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Simultaneous determination of enantioselective plasma protein binding of aminohydantoins by ultrafiltration and chiral high-performance liquid chromatography

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Abstract

Chiral HPLC methods were developed and utilized for the simultaneous determination of plasma protein binding of enantiomers of two racemic aminohydantoin compounds. Reversed-phase HPLC with the use of a polysaccharide-type chiral stationary phase column was employed for the separation and quantitation of the enantiomers of the two compounds with detection limits in the range 5–10 ng/ml in the plasma matrix. The chiral HPLC methods were selective, sensitive and reproducible. The *R* and *S* enantiomers of both compounds were baseline-resolved under the chromatographic conditions employed. Ultrafiltration techniques were applied to determining the plasma protein binding for each enantiomer in rat, dog and human plasma. The results clearly show stereoselective binding of the two enantiomers of each compound with higher protein binding of the *R* enantiomer than the *S* enantiomer in rat, dog and human plasma. Binding association constants were also determined to be in the range $1.01–14.0 \cdot 10^4\ M^{-1}$ at 37°C. Both the protein binding percentage and binding association constant were enantioselective and species-dependent. Such information is important for a clear understanding of the differences in biological activity as well as in pharmacokinetic and pharmacodynamic properties between the two enantiomers of each compound in the drug discovery and development process.

Keywords: Binding constants; Plasma protein binding; Hydantoin; Proteins; Aminohydantoins

1. Introduction

Aminohydantoins have been explored as potential drug candidates for the treatment of cardiac, anti-infective and musculoskeletal diseases [1]. Many of these compounds are enantiomers which are expected to show significant differences in drug efficacy and safety as they may have different pharmacology and toxicology in a stereospecific biological environment. For a given biological target, one enantiomer can be efficacious and safe while the other can be inactive and toxic. When a chiral drug is developed, the differences in biological activities as well as in the pharmacokinetic and pharmacodynamic processes of absorption, distribution, metabolism, excretion and response, must be shown, even when the physicochemical properties of enantiomers are equivalent [2].

Plasma protein binding has an important effect on the pharmacology and toxicology of drugs. The

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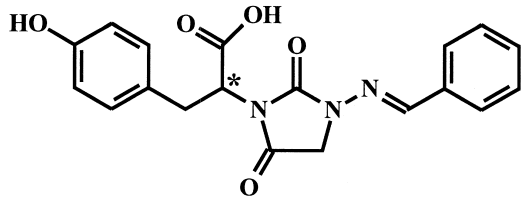
extent of protein binding may determine the relative concentrations of drugs that are available for the drug–receptor interaction at the site of action. The extent of drug binding to plasma proteins varies among different types of drugs. The protein binding can be very concentration-dependent which has a profound effect on dosage adjustments to target total drug concentration within the therapeutic range defined for total drug. Therefore, it is important to know the extent of protein binding for each enantiomer of a chiral compound. The plasma protein binding of enantiomers of chiral drugs have been reviewed in the literature [3].

The drug candidates selected for this study are two racemic aminohydantoin compounds. Many plasma protein binding studies have focused on the binding of individual plasma protein components. Although it is important to understand the contribution of each isolated plasma protein to drug binding, the data obtained with individual components of plasma are not applicable to the clinical situation. For this reason, whole plasma was used for this work. Among a variety of plasma protein binding techniques utilized today [4], ultrafiltration was chosen for this study because of its speed, simplicity, and accuracy [5]. The chiral high-performance liquid chromatography (HPLC) separations of several hydantoin compounds have been reported, employing a mobile phase containing cyclodextrin as a chiral selector [6,7], a cyclodextrin-bond chiral column [8], or an α_1-acid glycoprotein column [9]. This paper reports the development and application of chiral HPLC methods using a polysaccharide-type chiral stationary phase column in conjunction with ultrafiltration for the simultaneous determination of the plasma protein binding of each enantiomer of two aminohydantoins in rat, dog and human plasma.



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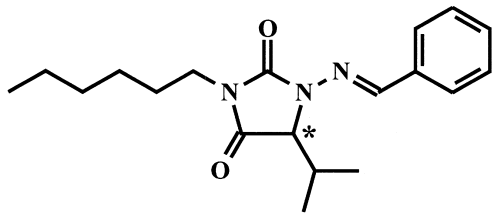

II

Fig. 1. Structures of aminohydantoin compounds I and II (the chiral centers are indicated by *).

