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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TOLMETIN, TOLMETIN GLUCURONIDE AND ITS ISOMERIC CONJUGATES IN PLASMA AND URINE

MARTHA L. HYNECK★, PHILIP C. SMITH★★, ELISABETH UNSELD and LESLIE Z. BENET*

Department of Pharmacy, School of Pharmacy, University of California, San Francisco, San Francisco, CA 94143-0446 (U.S.A.)

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SUMMARY

A rapid and sensitive analytical procedure is described for the simultaneous measurement of tolmetin (T), tolmetin glucuronide (1β-TG) and the isomers of tolmetin glucuronide in plasma and urine. A reversed-phase liquid chromatographic system is used with an ion-pairing mobile phase of methanol–tetrabutylammonium hydrogensulfate buffered to pH 4.5 and kept at a constant temperature of 50°C. Detection is by UV at 313 nm. Plasma (0.5 ml) and urine (0.1 ml) are collected in pre-cooled containers and immediately adjusted to pH 3.0 to minimize TG isomerization and hydrolysis. Samples are then deproteinated with acetonitrile, the supernatant is evaporated to dryness and reconstituted in an acetate buffer (pH 4.5), and 50 μl are injected onto the system. Using zomepirac as the internal standard, the measurable, linear concentration ranges are 0.05–50 μg/ml for T in plasma and 0.025–50 μg/ml for T in urine. Chromatographic peaks representing T,1β-TG and three isomers of TG were identified, all with retention times less than 10 min. The need for special handling of biological samples is discussed.

INTRODUCTION

Tolmetin (T), 1-methyl-5-*p*-toluoylpyrrole-2-acetic acid (Fig. 1), is a non-steroidal anti-inflammatory drug used in the treatment of rheumatoid arthritis [1]. Reports of the chemistry, pharmacology and pharmacokinetics of T have appeared in the literature [2–8]. T is oxidized to methylcarboxybenzoylpyrrole acetic acid (MCPA) and to tolmetin (acyl) glucuronide (TG). Plasma concentrations of tolmetin following the administration of a single 400-mg capsule range

★Present address: College of Pharmacy, The University of Michigan, Ann Arbor, MI 48109-1065, U.S.A.
★★Present address: Building 37, Room 2C-04, National Cancer Institute, NIH, Bethesda, MD 20892, U.S.A.

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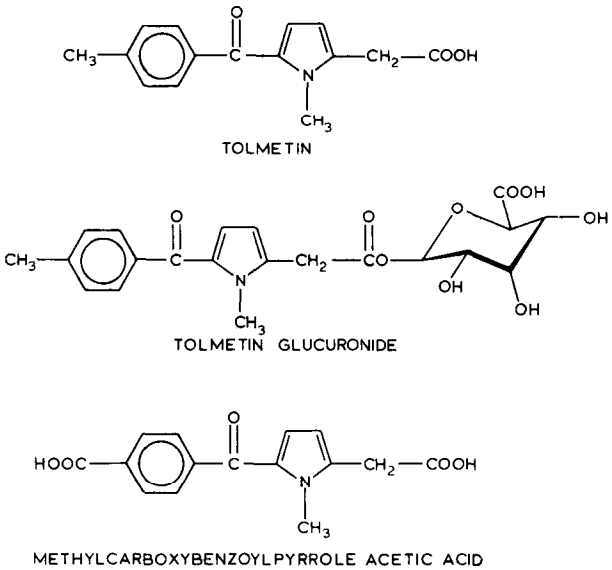

Fig. 1. Chemical structures of tolmetin and its major metabolites.

from a peak of 30–50 μg/ml to a minimum of 0.1–0.4 μg/ml by the end of a 12-h dosing interval [2, 4, 6, 7]. The disposition of 1β-tolmetin glucuronide (1β-TG) and its isomeric conjugates (TG-iso) has not been described previously due to the lack of a sensitive assay which can quantitate these metabolites.

Previous work from this laboratory [9] and by others [10–15] has demonstrated that ester glucuronides are unstable at physiological pH. The instability of the glucuronides of zomepirac [9], clofibric acid [10], diflunisal [11], probenecid [12], valproic acid [13], furosemide [14] and bilirubin [15] has been documented in the literature. Glucuronide ester metabolites can undergo base-catalyzed intramolecular acyl migration to form isomers, and can be hydrolyzed back to the parent drug. Unless proper precautions are taken, both acyl migration and hydrolysis can occur during sample handling and drug analysis, leading to inaccurate descriptions of the disposition of tolmetin and its metabolites.

