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Stereoselective Binding Properties of Naproxen Glucuronide Diastereomers to Proteins

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The stability of naproxen glucuronide (NAP-G) diastereomers was investigated in buffer, 0.3% and 3% human serum albumin (HSA) solutions, and human plasma. R-*NAP-G was found to be less stable in phosphate buffer than its* S-*diastereomer, whereas incubation media containing protein in general increased the degradation rate of NAP-G but also caused a change of the stereoselective stability where the* R-*NAP-G was more stable than* S-*NAP-G. Reversible binding of NAP-Gs to HSA (0.3%) was investigated and compared with the corresponding properties of naproxen (NAP) enantiomers. NAP-G diastereomers exibited a considerable and stereoselective affinity to HSA, although less than that observed for the NAP enantiomers.* In vitro *irreversible binding of NAP-Gs to HSA, human and rat plasma proteins was also investigated. Irreversible binding was higher for* R-*NAP-G (50 μM) than for* S-*NAP-G (50 μM) in all incubation media. This stereoselective difference was observed with HSA containing medium as well as in rat and human plasma. Incubation with unconjugated NAP did not lead to irreversible binding. Preincubation of HSA with acetylsalicylic acid (~11 mM) and glucuronic acid (50 mM) decreased the extent of irreversible binding suggesting involvement of lysine residues for covalent binding. Preincubation with* S-*NAP also decreased the irreversible binding yield.*

KEY WORDS: naproxen; naproxen glucuronide; stereoselective binding; covalent binding; reversible binding; degradation rates, acyl glucuronides.

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INTRODUCTION

Naproxen is a nonsteroidal antiinflammatory drug (NSAID) of the aryl-alkyl carboxylic acid class which is used mostly as an analgesic and in the treatment of rheumatic diseases. Although NSAIDs are widely used and highly useful, they can cause unexplained side effects. Many NSAIDs are chiral. Even though only one enantiomer (usually the *S*-) may have significant antiinflammatory activity, the drugs are usually marketed as racemates—with the notable exception of naproxen which is sold as its *S*-enantiomer [*S*-(+)-6-methoxy-(α-methyl)-2-naphthalene acetic acid].

An important biotransformation pathway for naproxen (50–60%) is conversion to the corresponding β-1-*O*-acyl glucuronide (Fig. 1), other metabolites being 6-desmethyl-NAP and its conjugates (1). Acyl glucuronides react with nucleophiles and are unstable at physiologic pH, undergoing hydrolysis and acyl migration (2–4). They can react with proteins, such as human serum albumin (HSA), to form two types of covalent adducts (5,6). In one type of adduct the aglycone moiety becomes bound directly to the protein by an amide or ester linkage with elision of the glucuronic acid moiety. In the other type of adduct the glucuronic acid moiety forms a Schiff base with a primary amino group on the protein and both the aglycone and

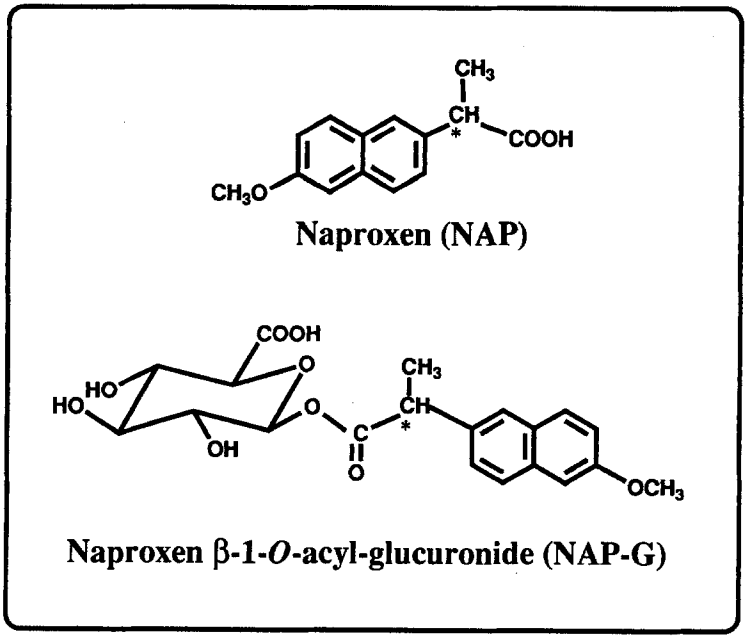

Fig. 1. Chemical structures of naproxen and naproxen β-1-*O*-acyl glucuronide.

the carbohydrate elements of the glucuronide are present in the final protein product. These adducts have been suggested to play a role in causing the hypersensitivity reactions associated with certain acidic NSAIDs (4).

Irreversible binding of acyl glucuronides to plasma proteins may be dependent on reversible binding. The reversible binding of carprofen acyl glucuonide has been studied (7), but there have been few studies on the reversible binding properties of other acyl glucuronides (8,9) and no studies on stereoselective interactions of acyl glucuronide diastereomers.

In this study, we compared the relative stabilities of glucuronides of *R*- and *S*-NAP and investigated their reversible binding to HSA, and irreversible binding to HSA, human plasma, and rat plasma proteins *in vitro*. Our results show that the chirality of the asymmetric center in naproxen has a substantial effect on the stabilities of the corresponding glucuronides as well as on the reversible and irreversible binding of the glucuronides to proteins.

