In Vitro Regioselective Stability of β-1-*O*- and 2-*O*-Acyl Glucuronides of Naproxen and Their Covalent Binding to Human Serum Albumin

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Abstract □ β-1-*O*- (NAG) and 2-*O*-glucuronides (2-isomer) of (*S*)-naproxen (NA) were prepared to determine which positional isomer(s) of the acyl glucuronide of NA is responsible for forming covalent adducts with human serum albumin (HSA). Their comparative stability and covalent binding adduct formation with HSA were investigated at pH 7.4 and at 37 °C. NA and its acyl glucuronides were simultaneously determined by HPLC. Three positional isomers were formed successively after incubation of NAG in the buffer only. However, when NAG was incubated with HSA (30 mg/mL), isomers other than the 2-isomer were formed in little or negligible quantities. In HSA solution, NAG ($k_d = 2.08 \pm 0.08$ h^{-1}) was four times less stable than 2-isomer ($k_d = 0.51 \pm 0.02$ h^{-1}). NAG was degraded by hydrolysis ($k_{hyd} = 1.01 \pm 0.10$ h^{-1}) and isomerization ($k_{iso} = 1.07 \pm 0.07$ h^{-1}) to the same extent; however, hydrolysis was predominant for the 2-isomer ($k_d = 0.51 \pm 0.02$ h^{-1}). The incubation of both NAG and 2-isomer with HSA led to the formation of a covalent adduct; however, the adduct formation from the 2-isomer proceeded more slowly than that from NAG. The present results suggest that the covalent binding of NA to HSA via its acyl glucuronides proceeds through both transacylation (direct nucleophilic displacement) and glycation mechanisms; NAG rapidly forms an adduct that may be unstable, and the protein adduct from the 2-*O*-acyl glucuronide is as important for the covalent binding as those from the 1-*O*-acyl glucuronides.

Many acidic drugs with carboxylic acid functions are metabolized to reactive acyl glucuronides, which are susceptible to hydrolysis, isomerization (intramolecular acyl migration), and irreversible (covalent) binding to proteins under physiological conditions.[1,2] These reactions are considered to proceed through transacylations to the hydroxyl ion, to a different OH-group of the glucuronic acid moiety and to a nucleophilic group on the protein molecule, respectively, as suggested in an oxaprozin glucuronide study.[3] Formation of irreversible binding of carboxylic acid-containing drugs, such as nonsteroidal antiinflammatory drugs (NSAIDs), via their acyl glucuronide metabolites, is now well recognized and is a potential cause of hypersensitivity.[2,4,5] The exact mechanism underlying irreversible binding to proteins, however, has not yet been clarified due to the instability of acyl glucuronides. Two possible mechanisms can be considered: transacylation and glycation mechanisms.[6,7] Based on these proposed mechanisms, two different types of adduct can be formed: one is a transacylation adduct, where aglycon binds, via displacement of the glucuronic acid moiety from acyl glucuronide, directly to the protein NH_2, SH, or OH groups to yield an irreversibly bound adduct of aglycon linked to the protein by an amide, thioester, or ester linkage; the other is a rearrangement/glycation adduct, where acyl migration of the glucuronide has occurred, allowing the open chain form of the glucuronic acid moiety to react with an amino group to form an imine (Schiff's base). Subsequent Amadori rearrangement can then yield a more stable ketamine derivative.

The extent of covalent adduct formation via the acyl glucuronides varies from their parent NSAIDs. Benet et al.[6,8] showed that a striking correlation was observed between the extent of irreversible binding to protein and the degradation rate constant of acyl glucuronides. This strongly suggests that covalent adduct formation may reflect the reactivity of the corresponding acyl glucuronide. Although the degradation and covalent binding have been simultaneously investigated for some β-1-*O*-acyl glucuronides of NSAIDs,[9–11] it is not clear which ester(s) contributes to the formation of covalent binding to proteins. If the transacylation mechanism is predominant for the formation of irreversible binding to proteins, β-1-*O*-acyl glucuronide should relate mainly to the formation; if the Schiff's base mechanism is predominant, positional isomer(s) of β-1-*O*-glucuronide should contribute to the formation of covalent binding. In vitro stereoselective stability and binding properties of β-1-*O*-acyl glucuronides of (*S*)- and (*R*)-naproxen (NA) and in vivo stereoselective disposition of naproxen β-1-*O*-acyl glucuronide (NAG), using rats, have been reported by Benet et al.[8,12] However, the individual contributions of both hydrolysis and transacylation to the overall degradation of NAG, and the stability of positional isomers of NAG and their contribution to covalent protein binding, remain unclear.

In this study, we isolated the 2-*O*-acyl positional isomer (2-isomer) of (*S*)-NAG, in which the acyl-containing aglycon migrates from the glycosidic linkage at C-1 to a new ester linkage with a hydroxyl group at C-2 of the glucuronic acid moiety, and investigated its reactivity with human serum albumin (HSA) comparing it with that of (*S*)-NAG to test which possible mechanism is dominant for the formation of covalent binding. Our results show that (1) in the presence of HSA, NAG is subjected to both hydrolysis to NA and isomerization to the 2-isomer to the same extent, while further isomerization to the 3- and 4-*O*-acyl positional isomers is negligible, and (2) the covalent adduct of NA is formed mainly from 1-*O*-acyl- and 2-*O*-acyl glucuronides, not 3-*O*-acyl and 4-*O*-acyl glucuronides; the former may be unstable and act as an intermediate for HSA-catalyzed hydrolysis while the latter may be a stable adduct, which will be important for immunoreactive side-effects.

