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Characterization of the in vitro atropisomeric interconversion rates of an endothelin A antagonist by enantioselective liquid chromatography

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Abstract

Substituted biphenyl **I** (BMS-207940), a selective antagonist of the endothelin A (ET_A) receptor, has been proposed for the treatment of congestive heart failure. The structure of **I** possesses a stereogenic axis due to the hindered rotation about the biphenyl bond in the presence of its large *ortho*-substituents. As a result, **I** exhibits atropisomerism in which two nonplanar, axially enantiomers exist, which will be generically referred to as isomers A and B. Within the pharmaceutical industry, both from a scientific and regulatory point of view, characterization of enantiomeric drugs has become an important step in the development process. To investigate the configurational stability of **I** atropisomers, normal phase enantiomeric LC with tandem UV and laser polarimetric detection was used under pseudo-physiological conditions: first in a simple aqueous medium at 37 °C, and then in human serum at 37 °C. Kinetic studies indicated that the half-life of **I** enantiomerization in an aqueous medium at 37 °C was ca. 15 h. Enantiomerization of **I** atropisomers was greatly accelerated in the presence of human serum and human serum albumin, and the rate of enantiomerization depended on the concentration of **I**. The sera-concentration-dependent enantiomerization behavior of **I** strongly suggests a restricted site-specific substrate/**I** interaction mechanism. It was therefore demonstrated that atropisomeric interconversion studies for the compound studied required consideration of temperature, presence of plasma proteins, and drug concentration to account for the kinetic data.

Keywords: Atropisomers; Enantiomerization; Biphenyl; Kinetics; Serum; Drug–serum interaction

1. Introduction

Conventional carbon-centered enantiomerization has become of major importance in pharmaceutical drug development over the last twenty years. Although enantiomeric forms have been long known to exist structurally and their interconversion rates examined either theoretically or empirical more recently [1–10], attention to the relative bioactivity of the enantiomers was often not addressed. More recently, drug manufacturers have investigated the pharmacological profiles of the individual isomers, and in some cases, found that the bioactivity of the drug substance could be wholly or substantially attributed to a single enantiomer. While synthetic efforts to produce pure enantiomeric forms might be challenging, the resulting molecules were usually very stable with respect to inter-form conversion. A less obvious structure–activity complication is presented by *atropisomerism*, in which free rotation about a single bond can be sufficiently hindered to allow isolation of the stereoisomers. Atropisomerism can occur in a variety of systems—representatives would be aromatic amides, anilides, substituted styrenes, and the vancomycin antibiotics. Substituted biaryl compounds are found extensively both in natural products and as subunits of drug entities [11]. An important subclass is the *ortho*-tetrasubstituted biphenyls, which possess an axis coinciding with the single sigma bond connecting the ring structures. If the *ortho*-substituents offer sufficient steric hindrance, free rotation is

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impeded, and if the hindrance is severe, isolatable atropisomers may result.

Atropisomeric interconversion may be observed in situ by NMR. Examples of this include the *ortho*-methyl groups adjacent to the sulfonate groups in a porphyrin series [12] and the interflavanoid linkages in tannins [13]. LC, GC, and CE analyses of atropisomers require the non-trivial separation of the enantiomers as a first step. Polychlorinated biphenyls (PCBs) present an impressive separation challenge for both LC [14] (19 components) and enantiomeric GC [15] (41 components). Separations of rotamers by LC of compounds of pharmaceutical interest include the anticancer drug aplidine [16] and the antihypertensives lisinopril and enalapril [17], the contrast agent Iotetrol [18], and digoxin [19]. The widespread use of Chiralpak AD and OD columns in the normal-phase mode prompted an analyte-stationary phase interaction study using *N*-arylthiazolin-2-(thia)-one as the probe molecule [20]. CE has been used as an enantiomeric separatory technique for studying the hindered rotation around the proline amide bond in enalapril [21], and in the hypnotic and anticonvulsant methaqualon [22]. A representative of the aforementioned *ortho*-tetrasubstituted biphenyl class, namely, atropisomeric 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate has been used as a model compound to investigate the enantiomeric selector properties of non-cyclic malto-and cello-oligosaccharides and disaccharides [23]. Less employed techniques include determination of rotamers of the fluorescein derivatives of hydrocortisone and oestriol by fluorescent polar immunoassay [24]. Since it is always desirable to expose patients only with active drug substance in the lowest amount possible to avoid potential toxicities, understanding the sterogenicity issues of any potential drug is crucial. Thus, for drug substances that exhibit atropisomerism, a determination of the stability of the enantiomeric forms is required to ascertain whether development of a single enantiomer is feasible.

