. Ahmed, R. Bragg, J. Clayden, L. Lai, C. McCarthy, J. Pink, N. Westlund, S. Yasin, Tetrahedron 54 (1998) 13277–13294.
[10] W. Stewart, T. Siddall, Chem. Rev. 70 (1970) 517–551.
[11] J. Oxelbark, S. Allenmark, J. Org. Chem. 64 (1999) 1483–1486.
[12] H. Niemeyer, Tetrahedron 33 (1977) 2267–2270.
[13] C. Pepper, H. Smith, K. Barrell, P. Nicholls, M. Hewlins, Chirality 6 (1994) 400–404.
[14] R. Baechler, K. Mislow, J. Am. Chem. Soc. 92 (1970) 3090–3093.
[15] S. Yang, X. Lu, Chirality 4 (1992) 443–446.
[16] G. Severin, Chirality 4 (1992) 222–226.
[17] J. Caldwell, I. Wainer, Hum. Psycho. Clin. Exp. 16 (2001) S105–S107.
[18] K. Mislow, Chirality 14 (2002) 126–134.
[19] F. Gasparrini, D. Misiti, M. Pierini, C. Villani, Tetrahedron: Assymetry 8 (1997) 2069–2073.
[20] J. Krupcik, P. Oswald, P. Majek, P. Sandra, D. Armstrong, J. Chromatogr. A 1000 (2003) 779–800.
[21] O. Trapp, G. Schoetz, V. Schurig, Chirality 13 (2001) 403–414.
[22] K. Cabrera, M. Jung, M. Fluck, V. Schurig, J. Chromatogr. A 731 (1996) 315–321.
[23] O. Trapp, V. Schurig, Computers and Chemistry 25 (2001) 187–195.
[24] O. Trapp, V. Schurig, J. Chromatogr. A 911 (2001) 167–175.
[25] O. Trapp, S. Caccamese, C. Schmidt, V. Bohmer, V. Schurig, Tetrahedron: Assymetry 12 (2001) 1395–1398.
[26] V. Meyer, Chirality 7 (1995) 567–571.
[27] O. Gyllenhaal, J. Chromatogr. A 1042 (2004) 173–180.
[28] P. Mukherjee, S. Cook, J. Pharm. Biomed. Anal. 41 (2006) 1287–1292.
[29] P. Mukherjee, J. Pharm. Biomed. Anal. 43 (2007) 464–470.
[30] P. Marriott, K. Aryusuk, R. Shellie, D. Ryan, K. Krisnangkura, V. Schurig, O. Trapp, J. Chromatogr. A 1033 (2004) 135–143.
[31] C. Wolf, W. Pirkle, C. Welch, D. Hochmuth, W. Konig, G. Chee, J. Charlton, J. Org. Chem. 62 (1997) 5208–5210.
[32] P. Oswald, K. Desmet, P. Sandra, J. Krupcik, D. Armstrong, Chirality 14 (2002) 334–339.
[33] P. Oswald, K. Desmet, P. Sandra, J. Krupcik, P. Majek, D. Armstrong, J. Chromatogr. A 779 (2002) 283–295.
[34] J. Veciana, M. Crespo, Angew. Chem. Int. Ed. Engl. 30 (1991) 74–76.