Experimental Section

Materials—(*S*)-NA (enantiomeric excess > 0.98), phenylmethylsulfonyl fluoride, and fatty acid free-HSA (fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). The molecular weight of HSA was assumed to be 69000.[13] Tryptophol [2-(3-

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Table 1—List of the H-1 Chemical Shift of Naproxen Glucuronide and Their Related Compounds

compound	H-1	H-3	H-4	H-5	H-7	H-8	$-CH_3$	-CH-	$-OCH_3$	H-1′	H-2′	H-3′	H-4′	H-5′
(*S*)-NA	7.69 (m)	7.425 (dd, 2.0, 9.0)	7.69 (m)	7.13 (m)	7.13 (m)	7.69 (m)	1.583 (d, 6.7)	3.866 (q, 7.2)	3.905 (s)					
GlcA (α-isomer)										5.315 (d, 3.4)	3.63 (m)	3.784 (dd, 9.1)	3.63 (m)	4.060[a] (d, 9.9)
GlcA (β-isomer)										4.741 (d, 8.2)	3.343 (dd, 8.5)	3.578 (dd, 9.6)	3.63 (m)	4.383[a] (d, 10.0)
(*S*)-NAG	7.715 (d, 1.3)	7.425 (dd, 1.9, 8.0)	7.76 (bd, 8.9)	7.253 (d, 2.6)	7.140 (dd, 2.0, 9.0)	7.76 (bd, 8.9)	1.560 (d, 7.4)	4.036 (q, 6.9)	3.900 (s)	5.522 (d, 8.2)	3.432 (dd, 8.0, 9.4)	3.451 (dd, 9.4, 11.6)	3.520 (d, 8.8)	3.730 (d, 8.9)
S-2-Isomer	7.740 (d, 1.9)	7.456 (dd, 1.9, 8.5)	7.757 (d, 9.0)	7.246 (d, 2.4)	7.133 (dd, 2.4, 9.2)	7.757 (d, 9.0)	1.570 (d, 6.8)	4.003 (q, 7.0)	3.905 (s)	5.345 (d, 3.4)	4.652 (dd, 3.4, 9.9)	3.878 (d, 9.5)	3.556 (d, 9.6)	4.088 (d, 9.6)

[a] Signals are interchangeable.

indolyl)ethanol] was obtained from Tokyo Kasei Co. (Tokyo, Japan). Other chemicals used were of analytical or HPLC grade.

Preparation of Glucuronides of NA—(*S*)-NAG was extracted from human urine, which was collected following a 600 mg oral administration of (*S*)-NA (Naixan, Tanabe Pharmaceutical Co., Tokyo, Japan). Urine was collected over 24 h into a vessel containing phosphoric acid to stabilize the NAG by lowering the pH to 3−4. The urine was treated as described by Smith et al.,[14] with minor modifications. The extract was then separated by liquid-chromatography with a Cosmosil 75C18-OPN column (Nacalai Tesque Inc., Kyoto, Japan). NAG was eluted from the column with the stepwise gradient of acetonitrile/0.05 M acetic acid, 10:90, 20:80, and 30:70 (v/v). After the fractions containing NAG were evaporated with a rotary evaporator at 30 °C, the remaining aqueous solution was lyophilized. The 2-*O*-acyl positional isomer (2-isomer) of (*S*)-NAG was prepared after 6-h incubation of (*S*)-NAG in 0.1 M phosphate buffer at pH 7.4 and at 37 °C. 2-Isomer extracted from the solution was purified by Sephadex LH-20 using 50% methanol as an eluting solvent. The purity of the glucuronides obtained was determined by analytical HPLC and exhibited homogeneous properties (>98%) at a UV wavelength of 254 nm, with the remaining fraction represented by polar impurities that did not yield NA when treated with 1 M NaOH for 1 h. The structures of the glucuronides were confirmed by ^{1}H NMR and ^{1}H−^{1}H shift correlation NMR. NMR spectra were obtained on a JEOL GX-500 at 500 MHz using deuterated methanol, with tetramethylsilane as an external reference.

Analysis of NA and Its Glucuronides Using HPLC—Analyses of NA and NAGs were performed according to the method described by Bischer et al.[8] using a Shimadzu LC6AD solvent delivery system (Shimadzu Co., Kyoto, Japan), a Shimadzu RF-535 fluorescence monitor, a Shimadzu C−R4A Chromatopac integrator, and a Cosmosil 5C18-AR column (5 μm particle size, 4.6 × 250 mm, Nacalai Tesque Inc., Kyoto, Japan).

Stability Studies—NAG (35−40 μM) was incubated at 37 °C in 0.1 M sodium phosphate buffer (pH 7.4), and 50 μL aliquots were collected at timed intervals for analysis. Similar incubations using (*S*)-NAG (ca 35 and 135 μM) and *S*-2-isomer (ca. 30 μM) were also run in the presence of 30 mg/mL (435 μM) HSA. Aliquots were immediately mixed with 10 μL of 17% phosphoric acid and 50 μL of acetonitrile containing 4 μg/mL of tryptophol as an internal standard. The precipitated protein was removed by centrifugation, and a 3 μL-portion of the supernatant was analyzed by HPLC.

Determination of in Vitro Covalent Binding to HSA—NAG or 2-isomer was incubated at 37 °C for 30 h in 0.1 M phosphate buffer containing 30 mg/mL HSA, as described above. Aliquots (300 μL) of each reaction mixture were taken at different time points to assay the covalently bound drug. The binding yield for irreversible binding of the glucuronides was quantified using the method of Bischer et al.[8]

Data Analysis—The rate constants for hydrolysis and isomerization of the glucuronides were estimated using the model shown in Figure 1. The calculation for the kinetic parameters was performed with a nonlinear least-squares fitting program, WinNonlin (Scientific Consulting, Inc., Apex, NC).