MATERIALS AND METHODS

Chemicals

S-NAP (enantiomeric excess >0.98), phenylmethylsulfonyl fluoride (PMSF), β-glucuronidase (from bovine liver, Type B-1), HSA (essentially fatty acid free fraction 5), D-glucuronic acid (GA), D-glucose (Gluc), uridine diphosphoglucuronic acid (UDPGA), acetyl salicylic acid (ASA), α-methylbenzylamine, tryptophol, and homotryptophol were purchased from Sigma Chemical Co. (St. Louis, MO). *R*-NAP (enantiomeric excess >0.97) was provided by Syntex Laboratories (Palo Alto, CA) and *R*-flunoxaprofen was obtained from Ravizza Muggio, Italy. The enantiomeric purities of *R*- and *S*-NAP were determined by HPLC of their α-*S*-methylbenzylamine amides (10). Pooled human plasma was obtained from University of California, San Francisco Hospital Blood Bank, and pooled rat plasma from Sprague-Dawley rats. Other chemicals used were of analytical or HPLC grade.

High Performance Liquid Chromatography

Analyses of NAP and NAP-G were performed using a Beckman 114M solvent delivery system with a 421A controller (Beckman Instruments, Berkeley, CA), a Shimadzu RF-535 fluorescence monitor (excitation 275 nm, emission 355 nm) (Shimadzu Corp., Kyoto, Japan), a HP3390A or a HP3395A integrator (Hewlett-Packard Co., Avondale, PA), 5-μ Ultrasphere ODS columns (Beckman Instruments), and a mobile phase containing 25% acetonitrile, 66 mM ammonium acetate solution (v/v) with a final pH of 6.0 adjusted with glacial acetic acid (11). The column for preparative HPLC was 10 × 250 mm and the flow rate 3 ml/min. The column for analytical

HPLC was 4.5 × 250 mm and the flow rate 1 ml/min. With the latter column, retention times for *S*-, *R*-NAP-G, and NAP were ~9.9, 11.5, and 19.1 min, respectively. The retention times of the putative NAP-G positional isomers were ~12.1, 13.2, and 14.1 min. The resolution (1.1) between the β-1-NAP-G peak and the closest positional isomer was adequate for quantitative analysis. Homotryptophol (retention time 18.5 min) was used as internal standard for analyses of NAP-G, and tryptophol (retention time 10.9 min) for analyses of NAP. Sodium phosphate buffer (pH 7.4) spiked with *S*-NAP-G or *R*-NAP-G was used to establish calibration curves. Control studies on rat plasma or HSA solutions spiked with 0.16 μg/ml *S*-NAP and worked up as for the experimental samples showed that recoveries exceeded 95%. After addition of the internal standard, the buffered solutions were acidified similar to the samples. The lowest measured concentration for NAP was 0.02 μg/ml (0.1 μM) and for NAP-G 0.17 μg/ml (0.4 μM).

For irreversible binding studies, the HPLC conditions were modified as follows: In order to decrease the retention time of internal standard, flunoxaprofen, and to minimize interferences from plasma, the acetonitrile concentration was increased to 30% and detection was performed at excitation and emission wavelengths of 315 nm and 355 nm, respectively. NAP enantiomers were eluted at 9.0 min and flunoxaprofen at 16.4 min. The lowest measured concentration in the above studies was 0.005 μg/ml.

Preparation of Glucuronides

R-β-1-NAP-G was prepared *in vitro* using washed sheep liver microsomes (12). Briefly, 10 mg microsomal protein/ml, 100 mM Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 20 mM saccharic acid 1,4–lactone, 2 mM PMSF, 0.2% Triton X-100 and 1 mM *R*-NAP were preincubated for 5 min at 37°C and incubated for 1.5 hr at 37°C after addition of UDPGA. (Longer incubation resulted in formation of acyl migration products of *R*-NAP-G.) *S*-β-1-NAP-G was extracted from human urine after oral administration of *S*-NAP. The freshly voided urine was acidified (pH 2–3) at once with phosphoric acid and kept frozen until extracted.

Glucuronides extracted from urine or microsomal incubation mixtures were purified by preparative TLC (Silica, 0.5 mm, E. Merck, Darmstadt, Germany) using methanol: chloroform: acetic acid (25: 75: 3) as irrigant and bands were visualized with 355 nm light. The band at $R_f = 0.2$–0.3 was extracted with methanol. This mixture was filtered and the methanol evaporated. The residue was redissolved in a minimal volume of methanol. This solution was brought to turbidity by addition of isopropanol and the glucuronide precipitated with diethyl either at −20°C. When necessary, further purification to remove acyl migration products was done by preparative

HPLC. Eluted glucuronide fractions were acidified immediately to pH 4.2 with acetic acid, organic solvent was evaporated under reduced pressure at room temperature, and the residual aqueous phase was freeze-dried. The identities of *R*- and *S*-NAP-G were confirmed by negative ion liquid secondary ion MS, using cesium as ion source, and HPLC. The identity of *S*-NAP-G was also confirmed by ^1H-NMR. The NMR and MS data are presented elsewhere (13). Further purification and combustion analyses were not attempted because of the limited amounts of glucuronides available. Quantitation of acyl glucuronides was performed by cleavage with β-1-glucuronidase and HPLC determination of liberated NAP using tryptophol as the internal standard. The isomeric purity of each diastereomer, by HPLC, was about 98%.

