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SIMULTANEOUS DETERMINATION OF ZOMEPIRAC AND ITS MAJOR METABOLITE ZOMEPIRAC GLUCURONIDE IN HUMAN PLASMA AND URINE

PIM N.J. LANGENDIJK*, PHILIP C. SMITH, JIRO HASEGAWA** and LESLIE Z. BENET*

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

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SUMMARY

A method is described for the simultaneous determination of zomepirac and its primary metabolite, zomepirac glucuronide, in plasma and urine. Reversed-phase liquid chromatography is used with an ion-pairing mobile phase of methanol—tetrabutylammonium hydrogen sulfate. Detection is by UV at 313 nm. Biological samples are cooled immediately, then adjusted to pH 3 to avoid zomepirac glucuronide degradation. Samples (0.5 ml) are then deproteinated with acetonitrile or acetone, the supernatant concentrated and dissolved in acetonitrile—acetate buffer, with up to one half of the sample injected onto the LC system. Recovery is greater than 70% and reproducible. The measurable concentration range is linear from 0.05 to 200 μg/ml. Total elution time of the assay is less than 10 min. Selectivity of zomepirac and zomepirac glucuronide is optimized. Sample preparation prior to analysis so as to prevent zomepirac glucuronide degradation is emphasized.

INTRODUCTION

Zomepirac (Z), 5-(4-chlorobenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetic acid (Fig. 1) is a nonsteroidal anti-inflammatory drug (NSAID) with analgesic effects [1]. Its major metabolite in man is the glucuronic acid conjugate, zomepirac glucuronide (ZG) [2, 3]. A recent report from our laboratory has shown that ZG is unstable at physiological pH in vitro [4]. Preliminary results suggest that ZG is present in plasma at concentrations near that of the parent

*Present address: Department of Pharmacology, Subfaculty of Pharmacy, Sylvius Laboratories, State University of Leiden, P.O. Box 9503, 2300 RA Leiden, The Netherlands.
**Present address: Eisai Co. Ltd., 6-10 Koishikawa 4-chome, Bunkyoko, Tokyo 113, Japan.

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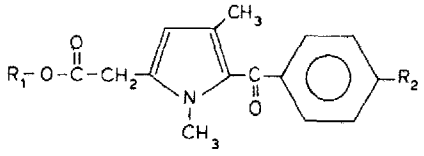

Compound	R_1	R_2
ZG	Glucuronic acid	CL
Z	H	CL
IS	H	OCH_3

Fig. 1. Structures of zomepirac, Z; zomepirac glucuronide, ZG; and the internal standard, IS.

Z. Interest in studying the disposition of ZG led to our development of an analytical method for direct measurement of intact ZG and Z in plasma and urine.

Several methods have been published for determination of Z in plasma and urine. Normal-phase [5], reversed-phase [6—8] and thin-layer chromatography (TLC) followed by gas chromatography—mass spectrometry (GC—MS) [9] have been used. Only one method included a direct analysis of ZG [8]. Using ^{14}C-labeled Z, ZG has been measured following TLC separation [2]. None of the above methods consider the possibility of ZG degradation which can be significant at physiological pH [4]. Not only do all but one of the previous methods not measure ZG, but because of ZG instability, these methods may be overestimating free Z concentrations.

In addition to simple hydrolytic cleavage of ZG to Z, our laboratory has documented the problem of intramolecular acyl migration of ZG [4]. This phenomenon is increasingly becoming recognized as a problem for acyl glucuronides with reports of acyl migration occurring with bilirubin [10], clofibric acid [11], probenecid [12], isoxepac [13], an investigational NSAID from Wyeth [14] and diflunisal [15]. The isomeric products formed from acyl migration of ZG have different liquid chromatographic (LC) retention times. No specific attempt was made to resolve the isomeric conjugates during development of the present LC method, although partial resolution occurred. As described below, the isomers are simply combined as total conjugates in our analysis.

