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Stereoselective Plasma Protein Binding of Ibuprofen Enantiomers

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Summary. We have developed a novel and reproducible method for determining the plasma protein binding of the two ibuprofen enantiomers in the presence of each other. The method involves the use of radiolabelled racemic ibuprofen, equilibrium dialysis, derivatization of the enantiomers to diastereomeric amides, high-performance liquid chromatography, and radiochemical analysis.

We have determined the plasma protein binding of R(−)- and S(+)-ibuprofen in 6 healthy male volunteers after the oral administration of 800 mg racemic ibuprofen.

The mean time-averaged percentage unbound of the R(−)-enantiomer, 0.419 was significantly less than that of the S(+)-enantiomer, 0.643, consistent with stereoselective plasma protein binding.

The percentage unbound of each ibuprofen enantiomer was concentration-dependent over the therapeutic concentration range and was influenced by the presence of its optical antipode.

Key words: ibuprofen; enantiomers, stereoselective protein binding, pharmacokinetics

The non-steroidal anti-inflammatory drug ibuprofen [RS-2-(4-isobutyl-phenyl) propionic acid] contains a chiral carbon atom on the propionic acid side-chain, and as such exists in two mirror-image forms, or enantiomers. Ibuprofen is used clinically in its racemic form, i.e. as an equal mixture of its R(−)- and S(+)-enantiomers. The clinical pharmacology of ibuprofen is complicated by the fact that while the S(+)-enantiomer is virtually solely responsible for the desired pharmacological effects [1], one of the elimination pathways of the less active enantiomer, R(−)-ibuprofen, is unidirectional metabolic inversion to S(+)-ibuprofen [2, 3].

For ibuprofen the fraction unbound in plasma is an important determinant not only of its primary pharmacokinetic properties (apparent volume of distribution and clearance) [4], but also of its pharmacological activity, since it is generally accepted that drug effect is related more closely to unbound than to total plasma concentrations [4]. Data from non-stereoselective plasma protein binding studies have shown that ibuprofen is about 98 to 99% bound at therapeutic concentrations [5], and this suggests that any difference between the enantiomers in the fraction bound is minor. However, an important implication of the high degree of binding for the unresolved drug is that even a small difference in the fraction bound between the enantiomers may represent a substantial difference in their respective unbound fractions. An additional consideration is that the enantiomers of ibuprofen may compete for protein binding sites [3, 6, 7]. Hence, methods are needed which measure the plasma protein binding of the individual enantiomers of ibuprofen in the presence of each other.

There are only a few methods available for studying the plasma binding of the enantiomers of 2-phenylpropionic acid derivatives when present together in the same sample. Jones et al. [7] studied the binding of R(−)- and S(+)-2-phenylpropionic acid in rabbit plasma using the individual radiolabelled enantiomers. A potential problem in applying this approach to other chiral drugs is that the radiolabelled material made available to investigators is in general racemic, and there are numerous problems associated with the optical resolution of milligram quantities of radiolabelled racemate, whilst maintaining sufficiently high purity and specific activity.

In this paper we describe a novel method which

permits the reproducible, simultaneous determination of the plasma protein binding of R(−)-ibuprofen and S(+)-ibuprofen. We also present results which suggest that the plasma protein binding of ibuprofen is stereoselective and that there is a mutual effect of each enantiomer on the plasma binding of the other.

Material and Methods

Plasma Protein Binding Methods

Materials

R(−)-ibuprofen, S(+)-ibuprofen and radiolabelled ^{14}C-RS-ibuprofen (specific activity 25 mCi·g^{-1}) were all generously supplied by Boots Co. (Nottingham, UK). S(−)-1-phenylethylamine was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and thionyl chloride from Fluka AG (Buchs, Switzerland). High-performance liquid chromatography (HPLC) grade heptane, acetonitrile, and methanol were purchased from Waters Associates (Milford, MA, USA), and dichloromethane GR from Merck (Darmstadt, FRG). Isopropanol, sulphuric acid, sodium acid phosphate, disodium hydrogen phosphate, and sodium chloride were all of analytical reagent grade. Isotonic phosphate buffer (pH 7.4) was prepared with 0.067 mol·l^{-1} disodium hydrogen phosphate, 0.067 mol·l^{-1} sodium acid phosphate, and sodium chloride. The dialysis membrane (Spectropor 2, molecular weight cut-off 12000–14000, Spectrum Medical Industries Inc) was prepared for use by washing three times with purified water and then soaking for 24 h in the isotonic phosphate buffer. Human serum albumin (Calbiochem, fraction V, 12666) was dissolved in isotonic phosphate buffer to produce a final concentration of 40 g·l^{-1} (4% HSA). Liquid scintillation counting was performed with either aqueous (Amersham ACS II) or non-aqueous (Beckman Ready-Solv NA) counting scintillant, depending on the nature of the samples.