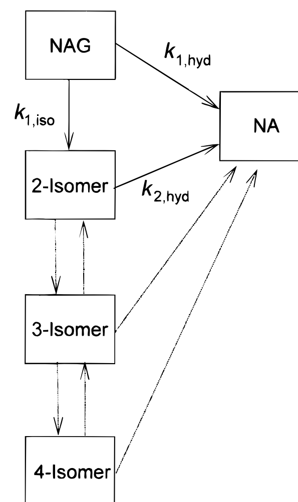


Figure 1—Kinetic model employed for the estimation of rate constants of hydrolysis and isomerization of NAG in HSA solution: $k_{1,hyd}$ and $k_{1,iso}$ are the first-order rate constants of hydrolysis to NA and isomerization to the 2-isomer from NAG, respectively. $k_{2,hyd}$ is the first-order rate constant of hydrolysis to NA from the 2-isomer.

Results

NMR Spectra of NAG and Its 2-*O*-Regioisomer—Identification of the purified acyl glucuronides was achieved using proton NMR analysis. Spectral features of NAG were similar to those reported by Wilson and Nicholson,[16] although confirmation of each proton signal was not performed. The results are summarized in Table 1, in which ^{1}H signals observed for GlcA in deuterated water and NA in deuterated methanol are also included as the reference compounds. A one-proton signal observed at 5.522 ppm as a doublet was easily assigned to H-1′ of the GlcA residue. The large coupling constant (J = 8.2 Hz) suggested that D-GlcA was bound to NA through a β-linkage. Due to lack of protons at the C-6 position, the proton signal (H-5′) attached to C-5 was also clearly observed as a doublet at a lower field (3.730 ppm). Starting from these characteristic signals of H-1′ and H-5′ of GlcA, all the proton signals were easily assigned by using the ^{1}H−^{1}H shift correlation spectra (data not shown).

The most abundant compound, 2-isomer, after 6-h incubation of NAG at pH 7.4 and at 37 °C was obtained in an almost pure state (>97% by HPLC). The ^{1}H−^{1}H shift correlation spectra of the 2-isomer is shown in Figure 2, which indicates the presence of a small amount of other isomers. Signals of GlcA moiety were assigned in Figure 2. The H-1′ signal of GlcA was observed at 5.345 ppm as a doublet with a small coupling constant (J = 3.4 Hz), indicating that the isomer was an α-isomer. The H-1′ signal of the corresponding β-isomer was not observed, probably due to overlap with a signal of HOD.[17] However, we were able to assign all of the signals from the ^{1}H−^{1}H shift correlation spectra. The H-2′ signal was observed at 4.652 ppm, which is 1.22 ppm lower than that of NAG. This

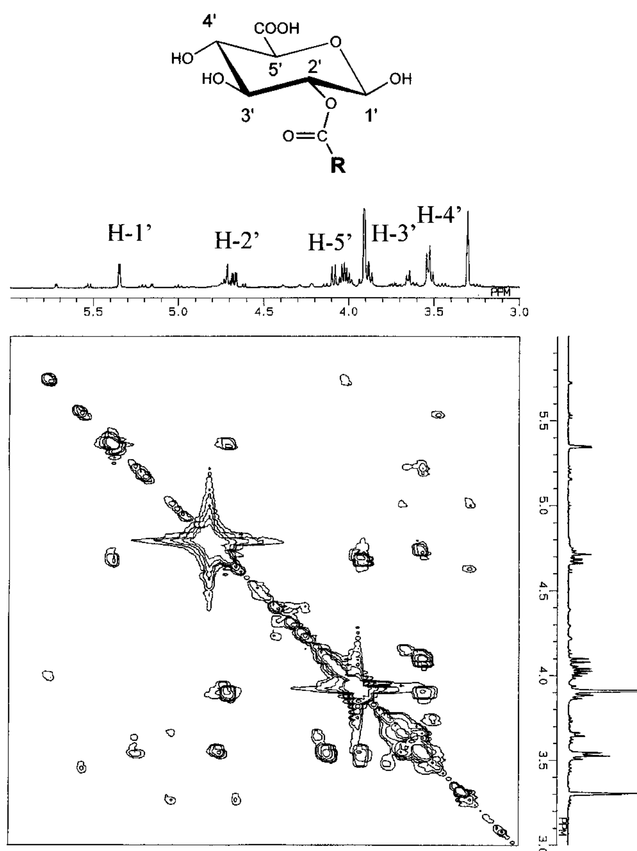


Figure 2—^1H–^1H shift correlation spectra of the *S*-2-isomer.

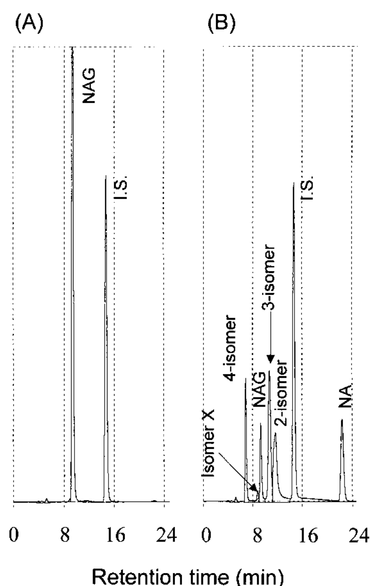


Figure 3—HPLC chromatograms of product mixtures from incubation of NAG after incubation in 0.1 M phosphate buffer (pH 7.4) at 37 °C for 30 s (A) and 6 h (B). Isomer X is an unidentified minor isomer which was hydrolyzed by 1 M NaOH but not β-glucuronidase. I.S. is tryptophol and was used as an internal standard. Retention times of NAG, 2-, 3-, 4-isomers, isomer X, and NA are 9.2, 11.6, 10.8, 7.0, 8.8 and 22.2 min, respectively.

