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# Stereoselective Esterase Activity of Human Serum Albumin toward Ketoprofen Glucuronide

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## SUMMARY

Many carboxylic acid-containing drugs undergo conjugation with D-glucuronic acid in humans, leading to the formation of acyl glucuronides, which are excreted into urine. However, these metabolites can be hydrolyzed back to the parent aglycon; this reaction can be accelerated by human serum albumin (HSA). Although this phenomenon of interaction between the acyl glucuronide and HSA has been described for various drugs, the kinetics of the protein have not been characterized. The aim of this study was to investigate the HSA-mediated mechanism involved in the *in vitro* hydrolysis by albumin of the acyl glucuronides of (*R*)- and (*S*)-ketoprofen (a nonsteroidal anti-inflammatory drug), as model compounds. The conjugates of both ketoprofen enantiomers were incubated, separately or together, with increasing concentrations of albumin (14.5–145 μM) at pH 7.4 and 37°. The reaction followed Michaelis-Menten kinetics and was stereoselective; the (*R*)-ketoprofen glucuronide was a better substrate than the *S*-conjugate. To identify the HSA domain involved in the hydrolysis reaction, specific probes of HSA binding sites were used as potential inhibitors. These probes, added at an equimolar probe/glucuronide ratio (145 μM), slightly decreased the hydrolysis (by up to 30%). They affected the reversible binding of (*R*)-ketoprofen glucuronide to HSA, as shown by CD studies. Because iodoacetic acid did not modify the single free cysteine residue on HSA, this amino acid residue cannot be the reactive one. In addition, the chemical modification of a single tyrosine residue (probably Tyr-411) on HSA by diisopropyl fluorophosphate significantly but weakly affected the hydrolysis of (*R*)-ketoprofen glucuronide, suggesting that this residue also is not involved in the catalysis. In contrast, the *R*-conjugate was not bound to modified albumin, as revealed in CD experiments. These results support the existence of distinct sites on HSA for reversible binding and hydrolysis of (*R*)-ketoprofen glucuronide.

Conjugation of drugs with D-glucuronic acid is generally believed to lead to readily excreted, pharmacologically inactive products. However, several recent reports suggest that this process does not always correspond to a detoxication pathway (1). Acyl glucuronides, in contrast to ether glucuronides, are unstable under physiological conditions, because of their susceptibility to nucleophilic attack; they can undergo hydrolysis and intramolecular acyl migration (1, 2). These two phenomena and their extents vary greatly from one compound to another and have been described for a number of carboxylic drugs, including fenoprofen (3), oxaprozin (4), flurbiprofen (5), tolmetin (6), ketoprofen (7, 8), carprofen (9), and diflunisal (10).

The lack of stability of these metabolites may affect the *in vivo* fate of the drug. Hydrolysis back to the parent aglycon can lead to a futile cycle, as has been reported for clofibric acid, whose plasma clearance is a function of conjugation and competition between deconjugation of the acyl glucuronide and its renal clearance (11, 12). If renal excretion is impaired, more of the conjugate becomes available for hydrolysis and the elimination of the drug from plasma is decreased. Studies with patients with renal dysfunction who are treated with a drug such as clofibrate (13), ketoprofen (14, 15), or naproxen (16) have indeed shown reduced plasma clearance of the drug.

Except for naproxen, the 2-arylpropionates, a class of nonsteroidal anti-inflammatory drugs, are used clinically as racemic mixtures of the pharmacologically active *S*-enantiomer and the inactive or less active *R*-enantiomer. These drugs are often metabolized to their acyl glucuronides, and this biotransformation may be stereoselective (17–19). They can also undergo chiral and unidirectional inversion from the *R*-isomer to its antipode (20). These two biological reactions can

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**ABBREVIATIONS:** HSA, human serum albumin; DIFP, diisopropyl fluorophosphate; HPLC, high performance liquid chromatography; CD, circular dichroism.

alter the ratio of *R*/*S*-enantiomers and may thus increase or decrease the fraction of the active form. In addition, enantioselective hydrolysis has been reported for acyl glucuronides and may also contribute to variation of this ratio (3, 5, 7–9). For example, after administration of racemic 2-phenylpropionic acid the plasma concentration of the *S*-enantiomer was higher in animals with experimental renal failure than in controls (21).

The extent of hydrolysis may be influenced by the presence of proteins, such as esterases, in the medium (12). Previous studies suggested that albumin may play a major role in the degradation of glucuronides because it may catalyze the hydrolysis reaction (3–5, 7–9). HSA, a major plasma protein, transports many drugs and endogenous compounds, for which various binding sites have been identified using specific probes (22, 23).