While interconversion rates of atropisomers are important in any matrix, the ideal medium of choice for pharmacological studies would be blood serum or plasma and the temperature would be 37 °C. Both the atropisomeric separation and the kinetics of interconversion are known to be temperature dependent [17,25,26]. A further complication for in vitro studies is the potential interaction between blood protein molecules, such as albumin, and the atropisomeric forms, potentially changing the kinetics of interconversion [27–31]. However, it has been often assumed, despite these complexities, that meaningful interconversion data may be deduced by extrapolation of rate values determined in essential pure solutions. The purpose of this paper is *not* to add to the fundamental knowledge of atropisomer chemistry, but rather to demonstrate that conventional calculational assumptions (extrapolation of interconversion data from room temperature, organic phase, relatively high concentration solutions), when applied to the atropisomeric interconversion of the biphenyl BMS-207940 [33], would have yielded unreliable data.

2. Experimental

2.1. Reagents and material

HPLC-grade *n*-hexane, 2-propanol, trifluoroacetic acid (TFA), triethylamine (TEA), sodium chloride, hydrochloric acid, and acetic acid were obtained from EM Science (Gibbstown, NJ, USA). Human serum, human serum albumin (HSA) and Tris buffer (pH 7.4) were obtained from Sigma (St. Louis, MO, USA). Ammonium acetate was purchased from Aldrich (Milwaukee, WI, USA), and sodium citrate buffer (pH 2.5) was obtained from Fluka (Buchs, Switzerland). Compound **I** (BMS-207940 [26] was provided by Bristol-Myers Squibb (Princeton, NJ, USA) (Fig. 1).

2.2. HPLC instrumentation and method

The LC system used in this investigation consisted of an Alliance 2690 system (Waters, Milford, MA, USA), a Model 996 photodiode array detector (Waters, Milford, MA, USA), and a Model ALP-2000 Advanced Laser Polarimeter (PDR, Lake Park, FL, USA). Data was collected with Millennium software (Waters, Milford, MA, USA). Normal-phase enantiomeric separations were carried out on a Chiralcel OD-H column (250 mm × 4.6 mm I.D., 5 μm particle size; Chiral Technologies, Exton, PA, USA). The mobile phase consisted of 80% *n*-hexane and 20% 2-propanol with 0.1% TFA and 0.1% TEA at a flow rate of 1.0 mL/min. The column temperature was maintained at 15 °C to minimize on-column interconversion. Polarimetric detection carried out on the PDR Advanced Laser Polarimeter was at a wavelength of 670 nm. The UV detection wavelength was 280 nm. For the interconversion kinetic studies, enantiomers resolved by normal-phase LC were collected and dried under vacuum. The individual enantiomers were reconstituted in 80% water/20% 2-propanol for the studies in aqueous solution and analyzed by normal-phase enantiomeric LC at 37 °C at fixed intervals for up to 10 h after dissolution. Simulated gastric fluid without pepsin was prepared according to USP 24. Buffers at pH 6.5, pH 5.1 and 4.8 were made by adjusting the pH of a 25 mM ammonium acetate solution with acetic acid. For the interconversion study in human serum and HSA, the enan-

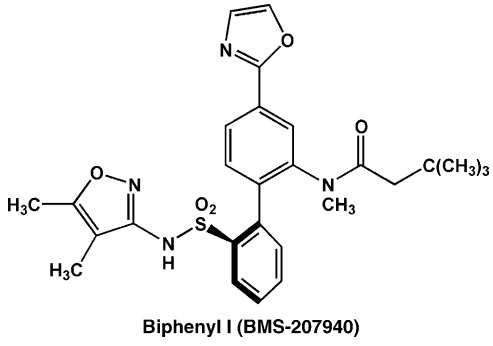

Fig. 1. Structure of **I**.

tiomers were reconstituted in human serum and HSA at the desired concentrations. The enantiomer-serum mixture was maintained at 37 °C and sampled at defined time points. After removal of serum proteins by 2-propanol precipitation, the supernatant was analyzed by normal-phase enantiomeric LC.

