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Marked Enantioselective Protein Binding in Humans of Ketorolac In Vitro: Elucidation of Enantiomer Unbound Fractions Following Facile Synthesis and Direct Chiral HPLC Resolution of Tritium-Labelled Ketorolac

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ABSTRACT The protein binding of the enantiomers of the nonopiate analgesic, ketorolac, was investigated in vitro using human plasma and solutions of human serum albumin (HSA) at physiological pH and temperature. In order to detect the very low levels of unbound enantiomers in protein solutions, tritium-labelled *rac*-ketorolac was synthesised by regiospecific isotopic exchange of the parent drug with tritiated water as the isotope donor. Radiochemical purification of this compound by reversed-phase HPLC followed by direct resolution using a chiral α_1-acid glycoprotein (Chiral-AGP) HPLC column afforded labelled enantiomers of high specific activity. The in vitro use of (R)- and (S)-[$^3H_4$]ketorolac enabled reproducible radiometric detection of enantiomers in protein solution ultrafiltrate. The unbound fractions of (R)- and (S)-ketorolac [*fu*(R) and *fu*(S), respectively] were determined when drug was added to various plasma or albumin solutions as either the separate enantiomers or as the racemate. Over an enantiomeric plasma concentration range of 2.0–15.0 μg/ml, *fu*(S) (mean range: 1.572–1.795%) was more than 2-fold greater ($P < 0.001$) than *fu*(R) (mean range: 0.565–0.674%). Both *fu*(R) and *fu*(S) were constant over this concentration range, and each was unaffected by the presence of the corresponding antipode ($P > 0.05$). At a concentration of 2.0 μg/ml in 40.0 g/liter fatty acid-free HSA, *fu*(R) and *fu*(S) were approximately 0.5 and 1.1%, respectively, and both values declined with increasing concentrations of the long chain fatty acid, oleic acid. We have previously shown that the pharmacokinetics of ketorolac in humans are markedly enantioselective and suggest in this report that these differences are largely the result of substantial differences in the protein binding of ketorolac enantiomers. These findings stress the importance of monitoring the unbound concentrations of the enantiomers of chiral drugs if correct interpretations are to be made of enantioselective pharmacokinetic data.

KEY WORDS: ketorolac, nonsteroidal antiinflammatory drugs, chiral drugs, enantiomers, protein binding, human serum albumin, enantioselectivity, pharmacokinetics, fatty acids, oleic acid

Ketorolac is a nonopiate agent with potent analgesic and moderate antiinflammatory activity[1] and is marketed for clinical use as a racemate. It is believed to exert its pharmacological effects via inhibition of prostaglandin synthesis peripheral to the central nervous system in a highly enantioselective manner.[2] The (S)-enantiomer has been shown in animal studies to be responsible for most of the activity of ketorolac.[2] While no concentration-effect data currently exist for the separate enantiomers of ketorolac in humans, based on in vitro pharmacodynamic data for pharmacologically related 2-arylpropionic acid nonsteroidal antiinflammatory drug (NSAID) enantiomers,[3] high eudismic ratios are likely to occur in humans for the prostaglandin-dependent actions of ketorolac. Consequently, the concentrations of individual ketorolac enantiomers should be monitored if meaningful pharmacological data are to be obtained.

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In common with other NSAIDs, ketorolac has been characterised in humans (in terms of unresolved drug) as a restrictively cleared, low hepatic extraction ratio drug which is highly bound to plasma protein.[4–6] Thus the total (bound plus unbound) plasma clearance of ketorolac will be dependent on the unbound fraction in plasma.[7] Accordingly, in a previous communication we were unable to ascribe mechanisms for the observed enantioselective pharmacokinetics of parenterally administered drug in humans in the absence of protein binding data for the individual isomers.[8] The 2-fold higher total clearance of (S)-ketorolac relative to its antipode observed in humans[8] could equally well be due to differences in the unbound fraction as intrinsic unbound clearance of the enantiomers.