EXPERIMENTAL

Developmental work

Methanol, acetonitrile and tetrahydrofuran were considered the organic modifiers of choice in this investigation. After preliminary tests only methanol proved to have a suitable selectivity between Z, ZG and the internal standard as was determined by measuring selectivity, $\alpha = K'_Z/K'_{ZG}$, where K' is the capacity factor. Further tests were done with methanol in order to obtain the most optimal mobile phase. Data are illustrated in Fig. 2. As is known from previous investigations in our laboratory [4], ZG is most stable

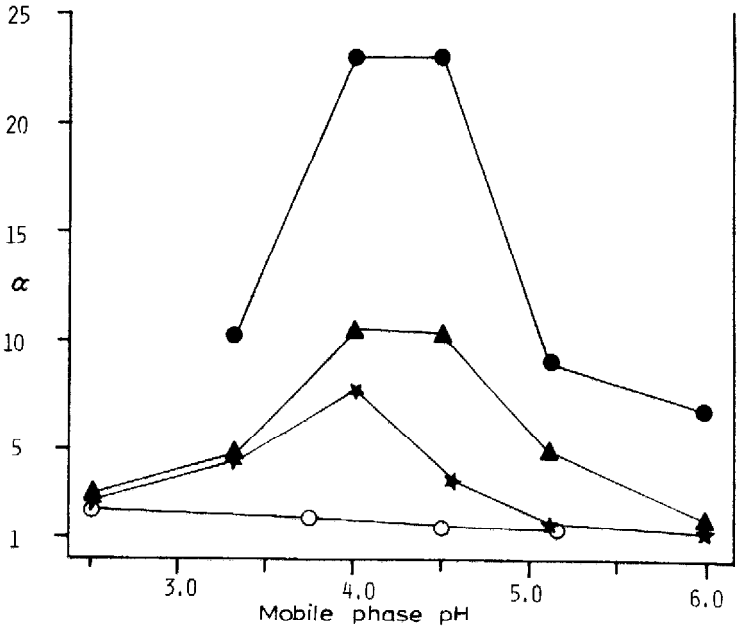

Fig. 2. Selectivity (Z—ZG) as a function of pH and mobile phase organic solvent. •, 40% acetonitrile; ⋆, 50% methanol; ▲, 30% tetrahydrofuran; ○, 58% methanol—ion pair. Column conditions and the ion-pairing buffer are as described in the text. All other buffers were 0.01 *M* acetate.

between a pH of 2 and 5. Because of this and because of a more favorable selectivity with the internal standard, the mobile phase was chosen to be 58% methanol—0.01 *M* tetrabutylammonium hydrogen sulfate and 0.05 *M* sodium acetate at pH 4.5. The facts that acetonitrile—water enhanced the stability of ZG and methanol—water caused loss of ZG to yield the methyl ester [4], accounted for the former being the solvent in which samples were reconstituted prior to injection. The short retention on the column (< 10 min) caused no significant loss of ZG due to the formation of the methyl ester in the methanol—water mobile phase.

Reagents and materials

Zomepirac sodium · $2H_2O$ and 5-(4-methoxybenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetic acid, the methoxy analogue of zomepirac used as the internal standard (IS) (Fig. 1), were kindly supplied by McNeil Pharmaceutical (Springhouse, PA, U.S.A.). ZG was obtained by extraction and purification from urine [4]. Methanol, acetone and acetonitrile, all HPLC grade, were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). All other chemical reagents were of analytical grade. Quantities less than 100 μl were pieptted with glass syringes from Hamilton (Reno, NV, U.S.A.).

Sample preparation

A stock solution of zomepirac sodium · $2H_2O$ was prepared by dissolving 100 mg into 100 ml methanol. By appropriate dilution with methanol, standard solutions of 100 μg/ml and 10 μg/ml were also obtained. A stock solution of ZG was prepared by dissolving 25 mg of this compound into 25 ml

50% acetonitrile—0.01 *M* phosphate, pH 2. By appropriate dilution with 50% acetonitrile—0.01 *M* phosphate, pH 2, standard solutions of 100 μg/ml and 10 μg/ml were also obtained. A stock solution of IS was prepared by dissolving 50 mg of this compound into 100 ml acetonitrile. All stock and standard solutions were stored at −20°C. Standard curves at concentrations of 0.1, 0.3, 0.6, 1.0, 5.0 and 10.0 μg/ml for Z and ZG with a concentration of 3.0 μg/ml for the internal standard, IS, were prepared in plasma. Standard curves with concentrations of 0.1, 0.3, 0.6, 1.0, 5.0 and 10.0 μg/ml for Z, 1.0, 5.0, 10,0, 50.0, 100.0 and 200.0 μg/ml for ZG and a concentration of 10.0 μg/ml for IS were prepared in urine. The standard curves were prepared with blank plasma or urine, buffered to pH 2—4 with phosphoric acid and spiked with a small amount (2.5—100 μl) of the appropriate dilution of the standard solutions.