3. Results and discussion

As was previously noted, a number of techniques have been used to separate, and to a lesser degree, to determine the interconversion rates of physiologically-important atropisomers. Any such in vitro drug kinetic study is ultimately directed towards predicting in vivo interconversion rates, which may be difficult or impossible to reliably and directly obtain. Although the in vivo matrix (e.g., human sera) often presents conditions that are distinct from the in vitro conditions, it has often assumed that the differences (e.g., temperature, presence of protein and their concentration) can reliably be accounted for by calculation and/or assumption. Hence, an atropisomeric interconversion rate determined in an organic solvent at room temperature might be assumed to predict the in vivo interconversion rate, independent of the potential effects of protein and drug concentration. An important conclusion of this paper demonstrates that this line of assumptions can yield unacceptable and inaccurate results.

I atropisomers were separated by normal phase enantiomeric LC with tandem ultraviolet and laser polarimetric detection. The chromatograms, as shown in Fig. 2, demonstrated the separation of the two enantiomeric forms as did the opposite rotation of the plane-polarized light by each atropisomer: negative peak deflection (levorotatory) for the enantiomer designated A and positive peak deflection (dextrorotatory) for enantiomer B. Tandem detection allows not only for identification of polarization at 670 nm, but also offers assurances that the peaks separated are atropisomers of each other and not a single isomer and an unrelated species.

Pure isomer A and B were collected on a semi-preparative scale using conditions scaled up in the classical manner from the atropisomeric determination. Approximately 50 mg of >99% pure isomer A was collected prior to interconversion studies. The atropisomeric interconversion of **I** was monitored over time by normal phase enantiomeric LC. The formation of a racemate from pure enantiomer B or A in aqueous solution was studied at 37 °C to mimic physiological temperature. Fig. 3 illustrates the interconversion of **I** enantiomer A into a racemic mixture of isomer B and A. The enantiomeric excess (ee) was calculated by the standard definition:

$$\text{ee} = \frac{\text{major enantiomer} - \text{minor enantiomer}}{\text{major enantiomer} + \text{minor enantiomer}} \times 100\%$$

A kinetic plot of **I** atropisomeric interconversion in an aqueous solution at 37 °C is shown in Fig. 4. Since the interconversion of **I** atropisomers appeared to follow first-order reaction kinetics, the interconversion half-life of was calculated from the formula $t_{1/2} = -\ln 2/k$, where k, the pseudo-first order rate constant, is equal to the slope. The interconversion half-life of **I** (A and B) in the aqueous medium at 37 °C was ca. 15.2 h with a standard deviation of 0.8 h (both forward and backward approaches to equilibrium, four independent kinetic determinations). We next studied the effect of low pH on the atropisomeric interconversion half-life, since one could be concerned with accelerated interconversion in the low pH environment of the stomach. To study this, a kinetic determination was carried out in simulated gastric fluid. The interconversion half-life of **I** in simulated gastric fluid was determined to be 15.8 h, which was experimentally equivalent to the results obtained in the pH neutral solution.

To study the effect of both physiological temperature *and* the presence of protein, enantiomerization of **I** atropisomers was studied using **I** (A or B) at a concentration of 400 μg/mL in human serum at 37 °C. A representative kinetic plot is shown in Fig. 5. The interconversion rate was significantly faster in a human serum matrix when compared to the "pseudo-physiological" non-protein-containing medium previously discussed, with a half-life of 2.5 h. The facilitation/inhibition of atropisomeric interconversion in the presence of serum proteins has been previously reported [20–24]. The protein α_1-acid glycoprotein (AGP; orosomucoid) and HSA have been postulated or inferred to affect isomer interconversion, whereas lipoprotein and globulin fractions seem to exhibit little or no effect. These effects are apparently seen in atropisomeric interconversions, *cis–trans* conversions, and inversion of configuration, but the general trend is not straightforward.