Radiochemical Purification of ^{14}C-RS-Ibuprofen

Before use the racemic radiolabelled ibuprofen was purified by reversed-phase HPLC. On each occasion purification was performed no more than 2 days before the start of binding investigations. A Waters Associates 501 HPLC pump connected to a Waters model U6K manual injector was used to deliver the mobile phase (acetonitrile:water, adjusted to pH 2.4 with phosphoric acid, 60:40) at a constant rate of 1.5 ml·min^{-1} to a reversed-phase chromatographic column (250 mm × 4.6 mm i.d., C_{18}, 10 µm particle size; Alltech, Homebush, Aust). A Waters Lamda Max 481 LC spectrophotometric detector set at 216 nm was used to monitor the column effluent.

Aliquots (100 µl) of a methanolic solution of ^{14}C-ibuprofen (2 mg in 5 ml) were injected onto the column. The eluate fraction (2–3 ml) corresponding to the peak representing ibuprofen (retention time 7 min) was collected into a tube and placed in a water bath set at 50°C under a steady flow of purified nitrogen, to reduce the volume to about 0.5 ml. After the addition of 200 µl of 2 mol·l^{-1} sulphuric acid and 5 ml of 5% isopropanol in heptane, the tube was vortex-mixed for 1 min and centrifuged at 1000 *g* for 5 min. The organic phase was removed and evaporated to dryness at 50°C under a steady flow of nitrogen. The residue of ^{14}C-ibuprofen was reconstituted in 4% HSA to produce a final ibuprofen concentration of approximately 7 µg in 20 µl. The purification process yielded an overall recovery of 96% and this figure was used to determine the amount of the methanolic ^{14}C-ibuprofen solution which needed to be purified for a given set of plasma binding experiments.

Equilibrium Dialysis

The equilibrium dialysis unit (total capacity about 8 ml), constructed of clear perspex, consisted of two identical compartments separated by a single layer of dialysis membrane. Into one compartment was placed 3.5 ml of the plasma sample under investigation, containing known concentrations of non-radiolabelled R(−)-ibuprofen and/or non-radiolabelled S(+)-ibuprofen, together with a 20 µl aliquot of the solution of purified ^{14}C-ibuprofen in 4% HSA. Into the opposing compartment was placed 3.5 ml of isotonic pH 7.4 phosphate buffer. The access ports to the two compartments were tightly sealed and the dialysis unit was agitated (one 6 cm oscillation per sec) at 37°C for an experimentally-determined equilibration time of 16 to 17 h. The contents of each compartment were then removed and placed into separate collection vials.

Determination of the Distribution of Radiolabelled Ibuprofen Between the Equilibrated Buffer and Plasma

After equilibration a 400 µl aliquot of the plasma and buffer was placed into separate liquid scintillation vials along with 10 ml of aqueous scintillation fluid. The concentration of radioactivity (expressed as disintegrations per min (dpm) per ml of fluid counted) within the buffer (dpm·ml^{-1}-B) and plasma (dpm·ml^{-1}-P) was determined with a Packard

Model 2003 Tricarb Scintillation Spectrometer. The channels-ratio method was used for quench correction and appropriate blanks for background subtraction.