Enantioselective protein binding of chiral NSAIDs in humans has been observed for a number of congeners although recorded differences between enantiomeric unbound fractions in plasma or human serum albumin (HSA) solutions are typically small. Examples of such compounds showing differences include flurbiprofen,[9] ketoprofen,[10,11] ibuprofen,[12] and etodolac.[13] In order to examine the in vitro protein binding of (R)- and (S)-ketorolac, we developed a simple method for the production of tritium-labelled racemic drug which was subsequently purified radiochemically and resolved directly by achiral and chiral high-performance liquid chromatography (HPLC), respectively. Various aspects of the protein binding of the isomers were examined including linearity, the influence of each enantiomer on the binding of the corresponding antipode, and the effect of increasing concentrations of oleic acid. We observed highly enantioselective in vitro protein binding of ketorolac and suggest that these differences largely account for the enantioselective disposition (in terms of total drug) which we have observed previously in humans dosed with *rac*-ketorolac.[8]

MATERIALS AND METHODS

Materials

Racemic ketorolac free acid (unlabelled) was a gift from Syntex Research (Palo Alto, CA). Separate unlabelled enantiomers of ketorolac [enantiomeric excess (ee) > 96% for each] were obtained by direct resolution of racemic drug using the chiral HPLC system detailed below for the chromatographic enantioseparation of tritium-labelled racemic drug. HPLC-grade solvents were obtained from BDH Chemicals (Poole, England); oleic acid (sodium salt, 99% pure by GC), *N,N*-dimethyloctylamine, and essentially fatty acid-free HSA (cat. #A-1887) were obtained from Sigma Chemical Co. (St. Louis, MO). Tritium-labelled water (5.0 Ci/ml) and palladium black, both used for the synthesis of *rac*-[$^3H_4$]ketorolac (vide infra), were procured from Du Pont (Melbourne, Australia) and Aldrich (Milwaukee, WI), respectively.

Instruments

Chiral and achiral HPLC were performed at ambient temperature using a Waters and Associates system (Milford, MA) incorporating a Model 510 pump, Model 712 Wisp™ autoinjector, Model 490 variable wavelength UV-absorbance detector and Millennium™ 2010 Chromatography Manager. HPLC-eluate fraction collection was achieved with a Gilson Model 202 instrument (Villiers le Bel, France). Achiral reversed-phase chromatography was carried out with a radially compressed phenyl bonded phase cartridge (4 μm particle size, 100 × 8 mm i.d., Nova-Pak™, Waters Assoc.) and direct chromatographic separation of ketorolac enantiomers was performed with a Chiral-AGP HPLC column (100 × 4 mm i.d., Chromtech, Norsborg, Sweden).

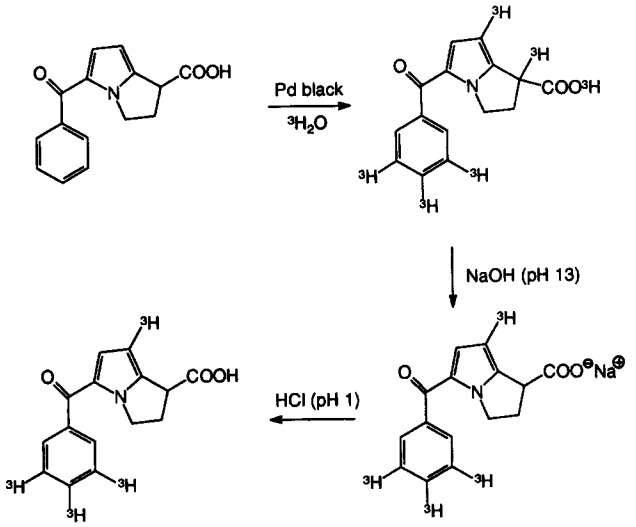

Fig. 1. Scheme for the synthesis of *rac*-[$^3H_4$]ketorolac (*rac*-5-[3′,4′,5′-$^3H_3$]benzoyl-[7-^3H]-1,2-dihydro-3*H*-pyrrolo[1,2*a*]pyrrole-1-carboxylic acid).

