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Research Article

Irreversible Binding of Tolmetin Glucuronic Acid Esters to Albumin *in Vitro*

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Tolmetin glucuronide (TG), extracted and purified from human urine, was incubated with albumin *in vitro*. The degradation profile and irreversible binding to protein were investigated and kinetic parameters calculated. Standard conditions were as follows: TG, 30 μg/ml; human serum albumin (HSA), 3%; pH 7.45; 37°C. Lower pH enhanced TG stability and reduced both the extent and the rate of irreversible binding. HSA also increased TG stability, compared to protein-free buffer, but the opposite was observed with bovine serum albumin (BSA). With BSA, irreversible binding was much less, but the rate of adduct formation was the same as with HSA. Essentially fatty acid free HSA behaved similarly to HSA. Preincubation of HSA with warfarin, or diazepam, or an excess of tolmetin, did not influence irreversible binding significantly. In buffer, acyl migration led predominantly to one isomer. This isomer bound irreversibly to HSA, although more slowly and to a lesser extent than the β1-isomer. Incubation of TG with poly-L-lysine also resulted in irreversible binding but to a lesser extent than with HSA. Our results suggest that there is more than one binding mechanism, with the preferential pathway a function of the isomers present and the experimental conditions.

KEY WORDS: tolmetin; irreversible binding; acyl glucuronide; acyl migration; serum albumin.

INTRODUCTION

Tolmetin is a nonsteroidal antiinflammatory drug (NSAID) of the aryl acetic acid class, currently used in the treatment of rheumatoid arthritis (1,2). It is metabolized via two principal pathways (Fig. 1). The major route is oxidation of the methyl group in the 4 position of the phenyl ring, leading to 1-methyl-5-[4-carboxybenzoyl]-1H-pyrrole-2-acetic acid (MCPA). The second route is conjugation with glucuronic acid, leading to tolmetin β-1-glucuronide (TGβ1) (3–5). Acyl glucuronides are unstable, undergoing hydrolysis and isomerization (by acyl migration), even in mildly alkaline or physiological environments (6–13).

Several aryl alkanoic NSAIDs analogous to tolmetin have been withdrawn from the market, because of toxicity and/or allergic reactions (14). Immunologic reactions to tolmetin have also been described (15). Structurally, tolmetin resembles zomepirac, which was withdrawn after several drug-related deaths had been reported (16).

Covalent binding to endogenous macromolecules is considered an important cause of toxicity and anaphylactic reaction to xenobiotics (8,17–19). Irreversible binding of glucuronide metabolites to plasma proteins *in vitro* and/or *in vivo* has been documented for bilirubin (9,20), zomepirac (21), oxaprozin (22,23), flufenamic acid, indomethacin, clofibric acid, and benoxaprofen (24). Recently we demonstrated irreversible binding *in vivo* for tolmetin to plasma proteins in young (25) and elderly volunteers with accumulation of the adduct in the case of multiple dosing (26).

Despite a growing interest in the irreversible binding of acyl glucuronides to proteins, the mechanism of the reaction is unclear. Two general mechanisms have been proposed (27). In one, binding occurs by nucleophilic attack of a protein functional group at the acyl carbonyl of the glucuronide. Protein nucleophiles that have been suggested are lysine ε-amino groups (9,28), cysteine sulfhydryl residues (24,29,30), and the tyrosine hydroxyl group (22,23). In this mechanism the glucuronyl moiety acts as a good leaving group, activating the parent compound. At the end of the reaction only the drug, without glucuronic acid, remains irreversibly bound to protein. In the other mechanism the aglycone migrates from the 1 hydroxyl group of the sugar to the 2, 3, or 4 hydroxyl group, allowing the glucuronic acid moiety to undergo ring-chain tautomerism. The aldehyde group of the ring-opened tautomer then condenses with a lysine or histidine group on the protein to form an imine. This mechanism, first proposed by us to explain zomepirac binding (21), is rather analogous to that involved in the glycosylation of proteins (31). At the completion of the reaction, the irreversibly bound product still contains a glucuronic acid, acting as a covalent link between protein and drug (27).

