Journal of Chromatography, 231 (1982) 333—339
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1313

SIMULTANEOUS DETERMINATION OF GLUCURONIDES OF TRIMETOZINE IN HUMAN URINE BY GAS CHROMATOGRAPHY

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(First received November 17th, 1981; revised manuscript received April 9th, 1982)

SUMMARY

A gas chromatographic method for the simultaneous determination of four glucuronides (metabolites) of trimetozine excreted in human urine is described. The method involves pretreatment of the urine specimen [i.e. removal of interfering substances by solvent extraction, desalting on an ion-exchange (Amberlite XAD-2) column], and permethylation of glucuronides by reaction with methylsulfinyl carbanion and methyl iodide. The permethylated derivatives were submitted to gas chromatographic separation on an OV-17 column, and their structures were investigated by subsequent gas chromatographic—mass spectrometric analysis. The minimum detectable concentration of each glucuronide is 5 μg/ml when 1 ml of urine is used. The utility of the present method is successfully demonstrated by determining the urinary concentration of four glucuronides following oral administration of trimetozine to human subjects.

INTRODUCTION

Glucuronide formation is one of the detoxication processes of drug metabolism in man. Trimetozine, N-(3,4,5-trimethoxybenzoyl)morpholine, is an antidepressant agent, which is known to be biotransformed in man into four glucuronides [1]. The structures of the metabolites excreted in urine are shown in Fig. 1.

Quantitation of the glucuronides of drugs in general has been performed by determination of the aglycone released by enzymatic or chemical hydrolysis. Such an indirect method, however, suffers the disadvantage that the enzymatic hydrolysis is often influenced by the presence of inhibitors in urine, and that the stability of the aglycone in the reaction medium of chemical hydrolysis is sometimes a prerequisite.

Marcucci et al. [2] and Nakagawa et al. [3] reported alternative methods, in which glucuronides were converted to their methyltrimethylsilyl de-

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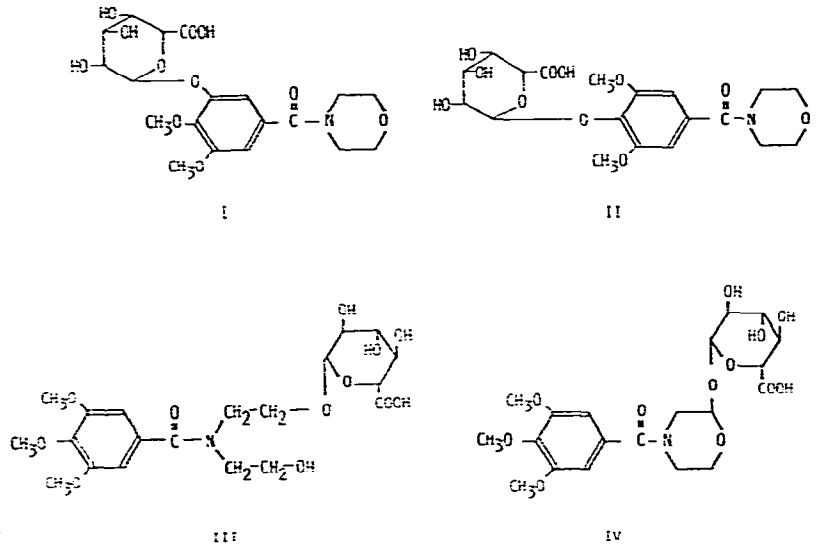

Fig. 1. Chemical structures of I, II, III and IV.

rivatives and determined by gas chromatography (GC). The present paper describes a GC method for the simultaneous determination of four glucuronides of trimetozine after permethylation of hydroxyl and carboxyl groups in the glucuronic acid moiety.

EXPERIMENTAL

Reagents and materials

The reagents used were a 50% dispersion of sodium hydride in oil (Metal Hydrides Inc., U.S.A.), methanol, ethyl acetate and diethyl ether of special grade for pesticide analysis (Wako, Osaka, Japan), chloroform of spectral grade (Nakarai, Kyoto, Japan), methyl iodide, acetic acid, sodium chloride and petroleum ether of reagent grade (Nakarai), and dimethyl sulfoxide of spectral grade (Merck, Darmstadt, G.F.R.).

Tricaprin used as an external standard, and 6-bromo-2-naphthyl-β-D-glucuronide used as an internal standard, were obtained from Sigma (St. Louis, MO, U.S.A.). The internal and external standards were used as an aqueous solution (50 μg/ml) and a chloroform solution (25 μg/ml), respectively.

Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, U.S.A.) was thoroughly washed successively with two volumes of distilled water and methanol. The resin particles floating on the surface were discarded by decantation. The resin was then transferred to a glass column and washed successively with 5 volumes of distilled water, methanol, distilled water, methanol containing 2% acetic acid, and distilled water, in this order.