Synthesis of rac-[$^3H_4$]Ketorolac

The scheme is depicted in Figure 1. Palladium black (15 mg) was weighed into a 2-ml, screw cap Wheaton vial to which was added *rac*-ketorolac (55 mg). Tritiated water (0.2 ml, 1.0 Ci) was immediately added and the vial contents vortex-mixed prior to deaeration with a vacuum (−40 kPa). The vial was purged with nitrogen, firmly capped, and then immersed in a preheated oil bath at 150°C for 20 h. After this time the vial was cooled to ambient temperature and the contents broken up with a fine spatula before transfer into a glass separatory funnel using 1.0 *M* sodium hydroxide (10 ml). This solution was left to stand for 15 min after which it was washed with dichloromethane (3 × 5 ml) and the basic (pH 13) aqueous layer acidified to pH 1–2 with 35% (w/w) hydrochloric acid. The acidified solution was left to stand for a further 15 min and then extracted with dichloromethane (3 × 5 ml). The dichloromethane extracts were combined and reextracted with 1.0 *M* sodium hydroxide (3 × 5 ml). The basic layers were combined, left to stand for 30 min and acidified to pH 0.5 as before. The acidic solution was extracted with dichloromethane (3 × 5 ml), the organic extracts combined and dried with anhydrous magnesium sulphate. The solution was filtered and the solvent removed (45°C) under a purified nitrogen stream (TurboVap™ evaporator, Zymark, Hopkinton, MA) to give 10 mg of *rac*-[$^3H_4$]ketorolac {*rac*-5-[3′,4′,5′-$^3H_3$]-benzoyl-[7-^3H]-1,2-dihydro-3*H*-pyrrolo[1,2*a*]pyrrole-1-carboxylic acid} of specific activity 3.0 Ci/mmol as an off-white solid.

The synthetic procedure and the elucidation of the tritium location were performed in the first instance with deuterium

oxide exchange and high-field ^{1}H-NMR studies. A sample of ketorolac (10 mg) was heated at 150°C for 18.5 h with Pd black (3 mg) and 0.2 ml of D_2O (Aldrich). As compared with the ^{1}H-NMR spectrum of authentic ketorolac,[4] the ^{1}H-NMR spectrum of the isolated product showed approximately 60% deuterium incorporation at the *meta* and *para* positions of the benzoyl moiety and approximately 100% deuterium incorporation at the C7 pyrrole position. The C1 position, which is alpha to the carboxyl group, was also completely deuterated. Some minor exchange at the C6 pyrrole position was also apparent. Given the lability of deuterium at C1, it was removed by treating the sample with base to pH 12 and acidifying to pH 1. The ^{1}H-NMR spectrum of the material obtained after this procedure showed that the C7 position was still completely deuterated and each of the *meta* and *para* positions of the benzoyl group were ~60% deuterated. The C1 positon was completely reprotonated. At the higher concentrations, as used for the introduction of tritium, almost complete exchange of the *meta* and *para* protons and an increase in the deuteration of the C6 position occurred.

Radiochemical Purification and Resolution of rac-[$^3H_4$]Ketorolac

Individual batches of tritiated racemic ketorolac were first purified by reversed-phase HPLC by injecting 10-μg aliquots dissolved in mobile phase (40% acetonitrile in 20 m*M* sodium acetate buffer; pH 3.0) onto the achiral column detailed above (operated at ambient temperature). The mobile phase was pumped at 1.5 ml/min and unresolved ketorolac eluted at circa 6.5 min. Corresponding eluate fractions (eluted 20 min apart) were collected in separate glass culture tubes, 0.5 ml of 2.0 *M* sulphuric acid and 5 ml of 20% ethyl acetate in *n*-hexane were added to each, and the tubes vortex-mixed prior to centrifugation (2000*g*, 10 min). The organic layers were pooled and evaporated to dryness at 45°C under a nitrogen stream.

This purified *rac*-[$^3H_4$]ketorolac (typically 100 μg per batch) was initially dissolved in 200 μl of methanol followed by 4.0 ml of mobile phase used for the direct chromatographic resolution of ketorolac (2.0% propan-2-ol, 1.0 m*M* *N,N*-dimethyloctylamine in 10 m*M* phosphate buffer; pH 6.5). Aliquots (200 μl) were injected onto the Chiral-AGP column (vide supra) operated at ambient temperature and eluted at a flow rate of 0.8 ml/min. (R)-[$^3H_4$]- and (S)-[$^3H_4$]Ketorolac eluted as baseline resolved peaks at 2.8 and 4.8 min, respectively. Corresponding enantiomeric eluate fractions were collected separately with 20 min between injections. Tritiated ketorolac enantiomers were extracted from mobile phase using the same method as described above for the purification of racemic drug. Following evaporation of the organic solvent, each enantiomer residue was dissolved in 1.0 ml of acetonitrile and this solution was then analysed using the chiral HPLC assay. The efficiency of the combined resolution, fractionation, and extraction steps was approximately 80%. The tritiated enantiomer solutions (ee > 96%) were stored at −20°C and used for protein binding experiments within 48 h of purification and resolution.

