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Note

Reversed-phase high-performance liquid chromatographic assay for zomepirac in urine

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Zomepirac sodium, sodium 5-(4-chlorobenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetate dihydrate, is an orally active, non-narcotic analgesic agent [1—3]. Prior publications from these laboratories have detailed the pharmacokinetics and disposition of zomepirac in man [4, 5] and animals [5, 6]. These studies utilized a normal-phase high-performance liquid chromatographic (HPLC) assay for zomepirac in plasma [7].

Recently, a reversed-phase HPLC assay for zomepirac in plasma and serum was published by Welch et al. [8]. However, neither of these assays are directly applicable for analysis of zomepirac in urine since they both utilize a diethyl ether extraction step at neutral pH prior to the acidic ether extraction. Such a prewash step is effective at removing interference peaks when the drug is highly bound to plasma protein, but extracts large amounts of drug from urine where the drug is not bound to protein.

Since urinary excretion accounts for 5% of the dose as zomepirac and 80% as zomepirac glucuronide in man, a rapid, sensitive and reproducible assay in urine was required for these chemicals. Thus, a reversed-phase HPLC assay was developed for zomepirac and hydrolyzed zomepirac conjugates in urine with the goal of minimal sample manipulation, adequate sensitivity and wide dynamic range.

MATERIALS AND METHODS

Reagents

Zomepirac sodium and the internal standard, 5-(4-chlorobenzoyl)-1,4,α-trimethyl-1H-pyrrole-2-acetic acid, were supplied by McNeil Pharmaceutical (Spring House, PA, U.S.A.). Methanol and water were distilled in glass grade from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Hexane and isoprop-

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anol were reagent grade from Mallinckrodt (St. Louis, MO, U.S.A.). Sodium acetate (J.T. Baker, Phillipsburg, NJ, U.S.A.) and glacial acetic acid (Mallinckrodt) were used to make 0.01 *M* acetate buffer (pH 4.0).

Chromatography

A Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery system was utilized with a U6K injector and a Model 440 UV detector (254 nm). Separation of components was effected with a 25 cm × 4.6 mm I.D. Whatman (Clifton, NJ, U.S.A.) Partisil-10 ODS (10 μm) column with a 6.5 cm × 4.1 mm I.D. Bondapak C_{18}/Corasil (Waters Assoc.) pre-column. The mobile phase was a methanol—0.01 *M* acetate buffer (pH 4.0) (45:55, v/v) solution run at 2.0 ml/min.

Procedure

Zomepirac. Urine (0.5 ml) is added to a 15-ml glass screw-top centrifuge tube with PTFE-cap liners. To the urine is added 0.5 ml of a methanol solution (240 μg/ml) of the internal standard. The sample is vortexed, allowed to stand for 15 min, centrifuged and aliquots (20 μl) of the supernatant are injected directly into the chromatograph.

Zomepirac conjugates. Urine (0.5 ml) is placed in a 15-ml glass screw-top centrifuge tube with PTFE-cap liners, after which 25 μl of 6 *N* sodium hydroxide solution are added. The solution is vortexed and allowed to stand for 1 h. Then 27 μl of 6 *N* hydrochloric acid solution are added, the solution is vortexed, and the internal standard solution (0.5 ml) is added. The remainder of the procedure is as described for zomepirac.

Calibration

Daily standards are prepared over the range of 4—1000 μg/ml. Zomepirac sodium (6.0 mg) is dissolved in 50 ml of methanol, appropriate aliquots are pipetted into centrifuge tubes, evaporated to dryness and reconstituted with 0.5 ml of water. Sample treatment then begins as previously described. Seeded quality control urine samples were prepared by adding appropriate aliquots of the methanol solution of zomepirac to glass bottles, evaporating to dryness and reconstituting with 100 ml of human urine. Concentrations of 800, 100 and 10 μg/ml were prepared.