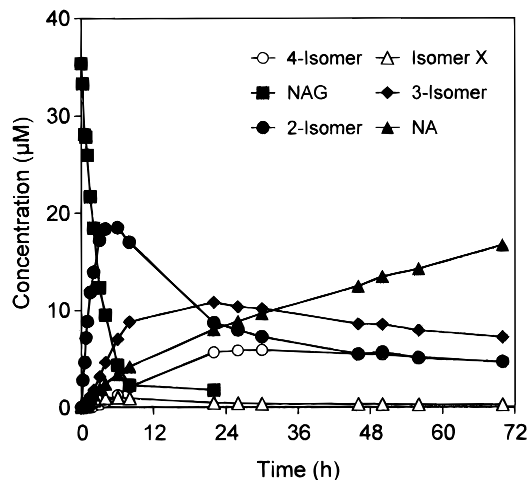


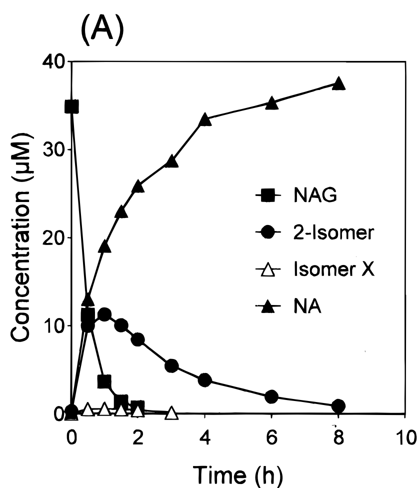
Figure 4—Time courses of rearrangement and hydrolysis of (*S*)-NAG in 0.1 M phosphate buffer (pH 7.4) at 37 °C. Only mean values ($n = 3$) are shown for the sake of visual clarity. Coefficients of variation for the data points ranged from 1 to 25%. Isomer X is an unidentified minor isomer which was hydrolyzed by 1 M NaOH but not β-glucuronidase.

shift obviously indicated that the hydroxyl group attached at C-2 of GlcA was substituted with NA.

Degradation of NAG and Its Regioisomer—(*S*)-NAG was incubated at pH 7.4 and at 37 °C in 0.1 M phosphate buffer for 72 h. The HPLC chromatograms of the incubation medium of NAG are shown in Figure 3. The time dependence of the degradation of (*S*)-NAG and the appearance of its isomers and hydrolyzed NA is shown in Figure 4. As has been reported for several other 1-*O*-acyl glucuronides, (*S*)-NAG underwent nonenzymatic isomerization (acyl migration) and hydrolysis at pH 7.4. In HSA-free buffer, (*S*)-NAG was subjected predominantly to acyl migration resulting in a rapid appearance of the 2-*O*-acyl isomer (2-isomer) and then gradual formation of other isomers (at least three peaks detected by HPLC). Hydrolysis of 1-*O*-glucuronide and/or its isomers to NA was slow when compared with isomerization. After 72-h incubation, three regioisomers, including the 2-isomer, equilibrated to almost the same concentration; about half of the initial amount of NAG was hydrolyzed to NA. The apparent degradation rate constant ($k_{1,\mathrm{deg}}$) for (*S*)-NAG was 0.343 ± 0.010 h^{-1} in HSA-free buffer.

The time dependence of degradation of the β-1-*O*- and 2-*O*-glucuronides of (*S*)-NA was investigated in the presence of 30 mg/mL of HSA. The results are shown in Figure 5, parts A and B, respectively. For comparison, the logarithmic plots for degradation of the glucuronides of NA in HSA solution are presented in Figure 6 as well as the result obtained in HSA-free buffer. As shown in Figure 6, the incubation of NAG with HSA at pH 7.4 resulted in a more rapid degradation when compared with the corresponding rate constant in HSA-free buffered solution, showing that HSA plays a major role in the degradation of NAG. In the presence of HSA, the contribution of hydrolysis to total degradation of NAG increased when compared with that in HSA-free buffer, as seen by the rapid appearance of NA. NAG was rapidly hydrolyzed to NA and isomerized to the 2-isomer, but no significant further acyl migration was observed in the buffer containing HSA (Figure 5A). This confirmed that further acyl migration of the 2-isomer to form the 3- and 4-isomers was negligible in the presence of HSA. The degradation rate constant ($k_{2,\mathrm{deg}} = 0.514 \pm 0.015$ h^{-1}) was extremely slow for 2-isomer when compared with that for its β-1-*O*-acyl conjugates ($k_{1,\mathrm{deg}} = 2.08 \pm 0.08$ h^{-1}), as shown in Figure 6. The 2-isomer was almost entirely hydrolyzed before further rearrangement, although only a small amount of the isomers was detected (Figure 5B). A higher initial concentration of (*S*)-NAG (mean 134 μM) resulted in a similar degradation pattern to the lower concentration experiments (data not shown).