Several analytical procedures to determine T and MCPA concentrations in plasma and urine have been published. Previous reports have described assays using reversed-phase high-performance liquid chromatography (HPLC) [16–19], gas chromatography [20, 21], spectrophotometry [20] and radiolabeling (^{14}C) [2]. However, in most of these assays the lower limits of detection are not sufficiently sensitive to allow for appropriate pharmacokinetic analysis. And, in addition to the fact that some of the procedures are tedious to perform, none of the above assays allow for the resolution and accurate quantitation of 1β-TG or TG-iso.

Therefore, since we were interested in examining the disposition and stability of T and TG, we developed a rapid and sensitive analytical HPLC method to simultaneously measure T, its acyl glucuronide metabolite and the isomeric conjugates of 1β-TG in human plasma and urine incorporating sample handling pro-

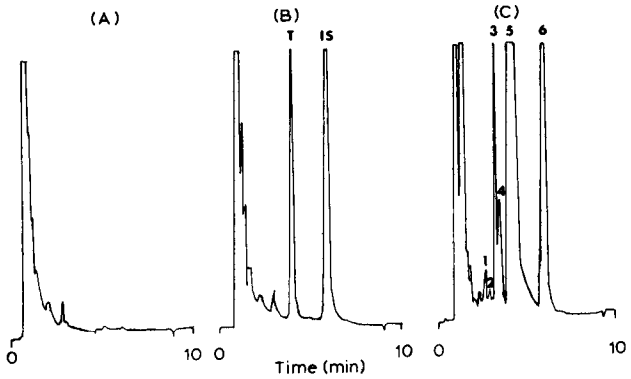

Fig. 2. Sample plasma chromatograms. (A) Plasma blank; (B) plasma blank spiked with 1 μg/ml T and 2 μg/ml I.S.; (C) plasma sample taken 90 min following administration of a 400-mg oral dose of T, demonstrating peaks of T (5), 1β-TG (3), three potative TG-iso peaks (1, 2 and 4) and I.S. (6).

cedures which minimize acyl migration and hydrolysis. From these studies, we conclusively show that isomeric conjugates are present in plasma and urine of humans after an oral dose of T.

EXPERIMENTAL

Chemicals and reagents

Tolmetin sodium·$2H_2O$ and zomepirac sodium·$2H_2O$, the internal standard (I.S.), were kindly supplied by McNeil Pharmaceutical (Springhouse, PA, U.S.A.). Methanol and acetonitrile (HPLC grade) were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). β-Glucuronidase from bovine liver was obtained from Sigma (St. Louis, MO, U.S.A.). All other chemical reagents were of analytical grade.

Chromatography

The HPLC system was equipped with the following components: Altex Model 110A pump, Waters Model 710A WISP automatic injector, Waters Model 440A fixed-wavelength UV detector set at 313 nm, Hewlett-Packard Model 3390A integrator and Beckman Ultrasphere C_8 reversed-phase column (5 μm particle size, 15 cm × 4.6 mm I.D.). Column temperature was held constant at 50°C by circulating water from a constant temperature water-bath through coils of tygon tubing wrapped around the column.

The mobile phase was methanol–0.01 *M* tetrabutylammonium hydrogensulfate (50:50, v/v) containing 0.05 *M* sodium acetate (pH 4.5) and was prepared fresh daily. With a flow-rate of 1.7 ml/min, retention times for T, 1β-TG, TG isomers identified as fractions 1, 2, and 4 and I.S. were 5.3, 4.0, 3.2, 3.5, 4.4 and 8.0 min, respectively (Figs. 2 and 3). MCPA did not interfere with T, 1β-TG or TG-iso in that MCPA eluted either with (plasma) or close to (urine) the solvent front (Figs. 2 and 3). By changing the mobile phase to 36% methanol, the retention time for MCPA becomes 5 min, while the retention times for T, 1β-TG and TG-iso become greater than 20 min.

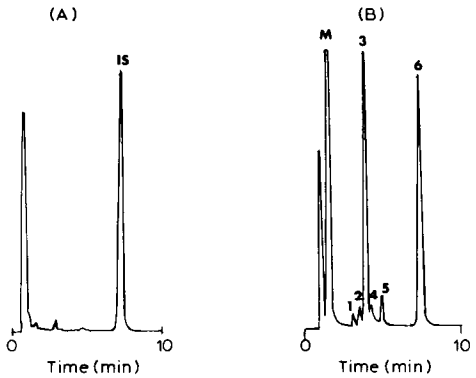

Fig. 3. Sample urine chromatograms. (A) Urine blank spiked with 10 μg/ml I.S.; (B) urine sample, 2–4 h collection, following administration of a 400-mg oral dose of T. Peak designations as listed in Fig. 2 with the MCPA peak indicated as M.