Protein Binding Experiments

The in vitro protein binding of ketorolac enantiomers was determined in plasma harvested in the morning from each of six healthy nonfasting volunteers, none of whom was taking any medication. The mean (± SD) age and plasma albumin concentration were 36.7 ± 11.0 years and 39.7 ± 3.1 g/liter, respectively. All subjects had plasma electrolyte concentrations and liver and kidney functions within normal ranges. Plasma was obtained by centrifugation (2000*g*, 10 min) of heparinized blood sampled via venepuncture of an arm vein and was used for determining the binding of keterolac enantiomers within 2 h of collection.

Aliquots (1.0 ml) of plasma adjusted to 37°C and pH 7.4 with 2.5% (v/v) orthophosphoric acid were added to glass culture tubes containing (R)-ketorolac alone, (S)-ketorolac alone, and *rac*-ketorolac over an enantiomeric concentration range (labelled plus unlabelled compound) of 2.0–15.0 μg/ml (4.0–30.0 μg/ml of racemic drug in the last case). A constant 2.0 μg/ml of either tritiated enantiomer was present to enable radiometric detection of unbound moieties. In the case of binding experiments conducted with *rac*-ketorolac, each labelled enantiomer was spiked separately into plasma to permit measurement of each unbound ketorolac enantiomer. Sufficient nonradiolabelled enantiomeric and/or racemic ketorolac was added as appropriate volumes of methanolic solutions of the free acids and together with the added labelled isomers was evaporated to dryness under a nitrogen stream prior to the addition of plasma. Samples were gently mixed and incubated for 30 min at 37°C in an oscillating water bath (20 Hz). Subsequently, 50 μl was removed for determining total (bound plus unbound) dpm prior to loading the remaining plasma volume into a Centrifree® ultrafiltration device (Amicon, Beverly, MA) and centrifugation (2000*g*, 30 min, 37°C) in a prewarmed Model Suprafuge 22 equipped with a HFA 20.16 fixed-angle rotor (Osterode, Germany). Two hundred and fifty microliters of the ultrafiltrate was counted for determining unbound dpm. The unbound fraction (*fu*) in plasma for each ketorolac enantiomer was calculated as *fu* = (unbound dpm/ml) ÷ (total dpm/ml). Liquid scintillation counting of samples was carried out after the addition of 10 ml of Formula 989 scintillant (Du Pont, Melbourne, Australia) with quench correction performed using the external standard method.

The protein binding of ketorolac enantiomers was also determined in 40.0 g/liter HSA (essentially fatty acid-free fraction V) in 0.067 *M* phosphate buffer (37°C, pH 7.4) by an identical protocol as that described above for human plasma. Further, the influence of oleic acid on the unbound fraction of (R)- and (S)-ketorolac (present either alone or together) in 40.0 g/liter HSA was investigated. Sodium oleate was added (2.0 m*M*) with constant stirring to a 40.0 g/liter solution of fatty acid-free HSA at 37°C and subsequently diluted with appropriate volumes of fatty acid-free HSA to give a final oleic acid concentration range of 0–2.0 m*M*. A single ketorolac enantiomer concentration (2.0 μg/ml) was used with these experiments.

Statistical Analysis

Data are presented as the arithmetic mean ± SD. Comparison of *fu* for each enantiomer, the influence of the corresponding antipode, and increasing plasma concentration on *fu* were evaluated using analysis of variance. Multiple comparison procedures were performed using the Tukey–Kramer

post test (Instat 2.0, GraphPad Software Inc., San Diego, CA).

RESULTS AND DISCUSSION

Performance of the Method

As discussed in a previous communication,[14] when radiolabelled ligands are used for assessing the protein binding of xenobiotics these ligands must be free of radiochemical impurities. An initial purification of *rac*-[$^3H_4$]ketorolac was performed using reversed-phase achiral HPLC (Fig. 2a). This material was subsequently resolved directly by a Chiral-AGP stationary phase. To verify that the resulting labelled enantiomers were free of radiochemical impurities both (R)- and (S)-[$^3H_4$]ketorolac were separately reinjected onto the chiral HPLC system and timed eluate fractions were collected and counted for radioactivity. There were no detectable radiometric signals except for authentic labelled enantiomers of ketorolac (Fig. 2b).

