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STEREOSELECTIVE BINDING OF THE GLUCURONIDE OF KETOPROFEN ENANTIOMERS TO HUMAN SERUM ALBUMIN

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Abstract—Since acyl glucuronides are known to undergo deconjugation, especially in the presence of human serum albumin (HSA), only a few reports have described their reversible binding to plasma proteins. The aim of this study was to investigate the reversible binding of *R* and *S* ketoprofen glucuronides to HSA by a rapid technique, such as ultraviolet circular dichroism. Binding of *R* ketoprofen glucuronide only induced an extrinsic Cotton effect at 340 nm. Scatchard plot analysis revealed that *R* ketoprofen and its glucuronide are bound to one site of albumin with an association constant of 28.1×10^4 and 6.1×10^4 M^{-1}, respectively. Modification of one tyrosine residue by diisopropylfluorophosphate prevented the access of ligands to sites I and II of albumin, and also fully inhibited the binding of *R* ketoprofen and that of its conjugate. Displacement experiments with specific probes of albumin binding sites suggested that *R* ketoprofen and the glucuronide are bound to site II rather than site I. However, *R* ketoprofen was not displaced by its conjugate. *S* ketoprofen glucuronide is also bound to HSA, since it decreased the binding of the antipode conjugate. However, the binding of this metabolite to albumin did not induce an extrinsic Cotton effect large enough to determine the binding constants. D-Glucuronic acid did not bind to sites I or II of albumin. This moiety is likely responsible for the lower affinity of HSA for the *R* ketoprofen glucuronide when compared to that for *R* ketoprofen, due to the hydrophilicity and/or the bulkiness of this group.

Key words: acyl glucuronide; nonsteroidal anti-inflammatory drugs; reversible binding; circular dichroism; human serum albumin

Many NSAIDs‖ are predominantly cleared as acyl glucuronides in humans [1]. Conjugation of xenobiotics with D-glucuronic acid catalysed by UDP-glucuronosyltransferases has been assumed to act as a detoxication pathway by reducing their lipophilicity. However, acyl glucuronides unlike ether glucuronides, are labile under physiological conditions [2, 3]. They can undergo hydrolysis, releasing the parent aglycon, as well as intramolecular rearrangement. This latter phenomenon proceeds *via* acyl migration of the drug moiety from the carbon 1 of the glucuronic acid ring to the other positions, leading to isomers not cleaved by β glucuronidase. In addition, drug–protein adducts may be formed after covalent binding of acyl glucuronides to proteins [3]. Each of these processes has been reported for a number of drugs including fenoprofen [4], oxaprozin [5], carprofen [6], ketoprofen [7, 8], zomepirac [9, 10] and tolmetin [11].

NSAIDs show a high degree of binding to HSA (generally 99%, and 80% for salicylate) [12]. Their binding characteristics to plasma proteins determine not only their pharmacological activity, but also their pharmacokinetic properties. The binding of acyl glucuronides to proteins can explain the variation in the renal clearance of the drugs. Precise knowledge about the properties and location of the HSA binding sites for these metabolites would also help understand and predict drug–glucuronide interactions. Several studies have described the reversible binding to plasma proteins [8, 11, 13–17]. Since HSA exhibits an esterase activity towards glucuronoconjugates [2–9] in addition to spontaneous hydrolysis, their free fractions have to be determined rapidly.

Ketoprofen [*R*, *S* 2-(3-benzoylphenyl)propionic acid] is an NSAID mainly excreted as acyl glucuronide in humans [18]. Significant concentrations of ketoprofen conjugates have been detected in the plasma of elderly patients with impaired renal function [19] and in young healthy subjects after co-administration of probenecid [20]. In the present study, the reversible binding of ketoprofen acyl glucuronide to HSA was examined using circular dichroism and then compared to that of the aglycon. An attempt was made to locate the binding site of the glucuronoconjugate on the protein using albumin modified with DIFP and specific probes of HSA

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‖ Abbreviations: HSA, human serum albumin; NSAID, nonsteroidal anti-inflammatory drug; DIFP, diisopropylfluorophosphate.

binding sites. The binding of D-glucuronic acid to HSA was also studied to elucidate the contribution of this moiety to glucuronide binding.