Determination of the Enantiomeric Composition of Radiolabelled Ibuprofen in Equilibrated Buffer and Plasma

A. Conversion of Radiolabelled Ibuprofen Enantiomers to Diastereomeric Amides. Another portion (2 ml) of the equilibrated buffer was placed into a 20 ml screw-topped test-tube along with 500 μl of 2 mol·l^{-1} sulphuric acid, 10 ml of 5% isopropanol in heptane, and a 100 μl aliquot of racemic non-radiolabelled ibuprofen (100 mg·l^{-1} in methanol), the latter to serve as a carrier. After vortex-mixing for 1 min and centrifugation at 1000 *g* for 5 min, the organic phase was removed, placed into a clean 20 ml screw-topped test-tube and evaporated to dryness as described above. A 100 μl aliquot of freshly prepared thionyl chloride solution (1% in dichloromethane) was added to the tube, which was then securely sealed with a PTFE-lined screw-cap. The contents were then briefly vortex-mixed and heated for 1 h in a thermostatically controlled heating block set at 70° C. The tube was then removed and left to cool at room temperature for 15 min, after which a 500 μl aliquot of a freshly prepared solution of S(−)-1-phenylethylamine (1% in dichloromethane) was added. The tube was then resealed, briefly vortex-mixed, and left to stand at room temperature for 20 min. Subsequently, 500 μl of 2 mol·l^{-1} sulphuric acid was added along with 5 ml of heptane, and the contents of the tube were vortex-mixed for 1 min and centrifuged at 1000 *g* for 5 min. Most of the organic phase was placed into a 10 ml tapered test-tube and evaporated to dryness as described above. The dried residue was reconstituted in 100 μl of 2.5% isopropanol in heptane, and after a brief vortex-mix an aliquot (50–90 μl) was injected onto a normal-phase chromatographic system.

B. Chromatographic Resolution of the Diastereomeric Amides of R(−)-Ibuprofen and S(+)-Ibuprofen. The chromatographic pump, injector, and detector used for resolving the diastereomeric amides of ibuprofen were the same as those used for the purification of the radiolabelled ibuprofen. The mobile phase (isopropanol: heptane, 2.5:97.5) was pumped through a normal-phase silica column (Hibar Lichrosorb Si60, 250 mm × 4 mm i.d., 5 μm particle size; Merck) at a constant rate of 2 ml·min^{-1}. The column effluent was monitored at a wavelength of 216 nm. With the normal-phase chromatographic conditions used, the diastereomeric amides of R(−)-ibuprofen and S(+)-ibuprofen were well resolved and had approximate retention times of 3 and 7 min respectively. The addition of carrier non-radiolabelled racemic ibuprofen in a previous step ensured visualization of peaks for the diastereomeric amides as well as minimizing potential sorptive losses of the radiolabelled compounds. The eluate fractions corresponding to the amides of R(−)-ibuprofen and S(+)-ibuprofen were collected directly into separate liquid scintillation vials, and after the addition of 10 ml of non-aqueous liquid scintillation fluid the radioactivity associated with each HPLC fraction (dpm R-B and dpm S-B, respectively) was determined.

A 200 μl aliquot of the equilibrated plasma was put through the extraction, derivatization, and chromatographic procedures along with the corresponding buffer sample. Once again eluate fractions were collected and the radioactivity associated with each diastereomeric amide (dpm R-P and dpm S-P, respectively) was determined.

Calculation of the Unbound Fraction of R(−)-Ibuprofen and S(+)-Ibuprofen

The unbound fraction of R(−)-ibuprofen ($f_{U,R}$) in the plasma sample under investigation was determined from Eq. (1).

$$f_{U,R} = \frac{\text{dpm}\cdot\text{ml}^{-1}\text{-B} \times \text{dpm R-B}/(\text{dpm R-B} + \text{dpm S-B})}{\text{dpm}\cdot\text{ml}^{-1}\text{-P} \times \text{dpm R-P}/(\text{dpm R-P} + \text{dpm S-P})} \quad (1)$$

The unbound fraction of S(+)-ibuprofen ($f_{U,S}$) in the plasma sample was determined in a similar manner from Eq. (2).

$$f_{U,S} = \frac{\text{dpm}\cdot\text{ml}^{-1}\text{-B} \times \text{dpm S-B}/(\text{dpm S-B} + \text{dpm R-B})}{\text{dpm}\cdot\text{ml}^{-1}\text{-P} \times \text{dpm S-P}/(\text{dpm S-P} + \text{dpm R-P})} \quad (2)$$

The concentrations of R(−)-ibuprofen and S(+)-ibuprofen unbound in the plasma sample ($C_{U,R}$ and $C_{U,S}$ respectively) were determined from Eqs. (3) and (4).

$$C_{U,R} = f_{U,R} \times C_R \quad (3)$$

$$C_{U,S} = f_{U,S} \times C_S \quad (4)$$

where C_R and C_S represent the predialysis plasma concentrations of R(−)- and S(+)-ibuprofen, respectively.