Stability Studies

NAP-G diastereomers (32.7–58.9 μM) were incubated at 37°C in 0.1 M sodium phosphate buffer (pH 7.4) and 100 μl aliquotes were removed at timed intervals for analysis. Similar incubations were also run in the presence of 3 mg/ml (0.3%, 45 μM) or 30 mg/ml (3%, 450 μM) HSA, and in human plasma. Aliquots were mixed with 200 μl acetonitrile (containing 12 μl of 8 μg/ml homotryptophol internal standard and 5 μl of 10% sulfuric acid to stabilize the acyl glucuronides), precipitated protein was removed by centrifugation and 25 μl of the supernatant was analyzed immediately by HPLC.

Reversible Protein Binding Studies *In Vitro*

Measured aliquots of freshly prepared solutions of NAP enantiomers or their glucuronides in methanol were evaporated to dryness and the residue, containing 5.75–184 μg NAP or 0.84–125.4 μg NAP-G, was dissolved in 1 ml 0.1 M sodium phosphate buffer, pH 7.4, containing 3 mg/ml (0.3%, 45 μM) HSA. The solutions were immediately transferred to the sample reservoir of a Centrifree Micropartition System (Amicon Div., W. R. Grace & Co., Danvers, MA), centrifuged at $1000 \times g$ (TJ-6R centrifuge, TA-24 rotor, Beckman Instruments, Palo Alto, CA) at ambient temperature, and a 150 µl aliquot of the filtrate was immediately acidified with 6 μl of 10% sulfuric acid, spiked with 20 μl internal standard (1.3 μg/ml tryptophol or 6 μg/ml homotryptophol in methanol), and stored at −20°C until HPLC analysis. Samples were run in triplicate. Although the time interval from dissolving the NAP-G in methanol to the final acidification of the filtrate was less than 30 min, about 5–7% of each β-1-*O*-acyl glucuronide degraded during the procedure. Control studies with solutions of NAP or NAP-G in

the absence of protein indicated no measureable adsorption of drug or drug glucuronide to the membrane.

Initial estimates of the binding parameters were obtained graphically by Scatchard plots of the binding data. These plots indicated that the binding parameters for NAP enantiomers could be estimated from

$$C_b = \frac{N_1 \cdot P \cdot C_f}{(K_{d,1} + C_f)} + \frac{N_2 \cdot P \cdot C_f}{(K_{d,2} + C_f)} \quad (1)$$

which assumes two different classes of independent binding sites (14,15), whereas binding parameters for NAP-G could be better estimated using the more general equation

$$C_b = \frac{N_1 \cdot P \cdot C_f}{(K_{d,1} + C_f)} + NS \cdot P \cdot C_f \quad (2)$$

where C_b is the concentration of drug bound to HSA, C_f is the free (unbound) drug concentration, and P is the concentration of HSA (molecular weight used for calculation 65,000). N_1 and N_2 are the numbers of primary and secondary class binding sites, respectively, and $K_{d,1}$ and $K_{d,2}$ are the corresponding dissociation constants. NS is the nonsaturable site (linear) binding parameter, within which is subsumed the number of nonsaturable binding sites per molecule. Binding parameters were calculated on a Macintosh computer with the iterative nonlinear regression program MINIM 1.8 using the Gauss-Newton-Marquardt algorithm minimizing the weighted sum of squared residual errors over all data points obtained in each experiment and evaluated by analysis of variance with the program Statworks.

Irreversible Binding of NAP-G to HSA and Plasma Proteins *In Vitro*

R- or *S*-NAP-G (20.9 μg/ml, 50 μM) were incubated at 37°C for 21.8 hr in 0.1 M phosphate buffer containing 30 mg/ml (3%, 450 μM) HSA, in rat plasma with and without 0.35 mg/ml (2 mM) PMSF (an esterase inhibitor), and in human plasma. Each experiment was performed in triplicate. To confirm the reproducibility of the experiments, the binding study in 3% HSA was repeated using two different lots of *R*- and *S*-NAP-G. The maximal binding yields for the different batches of each glucuronide diastereomer were within 5% of one another.

Aliquots (500 μl) of each reaction mixture were taken at different time points to assay covalently bound drug. The binding reaction was stopped by precipitating the protein by sequential addition of 1 vol of refrigerated isopropanol and 4 vol of acetonitrile:phosphoric acid (3:0.01, v/v). The binding yield for irreversible binding of NAP-G was quantified using a

slightly modified established method (16). The protein pellets obtained after centrifugation were washed several times (≥ 7; washing procedure: 10-min mixing followed by 20 min of sonication and 10-min centrifugation at $3000 \times g$) with 4 ml of methanol:ether (3:1, v/v) to remove the reversibly bound NAP and conjugates. The residual pellet, containing irreversibly bound NAP and/or NAP-G, was dissolved in 1 ml 0.5 M aqueous potassium hydroxide and incubated at approximately 80°C overnight to release NAP from the protein. The protein digest was spiked with 20 μl of 25 μg/ml flunoxaprofen internal standard, and acidified to pH 3 with 50 μl concentrated phosphoric acid. The acidified digest was extracted with 5 ml of methylene chloride, the organic phase was evaporated under nitrogen, and the residue was reconstituted with 200 μl mobile phase. Aliquots (25 μl) of this solution were injected onto the HPLC for quantification of NAP.