MATERIALS AND METHODS

Chemicals. The enantiomers of ketoprofen were a gift from Rhone Poulenc Rorer (Paris, France). HSA, essentially fatty acid free (fatty acids less than 0.005%) (batch 42H9313), dansylamide, dansylsarcosine, digoxin and L-tyrosine were purchased from the Sigma Chemical Co. (l'Isle d'Abeau, Chesnes, France). Warfarin was provided by Merrel-Dow (Levallois-Perret, France), phenylbutazone by Ciba-Geigy (Rueil-Malmaison, France), diazepam by Roche (Neuilly sur Seine, France) and ibuprofen by Boots-Pharma (Courbevoie, France). DIFP was obtained from Aldrich (l'Isle d'Abeau, Chesnes, France). The other chemicals used in the study were of analytical grade.

Synthesis of ketoprofen glucuronide. The glucuronide of each enantiomer was prepared *in vitro* from phenobarbital-treated rat liver microsomes immobilized in alginate beads as described previously [21]. Products were purified using semi-preparative HPLC with a Lichrosorb RP 18 (250 × 10 mm, 7 μm, MERCK) and the mobile phase consisted of acetonitrile/water/trifluoroacetic acid (80:120:0.08, by vol.) (pH = 2.2). Detection was performed at 256 nm and the eluted glucuronide was collected and lyophilized. The amount of product biosynthesized was determined after alkaline hydrolysis (2 M NaOH) and assay of the ketoprofen released, according to a method previously proposed [22]. The structure as ketoprofen glucuronide was characterized by mass spectrometry and ^{1}H-NMR [23].

Protein binding study. Protein binding of ketoprofen enantiomers and their glucuronides was evaluated *in vitro* by circular dichroism. Experiments were carried out with a Jobin Yvon IV dichrograph (Paris, France). Spectra were performed in the range of 280–450 nm with an optical pathlength of 10 mm.

Solutions of albumin (145 μM) and glucuronides were prepared in an isotonic Sörensen 0.067 M phosphate buffer (pH = 7.4) and ketoprofen enantiomers were dissolved in methanol (8 mM). The concentration of ligand varied from 1.45 to 290 μM in a volume below 5% of the total volume. Experiments were performed at 20° and in a short course (below 20 min) to limit degradation, intramolecular rearrangement and covalent binding of acyl glucuronide (less than 2%). The bound concentrations were determined by analysing the ellipticity at the maximum wavelength *versus* ligand concentration plot, according to the method suggested by Rosen [24].

The displacement of ketoprofen and its glucuronide by several specific probes of HSA binding sites was also investigated using circular dichroism. Binding of warfarin and dansylamide generated a positive band with a maximum at 335 and 330 nm, respectively, and diazepam and phenylbutazone a negative band at 320 and 315 nm, respectively. Ibuprofen or digoxin bound to albumin did not induce an extrinsic Cotton effect over the wavelength range studied. The protein was pre-incubated with the conjugate or ketoprofen at equimolar concentration (145 μM) prior to addition of increasing concentrations of probes (72.5; 145 and 290 μM). The same procedure was applied with D-glucuronic acid rather than ketoprofen or glucuronide. The effects observed with the probes were compared to a control experiment without probe. For the probes producing an extrinsic Cotton effect, ellipticities were measured at a wavelength for which only glucuronide or ketoprofen displayed a signal.

Chemical modification of HSA. A 145 μM HSA solution in 125 mM Tris–HCl buffer (pH = 8.2) was treated with a 10-fold molar excess of DIFP at 30° for 2 hr. Tyrosine residues in HSA were assayed according to the method of Murachi *et al.* [25]. The modified albumin solution was exhaustively dialysed (60 hr) against an isotonic Sörensen 0.067 M phosphate buffer (pH = 7.4). Protein concentration was determined using the Bicinchoninic Acid Protein Assay kit (Sigma) and the solution was diluted to 72.5 μM. The control experiment consisted of albumin subjected to identical treatment but without the chemical reagent. A binding study was carried out by circular dichroism with a freshly prepared protein solution and was compared both to control and DIFP modified albumin solutions using dansylamide, dansylsarcosine, *R* ketoprofen and its conjugate as ligands.

Mathematical analysis. The experimental data of binding were fitted according to the Scatchard model for one class of sites. The parameters involved, i.e. the number of binding sites, n, and the association constant for these sites, k, were determined with an optimization method by quadratic polynomial interpolation with the least squares criterion using the Siphar software (Version 3.3, Créteil, France) installed on an IBM AT microcomputer.

