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# STEREOSELECTIVE INTERACTIONS OF KETOPROFEN GLUCURONIDES WITH HUMAN PLASMA PROTEIN AND SERUM ALBUMIN

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**Abstract**—A clearance pathway common to many aryl alkanoic acids is the generation of renally eliminated ester glucuronides. These metabolites are susceptible to systemic hydrolysis which generates the parent aglycone. We have conducted *in vitro* studies with biosynthetic *R*- and *S*-ketoprofen glucuronides to elucidate the mechanism of this phenomenon. These conjugates were incubated in human plasma, various concentrations of human serum albumin (HSA) and protein-free buffer. It was apparent that albumin, rather than plasma esterases, catalysed the hydrolysis of the glucuronides. The albumin-catalysed hydrolysis of ketoprofen glucuronides was highly stereoselective. The mean (±SD) hydrolysis half-life of *R*-ketoprofen glucuronide in plasma (N = 4) at physiological pH and temperature was 1.37 (±0.30) hr. The corresponding value for *S*-ketoprofen glucuronide, 3.46 (±0.84) hr, was significantly different ($P < 0.005$). In contrast, synthetic ethyl esters of *R*- and *S*-ketoprofen were hydrolysed by plasma esterases, but not by HSA, and with little stereoselectivity. The reversible protein binding of ketoprofen glucuronides was determined at physiological pH and temperature by a rapid ultra-filtration method. The binding of *R*- and *S*-ketoprofen glucuronide to human plasma protein was independent of concentration ($P > 0.05$) over the range of 1–20 μg/mL. The mean (±SD) percentage unbound in plasma (N = 4) of *R*-ketoprofen glucuronide was 12.6 (±1.4) %. The corresponding value for *S*-ketoprofen glucuronide, 9.12 (±0.54) %, was significantly different ($P < 0.005$). *S*-Ketoprofen glucuronide was also more avidly protein bound in physiological concentrations of HSA. However, this stereoselectivity decreased in more dilute HSA solutions. Based on the hydrolysis and protein binding data for ketoprofen glucuronides, we propose the existence of separate binding and catalytic sites on the albumin molecule for these metabolites.

Ketoprofen is a non-steroidal anti-inflammatory drug (NSAID‖) and is eliminated predominantly (approximately 70% of administered dose in humans) as acyl (ester) glucuronides in urine [1] (see Fig. 1 for structure). This is a clearance process common to many NSAIDs including fenoprofen [2], indoprofen [3], carprofen [4] and pirprofen [5]. Glucuronoconjugation of xenobiotics has been assumed to lead to abolition of the pharmacological activity of the aglycone due to reduced lipophilicity and subsequent renal or biliary excretion of the conjugate [6].

A series of elegant experiments [7, 8] examined the disposition of clofibric acid in an animal model of diminished renal function and in animals coadministered probenecid. The existence of a cycle of reversible glucuronide conjugation for acyl glucuronides and their respective aglycones was deduced. This provided a mechanistic explanation for the reduced clearances of the predominantly non-renally cleared NSAIDs ketoprofen [9, 10], naproxen [11], ximoprofen [12] and benoxaprofen [13] in patients with renal dysfunction or in elderly patients in whom renal function was diminished. In such a cycle, net clearance is due to glucuronoconjugation and competition between elimination of glucuronide by renal and hydrolytic clearances. This cycle predicts that inhibition of the renal elimination of the acyl glucuronide will lead to a reduction in net drug clearance due to accumulation and deconjugation of the physiologically labile glucuronide metabolite [14].

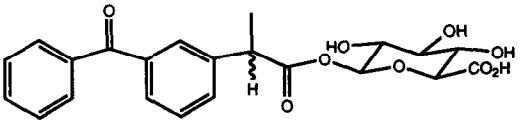


Fig. 1. Structure of 1-*O*-ketoprofen-β-D-glucuronide.

In addition to direct hydrolysis of the biosynthetic 1-*O*-acyl-β-glucuronide to the corresponding aglycone, intra-molecular rearrangement via acyl migration of the drug moiety to other positions on the glucuronic acid ring and subsequent hydrolysis of these isomers are potential sources of deconjugated drug [14, 15]. Moreover, a quantitatively minor competing pathway is covalent binding via the acyl glucuronide forming a drug–protein adduct [15]. Each of these processes involves potential regeneration of pharmacologically

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‖ Abbreviations: NSAID, non-steroidal anti-inflammatory drug; HSA, human serum albumin; PBS, isotonic phosphate-buffered (0.067 M, pH 7.4) saline.

active aglycone and has been reported for a number of NSAIDs [15] including fenoprofen (Ref. 16 and references to other compounds therein). Whereas the hydrolysis of covalent drug–protein adducts has been a quantitatively minor contributor to regenerated aglycone, the hydrolysis of positional isomers of glucuronides has been shown to be a significant pathway for fenoprofen [16], oxaprozin [17] and diflunisal [18].