2. Experimental

2.1. Materials

Two aminohydantoins (as shown in Fig. 1), 1-[(phenylmethylene)-amino]-3-[(±)-1-carboxy-2-(4-hydroxyphenyl)-ethyl]-imidazoline-2,4-dione (compound I) and 1-[(phenylmethylene)-amino]-3-*n*-hexyl-(±)-5-isopropyl-imidazolidine-2,4-dione (compound II), were obtained from Procter and Gamble Pharmaceuticals (Mason, OH, USA). HPLC-grade acetonitrile and formic acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Blank rat, dog and human plasma were obtained from Rockland (Gilbertsville, PA, USA). Phosphate-buffered saline (PBS, pH 7.4) was purchased from Life Technologies (Gaithersburg, MD, USA).

2.2. Preparation of standards and samples in plasma and PBS buffer

The stock solutions of compounds I and II were prepared by dissolving the respective compound in methanol to yield 1 mg/ml stock solutions. The stock solutions were used for the preparation of standards and samples for both plasma protein binding and non-specific filtration membrane binding. Appropriate amounts of each methanol stock

solution were taken, evaporated to dryness under nitrogen, and reconstituted in blank plasma and PBS buffer, respectively, to give plasma and PBS samples at 1, 10, 25 and 50 μg/ml. The PBS standards were prepared in a manner similar to that described for PBS samples to yield a series of standards ranging from 0.1 to 50 μg/ml. The plasma standards were made by reconstituting residues with blank plasma ultrafiltrate after evaporation of appropriate amounts of the methanol stock solution to give concentrations of 0.1 to 50 μg/ml. All samples were made in triplicate. The calibration curves were generated by analyzing the standard samples immediately after they were made. Quality control (QC) samples were prepared by spiking blank PBS buffer and respective blank plasma matrix with the compounds to yield three concentrations at 0.1, 5 and 50 μg/ml.

2.3. Ultrafiltration

Both plasma samples and PBS buffer samples were equilibrated at 37°C for 15 min prior to ultrafiltration. The ultrafiltration was performed using an Amicon centrifree micropartition device with the filter membrane of 30 000 M_r cut-off. Samples of 1 ml volume were centrifuged at 1800 *g* (37°C) for 15 min. The ultrafiltrates, approximately 200 to 300 μl, were then collected for HPLC analysis.

2.4. Equipment and chromatographic conditions

Chiral HPLC was performed on a Waters (Milford, MA, USA) Alliance HPLC/PDA system consisting of a 2690 separations module and a 996 photodiode array detector. The system was controlled by the Waters Millennium 2020 data system. A polysaccharide-type chiral stationary phase column (Chiralcel OJ-R, 150×4.6 mm I.D., 5 μm) obtained from Chiral Technologies (Exton, PA, USA) was utilized. A 20-min isocratic elution with a mobile phase consisting of acetonitrile–water–formic acid (30:70:0.1, v/v/v, for compound I; 55:45:0.1, v/v/v, for compound II) was employed. The flow-rate was 1.0 ml/min with UV detection at 288 nm for both compounds.

3. Results and discussion

3.1. Selectivity and linearity of calibration

Figs. 2 and 3 show the representative chromatograms of the chiral compounds I and II in blank human plasma and spiked samples. As demonstrated in Figs. 2 and 3, both chiral HPLC methods for the separation of the enantiomers of the two compounds were very selective. The *R* and *S* enantiomers of each compound were clearly well resolved from the matrix components under the chromatographic conditions employed. In addition, the two enantiomers from each of the two compounds were baseline-resolved from each other. The enantioselectivity (α) and resolution (R_s) of the two enantiomers were calculated from the chromatograms in rat, dog, and human plasma, as well as in PBS buffer. The α values ranged from 1.30 to 1.36 for compound I and 1.14 to 1.55 for compound II while the R_s values were from 2.11 to 2.32 for compound I and 1.45 to 1.57 for compound II. The calibration curves of the two enantiomers of each compound were linear from 0.1 to 50 μg/ml with correlation coefficients greater than 0.999.