Reproducibility

Human plasma obtained from a healthy drug-free man was spiked with non-radiolabelled racemic ibuprofen to produce concentrations of 5.0 and 50 mg·l^{-1}. Five aliquots (3.5 ml) of each of these plasma samples were analysed in replicate to assess the reproducibility of the method.

Table 1. Derived variables for R(−)-ibuprofen and S(+)-ibuprofen in 6 subjects who had taken an 800 mg dose of racemic ibuprofen

Subject	Age	Plasma albumin	$AUC_{U,R}$	$AUC_{U,S}$	AUC_R	AUC_S	$\overline{f_{U,R}}$	$\overline{f_{U,S}}$
	(years)	($g \cdot l^{-1}$)	($mg \cdot l^{-1} \cdot min$)				$\times 100$	
1	37	53	21.5	36.1	5841	6724	0.368	0.537
2	25	46	11.2	38.0	3055	6077	0.367	0.625
3	21	48	18.7	31.5	4283	4502	0.437	0.700
4	21	48	14.5	31.5	3261	5156	0.445	0.611
5	22	48	22.6	46.9	4543	5888	0.497	0.797
6	42	48	18.8	39.4	4739	6726	0.397	0.586
Mean	28	48.5	17.9	37.2	4287	5846	0.419	0.643
SD	9.2	2.4	4.31	5.76	1025	881	0.051	0.093
Significance[a]			$p=0.0004$		$p=0.011$		$p=0.0002$	

[a] The significance of differences between the values for the R(−)- and S(+)-enantiomers was assessed by paired Student's *t*-tests

Plasma Protein Binding Studies

Stereoselective Plasma Binding of R(−)-Ibuprofen and S(+)-Ibuprofen

The method was used to investigate the plasma unbound fractions of R(−)-ibuprofen and S(+)-ibuprofen in 6 healthy men, who were participating in a pharmacokinetic study investigating the effect of concurrent cimetidine treatment on the disposition of ibuprofen enantiomers. The study was approved by the institutional ethics committee and the subjects gave informed consent. Full details of the drug interaction aspects of the study will be reported separately. The plasma binding results reported here are those obtained in the control phase of the study, in which the subjects received ibuprofen only. The ages of the subjects and their plasma albumin concentrations are shown in Table 1. Each subject took a single 800 mg oral dose of racemic ibuprofen (2 × Brufen 400-mg tablets, Boots Co, Nottingham, UK) after an overnight fast. Blood samples were withdrawn from an arm vein immediately before and at the following times after drug administration: 10, 20, 30, and 45 min, 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 8, and 10 h.

The total (bound plus unbound) concentrations of R(−)-ibuprofen and S(+)-ibuprofen in each plasma sample were determined by a stereoselective method (Evans et al., in preparation). From these data five plasma samples from each volunteer were selected on the basis that they gave an accurate representation of the overall plasma concentration-time profile. The unbound concentrations of each enantiomer in these 5 plasma samples were determined by the method described. The area under the concentration-time curve (from time zero to the time of the last of the five data points) for total R(−)-ibuprofen and total S(+)-ibuprofen (AUC_R and AUC_S, respectively) and unbound R(−)-ibuprofen and unbound S(+)-ibuprofen ($AUC_{U,R}$ and $AUC_{U,S}$, respectively) were determined by linear and logarithmic trapezoidal methods (for pre- and post-maximum plasma concentration areas respectively). For each volunteer a time-averaged unbound fraction (f_U) for each enantiomer was derived from Eqs. (5) and (6).