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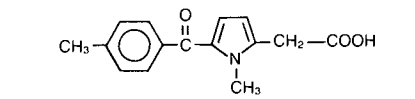
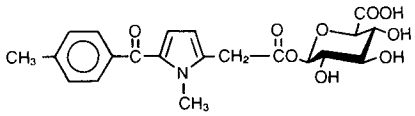
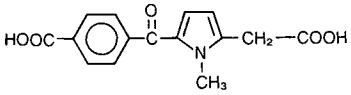


TOLMETIN



TOLMETIN GLUCURONIDE



METHYL-CARBOXYBENZOYL-PYRROLE-ACETIC ACID

Fig. 1. Chemical structures of tolmetin and its major metabolites.

Currently, it is not known which of these mechanisms is prinicipally responsible for the protein binding of acyl glucuronides; evidence for both exists.

In the present study we have examined the stability and albumin binding kinetics of tolmetin glucuronide and its isomers *in vitro,* with a view to comparing the reactivity of this important metabolite to that of other acyl glucuronides and obtaining information on the mechanism of the irreversible binding reaction.

MATERIALS AND METHODS

Materials

Human serum albumin fraction V, essentially fatty acid free human serum albumin (FAF-HSA), bovine serum albumin (BSA), and poly-L-lysine hydrobromide (molecular weight 30,000–70,000) were purchased from Sigma (St. Louis, MO). Tolmetin was obtained from McNeil Pharmaceutical (Springhouse,PA), diazepam from Hoffmann–LaRoche (Nutley, NJ), and warfarin sodium was USP grade. Other reagents were analytical or HPLC grade.

Isolation and Purification of Tolmetin Glucuronide from Urine

Because of the instability of acyl glucuronides, precautions were taken to avoid hydrolysis and isomerization (11,32) during the isolation and purification procedure. Urine of a volunteer, collected for 4 to 6 hr after ingestion of a 400-mg tolmetin capsule (Tolectin, McNeil Pharmaceutical, Springhouse, PA), was adjusted to pH 3 at once with phosphoric acid and frozen at −15°C. Upon thawing 200 ml of acidified urine was washed twice with an equal volume of CH_2Cl_2 and filtered. The filtrate was adjusted to pH 2.0 (H_3PO_4) and extracted twice with an equal volume of ethyl acetate. The combined extracts were dried for 2 hr with sodium sulfate, and the volume was reduced under vacuum at room temperature. The dry residue was reconstituted in 5 ml of 45% methanol in water, pH 4.5, and injected, as 25 to 50-μl portions, onto a semipreparative HPLC column (Ultrasphere ODS 5 μm, 10.0 mm × 25 cm) thermostated at 50°C. The mobile phase was 55% 0.05 *M* ammonium acetate buffer, pH 4.5, in methanol at a flow rate of 3 ml/min. Monitoring absorbance at 313 nm allowed collection of the fractions corresponding to TGβ1. The pH of each eluted fraction was decreased by the addition of 1 ml of glacial acetic acid per 15 ml of eluent, and fractions from different injections were combined and frozen if necessary. Methanol was removed at room temperature under vacuum and the residual aqueous solution was lyophilized.

The yield varied extensively among experiments but approximated 70 mg per 200 ml urine. Tolmetin glucuronide obtained by this procedure is abbreviated TG. The highest purity achieved, determined by our previously published HPLC procedure (33), was 97% TGβ1. The main impurity was tolmetin, resulting from hydrolysis of the glucuronide after purification.

Experimental Design

Protein solutions, 0.5 m*M* (3%, w/v), were prepared in sodium phosphate buffer 0.15 *M* (pH 7.45) unless otherwise specified. Potential inhibitors of reversible binding (warfarin and diazepam) were added as 200 μl isopropanol solutions at molar ratios (relative to albumin) of 3:4, 1:1, or 2:1, as indicated. Controls, i.e., no inhibitors added, also contained a similar isopropanol concentration. The effect of excess unconjugated tolmetin was studied at a concentration of 117 μ*M* (30 μg/ml), which corresponds to an average maximum plasma concentration after ingestion of 400 mg tolmetin (25). Solutions were stabilized at 37°C for 1 hr before adding 69.3 μ*M* TG (30 μg/ml) and incubations were conducted at 37°C. In one experiment, TG was dissolved in the buffer and incubated for 90 min before HSA was added. Timing for sampling began upon HSA addition. During the course of this work, three different batches of TG were prepared as described above and HSA was used from three different purchased lots. For a given batch of TG and a given lot of HSA, control irreversible binding values yielded coefficients of variation <15%. When different batches of TG were tested with the same lot of HSA, similar variabilities for control binding measurements were obtained. However, when different lots of HSA were tested, marked differences in control irreversible binding was observed. Therefore, each experiment described here was run using a single batch of TG and a single lot of HSA, allowing comparison with a reproducible control value. When different experiments are compared, percentages of control, rather than absolute amounts bound, are reported.