The aim of the present study was to examine the mechanisms involved in *in vitro* deconjugation of acyl glucuronoconjugates using ketoprofen glucuronides as model metabolites. Significant concentrations of ketoprofen glucuronides have been detected in the plasma of elderly patients with reduced renal function [19] and in young healthy subjects coadministered probenecid [20]. Specifically, we have studied the relationship between the reversible binding of these conjugates to human plasma or human serum albumin and net hydrolysis (i.e. hydrolysis of biosynthetic glucuronide and the positional isomers together with, possibly, hydrolysis of drug–protein adduct). A further aim was to investigate factors which might influence these processes. Moreover, since ketoprofen (in common with most NSAIDs possessing a chiral carbon) is marketed for clinical use as a racemate, we have examined the stereoselectivity of glucuronide net hydrolysis and reversible binding.

## MATERIALS AND METHODS

*Purification and characterisation of ketoprofen glucuronides.* The purification procedure for biosynthetic ketoprofen glucuronides has been described in detail elsewhere [21]. Characterization of the purified *R*- and *S*-ketoprofen glucuronides was carried out by both chemical and physical means. The extent of hydrolysis of the glucuronides by purified β-glucuronidase (type VII-A, Sigma Chemical Co., St Louis, MO, U.S.A.; 100 U/mL, 37°, 60 min) was identical to that of complete alkaline hydrolysis (1.0 M sodium hydroxide, 22°, 15 min). This suggested exclusive C-1 attachment of ketoprofen to the glucuronic acid moiety with a β-linkage; and consequently, a lack of regioisomeric compounds in the purified material. In addition, enantioselective analysis of the aglycone (*vide infra* and [22]) confirmed the proportion of *R*:*S* ketoprofen to be 44.6:55.4 following hydrolysis by either chemical or enzymatic means of the purified biosynthetic metabolites.

Confirmation of structure as 1-*O*-(*R*,*S*)-ketoprofen-β-D-glucopyranosiduronate (glucuronide) was obtained by high-field (300 MHz) $^1$H-NMR spectroscopy. Two overlapping doublets at 5.4δ (relative to tetramethylsilane) of unequal heights (corresponding to the enantiomeric ratio of aglycone) with coupling constants (5.7 and 6.3 Hz) the same for each and consistent with trans diaxial hydrogen atoms (anomeric protons) confirmed acyl attachment at C-1 of D-glucuronic acid in the β-configuration. Hydroxyl protons were not seen, presumably replaced by deuterium from the solvent ($CD_3OD$). Two overlapping doublets, again of unequal height, at 1.45 δ with coupling constants of 6.3 and 6.6 Hz corresponded to the α-methyl protons of the two diastereomers split by the corresponding α protons.

Fast-atom bombardment mass spectroscopy of glucuronides dispersed in thioglycerol showed fragments consistent with a glucuronide conjugate of ketoprofen. Major ions at *m/z* 453 and 276 corresponded to sodium adducts of both the molecular ion and fragment aglycone ion. In addition, fragment ions were recorded at *m/z* 253 (aglycone ion), 209 and 105.

*Synthesis of ketoprofen ethyl ester.* Racemic ketoprofen (200 mg, 0.79 mmol) in 5 mL of dichloromethane and 0.5 mL of thionyl chloride (previously distilled over quinoline and boiled linseed oil, successively) was refluxed for 2 hr, and the solvent and excess thionyl chloride removed under a stream of purified nitrogen. The residue was dissolved in 2 mL of dry toluene and evaporated to dryness; this azeotroping process was repeated.

The resulting crude racemic ketoprofen acid chloride was dissolved in 5 mL of dichloromethane and added dropwise with stirring (4 hr) over an ice-bath to 1 mL of anhydrous ethanol containing 20 μL of dry pyridine. The solution was filtered, evaporated to dryness and redissolved in 5 mL of dichloromethane. This solution was washed thrice with 10 mL of phosphate buffer (0.1 M, pH 6). The organic layer was dried (magnesium sulphate) and the solvent removed under a stream of nitrogen to yield ketoprofen ethyl ester (167 mg, 75%). A portion of this material was subjected to complete alkaline hydrolysis (1 M sodium hydroxide, 50°, 2 hr) and yielded an acid with physical properties identical to those of authentic racemic ketoprofen.

*Hydrolysis experiments.* The rates of net hydrolysis of the glucuronide or ethyl esters of *R*- and *S*-ketoprofen were determined in a number of different matrices. The concentrations of the diastereomeric glucuronides or enantiomeric ethyl esters were determined by a difference method. The concentration of each hydrolysed product (*R*- and *S*-ketoprofen) after a given incubation time, was subtracted from the initial (time zero) concentration of ester (expressed as ketoprofen equivalents) and the difference was subsequently normalized as a percentage of the initial ester concentration.