Standard curves were constructed from dried blank protein pellets (HSA or plasma proteins) to which *S*-NAP (0.05–1.25 μM) was added. Inter- and intraday coefficients of variation were determined by analyzing 6 samples of *S*-NAP at a concentration of 0.16 μg/ml (0.3 μM) on 5 different days. Intra- and interday coefficients of variation (*CV*) were less than 6% (intraday *CV* 5.9%, interday *CV*, 4.6%). NAP is stable under the basic conditions employed. Protein concentration in each of the hydrolysates was measured by Bicinchoninic Protein Assay (Pierce, Rockford, IL). Binding yields are reported as moles of NAP per mole of protein.

To investigate the effect of potential competitors on the extent of irreversible binding of NAP-G diastereomers, the competitors were preincubated with HSA or plasma (containing 0.01% NaN_3 to inhibit bacterial growth) at 37°C for 16.5 hr before addition of NAP-G to the solution. The experiments were performed at two different concentrations of the competitors. The extent of irreversible binding of NAP to protein was determined after 3.8 hr of reaction time for the lower concentrations and after 2.6 hr for the higher concentrations of competitors. By these times, maximum irreversible binding had already been reached. The potential competitors and their tested concentrations are as follow: acetylsalicylic acid (ASA; 1.1 mM, ~11 mMl, D-glucose (Gluc; 20 mM, 50 mM), D-glucuronic acid (GA; 20 mM, 50 mM). One concentration of 0.2 mM was used for *S*-NAP.

RESULTS

Stability of NAP-Gs *In Vitro*

As expected, both NAP-G diastereoisomers underwent spontaneous hydrolysis and isomerization (acyl migration) *in vitro* at physiologic pH. The overall degradation rates, reflecting both hydrolysis and acyl migration,

Table I. First-Order Degradation Rate Constants and Apparent Half-Lives of *R*-and *S*-Naproxen Glucuronides in Different Media at 37°C

	R-Nap-G[a]		*S*-Nap-G[a]	
Medium	k_{deg} (hr^{-1})[b]	$t_{1/2}$ (hr)	k_{deg} (hr^{-1})[b]	$t_{1/2}$ (hr)
0.1 M Phosphate buffer	0.75 ± 0.02[c]	0.92 ± 0.02[c]	0.40 ± 0.02	1.75 ± 0.07
0.3% HSA	0.71 ± 0.06[c]	1.05 ± 0.13[c]	0.89 ± 0.02	0.78 ± 0.01
3.0% HSA	1.05 ± 0.04[c]	0.66 ± 0.02[c]	1.55 ± 0.08	0.45 ± 0.02
Human plasma	1.58 ± 0.03[c]	0.44 ± 0.01[c]	1.78 ± 0.01	0.39 ± 0.00

[a] *R*-NAP-G = *R*-naproxen glucuronide, *S*-NAP-G = *S*-naproxen glucuronide, HSA = human serum albumin.
[b] Mean values ± SD (three to six experiments).
[c] Significantly different from that of *S*-NAP-G ($p < 0.01$).

for each compound were faster in human plasma and HSA solution than in protein-free buffer (Table I). *R*-NAP-G disappeared approximately two times faster than *S*-NAP-G in buffer, but in the presence of protein the disappearance rates for the two compounds were not as different. After 5.5-hr incubation in phosphate buffer, only about 1% of the original β-1-*R*-NAP-G and 11% of original β-1-*S*-NAP-G were present at the β-1-glucuronide. However, at the same time, the sum of the isomeric conjugates of *S*- or *R*-NAP accounted for approximately 70–80% of the initial total *S*-NAP-G or *R*-NAP-G concentrations. Considerable amounts of acyl migration isomers were still present after 22 hr of incubation in phosphate buffer. In contrast, essentially all of the starting material and most of the isomers had disappeared after 9-hr incubation of either *R*- or *S*-NAP-G in 3% HSA or plasma.

Reversible Binding of NAP Enantiomers and Their Acyl Glucuronides

Scatchard plots for the reversible binding of NAP enantiomers and NAP-G diastereomers to HSA are shown in Fig. 2, and calculated binding constants are given in Table II. The Scatchard plots for the NAP enantiomers indicate at least two different independent classes of binding sites (N_1 and N_2), each with its own dissociation constant ($K_{d,1}$ and $K_{d,2}$). The first class of binding sites can accommodate 1 molecule of drug per molecule of albumin and binds the *S*-enantiomer with higher affinity than the *R*-enantiomer. The second class of sites, which can bind up to 4 molecules of drug, does not exhibit a significant difference in affinity but tends to prefer the *S*-enantiomer. For the glucuronides, the high affinity binding site has a higher affinity for *R*-NAP-G than for *S*-NAP-G at the typical *S*-NAP-G concentrations ($<10\ \mu M$) that occur in healthy volunteers taking *S*-NAP (17). It is possible to predict how the glucuronide concentration influences its binding to protein by examining the Scatchard plots of the glucuronides. When the total acyl