Each aliquot of 0.5 ml plasma or urine was spiked with the internal standard (plasma: 3 μg/ml IS; urine: 10 μg/ml). Then 1.0 ml acetone or acetonitrile was added and protein precipitation was done by vortexing on a whirlmixer for 30 sec. The precipitate was separated by centrifugation at 2250 *g* (3000 rpm) for 10 min. The supernatant was removed into a clean tube and evaporated under a gentle stream of nitrogen at 30°C. In the case of plasma the pellet was not discarded, but once more treated with 1.0 ml acetonitrile or acetone, vortexed and centrifuged. The supernatant was combined with that of the first sample treatment. The residue was reconstituted into 0.25—1.0 ml 25% acetonitrile—0.5 *M* acetate, pH 4.5, then 50—100 μl were injected onto the high-performance liquid chromatography (HPLC) column. Sample preparation and assay were done on the same day.

Special precautions in sample handling were taken because of the fast degradation of ZG at higher temperatures and pH [4]. During clinical trials blood samples were immediately cooled in ice after collection, red blood cells were separated from plasma in a refrigerated centrifuge and the plasma was transferred into a vial, prebuffered with 10 μl concentrated phosphoric acid (15 *M*) per ml plasma and immediately frozen at −20°C. Urine was immediately buffered with 5 μl concentrated phosphoric acid per ml urine and frozen at −20°C. In addition, volunteers were kept on cranberry juice throughout the study in order to maintain the pH of their urine below 6.

Chromatography

The HPLC system used consisted of an Altex Model 110A pump, a Waters Model 710A Wisp automatic injector, a Waters Model 440A fixed-wavelength UV detector set at 313 nm and an Altex Ultrasphere ODS reversed-phase column (15 cm × 4.0 mm I.D. with 5-μm particles). Chromatograms were recorded on a Spectra-Physics Model SP4100 computing integrator. Area ratios relative to the internal standard, IS, were obtained. The ion-pairing mobile phase (58% methanol—0.01 *M* tetrabutylammonium hydrogen sulfate and 0.05 *M* sodium acetate, pH 4.5) was prepared fresh daily and was filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.). The column was conditioned with approximately 50 ml mobile phase prior to sample injection. The flow-rate was 1.3 ml/min. The retention times of IS, ZG and Z were approximately 4.5, 6.5 and 8.5 min, respectively (Figs. 3 and 4).

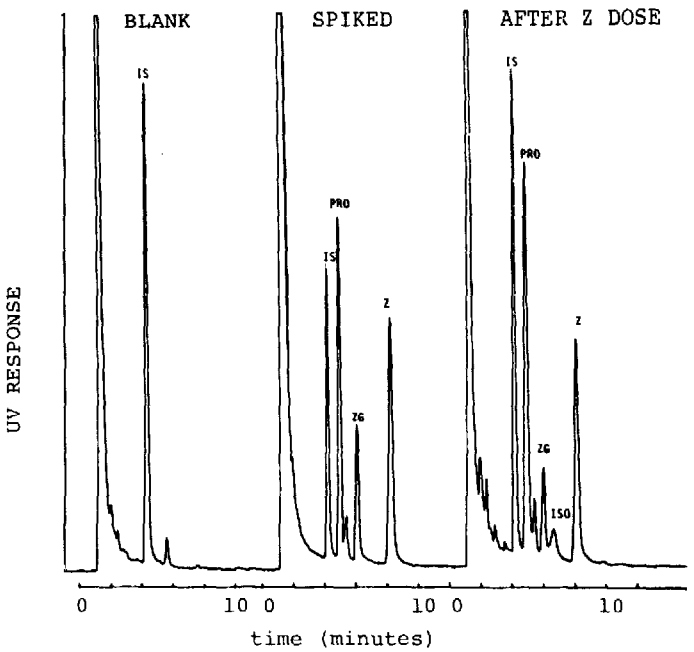

Fig. 3. Chromatograms of blank plasma spiked with 3 μg/ml IS; plasma spiked with IS and 1 μg/ml of Z and ZG with probenecid (PRO); and a plasma sample from a subject, pretreated with probenecid, 1 h after an oral dose of 100 mg of Z as its sodium salt. a.u.f.s. = 0.016.