RESULTS

The reversible binding of ketoprofen and its acyl glucuronide to HSA (145 μM) was investigated by circular dichroism.

The binding of each ketoprofen enantiomer to albumin generated a negative extrinsic Cotton effect at 342 nm whose extent depended on the ligand concentration used (Fig. 1a). According to the Scatchard model, both isomers are bound to approx. one site of HSA with a similar affinity, indicating that the protein lacked stereospecificity under the experimental conditions used (Table 1).

In contrast, the addition of *S* ketoprofen glucuronide to the albumin solution induced a positive band of very low intensity at 345 nm (data not shown). Thus, no further study could be carried out with this metabolite.

The binding of *R* ketoprofen glucuronide to HSA produced a large negative extrinsic Cotton effect with a maximum at 340 nm (Fig. 1b). The addition of various concentrations of conjugate (from 1.45 to 290 μM) to the 145 μM HSA solution resulted in an enhancement of ellipticity. The Scatchard plot of the binding data of *R* ketoprofen glucuronide is shown in Fig. 2b. The conjugate appeared to bind to approx. one site per albumin molecule with an association constant of 6.1×10^4 M^{-1} (Table 1).

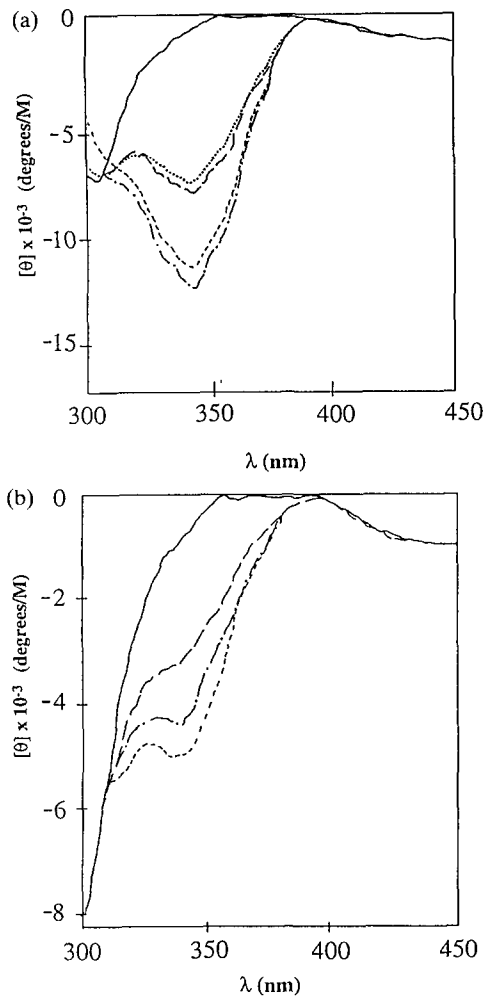

Fig. 1. Circular dichroism spectra of HSA (145 μM) alone (—) and in the presence of *R* and *S* ketoprofen (a) and of *R* ketoprofen glucuronide (b) at various concentrations. (a) (. . .), (----) for *R* ketoprofen at 72.5 and 145 gmM, respectively, and (--), (—.—) for *S* ketoprofen at the same concentrations, respectively. (b) (--), (—.—) and (----) at 72.5, 145 and 290 μM, respectively.

Table 1. Binding parameters for the ketoprofen enantiomers and for *R* ketoprofen glucuronide to HSA

Ligand	n	k (M^{-1}) × 10^4
R ketoprofen	0.86 ± 0.02	29.7 ± 3.6
S ketoprofen	0.92 ± 0.02	28.1 ± 4.2
R ketoprofen glucuronide	0.92 ± 0.03	6.1 ± 0.8

The study was carried out in a 145 μM HSA solution, with ligand concentrations ranging from 14.5 to 290 μM. k and n are the association constant and the number of saturable binding sites per albumin molecule, respectively. Values are means ± SD (two individuals curves with eight experimental points).

Albumin was treated with DIFP to identify the binding domain of *R* ketoprofen glucuronide on HSA. A 10-fold molar excess of this reagent allowed the modification of one tyrosine residue. The binding of *R* ketoprofen, its conjugate, dansylamide and dansylsarcosine was studied with the 72.5 μM modified HSA solution using circular dichroism. The protein modification itself did not generate a Cotton effect within the wavelength range studied. The binding of these ligands to modified HSA was fully inhibited when compared to the binding in the control albumin solution, whereas this latter and a freshly prepared protein solution exhibited the same behavior (data not shown).