In order to further study the atropisomeric interconversion rate enhancement in the presence of serum, additional studies were performed. In the first study, HSA was substituted for serum to determine if the apparent matrix-atropisomeric interaction was due to the albumin fraction or to a different protein subset. Whereas the interconversion half-life was 15.2 h (average of four independent determinations, inter- and intra-day, SD = 0.8 h) in the absence of HSA, the half-life was 1.2 h in the presence of HSA, in good agreement with the value obtained in intact serum. This result strongly suggested that HSA played a major role in the rate enhancement of the enantiomerization of the atropisomers of **I** in serum.

Since most often drugs are effective at relatively low concentrations compared to the amount of serum proteins present in the physiological matrix, we next examined the effect of varied concentrations of **I** atropisomers in a set serum matrix concentration. These studies were performed using 250, 130, 80, and 20 μg/mL (approximately 40 μM) of **I** at 37 °C in the presence of human serum. The interconversion half-lives and rate constants (k) are summarized in Table 1. If the interconversion rate law was truly pseudo-first order, it would be expected that the half-life would be independent of concentration. As the data indicates, the half-life decreases by a factor of at least 20-fold over a concentration range of 400–20 μg/mL of **I**. This data appears to be consistent with a restricted site drug/serum-binding model. In the case where

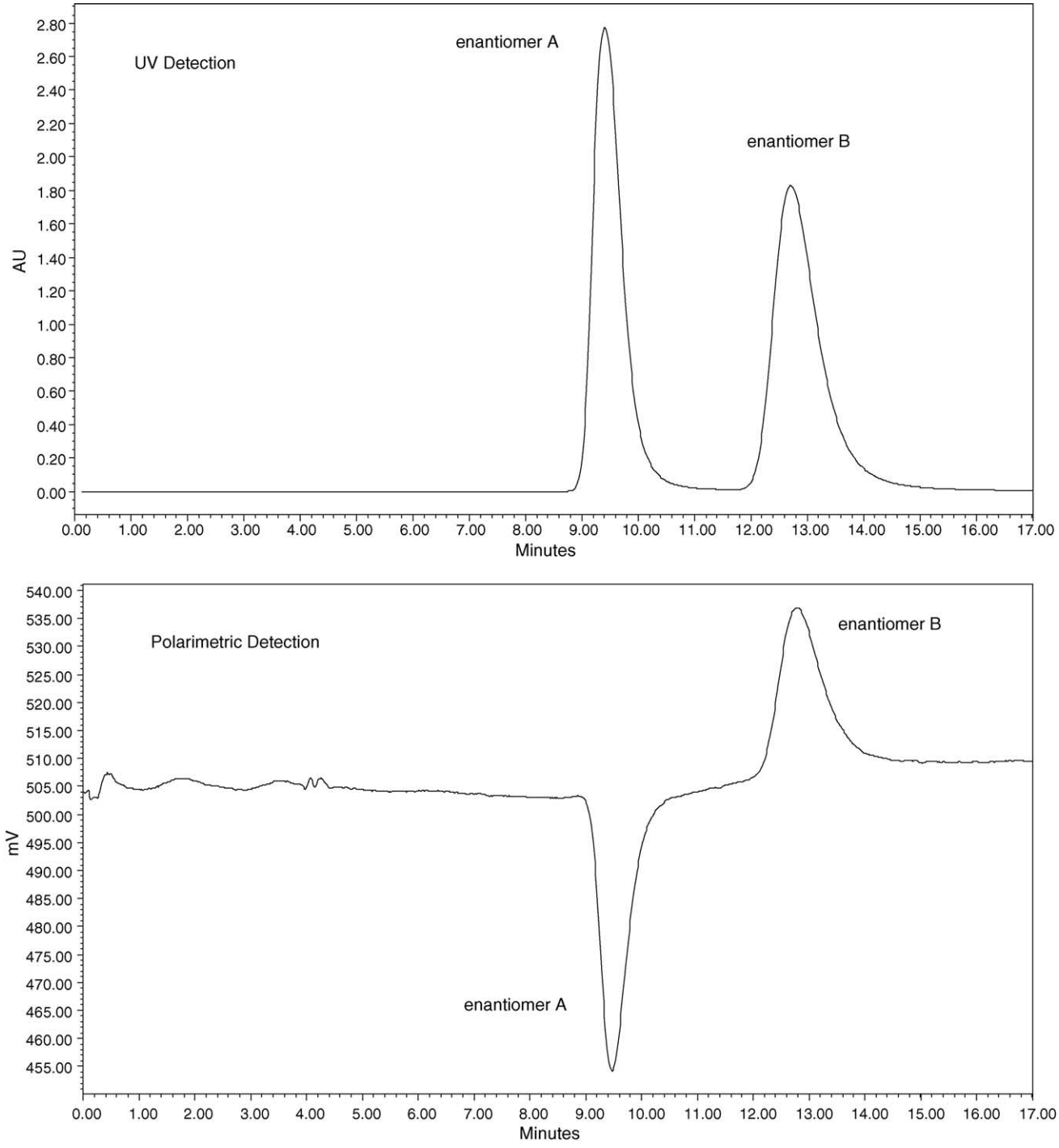

Fig. 2. Chromatographic separation of the atropisomeric forms of **I** by normal-phase LC with tandem UV/laser polarimetric detection.

Table 1
Interconversion half-life vs. the concentration of **I** in human serum at 37 °C

Concentration (μg/mL)	$t_{1/2}$ (h)	Substrate/**I** ratio	k (min^{-1})
20	<0.1	3000	>0.058
80	0.4	750	0.014
130	1.0	462	0.0058
250	2.1	240	0.0028
400	2.5	150	0.0023

the number of available protein sites in the albumin fraction of the serum that facilitate interconversion is less than the total number of drug molecules, the $t_{1/2}$ of interconversion will become drug concentration dependent until, in the limiting case of very high drug concentrations, the rate of interconversion becomes concentration independent. The concentration dependence is not due to incorrectly choosing a pseudo-first order mechanism, but rather it reflects the limiting of the in-