Kinetics Studies. Sampling times were 5, 10, 30, 45, 60, 90, 120, 180, 240, 300, 360, 480, and 1440 min. At each time, four aliquots (500 μl) were taken for duplicate HPLC assay of reversibly and irreversibly bound species, as previously described (25,33). Drug remaining with the precipitated protein pellet after exhaustive washing is defined as irreversibly bound (34). Reversibly bound concentrations refer to that which is free plus that reversibly bound to plasma proteins.

Single-Point Studies. Samples were taken as soon as TG had dissolved (control time zero) and 4 hr later. Three aliquots (500 μl) at time zero and six at 4 hr were taken for measurement of reversibly bound concentrations, and six aliquots were taken at 4 hr for the irreversible binding assay.

Calculations

Concentrations of irreversibly bound drug were calculated as the amount of tolmetin bound per milligram of protein multiplied by the protein concentration. AUCs (areas under the concentration versus time curves) were estimated by the log-linear trapezoidal procedure. Statistical analyses and linear and multilinear regressions were performed with SYSTAT (35), while nonlinear/multicompartmental fittings were obtained using NONMEM (36).

RESULTS

When TG is dissolved in buffer or albumin solutions at physiologic pH, it undergoes hydrolysis to the parent tolmetin, as well as isomerization. The latter occurs by acyl migration, the aglycone migrating sequentially from the 1 position of the glucuronic acid to the 2, 3, and 4 positions. The isomers also undergo hydrolysis to tolmetin. On HPLC three isomer peaks are detectable, designated A, B, and C in order of their retention times. The specific identities of these peaks have not been definitively established. However, because acyl migration is usually sequential, the time for each isomer to reach its peak concentration on incubation of TG probably reflects its position on the glucuronic acid. This has been confirmed for zomepirac and bilirubin glucuronide isomers (6,37). On this basis, we conclude that isomer A has the aglycone attached in the 4 position, isomer B in the 2 position, and isomer C in the 3 position.

Figure 2 shows measured and computer-fitted curves for the concentrations of reversibly bound species (T, TGβ1, isomers A and C) observed when TG was incubated with 0.5 m*M* HSA in buffer, pH 7.45, at 37°C ("control" conditions). Computer fitting was based on the model shown in Fig. 3, which was the best of several models considered. Isomer B was barely detectable and is not included. Drug loss due to irreversible protein binding was measurable, but at concentrations similar to isomer B, and this route of loss was also not included in the initial model depicted in Fig. 3, although

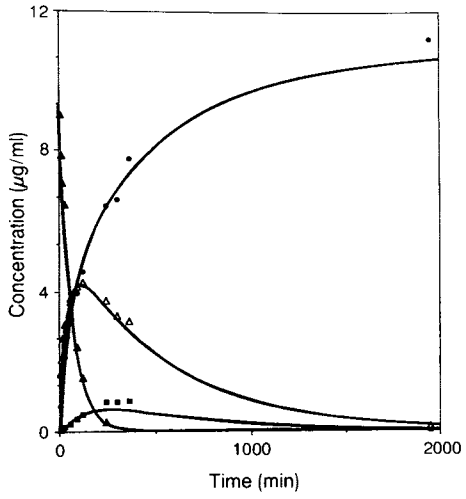


Fig. 2. Concentration versus time profiles for tolmetin (filled circles), TGβ1 (filled triangles), Isomers A (filled squares) and C (open triangles). Conditions are pH 7.45, HSA 3%, 37°C (= control). The lines are fitted by nonlinear simultaneous regression according to the model depicted in Fig. 3 (see text).