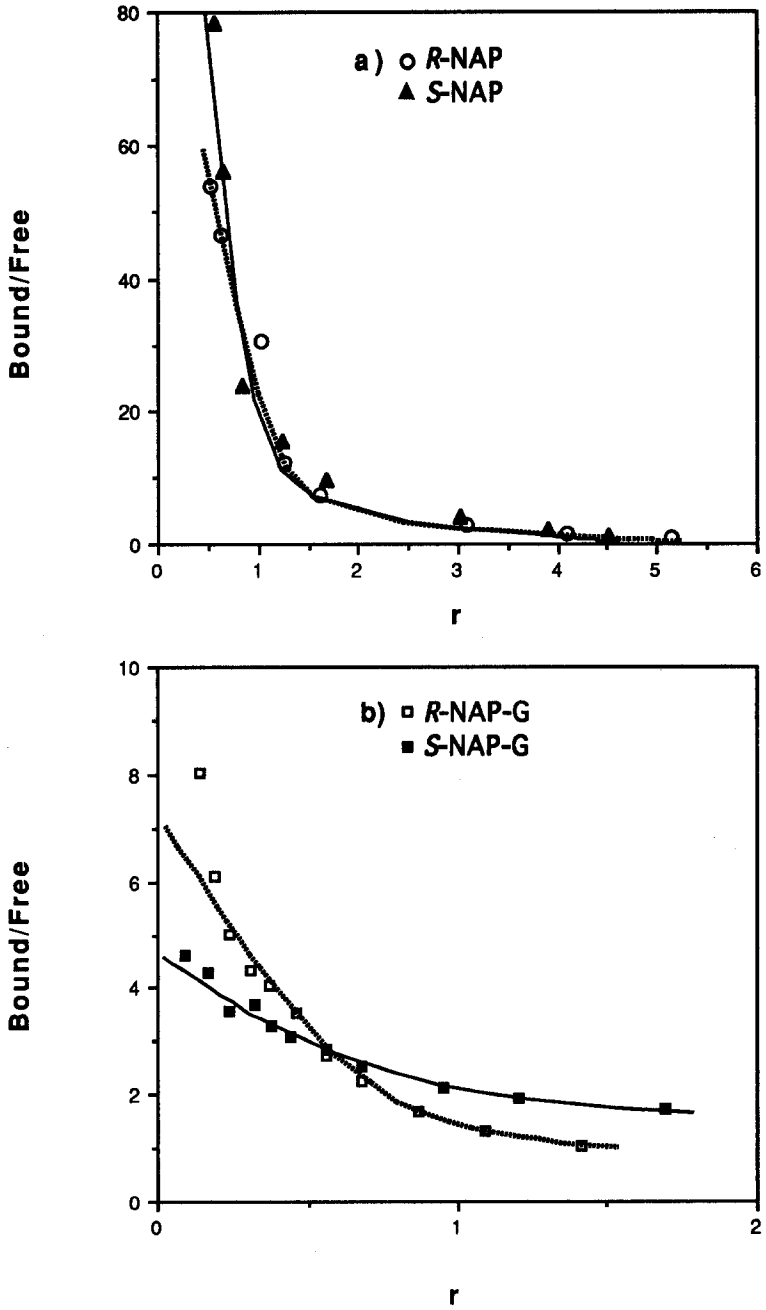

Fig. 2. Scatchard plots of the binding data of *R*- and *S*-NAP (a) and *R*- and *S*-NAP-G (b). The experiments were performed using fatty acid free HSA (3 mg/ml). Each point represents the mean value of three experiments.

Table II. Binding Parameters for Naproxen Enantiomers and Their Glucuronides to Fatty Acid Free Human Serum Albumin (3 mg/ml in 0.1 M Sodium Phosphate Buffer, pH 7.4) at Ambient Temperature[a]

			N_2	$K_{d,2}$ (μM)
	N_1	$K_{d,1}$ (μM)	NS (M^{-1})	
S-enantiomer	0.92 ± 0.10[b]	0.27 ± 0.05[b]	3.90 ± 0.29	49.8 ± 19.4
R-enantiomer	1.14 ± 0.06	0.55 ± 0.06	4.57 ± 0.40	85.5 ± 23.4
S-glucuronide	0.65 ± 0.07	8.26 ± 1.68[c]	0.025 ± 0.002[c]	
R-glucuronide	0.68 ± 0.08	4.56 ± 0.71	0.013 ± 0.002	

[a] Each reported number represents the mean ± SD from three independent experiments. N_1 and N_2 are the numbers of primary and secondary class binding sites, respectively, and $K_{d,1}$ and $K_{d,2}$ are the corresponding dissociation constants. *NS* is the nonsaturable site (linear) binding parameter.
[b] Significantly different from the value of the *R*-enantiomer ($p < 0.04$).
[c] Significantly different from the value of the *R*-glucuronide ($p < 0.04$).

glucuronide concentration increases above 30 μM ($r > 0.5$), the high-affinity low-capacity primary binding site region seems to become saturated and under these conditions *S*-NAP-G shows higher binding than its *R*-diastereomer.