$$\overline{f_{U,R}} = AUC_{U,R}/AUC_R \quad (5)$$

$$\overline{f_{U,S}} = AUC_{U,S}/AUC_S \quad (6)$$

The Effect of Each Enantiomer on the Plasma Binding of the Optical Antipode

An experiment was performed to investigate the effect of each ibuprofen enantiomer on the plasma protein binding of the other, over the range of concentrations likely to be encountered either clinically or in pharmacokinetic and pharmacodynamic studies. Plasma samples containing a range of concentrations of either R(−)-ibuprofen alone (5 to 50 $mg \cdot l^{-1}$), S(+)-ibuprofen alone (5 to 50 $mg \cdot l^{-1}$), or racemic ibuprofen (10 to 100 $mg \cdot l^{-1}$) were prepared by spiking drug-free human plasma, obtained from a healthy man, with non-radiolabelled R(−)- and/or S(+)-ibuprofen. The unbound fractions of the individual enantiomers in these plasma samples were determined.

Statistical Analysis

All group data are presented as arithmetic mean with standard deviation. Two-tailed paired Student's *t*-tests were used to test for significant differences.

Results and Discussion

It has been estimated that between 10 and 15% of all drugs are racemic mixtures of two or more stereoisomers [8]. In most cases, a comprehensive understanding of the clinical pharmacology of a chiral

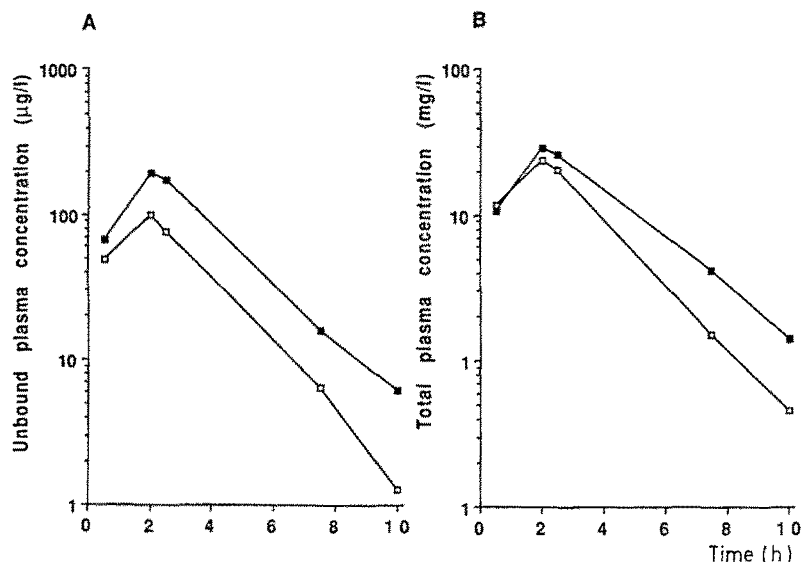

Fig. 1 A, B. Plasma concentration-time profiles of R(−)-ibuprofen (-□—□-) and S(+)-ibuprofen (-■—■-) in Subject 6. (**A**) unbound concentrations; (**B**) total concentrations

drug which is administered as its racemate requires the determination not only of the total concentrations (bound plus unbound) of the individual enantiomers in plasma, but also of their respective unbound concentrations. Cox et al. [9] recognized that the interpretation of results from hepatic clearance studies in the perfused rat liver for R(−)-ibuprofen and S(+)-ibuprofen was limited in the absence of protein binding data. Similarly, in order to fully elucidate the relationship between plasma drug concentration and drug effect for ibuprofen, and other chiral drugs administered as racemates, the unbound concentrations of the individual enantiomers must be measured. Consequently, methods are needed which permit the simultaneous determination of the plasma protein binding of drug enantiomers when present concurrently.