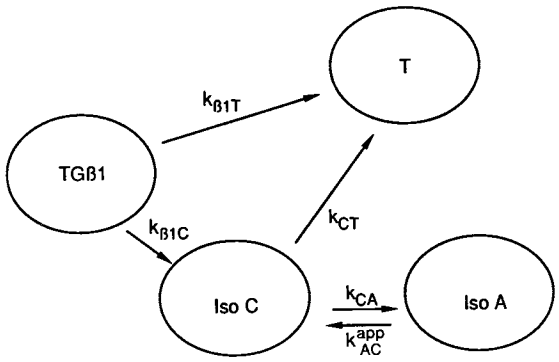


Fig. 3. Model employed in the fitting of the control experiment. T stands for tolmetin, TGβ1 for the 1β-glucuronide, and Iso A and C for two of the acyl migration isomers.

these concentrations are analyzed subsequently. Back-isomerization to the enzymatically formed β1-conjugate was considered to be negligible as has been found in all studies of acyl migration. Although isomer B peaked first, its concentrations were only briefly and marginally above the level of detection. Therefore, the throughput of a possible B compartment was considered extremely rapid and this intermediate was not included in the model. The rate constants for conversion of isomer A to either tolmetin or isomer C cannot be differentiated. However, on a semilogarithmic plot, all isomers (except TGβ1) declined in parallel, since the interconversion reactions are faster than hydrolysis. This rapid interconversion plus the fact that the highest isomer concentration is reached by isomer C suggests that conversion of isomer A to tolmetin is probably slower than conversion of isomer A to isomer C. Since isomer A concentrations were always significantly less than tolmetin concentrations (Fig. 2), the calculated apparent rate constant k_{AC}^{app} must equal the sum of the true rate constants k_{AC} and k_{AT}. Microconstants calculated for the model in Fig. 3 are reported in Table I. The β1-glucuronide is less stable than its isomers, and under control conditions its hydrolysis rate (defined by $k_{\beta 1T}$) is about the same as the rate of isomerization (defined by $k_{\beta 1C}$). Back-conversion from isomer A to isomer C is fast, and the rate-limiting step responsible for the parallel decline of the isomers is hydrolysis of isomer C (i.e., k_{CT}).

The disappearance of TGβ1 was examined under a number of conditions. The first-order rate constants for this disappearance, kβ1, are listed in Table II. This rate constant is actually the sum of the hydrolysis and isomerization rate

Table I. Rate Constants of Hydrolysis and Isomerization of Tolmetin Glucuronide[a] and Its Isomers in 3% HSA, pH 7.45, 37°C

	Rate constant[b] (10^{-3} min^{-1})	SE (10^{-3} min^{-1})	Coefficient of variation (%)
$k_{\beta 1C}$	7.91	0.36	4.6
$k_{\beta 1T}$	7.45	0.39	5.2
k_{CA}	1.81	0.30	16.6
k_{CT}	2.20	0.23	10.5
k_{AC}^{app}	9.8	1.5	15.6

[a] Initial tolmetin glucuronide concentration, 30 μg/ml.
[b] Rate constants refer to pathways in Fig. 3.

Table II. Effect of pH and Nature of the Protein on the Disappearance Rate Constant of Tolmetin Glucuronide ($k_{\beta 1}$), the Apparent Formation Rate Constant for Irreversible Binding (k_f), and the Extent of Irreversible Binding at 4 hr

Conditions	$k_{\beta 1}$ (±SE) (10^{-3} min^{-1})	k_f (±SE) (10^{-3} min^{-1})	Extent of binding (±SE) (% of control)
Control[a]	14.8 ± 0.9	12 ± 1	100 ± 26
pH 6.8	5.4 ± 0.1	3 ± 1	19 ± 5
pH 5.5	1.0 ± 0.1	4 ± 1	6.5 ± 1.7
Excess T[b]	18.2 ± 0.4	10 ± 3	84 ± 24
BSA	74 ± 2	78 ± 13	19 ± 5
Buffer	43.9 ± 0.5		
FAF-HSA[c]			98 ± 14

[a] 30 μg/ml TGβ1, 3% HSA, pH 7.45, 37°C.
[b] 30 μg/ml tolmetin added before TG.
[c] Four-hour sample only.

constants of TGβ1 (i.e., $k_{\beta 1T}$ and $k_{\beta 1C}$ in Fig. 3) plus the rate constant for formation of irreversible bound drug which results directly from the β1-glucuronide. The values listed in Table II were determined from computer fits of the data to a single exponential equation describing TGβ1 disappearance. As we recently demonstrated (32), decreasing the pH stabilized the β1-glucuronide, with $k_{\beta 1}$ at pH 5.5 decreased approximately 15-fold as compared to control conditions. Unexpectedly, disappearance of TGβ1 was faster in buffer than in HSA solution and faster still in the presence of BSA. The addition of excess tolmetin had no effect on the rate of TGβ1 disappearance.