The kinetic parameters of degradation reflecting both rearrangement and hydrolysis of each glucuronide were estimated based on the model shown in Figure 1. The



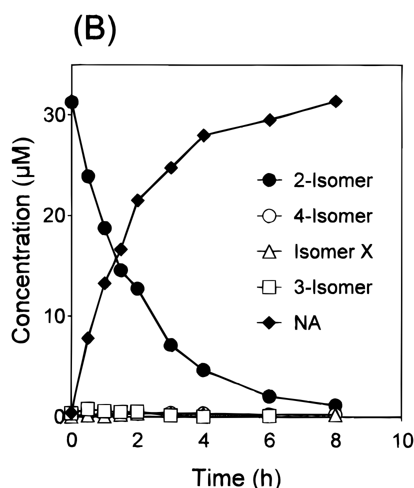


Figure 5—Time courses of rearrangement and hydrolysis of (*S*)-NAG (A) and *S*-2-Isomer (B) in 0.1 M phosphate buffer including 30 mg/mL of HSA (pH 7.4) at 37 °C. Only mean values ($n = 3$) are shown for the sake of visual clarity. Coefficients of variation for the data points ranged from 1 to 13%. Isomer X is an unidentified minor isomer which was hydrolyzed by 1 M NaOH but not β-glucuronidase.

parameters are shown in Table 2. The parameters were almost the same for the lower and higher initial concentrations, showing the linear kinetics in the degradation of β-1-glucuronides and its isomers under the conditions used. The contribution of hydrolysis of (*S*)-NAG to its total elimination was almost identical to the isomerization. The hydrolysis of the purified 2-isomer ($k_{2,\mathrm{hyd}} = 0.514 \pm 0.015$ h^{-1}) occurred at approximately the same rate as for the 2-isomer formed in the (*S*)-NAG incubation experiment ($k_{2,\mathrm{hyd}} = 0.446 \pm 0.043$ h^{-1}). The isomerization rate constant of (*S*)-NAG in the presence of HSA was also greater than its overall degradation rate constant in HSA-free solution, suggesting that HSA accelerated transacylation of (*S*)-NAG as well as its hydrolysis.

Irreversible Binding to HSA—The time dependence for irreversible binding formation of NA was investigated after incubation of NAG and 2-isomer at pH 7.4 and at 37 °C over 30 h. As shown in Figure 7, NAG rapidly produced a covalent adduct with HSA; the maximum yield was achieved after 1 h of incubation. The isomeric conjugate (2-isomer) also produced an irreversible binding adduct, although the maximum yield was achieved after ca. 8–10 h. The extent of irreversible binding yield from each of the glucuronides tested reached almost the same level, regardless of the differences in their stability. We calculated empirically the apparent first-order formation rate constant of covalent binding using the following exponential equation: $\mathrm{BY} = A(\mathrm{e}^{-k_{\mathrm{e}}t} - \mathrm{e}^{-k_{\mathrm{f}}t})$, where BY is the covalent binding yield, k_{e} and k_{f} being the apparent elimination and formation rate constants of the covalent adduct, respectively. The apparent formation rate constant ($k_{\mathrm{f}} = 0.684 \pm 0.086$ h^{-1}) for irreversible binding from the

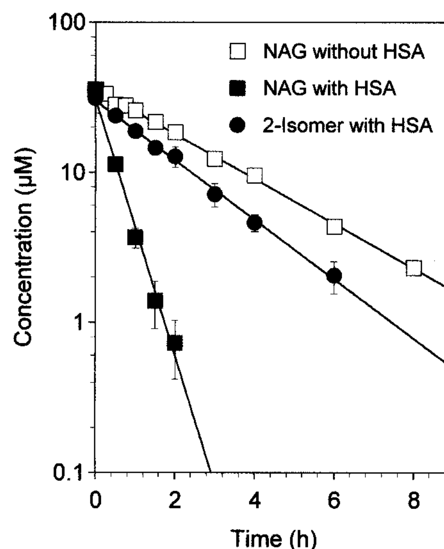


Figure 6—Logarithmic plots for degradation of NAG and 2-isomer without or with HSA. Each point and vertical bar represent the mean ± SD of three independent series. Error bar lines of NAG without HSA are within the outline of the symbol.

Table 2—Kinetic Parameters[a] for Hydrolysis and Isomerization of Glucuronides of NA in HSA Solution

	(*S*)-NAG		
parameter	lower (35 μM)[b]	higher (135 μM)	*S*-2-isomer (30 μM)
initial concentration (μM)	34.9 ± 0.3	134 ± 10	31.3 ± 6.4
$k_{1,\mathrm{hyd}}$ (h^{-1})	1.01 ± 0.10	0.860 ± 0.199	
$k_{1,\mathrm{iso}}$ (h^{-1})	1.07 ± 0.07	1.09 ± 0.02	
$k_{1,\mathrm{deg}}$ (h^{-1})[c]	2.08 ± 0.08	2.05 ± 0.06	
$k_{2,\mathrm{hyd}}$ (h^{-1})	0.446 ± 0.043	0.473 ± 0.012	0.514 ± 0.015

[a] Data are expressed as mean ± SD of three experiments. [b] Initial concentration. [c] $k_{1,\mathrm{deg}} = k_{1,\mathrm{hyd}} + k_{1,\mathrm{iso}}$.

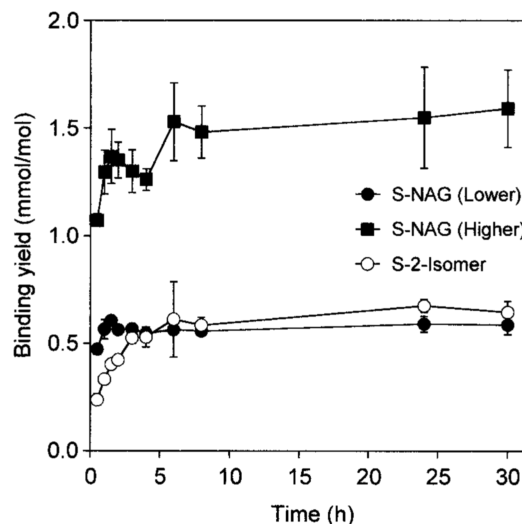


Figure 7—Time courses of irreversible binding of NA to HSA from acylglucuronides of NA in 0.1 M phosphate buffer (pH 7.4) at 37 °C. Each point and vertical bar represent the mean ± SD of three independent series.