3.2. Precision and accuracy

The method precision and accuracy were also investigated. The precision expressed as the relative standard deviation (RSD) based on eight repetitive injections at drug concentrations of 0.1, 5 and 50 μg/ml, was less than 3.3% (intra-day) and 5.6% (inter-day) for compound I and less than 4.7% (intra-day) and 6.2% (inter-day) for compound II, for the plasma extracts of all three species. The accuracy was found to be in the range 94–105% for compound I and 92–104% for compound II for all plasma and PBS buffer matrices at three drug concentration levels, 0.1, 5 and 50 μg/ml.

3.3. Limit of detection and analyte stability

The detection limit, at a signal-to-noise ratio of 3, in plasma of all three species was about 5 ng/ml for compound I and 10 ng/ml for compound II. The enantiomers of both compounds were found to be stable during the entire course of the study that

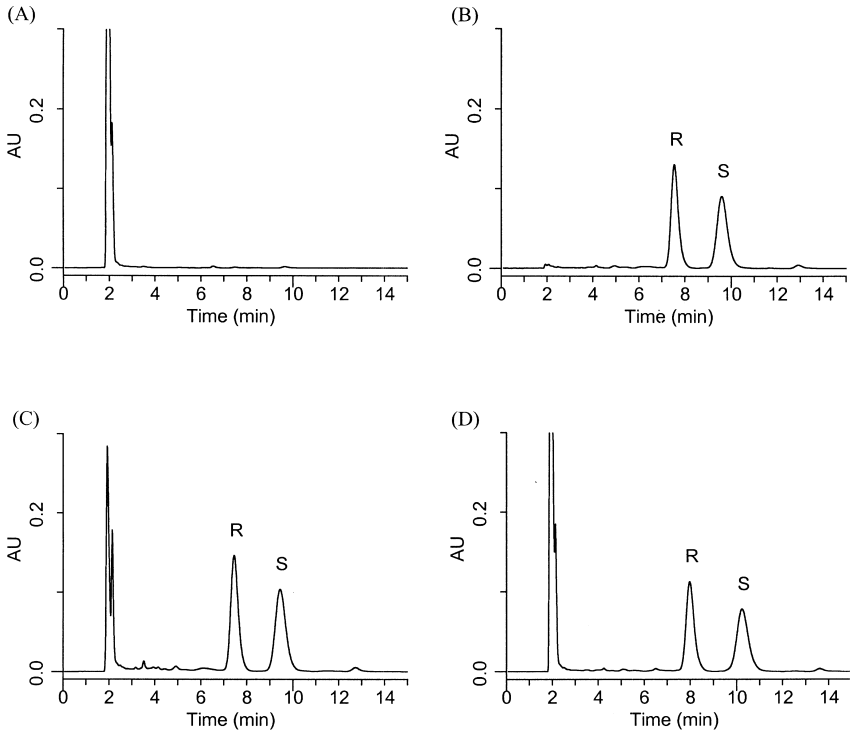

Fig. 2. Representative chromatograms of blank human plasma (A) and the samples spiked with 5 μg/ml compound I in PBS buffer (B), dog plasma (C), and human plasma (D) under the same chromatographic conditions.

included sample preparation, incubation, centrifugation and HPLC assay.

3.4. Plasma protein binding

The non-specific binding of the compounds to the filtration membrane was determined by comparing the analyte concentrations in PBS buffer before and after ultrafiltration. The results indicated that the filter membrane binding was in the range 1–5% for the four concentrations studied. Plasma protein binding at various concentrations was calculated using the concentrations determined from the plasma ultrafiltrates, corrected for filter membrane binding at the respective concentrations, according to the following equations,

$$\mathrm{PB} = 1 - (C_{\mathrm{f}}^{\mathrm{p}}/C_{\mathrm{uf}}^{\mathrm{p}})(1 + \mathrm{NSB}) \quad (1)$$

$$\mathrm{NSB} = 1 - C_{\mathrm{f}}^{\mathrm{b}}/C_{\mathrm{uf}}^{\mathrm{b}} \quad (2)$$

where PB and NSB are percent protein binding and non-specific binding. $C_{\mathrm{f}}^{\mathrm{p}}$, $C_{\mathrm{f}}^{\mathrm{b}}$and $C_{\mathrm{uf}}^{\mathrm{p}}$, $C_{\mathrm{uf}}^{\mathrm{b}}$ represent filtered and unfiltered drug concentrations in plasma and buffer, respectively. The results for the concentration-dependent binding of the enantiomers of compounds I and II in rat, dog and human plasma are listed in Table 1. As shown in the table, protein binding in plasma of all three species increased as the total analyte concentration decreased for both enantiomers of each compound. The binding experiments also revealed that at a given concentration, the binding was considerably higher in human plasma than in rat and dog plasma for both enantiomers of compound I. For compound II, the protein binding of the two enantiomers increased gradually from the rat to the dog to human. In general, plasma protein binding was greater for compound II than for compound I in all three species. The differences in enantioselective binding between two enantiomers of compound I were larger in rats and dogs than in humans. For compound II, no significant differences

