Research Article

Irreversible Binding of Tolmetin Glucuronic Acid Esters to Albumin in Vitro

Alain Munafò, Antony F. McDonagh, Philip C. Smith, and Leslie Z. Benet

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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, 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 β1-glucuronide (TGB1) (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Several aryl alkanoic NSAIDs analogous to tolmetin have been withdrawn from the market, because of toxicity and/or allergic reactions (14).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 Structurally, tolmetin resembles zomepirac, which was withdrawn after several drug-related deaths had been reported (16).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 sulphydryl 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).

1 Department of Pharmacy, School of Pharmacy, University of California, San Francisco, California 94143-0446.
2 Present address: Division of Clinical Pharmacology, Department of Internal Medicine, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland.
3 Liver Center, School of Medicine, University of California, San Francisco, California 94143-0538.
4 Present address: College of Pharmacy, PHR 4.214E, The University of Texas at Austin, Austin, Texas 78712.
5 To whom correspondence should be addressed.
Currently, it is not known which of these mechanisms is principally 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 tolnetin 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 (Tolmetin, 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 CH₂Cl₂ and filtered. The filtrate was adjusted to pH 2.0 (H₃PO₄) 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 mM (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 μM (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 μM 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.