For the net hydrolysis of the glucuronoconjugates of ketoprofen, the HPLC-purified diastereomeric glucuronides were added to various media to achieve a concentration of approximately 15 μg/mL (*ca.* 8.3 and 6.7 μg/mL of *S*- and *R*-ketoprofen glucuronide, respectively) expressed as ketoprofen equivalents. The media comprised; (i) fraction V human serum albumin (HSA) (Calbiochem, San Diego, CA, U.S.A.; lot 902736, 99.5% pure by electrophoresis) at concentrations of 40, 4.0 and 0.40 g/L in isotonic phosphate-buffered (0.067 M, pH 7.4) saline (PBS); (ii) essentially fatty acid-free fraction V HSA (Sigma; lot 118F9311) 4.0 g/L in PBS; and (iii) plasma obtained via arm vein venepuncture from four healthy volunteers. The media were adjusted to a final pH of 7.4 with orthophosphoric acid (2.5%) and temperature of 37° (oscillating waterbath, 20 cpm). In addition, the hydrolysis of ketoprofen glucuronides was determined in protein-free PBS at pH 7.4 and 9.0. The influence of warfarin sodium

(100 μM; Sigma) and diazepam (100 μM; Roche, Sydney, Australia) on the rates of hydrolysis of the diastereomeric glucuronides was examined in HSA solutions by pre-incubating with warfarin or diazepam for 15 min prior to addition to ketoprofen glucuronides. The experiments were performed throughout by adding fresh temperature- and pH-adjusted medium (10.0 mL) to a glass culture tube containing the glucuronides (from a methanolic solution evaporated to dryness under nitrogen immediately prior to addition of medium). The solution was mixed and rapidly aliquoted (1.00 mL) into individual glass tubes which were closed with PTFE-lined screw caps and returned to the waterbath. One of the aliquots of glucuronides was hydrolysed to completion (1.0 M sodium hydroxide, 100 μL, 15 min, 22°) to determine the initial (time zero) *R*- and *S*-ketoprofen glucuronide concentration. Thereafter, timed samples were removed from the waterbath and the hydrolysis reaction quenched (pH decreased to 3.0; [14]) with 0.025 mL of 2 M sulphuric acid. Incubations of glucuronides were carried out for up to 6 hr in the case of plasma and 40 g/L HSA, up to 8 hr for 4.0 g/L HSA and up to 24 hr for 0.4 g/L HSA and protein-free PBS solutions. The quench conditions were verified experimentally for ketoprofen glucuronides. The liberated aglycones were immediately extracted into hexane/ethyl acetate prior to enantioselective analysis as described below.

Hydrolysis experiments with the ethyl esters of ketoprofen were essentially the same as described for the glucuronides, with the following exceptions. The synthesized material spiked into media (15 μg/mL) was racemic and complete hydrolysis (time zero aliquot) was effected by 100 μL of 1 M sodium hydroxide at 50° for 2 hr. Fraction V HSA (40 g/L), human plasma from a single volunteer and protein-free PBS (all at 37° and pH 7.4) were the media tested. The influence of physostigmine (8 mM; Sigma) and sodium fluoride (120 mM; Ajax Chemical, Sydney, Australia) on the rates of hydrolysis of the enantiomeric ethyl esters was examined. Incubations of these esters were carried out for up to 6 hr.

*Protein binding of ketoprofen glucuronides.* The *in vitro* reversible protein binding of *R*- and *S*-ketoprofen glucuronide was determined in human plasma and in various concentrations of HSA (both fraction V and fatty acid free) with and without warfarin (100 μM) or diazepam (100 μM). Binding was determined at physiological pH and temperature. The unbound species were obtained by ultrafiltration (Centrifree$^R$, Amicon Division, Danvers, MA, U.S.A.) of 1-mL aliquots in a pre-warmed (37°) centrifuge (2000 *g*, 10 min; Heraeus Suprafuge 22 with HFA 20.16 fixed-angle rotor, Osterode, Germany). Less than 15 min elapsed between the addition of glucuronides to plasma or HSA and the acid-quenching of the corresponding ultra-filtrate. The concentrations of unbound diastereomeric glucuronides were determined by the difference method (*vide supra*). Typically, the aglycone concentration in the non-hydrolysed (acid-quenched) ultra-filtrate was less than 10% of that in the alkaline hydrolysed ultra-filtrate. There was no detectable sorption of glucuronides onto the ultra-filtration membrane.

In the experiments conducted with human plasma, the protein binding of *R*- and *S*-ketoprofen glucuronide (present together as 44.6% *R*- and 55.4% *S*-) was examined over the plasma concentration range of approximately 2–40 μg/mL (expressed as unresolved ketoprofen equivalents). Plasma (harvested within 1 hr of use) was obtained from each of four healthy volunteers, none of whom was taking any medication.