Assay validation

Validation of the HPLC assay was performed by comparison of results with those obtained by thin-layer radiochromatographic techniques. Two pooled urine samples were prepared from samples obtained from a male subject who had received a single 200-mg oral dose of [^{14}C] zomepirac sodium (100 μCi/dose).

Each pooled urine sample was assayed in quadruplicate by the HPLC assay as described and by the thin-layer chromatographic (TLC) assay as follows: 1 ml of urine is made basic with 25 μl of 6 *N* sodium hydroxide and incubated overnight at 37°C (hydrolysis step is omitted when assaying for unconjugated zomepirac). The sample was then neutralized with 27 μl of 6 *N* hydrochloric acid, and 50 μl of each sample were applied directly to a 20 cm × 5 cm silica

gel GF TLC plate (Analtech, Newark, DE, U.S.A.). Unlabelled zomepirac (50 μg) was co-chromatographed as a standard. The plates were developed in a solvent system of hexane—isopropanol—glacial acetic acid (90:9:1, v/v/v). The mean R_F value for zomepirac was 0.35. The plates were analyzed by removing a 2-cm silica gel zone at the R_F of zomepirac, transferring it to a liquid scintillation vial containing 0.5 ml of water, adding 10 ml of Biofluor (New England Nuclear, Boston, MA, U.S.A.) and assaying for total radioactivity by liquid scintillation spectrometry. Assay controls were two seeded urine samples at 6 and 100 μg/ml, respectively, assayed in duplicate on each analysis day.

Statistical approaches

Calibration curve data were fitted by least squares linear regression analysis of zomepirac/internal standard peak height ratio (weighted by 1/variance of peak height ratio) vs. zomepirac concentration. Results of the assay validation were compared by paired t-test with significance set at $p < 0.05$.

RESULTS AND DISCUSSION

Chromatography

Separation of zomepirac and the internal standard from the background components of urine was effected using a C_{18} reversed-phase column (10 μm particle size) and methanol—acetate buffer mobile phase. As can be seen in Fig. 1, there were no extraneous peaks observed in a chromatogram of blank human urine at the retention times of zomepirac (4.1 min) and the internal standard (6.2 min). Also, it is apparent that baseline resolution was achieved

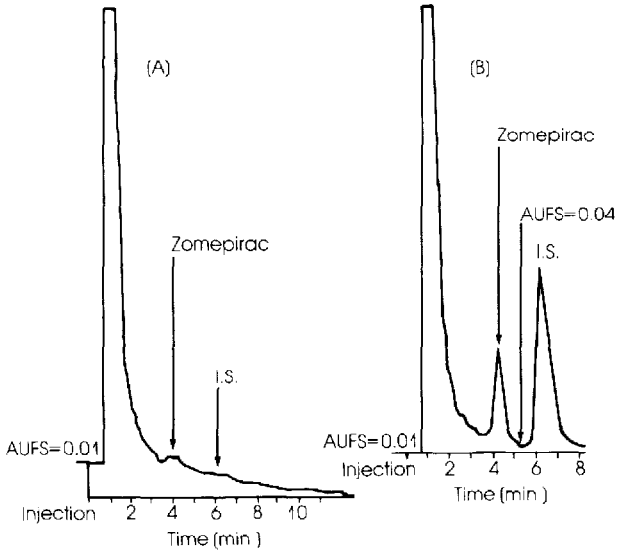

Fig. 1. Analysis of human urine by HPLC. Chromatograms of (A) blank human urine; and (B) human urine containing zomepirac (20 μg/ml) and the internal standard (I.S., 240 μg/ml).

for all components of interest as seen in the chromatogram of urine containing zomepirac and the internal standard. Minor alterations in the composition of the mobile phase will substantially affect the retention times of both zomepirac and the internal standard. Therefore, care must be taken to prepare the mobile phase in the same manner every day. The base hydrolysis step has no detectable effect on the chromatography.