The glucuronides of trimetozine (I, II, III and IV in Fig. 1) were isolated from the urine of a dog dosed with trimetozine, using the Amberlite XAD-2 column and thin-layer chromatography followed by recrystallization. The detailed procedure is found in a previous paper [1].

Methylsulfinyl carbanion solution was prepared as follows: 300 mg of sodium hydride were carefully rinsed three times each with 5 ml of anhydrous light petroleum (b.p. 30—60°C). A 5-ml portion of dimethyl sulfoxide was added to the sodium hydride and the mixture was heated at 75°C until evolution of hydrogen had ceased. The mixture was diluted six times with dimethyl sulfoxide.

Drug administration

Two healthy male adults, 26 and 32 years of age, weighing 59 and 53 kg, respectively, each received 200 mg of trimetozine as a capsule after a 16-h fast. The urine specimens were collected just before and at 3, 6, 12 and 24 h after administration.

Sample preparation

In a glass-stoppered test-tube were placed 1 ml of urine, 1 ml of 1 *N* hydrochloric acid and 0.5 g of sodium chloride. After shaking for 5 min, the mixture was washed with 10 ml of ethyl acetate. The trace of ethyl acetate remaining in the aqueous layer was completely removed by shaking with 10 ml of diethyl ether from the aqueous layer under reduced pressure at room temperature. A 1-ml aliquot of internal standard was added to the aqueous layer, and the mixture was placed on an Amberlite XAD-2 column (5 cm × 10 mm I.D.). A 20-ml portion of distilled water was first passed through the column for desalting and the glucuronides were eluted with 20 ml of methanol containing 1.5% acetic acid. The latter eluate was collected and evaporated to dryness in a flask under reduced pressure at 40°C. The residue was dissolved in 0.5 ml of the methylsulfinyl carbanion solution, and kept at room temperature for 1 min. Then 1.5 ml of methyl iodide were added to the reaction mixture which was kept at room temperature for 30 min. The mixture was shaken with 8 ml of distilled water and 12 ml of chloroform for 5 sec then transferred into a glass-stoppered test-tube. After centrifugation at 3000 rpm (1660 *g*) for 2.5 min, the chloroform layer was separated, washed three times with 8 ml of distilled water, and dehydrated with anhydrous sodium sulfate. The chloroform was evaporated to dryness in a flask under reduced pressure at 40°C. The residue was dissolved in 150 μl of chloroform, a 1-μl portion of which was injected into the gas chromatograph.

GC conditions

An Hitachi 073 gas chromatograph equipped with a flame-ionization detector was used. A U-shaped glass column (2 m × 3 mm I.D.) was packed with 1.5% OV-17 on Gas-Chrom Q AW DMCS (80—100 mesh). The column temperature was 285°C, the injection port and detector temperature 295°C; the carrier gas (nitrogen) flow-rate was 60 ml/min. The peak area was measured by a digital integrator (Takeda Riken TR-2213-A).

GC—mass spectrometry conditions

The GC—mass spectrometric (MS) analysis was carried out on an Hitachi 6MG gas chromatograph—mass spectrometer with following conditions: a

column of glass tubing (1 m × 3 mm I.D.) packed with 1.5% OV-17 on Chromosorb W AW DMCS (80—100 mesh); column temperature, 245°C; injection port temperature, 260°C; separator temperature, 260°C; ionization source temperature, 220°C; the carrier gas (helium) flow-rate, 60 ml/min; accelerating voltage, 3.5 kV; ionization energy, 20 eV; trap current, 60 μA.

RESULTS AND DISCUSSION

Recovery of glucuronides from the ion-exchange column

The effect of the elution solvent on the recovery of the four glucuronides (I, II, III, IV) and the internal standard from the Amberlite XAD-2 column was examined by changing the concentration of acetic acid (0 to 1.5% v/v) in methanol. The recoveries of glucuronides were determined by using internal standard added to the eluate; and the recovery of the internal standard itself was determined by using glucuronide I as the standard. The results indicated that the recoveries for all materials were less than 10% when eluted with methanol, but increased rapidly with initial increases in acetic acid concentration up to 0.2%, followed by gradual approaches to the plateau levels (92—100%) between 1.2 and 1.5%. From these results, methanol containing 1.5% acetic acid was chosen for the elution of glucuronides from the Amberlite XAD-2 column.