The binding of ketoprofen glucuronides, at a constant concentration of 15 μg/mL (8.3 and 6.7 μg/mL of *S*- and *R*-ketoprofen glucuronide, respectively) was investigated in the HSA solutions. When the influence of warfarin or diazepam on the binding of the glucuronides was investigated, HSA solutions

Table 1. *In vitro* net hydrolysis and reversible protein binding of *R*- and *S*-ketoprofen glucuronide in human plasma harvested from four healthy volunteers

| Subject (gender) | Age (years) | Serum albumin (g/l) | $T_{1/2,R}$ (hr) | $T_{1/2,S}$ (hr) | % unbound$_R^*$ | % unbound$_S^*$ |
|---|---|---|---|---|---|---|
| I (f) | 28 | 38 | 1.77 | 4.71 | 11.1 | 8.59 |
| II (m) | 54 | 39 | 1.11 | 2.93 | 14.4 | 9.76 |
| III (f) | 27 | 37 | 1.18 | 2.99 | 12.6 | 9.37 |
| IV (m) | 26 | 42 | 1.42 | 3.22 | 12.3 | 8.77 |
| Mean | | | 1.37† | 3.46 | 12.6‡ | 9.12 |
| SD | | | 0.30 | 0.84 | 1.4 | 0.54 |

* Analysis of variance indicated that there was no difference ($P > 0.05$) in % unbound$_R$ and % unbound$_S$ over the concentration range examined (approximately 1–20 μg/mL of ketoprofen equivalents) and therefore the values for each subject at the various concentrations were averaged.

† Statistically different ($P < 0.005$; unpaired *t*-test) from the corresponding value for *S*-ketoprofen glucuronide.

‡ Statistically different ($P < 0.005$; analysis of variance) from the corresponding value for *S*-ketoprofen glucuronide.

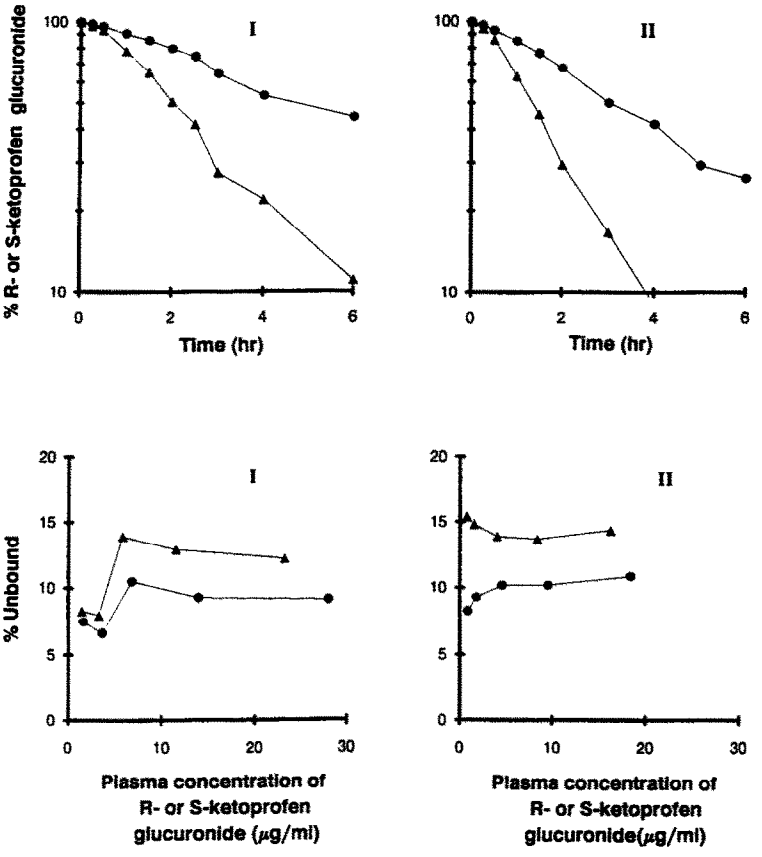


Fig. 2. Net hydrolysis and reversible protein binding at physiological pH and temperature of *R*-ketoprofen glucuronide (▲) and *S*-ketoprofen glucuronide (●) in human plasma from 2 subjects (I and II). The upper panels are semi-logarithmic plots of percentage glucuronide remaining after incubation of 15 μg/mL of glucuronides. The lower panels depict the plasma protein binding data for the glucuronides. Concentrations are expressed as ketoprofen equivalents.

were preincubated (15 min) with warfarin or diazepam prior to addition of the glucuronides.

