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Note

High-performance liquid chromatographic determination of 3-hydroxymethyl-dibenzo[*b*,*f*]thiepin 5,5-dioxide and its acid metabolite in human plasma and urine

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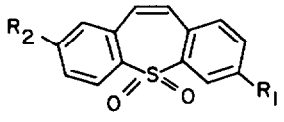
Prostaglandins are potent, biologically active agents with a wide spectrum of activity depending on species, organ, and prostaglandin involved. Activation of prostaglandin biosynthesis results in the production of unstable prostaglandin endoperoxides, prostaglandins G_2 and H_2. These in turn degrade to prostaglandin I_2 and thromboxane A_2 which are also unstable. Thromboxane A_2 is a strong contractor of vascular smooth muscle and induces platelet aggregation [1, 2].



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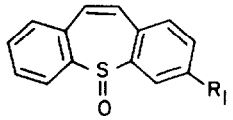

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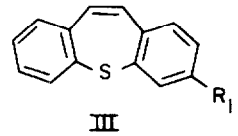

III

COMPOUND	STRUCTURE	R_2	R_1
1	I	H	CH_2OH
2	I	H	COOH
3	I	F	CH_2OH
4	I	H	CHO
5	I	H	$CONHCH_2COOH$
6	II		COOH
7	III		COOH

Fig. 1. Chemical formulae of compounds 1—7.

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3-Hydroxymethyl-dibenzo[*b*,*f*]thiepin 5,5-dioxide, 1 (see Fig. 1), has been found to antagonize the actions of thromboxane A_2 and other contractile prostaglandins in in vitro and in vivo studies [3]. Compound 2, the acid metabolite of 1, was also found to have a similar profile of pharmacological activity and may be approximately twice as potent in in vitro studies of platelet function [3].

A new high-performance liquid chromatographic (HPLC) method for the concurrent determination of compounds 1 and 2 in plasma and urine is reported. The method has been developed to determine the pharmacokinetic profile of 1 and 2 in man. Compounds 1 and 2 can be reliably quantitated with detection limits of 5 ng/ml in plasma and 1 μg/ml in urine. Only free 2 was found in plasma and urine from patients given 1. Free, Glusulase® and base-hydrolyzable concentrations of 2 were determined in urine from two subjects given different doses of 1.

MATERIALS AND METHODS

All reagents were analytical grade or better. Chromatographic solvents were HPLC grade. An Analytichem Vac-Elut® system (Harbor City, CA, U.S.A.) equipped with a Bond-Elut® 200-mg octadecylsilane cartridge was used for sample extraction. Glusulase was obtained from Endo Labs. (Garden City, NY, U.S.A.).

All standards were supplied by Merck Sharp & Dohme Research Labs. (Rahway, NJ, U.S.A.). Standards were dissolved in a mixture of acetonitrile—water (1:4, v/v) with the exception of compound 2, which was soluble in acetonitrile—1 *M* sodium acetate (pH = 6)—water (4:1:15, v/v/v). Stock standard solutions of 1 and 2 were 500 μg/ml and stock internal standard, 3, was 100 μg/ml.

Apparatus

The chromatographic separation was attained using a Rainin Gradient HPLC system (Woburn, MA, U.S.A.). Injections were made with a Perkin-Elmer ISS-100 autosampler (Norwalk, CT, U.S.A.). The column system was held at 50°C and included a Brownlee octadecylsilane, 5-μm, 3 cm × 4.6 mm precolumn (Santa Clara, CA, U.S.A.) and an Analytical Sciences octadecylsilane, 10-μm 30 cm × 4.6 mm analytical column (Santa Clara, CA, U.S.A.). Peaks were detected at excitation wavelength 315 nm and emission wavelength 390 nm with a Perkin-Elmer 650-10S fluorescence detector. The mobile phase consisted of 0.85% phosphoric acid solution (pH = 3.5)—acetonitrile (60:40), and was pumped at 2 ml/min. Data were generated by a Spectra-Physics SP4270 integrator (Santa Clara, CA, U.S.A.).

Clinical samples

Healthy male volunteers received single rising oral doses of compound 1 over the range of 250—2000 mg. Plasma was collected at 0, 1, 4, 10 and 24 h and urine was collected and pooled for time intervals of −1—0, 0—4, 4—10, 10—24 and 24—48 h. Plasma and urine samples were stored at −15°C until time of analysis.

Sample preparation and extraction of plasma

Plasma analyzed for free 1 and 2 was prepared by placing 0.25 ml of plasma, 0.1 ml of 1 *M* hydrochloric acid, 0.1 ml of working internal standard (10 μg/ml) and 0.75 ml of 0.85% phosphoric acid solution in a polypropylene tube.