Permethylation reaction

Permethylation of the glucuronides of trimetozine was achieved by a two-step reaction with methylsulfinyl carbanion and with methyl iodide. The time and temperature dependencies of the reaction were investigated using each of the four glucuronides isolated from the dog urine. The degree of permethylation of the glucuronides and internal standard were evaluated by using tricaprin as an external standard. In examining the time dependence, the reaction temperature was fixed at 25°C for both reaction steps, and in examining temperature dependence the reaction time was fixed at 1 min for the reaction with methylsulfinyl carbanion and at 30 min for the reaction with methyl iodide. The results of the time dependence for the first reaction indicated that glucuronides I, III and IV and the internal standard underwent rapid raction with methylsulfinyl carbanion to give constant yields of permethylated derivatives, whereas glucuronide II suffered a gradual decrease in yields, possibly due to partial decomposition. The results for time dependence of the reaction with methyl iodide indicated that the formation of all the permethylated glucuronides and internal standard was complete 10—30 min after initiation of the second reaction. The results for the effect of temperature on both reaction steps showed that there were almost no changes in the yields of permethylated derivatives between 15°C and 35°C.

Thus, the four glucuronides and the internal standard were permethylated by reaction with methylsulfinyl carbanion for 1 min at room temperature and with methyl iodide for 30 min at room temperature.

GC separation and calibration graph

The permethylated derivatives of the isolated glucuronides and internal

standard each gave a single peak on the gas chromatogram. Fig. 2 shows chromatograms of human urine treated as mentioned above. No interfering peak was observed on the chromatogram of control urine. The retention times of the glucuronides and internal standard are 9.9, 10.6, 12.0, 14.1 and 3.2 min, respectively.

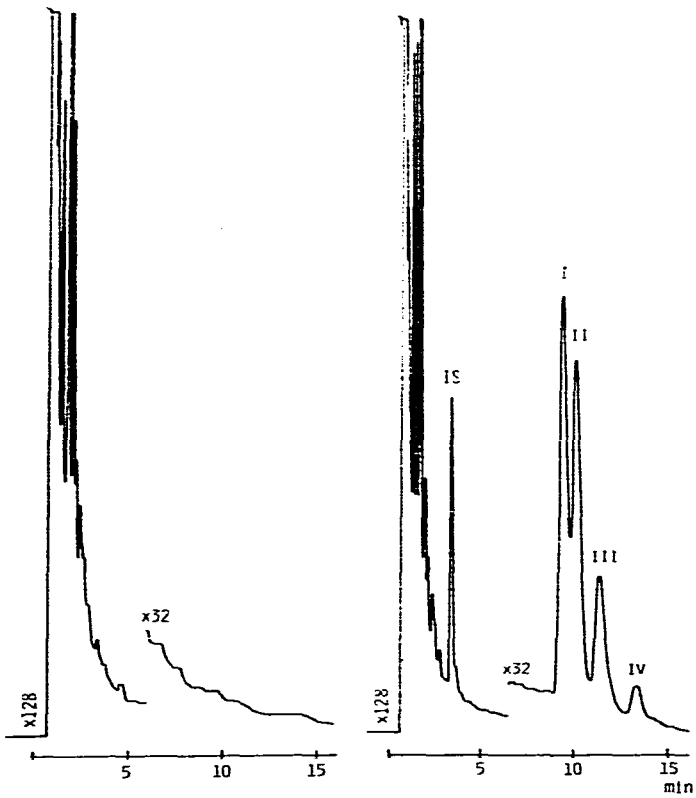

Fig. 2. Chromatograms of urine extracts. Left: blank urine. Right: urine from a volunteer who received 200 mg of trimetozine. IS = internal standard.

The calibration graphs were constructed using a 1-ml aliquot of urine containing 5—200 μg each of I, II, III and IV. The linear relationships were obtained between peak area ratio and sample weight (I, r=0.9995; II, r=0.9978; III, r=0.9986; IV, r=0.9993). The minimum detectable concentration of I, II, III and IV was 5 μg/ml when 1 ml of urine was used.

Mass spectrometry

The structures of the four permethylated glucuronides of trimetozine were confirmed from GC—MS spectra. Typical spectra are shown in Figs. 3 and 4; Table I summarizes the *m/e* values of characteristic peaks of other products. It is known that the fragment ions at *m/e* 75, 101, 141, 169 and 201 are characteristic of the permethylated glucuronic acid moiety [4, 5], and that the ions at *m/e* 116 and 232 are characteristic of the fragments