*Analytical methods.* In order to quantify the glucuronide or ethyl esters of *R*- and *S*-ketoprofen, samples (hydrolysis incubation media or ultra-filtrate) were alkali-hydrolysed (at time zero only, in the case of hydrolysis experiments) and corresponding matched or timed samples protected against hydrolysis (acid-quenched with 0.025 mL of 2 M sulphuric acid). The differences in the aglycone concentrations between the alkali-treated samples and those of acid-protected samples was taken as a measure of *R*- or *S*-ketoprofen: glucuronide or ethyl ester. The enantioselective HPLC analytical method employed for ketoprofen has been described in detail elsewhere [22]. In outline the method involved rapid solvent extraction of ketoprofen enantiomers together with internal standard (*S*-naproxen) with 10% ethyl acetate in *n*-hexane from acidified (pH 3) matrix. Samples were evaporated to dryness prior to conversion to the intermediate acid chlorides (thionyl chloride) and subsequent derivatization with *S*-1-phenylethylamine (Sigma) generated the diastereomeric *S*-1-phenylethylamides. These diastereomers of ketoprofen and internal standard were resolved on a silica (5 μm) column (250 × 4 mm i.d., SGE, Sydney, Australia) eluted with 8% isopropyl alchol in *n*-heptane at a flow rate of 1 mL/min. Quantification was performed by monitoring the column eluant for UV absorbance (254 nm). Calibration was carried out by spiking drug-free incubation media (hydrolysis experiments) or compound sodium lactate injection B.P. or PBS (binding ultra-filtrate from plasma or HSA, respectively) with racemic ketoprofen in the concentration range 0.31–20.00 μg/mL.

*Data analysis.* Half-lives ($T_{1/2}$) for the net hydrolysis of glucuronide or ethyl esters of ketoprofen were calculated for the apparent first-order processes in each of the media after performing least-squares linear regression analysis (from time zero) of the logarithmic transform of percentage remaining ester as a function of linear time. The correlation coefficient ($r$) was calculated for each incubation experiment.

The percentage unbound values of the glucuronides (% unbound) were calculated as the ultra-filtrate (unbound) concentration of *R*- or *S*-ketoprofen

Table 2. Net hydrolysis and reversible protein binding of *R*- and *S*-ketoprofen glucuronide (15 μg/mL of both metabolites: 6.7 of *R*- and 8.3 μg/mL of *S*-ketoprofen glucuronide) upon incubation in fraction V HSA, and net hydrolysis in protein-free PBS

| | HSA concentration in PBS (37°, pH 7.4) | | | Protein-free PBS (37°) | |
|---|---|---|---|---|---|
| | 40 g/L | 4.0 g/L | 0.4 g/L | pH 7.4 | pH 9.0 |
| $T_{1/2.R}$ (hr) | 1.68 | 2.70 | 5.47 | >24 | 3.91 |
| Correlation coefficient* | 0.986 | 0.998 | 0.996 | 0.998 | 0.998 |
| % unbound$_R$† | 16.9‡ | 68.0‡ | 94.5 | — | — |
| SD | 0.9 | 1.5 | 2.6 | | |
| $T_{1/2.S}$ (hr) | 6.75 | 7.34 | 17.5 | >24 | 3.48 |
| Correlation coefficient* | 0.993 | 0.997 | 0.999 | 0.995 | 0.996 |
| % unbound$_S$† | 11.7 | 60.4 | 93.4 | — | — |
| SD | 0.5 | 1.7 | 2.7 | | |

* Correlation coefficient ($r$) calculated for the linear regression of the logarithmic transformation of percentage glucuronide remaining as a function of time.

† Mean and SD of replicate determinations of HSA binding of each glucuronide (N = 6 for 40 and 4.0 g/L; N = 3 for 0.4 g/L).

‡ Statistically different ($P < 0.0001$; unpaired $t$-test) from the corresponding value for *S*-ketoprofen glucuronide.

glucuronide divided by the total (bound plus unbound) concentration of the respective glucuronide.

Analysis of variance or the Student's $t$-test was used, as appropriate, to assess the statistical significance of differences between groups. Differences were considered significant at $P < 0.05$.

## RESULTS

### *Hydrolysis of glucuronides to aglycones*

The apparent first-order net hydrolysis $T_{1/2}$ of *R*-ketoprofen glucuronide was less than 50% of the corresponding value for *S*-ketoprofen in human plasma at physiological pH and temperature (Table 1 and Fig. 2, upper panels). This rapid, stereoselective deconjugation reaction was unaffected by the presence of plasma esterase inhibitors (8 mM physostigmine or 120 mM sodium fluoride; data not shown). The $T_{1/2}$ values for the glucuronides following incubation in a physiological concentration of HSA (40 g/L in PBS; Table 2) were similar to those recorded in plasma. Whilst maintaining a constant initial concentration of glucuronides (15 μg/mL) and decreasing the incubate HSA concentration by successive orders of magnitude, $T_{1/2.R}$ and $T_{1/2.S}$ increased to a far lesser extent than the corresponding fall in HSA concentration (Table 2). The marked stereoselective nature of the glucuronoconjugate net hydrolysis was maintained in these less concentrated HSA solutions.