Some authors have observed an esterase activity of albumin toward acyl glucuronides, but none has attempted to characterize the mechanism of the reaction. Therefore, in this study, we examined the kinetics of the HSA-mediated hydrolysis of ketoprofen glucuronide for each enantiomer separately and in racemic mixtures at various albumin concentrations. Ketoprofen [(*R*,*S*)-2-(3-benzoylphenyl)propionic acid] is a nonsteroidal anti-inflammatory drug that is predominantly cleared as its acyl glucuronide (17, 24) and for which accelerated degradation has been described in the presence of HSA (7, 8). The reaction was also carried out in the presence of specific probes of binding sites, to locate the catalytic domain. The effect of these markers on the reversible binding between glucuronide and HSA was investigated using CD. Finally, we tried to modify the protein chemically with DIFP or iodoacetic acid, to identify the amino acid residue involved in the reaction.

## Materials and Methods

**Chemicals.** The chromatographic internal standard benzoyl-1,4-phenyl-2-butyric acid, ketoprofen, and its enantiomers were a gift from Rhône Poulenc Rorer (Paris, France). Iodoacetic acid, L-cysteine, 4,4′-dithiodipyridine, and DIFP were purchased from Aldrich (L'Isle d'Abeau Chesnes, France). Dansylamide, dansylsarcosine, digoxin, L-tyrosine, and HSA were from Sigma (L'Isle d'Abeau Chesnes, France). HSA was fraction V (purity, 99%), essentially fatty acid-free (<0.005% fatty acids) (batch 42H9313). Warfarin was a gift from Merrell-Dow (Levallois-Perret, France), phenylbutazone from Ciba-Geigy (Rueil-Malmaison, France), diazepam from Roche (Neuilly sur Seine, France), and ibuprofen from Boots-Pharma (Courbevoie, France). All reagents used in the study were of analytical grade. Acetonitrile in the mobile phase for HPLC was of chromatographic grade (SDS, Villeurbanne, France).

**Synthesis of ketoprofen glucuronide.** Racemic ketoprofen glucuronide was produced *in vitro* with phenobarbital-treated rat liver microsomes immobilized onto alginate beads, as described previously (25). Semipreparative HPLC with a Lichrosorb RP18 column (250 × 10 mm, 7 μm; Merck) was used to obtain large quantities of metabolite as described by Magdalou *et al.* (19), with minor modifications. The mobile phase consisted of acetonitrile/water/trifluoroacetic acid (80:120:0.08, v/v), pH 2.2, and the glucuronide was detected at 256 nm. The eluted glucuronide was collected and freeze-dried. The structure was characterized as ketoprofen glucuronide by mass spectrometry and by its susceptibility to hydrolysis by β-glucuronidase, as shown Chakir *et al.* (26). The same procedure was also used for each enantiomer separately.

**Kinetic study.** The glucuronide of (*R*)-, (*S*)-, or racemic ketoprofen was incubated in a single experiment in the presence of various concentrations of HSA (14.5, 29, 72.5, or 145 μM), in an isotonic 0.067 M phosphate buffer, pH 7.4, at 37°. The substrate concentrations ranged from 0.02 to 4.5 mM and were always higher than that of the albumin. At each sampling time (from 0 to 50 hr), aliquots (in duplicate) were taken and HCl (1 N) was added to stop the hydrolysis reaction. The ketoprofen released was assayed as described previously (27). Briefly, after addition of the internal standard, extraction with diethyl ether (3 ml), and evaporation of the organic solvent under nitrogen, the residue was reconstituted in the mobile phase (acetonitrile/0.3% acetic acid, 45:55, v/v; flow rate of 1 ml/min, at room temperature). The HPLC system consisted of a model 510 pump, a model 481 variable-wavelength UV absorbance detector, and a model 740 integrator, all from Waters Associates (St. Quentin en Yvelines, France). The column was a Radial Pak $C_{18}$, 8- × 150-mm, 3-μm column, also from Waters. Compounds were detected at 256 nm. The proportions of the two enantiomers were determined by stereoselective analysis of the released ketoprofen using an HSA chiral column (Shandon, Eragny, France). Their respective concentrations in the mixture were then deduced from the nonstereoselective assay. The eluent consisted of 0.05 M phosphate buffer, pH 7.0/acetonitrile/isopropanol/octanoic acid (82:15:3:0.005, v/v), at a flow rate of 0.8 ml/min.

The same experimental procedure was used in buffer, pH 7.4, without HSA, and the rate of spontaneous hydrolysis was subtracted from that obtained with the protein solutions to provide the actual esterase activity of albumin for each substrate concentration. Apparent kinetic constants ($V_{max}$ and $K_m$) were determined using least-squares regression analysis of double-reciprocal plots of initial rates versus substrate concentrations. An experiment was carried out with albumin (145 μM) that had been heated at 60° for 30 min before incubation with glucuronide (145 μM).