S-2-isomer was considerably smaller than that (k_f = 3.55 ± 0.39 h^{-1}) from β-1-glucuronide, indicating that protein adduct formation from the 2-isomer occurred at a slower rate than that from NAG.

To test whether the extent of irreversible binding depended on the NAG concentration, a higher concentration of NAG was incubated under the same conditions. The increase in initial concentration of NAG to 134 μM from 35 μM for (*S*)-NAG produced a proportional increase in maximum adduct formation. However, interestingly, a secondary increase in adduct formation followed by a plateau level was observed. This phenomenon was not observed in the lower NAG concentration experiment.

Discussion

The stability and irreversible binding to protein of β-1-*O*-acyl glucuronides of many other carboxylic acid drugs have been reported. In this study, we compared the reactivities of a model acyl glucuronide, NAG, and its 2-*O*-positional isomer (2-isomer) in the presence of HSA, to gain an insight into the favored mechanism for irreversible binding. The present study clearly showed the regioselective differences of acyl glucuronides of NA in their stability and the formation of covalent binding to HSA.

To perform the study, we initially isolated a positional isomer (2-*O*-acyl derivative) of (*S*)-NAG and recorded its one-dimensional and two-dimensional proton NMR spectra when compared with (*S*)-NAG. The NMR data of some 1-*O*-acyl glucuronides[17,18] and the positional isomers of 1-*O*-acyl glucuronides[16,19,20] have been reported. In this study, all the proton signals of the positional isomer of NAG were confirmed. The regioisomers of β-1-acyl glucuronide can exist as α- and β-anomers. From a comparison with the NMR spectrum of GlcA, the 2-isomer exists mainly as α-anomer. The NMR spectrum of the mixture of isomers, which was obtained 6 h after incubation of (*S*)-NAG, was also analyzed (data not shown). Although other positional isomers (3- and 4-isomers), unfortunately, could not be obtained in a pure state, signals due to the H-1′ protons of the isomers were isolated from other ring protons of the GlcA residue. Hence, these signals in the anomeric region (around 5 ppm) may be good markers for determining the relative abundance of each isomer.

In the HSA-free buffer solution, at least four isomers of NAG were detected after incubation of NAG (Figure 3). Like diflunisal acyl glucuronide,[11,21] the 2-isomer was formed first followed by formation of other positional isomers. Finally, three abundant isomers reached an almost equivalent concentration. Since the 2-isomer was generally subject to further acyl migration,[11,21] these isomers, which appeared later, were probably 3- and 4-isomers. Acyl migration in the glucuronic acid residue is considered to proceed through an ortho ester-like intermediate.[22] Thus, we conclude that acyl migration occurs through the sequence β-1-glucuronide → 2-isomer, 2-isomer → 3-isomer and 3-isomer → 4-isomer. Therefore, the peaks of the isomers on the HPLC chromatogram are assigned here based on their order of appearance, as reported by Faed.[1] A similar result was reported for flufenamic acid and benoxaprofen.[18] The unidentified small amount of esters is probably their anomeric isomers.

In the buffer including HSA, the 2-isomer as well as NA was predominantly formed after incubation of NAG, and the formed 2-isomer was sequentially hydrolyzed before further rearrangement. Unlike the case in HSA-free buffer, the levels of regioisomers other than 2-isomer were near or below the detection limit. Additionally, the stability experiment of the purified 2-isomer (Figure 5B) in HSA solution showed that the rearrangement from the 2-isomer to the 3-isomer or 4-isomer was hardly observed. Although we cannot account clearly for the observation of no further acyl migration of the 2-isomer to form a 3- or 4-isomer in HSA solution, HSA may retard the formation of the ortho ester-like intermediate between the C-2 and C-3 positions of GlcA, resulting in predominant hydrolysis of the 2-isomer in its degradation pathway. In the case of β-1-acyl glucuronides of diflunisal,[11] tolmetin,[9] and salicylic acid,[26] it has been reported that HSA seems to stabilize the glucuronides.

On the basis of the calculation of the individual rate constants using the model shown in Figure 1, (*S*)-NAG was decomposed by hydrolysis and intramolecular rearrangement almost to the same extent (Table 2). Our preliminary experiment using *R*-NAG showed that *R*-NAG was preferentially isomerized (unpublished data), suggesting that the HSA-mediated hydrolysis was predominant for (*S*)-NAG. In many other acyl glucuronides,[3,23−25] including NAG,[8] the catalytic role of HSA in the degradation (hydrolysis and intramolecular rearrangement) has been reported. Our calculations of the individual rate constants of NAG in HSA solution clarified that both the hydrolysis and acyl transformation rate constants of NAG were greater than the overall degradation rate constant (k_d = 0.343 ± 0.010 h^{-1}) in HSA-free solution, which should reflect acyl transformation and hydrolysis rate constants. This suggests the catalytic role of HSA not only in the hydrolysis of NAG but also in the isomerization of NAG. This may be due to the lowered free energy of ortho ester formation between the hydroxyl group at C-2 of the GlcA residue and the carboxyl group of NA by reversible binding to HSA, although we cannot explain exactly the enhancement effects of HSA on the isomerization of NAG.