Net hydrolysis of ketoprofen glucuronides in protein-free PBS was minimal at physiological pH and temperature. Rapid and largely non-stereoselective hydrolysis of the conjugates was observed when the pH of this solution increased to pH 9.0 (Table 2).

Results from the co-incubation of either warfarin sodium (100 μM) or diazepam (100 μM) with ketoprofen glucuronides in either fraction V of fatty acid-free HSA (4.0 g/L) are given in Table 3. Warfarin appeared to marginally increase $T_{1/2.S}$ in both HSA-type solutions and $T_{1/2.R}$ in fatty acid-free HSA. Co-incubations of glucuronides with diazepam gave rise to slight changes in $T_{1/2}$ values from control values, notably, an increase in $T_{1/2.R}$ in both HSA-type solutions, an increase in $T_{1/2.S}$ in fatty acid-free HSA and a decrease in $T_{1/2.S}$ in fraction V HSA.

### *Protein binding of the glucuronides*

The reversible binding of *R*- and *S*-ketoprofen glucuronide (present simultaneously) to human plasma protein was independent of glucuronide concentration ($P > 0.05$; analysis of variance) over the range of 1–20 μg/mL (depicted for two representative subjects, Fig. 2, lower panels). The mean (N = 4) % unbound of *R*-ketoprofen glucuronide was approximately 1.4 times that of the corresponding value for its diastereomer ($P < 0.005$; Table 1). When a constant amount of ketoprofen glucuronides was added to HSA solutions of varying strength, the stereoselective nature of the binding observed at 40 and 4.0 g/L was abolished in the most dilute HSA solution (0.4 g/L); the % unbound value for each diastereomer was approximately 95% in this solution (Table 2). Warfarin sodium (100 μM) displaced *S*-ketoprofen glucuronide in both fraction V and fatty acid-free HSA (4.0 g/L, $P < 0.001$) and displaced *R*-ketoprofen glucuronide in fatty acid-free HSA only ($P < 0.01$); diazepam (100 μM) displaced *R*-ketoprofen glucuronide in both types of HSA ($P < 0.05$ for fraction V and $P < 0.001$ for fatty acid-free) and *S*-ketoprofen glucuronide in fatty acid-free HSA only ($P < 0.001$). The magnitude of these changes were small in each case (Table 3).

### *Hydrolysis of the ethyl esters of ketoprofen*

The hydrolysis of the ethyl esters of *R*- and *S*-ketoprofen in human plasma from a single volunteer and the influence thereon of physostigmine (8 mM)

Table 3. Influence of warfarin (100 μM) and diazepam (100 μM) on the net hydrolysis and reversible protein binding (37°, pH 7.4) of *R*- and *S*-ketoprofen glucuronide (15 μg/mL of both metabolites: 6.7 μg/mL of *R*- and 8.3 μg/mL of *S*-ketoprofen glucuronide) in fraction V or fatty acid-free HSA (4.0 g/L)

| | Fraction V HSA | | | Fatty acid-free HSA | | |
|---|---|---|---|---|---|---|
| | Control | Warfarin | Diazepam | Control | Warfarin | Diazepam |
| $T_{1/2,R}$ (hr) | 2.70 | 2.82 | 3.04 | 2.86 | 3.19 | 3.00 |
| % unbound$_R$* | 68.0 | 69.8 | 70.4† | 55.8 | 57.7‡ | 61.0§ |
| SD | 1.5 | 1.4 | 1.9 | 1.3 | 1.1 | 0.7 |
| $T_{1/2,S}$ (hr) | 7.34 | 9.13 | 6.21 | 6.35 | 8.74 | 7.64 |
| % unbound$_S$* | 60.4 | 67.2§ | 61.6 | 52.5 | 58.0§ | 56.4§ |
| SD | 1.7 | 1.3 | 2.1 | 1.2 | 1.1 | 0.7 |

* Percentage unbound of R- and S-ketoprofen glucuronide expressed as the mean value and standard deviation from six replicate determinations.

Statistically different (†$P < 0.05$, ‡$P < 0.01$ and §$P < 0.001$: analysis of variance) from the corresponding value for the control sample (no warfarin or diazepam).

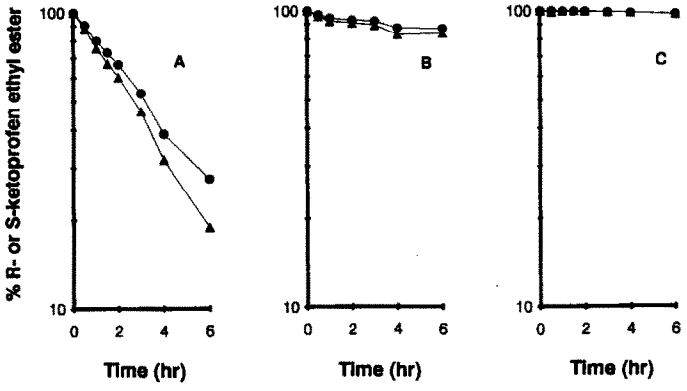


Fig. 3. Semi-logarithmic plots of the hydrolysis (pH 7.4, 37°) of *R*-ketoprofen ethyl ester (▲) and *S*-ketoprofen ethyl ester (●) in human plasma (expressed as percentage remaining ethyl ester) upon incubation of 15 μg/mL of racemic compound in each of: plasma (panel A), plasma spiked with 8 mM physostigmine (panel B) and plasma spiked with 120 mM sodium fluoride (panel C).