Irreversible Binding of NAP Acyl Glucuronides

Both diastereomers of NAP-G exhibit covalent binding to HSA and to proteins of rat and human plasma *in vitro*. The covalent binding profiles, maximal covalent binding yields and overall stabilities of the products varied according to the medium, as shown in Fig. 3, but covalent binding yields (μmol NAP/mmol protein) were consistently higher for *R*-NAP-G than for *S*-NAP-G (Fig. 3, Table III). The highest covalent binding for both diastereomers was observed in human plasma. Addition of PMSF, to block esterase activity, had no detectable effect on the extent of covalent binding to protein in rat plasma.

Incubation of HSA with D-glucose before incubation with NAP-G diastereomers had no significant effect on covalent binding of either diastereomer (Table IV), whereas preincubation with 50 mM D-glucuronic acid (Concentration II) markedly inhibited covalent binding of only the glucuronide of *R*-NAP. Pretreatment with 1.1 mM ASA had no significant effect, but pretreatment with a saturating concentration (approximately 11 mM) of ASA markedly diminished the covalent binding of both glucuronide diastereomers. Pretreatment with 0.2 mM *S*-NAP diminished the covalent binding of both diastereomers.

DISCUSSION

This study shows that the handedness of the chiral center in naproxen has a significant effect on the reversible binding of both naproxen itself and

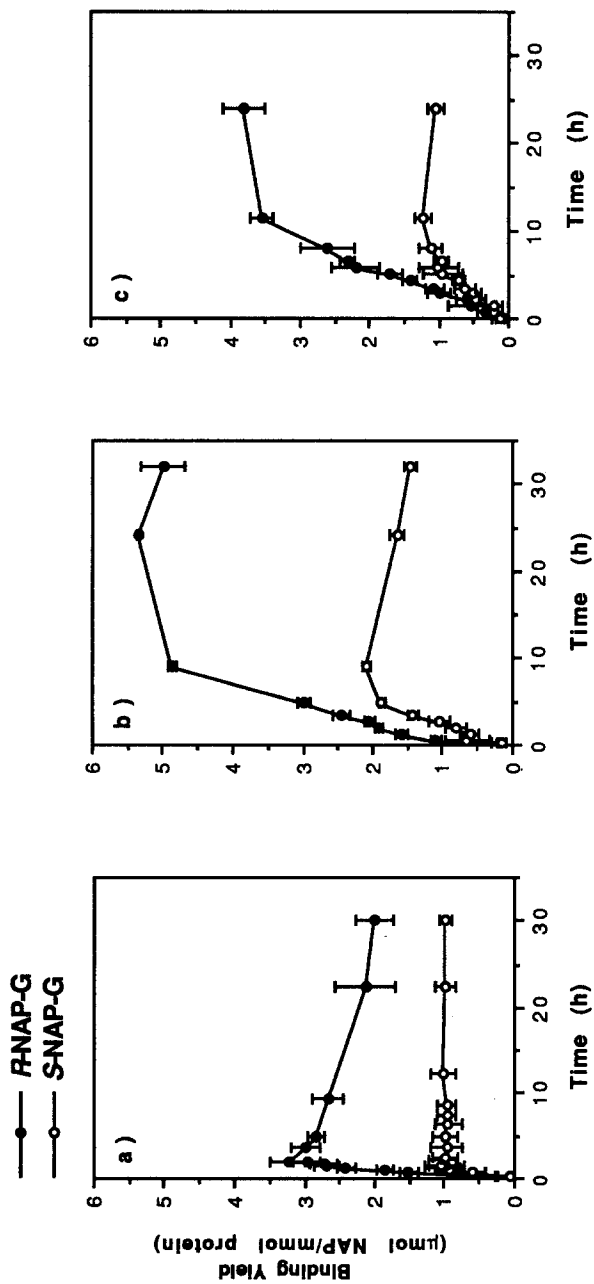

Fig. 3. *In vitro* covalent binding yield for *R*- and *S*-Nap-G in different incubation media vs. time: (a) 30 mg/ml HSA (in 0.1 M sodium phosphate buffer, pH 7.4); (b) human plasma; (c) rat plasma. Values are the mean of three to six experiments.

Table III. Maximum Binding Yield and t_{max} for Irreversible Binding of Naproxen Glucuronides to Human Serum Albumin and Plasma Proteins at 37°C[a]

	t_{max} (hr)		Maximum irreversible binding yield[b] (μmol/mmol protein)	
	R-NAP-G	*S*-NAP-G	*R*-NAP-G	*S*-NAP-G
3% HSA	2.0	1.3–1.5	3.23 ± 0.25	1.03 ± 0.19
Human plasma	24	9.0	5.35 ± 0.21	2.09 ± 0.06
Rat plasma	24	11.4	3.81 ± 0.29	1.23 ± 0.13
Rat plasma (PMSF[c])	24	11.4	3.82 ± 0.57	0.97 ± 0.05

[a] *R*-NAP-G = *R*-naproxen glucuronide, *S*-NAP-G = *S* naproxen glucuronide, HSA = human serum albumin.
[b] Mean ± SD ($n = 3$, $n = 6$ for HSA incubations).
[c] Phenylmethylsulfonyl fluoride (0.35 mg/ml, 2 mM).