After vortexing, the plasma sample was applied to a Bond-Elut octadecylsilane cartridge. The cartridge was pre-wet with 3 ml of methanol followed by 3 ml of water. The sample was passed through the cartridge and washed with 1 ml of water. Excess water was removed from the cartridge using the house vacuum. Methanol (1 ml) was passed through the cartridge and the eluate was collected into a tube containing 0.15 ml of 0.85% phosphoric acid solution. After vortexing and centrifugation, the sample was injected on the HPLC system.

Sample preparation and extraction of urine

Free concentrations of compounds 1 and 2 were determined in urine by placing 0.1 ml of urine, 0.1 ml of working internal standard (100 μg/ml), 0.1 ml of water and 0.75 ml of acetonitrile in a test tube. After vortexing and centrifugation, 0.10 ml was removed and diluted with 0.75 ml of mobile phase. The sample was then injected on the HPLC system.

Urine hydrolyzed by Glusulase was prepared by placing 0.1 ml of urine, 0.1 ml of internal standard (100 μg/ml), 0.1 ml of diluted Glusulase (activity ≈10000 U/ml) and 0.70 ml of 0.5 *M* sodium acetate (pH = 4.5) in a test tube and capping. Urine was incubated for 90 min at 40°C. After incubation, 0.1 ml of urine was diluted with 0.5 ml of acetonitrile and 0.25 ml of 0.85% phosphoric acid solution. The sample was then vortexed, centrifuged and injected on the HPLC system.

Urine for base hydrolysis was prepared by adding 0.1 ml of urine, 0.1 ml of working internal standard (100 μg/ml), 0.3 ml of 5 *M* sodium hydroxide solution and 0.5 ml of water to a test tube and capping. The sample was incubated for 90 min at 40°C. The sample was then diluted by placing 0.1 ml of hydrolyzed urine, 0.2 ml of hydrochloric acid and 0.75 ml of acetonitrile in a sample vial. After vortexing and centrifugation, the sample was injected on the HPLC system.

RESULTS AND DISCUSSION

An HPLC method using fluorescence detection has been developed for the concurrent determination of compounds 1 and 2 in plasma and urine. Owing to the natural fluorescing properties of structure I (see Fig. 1), the method was found to be more sensitive and selective than using UV detection at 254 nm [3]. Under the chromatographic conditions utilized, compound 1 was completely separated from its carboxylic acid metabolite (2), the internal standard (3) and other structurally related analogues (compounds 4—7) (Table I and Fig. 2A). For 1 and 2, the detection limit was 5 ng/ml in plasma and 1.0 μg/ml in urine.

Representative chromatograms of control and subject plasma and urine samples are shown in Fig. 2. No interfering peaks at the retention times of compounds 1, 2 or 3 were seen in control samples of plasma and urine.

TABLE I

CHROMATOGRAPHIC PROPERTIES OF COMPOUNDS 1—7 UNDER HPLC CONDITIONS

Compound	Retention time (min)
1	4.1
2	3.6
3	4.8
4	7.2
5	2.8
6	NF*
7	NF

*NF = no fluorescence at 100 μg/ml.

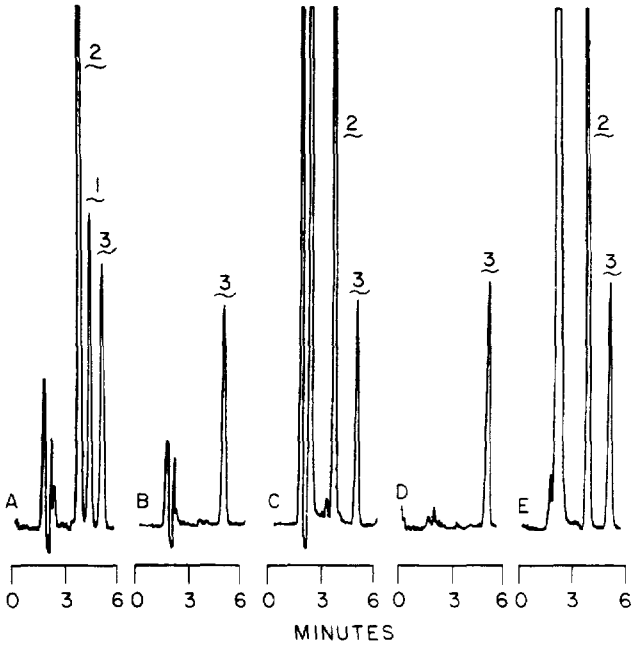

Fig. 2. Representative chromatograms of (A) standards; (B) predose plasma spiked with 0.1 ml of 10 μg/ml internal standard; (C) plasma collected after administration of compound 1 to human subject; (D) predose urine spiked with 0.1 ml of 100 μg/ml internal standard; and (E) urine collected after administration of compound 1 to human subject.