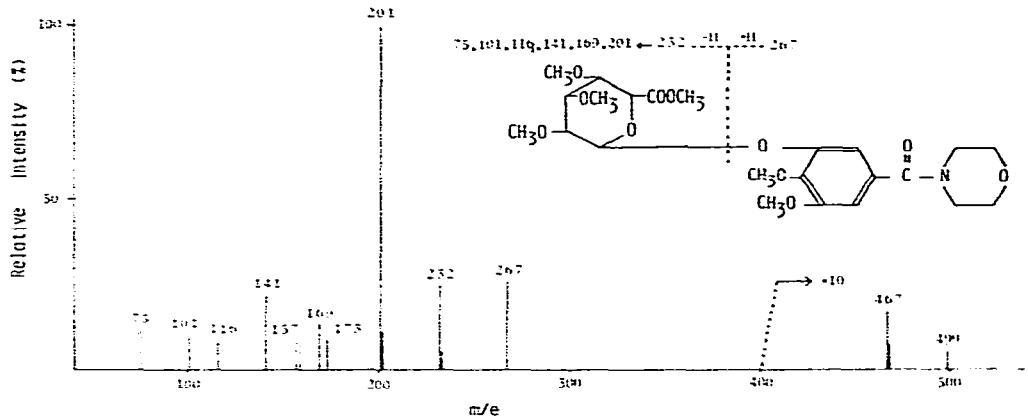

Fig. 3. Mass spectrum of I derivative.

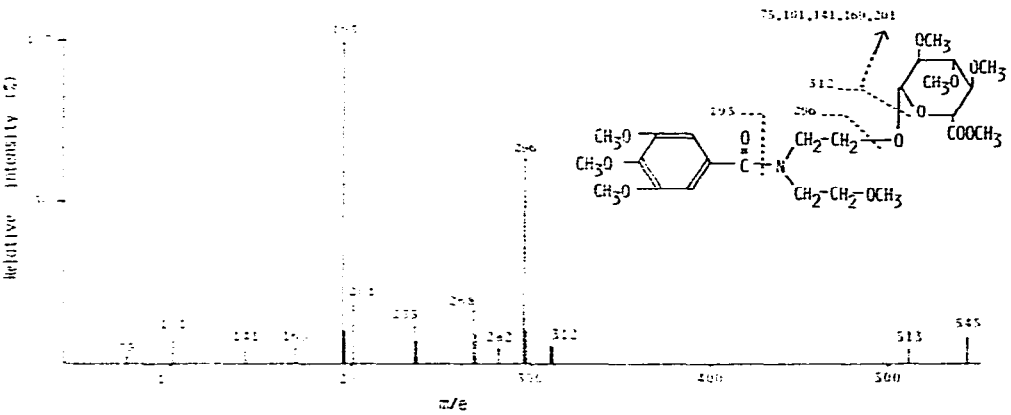

Fig. 4. Mass spectrum of III derivative.

TABLE I

MASS SPECTRA OF PERMETHYLATED DERIVATIVES OF GLUCURONIDES

Glucuronide	Diagnostic ions, *m/e* (relative intensity, %)						
II	75(14.0)	101(9.2)	116(9.1)	141(21.2)	157(9.6)	169(13.8)	173(13.2)
	201(100)	232(37.2)	267(21.5)	499(0.1)			
IV	75(5.8)	101(7.2)	141(4.1)	169(5.4)	195(100)	201(29.6)	280(18.3)
	297(10.1)	529(6.1)					
Internal	75(21.2)	101(19.8)	116(18.1)	141(19.7)	157(7.1)	169(18.0)	201(100)
standard	222(2.1)	224(2.0)	422(0.2)	424(0.2)	454(0.4)	456(0.4)	

of permethylated phenolic glucuronide; also that the ion *m/e* M − 249 is due to the fragment of permethylated aliphatic glucuronide [4, 5]. Compared with these findings, the present results readily show that the derivatives of I, II and internal standard are permethylated phenolic glucuronides, and the derivatives of II and IV are permethylated aliphatic glucuronides.

Urinary excretion in humans

The present method was applied to the determination of four glucuronides of trimetozine excreted in urine after oral doses of 200 mg of trimetozine as a capsule to human subjects. The results (Table II) show that an average of 23% of the administered amount was excreted in the urine as total glucuronides within 24 h.

TABLE II

URINARY EXCRETION OF GLUCURONIDES I, II, III and IV

Values are given in mg.

	h	I	II	III	IV
Volunteer A	0—3	3.1	2.0	ND*	ND
	3—6	7.5	5.6	0.8	0.7
	6—12	14.6	13.0	5.8	3.1
	12—24	8.9	7.0	5.0	1.2
Total		34.1	27.6	11.6	5.0
Trimetozine equivalent		21.6	17.5	6.9	3.0
Volunteer B	0—3	5.0	2.3	ND	ND
	3—6	11.3	6.4	1.2	0.9
	6—12	13.3	8.7	3.5	1.5
	12—24	7.0	4.0	3.0	0.7
Total		36.6	21.4	7.7	3.1
Trimetozine equivalent		23.2	13.6	4.6	1.8

*ND = not determined.

ACKNOWLEDGEMENTS

The authors wish to thank Professor T. Uno and Dr. Nakagawa of the Faculty of Pharmaceutical Sciences, Kyoto University, for many suggestions during this work, and Mr. T. Yamamoto and Mr. N. Kazikawa for isolation of the glucuronides of trimetozine from urine.

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