Development of the Method

The radiochemical purity of the ^{14}C-ibuprofen as received was determined chromatographically to be in the order of 98%. However, for drugs which are highly protein-bound, such as the 2-phenylpropionic acid derivatives, even small amounts of poorly protein-bound radiochemical impurities can lead to spurious estimates of the unbound fraction [10, 11]. In order to assess the potential for this artefact in the present work, the plasma protein binding of racemic ibuprofen was measured non-stereoselectively by radiochemical analysis with non-purified ^{14}C-ibuprofen (ie as received) and the results were compared with those obtained by HPLC analysis. Drug-free plasma obtained from a healthy man was spiked with non-radiolabelled racemic ibuprofen to produce a final concentration of 50 $mg \cdot l^{-1}$ (a sufficiently high concentration to measure the protein binding of ibuprofen non-stereoselectively by HPLC). After the addition of non-purified ^{14}-C-ibuprofen and equilibrium dialysis, the mean ($n = 3$) percentage unbound of ibuprofen, determined radiochemically, was 1.68 (0.038)%, and that determined by HPLC was 0.739 (0.055)%. The experiment was repeated with ^{14}C-ibuprofen that had been purified by the method described. On this occasion the unbound percentages, determined radiochemically and by HPLC were 0.725 (0.034)% and 0.714 (0.035)% respectively. These results confirmed the need for routine purification of the ^{14}C-ibuprofen before use, to remove low plasma-binding radiochemical impurities.

A number of workers have investigated the plasma protein binding of ibuprofen with the racemic radiolabelled form of the drug. However, in all of these studies the methods used did not discriminate between the individual enantiomers. Aarons et al. [5] added racemic radiolabelled ibuprofen to drug-free plasma obtained from healthy volunteers and found that at concentrations of 1.25 and 51.4 $mg \cdot l^{-1}$ the mean percentages unbound of ibuprofen were 1.25% and 1.64% respectively. Gallo et al. [12] investigated the kinetics of ibuprofen in the plasma and synovial fluid of arthritic patients. In their study racemic radiolabelled ibuprofen was added to the plasma and synovial fluid collected from patients who had received an oral dose of racemic ibuprofen. The percentages of ibuprofen unbound in the plasma of these patients ranged from 1.54% to 2.53%. In both of these studies the values reported are higher than those obtained in our laboratory, although in the case of the data presented by Gallo et al. [12] the

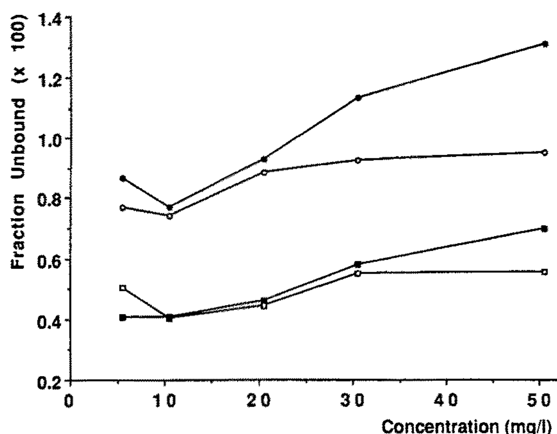

Fig. 2. Plots of the fraction unbound versus the plasma concentration of R(−)-ibuprofen both alone (-□—□-) and in the presence of an equal concentration of S(+)-ibuprofen (-■—■-); and of S(+)-ibuprofen both alone (-○—○-) and in the presence of an equal concentration of R(−)-ibuprofen (-●—●-)

higher values may have been related to the disease state. Both groups seemingly overlooked the possibility of radiochemical contamination of the radiolabelled sample. Using radiochemically pure ^{14}C-ibuprofen Lockwood et al. [13] found that the percentages unbound of unresolved ibuprofen in healthy subjects ranged from 0.419% to 0.933% after the oral administration of a range of doses of the racemate.

The concentration-dependent plasma protein binding of ibuprofen [5] was a major factor influencing the choice of the quantity of ^{14}C-ibuprofen to spike into each dialysis cell. The concentration used ($2.0\ mg \cdot l^{-1}$, i.e. $1.0\ mg \cdot l^{-1}$ of each enantiomer) was sufficient for assay sensitivity and represented a small percentage increase in the concentration of each enantiomer at the level at which concentration-dependent protein binding becomes important (above $10–15\ mg \cdot l^{-1}$ for each enantiomer: Fig. 2 and unpublished results).

Equilibrium dialysis is typically associated with a net movement of fluid from the buffer compartment to the plasma compartment. The resultant dilution of the plasma can influence the estimated unbound fraction of the ligand under investigation [14]. In some cases variable volume shifts of up to 80% have been reported [15]. With our apparatus and method the net movement of fluid was minimal (8.9 (3.1)%, $n=20$). Hence, since routine correction of unbound fraction for fluid shift involves additional measurements for individual dialysis cells, which themselves are a potential source of error, no correction factors were applied [14].