We compared the stability and covalent binding formation of the 1-*O*- and 2-*O*-acyl glucuronides of (*S*)-NA. The 2-isomer, which was more stable than NAG, formed a protein adduct more slowly when compared with NAG. NAG, which forms the protein adduct most easily, achieved a maximum binding within 1 h of incubation. Although evidence exists for the two major mechanisms of covalent binding, it has not been clarified as to which of these is principally responsible.[28−32] If a covalent adduct is formed predominantly from the regioisomers of NAG, the formation rate of the adduct should be faster for the 2-isomer than for NAG, since no acyl migration time is required. Consequently, our results suggest that NAG can directly bind to the protein. Interestingly, the protein adduct formation temporarily declined, and a secondary increase in the formation was observed after incubation at a higher concentration of NAG. On the basis of comparison of the time dependence of degradation of NAG with the pattern of adduct formation, the covalent adduct in the earlier stage could be formed by β-1-acyl glucuronide, and the secondary peak of binding formation may arise from the formed 2-isomer. Additionally, in the case of NAG, 3-*O*- and 4-*O*-acyl glucuronides contribute little to the adduct formation under the conditions used. Finally, the covalent binding of NA to HSA seems to proceed via its acyl glucuronides in both the transacylation and glycation mechanisms. In the case of tolmetin, not only the β-1-isomer of tolmetin glucuronide but also other isomers can react with protein to give irreversibly linked products.[9] In this study, β-1-*O*- and 2-*O*-acyl glucuronides of NA clearly contribute the formation of protein adduct of NA. We quantified the regioselective difference in stability and covalent binding of acyl glucuronides of (*S*)-NA, although the stereoselective difference of *R*- and *S*-β-1-*O*-acyl glucuronides of NA in the protein adduct formation have been reported.[8]

Smith et al.[19] have reported that isomeric conjugates of zomepirac glucuronide occur via imine formation between

the free aldehyde of the open-chain GlcA and protein, followed by an Amadori rearrangement to the more stable adduct. The data suggest that although NAG can directly and rapidly form an adduct that is unstable and hydrolyzed almost as quickly as it is formed, intramolecular transacylation may well be a prerequisite for formation of a stable adduct. Presumably, the rapid complex formation of β-1-*O*-acyl glucuronide with HSA may assist in the HSA-mediated hydrolysis of NAG. We speculate that hydrolysis of acyl glucuronides may proceed through an acylated intermediate (acyl-HSA intermediate) such as an ester with a serin residue, thioester with a cysteine residue and/or acylimidazole with a histidine residue in HSA.[33] In the second step, the acyl-HSA intermediate is deacylated by hydrolysis. Thus, this transient adduct may be less important than the persistent one that arises from the 2-*O*-acyl glucuronide.

In conclusion, the present results indicate that covalent adduct formation proceeds via the proposed two kinds of mechanisms, transacylation and imine formation, according to the degree of formation of isomeric forms of the acyl glucuronide of NA.