The effects of probes known to bind to specific HSA sites on the rate of hydrolysis of the diastereomeric glucuronide were examined in a 145 μM albumin solution by preincubation with the probe (dansylamide, dansylsarcosine, phenylbutazone, warfarin, diazepam, ibuprofen, or digoxin) before the addition of ketoprofen conjugate. Ligands (probes and glucuronide) were at the same molar concentration as HSA. The ketoprofen released after a 4-hr incubation was assayed as described previously and was compared with a control without probes.

**Chemical modifications of HSA.** We attempted to determine the amino acid residue involved in hydrolysis by modifying cysteine or tyrosine residues with iodoacetic acid or DIFP, respectively. Albumin at various concentrations (36.75, 72.5, or 145 μM) in 0.1 M Tris·HCl buffer, pH 8.2, was treated with a 50–700-fold molar excess of iodoacetic acid at 25° for 2 hr. The number of thiol groups was evaluated according to the method of Grassetti and Murray (28). The procedure was based on the formation of 4-thiopyridone after reaction between thiol groups and 4,4′-dithiodipyridine. The UV absorption of the product was measured at 324 nm. The calibration curve obtained by adding 4,4′-dithiodipyridine at 0.1 mM was linear for concentrations of amino acid residue ranging from 0.0025 to 0.1 mM.

Tyrosine residues of a 145 μM HSA solution in 125 mM Tris·HCl buffer, pH 8.2, reacted with a 5–100-fold molar excess of DIFP at 30° for 2 hr. The number of phenolic groups was then monitored at 295 nm, according to the method of Murachi *et al.* (29). A protein aliquot (700 μl) was added to 0.05 M sodium acetate buffer, pH 5.2 (1550 μl). Subsequent addition (250 μl) of 0.1 M NaOH or 0.1 M sodium acetate, pH 5.2, determined whether the phenolic groups were ionized. The difference in absorbance between the two pH conditions indicated the amount of tyrosine residues.

Albumin derivative solutions were exhaustively dialyzed (for 60 hr) against an isotonic 0.067 M phosphate buffer, pH 7.4. Protein concentrations were calculated using the bicinchoninic acid protein assay kit (Sigma), and albumin solutions were diluted to 72.5 μM. The number of modified residues was titrated again to ensure that dialysis had no effect on modification. The control experiment consisted of subjecting albumin to identical experimental conditions,

except that no chemical reagent was used. The protein preparations (control and modified albumin) were incubated in triplicate with (*R*)-ketoprofen glucuronide at 77.5 μM, and hydrolysis was followed for 4 hr as described previously.

**CD study.** CD experiments were conducted with a Jobin Yvon IV dichrograph (Paris, France) at a temperature of 20°, to limit the degradation of the glucuronide (<2%), and in the range of 280–450 nm, with an optical pathlength of 10 mm and $2 \times 10^{-6}$ sensitivity. A 145 μM albumin solution was prepared in an isotonic 0.067 M phosphate buffer, pH 7.4. Ligands (glucuronides and specific binding probes, i.e., dansylamide, phenylbutazone, warfarin, digoxin, ibuprofen, and diazepam) were used at 145 μM. The binding of (*R*)-ketoprofen glucuronide to albumin induced a negative band at 340 nm. Ellipticities obtained with and without probes were compared to determine the influence of probes on (*R*)-ketoprofen conjugate binding. The same procedure was carried out with DIFP-modified albumin at 72.5 μM and dansylamide, dansylsarcosine, and (*R*)-ketoprofen as specific probes.

## Results

**HSA-mediated hydrolysis of ketoprofen glucuronide.** When the glucuronide of (*R*)-, (*S*)-, or racemic ketoprofen was incubated in phosphate buffer alone, the rate of spontaneous hydrolysis was proportional to the initial substrate concentration and corresponded to 6% of the total hydrolysis after 50 hr of incubation. A slight stereoselective deconjugation of ketoprofen glucuronide was apparent; the *R*-enantiomer glucuronoconjugate, separately or in the racemate, appeared to be more stable than its antipode (1.5-fold) (data not shown).

In the presence of HSA, the extent of hydrolysis of the conjugates of both enantiomers increased. For each concentration of substrate and of protein, the kinetics observed over 50 hr displayed a typical pattern with a linear part (Fig. 1). During this linear period, a small fraction of the substrate was consumed (<10%) and initial rates were measured using at least four experimental points.