Table IV. Effect of Competitors on the Extent of Irreversible Binding of Naproxen Glucuronides (50 μM) to Human Serum Albumin (450 μM)[a]

	Concentration I[b] % of control		Concentration II[c] % of control	
	R-NAP-G	*S*-NAP-G	*R*-NAP-G	*S*-NAP-G
Control	100 ± 5.4	100 ± 8.1	100.0 ± 3.8	100.0 ± 1.3
D-Gluc	91.6 ± 7.1	87.8 ± 9.4	100.8 ± 3.3	106.1 ± 2.6
D-GA	101.5 ± 5.9	86.6 ± 7.1	59.9 ± 1.5[d]	95.9 ± 2.8
ASA	95.2 ± 4.4	86.9 ± 6.8	8.7 ± 0.8[d]	18.1 ± 0.5[d]
S-NAP	79.6 ± 5.8[d]	82.1 ± 3.0[d]		

[a] *R*-NAP-G = *R*-naproxen glucuronide, *S*-NAP-G = *S*-naproxen glucuornide, D-Gluc = D-glucose, D-GA = D-glucuronic acid, ASA = acetylsalicylic acid. Values are the mean ± SD of three to five experiments.
[b] Concentrations: D-Gluc 20 mM, D-GA 20 mM, ASA 1.1 mM, *S*-Nap 0.2 mM.
[c] Concentrations: D-Gluc 50 mM, D-GA 50 mM, ASA (≈11 mM).
[d] Significantly different from control, $p < 0.01$.

its acyl glucuronides, as well as on the stability of the glucuronides and their irreversible binding to serum albumin. Our data show that *S*-NAP has a higher affinity for HSA than *R*-NAP, as previously found for most, but not all, acidic drugs with a chiral center adjacent to the carboxyl carbon (18) including naproxen (15,19). Using equilibrium dialysis, Runkel *et al.* (19) reported that *S*-NAP is more than 99% bound at a concentration of ~50 μg/ml (~200 μM) in plasma, and Mortensen *et al.* (15) found >99.9% of *S*-NAP (9.2 μg/ml, 40 μM) to be bound to HSA (4.5%, 660 μM). From our data we calculate that both NAP enantiomers are 99.9% bound to HSA at the drug and protein concentrations used by Mortensen *et al.* (15). This excellent agreement establishes the validity of the ultrafiltration binding assay used in our studies.

Measurement of the reversible binding of the acyl glucuronides to albumin was hampered by the inherent instability of the glucuronides and the esterase-like activity of albumin which accelerates their hydrolytic decomposition. However, by working at low (3 mg/ml), subphysiological, albumin concentrations and using a rapid ultrafiltration method, we were able to determine binding constants for the individual NAP-G diastereomers. The glucuronides of *R*- and *S*-NAP showed high, stereoselective, reversible binding to HSA, but, as anticipated, they had a lower affinity for the protein than the respective parent *R*- and *S*-aglycone enantiomers. At an HSA concentration close to physiologic (40 mg/ml, 4%, 600 μM) and acyl glucuronide concentrations less than 10 μg/ml, which is a concentration that has been found in plasma in naproxen recipients, the calculated binding of each β-1-*O*-acyl glucuronide is greater than 98% for *S*- and 99% for the *R*-diastereomers. Such high binding for an acyl glucuronide is exceeded only by carprofen (7).

At acyl glucuronide concentrations below 30 μM, which is within the concentration range observed following oral drug doses, the K_d values of the *R*-diastereomer are smaller than those of the *S*-diastereomer, indicating that the *R*-diastereomer has the highest affinity for HSA. For fenoprofen (20), it is also the glucuronide of the *R*-enantiomer that binds with highest affinity to albumin, in contrast to carprofen glucuronide (7), for which the diastereomer derived from the *S*-enantiomer binds most strongly.

Previous studies have shown that the decomposition of acyl glucuronides *in vitro*, via hydrolysis and acyl migration, is affected by several factors, including pH, temperature, and solvent (4), and that glucuronides of *R*-benoxaprofen (21), *R*-carprofen (7), *R*-flunoxaprofen (22), and *R*-fenoprofen (12) are less stable than glucuronides of the corresponding *S*-enantiomers. We observed similar stereoselectivity for naproxen glucuronides. In buffer *R*-NAP-G was less stable than *S*-NAP-G (k_{deg} $R/S = 1.88$). In 3% HSA and in plasma the decomposition of both NAP-G diastereomers was accelerated because of the esterase activity of HSA and *R*-NAP-G became the less stable diastereomer (k_{deg} $R/S = 0.9$ in 3% HSA and 0.89 in plasma, see Table I). In contrast to these findings, Iwakawa *et al.* (7) reported that glucuronides of *R*- and *S*-carprofen are more stable in the presence of HSA (2 mg/ml, 30 μM) than in phosphate buffer (pH 7.4). The effect of HSA on NAP-G decomposition was dependent on the HSA concentration. In 0.3% HSA *R*- and *S*-NAP-G diastereoisomers decomposed at similar rates and the rate of decomposition of the *R*-NAP-G diastereomer was not significantly different from that in buffer. We used this observation to our advantage in measuring the reversible binding of the glucuronide diastereomers to albumin.

The acyl glucuronides of both *R*- and *S*-naproxen reacted with proteins *in vitro* to form adducts containing irreversibly bound drug. The highest irreversible binding yields were obtained when the glucuronides were incubated in human plasma. Unexpectedly, coincubation with an esterase inhibitor (PMSF) did not increase binding yields significantly when rat plasma was the reaction medium. Covalent binding yields were higher for *R*-NAP-G than for *S*-NAP-G in all the protein-containing media examined (see Table III) and the yield/time profiles were different for the two diastereomers in a given medium (see Fig. 3), demonstrating that the formation of the modified proteins is sensitive to the stereochemistry of the attacking glucuronide.