Standard curve preparation

Stock solutions of T were prepared by dissolving the equivalent of 125 mg of tolmetin acid in 100 ml of methanol (1250 μg/ml). Standard solutions in methanol were made by serial dilution of this stock solution in methanol to final concentrations of 125, 12.5 and 1.25 μg/ml. Stock solution A of the internal standard was prepared by dissolving the equivalent of 20 mg of zomepirac acid in 100 ml of methanol (200 μg/ml). The stock I.S. solution B (20 μg/ml) was made as a 1:10 dilution in methanol of stock solution A. Standard curves of T in plasma were run each day at concentrations from 0.05 to 50 μg/ml, together with 2.0 μg/ml I.S. Standard curves of T in urine were run at concentrations from 0.025 to 50 μg/ml, together with 10 μg/ml I.S.

Sample preparation

Aliquots of plasma (0.5 ml) or urine (0.1 ml) were adjusted to pH 3.0 by the addition of concentrated phosphoric acid immediately following blood withdrawal and urine collection from the subjects. Plasma and urine samples were then maintained in a frozen state at −15 °C prior to analysis. Samples were spiked with 50 μl of internal standard using stock solution A for the plasma assay and stock solution B for the urine assay. Since the concentration of tolmetin in the urine is very high during most of a dosing interval, urine samples and blank samples for the standard curves were diluted 1:10 with distilled water prior to withdrawing the aliquot for assay. Plasma and urine samples, together with the standard curve samples, were vortexed (10 s), deproteinated with either 1.0 ml (urine) or 1.5 ml (plasma) acetonitrile and vortexed again for 60 s. The precipitate was separated by centrifugation at 2250 *g* (3000 rpm) for 10 min. The supernatant was then transferred to another test tube and evaporated to dryness under a stream of nitrogen at 30 °C. The residue was reconstituted into 300 μl acetonitrile–0.5 *M* sodium acetate, pH 4.5 (25:75, v/v), transferred to a WISP vial, and 50 μl were injected onto the HPLC column. All samples were prepared and analyzed on the same day.

Since previous work from this laboratory has demonstrated that acyl migration and hydrolysis of glucuronides can occur at physiological pH [9], special handling procedures were implemented during the collection of plasma and urine samples. Blood samples were taken directly into heparinized, preiced Vacutainers® and centrifuged immediately at 10°C. Plasma (3–4 ml) was immediately transferred to vials containing concentrated phosphoric acid (10 μl/ml of plasma) so that the resulting pH was in the 2–4 range. The pH of urine aliquots was adjusted to the 2–4 range using phosphoric acid immediately after collection. Overnight urine specimens were collected in plastic vessels which contained phosphoric acid.

Identification of TG and TG isomers

To identify the TG and putative TG-iso peaks in the chromatogram, urine samples from subjects who had ingested T were assayed as described above and compared to chromatograms from blank urine spiked with either T or IS. As can be seen in Figs. 2 and 3, four additional peaks can be identified and were consistently present when T was present in vivo. Since 1β-TG and TG-iso, as esters, would be labile to hydrolysis when exposed to strong basic conditions but only the 1β-TG would be susceptible to β-glucuronidase [9], urine samples were exposed to basic conditions (pH 7.4) and β-glucuronidase (5000 U) on separate occasions and HPLC analysis was used to follow the resulting reactions. From these experiments it became obvious which of the four peaks was 1β-TG (Figs. 2 and 3). The stability of 1β-TG as well as the acyl migration of TG as a function of pH and time will be presented in detail elsewhere.

Quantitation

Area ratios of T/I.S. were plotted against concentrations of T; similar concentration-to-area ratios were determined for TG, assuming an identical molar extinction coefficient as for T. We previously found that zomepirac and zomepirac glucuronides exhibited isomolar absorbancy [22]. A transformed least-squares regression (1/concentration) was performed on the data [23]. Linearity of the plasma standard curve samples was tested from 0.05 to 50 μg/ml, and the urine standard curve was tested from 0.025 to 50 μg/ml.