For each of the reactions in which TGβ1 disappearance was determined, the time course of irreversible protein bound drug concentration was measured (except for the buffer experiment where no protein was present). A plot of average measurements for the control experiments is presented in Fig. 4. The bound drug concentrations, C_b, were fitted to the empiric equation:

$$C_b = Z(e^{-k_d t} - e^{-k_f t}) \quad (1)$$

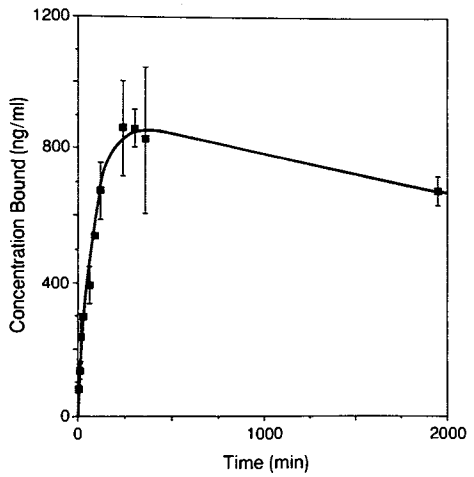


Fig. 4. Concentration of tolmetin irreversibly bound to protein versus time. The line is fitted according to Eq. (1). Same conditions as in Fig. 2.

where k_d and k_f are, respectively, the degradation and apparent formation rate constants for the irreversibly bound adduct, and Z is a constant. Since only a few measurements were obtained during the decreasing adduct concentration phase, estimates of k_d may not be reliable, but the apparent formation rate constant, k_f, should be precise and independent of the relatively low k_d value. Values for k_f are listed in Table II and they are quite similar to the $k_{\beta 1}$ estimates, suggesting that the rate-limiting step for irreversible binding is the disappearance of TGβ1. Measures of the extent of irreversible binding at 4 hr relative to control are also presented in Table II. The addition of excess tolmetin and the use of essentially fatty acid free HSA did not affect the extent of binding. Lowering the pH resulted in a decreased extent of binding at 4 hr which roughly paralleled the decrease in the rate of disappearance of TGβ1. After 90 min in buffer alone, TGβ1 was almost completely converted to isomer C, with some isomer A, and only a little hydrolysis to tolmetin. When HSA was added at this point irreversible binding still occurred, although more slowly than under the control conditions when HSA was present from the beginning of the incubation. At and after 1 hr, irreversible binding averaged 28% of the control value (not shown in Table II).

The rate of formation of total adducts, dX_b/dt, can be described, by analogy with multicompartmental pharmacokinetics, as the sum of products of concentrations, C, and formation clearances, CL_{ib}, of an irreversibly bound species, b, from various glucuronides:

$$\frac{dX_b}{dt} = CL_{\beta 1b}C_{\beta 1} + CL_{Ab}C_A + CL_{Bb}C_B + CL_{Cb}C_C \quad (2)$$

where β1 indicates the parent TGβ1 and A, B, and C the migration isomers. Degradation of the adduct is negligible under the conditions utilized here and can be ignored. Integrating from time zero to t gives

$$\int_0^t dX_b = CL_{\beta 1b}\int_0^t C_{\beta 1}dt + CL_{Ab}\int_0^t C_A dt + CL_{Bb}\int_0^t C_B dt + CL_{Cb}\int_0^t C_C dt \quad (3)$$

or

$$X_b = CL_{\beta 1b}AUC_{\beta 1} + CL_{Ab}AUC_A + CL_{Bb}AUC_B + CL_{Cb}AUC_C \quad (4)$$

where AUC denotes the cumulative area under the concentration vs time curve for each acyl glucuronide.