Drug-related peaks observed in high-dose urine were also separable from compound 2 under the conditions described. However, these peaks interfered with 2 when samples were analyzed under fast HPLC conditions using a Regis octadecylsilane, 3-μm, 3 cm × 4.6 mm Little Champ column (Morton Grove, IL, U.S.A.) and a mobile phase of 0.85% phosphoric acid (pH = 4.5)—acetonitrile (70:30).

Since compound 1 was not identified in any samples analyzed, 2 could feasibly be moved from interfering peaks and closer to the internal standard by altering the pH of the mobile phase. This would make the fast HPLC method suitable for analysis of samples, thus reducing the run time by 50%. Future work may include validation of this fast HPLC method.

Plasma was extracted using a Bond-Elut cartridge with octadecylsilane packing. Extraction with cartridges was found to be more efficient than protein precipitation methods. The small precipitate particles not removed by centrifugation required microfiltration of samples. This step was found to be more time-consuming than sample extraction with cartridges. Recovery using extraction cartridges was greater than 90% for compounds 1 and 2 spiked in control plasma and was comparable to that observed using protein precipitation methods.

Methods for determining free 1 and 2 in plasma and urine were evaluated for intra-day and inter-day variation. Standard curves in plasma ranged from 5 to 500 ng/ml. Coefficients of variation were less than 4% for each point of the curve. The inter-day correlation (n = 6) calculated using linear regression was r^2 = 0.9999 for both 1 and 2. In urine, standard curves ranged from 1 to 500 μg/ml with an inter-day correlation (n = 6) of r^2 = 1.0000 for both 1 and 2. Coefficients of variation (n = 6) were less than 3.5% for every value on the standard curve.

Quality control samples were prepared at the high and low ends of all standard curves. In plasma and urine, coefficients of variation (n = 6) were less than 2.7% for quality control samples. Analysis of quality control samples over a period of one month showed compounds 1 and 2 to be stable when stored at −15° C in plasma or urine.

Retention times of unidentified drug-related peaks observed in chromatograms of urine from high-dose treatments did not match with retention times of standards 4—7 (Table I). Glusulase, an enzyme preparation specific for hydrolysis of β-glucuronide and sulfate conjugates, was used to evaluate β-

TABLE II

URINE RECOVERY (mg) OF COMPOUND 2 FOLLOWING NO, GLUSULASE, OR BASE HYDROLYSIS

Dose (mg)	Subject	No hydrolysis	Glusulase hydrolysis	Base hydrolysis
500	No. 7	51.14	219.66	383.80
1000	No. 12	77.13	190.62	454.98
Low quality control		47.71	46.89	47.97
High quality control		256.18	248.71	253.28

TABLE III

PLASMA CONCENTRATION AND URINE RECOVERY OF COMPOUND 2 FOLLOWING SINGLE DOSES OF COMPOUND 1

Dose (mg)	Subject No.	Plasma concentration (μg/ml)					Urine recovery (mg)					
		Time (h)					Time interval (h)					
		0	1	4	10	24	0	0—4	4—10	10—24	24—48	Total
250	3	0	0.41	0.10	0.11	0.05	0	12.48	1.52	2.33	NC*	16.33
500	1	0	0.17	0.61	0.14	0.11	0	19.57	12.98	5.01	NC	37.56
1000	9	0	2.48	0.29	0.22	0.32	0	5.32	11.97	41.50	34.06	92.85
2000	16	0	4.11	0.65	0.57	0.46	0	86.77	17.85	27.65	73.12	205.39

*NC = samples were not collected.

glucuronide conjugation. Sodium hydroxide was used to evaluate base-hydrolyzable conjugates including β-glucuronide.

Both chemical and enzymatic hydrolysis of subject urine resulted in an increase of compound 2 (Table II). Treatment of quality control samples under each of these conditions resulted in no change in concentration of 2. The increase in 2 observed following Glusulase treatment is assumed to be due to hydrolysis of the β-glucuronide conjugate since sulfate conjugation is improbable. Base hydrolysis resulted in a greater increase in 2 than Glusulase hydrolysis and complete removal of drug-related peaks observed near the solvent front. This suggests that base-hydrolyzable metabolites in addition to the β-glucuronide exist. Further work is needed to identify and characterize these metabolites.

The methods developed for analysis of compounds 1 and 2 in plasma and urine have been shown to be reproducible and reliable. Table III shows levels of 2 in plasma and urine from selected subjects participating in an active rising oral dose study of 1.

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