The displacement of *R* ketoprofen glucuronide by digoxin, ibuprofen, diazepam, phenylbutazone, warfarin and dansylamide was also examined in order to locate the binding site of the glucuronide on albumin and was compared to that obtained for *R* ketoprofen. The binding extent of *R* ketoprofen glucuronide was slightly affected by digoxin and completely inhibited by diazepam (145 μM) or by dansylamide (290 μM) (Fig. 3a). Ibuprofen and phenylbutazone had an intermediate effect, whereas warfarin had no significant displacement effect. The *S* ketoprofen conjugate also decreased the binding extent of the other diastereoisomer to HSA. *R* ketoprofen was strongly displaced by ibuprofen and diazepam, but not by phenylbutazone, digoxin and *R* ketoprofen glucuronide (Fig. 3b).

Finally, binding experiments were carried out with D-glucuronic acid as a ligand. No extrinsic Cotton effect was obtained in the wavelength range analysed and no displacing effect was observed for phenylbutazone, warfarin, dansylamide or dansylsarcosine (data not shown).

DISCUSSION

Protein binding studies, usually carried out by equilibrium dialysis, provide a characterization of all classes of sites. However, the instability of acyl glucuronides, especially in the presence of HSA, and their ability to form covalent adducts, require faster methods to determine their protein binding. The formation of an asymmetrical complex between the protein and the ligand induce an extrinsic Cotton effect in the wavelength range of the ultraviolet absorption bands. Circular dichroism is then a suitable technique for estimating the bound concentration of a compound. Sjöholm and Sjödin [26] suggested that these complexes with k above 10^4 M^{-1} can generate a strong optical activity, and thus, measurements of the induced ellipticity may be treated assuming one independent class of binding sites.

In the present study, the binding of *R* and *S* ketoprofen to HSA showed that the protein was not stereoselective. However, Rendic *et al.* [27] found a stronger binding of the *S* isomer by circular dicroism with the extent of ellipticity decreasing in the rank *S* form > *R* form. However, these authors studied the binding of each enantiomer at one molar ligand-to-protein ratio only (0.2) and for a 14.5 μM HSA solution. Moreover, a difference in ellipticity extent for both ligands is not necessarily directly related to