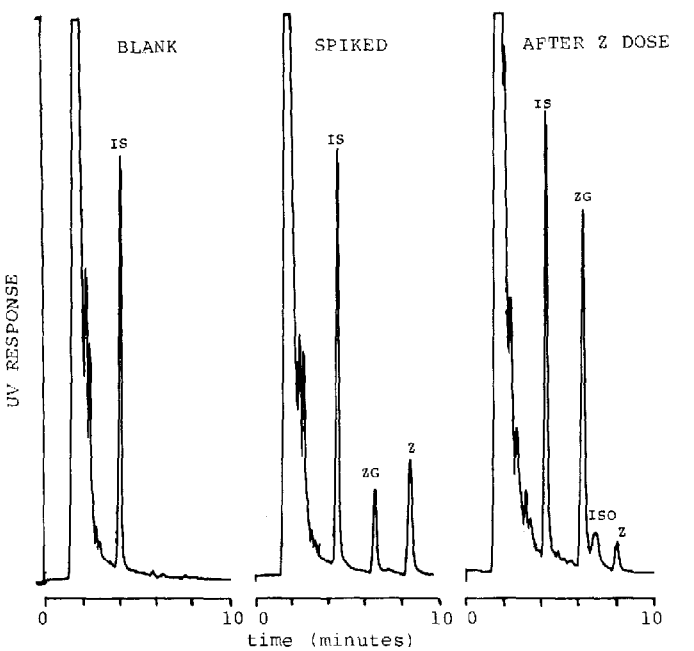

Fig. 4. Chromatograms of blank urine spiked with 10 μg/ml IS; urine spiked with IS and 5 μg/ml Z and ZG; and a urine sample from a subject collected between 8 and 12 h after an oral dose of 100 mg Z as its sodium salt. a.u.f.s. = 0.016.

Quantitation

Standard curves for Z and ZG in plasma and urine have been prepared according to the procedure described above. Area ratios of Z and ZG to the internal standard were plotted against concentrations of Z and ZG. A weighted (1/concentration) least-squares regression analysis was performed on the data. Linearity was tested and confirmed in the concentration range of at least 0.05–30 μg/ml for Z and ZG in plasma and 0.1–200.0 μg/ml for Z and ZG in urine.

RESULTS AND DISCUSSION

Recovery and variability

Recoveries were estimated by calculating the ratio of the slopes of standard curves obtained by protein precipitation, to untreated aqueous standard curves for three independently prepared sets of standards. An external standard was used to determine recoveries. Recoveries (± S.D.) were 74 ± 5% and 82 ± 7% for ZG and Z, respectively, in plasma over the concentration range of 0.1–10.0 μg/ml. In urine recoveries were 90 ± 3% and 95 ± 2% for ZG and Z, respectively, over the concentration range of the urine standard curves.

Intraday variability was calculated from the area ratios at three different concentrations: 0.1, 1.0 and 10.0 μg/ml in plasma and 0.5, 5.0 and 50.0 μg/ml in urine for both Z and ZG. Data are shown in Table I.

Interday variability is given in Table II, where the mean slopes of six,

TABLE I

INTRADAY VARIABILITY IN PLASMA AND URINE AT LOW, MEDIUM AND HIGH CONCENTRATIONS

In all cases $n = 6$.

	Concentration (μg/ml)	C.V. (%)	
		ZG	Z
Plasma	0.10	10.2	14.8
	1.0	12.4	11.8
	10.0	3.72	2.53
Urine	0.50	2.92	1.50
	5.0	0.45	0.40
	50.0	2.01	1.61

TABLE II

INTERDAY VARIABILITY OF THE STANDARD CURVES FOR PLASMA

In all cases $n = 6$.

	Mean slope	S.D.	C.V. (%)
ZG	5.38	0.359	6.67
Z	3.82	0.228	5.98

independently prepared, standard curves for Z and ZG in plasma are given together with the standard deviation and variability. These standard curves were prepared over a two-month time period.

Degradation and stability

Precautions undertaken to maintain an acidic urine pH and the immediate further acidification of plasma and urine samples together with the work-up procedure minimized the degradation of ZG in spiked samples due to sample handling, storage and work-up to less than 4%. However, analysis of clinical samples yielded greater fractions of the ZG degradation products. This could only result from degradation of ZG to the acyl migration products under the apparent in vivo physiological conditions. This degradation was accounted for by measuring the isomers peak (ISO, Figs. 3 and 4) and ZG as total conjugates. Degradation of glucuronides is not an unusual phenomenon among NSAIDs [13—16] nor other drugs that are extensively conjugated to acyl glucuronides [10—12]. However, degradation of NSAID glucuronides in vivo has not been extensively documented.

With Z no noticeable degradation occurred during sample handling, storage and sample preparation. In addition, the stock solutions of Z did not show significant changes in content over a period of five months. A small loss of ZG did occur in the stock solutions (3.2%) over a three-month period. A correction for this loss was made in each batch of samples that was analyzed. The stock solution of IS, the internal standard, also showed a slight degradation of approximately 2% per month. This, however, did not influence the assay since a fresh internal standard solution was prepared for each set of samples that was analyzed.