RESULTS AND DISCUSSION

Accuracy and variability

Average measured concentrations and intra-day variability for T in plasma and urine were calculated from the area ratios at 0.50, 5.0 and 50.0 μg/ml representing low, medium and high concentrations within the standard curve for each biological fluid. Data are shown in Table I. Inter-day variability of the assay was determined by taking the mean, standard deviation and coefficient of variation of six independently prepared standard curves for T in both plasma and urine (Table II).

TABLE I

ACCURACY, PRECISION AND INTRA-DAY VARIABILITY OF TOLMETIN ASSAY IN PLASMA AND URINE ($n=5$)

Spiked concentration (μg/ml)	Average measured concentration (μg/ml)		Coefficient of variation (%)	
	Plasma	Urine	Plasma	Urine
0.50	0.49	0.51	3.62	2.02
5.0	4.97	5.07	3.48	1.55
50.0	52.0	50.9	3.59	0.64

Degradation and stability

The stability of T and 1β-TG over time when stored at $-15\,^{\circ}$C was determined by analyses of frozen urine samples from subjects who had ingested the drug; samples were assayed within two weeks after the collection was completed and again approximately six months later. No decrease in T or 1β-TG concentration was found. Since the HPLC column was warmed to $50\,^{\circ}$C throughout the analysis, the effect of heat on the stability of T and TG was evaluated by placing samples, which had been processed through the drying and reconstitution steps, into a $50\,^{\circ}$C bath for up to 30 min. No degradation of T or TG was noted.

Application

This assay has been used to analyze plasma and urine samples from a clinical study in healthy volunteers that was undertaken to describe the disposition of T and the transformation of 1β-TG and TG-iso. A representative plasma curve from one subject can be seen in Fig. 4. Participants were asked not to ingest other drugs for one week prior to initiation of the study; under these conditions, no interference with the assay was noted.

In addition, experiments to examine the stability of 1β-TG and TG-iso as a function of pH and time were carried out using this assay. Both the acyl migration and hydrolysis of 1β-TG and the other isomers were followed for 24 h. These results indicate that previous studies describing the disposition of TG may be in error. Previous pharmacokinetic studies depended upon β-glucuronidase hydrolysis of TG to T as the means to quantitate TG. Since migratory products of TG are not susceptible to β-glucuronidase, yet represent a large percentage of the TG that is present in clinical samples, substantial error could result from such an analysis. The analytical problems associated with acyl migration and hydrolysis

TABLE II

INTER-DAY VARIABILITY OF THE STANDARD CURVES FOR PLASMA AND URINE ($n=6$)

Sample	Mean slope	S.D.	Coefficient of variation (%)
Plasma	0.7776	0.030	3.90
Urine	0.1740	0.006	3.45

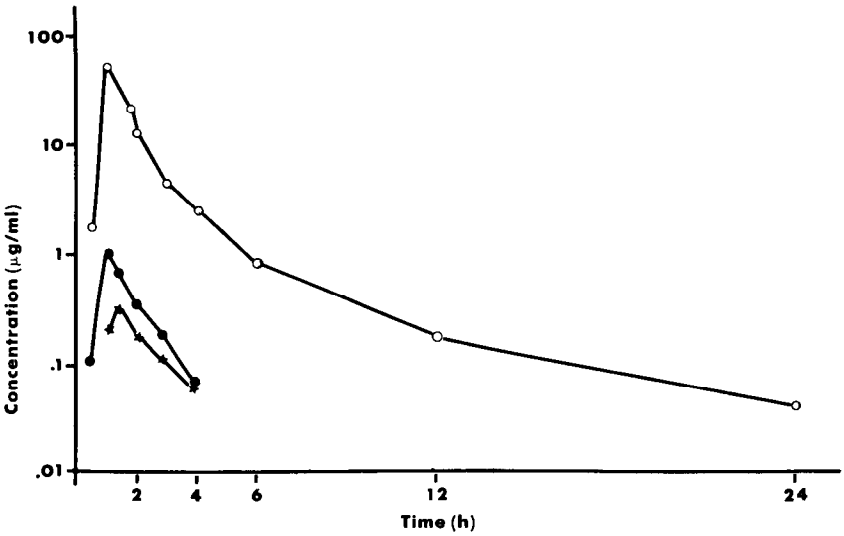

Fig. 4. Plasma concentration-time curves from a subject following ingestion of 400 mg T. Curves describe the time course of tolmetin (○), 1β-tolmetin glucuronide (●) and the sum of the isomers of tolmetin glucuronide (★).

are avoided by using the assay described here since special handling procedures are incorporated and the 1β-TG and TG-iso are measured directly.

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