The rates of hydrolysis increased hyperbolically as a function of substrate concentrations for the glucuronides of both enantiomers and for each protein concentration (Fig. 2A). These results suggest that the reaction of ketoprofen glucuronide with HSA proceeds via the formation of a Michaelis-Menten-type complex. Apparent kinetic constants ($V_{max}$ and $K_m$) were then determined by double-reciprocal plot analysis

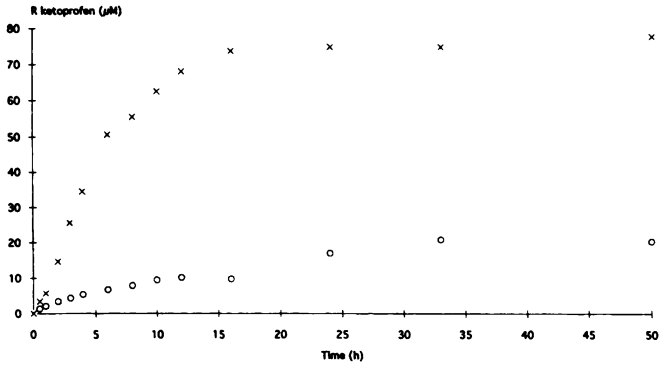


**Fig. 1.** Kinetics of hydrolysis of (*R*)-ketoprofen glucuronide with (×) and without (○) HSA. The (*R*)-ketoprofen conjugate (295 μM) was incubated in a single experiment at 37° in a 0.067 M phosphate buffer, pH 7.4, with or without HSA (145 μM). At each sampling time, the ketoprofen released was assayed in duplicate as described in Materials and Methods.

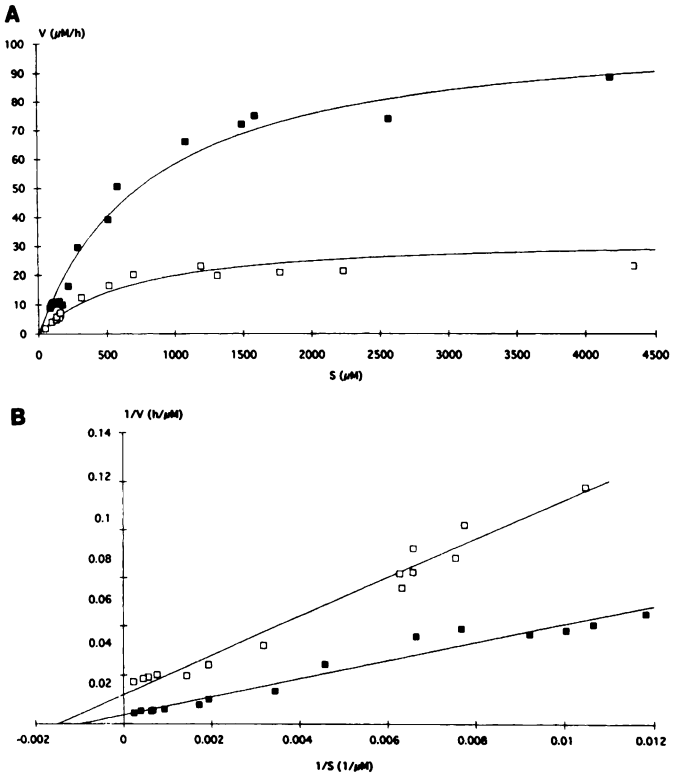


**Fig. 2.** Initial rates versus substrate concentrations for the hydrolysis by HSA of the glucuronide of (*S*)-ketoprofen (□) and (*R*)-ketoprofen (■) (A) and double-reciprocal plot of initial rates and substrate concentrations (B). A, *Curves* were drawn using Michaelis-Menten parameters determined by linear least-squares regression analysis of a double-reciprocal plot of initial rates versus substrate concentrations. B, *Lines* are the least-squares regression lines. Various glucuronide concentrations (0.1–4.5 mM) were incubated in a single experiment with a 72.5 μM HSA solution at pH 7.4 and 37°. The ketoprofen released was assayed in duplicate as described in Materials and Methods. Values of initial rates have been corrected for spontaneous hydrolysis in buffer alone and were determined using at least four experimental points.

(Fig. 2B, for example). In the racemate at all protein concentrations, and separately for the lowest HSA concentration, the esterase activity toward (*S*)-ketoprofen glucuronide was too low, compared with spontaneous hydrolysis. Under these conditions, no parameters were determined for this glucuronoconjugate.

For the (*S*)-ketoprofen glucuronide, the affinity of each HSA molecule increased with albumin concentration, as suggested from the $K_m$ values (Table 1). $V_{max}$ was not related to the protein concentration.

The velocity ($k_{cat} = V_{max}/K_m$) for (*R*)-ketoprofen glucuronide was not affected by the protein concentration. The apparent kinetic constants for the metabolite of (*R*)-ketoprofen in racemate and alone were also compared (Table 1). The presence of the (*S*)-ketoprofen glucuronide induced a decrease of $K_m$ and $V_{max}$ values but not of $V_{max}/K_m$.

To elucidate the inhibition mechanism, hydrolysis was investigated for various ratios of (*R*)/(*S*)-ketoprofen glucuronoconjugates. The conjugate of (*R*)-ketoprofen at 760 μM was mixed with that of (*S*)-ketoprofen at 760 and 2000 μM in a 72.5 μM HSA solution. The rates of hydrolysis of (*R*)-ketoprofen glucuronide varied from 52 μM/hr when assayed alone to 38 and 32 μM/hr, respectively, with (*S*)-ketoprofen at these concentrations.