The reproducibility of the method for determining the in vitro protein binding of (R)- and (S)-ketorolac was assessed by separately spiking the plasma from one subject with 2.0 μg/ml of purified tritium-labelled enantiomers. The mean (± SD) fraction unbound (expressed as a percentage) for eight replicate determinations of unbound (R)-ketorolac in plasma was 0.458 (± 0.030)%. The corresponding value (n = 8) for unbound (S)-ketorolac was 1.163 (± 0.036)%. Preliminary recovery experiments, conducted with protein-free solutions, confirmed that there was negligible sorption of ketorolac enantiomers onto the ultrafiltration membrane. Based on (1) our earlier observations of identical ketoprofen protein binding in both serum[15] and heparinized plasma,[14] and (2) an extensive examination of drug protein binding which showed a lack of effect of heparin on unbound fraction determinations,[16] it was assumed that using heparinized tubes for blood collection did not modify the protein binding of ketorolac isomers.

Initially, we attempted to synthesize ^{14}C-labelled *rac*-ketorolac using the patented method described for the commercial production of racemic drug.[17] Acylation was attempted with [7-^{14}C]-*N*,*N*-dimethylbenzamide prepared from [7-^{14}C]benzoic acid. However, for reasons which are yet to be elucidated, only minute amounts of recognisable material were obtained. We confirmed that the reaction proceeded as previously described[17] when unlabelled reactant was used. To obtain a sample of labelled ketorolac, attention was therefore focused on developing a method for the catalytic tritiation of commercial *rac*-ketorolac. Catalytic exchange with tritiated water using a noble metal catalyst and exchange with tritiated water in the presence of a strong electrophile, such as trifluoroacetic acid, are the most common methods of exchange. Syntheses were conducted initially using deuterated water since in this way both the site and extent of deuteration, and ultimately of tritiation, could be followed by conventional ^{1}H-NMR (^{3}H-NMR facilities were not available to us). Using D_2O and the optimised synthetic conditions described above and depicted in Figure 1, nearly complete deuterium incorporation occurred at each of the *meta* and *para* positions of the benzoyl moiety and 100% deuterium incorporation at the C7 pyrrole position. Both the carboxylate group and the C1 group were completely deuterated after the exchange reaction. Complete reprotonation occurred at these two labile positions following the successive alkali and acid wash steps.

