

STEREOSELECTIVE BINDING OF THE GLUCURONIDE CONJUGATES OF CARPROFEN ENANTIOMERS TO HUMAN SERUM ALBUMIN

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Abstract—The stereoselective binding of carprofen enantiomers and carprofen glucuronide diastereomers to human serum albumin (HSA) was studied using an ultrafiltration method. Carprofen glucuronides exhibit a considerable and stereoselective affinity to HSA, although less than that seen for the parent enantiomers. The (*S*)-glucuronide showed a higher binding affinity to HSA than the (*R*)-glucuronide. The (*S*)-enantiomer of unmetabolized carprofen was bound to fatty acid free HSA to a much greater extent than the (*R*)-enantiomer. Warfarin reduced the binding of the glucuronides to a greater extent than did diazepam, but diazepam displaced the unconjugated enantiomers to a greater extent than did warfarin. These results suggest differences in binding region between the carprofen enantiomers and their glucuronides on the albumin molecule.

Carprofen is a non-steroidal anti-inflammatory drug (NSAID) of the 2-arylpropionic acid class having a chiral carbon. It has been marketed in Europe for more than 5 years. Carprofen was approved by the FDA in 1988 but as yet has not been marketed in the United States. Most NSAIDs show a high degree of binding to albumin, and their binding characteristics to plasma proteins are primary determinants of their pharmacokinetic properties [1]. Recent papers [2–4] have described the stereoselective binding of phenylpropionic acids to plasma proteins in rabbits and rats. Whitlam and Brown [5] reported extremely high binding (99.9%) of racemic carprofen to 1% human serum albumin (HSA) using an ultrafiltration method. However, stereoselectivity of the binding of carprofen enantiomers has not been described. In this study we examined stereoselective binding of carprofen enantiomers to HSA, and also investigated the binding of their acyl glucuronide metabolites to plasma proteins.

In our previous studies [6, 7], a low renal clearance (less than 50 mL/min) for carprofen acyl glucuronides was reported. The renal clearances of other acyl glucuronides of NSAIDs have been shown to be much higher than the glomerular filtration rate, e.g. ketoprofen glucuronide 540 mL/min [8], zomepirac glucuronide 400 mL/min [9], and tolmetin glucuronide 950 mL/min [10]. One reason for the low renal clearance of carprofen glucuronides may be binding to plasma proteins. Stereoselective protein binding of the ether glucuronides of oxazepam has been reported [11], with the glucuronide of (*S*)-oxazepam exhibiting a higher binding to human albumin than that of (*R*)-oxazepam glucuronide. In the case of ester glucuronides, only a few studies describe any reversible binding to plasma proteins [12, 13], and no stereoselectivity has been reported. One of the major difficulties in studying reversible binding

of acyl glucuronides is their instability at physiological pH [14–16]. Since it is necessary to separate unbound glucuronide rapidly, the equilibrium dialysis method is unsuitable for quantitating protein binding of acyl glucuronides. In this study we have used an ultrafiltration method for the separation of the unbound forms and studied the stereoselective binding of carprofen enantiomers and their glucuronides to human serum albumin (HSA) to elucidate the role of protein binding in the stereoselective disposition of carprofen in humans.

MATERIALS AND METHODS

Chemicals. The racemate and enantiomers of carprofen (as analytical standards) were supplied by the Hoffmann-La Roche Co. (Nutley, NJ). Carprofen enantiomers were prepared via formation of their diastereomeric salts with (*R*)-(+)- or (*S*)-(–)- α -methylbenzylamine and fractionated crystallization from acetone. The optical purity of (*R*)-carprofen was 98.8% and the (*S*)-enantiomer contained 3.1% of (*R*)-antipode. Human serum albumins, essentially fatty acid free and fraction V, clomifen, acetyl-digitoxin and probenecid were obtained from the Sigma Chemical Co. (St Louis, MO). Carprofen glucuronides were prepared from human urine after oral administration of each carprofen enantiomer [6]. The optical purity of (*R*)-carprofen glucuronide was 95.5% and the (*S*)-carprofen glucuronide contained less than 4% of (*R*)-carprofen glucuronide. Warfarin and diazepam were USP standards. (*S*)-Naproxen, the internal standard, was provided by Syntex Laboratories (Palo Alto, CA). Other chemicals were of analytical grade.