Dividing both sides of Eq. (4) by the volume of the reaction solution transforms the amount term into a concentration, and clearances into rate constants:

$$C_b = k_{\beta 1b}AUC_{\beta 1} + k_{Ab}AUC_A + k_{Bb}AUC_B + k_{Cb}AUC_C \quad (5)$$

providing a multilinear relationship between the concentration of irreversibly bound tolmetin at time t and the corresponding AUCs. The coefficients in this equation are the true rate constants for each pathway. Unfortunately, the AUCs for isomer A and B were quite variable and yielded relatively low values based on measurements near the limit

of detection. Incorporating them as separate terms in the regression led to large errors. Therefore, Eq. (5) was simplified by including all isomer irreversible binding within a single constant, $k_{iso,b}$:

$$C_b = k_{\beta 1b}AUC_{\beta 1} + k_{iso,b}\Sigma AUC_{iso} \quad (6)$$

The total AUC of the isomers was small compared to the AUC for TGβ1, suggesting that the contribution of the isomers to the formation of the irreversible adduct might be insignificant. To test this we used three criteria. First, the calculated coefficient of the AUC_{iso} term was tested for a significant difference from zero, using a two-tailed t test at $P < 0.05$ (38). Second, the Akaike information criterion (AIC) was calculated (39). Third, the significance of the decrease in the sum of squared deviations was tested with an F test (40). The results of the multilinear regressions, accompanied by the results of the three criteria, are summarized in Table III. Correspondence between the three tests was good, except in the control study, where the t test for AUC_{iso} coefficient and F test for the sum of squared deviations contradicted the AIC. At pH 6.8, the isomers seem to make a significant contribution to the binding, whereas under other conditions they do not. The negative value for $k_{iso,b}$ at pH 5.5 has no physical meaning and cannot be attributed to a mechanistic explanation.

In Fig. 5, the concentrations of irreversibly bound drug are plotted versus the cumulative AUCs of TGβ1 for several incubation conditions. All curves except for the pH 6.8 study are approximately linear, reflecting the minimal contribution of the isomers to binding. The slopes of the regression lines are equal to $k_{\beta 1b}$. Values of $k_{\beta 1b}$ for the control, the excess of tolmetin, and the BSA experiments are not significantly different. The pH 6.8 study did not yield a linear relation, consistent with the conclusion (Table III) that the isomers do contribute to irreversible binding at this pH.

A separate study was carried out to compare the rate of adduct formation from β1-glucuronide with that from isomer C. For this study a solution of isomer C was generated by incubating TG in albumin-free buffer for 90 min. This solution was then used without further purification. This experiment was carried out after our original supplies of TGβ1 and HSA were exhausted, and the control value with the new batches of TGβ1 and HSA was lower than the reported for the control in Table III. However, the rate of formation of irreversible binding from isomer C ($k_{iso,b}$) was only one-tenth (0.10 ± 0.015) of its control ($k_{\beta 1b}$), indicating that binding from isomer C occurs at a 10-fold slower rate than from TGβ1. The extent of irreversible binding from isomer C was 28% of that found in the control experiments.

Table III. Adduct Formation Rate Constant Obtained by Multilinear Regression

Conditions[a]	$k_{\beta 1b}$ (10^{-3} min^{-1})	$k_{iso,b}$ (10^{-3} min^{-1})	Criteria for including a second linear term[d]		
			t test	Akaike	F test
Control	1.08 (±0.05)[b]		−	+	−
pH 6.8	0.102 (±0.011)	0.022 (±0.009)	+	+	+
pH 5.5	0.028 (±0.004)	−0.022[c] (±0.007)	+	+	+
Excess T	0.79 (±0.08)		−	−	−
BSA	1.20 (±0.05)		−	−	−

[a] As differs from the control (=3% HSA, pH 7.45, 37°C). Abbreviations are defined in the text.
[b] Standard error.
[c] A negative coefficient has no physical meaning. When ΣAUC_{iso} is not taken into account, $k_{\beta 1b}$ becomes 0.017 (±0.002) 10^{-3} min^{-1}.
[d] See text for definition of the tests utilized.

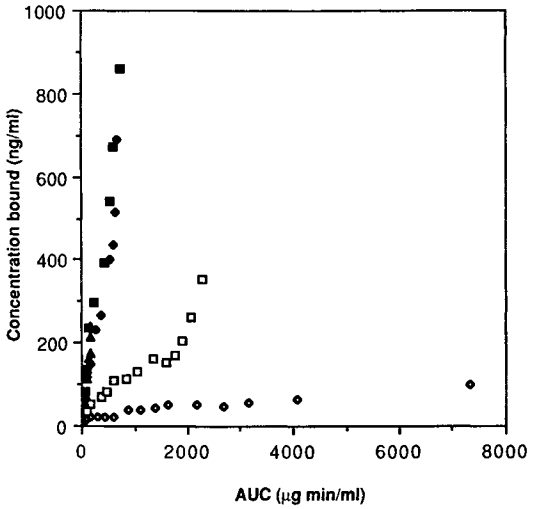


Fig. 5. Concentrations of tolmetin irreversibly bound to protein versus AUCs of TGβ1. Conditions were as follows: control HSA, pH 7.45 (filled squares), pH 6.8 (open squares), and 5.5 (open diamonds); BSA (filled triangles); excess of tolmetin (filled diamonds).