References and Notes

1. Faed, E. M. Properties of acyl glucuronides: Implications for studies of the pharmacokinetics and metabolism of acidic drugs. *Drug Metab. Rev.* **1984**, *15*, 1213–1249.
2. Spahn-Langguth, H.; Benet, L. Z. Acyl glucuronides revisited: Is the glucuronidation process a toxification as well as a detoxification mechanism? *Drug Metab. Rev.* **1992**, *24*, 5–48.
3. Ruelius, H. W.; Kirkman, S. K.; Young, E. M.; Janssen, F. W. Reactions of oxaprozin-1-O-acyl glucuronide in solutions of human plasma and albumin. *Adv. Exp. Med. Biol.* **1986**, *197*, 431–441.
4. Park, B. K.; Kitteringham, N. R. Drug–protein conjugation and its immunological consequences. *Drug Metab. Rev.* **1990**, *22*, 87–144.
5. Worrall, S.; Dickinson, R. G. Rat serum albumin modified by diflunisal acyl glucuronide is immunogenic in rats. *Life Sci.* **1995**, *56*, 1921–1930.
6. Benet, L. Z.; Spahn-Langguth, H.; Iwakawa, S.; Volland, C.; Mizuma, T.; Mayer, S.; Mutschler, E.; Lin, E. T. Predictability of the covalent binding of acidic drugs in man. *Life Sci.* **1993**, *53*, 141–146.
7. Benet, L. Z.; Spahn, H. Acyl migration and covalent binding of drug glucuronides—potential toxicity mediators. In *Cellular and Molecular Aspects of Glucuronidation*, Proceedings of the Workshop, Montpellier France, 27–29 April 1988 Siest, G., Magdalou, J., Burchell, B., Eds.; John Libbey Eurotext Ltd.: Montrouge, France, 1988; Vol. 173, pp 261–269.
8. Bischer, A.; Zia-Amirhosseini, P.; Iwaki, M.; McDonagh, A. F.; Benet, L. Z. Stereoselective binding properties of naproxen glucuronide diastereomers to proteins. *J. Pharmacokinet. Biopharm.* **1995**, *23*, 379–395.
9. Munafo, A.; McDonagh, A. F., Smith, P. C.; Benet, L. Z. Irreversible binding of tolmetin glucuronic acid esters to albumin in vitro. *Pharm. Res.* **1990**, *7*, 21–27.
10. Grubb, N.; Weil, A.; Caldwell, J. Studies on the in vitro reactivity of clofibryl and fenofibryl glucuronides. Evidence for protein binding via a Schiff's base mechanism. *Biochem. Pharmacol.* **1993**, *46*, 357–364.
11. Williams, A. M.; Dickinson, R. G. Studies on the reactivity of acyl glucuronides—VI. Modulation of reversible and covalent interaction of diflunisal acyl glucuronide and its isomers with human plasma protein in vitro. *Biochem. Pharmacol.* **1994**, *47*, 457–467.
12. Ikeda, K.; Kurono, Y.; Ozeki, Y.; Yotsuyanagi, T. Effects of drug binding on esterase activity of human serum albumin. Dissociation constants of the complexes between the protein and drugs such as N-arylanthranilic acid, coumarin derivatives and prostaglandins. *Chem. Pharm. Bull.* **1979**, *27*, 7, 80–87.
13. Iwaki, M.; Bischer, A.; Nguyen, A. C., McDonagh, A. F.; Benet, L. Z. Stereoselective Disposition of Naproxen Glucuronide in the Rat. *Drug Metab. Dispos.* **1995**, *23*, 1099–1103.
14. Smith, P. C.; Song, W. Q.; Rodriguez, R. J. Covalent binding of etodolac acyl glucuronide to albumin in vitro. *Drug Metab. Dispos.* **1992**, *20*, 962–965.
15. Wilson, I. D.; Nicholson, J. K. Solid-phase extraction chromatography and NMR spectroscopy (SPEC NMR) for the rapid identification of drug metabolites in urine. *J. Pharm. Biomed. Anal.* **1988**, *6*, 151–165.
16. Sidelmann, U. G.; Gavaghan, C.; Carless, H. A. J.; Spraul, M.; Hofmann, M.; Lindon, J. C.; Wilson, I. D.; Nicholson, J. K. 750-MHz Directly coupled HPLC NMR: Application to the sequential characterization of the positional isomers and anomers of 2-, 3-, and 4-fluorobenzoic acid glucuronides in equilibrium mixtures. *Anal. Chem.* **1995**, *67*, 7, 4441–4445.
17. Bradow, G.; Kan, L.-S.; Fenselau, C. Studies of intramolecular rearrangement of acyl-linked glucuronides using salicylic acid, flufenamic acid, and (*S*)- and (*R*)-benoxaprofen and confirmation of isomerization in acyl-linked Δ9-11-carboxytetrahydrocannabinol glucuronide. *Chem. Res. Toxicol.* **1989**, *2*, 316–324.
18. McGurk, K. A.; Remmel, R. P.; Hosagrahara, V. P.; Tosh, D.; Burchell, B. Reactivity of mefenamic acid 1-*O*-acyl glucuronide with proteins in vitro and ex vivo. *Drug Metab. Dispos.* **1996**, *24*, 842–849.
19. Smith, P. C.; Benet, L. Z. Characterization of the isomeric esters of zomepirac glucuronide by proton NMR. *Drug Metab. Dispos.* **1986**, *14*, 503–505.
20. Sidelmann, U.; Hansen, S. H.; Gavaghan, C.; Nicholls, A. W.; Carless, H. A.; Lindon, J. C.; Wilson, I. D.; Nicholson, J. K. Development of a simple liquid chromatographic method for the separation of mixtures of positional isomers and anomers of synthetic 2-, 3- and 4-fluorobenzoic acid glucuronides formed via acyl migration reactions. *J Chromatogr. B Biomed. Appl.* **1996**, *685*, 113–122.
21. Dickinson, R. G.; King, A. R. Studies on the reactivity of acyl glucuronides- -II. Interaction of diflunisal acyl glucuronide and its isomers with human serum albumin in vitro. *Biochem. Pharmacol.* **1991**, *42*, 2301–2306.
22. Takiura, K.; Honda, S.; Endo, T.; Kakehi, K. Studies of oligosaccharides. IX. Synthesis of gentio-oligosaccharides by block condensation. *Chem. Pharm. Bull.* **1972**, *20*, 438–442.
23. Dubois, N.; Lapicque, F.; Maurice, M. H.; Pritchard, M., Gigleux, S. F.; Magdalou, J.; Abiteboul, M.; Siest, G.; Netter, P. In vitro irreversible binding of ketoprofen glucuronide to plasma proteins. *Drug Metab. Dispos.* **1993**, *21*, 617–623.
24. Hayball, P. J.; Nation, R. L.; Bochner, F. Stereoselective inteactions of ketoprofen glucuronides with hyman plasma protein and serum albumin. *Biochem. Pharmacol.* **1992**, *44*, 291–299.
25. Volland, C.; Sun, H.; Dammeyer, J.; Benet, L. Z. Stereoselective degradation of the fenoprofen acyl glucuronide enantiomers and irreversible binding to plasma protein. *Drug Metab. Dispos.* **1991**, *19*, 1080–1086.
26. Dickinson, R. G.; Baker, P. V.; King, A. R. Studies on the reactivity of acyl glucuronides—VII. Salicyl acyl glucuronide reactivity in vitro and covalent binding of salicylic acid to plasma protein of humans taking aspirin. *Biochem. Pharmacol.* **1994**, *47*, 469–476.
27. Rawn, J. Enzyme catalysis and enzyme kinetics. In *Biochemistry*; Neil Patterson Publishers: Burlington, NC, 1989; pp 158–165.
28. Smith, P. C.; McDonagh; A. F.; Benet, L. Z. Irreversible binding of zomepirac to plasma protein in vitro and in vivo. *J. Clin. Invest.* **1986**, *77*, 934–939.
29. Wells, D. S.; Janssen, F. W.; Ruelius, H. W. Interactions between oxaprozin glucuronide and human serum albumin. *Xenobiotica* **1987**, *17*, 1437–1449.
30. van Breemen, R. B.; Fenselau, C. Acylation of albumin by 1-O-acyl glucuronides. *Drug Metab. Dispos.* **1985**, *13*, 318–320.
31. Ding, A.; Ojingwa, J. C.; McDonagh, A. F.; Burlingame, A. L.; Benet, L. Z. Evidence for covalent binding of acyl glucuronides to serum albumin via an imine mechanism as revealed by tandem mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3797–3801.
32. Smith, P. C.; Liu, J. H. Covalent binding of suprofen acyl glucuronide to albumin in vitro. *Xenobiotica* **1993**, *23*, 337–348.
33. Kuchel, P.; Ralston, G. *Theory and problems of biochemistry* (Japanese edition); Ohmu Publisher: Tokyo, 1988; p 22.

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