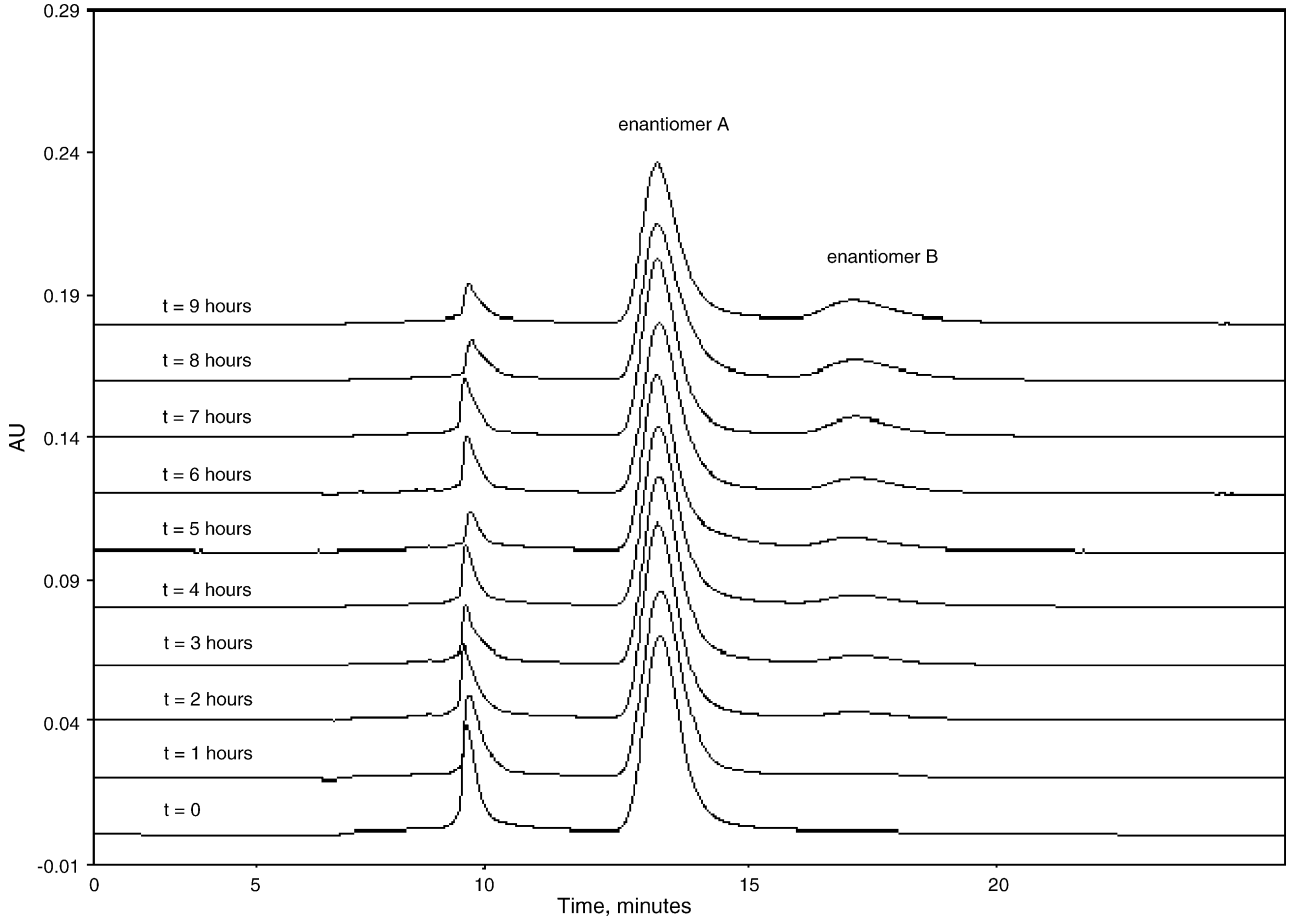

Fig. 3. Atropisomeric interconversion of enantiomer A of **I** in aqueous solution at 37 °C.

terconversion rate by the drug to protein active site ratio. The assumption of pseudo-first order kinetics is valid only when the interconversion is not limited by the number of protein sites on the albumin that facilitates a change in enantiomerization.

Although the existence of atropisomers has been known for some time, it appears that fundamental