To test whether other drugs with a high reversible affinity for albumin binding sites inhibit the irreversible binding, HSA was incubated with warfarin sodium and/or diazepam prior to TG addition. Both warfarin sodium and diazepam were dissolved in 200 μl isopropanol and the study was run versus a control containing 200 μl isopropanol. The concentrations irreversibly bound after 4 hr of incubation are plotted as a percentage of the control in Fig. 6. Values have been normalized for the total concentration of glucuronic acid esters present at time zero. The slight decrease in the binding is marginally significant [$P = 0.032$, ANOVA; but $\alpha_T = 0.08$ with Student–Newman–Keuls test for the largest observed difference, i.e., between the control and 2:1 W (see Fig. 6), while $P = 0.13$ when calculated with the Bonferroni t test with pooled variance] (38).

To investigate whether a lysine residue on HSA might be the binding site for glucuronides, TG was incubated with poly-L-lysine hydrobromide. After 4 hr, statistically significant binding to poly-lysine was observed ($P < 0.0005$, two-tailed t test), but this represented only about 12% of the control with HSA.

Finally, in all of the above experiments, tolmetin controls (in place of tolmetin glucuronide) were performed. With our procedure, which involves exhaustive washing of the precipitated protein pellet, no irreversible binding of tolmetin was ever detected.

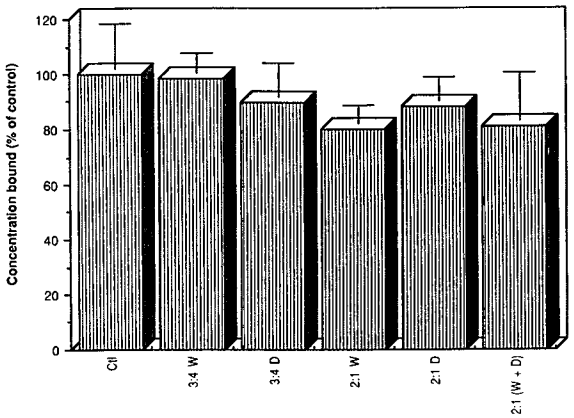


Fig. 6. Inhibition of irreversible binding by warfarin sodium (W) and diazepam (D) at various molar ratios of inhibitor to albumin. Results (mean of six experiments) are expressed as a percentage of the control (Ctl). Vertical lines indicate standard deviations.

DISCUSSION

In this study, we investigated the kinetics of hydrolysis and isomerization of tolmetin glucuronide and its acyl migration isomers and the kinetics of their irreversible binding to albumin. At pH 7.4, the rate-limiting step for irreversible binding is disappearance of TGβ1, i.e., $k_{\beta 1}$, which appears to be almost equally divided between hydrolysis to T and formation of migration isomers (Table I). The model depicted in Fig. 2 also yields an almost-equal split between hydrolysis and acyl migration for isomer C at pH 7.4. Like other acyl glucuronides (11), tolmetin glucuronide, and probably its isomers, was more stable in solution at slightly acidic pH than at pH 7.4, and irreversible binding with albumin occurred more slowly and to a lesser extent at acidic pH's (Table II). Replacing HSA with BSA in incubation mixtures did not affect the rate of irreversible binding but reduced its extent and accelerated the disappearance of TGβ1. Previous studies have suggested that HSA catalyzes the hydrolysis of oxaprozin glucuronide (22). Disappearance of TGβ1 was fivefold faster in the presence of BSA and threefold faster in protein-free buffer than in the presence of HSA (Table II). Therefore, if there is any protein catalysis of tolmetin glucuronide hydrolysis, it is exhibited by BSA, rather than HSA.

The faster TGβ1 disappearance caused by BSA apparently has no effect on the rate of adduct formation (Table III). The amount of drug that becomes irreversibly bound depends only on the exposure of the protein to the binding precursor, mainly TGβ1. Exposure is measured by the AUC, i.e., the integral of the product of concentration and time. For this reason, in the plot of the concentration of irreversibly bound drug versus AUC of TGβ1 (Fig. 5), the lines for the control and BSA experiments, and also for the excess tolmetin experiment, are superimposable.