TABLE 1

**Apparent kinetic parameters for the hydrolysis of the glucuronides of (*R*)- and (*S*)-ketoprofen separately (*R* and *S*, respectively) or in the racemate ($R_{rac}$) and of racemic ketoprofen (rac), with various concentrations of HSA**

The kinetic parameters for the esterase activity of HSA toward (*S*)-ketoprofen glucuronide in the racemate were too low to be measured. Values were determined using the assays described in Materials and Methods, with linear least-squares regression analysis of double-reciprocal plots of initial rates versus substrate concentrations.

| | 14.5 μM HSA | 29 μM HSA | 72.5 μM HSA | 145 μM HSA |
|---|---|---|---|---|
| rac | | | | |
| $K_m$ (μM) | 347.1 | 794.4 | 1226.4 | 1207.6 |
| $V_{max}$ (μM/hr) | 17.82 | 46.82 | 88.51 | 101.1 |
| $V_{max}/K_m$ ($hr^{-1}$) | 0.051 | 0.059 | 0.072 | 0.084 |
| $n$[a] | 9 | 12 | 11 | 10 |
| $r$ | 0.987 | 0.973 | 0.994 | 0.992 |
| $p$ | 0.001 | 0.001 | 0.001 | 0.001 |
| *R* | | | | |
| $K_m$ (μM) | 434.0 | 461.9 | 1006.4 | 2126.6 |
| $V_{max}$ (μM/hr) | 29.03 | 44.70 | 107.8 | 216.1 |
| $V_{max}/K_m$ ($hr^{-1}$) | 0.067 | 0.097 | 0.107 | 0.102 |
| $n$ | 13 | 10 | 15 | 15 |
| $r$ | 0.995 | 0.993 | 0.979 | 0.979 |
| $p$ | 0.001 | 0.001 | 0.001 | 0.001 |
| $R_{rac}$ | | | | |
| $K_m$ (μM) | 110.0 | 292.0 | 917.1 | 930.3 |
| $V_{max}$ (μM/hr) | 12.38 | 27.56 | 87.74 | 95.57 |
| $V_{max}/K_m$ ($hr^{-1}$) | 0.113 | 0.094 | 0.096 | 0.103 |
| $n$ | 6 | 11 | 10 | 8 |
| $r$ | 0.996 | 0.971 | 0.981 | 0.989 |
| $p$ | 0.001 | 0.001 | 0.001 | 0.001 |
| *S* | | | | |
| $K_m$ (μM) | ND[b] | 559.3 | 670.28 | 515.9 |
| $V_{max}$ (μM/hr) | ND | 18.22 | 33.25 | 46.98 |
| $V_{max}/K_m$ ($hr^{-1}$) | ND | 0.033 | 0.050 | 0.091 |
| $n$ | ND | 10 | 14 | 7 |
| $r$ | ND | 0.926 | 0.986 | 0.991 |
| $p$ | ND | 0.001 | 0.001 | 0.001 |

[a] $n$, number of experimental points; $r$, correlation coefficient; $p$, associated probability.
[b] ND, not determined.

Finally, albumin also exhibited stereoselectivity toward hydrolysis of ketoprofen glucuronide; although the $K_m$ for the *R*-enantiomer was high, its $V_{max}$ was 3- or 4-fold higher than that of its antipode for all protein concentrations (Table 1).

To confirm the source of the esterase activity, HSA was heated at 60° for 30 min. The hydrolysis by the protein was fully inactivated (data not shown).

**Influence of specific binding probes on the hydrolysis of ketoprofen glucuronide.** The hydrolysis of glucuronide by HSA was carried out in the presence of conjugate and specific probes for HSA binding sites, at the same molar concentration as albumin (145 μM), for 4 hr. These probes did not affect spontaneous hydrolysis of glucuronide, as shown by a control experiment without HSA (data not shown). The inactivation of the reaction by probes was low and quite variable, depending on the probe and the ketoprofen isomer; it was higher for the conjugate of (*S*)-ketoprofen than for its isomer (Fig. 3). The hydrolysis of (*S*)-ketoprofen glucuronide was significantly inhibited by dansylamide, warfarin, and digoxin (90% of control) and by phenylbutazone and diazepam (70% of control). Dansylsarcosine and ibuprofen had a significant effect on the hydrolysis of both metabolites. For the *R*-enantiomer, the reaction exhibited no inhibition with the other probes. Studies using a 72.5 μM HSA solution, probes at 70 μM, and glucuronide at the same or lower molar concentrations gave similar results (data not shown).

**Influence of specific probes on glucuronide binding to HSA investigated with CD.** The reversible binding of the glucuronide of (*R*)-ketoprofen to HSA displayed a negative Cotton effect at 340 nm, different from that of (*R*)-ketoprofen itself. The existence of such a signal allowed us to investigate the binding of this ligand to HSA in the presence of specific probes. Substrate, probes, and protein were used at equimolar concentrations (145 μM). After binding of the metabolite to HSA, the extent of the observed extrinsic Cotton effect decreased when the probes, except warfarin, were added to the mixture (Table 2). These results suggest that the conjugate was displaced from its binding site.