or sodium fluoride (120 mM) are depicted in Fig. 3. In the absence of esterase inhibitors the apparent first-order hydrolysis $T_{1/2}$ values for the *R*- and *S*-enantiomers were 2.48 hr ($r = 0.999$) and 3.14 hr ($r = 0.999$), respectively (Fig. 3, panel A). Both physostigmine and fluoride were effective in blocking ethyl ester hydrolysis (Fig. 3, panels B & C). There was negligible hydrolysis of these esters at physiological pH and temperature in either HSA solutions or in protein-free PBS solutions (data not shown).

## DISCUSSION

These data demonstrate the pivotal role of albumin in the rapid, stereoselective net hydrolysis of the glucuronoconjugates of ketoprofen *in vitro*. The similarity between plasma and HSA solution (40 g/L) suggests that albumin may be the major source of hydrolytic activity in plasma (Tables 1 and 2). Recent studies examining the hydrolysis of conjugates of fenoprofen [16], flurbiprofen [23] and carprofen [24] attributed similar importance to albumin. Of interest, Knadler and Hall [23] noted preferential hydrolysis of the conjugate of *S*-flurbiprofen in contrast to our studies with ketoprofen and previous studies with fenoprofen [16] and carprofen [24] where the rate of conjugate hydrolysis was faster for the respective *R*-acyl glucuronide. In contrast to glucuronide hydrolysis, HSA solutions (40 g/L) did not effect detectable ketoprofen ethyl ester hydrolysis after 6-hr incubations at physiological pH and temperature (data not shown). However, incubations of ethyl ester with plasma led to significant hydrolysis (Fig. 3, panel A). The marked degree of stereoselectivity elicited by plasma on ketoprofen glucuronide hydrolysis (Fig. 2, upper panels) was in contrast to our studies with ketoprofen ethyl esters. In the latter case, this plasma-derived esterase activity was largely indiscriminant of the stereochemical configuration of the acyl moiety.

Human serum albumin has been shown to exhibit *in vitro* hydrolytic activity towards the acyl glucuronides of fenoprofen [16], oxaprozin [17, 25]

and carprofen [24], and towards cinnamoylimidazoles [26] and phenyl esters [27]. In contrast, an *in vivo* study by Rowe and Meffin [28] suggested that plasma esterases were responsible for the hydrolysis of clofibric acid glucuronide based on experiments with the irreversible esterase inhibitor, diisopropylfluorophosphate. These workers administered very high doses of the inhibitor to anaesthetized rabbits (approximately 30-fold that of the $LD_{50}$ in conscious animals) and observed an increase in clearance of clofibric acid, presumed to be due to blockade of esterase-mediated hydrolysis of the glucuronide. Such a large dose of diisopropylfluorophosphate could conceivably lead to alkylphosphorylation of albumin, as observed *in vitro* by Wells *et al.* [25]. This might implicate albumin as the macromolecule responsible for deconjugating clofibric acid in the above study [28]. Further, Lin *et al.* [29] observed a lack of effect of an alternative esterase inhibitor (phenylmethylsulphonyl fluoride) on the clearance of diflunisal (subject to significant acyl glucuronide formation) in rats with experimentally induced renal failure. Moreover, when we incubated ketoprofen glucuronides in plasma spiked with classical esterase inhibitors (physostigmine and sodium fluoride) the rates of net hydrolysis of ketoprofen glucurononconjugates were unaffected (data not shown). Indeed we verified that these agents were potent inhibitors of plasma esterases by observing a marked reduction in the rate of hydrolysis of ketoprofen ethyl ester (Fig. 3).

It is interesting to note that the reversible binding of the glucuronide conjugates of ketoprofen to HSA and plasma exhibited significant stereoselectivity, while in a recent study [21] we observed a lack of stereoselectivity in the binding of ketoprofen aglycones to plasma. In the case of carprofen, stereoselectivity was observed, with respect to HSA binding, with both the carprofen glucuronide conjugates and the progenitive aglycones [30]. It is likely that incubations of ketoprofen glucuronides in protein solutions leads to the generation of covalent ketoprofen–protein adducts (irreversibly bound drug) based on previous *in vitro* studies with acyl glucuronides of other NSAIDs including fenoprofen [16], diflunisal [18] and zomepirac [31]. The degree to which acyl glucuronides form covalent adducts with proteins is quantitatively minor (1% or less for the three examples cited above) and time dependent. We determined the degree of reversible protein binding of ketoprofen glucuronides in solutions which had been incubated for less than 15 min. Thus our estimates of reversible binding would be unlikely to be influenced by covalent drug–protein adduct generation (presumptively) following *in vitro* incubation of ketoprofen glucuronides.