Because enantiomers have identical physicochemical properties, their resolution requires the involvement of discriminatory diastereomeric relationships. In the present case, the enantiomers of ibuprofen were derivatized with S(−)-1-phenylethylamine to form diastereomeric amides (Evans et al., in preparation). When racemic radiolabelled ibuprofen was derivatized and chromatographed on the normal-phase system, the ratio of the radioactivity associated with each collected HPLC eluate fraction was 1.00 (0.028); ($n=6$). This result confirms that the derivatization conditions gave rise to equal quantities of each diastereomer, which is imperative if Eqs. (1) and (2) are to be valid. After injection onto the normal-phase column of a derivatized sample of racemic radiolabelled ibuprofen there was no evidence of late eluting radioactive material.

Although there was a difference between the enantiomers in the percentage unbound in plasma (see below), since the binding of each was extensive, there was negligible loss of each enantiomer to the buffer compartment during dialysis. As a result the post-dialysis plasma concentrations of radiolabelled R(−)-ibuprofen and S(+)-ibuprofen were virtually identical. For this reason it was standard practice, when a large number of samples were being processed, to determine dpm R-P and dpm S-P for a selection of the post-dialysis plasma samples only. The mean ($n=24$) ratio of dpm R-P to dpm S-P returned by these samples was 1.008 (0.020). The relationships [dpm R-P/(dpm R-P + dpm S-P)] and [dpm S-P/(dpm S-P + dpm R-P)] therefore become 0.50 and Eqs. (1) and (2) are simplified accordingly.

The reproducibility of the method for determining the unbound fractions of ibuprofen enantiomers in human plasma was assessed. At a racemic ibuprofen concentration of $5.0\ mg \cdot l^{-1}$ the mean percentage unbound ($n=5$) of R(−)-ibuprofen was 0.392 (0.044)% and that of S(+)-ibuprofen was 0.600 (0.070)%. At $50\ mg \cdot l^{-1}$ the unbound percentages ($n=5$) were 0.595 (0.079)% and 0.877 (0.078)% respectively. Thus, although several steps are involved in the performance of the method, the overall reproducibility is good.

Applications of the Method

We used these methods to investigate the plasma concentration-time course of R(−)- and S(+)-ibuprofen in 6 healthy male volunteers who had taken a single oral dose of racemic ibuprofen (800 mg). The plasma concentration-time profiles of unbound R(−)-ibuprofen and unbound S(+)-ibuprofen in a representative volunteer are presented in Fig. 1 A. The corresponding total plasma concentration-time profiles of each enantiomer are presented in Fig. 1 B.

The derived values, AUC_R, AUC_S, $AUC_{U,R}$, $AUC_{U,S}$, $\overline{f_{U,R}}$ and $\overline{f_{U,S}}$ for each volunteer are presented in Table 1. The unbound fractions are expressed as time-averaged values, because the plasma binding of the enantiomers is concentration-dependent (see below). In all subjects $\overline{f_{U,R}}$ was less than $\overline{f_{U,S}}$. The time-averaged unbound fractions of the two enantiomers were closely related ($r = 0.841$, $p < 0.05$).

Because the plasma protein binding of ibuprofen is concentration dependent it might be argued that the higher time-averaged unbound fraction of S(+)-ibuprofen is due to the fact that after oral administration the plasma concentrations of this enantiomer usually exceed those of R(−)- ibuprofen, especially in post-peak samples. The total plasma concentration of R(−)-ibuprofen was greater than that of S(+)-ibuprofen in ten of the thirty plasma samples analyzed. However, in all of these plasma samples the unbound fraction of S(+)-ibuprofen was greater than that of R(−)-ibuprofen. The mean percentage unbound of the R(−)-enantiomer, 0.450 (0.060)%, was significantly less ($p = 0.0001$) than that of the S(+)-enantiomer, 0.774 (0.20)%. These data clearly indicate that the binding difference between the enantiomers was not merely a reflection of total concentration differences.