Protein binding study. For the separation of the unbound species, the Centrifree Micropartition System (Amicon Div., W.R. Grace & Co., Danvers, MA) was used. Each of the carprofen enantiomers or their glucuronides was dissolved in Krebs–Henseleit

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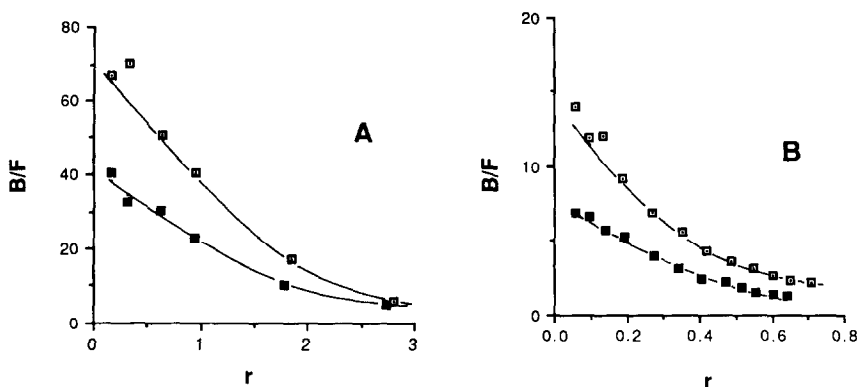


Fig. 1. Scatchard plots of binding data of carprofen enantiomers and their glucuronides: (A) enantiomer binding [(*S*)-form □, (*R*)-form ■]; (B) glucuronide binding [(*S*)-form □, (*R*)-form ■]. Fatty acid free HSA concentration was 2 mg/mL. Each point is a mean of three experiments.

Table 1. Binding parameters for the carprofen enantiomers and their glucuronides to fatty acid free human serum albumin

	<i>N</i>	<i>K_d</i> (μM)	<i>NS</i> (M ⁻¹)
(<i>S</i>)-Enantiomer	2.12 ± 0.07	0.93 ± 0.06	0.057 ± 0.005
(<i>R</i>)-Enantiomer	1.80 ± 0.06*	1.37 ± 0.08*	0.066 ± 0.004
(<i>S</i>)-Glucuronide	0.46 ± 0.02	1.05 ± 0.11	0.029 ± 0.002
(<i>R</i>)-Glucuronide	0.47 ± 0.02	1.99 ± 0.18*	0.015 ± 0.001*

Each value is the mean ± SD from three experiments.

K_d and *N* are the dissociation constant and the number of saturable binding sites per albumin molecule respectively. *NS* is the nonsaturable site (linear) binding parameter.

* Significantly different from the value of the antipode (*P* < 0.02).

phosphate buffer (pH 7.4) containing 2 mg/mL HSA. The concentration range for each enantiomer was 5–100 μM and that for each glucuronide was 1–30 μM. The concentration of HSA was 30 μM. One milliliter of sample was applied to the sample reservoir and centrifuged at 1000 *g* for 5 min (TJ-6R centrifuge, TA-24 rotor, Beckman Instruments, Palo Alto, CA) at room temperature. The filtrate was acidified with 2 M acetic acid as soon as possible to prevent hydrolysis of carprofen glucuronides, and the unbound concentration was determined according to the previously described method [6]. Briefly, carprofen diastereomeric glucuronides were determined directly by HPLC using a gradient program. Mobile phase composition varied as follows: from 0 to 15 min, 35% acetonitrile and 9 mM tetrabutylammonium chloride (TBA); for 15 to 20 min, 50% acetonitrile and 5 mM TBA; and from 20 to 30 min, 35% acetonitrile and 9 mM TBA with a total run time of 30 min. An Altex ultrasphere ODS (5 μm particle size, 4.6/150 mm) column was used with a flow rate of 2 mL/min. The excitation and emission wavelengths for the fluorescence detector were 290 and 365 nm respectively. To increase the sensitivity, unbound carprofen enantiomer concentrations were determined without diastereomeric derivitization using a pirkle D-naphthylethylamine column (5 μm, 4.6/250 mm) after derivitization of the enantiomers with 4-chloroaniline. The separation factor was 1.09 and the resolution factor was 1.40.