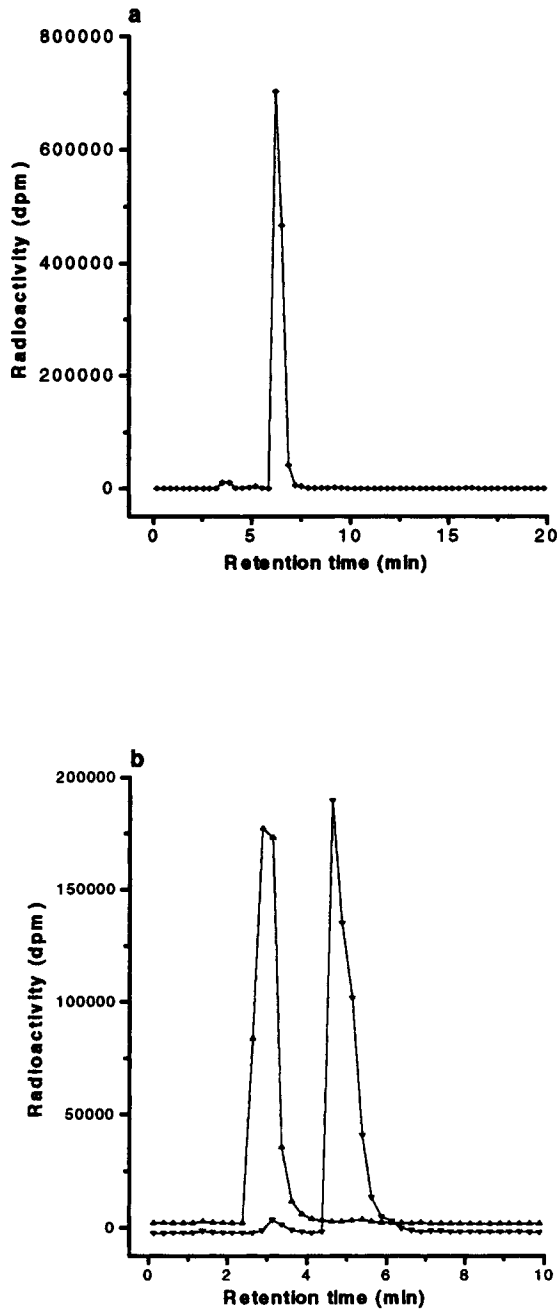

Fig. 2. Radiochromatograms depicting (a) achiral HPLC of 1.8 μg of crude *rac*-[$^3H_4$]ketorolac (♦), and (b) Chiral-AGP HPLC following separate injection of 1.5 μg of purified and resolved (R)-[$^3H_4$]ketorolac (▲) and (S)-[$^3H_4$]ketorolac (▼) (data are vertically displaced for graphic clarity). Twenty and 15 sec eluate fractions were collected and liquid scintillation counting performed to construct (a) and (b), respectively.

Binding of Ketorolac Enantiomers

The fractions unbound of (R)- and (S)-ketorolac in vitro in plasma at physiological pH and temperature and harvested

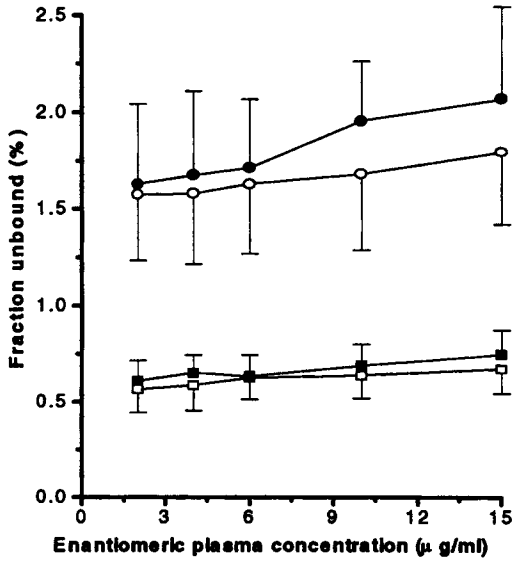

Fig. 3. The mean (SD bars) fraction unbound of (S)-ketorolac (●, ○) and (R)-ketorolac (■, □) in vitro in plasma at 37°C and pH 7.4 obtained from six healthy volunteers spiked with separate enantiomers (open symbols) and racemate (closed symbols).

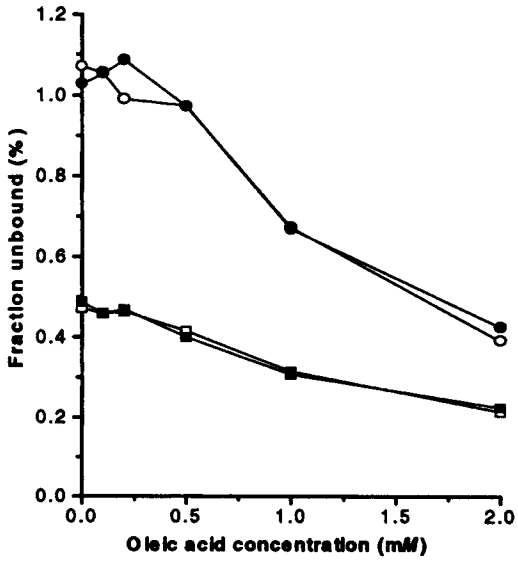

Fig. 4. The influence of varying concentrations of oleic acid on the fraction unbound of 2.0 μg/ml of (S)-ketorolac. (●, ○) and 2.0 μg/ml of (R)-ketorolac (■, □) in 40.0 g/liter HSA. Open symbols refer to ketorolac added as separate enantiomers and closed symbols to drug added as the racemate.