Calibration and sensitivity

Calibration of the assay was performed daily using standards prepared over the range of 4.0—1000 μg/ml. Accuracy of the daily analyses was ensured by analyzing duplicate aliquots (0.5 ml) of the seeded urine pools. The daily calibration curves were linear and passed through the origin using 1/variance of the peak height ratio as the weighting factor. The regression line was described by the parameters: slope = 0.0065 ± 0.00003; intercept = 0.0036 ± 0.00064; $r^2 = 0.9995$.

Table I presents data on the reproducibility and accuracy of the assay.

TABLE I

REPRODUCIBILITY AND ACCURACY OF THE HPLC ASSAY FOR ZOMEPIRAC IN URINE

The average coefficient of variation of the curve of concentration vs. normalized peak height ratio at 4—1000 μg/ml (1 per day, 22 days, 6 points per range) is 3.38 ± 0.86%

	Zomepirac concentration (μg/ml)			
	4.0	10	100	1000
Precision				
C.V. (%)				
Intra-day (n = 6)	5.1	2.1	1.6	4.6
Inter-day (n = 22)	5.3	3.4	3.3	2.5
Bias				
Deviation of mean from amount spiked (%)				
Intra-day (n = 6)	−1.25	−0.20	+3.00	−6.2
Inter-day (n = 22)	−1.25	−0.23	+1.60	−7.1

Mean inter- and intra-day variability was less than 10% and accuracy was within 10%. Accurate quantitation was performed down to 4 μg/ml. Sensitivity could be enhanced by decreasing the amount of internal standard added and constructing a more limited calibration curve. Under these conditions the assay is capable of accurately quantitating as little as 0.2 μg/ml. Preliminary work has demonstrated that detection at 313 nm in addition to detection at 254 nm provides adequate sensitivity with less background interference. Also, the assay published by Welch et al. [8], has demonstrated the utility of detection at 320 nm.

Validation

The accuracy of the assay was also ascertained by comparison of assay results with a thin-layer radiochromatographic assay. Pooled urine samples were prepared from specimens obtained in a clinical metabolic study. Assays for zomepirac and hydrolyzed zomepirac conjugate (the acylglucuronide) were performed using the reversed-phase HPLC assay and the TLC assay [9]. The accuracy and reproducibility of the TLC assay for zomepirac are demonstrated in Table II. The TLC assay for zomepirac caused little or no hydrolysis

TABLE II

VALIDATION OF THE TLC ANALYSIS OF ZOMEPIRAC IN URINE

Seeded zomepirac concentration (μg/ml)	*n*	Concentration found (μg/ml) (mean ± S.D.)	Recovery (%) (mean ± S.D.)
6.0	6	6.16 ± 0.34	102.6 ± 5.7
100	8	96.3 ± 5.6	96.3 ± 5.6

of the glucuronide conjugate unless preceded by the base hydrolysis step. A comparison of assay results by the HPLC and TLC assays is presented in Table III. As can be seen, the analysis for zomepirac agrees reasonably well between the two assays although the HPLC assay results were statistically significantly ($p < 0.05$) higher. The assays for zomepirac plus zomepirac glucuronide are in excellent agreement and no statistically significant differences exist. The HPLC assay has subsequently been used to assay well over 2000 human and monkey urine samples.

TABLE III

RECOVERY OF ZOMEPIRAC IN 0—24 h HUMAN URINE ANALYZED BY HPLC AND TLC

Each sample was assayed in quadruplicate by both assay procedures.

Pooled urine sample	Zomepirac (μg/ml) (mean ± S.D.)		Zomepirac + conjugates (μg/ml) (mean ± S.D.)	
	HPLC*	TLC	HPLC	TLC
A	26.7 ± 2.3	19.5 ± 1.3	367 ± 12	353 ± 51
B	16.9 ± 1.1	13.6 ± 1.4	152 ± 14	151 ± 13

*Value different from TLC assay ($p < 0.05$).

CONCLUSION

A rapid, reproducible and accurate HPLC assay for zomepirac in urine has

been described. Addition of a base hydrolysis step allows for quantitation of zomepirac glucuronide, the major urinary metabolite.

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