The (*S*)-ketoprofen glucuronide was not tested in this way because its binding produced a band of very low intensity. However, its presence reduced the extrinsic Cotton effect of the *R*-conjugate/HSA complex, indicating a displacement of the (*R*)-ketoprofen glucuronide from its binding site.

**Chemical modifications of albumin.** A tyrosine or cysteine residue is often involved in catalysis by esterases (30). Therefore, HSA was treated with iodoacetic acid or DIFP to evaluate the effect of such modifications on the hydrolysis of ketoprofen glucuronide. When albumin was mixed with iodoacetic acid at various molar excesses the number of cysteine residues titrated was not affected by the reaction, and hydrolysis with the albumin treated with iodoacetic acid was therefore not studied further.

For HSA at 145 μM, 8 tyrosine residues/mol of unmodified albumin were detected. A 10-fold molar excess of DIFP caused 1 tyrosine residue/mol of protein to be modified (data not shown). The influence of such modification on hydrolysis was examined for the best substrate, i.e., the glucuronide of (*R*)-ketoprofen, and compared with results for control HSA incubated similarly but without DIFP. The extent of hydro-

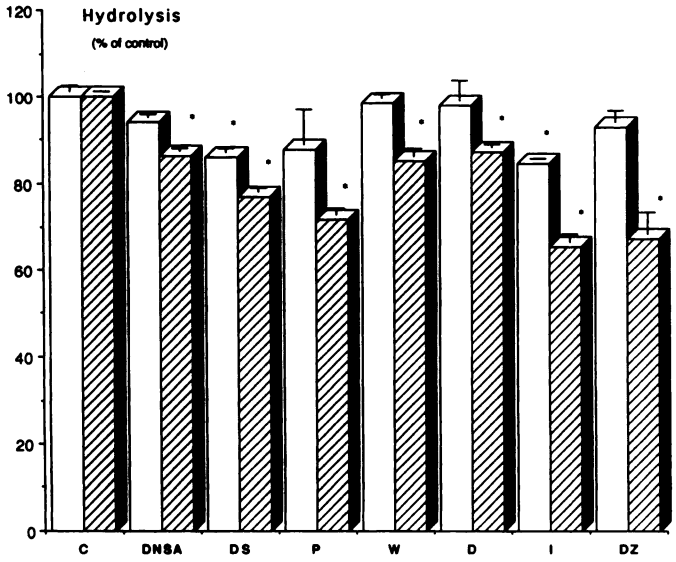


**Fig. 3.** Effects of addition of various specific binding probes on hydrolysis of (*R*)-ketoprofen (□) and (*S*)-ketoprofen (▨) glucuronide by HSA. *C*, Control; *DNSA*, dansylamide; *DS*, dansylsarcosine; *P*, phenylbutazone; *W*, warfarin; *D*, digoxin; *I*, ibuprofen; *DZ*, diazepam. *, $p < 5\%$ between probe and control (Student's *t* test) (two experiments). *Errors bars*, standard deviation. Values are expressed as percentages of a control without probe and are averages of replicate samples in two experiments. Probes (145 μM) were preincubated with HSA (145 μM) before addition of conjugate (145 μM). Assays were performed in duplicate, as described in Materials and Methods.

lysis was inhibited by 13% when HSA was treated with DIFP.

The effect of tyrosine alteration on the binding of (*R*)-ketoprofen, its conjugate, and two probes was investigated using CD. Dansylamide and dansylsarcosine were chosen for their large positive bands at 330 and 340 nm, respectively. The extent of binding of dansylamide or dansylsarcosine to control albumin was similar to that obtained with a fresh protein solution, indicating that exhaustive dialysis did not affect the protein sites. However, when one of these probes was added to the modified albumin, the extrinsic Cotton effect completely disappeared. The same effect was observed with (*R*)-ketoprofen and its glucuronide, even for high ligand concentrations.

## Discussion

The hydrolysis by HSA of ketoprofen glucuronoconjugates followed Michaelis-Menten-type kinetics, allowing the determination of apparent kinetic constants ($V_{max}$ and $K_m$). The catalytic reaction was actually mediated by HSA and probably not by contaminants, because albumin purity was >99% and no effect was observed with heat-treated albumin. Experiments were carried out at various albumin concentrations, because it has been suggested that aggregation of this protein might prevent access to the site of hydrolysis (7, 31).