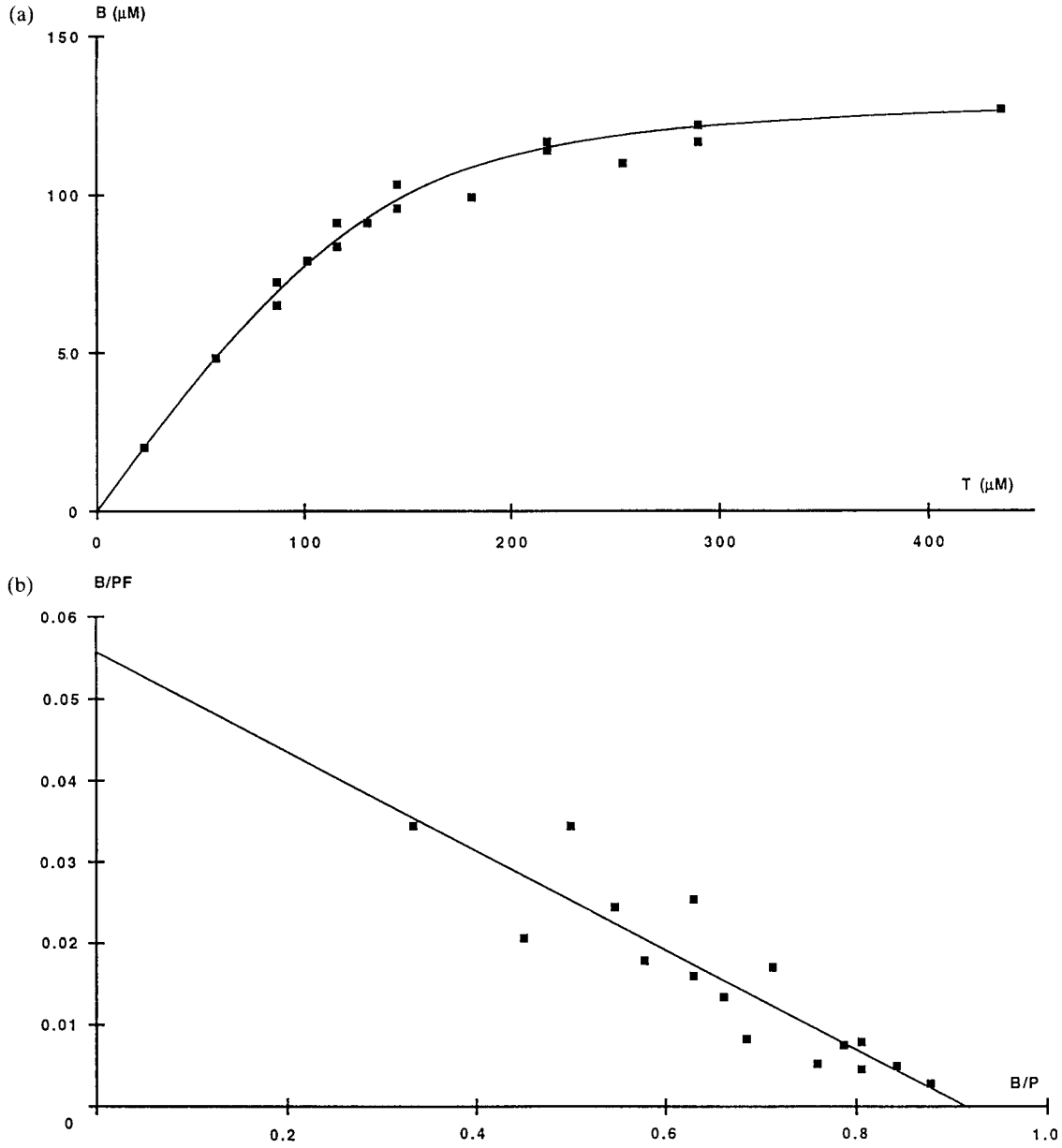

Fig. 2. Protein binding of *R* ketoprofen glucuronide to HSA (145 μM). (a) Variation of the bound concentration with the total concentration of conjugate. (b) Scatchard plot of binding data. B, F and T are the bound, free and total concentrations of conjugate, respectively. P corresponds to the albumin concentration. (—) optimization fitting by the Scatchard model.

a difference in binding. Rendic *et al.* [28] also investigated the binding of *R* and *S* ketoprofen by gel filtration. The stereoselectivity observed with HSA at 14.5 μM disappeared at 145 μM. A previous report has suggested that the concentration of 145 μM could be the crossover point for which no stereoselectivity was observed, since an inversion of stereoselectivity was obtained for HSA solutions at 14.5 and 290 μM [29].

The affinity of HSA for *R* ketoprofen glucuronide was high enough to generate a band and allow the determination of the binding constants. The spectrum was distinct from that of *R* ketoprofen itself. Our results revealed one binding site for the conjugate with an affinity lower than for *R* ketoprofen itself. The binding was significant since with an equimolar concentration of glucuronide relative to albumin, the fraction bound to protein still exceeded 70% (Fig. 2a). The glucuronides of oxaprozin [5], fenoprofen [14], valproic acid [17], suprofen and zomepirac [15] also exhibited considerable binding to HSA. Previous reports have also determined the binding parameters for the glucuronides of tolmetin [16] and carprofen [13]. For the former, the use