We were initially led to suspect that the site on the albumin molecule at which ketoprofen glucuronide hydrolysis was taking place might be the same site at which the glucuronides bind, as suggested for oxaprozin glucuronide [17, 25]. However, we observed significant and stereoselective binding of ketoprofen glucuronides to human plasma (Table 1 and Fig. 2) and physiological concentrations of HSA (Table 2); the eudismic ratio for the % unbound values was opposite to that for the $T_{1/2}$ values for the hydrolysis of the conjugates. This suggested to us the possible existence of a binding site distinct from the hydrolytic or catalytic site on the albumin molecule with respect to ketoprofen glucuronides. Indeed, incubations of ketoprofen glucuronides (15 μg/mL) in very dilute HSA solutions (0.4 g/L or 6 μM) where the ligand to albumin molar ratio was approximately 5:1 for each diastereomer, showed low, non-stereoselective binding yet markedly stereoselective conjugate hydrolysis (Table 2). It was also evident from these data (Table 2) that as binding of the glucuronides to HSA decreased (in successively more dilute protein solutions), more of these metabolites became available (as unbound glucuronides) for engagement with the catalytic site(s) on HSA molecules (and subsequent hydrolysis to their respective aglycones). A recent study with diflunisal acyl glucuronide [18] supports the alternative hypothesis of separate catalytic and binding sites on the albumin molecule. It is apparent that diflunisal acyl glucuronide, an extremely highly bound conjugate, is partially protected from hydrolysis in HSA solutions by virtue of this binding site being distinct (and a significant sink for glucuronide) from the catalytic site. Furthermore, the preferential deconjugation of *R*-carprofen glucuronide by HSA [24] and the greater percentage unbound value for this diastereomer compared to *S*-carprofen glucuronide [30] provides additional support for the hypothesis. It is possible that oxaprozin glucuronide binds to, and is degraded at, a common site on the albumin molecule. Wells *et al.* [25] reported this to be the benzodiazepine site, or Site II as classified by Sudlow *et al.* [32]. In contrast, carprofen glucuronides have been reported [30] to bind preferentially to the warfarin site (Site I).

We observed minor displacement of *R*- and *S*-ketoprofen glucuronide (*ca* 30 μM) in fraction V and fatty acid-free HSA (4.0 g/L or 60 μM) by warfarin (100 μM) or diazepam (100 μM) (Table 3). In parallel, we recorded slight increases in the corresponding conjugate hydrolysis $T_{1/2}$ values in those solutions where displacement had taken place. While these changes were subtle they are still consistent with the concept of separate hydrolytic and binding sites on the albumin molecule. It is possible that warfarin and diazepam exert an allosteric effect on the hypothesized separate catalytic site(s). Alternatively, these competing ligands might bind to more than one site on the albumin molecule, including the catalytic site(s).

The rearrangement of acyl glucuronides from the biosynthetic 1-*O*-β-isomer to other positional isomers has been demonstrated for a number of NSAIDs and other carboxylic acids including clofibric acid [33], valproic acid [34], oxaprozin [25], fenoprofen (Ref. 16 and references to other NSAIDs therein). While there are no reports in the literature, presumptively, ketoprofen glucuronides undergo such reactions. Thus, the part of our study concerned with glucuronide hydrolysis (analysis by the difference method) describes this process as the net result of hydrolysis of all possible glucuronide isomers. Caution is required in the interpretation of, for instance, the very low net hydrolysis of

conjugates in protein-free PBS at physiological temperature and pH (Table 2). These data might belie rapid 1-*O*-β-glucuronide degradation to other regioisomers. Fundamentally, we have attempted to describe the hydrolysis of ketoprofen glucuronides to the enantiomeric aglycones (pharmacologically active in the case of the *S*-enantiomer). This process potentially incorporates as yet unidentified intermediate pathways of glucuronide transacylation reactions, mutarotation and lactonization [14, 15].

In summary, acyl glucuronides are polar compounds generally restricted to the vascular and interstitial fluid compartments; regions containing significant concentrations of albumin. Thus, interactions of such metabolites with individual components of these compartments are of particular importance in determining the disposition of both metabolite and pharmacologically active aglycone. Our study implicates albumin to be of fundamental importance in the disposition of ketoprofen. Moreover, we suggest that this macromolecule possesses separate binding and catalytic sites for the acyl glucuronides of ketoprofen. The significant renal clearance of ketoprofen glucuronides [20] implies that in subjects with diminished renal function, the interaction of these accumulated glucuronides with albumin becomes of major pharmacological significance.

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