The detection limit for the glucuronides and enantiomers was 10 ng/mL. Adsorption of drugs to the filtration membrane was negligible. The total time required from the initial dissolution of glucuronide until the acidification of the filtrate was less than 40 min. Stability studies in 2 mg/mL HSA solution at room temperature indicated that the degradation half-lives for the carprofen glucuronides were more than 12 hr. At 37° the half-lives decreased to less than 4 hr [17].

The data were analyzed according to the following equation:

$$C_b = N \cdot P \cdot C_f / (K_d + C_f) + NS \cdot P \cdot C_f$$

where *C_b* is the concentration of drug bound to HSA, *C_f* is the unbound drug concentration, and *P* is the concentration of HSA. *K_d* and *N* are the dissociation constant and the number of saturable binding sites per albumin molecule respectively. *NS* is the nonsaturable site (linear) binding parameter. The number of nonsaturable binding sites per molecule is subsumed within this parameter.

RESULTS

In a preliminary study we had observed high binding (more than 99%) of carprofen enantiomers to HSA. To characterize the binding properties of carprofen enantiomers and their glucuronides under

Table 2. Binding of carprofen enantiomers and their glucuronides to human serum albumin

	% Unbound	
	Fatty acid free albumin	Fraction V albumin
(<i>S</i>)-Enantiomer	1.72 ± 0.36	2.12 ± 0.19
(<i>R</i>)-Enantiomer	2.16 ± 0.35*	1.38 ± 0.06*†
(<i>S</i>)-Glucuronide	8.0 ± 1.8	15.6 ± 1.0†
(<i>R</i>)-Glucuronide	14.2 ± 3.2*	24.5 ± 0.7*†

Each value is the mean ± SD from three experiments.

* Significantly different from the value of the antipode ($P < 0.02$).

† Significantly different from the value for binding to fatty acid free albumin ($P < 0.01$).

identical conditions, we examined the protein binding of each of the four compounds at 2 mg/mL (30 μ M) of fatty acid free HSA.

Scatchard plots of the binding data of the carprofen enantiomers and the carprofen glucuronide diastereomers are shown in Fig. 1. The binding of the (*S*)-enantiomer of carprofen was higher than that of the (*R*)-antipode. The (*S*)-glucuronide was also bound to fatty acid free HSA to a much greater extent than the (*R*)-glucuronide. These binding parameters are presented in Table 1. Major differences in the binding parameters of the stereospecific compounds were observed. The dissociation constant (K_d) of the carprofen (*S*)-enantiomer was less than 70% of that of the (*R*)-enantiomer. The K_d value of (*S*)-glucuronide was less than 55% of that of (*R*)-glucuronide. These binding differences between the glucuronides were also observed at a concentration range of 10–30 mg/mL HSA. When fraction V human serum albumin was used, the binding of the (*S*)-enantiomer was not significantly different from that for the binding to fatty acid free albumin (Table 2). But the binding of the (*R*)-enantiomer was increased when incubated with fraction V albumin. The binding of both glucuronides was decreased during incubation with fraction V albumin, with the (*S*)-glucuronide exhibiting higher binding than the (*R*)-glucuronide.