Application

This assay has been used successfully in a clinical study that investigated the influence of a steady-state drug level of probenecid on Z pharmacokinetics. Probenecid did not interfere with the plasma assay and could be measured together with Z and ZG. Plasma levels of probenecid were in the range of 20—100 μg/ml, much higher than Z or ZG. Because of a much lower molar extinction coefficient for probenecid at 313 nm compared to Z and ZG, the probenecid peak heights were of the same order of magnitude as for Z and ZG. No interference peaks due to endogenous compounds were observed with the clinical samples. In Fig. 5 typical plasma concentration—time profiles of Z and ZG with concomitant probenecid dosing are illustrated for one subject. Plasma concentration—time profiles of Z and ZG after ingestion of 100 mg Z with and without probenecid could be followed up to 25 h after Z administration. In all cases this part of the curve accounted for more than 95% of the total area under the curve.

Probenecid did cause problems with the analysis of urine. Putative metabolites of probenecid in urine eluted before probenecid, interfering with the IS measurement. When this occurred quantitation was done by normalizing peak areas with constant injection volumes. When probenecid metabolites were not interfering the use of constant injection volumes produced reproducible IS areas sufficient for quantitation. The simplicity of the sample preparation for

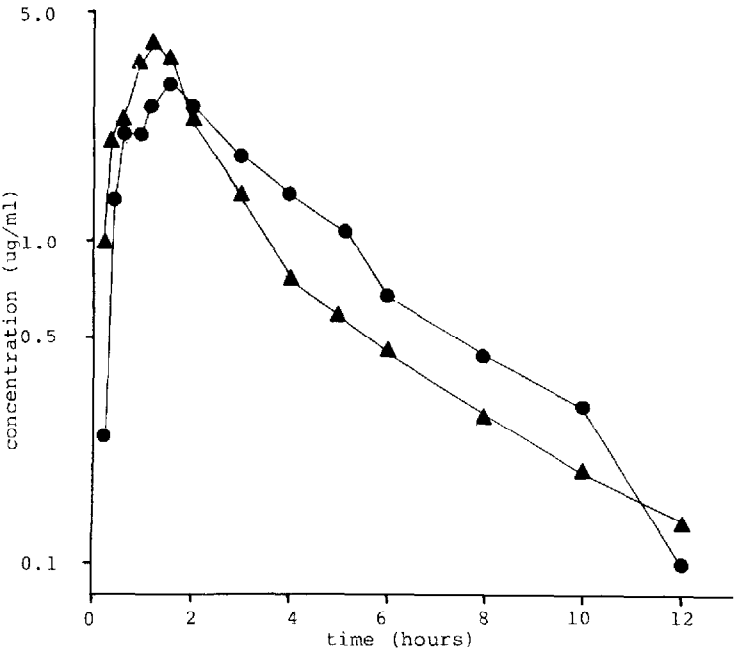

Fig. 5. Concentration vs. time of Z (▲) and ZG (●) in the plasma of a subject who had received 100 mg Z orally as its sodium salt after previous administration of 500 mg probenecid twice daily for four days. Ordinate is a log scale.

urine and the absence of protein in urine accounts for the reliability of this method of quantitation.

This assay was applied in a controlled clinical study. Volunteers were instructed not to take other drugs two weeks prior to and during the study. Other applications of this assay may involve patients who may also take products containing acetaminophen or salicylate. Urine samples collected from subjects after oral doses of acetaminophen and salicylate did not show any interference with this assay method.

Acyl migration

This direct injection assay method for Z and ZG has great advantages over previous reports. Although in a recent report ZG and Z are analyzed simultaneously in urine, no special precautions were taken prior to analysis to prevent loss of ZG [8]. These authors suggest that an anomalous peak in urine may be the putative hydroxylated metabolite of Z. We believe it likely that this peak results from an isomeric conjugate of ZG due to acyl migration. The fact that ZG is degrading, forming other isomers (not susceptible to β-glucuronidase) prior to liberation of the aglycone [4], means that estimation of the glucuronide simply by following formation of the free aglycone will result in error. To what extent degradation of glucuronides is affecting the results of clinical studies with drugs such as Z is not known, because usually no detailed information is given on sample handling or stability prior to analysis. The importance of this is emphasized in this assay and should be considered for further NSAID assays, since degradation of acyl glucuronides is probably a common occurrence among these drugs.

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