To investigate the effect of each enantiomer on the plasma protein binding of the other an in-vitro experiment was performed with drug-free plasma obtained from a healthy volunteer. Fig. 2 shows the unbound fraction of each ibuprofen enantiomer over the concentration range 5 to 50 $mg \cdot l^{-1}$ in the absence and in the presence of an equal concentration of the optical antipode. A number of observations can be made. Firstly, over the range of concentrations examined the percentage unbound of R(−)-ibuprofen was less than that of S(+)-ibuprofen, irrespective of whether the two enantiomers were alone or in the presence of each other. This first observation is consistent with the results from the disposition study and the reproducibility data reported above. Secondly, in keeping with earlier reports on the plasma binding of unresolved ibuprofen [5, 13], the plasma protein binding of each enantiomer demonstrated concentration-dependence. Further, it can be seen that the presence of one enantiomer influenced the plasma protein binding of the other. For example, the percentage unbound of the active enantiomer, S(+)-ibuprofen, at a concentration of 50 $mg \cdot l^{-1}$, was 0.905% in the absence of R(−)-ibuprofen, whereas in the presence of an equal concentration of R(−)-ibuprofen the percentage unbound was 1.11%. Recently Lee et al. [3] studied the stereoselective disposition of ibuprofen enantiomers in man and found that after the administration of the racemate both enantiomers had AUCs for total plasma concentrations which were lower than those predicted from the separate administration of the individual enantiomers. To explain this phenomenon Lee et al. [3] proposed an enantiomer-enantiomer interaction at the plasma protein binding level. The data presented in Fig. 2 add support to such a proposal.

Stereoselective plasma protein binding has been reported for a number of drugs, including warfarin [16], chloroquine [17], and verapamil [18]. The results of the present investigation show that in healthy individuals the plasma protein binding of ibuprofen also is stereoselective. After the administration of the racemate the absolute difference between the enantiomers in the percentage bound in plasma is minor (approximately 99.4% bound for S(+)-ibuprofen versus 99.6% bound for R(−)-ibuprofen). However, the percentage unbound of the pharmacologically active enantiomer, S(+)-ibuprofen, is on average 53.6 (12.1)% greater than that of its optical antipode. Jones et al. [7] have reported a qualitatively similar stereoselectivity in the protein binding of R(−)- and S(+)-2-phenylpropionic acid in rabbit plasma. Rendic et al. [19] have studied the binding of the individual enantiomers of ketoprofen to human serum albumin by the gel permeation filtration technique. Although the binding of the enantiomers in the presence of one another could not be studied, this group found R(−)-ketoprofen to be more highly bound to human serum albumin than S(+)-ketoprofen. Knadler et al. [20] have reported briefly (in abstract) the results of an investigation on the plasma binding of R(−)- and S(+)-flurbiprofen. In contrast to ibuprofen, ketoprofen, and 2-phenylpropionic acid, the mean percent unbound of R(−)-flurbiprofen (0.082) was greater than that of S(+)-flurbiprofen (0.048) over the concentration range of 1 to 26 $mg \cdot l^{-1}$.

Ibuprofen is bound with high affinity to binding site II (the benzodiazepine site) on the human serum albumin molecule [21]. This site has been shown previously to exhibit a high degree of stereoselectivity in its binding of the enantiomers of dl-tryptophan [22] and oxazepam hemisuccinate [22, 23]. Data presented here suggest that the benzodiazepine site may also exhibit stereoselectivity in its binding of ibuprofen enantiomers, although the extent of such stereoselectivity remains to be fully determined.

In summary, a novel approach has been described for simultaneously determining the protein binding of ibuprofen enantiomers in plasma. The approach outlined could be adapted to examine the stereoselective plasma protein binding of other chiral drugs which are administered as racemates. It

has been demonstrated that the binding of ibuprofen enantiomers to plasma proteins is concentration-dependent and stereoselective, R(−)-ibuprofen binding to a greater extent than S(+)-ibuprofen over the clinically encountered concentration range. Furthermore, at a given concentration the plasma protein binding of each enantiomer appears to be influenced by the presence of the other. Consequently, pharmacokinetic and concentration-effect studies involving ibuprofen must consider not only the total plasma concentrations of the individual enantiomers but also their unbound concentrations.

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