The velocity of the HSA reaction varied according to the substrate and especially according to the structure of the acyl group. The protein can protect acyl glucuronides against hydrolysis, as has been reported for tolmetin and diflunisal conjugates (6, 10). Moreover, albumin has no esterase activity for the ethyl ester of oxaprozin or ketoprofen (4, 8). In the present study, the esterase activity was dependent on the

TABLE 2

**Effects of various ligands on the reversible binding of (*R*)-ketoprofen glucuronide to HSA, using CD**

Binding values are expressed as a percentage of the control with no ligand added, as described in Materials and Methods. Glucuronide (145 μM) was incubated with a 145 μM HSA solution and 145 μM specific probe at 20°.

| Ligand | Binding | Specific binding site |
|---|---|---|
| | *% of control* | |
| Dansylamide | 41.9 | Site I |
| Phenylbutazone | 65.1 | Site I |
| Warfarin | 111.7 | Site I |
| Ibuprofen | 67.3 | Site II |
| Diazepam | 0 | Site II |
| Digoxin | 79.7 | Site III |
| (*S*)-Ketoprofen glucuronide | 63.9 | ND[a] |

[a] ND, not determined.

stereochemical configuration of the acyl moiety, because (*S*)-ketoprofen glucuronide was a poorer substrate than its diastereoisomer. Such stereoselectivity for the glucuronide of ketoprofen was also observed by Hayball *et al.* (8) and has been reported for fenoprofen (3) and carprofen (9), but the opposite has been found for flurbiprofen glucuronoconjugate (5). Interestingly, the buffer alone also exhibited a stereoselective hydrolysis, but to a lesser extent than in albumin solution and with an opposite preference. The same result has been found with fenoprofen (3).

Although the $k_{cat}$ values ($V_{max}/K_m$) were not affected by either the albumin concentration or the presence of the other diastereoisomer, the apparent kinetic constants were both altered. The $K_m$ and $V_{max}$ values for (*R*)-ketoprofen glucuronide increased with the protein concentration, probably because of an enhancement of the number of catalytic sites. However, the $K_m$ value decreased for the (*R*)-ketoprofen conjugate when it was mixed with the glucuronide of its antipode. CD studies showed that the (*S*)-ketoprofen glucuronide displaced the (*R*)-ketoprofen conjugate from its binding site. This effect could increase the free fraction available for hydrolysis, inducing then a decrease in the $K_m$ of the (*R*)-ketoprofen conjugate. Moreover, the binding of (*S*)-ketoprofen glucuronoconjugate to albumin may alter the protein conformation, thus enhancing the affinity of each HSA molecule and decreasing the hydrolysis rate for the conjugate of its antipode. A competition study could not be carried out because the extent of hydrolysis of (*R*)-ketoprofen glucuronide was not reduced enough after addition of (*S*)-ketoprofen conjugate at high concentrations. Albumin also catalyzed the hydrolysis of the glucuronoconjugate of (*S*)-ketoprofen, but to a lesser extent. Its affinity per mole of HSA increased with protein concentration, in contrast to that of (*R*)-ketoprofen glucuronide. It is possible that aggregation of albumin (31) produces a conformational change that increases the reversible binding of the glucuronide of (*S*)-ketoprofen but not that of its isomer. The racemate has an intermediate behavior because it is a mixture of a good substrate and a poor substrate.

Because HSA binding sites are well characterized, we attempted to locate by competition the domain responsible for glucuronide hydrolysis, using specific binding probes, i.e., dansylsarcosine, ibuprofen, and diazepam for site II, dansylamide, warfarin, and phenylbutazone for site I, and digoxin for site III (22, 23). Probes were used at the same molar concentrations as albumin, so that one molecule bound to one

site. The inactivation was low and was not large enough for a complete inhibition study. Although (*S*)-ketoprofen glucuronide was a poor substrate, its hydrolysis was more inhibited by these probes than was that of its antipode. Moreover, because the (*R*)-ketoprofen metabolite has a greater $K_m$ than does the (*S*)-ketoprofen metabolite, the hydrolysis of the (*R*)-ketoprofen metabolite should have been more affected by ligands. The probes should affect primarily the hydrolysis rate.

A relationship between the binding process and the hydrolysis reaction was sought by evaluating the reversible binding of the conjugates. Only the binding of (*R*)-ketoprofen acyl glucuronide to HSA in the presence of probes was investigated by CD measurements; warfarin had no effect, in contrast to diazepam, ibuprofen, phenylbutazone, dansylamide, and digoxin. The experiment was not conducted with dansylsarcosine because its binding to HSA generated an extrinsic Cotton effect at exactly the same wavelength as the substrate (340 nm). Although ibuprofen and diazepam were bound to the same site, ibuprofen had a weaker displacing effect on metabolite binding than did diazepam. In fact, ibuprofen is known to also bind to other sites than its primary one. Except for warfarin, percentages of inhibition of reversible binding were greater than those found for hydrolysis. These findings suggest that (*R*)-ketoprofen glucuronide is hydrolyzed by HSA at a site distinct from its reversible binding site.