The carprofen enantiomers appeared to react with approximately two binding sites per fatty acid free albumin molecule, while for the glucuronides each diastereomer bound approximately two albumin molecules. At 2 mg/mL fatty acid free HSA, the percent unbound of the glucuronide diastereomers was approximately 5- to 7-fold greater than the corresponding carprofen enantiomers (Table 2). However, the extent of carprofen glucuronide albumin binding was significantly greater than that previously reported for any other acyl glucuronide [12, 13]. To elucidate the characteristics of the binding regions of the enantiomers and the glucuronides on the albumin molecule, the displacement effects of diazepam, probenecid, clomifen, acetyldigitoxin and warfarin were studied. Diazepam (0.1 mM) displaced both carprofen enantiomers with the unbound fraction of these enantiomers increased about 2-fold (Table 3). Probenecid (0.1 mM) also inhibited the binding of carprofen enantiomers (data not shown). The bind-

ing of carprofen enantiomers was not inhibited by the presence of clomifen (0.1 mM) or acetyldigitoxin (0.1 mM) (data not shown). Warfarin had no significant displacement effect on the carprofen enantiomers. In contrast, the binding of the two glucuronides was reduced by the addition of warfarin, but the displacing effect of diazepam on the glucuronides was insignificant (Table 3). A significant increase in the unbound fraction of the glucuronides was observed when racemic carprofen (0.1 mM) was added. But at a lower concentration (0.03 mM) of carprofen enantiomers, inhibition of glucuronide binding was not observed (Table 4).

DISCUSSION

This study revealed stereoselective binding to human serum albumin for both the carprofen enantiomers and the carprofen glucuronides. It was necessary to carry out the binding studies at albumin concentrations (2 mg/mL or 30 μ M) considerably less than found under physiologic conditions (35–50 mg/mL) for a number of reasons: (a) carprofen has a limited solubility in aqueous medium; (b) preliminary results indicated that albumin exhibits esterase-like activity and can hydrolyze the glucuronides of carprofen as previously described for oxaprozin glucuronide [13], and (c) only limited supplies of the pure carprofen enantiomers and glucuronide diastereomers were available. We used an ultrafiltration method to quantitate the unbound concentrations of each of the four stereoisomers to compare the binding characteristics of the parent enantiomers and their metabolites under the same conditions. The method minimized the degradation of the acyl glucuronides during the procedure to separate the unbound forms. Direct analyses of carprofen and glucuronide concentrations indicated that less than 4% of the glucuronides were degraded during the ultrafiltration and sample handling processes.

Whitlam and Brown [5] reported the bound percent of racemic carprofen (12.71 μ g/mL) to 10 mg/mL HSA (fraction V) to be 99.90 and 99.85% using ultrafiltration and equilibrium dialysis methods respectively. From the binding data found here, we calculate the bound percents for the carprofen enantiomers to be 99.92% for (*S*)-carprofen and

Table 3. Effects of warfarin and diazepam on the binding of carprofen enantiomers and their glucuronides to fatty acid free human serum albumin

	% Unbound		
	Control	+Diazepam	+Warfarin
(<i>S</i>)-Enantiomer	1.72 ± 0.36	3.92 ± 0.19*	1.40 ± 0.21
(<i>R</i>)-Enantiomer	2.16 ± 0.35	4.12 ± 0.39*	1.92 ± 0.12
(<i>S</i>)-Glucuronide	8.0 ± 1.8	6.4 ± 1.4	28.8 ± 2.9*
(<i>R</i>)-Glucuronide	14.2 ± 3.2	10.0 ± 0.3	39.9 ± 1.9*

The concentration of displacer was 0.1 mM. The concentration of fatty acid free HSA was 2 mg/mL (30 μ M). The concentration of the enantiomers was 5 μ M, and that of the glucuronides was 1 μ M. Each value is the mean \pm SD from three experiments.

* Significantly different from control value ($P < 0.01$).

Table 4. Effect of carprofen on the binding of carprofen glucuronides to fatty acid free human serum albumin

	% Unbound			
	Control	+(<i>S</i>)-Enantiomer (30 μ M)	+(<i>R</i>)-Enantiomer (30 μ M)	+ Racemate (100 μ M)
(<i>S</i>)-Glucuronide	8.9 ± 1.4	9.1 ± 1.2	7.0 ± 0.3	18.0 ± 1.9*
(<i>R</i>)-Glucuronide	12.8 ± 2.4	10.9 ± 0.8	12.4 ± 1.5	28.1 ± 2.0*

The concentration of fatty acid free HSA was 2 mg/mL (30 μ M). The concentration of the glucuronide was 1 μ M. Each value is the mean \pm SD from three experiments.