When TG was incubated in protein-free buffer for 90 min, the β1-glucuronide disappeared and isomer C became the predominant glucuronic acid ester, with less than 20% as isomers A and B. When HSA was incubated with this mixture irreversible binding still occurred, although more slowly and to a lesser extent than when HSA was incubated with unrearranged TG. Back-isomerization to TGβ1 was not detected. These observations indicate that attachment of the aglycone to the β1-position of the sugar is not an absolute requirement for covalent binding, as we previously demonstrated with zomepirac glucuronide (21). Interestingly, the reactivity of TGβ1 was significantly greater than that for isomer C. The model depicted in Fig. 3 suggests that both TGβ1 and isomer C can both isomerize and be hydrolyzed to the parent aglycone. However, the difference in reactivity appears to show a rank-order correlation with irreversible binding. The imine mechanism previously proposed (21) would suggest that isomer C would lead to irreversible binding (Smith, Benet, and McDonagh, submitted for publication). Yet if that mechanism was operative, it is difficult to explain why the reactivity of isomer C was less than that for TGβ1. Thus, imine formation with the protein may not be the only pathway for irreversible binding of tolmetin glucuronide. Consistent with the imine mechanism, we have observed in preliminary studies not described here that adding the imine-trapping reagent NaCN to incubation mixtures containing TG and HSA increases the extent of irreversible binding dramatically, as was seen previously for zomepirac (21). That free sulfhydryl groups are not required for irreversible binding (29) was demonstrated by incubating TG with poly-lysine. Although irreversible binding was observed, poly-lysine was significantly less reactive than HSA.

Presumably, reversible binding of the glucuronide to protein accompanies or precedes irreversible binding. Therefore, agents with a high affinity for the same binding site may diminish irreversible binding. To test this hypothesis, we studied the effect of several compounds which bind to albumin. Because the location of the tolmetin glucuronide binding site on albumin is not known, we used as potential inhibitors warfarin and diazepam, which bind to different specific sites on the albumin molecule (41), and tolmetin, which is greater than 99% reversibly bound to albumin under the incubation conditions (42). We also compared Cohn fraction V HSA, which contains bound fatty acids, with fatty acid free HSA as the substrate for irreversible binding. None of the potential inhibitors decreased irreversible binding significantly, suggesting that tolmetin glucuronide does not bind to or react with HSA at the warfarin, diazepam, tolmetin, or fatty acid binding sites. In this respect, irreversible binding of tolmetin glucuronide is different from that of oxaprozin glucuronide, which is markedly inhibited by the aglycone and diazepam but not by warfarin (22). Using defatted, in place of nondefatted, HSA did not enhance irreversible binding of tolmetin glucuronide, as reported for oxaprozin glucuronide (22). In the presence of excess tolmetin there was a small, although not statistically significant, decrease in the amount of adduct formation. If this really reflects inhibition, it must be competitive, rather than noncompetitive inhibition, since there was no change in the apparent rate of adduct formation (Fig. 5).

These studies confirm that tolmetin glucuronide is a reactive metabolite. Although it readily undergoes hydrolysis, isomerization, and irreversible binding to albumin, in common with glucuronides of other nonsteroidal antiinflammatory agents (21–24), its stability and reactivity with serum albumin are clearly not identical to those of other glucuronides that have been studied. In particular, in its reaction with albumin, tolmetin glucuronide shows marked differences from oxaprozin glucuronide but, not surprisingly,

strong similarities to its structural analogue, zomepirac glucuronide. Our results demonstrate that not only the β1 isomer of tolmetin glucuronide, but also isomers derived from this by migration of the aglycone around the sugar, can react with protein to give irreversibly linked products, as previously shown for zomepirac glucuronide (21). However, as with zomepirac glucuronide, the β1 isomer of tolmerin glucuronide seems to be more reactive than its isomers. The formation of irreversible protein complexes from acyl glucuronides appears to be sensitive to the structure of the aglycone, the nature of the protein, and the environmental conditions. Our present data suggest that irreversible binding can occur by at least two mechanisms and that a particular acyl glucuronide may react covalently with albumin by more than one route, depending on the conditions.

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