The binding of (*S*)-ketoprofen glucuronide to HSA was not investigated using CD because it did not induce an extrinsic Cotton effect. However, this conjugate affected the binding of its antipode, as shown by the decrease of ellipticity induced by the formation of a complex between albumin and (*R*)-ketoprofen glucuronide. Nevertheless, the apparent kinetic constants revealed a better affinity for (*R*)-ketoprofen conjugate when it was associated with the other isomer. Therefore, using CD, we observed a binding process for (*R*)-ketoprofen metabolite that was not related to the hydrolysis reaction.

The use of specific probes did not allow us to locate the domain on HSA involved in the hydrolysis, because none inactivated the reaction completely. Therefore, we attempted to modify albumin with two chemical reagents. Proteases and esterases contain in their active center a serine or cysteine residue (30). Unfortunaly, even a large molar excess of iodoacetic acid did not produce any notable alteration of the single free cysteine residue in HSA (32). This residue must be inside the protein structure, inaccessible to the reagent, and is therefore probably not involved in the hydrolysis of ketoprofen glucuronide.

Means and Wu (33) reported that DIFP reacted with a single reactive tyrosine residue in HSA. Similar results were obtained in our study, in which a 10-fold molar excess of DIFP did alter 1 tyrosine residue/mol of protein. Means and Wu (33) used the same excess of reagent to incorporate 0.97 equivalents of label from $^3$H-labeled DIFP into HSA. Among the 18 tyrosine residues of HSA, nine are close enough to the surface of the protein to be accessible to small molecules and only a few seems to be fully exposed (34). In our study, 8 tyrosine residues/molecule of native albumin were detected. Although the tyrosine residue in albumin reacted with DIFP, no reaction was observed with a tyrosine solution (data not shown), which suggests that the neighboring amino acid residues increase the reactivity of the tyrosine residue in the protein. Means and Wu (33) described the effect of ionic strength on the reaction of DIFP with HSA. The reactive tyrosine residue of albumin thus appears to be located on the surface of the protein, in a largely nonpolar environment adjacent to several positively charged groups. These cationic groups might account for both its stabilized reactive anionic form and the abnormally low p$K_a$ value of the phenolic group (about 8 instead of 10). We did not study the pH dependence of the reaction between glucuronide and albumin, to specify the p$K_a$ of the reactive amino acid residue, because of the instability of the conjugates at high pH. Fehske *et al.* (35) showed the existence of one tyrosine residue (probably Tyr-411) whose reactivity toward tetranitromethane, DIFP, and *p*-nitrophenyl ester was about 20 times that of the other tyrosine residues. This residue was probably modified in our experiment with DIFP.

Because this amino acid is located near sites I and II (32), the binding of dansylamide, dansylsarcosine, and (*R*)-ketoprofen to DIFP-modified albumin was evaluated using CD. The extrinsic Cotton effect for these ligands disappeared even at high concentrations. Exhaustive dialysis did not explain these results; the extent of ellipticity was the same for control HSA and HSA from a fresh solution. After alteration of a tyrosine residue on HSA with tetranitromethane, Fehske *et al.* (35) observed a large reduction of extrinsic Cotton effects of ligands bound to site II only. Substitution with the bulky diisopropyl phosphate group led to a modification, thus preventing access to sites I and II. Moreover, the alteration of the tyrosine residue also inhibited the binding of (*R*)-ketoprofen glucuronide.

The inactivation of the tyrosine residue significantly but weakly inhibited the hydrolysis of (*R*)-ketoprofen conjugate; 87% of the reaction remained after treatment with DIFP. Therefore, Tyr-411 was not involved in the catalysis. Wells *et al.* (4) obtained different results for oxaprozin glucuronide; chemical modification of a single tyrosine residue with DIFP caused complete loss of esterase activity of HSA.

These data suggest again that the reversible binding site is distinct from the site of hydrolysis; albumin alteration has a strong effect on reversible binding of the (*R*)-ketoprofen conjugate to HSA, in contrast to its hydrolysis. This result confirms the hypothesis of Hayball *et al.* (8). Previous studies have reported the protective effect of HSA toward glucuronides of diflunisal and tolmetin; the conjugate probably binds to the reversible binding site with a higher affinity, compared with the site of hydrolysis. Our study leads to an opposite conclusion in the case of ketoprofen glucuronide.

In conclusion, the hydrolysis reaction catalyzed by HSA was shown to proceed via the formation of a Michaelis-Menten-type complex. The protein exhibited substrate stereospecificity, because reaction with the (*R*)-ketoprofen conjugate had the highest velocity. This stereoselective hydrolysis is likely to affect the ketoprofen *R/S* ratio *in vivo*. Specific binding probes slightly inactivated the metabolite hydrolysis, but some of them inhibited (*R*)-ketoprofen glucuronide binding to HSA. Tyr-411 was probably not involved in the esterase activity but was located in the reversible binding site of (*R*)-ketoprofen glucuronide.

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