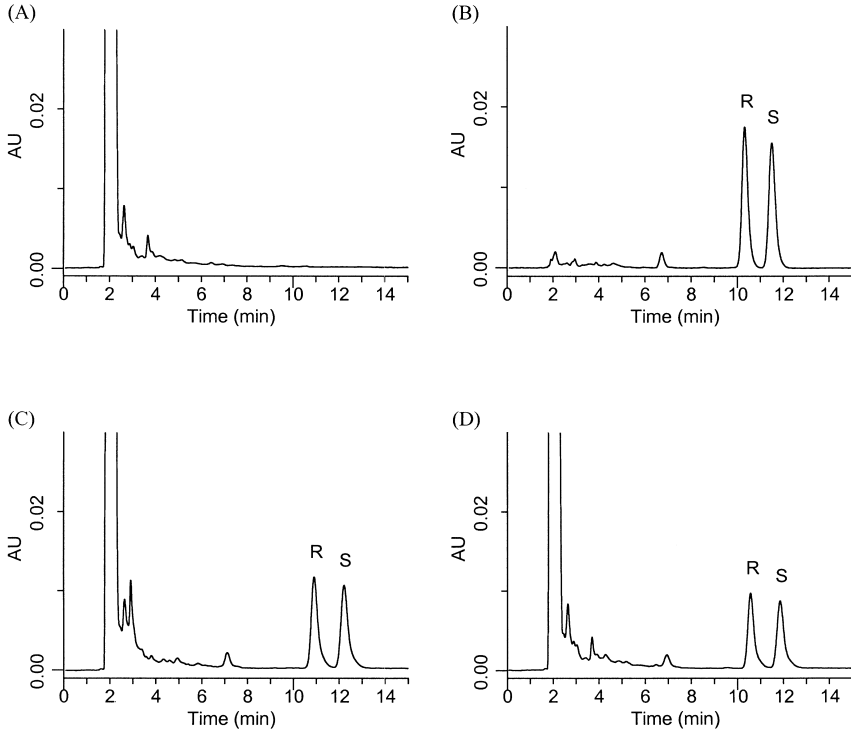

Fig. 3. Representative chromatograms of blank human plasma (A) and the samples spiked with 1 μg/ml compound II in PBS buffer (B), rat plasma (C), and human plasma (D) under the same chromatographic conditions.

Table 1
Plasma protein binding of *R* and *S* enantiomers of compounds I and II in rat, dog and human plasma at 37°C as determined by ultrafiltration and chiral HPLC (mean±SD, $n=3$)

Species	Concentration (μg/ml)	Plasma protein bound (%)				Association constant (10^4 M^{-1})			
		Compound I		Compound II		Compound I		Compound II	
		R	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Rat	50	66.9±0.2	54.1±0.2	91.6±0.2	88.0±0.3				
	25	71.8±0.3	60.0±0.2	93.2±0.1	90.2±0.2	1.21±0.15	1.01±0.10	3.89±0.46	2.74±0.40
	10	73.4±0.2	62.6±0.2	93.6±0.2	90.7±0.2				
	1	76.0±0.3	65.9±0.4	94.3±0.4	91.8±0.3				
Dog	50	67.1±0.3	58.0±0.3	92.0±0.2	88.4±0.2				
	25	74.4±0.2	66.2±0.2	93.9±0.3	90.5±0.2	1.68±0.37	1.34±0.16	5.07±0.79	2.83±0.23
	10	75.8±0.3	68.1±0.2	94.2±0.2	91.2±0.3				
	1	78.4±0.4	71.2±0.5	95.0±0.4	92.0±0.5				
Human	50	91.4±0.2	88.4±0.4	95.1±0.4	90.5±0.3				
	25	94.6±0.3	92.2±0.3	96.9±0.3	92.7±0.3	10.8±0.82	6.42±0.73	14.0±0.94	4.61±0.69
	10	95.2±0.3	92.9±0.4	97.1±0.5	93.2±0.4				
	1	96.1±0.6	94.0±0.5	97.6±0.6	94.2±0.5				