Competition experiments designed to locate where on the protein the glucuronides react and to determine whether reversible binding is a prerequisite for irreversible binding (see Table IV) gave ambiguous results. Preincubation of HSA (450 μM) with *S*-NAP (200 μM) followed by coincubation with *S*- or *R*-NAP-G (50 μM) decreased the extent of irreversible binding by about 20%, which suggests that the binding site for *S*-NAP is also an important site for irreversible binding. Irreversible binding was inhibited more markedly by preincubation with acetylsalicylic acid (Table IV) but the effect was only detectable at high, nonphysiologic, competitor concentrations. Acetylsalicylic acid is known to acetylate reactive nucleophilic sites on proteins (23) and has been shown to inhibit covalent binding of oxaprozin (24) and zomepirac glucuronides (25) to albumin. However, the inhibitory effect of ASA at high concentrations may also have been caused by reversible binding of salicyclic acid generated by adventitious hydrolysis during incubation. High concentrations of glucose, which reacts with primary amino groups on albumin, had no detectable effect on irreversible binding of the NAP-G diastereomers. Nor did 20 mM glucuronic acid, which also reacts with albumin (26). Curiously, preincubation with glucuronic acid at higher concentration (50 mM) markedly reduced the irreversible binding of *R*-NAP-G, but not *S*-NAP-G, a stereoselective effect we are unable to explain.

A synthesis of published data (27) on the covalent binding of several acyl glucuronides indicates that there is a good linear correlation between the apparent first-order disappearance rate constant for an acyl glucuronide in buffer, which is a measure of its chemical reactivity, and the maximum irreversible binding yield observed when the glucuronide is incubated with HSA *in vitro* (Fig. 4). Acyl glucuronides of α-unsubstituted acetic acid derivatives such as tolmetin and zomepirac exhibit the highest covalent binding, followed by the more stable glucuronides of mono α-substituted acetic acids, such as carprofen and fenoprofen. Lowest irreversible binding is observed for the most stable fully substituted α-acetic acids, beclobric acid and furosemide (27). The data obtained in this study show that the

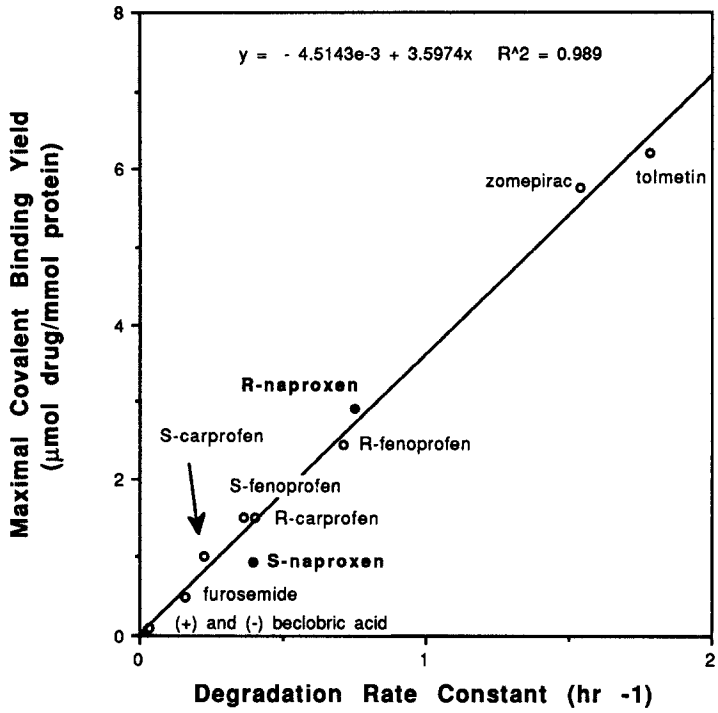

Fig. 4. Plot of maximum covalent binding yield (μmol drug/mmol protein) vs. degradation rate constant (hr^{-1}) for the *in vitro* incubation of various acyl glucuronides in the presence of human serum albumin. Degradation rates reflect both acyl migration and hydrolysis. Redrawn from ref. 27 with the addition of points for *R*- and *S*-naproxen.

diastereomers of NAP-G also fit this linear correlation (Fig. 4). It has been suggested that irreversible binding of acidic drugs to plasma proteins via acyl glucuronide metabolites may have toxicological significance (4). The linear correlation shown in Fig. 4 may be useful for predicting from easily obtained stability data which glucuronide-forming acidic drugs are most likely to lead to relatively high covalent binding *in vivo*. The data in this paper show that irreversible binding is stereoselective and that for chiral or racemic drugs one enantiomer may produce more covalently modified protein, and thus be potentially more immunotoxic than the other. As shown here for naproxen, the enantiomer that results in highest covalent binding is not necessarily the enantiomer that is clinically most active. If covalent binding can lead to significant immunotoxicity, administration of the optically pure clinically active enantiomer on its own might be more prudent than administration of the racemate, as in the case for the commercially available naproxen formulations.

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