protein-isomer mechanistic studies have not been extensive. In order to attempt to evaluate the restricted-site model hypothesis proposed here, our data from Table 1 was plotted in Fig. 6 as

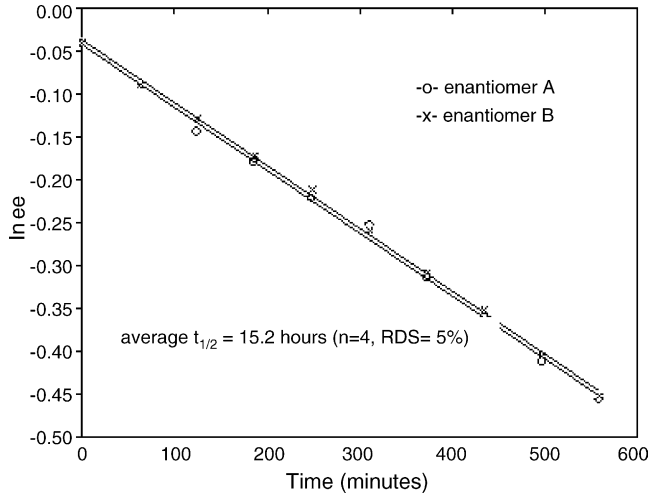

Fig. 4. Kinetic determination of atropisomeric interconversion of **I** in aqueous solution at 37 °C.

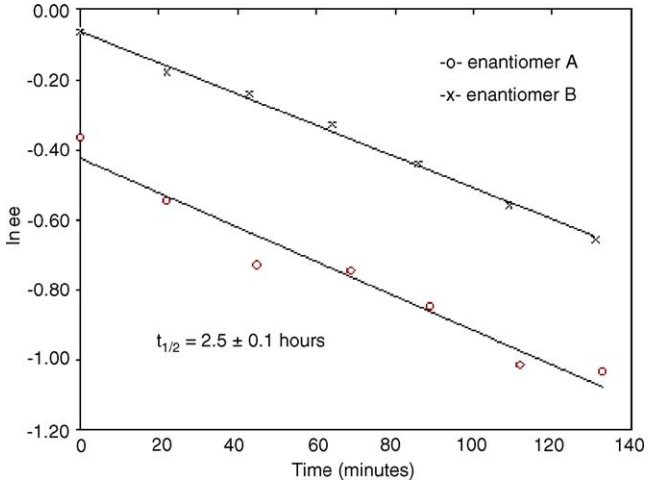

Fig. 5. Kinetic determination of atropisomeric interconversion of **I** (ca. 400 μg/mL) in human serum at 37 °C.