in enantioselective binding from different species were observed. Overall, the *R* enantiomer exhibited higher protein binding than the *S* enantiomer for both compounds in all three species. Although the differences in protein binding percentage between the two enantiomers may be relatively small for both compounds in human plasma in the drug concentration range studied, the clinical significance would greatly increase as the protein binding for one enantiomer approaches 98% or higher at lower total drug concentrations. In those cases, because the volume of distribution and total clearance of the drug are directly proportional to the free fraction of the drug in plasma, these two pharmacokinetic parameters would be considerably altered, leading to a significant difference in intensity of drug action and toxicity between the two enantiomers. In general, the findings on the enantioselectivity and interspecies dependencies should be useful when these drug candidates are tested in animal models and human trials, especially when they have narrow therapeutic ranges.

For a better understanding of the binding of these compounds to plasma proteins, the protein binding association constant (K) – an indication of affinity of a drug for proteins, was derived based on the modified Scatchard equation [10]. By assuming that only one predominant plasma protein is involved in the binding, the association constants can be obtained according to:

$$C_b/C_{ub} = -KC_b + \nu KP_t \quad (3)$$

where C_b and C_{ub} are protein bound and unbound drug concentrations, ν the number of independent binding sites, and P_t the total protein concentration. Fig. 4 shows the modified Scatchard plots for the *R* and *S* enantiomers of compound I in rat plasma at 37°C. Based on the four drug concentrations measured, the protein binding association constants for the two enantiomers of each compound in rat, dog and human plasma were calculated and listed in Table 1. For both compounds, the binding association constant was highest in human plasma and lowest in rat plasma for both enantiomers. In addition, the binding constant was greater for the *R* enantiomer than for the *S* enantiomer for both compounds in the plasma of all three species,

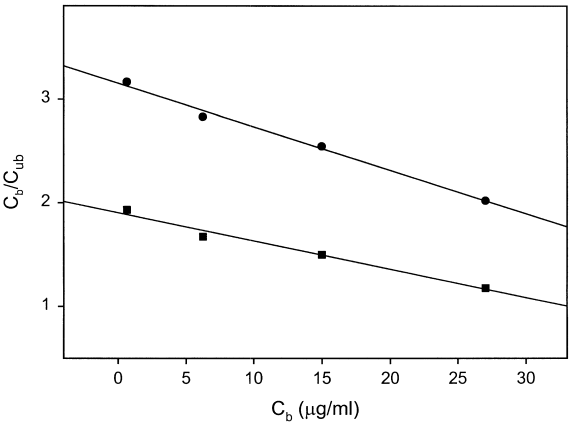

Fig. 4. The modified Scatchard plots for the *R* enantiomer (●) and *S* enantiomer (■) of compound I in rat plasma at 37°C.

indicating stronger affinity of the *R* enantiomers for the plasma proteins of these species. The protein binding association constants of the enantiomers in rat, dog, and human plasma were estimated to be in the range of $1.01–10.8 \cdot 10^4\ M^{-1}$ for compound I and $2.74–14.0 \cdot 10^4\ M^{-1}$ for compound II. The differences in enantioselectivity in protein binding association constant should be of great importance when there is a competition for the protein binding sites between the parent drug and other substances such as its metabolites or other drugs. In this instance, the parent drug may be displaced from the binding sites by other substances with higher affinity for plasma proteins, causing unexpected clinical consequences.

4. Conclusions

The ultrafiltration technique has been utilized to determine the plasma protein binding of the enantiomers of two aminohydantoin compounds. Chiral HPLC–UV methods have been developed and employed to measure the drug levels in the buffer and plasma ultrafiltrates. The chiral HPLC methods were selective, sensitive and reproducible. The *R* and *S* enantiomers of both compounds were baseline-resolved under the chromatographic conditions employed. For both compounds, the protein binding percentage and binding association constant were found to be greater for the *R* enantiomer than for the *S* enantiomer. Additionally, the plasma protein bind-

ing was species- and concentration-dependent, with higher protein binding in human plasma than in rat and dog plasma. For both compounds, the estimated protein binding association constants were in the range $1.01–14.0 \cdot 10^4\ M^{-1}$ at 37°C.

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