TABLE 1. The fractions unbound (expressed as percentages) of (R)- and (S)-ketorolac in fatty acid-free HSA (40.0 g/liter, pH 7.4, 37°C) when drug was added either as separate enantiomers or as the racemate

Ketorolac enantiomer concentration (μg/ml)	Enantiomers present alone		Enantiomers together (as racemate)	
	fu(R) (%)	*fu*(S) (%)	*fu*(R) (%)	*fu*(S) (%)
2.0	0.484	1.061	0.493	1.060
4.0	0.515	1.073	0.488	1.013
6.0	0.514	1.134	0.515	1.066
10.0	0.564	1.100	0.524	1.118
15.0	0.599	1.067	0.576	1.188

from healthy volunteers is depicted in Figure 3. At all enantiomeric plasma concentrations of ketorolac, the unbound fraction of (S)-ketorolac was more than 2-fold greater than the unbound fraction of (R)-ketorolac when drug was spiked into plasma as either separate enantiomers or as racemate ($P < 0.001$). There was no difference ($P > 0.05$) between unbound fractions for each enantiomer when drug was present in plasma as the pure enantiomer compared to drug present as the racemate. This indicated that over the concentration range examined the unbound fraction of each enantiomer was unaffected by the corresponding optical antipode (Fig. 3). Furthermore, the unbound fraction of each enantiomer was not different ($P > 0.05$) across an enantiomeric plasma concentration range of 2.0–15.0 μg/ml.

Table 1 lists the fractions unbound of (R)- and (S)-ketorolac in fatty acid-free HSA (40 g/liter), present both alone and as racemic drug over a drug concentration range of 2.0–15.0 μg/ml (expressed in terms of enantiomeric drug). The influence of increasing concentrations of oleic acid on the unbound fraction of each ketorolac enantiomer in 40.0 g/liter HSA at physiological pH and temperature is illustrated in Figure 4. At an oleic acid concentration of 2.0 m*M* the unbound fractions of (R)- and (S)-ketorolac in albumin solutions spiked with a constant 2.0 μg/ml of pure enantiomers were approximately half that observed in fatty acid-free HSA (Fig. 4).

This report describes for the first time the protein binding of the separate enantiomers of ketorolac. To our knowledge, a single in vitro study has described the protein binding of ketorolac in terms of unresolved drug.[4] This previous study reported an unbound fraction in human plasma of 0.8% (determined following equilibrium dialysis of plasma spiked with ^{14}C-labelled *rac*-ketorolac) which compares with ~1.0% extrapolated from our data for unresolved drug. Consistent with our findings, Mroszczak and co-workers[4] reported that the unbound fraction of (unresolved) ketorolac in human plasma was constant over a racemic drug concentration range of 0.50–10.0 μg/ml. In the absence of an analytical method capable of measuring the very low concentrations of nonradiolabelled drug in plasma ultrafiltrate, we were unable to verify that the binding of tritiated enantiomers was identical to that of unlabelled isomers. However, to our knowledge, there have been no documented examples of modification of drug binding due to an isotope effect. Moreover, given the similarity between our estimate of unresolved ketorolac protein binding using tritiated drug and that provided with ^{14}C-labelled drug,[4] we envisage that the binding characteristics of ketorolac should not be modified due to the incorporation of radiolabelled atoms.

It would have been desirable to have examined the protein binding of ketorolac isomers using in vivo methods, however, this was not possible for two main reasons. First, we wished

to examine enantiomer protein binding when the enantiomers were present alone in plasma and when the enantiomers were present together in the one sample to investigate possible interactions between (R)- and (S)-ketorolac for plasma protein binding sites. Since pure enantiomers of ketorolac are not currently available for administration to humans, ex vivo plasma samples containing a single enantiomer are unobtainable. Furthermore, the minimum plasma concentration of the tritiated ligand required for the detection of the (R)-unbound species was 2.0 μg/ml. If added to an ex vivo plasma aliquot this would significantly elevate the total isomer concentration over values typically seen after therapeutic dosing with ketorolac.[8] In light of the negligible plasma concentrations of metabolites of ketorolac in healthy volunteers dosed with this drug,[4] the use of metabolite-free plasma for estimating enantiomer protein binding in vivo would appear to be justified.