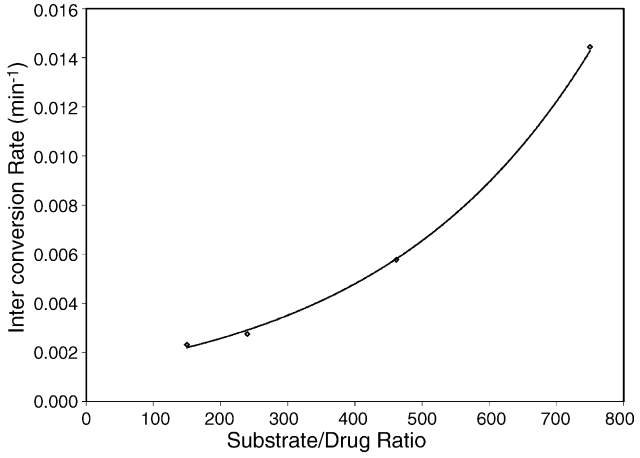

Fig. 6. Atropisomeric interconversion rate dependence of substrate (serum)/**I** ratio.

the interconversion rate versus the substrate/drug ratio. Kinetically, this is a representation of a restricted-site interaction model [32]. As the protein substrate/drug ratio increases, the ratio of protein facilitation sites to total drug present increases. To a first approximation, at the point when the number of restricted sites matches the drug concentration, the reaction rate becomes drug concentration independent, as mentioned previously. Therefore, this representation predicts a leveling of the interconversion rate versus substrate/drug ratio curve (at low abscissa values) as seen in Fig. 6. However, in in vivo systems, in which the protein substrate/drug ratio is at least two orders of magnitude greater than previously discussed, the model predicts an interconversion half-life no greater than the half-life determined empirically at 20 μg/mL (<5 min). Since the effective human doses/serum concentrations of the drug are likely to be below 20 μg/mL, one could possibly expect an even substantially shorter interconversion time in humans.

If a specific interaction between certain sites on albumin proteins and **I** facilitates interconversion of atropisomers, then it might be expected that any process *decreasing* that interaction should slow the rate, and therefore lengthen the reaction half-life of enantiomerization [34]. Since both **I** and HSA can be protonated, a study was performed to evaluate the effect of pH on interconversion rate. From simple coulombic considerations and inferences from empirical bioanalytical sample preparation protocols, it would be expected that the lowering of pH might decrease protein–drug interactions. If albumin proteins were truly responsible for our observed interconversion rate enhancement, the reaction half-life might increase in this scenario. In this study, at a concentration of 30 μg/mL of **I** in the presence of human serum, the pH of the serum was adjusted from the physiological value of 7.4 downward to a pH of 2.5, and the interconversion half-lives were determined at 37 °C. The pH values and interconversion half-lives from this study were: pH 7.4, 2 h; pH 6.5, 0.7 h; pH 5.1, 6 h; pH 4.8, 9 h; pH 2.5, 10 h. The interconversion half-life increased ca. five-fold from a pH range of 7.4–2.5, consistent with the current model. It is to be noted that the pH dependence of the interconversion reaction in serum albumin in no ways *proves* anything about the mechanism; it is simply consistent. Other pH effects, such as protein conformational changes, could be involved. However, it is to be noted that kinetic studies in the *absence of albumin* yielded equivalent half-life independent of the pH of the aqueous reaction medium.

4. Conclusions

A substituted biphenyl possessing antagonist activity towards the endothelin A receptor (BMS-207940, **I**) was studied to determine the kinetics and physiological relevance of atropisomeric interconversion. It was demonstrated that the half-life of interconversion was affected dramatically by the presence of human serum proteins and data was presented that supported the hypothesis that the proteins responsible for enhanced interconversion were contained in the albumin fraction. It was further shown that the half-life was not independent of drug concentration, as might be expected, but rather the half-life decreased significantly as the drug concentration decreased. This result suggested, in conjunction with other studies, that the albumin protein–drug interaction sites were not sufficiently plentiful (i.e., restricted) to support pseudo-first order kinetics of interconversion, even at relatively low drug concentrations. This set of studies suggests that atropisomeric interconversion studies need to be performed in the presence of human serum. Furthermore, these studies need to utilize physiologically-meaningful drug concentrations before interpolating the data to in vivo systems.

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