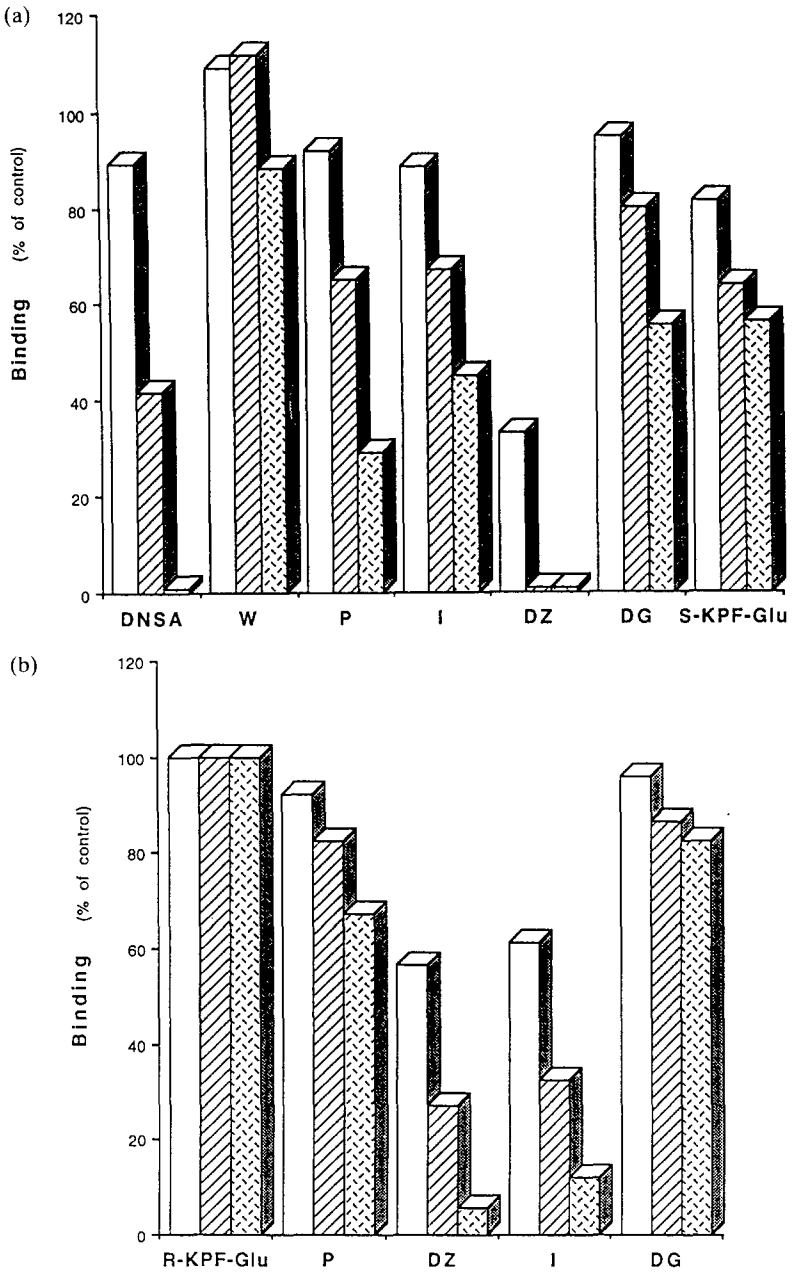

Fig. 3. Effect of addition of site specific probes on the binding of *R* ketoprofen glucuronide (a) and *R* ketoprofen (b) to HSA. DNSA, dansylamide; W, warfarin; P, phenylbutazone; DZ, diazepam; I, ibuprofen; DG, digoxin; R-KPF-Glu, *R* ketoprofen glucuronide; S-KPF-Glu, *S* ketoprofen glucuronide. *R* ketoprofen glucuronide or *R* ketoprofen was pre-incubated with HSA at equimolar concentration (145 μM) prior to addition of increasing concentrations of probes [(□) 72.5 μM (▨) 145 μM and (▤) 290 μM].

of the ultrafiltration technique resulted in the discrimination of two binding sites having affinity constants of 7.8×10^5 M^{-1} and 7.7×10^4 M^{-1} [16]. By ultrafiltration Iwakawa *et al.* [13] also found one binding site with an association constant of 9.5×10^5 M^{-1} and 5.0×10^5 M^{-1} for *S* and *R* carprofen glucuronides, respectively. Interestingly, HSA presented an esterase activity towards the conjugates of carprofen [6] and ketoprofen [7, 8], but not towards that of tolmetin [11]. In fact, the existence of two reversible binding sites with high affinity may protect the tolmetin conjugate from hydrolysis at the catalytic site, in contrast to carprofen and ketoprofen glucuronides.

The binding of *S* ketoprofen glucuronide to albumin was not studied by circular dichroism because it produced a very low extrinsic Cotton effect in contrast to the parent aglycon. The weakness

of such an effect does not mean that the molecule does not form a complex with the protein. Ellipticity only reflects asymmetry and a compound can bind to a protein without inducing this asymmetry. Moreover, the binding of *S* ketoprofen conjugate could generate a band in the wavelength range of the intrinsic Cotton effect of albumin, so that the ellipticity of the complex could not be measured. The presence of *S* ketoprofen glucuronide affected the binding of the other diastereoisomer, as shown from displacement experiments. Hayball *et al.* [8] have investigated the stereoselective binding of ketoprofen glucuronide to HSA by ultrafiltration. They reported a lower bound fraction (44%) than we observed (94%) for a molar *R* ketoprofen glucuronide to albumin ratio of 0.5. Their value probably differed from ours because of the different experimental conditions: they used glucuronide of *R* and *S* ketoprofen in mixture at equimolar concentration. In the present study, the binding of *R* ketoprofen glucuronide under the same conditions was only 60% of control, assuming that both diastereoisomers compete with each other for their HSA binding.