The highly significant differences reported previously for the plasma clearance and steady-state volume of distribution of ketorolac enantiomers in healthy volunteers dosed with im *rac*-ketorolac[8] were described in terms of total (bound plus unbound) isomers. Since ketorolac has a low hepatic extraction ratio, is cleared almost exclusively by metabolism and the systemic bioavailability of im drug is complete,[6] the AUC (bound plus unbound) after a single im dose will be inversely related to both (1) the unbound fraction in plasma, and (2) intrinsic metabolic clearance.[7] Accordingly, it was not possible previously[8] to attribute enantiomeric differences in ketorolac's disposition to enantioselective protein binding and/or intrinsic clearance. Extrapolating the in vitro plasma protein binding data herein, it would appear that the circa 2-fold higher total plasma clearance of (S)-ketorolac[8] was largely the result of a difference between the unbound fraction of (S)-ketorolac compared to that of its antipode. While the peak plasma concentrations of ketorolac enantiomers achieved in humans dosed with 30 mg of *rac*-ketorolac tromethamine approximate the lower end of the enantiomer spike concentrations used in this in vitro study, the unbound fraction of each enantiomer in plasma is linear from 2.0 to 15.0 μg/ml (Fig. 3). Consequently, the unbound fractions of (R)- and (S)-ketorolac in plasma determined at these higher total isomeric concentrations in vitro would not be expected to differ from corresponding ex vivo values at therapeutic drug concentrations.

Recently, apparent (S)- to (R)-ketorolac interconversion was reported (in abstract form) in healthy volunteers dosed with separate enantiomers of the drug.[18] Interestingly, there was no recorded (R)- to (S)-chiral inversion, a metabolic biotransformation common to many 2-arylpropionate NSAID congeners. The minimal extent of this reported (S)- to (R)-ketorolac conversion [7.6% of the dose of the (S)-enantiomer[18]], would not be expected to impact significantly on either the derivation of enantiomeric pharmacokinetic parameters following racemate dosing[8] or on our mechanistic interpretation of these data in terms of ketorolac's enantioselective plasma protein binding (vide supra).

Similar to our observations with human plasma solutions, we observed enantioselective protein binding of ketorolac in HSA solutions at physiological concentrations with an approximately 2-fold higher unbound fraction for the (S)-enantiomer (Fig. 4 and Table 1). This suggests that the principal source of enantioselectivity in the plasma binding of ketorolac is albumin, which is consistent with generally held conceptions about the protein binding of acidic ligands.[19] The magnitude of enantiomeric *fu* values of ketorolac were similar in solutions of physiological concentrations of HSA compared to plasma. By comparison, it has been suggested by us and others[10,14] that the protein binding of ketoprofen enantiomers is dependent on the protein source. Indeed, Dubois et al.[10] observed opposite enantioselectivity in the binding of ketoprofen to plasma protein compared to albumin.

Long-chain fatty acids have been shown to modify allosterically the binding to albumin of a number of drugs,[20] including the enantiomers of the progenitive 2-arylpropionate NSAID, 2-phenylpropionic acid.[21] When we examined the influence of increasing concentrations of oleic acid on ketorolac unbound fractions in albumin solutions, enhanced protein binding of both enantiomers was observed (Fig. 4). Moreover, there was no evidence of a change in binding enantioselectivity in the presence of increasing oleic acid concentration; the *fu*(S) to *fu*(R) ratio remained the same (Fig. 4). The maximal effect on ketorolac isomer binding was observed at the uppermost oleic acid concentration used where the oleic acid:albumin molar ratio was approximately 3.3:1. Similarly, Wanwimolruk et al.[22] have shown that an increasing endogenous fatty acid concentration was associated with an increase in the plasma protein binding of both indomethacin and warfarin. While we took no specific precautions to prevent release of fatty acids in vitro during the plasma protein binding experiments, harvesting of plasma and determination of *fu*(R) and *fu*(S) in each volunteer were conducted under identical conditions and completed within 2 h of blood collection. Experiments where oleic acid was added to fatty acid-free albumin solutions, while not necessarily an accurate representation of the situation in vivo, clearly showed that fluctuations in fatty acid concentration altered the protein binding of both (R)- and (S)-ketorolac.

In summary, the protein binding of ketorolac is highly enantioselective and in light of these differences caution is needed when interpreting enantioselective pharmacokinetic data expressed in terms of total (bound plus unbound) isomer concentrations. From these in vitro studies, enantioselectivity in the unbound fraction of ketorolac in plasma could largely explain the differences in the clinical pharmacokinetics of (R)- and (S)-ketorolac observed previously.[8] The fractions unbound in albumin solutions of both enantiomers were modified in the presence of oleic acid; however, the clinical significance of these observations remains to be elucidated and is under further investigation in our laboratory.

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