* Significantly different from control value ($P < 0.01$).

99.43% for the (*R*)-enantiomer at the drug and protein concentrations utilized by Whitlam and Brown [5]. These calculated binding values for the enantiomers are almost identical to those experimentally found by Whitlam and Brown for the racemic mixture.

The K_d values (Table 1) were similar for the four compounds, but the numbers of binding sites for the glucuronides were approximately one-fourth those of the carprofen enantiomers. The (*S*)-configurations of both the carprofen enantiomer and its glucuronide had higher affinities to fatty acid free HSA, a finding consistent with the predominance of reports concerning the binding properties of acidic drugs having a chiral carbon [18].

Fatty acid can modulate the binding of various drugs to albumin [19]. Jones *et al.* [4] reported an inhibitory effect of fatty acid (oleic acid) on the stereoselective binding of 2-phenylpropionic acid to rabbit albumin. These workers indicated in preliminary binding studies that human plasma also showed greater binding of the (*R*)-enantiomer than the (*S*)-enantiomer to plasma proteins. Our results (Table 2) also showed a reversal of the strength of binding for the (*R*)-carprofen enantiomer to fraction V versus fatty acid free albumin. The binding difference for the (*R*)-enantiomer may be due to conformational changes in albumin structure due to fatty acids. For the glucuronides, binding was less for both (*R*) and (*S*) conjugates with fraction V albumin versus fatty acid free protein. At an HSA concentration of 40 mg/mL (0.6 mM) and when concentrations of each glucuronide are less than 10 μ g/

mL, the calculated binding of each glucuronide is greater than 99%. This high fraction bound for carprofen glucuronides exceeds any previous report for glucuronide conjugates. In fact, many glucuronides have been found to exhibit no binding to plasma proteins. Thus, a major reason for the low renal clearance of carprofen glucuronides in humans [6, 7] may be this high binding to plasma albumin.

There are two major drug-binding regions on the albumin molecule [20]. One is the warfarin site (warfarin and azopropazone binding area), also called site I. The other is the diazepam site (indole and benzodiazepine binding area), also called site II. The presence of tamoxifen and digitoxin binding sites on the HSA molecule was also reported [21]. Little inhibitory effect on the binding of carprofen enantiomers to HSA was observed for clomifen which binds to the tamoxifen binding site or acetyldigitoxin which binds to the digitoxin binding site. Because of limited supplies of the diastereomeric glucuronide conjugates, competition for the tamoxifen and digitoxin binding sites was not investigated.

The effects of warfarin and diazepam on the protein binding of the parent enantiomers and the diastereomeric glucuronic acid conjugates were different. Sudlow *et al.* [22] previously indicated that carprofen (Ro 20-5720) occupies the diazepam binding site. Our work supports this finding, where as demonstrated in Table 3 the addition of diazepam significantly decreased the binding of both enantiomers to HSA, while warfarin did not inhibit the binding of the enantiomers. In contrast, the binding of the carprofen glucuronides to HSA was reduced

by warfarin, but not by diazepam (Table 3). Low concentrations of carprofen (mole ratio to HSA 1:1) did not inhibit the binding of carprofen glucuronides (Table 4). However, an effect was noted when the concentrations of racemate was increased to 100 μ M (mole ratio to HSA 3.3:1). This latter result could be due to an overlap of the diazepam and warfarin binding sites as suggested by Bruderlein and Bernstein in examining the binding of cyclic analogs of tryptophan [23].

In conclusion, the results presented here suggest that the plasma protein binding of acyl glucuronides may be significantly greater than previously observed. At glucuronide concentrations measured *in vivo* following carprofen dosing to healthy volunteers, greater than 99% of the conjugates would be bound reversibly to plasma proteins. The *S*-configurations of both the carprofen enantiomer and its glucuronide had higher affinities to HSA than the corresponding antipodes. Competitive binding studies suggest that there is a difference in binding regions (areas) on the albumin molecule for the enantiomers versus the glucuronides of carprofen.

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