Chemical modifications of HSA were used in an attempt to determine the involvement of a residue in the glucuronide binding site. The alteration of one tyrosine residue by DIFP fully inhibited the binding of specific probes of sites I and II as well as the binding of *R* ketoprofen and its glucuronide. Fehske *et al.* [30, 31] showed that one tyrosine residue, located in the indole and benzodiazepine binding site of HSA (the so-called site II according to Südlow [32]) was highly reactive towards tetranitromethane and DIFP. Thus, its modification by tetranitromethane strongly affected the binding of diazepam but not that of warfarin [31, 33]. These findings suggested that the metabolite is bound to one of these sites, but did not specify the exact site involved. Substitution with the bulkier diisopropylphosphate group led to a protein modification which prevented access to sites I and II.

Specific probes of HSA sites I and II were also used to study their influence on the binding extent of *R* ketoprofen and its conjugate. Dansylamide, warfarin and phenylbutazone were chosen for their binding specificity to site I, ibuprofen and diazepam for site II, and digoxin for site III [32, 34–36]. Although circular dichroism spectra indicated the formation of a complex at one binding site, probes of both sites had different effects on glucuronide binding. Probes of site II produced a strong displacement of *R* ketoprofen conjugate from its binding site. Although ibuprofen and diazepam were bound to the same site, ibuprofen had a lesser influence than diazepam on the metabolite binding. Ibuprofen could also bind to other sites apart from its primary binding site and not occupied by the metabolite. In contrast, this probe induced a strong displacing effect for *R* ketoprofen since both ligands bound primarily to site II and secondarily to site I, and so compete with each other [34, 36]. Among specific probes of site I, warfarin did not affect the binding extent of *R* ketoprofen glucuronide. These results suggest that the metabolite would bind to site II rather than site I. The effect observed with phenylbutazone and dansylamide may be attributed to a conformational change of HSA upon their binding to the protein which led to a somewhat strong decrease in the binding extent of the glucuronide to site II. This conformational alteration was not great enough to produce a displacement of *R* ketoprofen probably because of a higher affinity of HSA. Hayball *et al.* [8] observed a minor displacement of *R* ketoprofen glucuronide by warfarin or diazepam. However, in contrast to the present study, albumin was preincubated with probes prior to addition of the conjugate and the affinity of the metabolite was probably too low to displace diazepam. Although circular dichroism allows the characterization of one class of sites, ultrafiltration gives information on binding to the whole albumin. Under these experimental conditions, when a probe is bound to its specific site, *R* ketoprofen glucuronide could bind to other sites. Various ligands were also used to investigate the binding of glucuronide of oxaprozin [5], carprofen [13] or tolmetin [16]. Diazepam inhibited the binding of the conjugates of tolmetin and oxaprozin, but not that of carprofen. Among these metabolites, only carprofen glucuronide was displaced by warfarin. In these reports, the aglycon itself was used as the displacing ligand. Carprofen and tolmetin had no displacing effect on their respective conjugates whereas oxaprozin reduced the binding of its metabolite. In this latter case, binding interactions between the drug and its conjugate were able to occur *in vivo* and could account for discrepancies between results of protein binding studies performed *in vitro* in plasma samples where no metabolite was present, and after administration of the drug *ex vivo*. Brouwer *et al.* [17] observed *ex vivo* a stronger binding for the glucuronide of valproic acid than for the drug. They concluded that the metabolite may displace the aglycon from its high affinity, low capacity serum binding site, since valproic acid bound to only one class of sites *ex vivo* instead of two classes *in vitro*.

In conclusion, no binding site on HSA was described as specific to various glucuronides. Although D-glucuronic acid did not bind to HSA, the conjugate did bind with low affinity or to a site distinct from that of the parent drug, probably either because of its large steric bulk or its hydrophilic properties. The present study suggests that the protein binding of *R* ketoprofen glucuronide may be significant and could be affected by the presence of the metabolite of *S* ketoprofen. However, its affinity is too weak to displace ketoprofen, and therefore, it is unlikely that the metabolite will increase the free fraction of the drug *in vivo*.

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