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DETERMINATION OF TRIPAMIDE AND ITS METABOLITES IN PLASMA, RED BLOOD CELLS AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A procedure for the determination of tripamide and its hydroxylated metabolites in plasma, red blood cells and urine by reversed-phase high-performance liquid chromatography is described.

The concentrations in red blood cells showed a monophasic decline and the half-life was 9.5 h. The concentration in red blood cells was markedly higher than that in plasma, showing that 95—98% of the drug is present in whole blood, after a dose of tripamide (90 mg) in man. The specificity and sensitivity of this procedure appear to be satisfactory for pharmacokinetic studies.

INTRODUCTION

Tripamide, N-(4-aza-*endo*-tricyclo[5.2.1.$0^{2,6}$]-decan-4-yl)-4-chloro-3-sulfamoylbenzamide, is a newly developed antihypertensive drug [1, 2] for which the clinical effect has also been reported [3—5]. Metabolic studies after administration of [^{14}C]tripamide to rats showed that the compound is extensively incorporated into red blood cells, resulting in low plasma concentration. In vitro experiments also show that the compound is rapidly incorporated into red blood cells of humans. Therefore, the present method is proposed for the measurement of the concentration of tripamide in plasma and red blood cells.

The drug is also rapidly and extensively metabolized by amide hydrolysis and ring hydroxylation. Although the major metabolic pathway is the hydrolysis of the amide of tripamide in rats [6], hydroxylation on the 3 or 8 position of the tricyclodecane ring seems to be an important pathway on the basis of the possible pharmacological significance of the hydroxylated compound.

It has been already reported that sulfonamide-like compounds, such as

hydrochlorothiazide [7], chlorothiazide [8], polythiazide [9], mefruside [10] and furosemide [11], can be analyzed by high-performance liquid chromatography (HPLC). Therefore, this method for the analysis of tripamide and its metabolites in biological fluids was developed. The method which was sensitive enough for the determination of tripamide in kinetic studies was established and preliminary pharmacokinetics of tripamide in healthy volunteers are presented in this paper.

MATERIALS AND METHODS

Reagents

The chemical structures of tripamide, the hydroxylated metabolites and internal standard for the determination of unchanged drug in plasma, red blood cells and urine are shown in Fig. 1. These were prepared in our laboratory as previously reported [12] and two hydroxylated metabolites (3-hydroxy- and 8-hydroxytripamide) were biosynthesized as previously described [6]. Acetic acid (Kanto Chemical Co., Tokyo, Japan) and methanol (Wako Pure Chemical Industry Co., Osaka, Japan) were used.

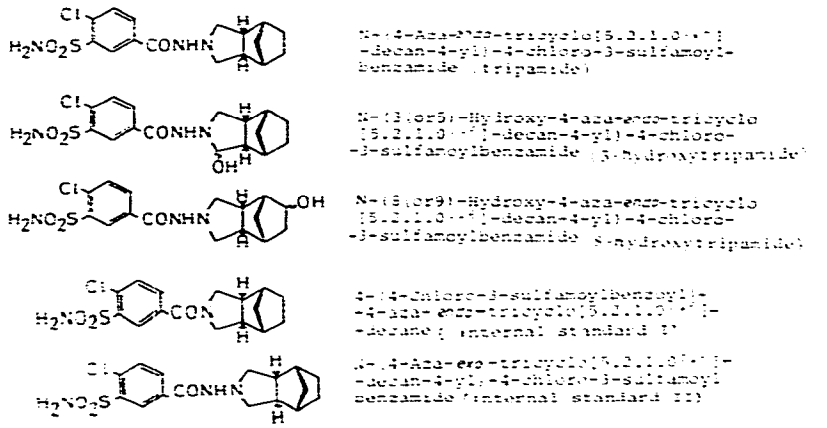

Fig. 1. The chemical structures of tripamide, hydroxylated metabolites, and of internal standards I and II used in the analysis of the blood samples and urine samples, respectively.

Apparatus, column and solvent

An Altex Model 100A high-performance liquid chromatograph, equipped with a variable-wavelength spectrophotometric detector (Jasco Uvidec Model 100-II, Japan Spectroscopic Co.) was used. The chromatographic column, solvent and temperature are shown in Table I.

Human subjects

To three healthy male volunteers (aged 36, 42, and 47 years, weight 62.0, 62.5, and 65.7 kg, respectively), tripamide was administered orally 1 h after a meal at a dose of 90 mg as Normonal tablets (Eisai Co., Tokyo, Japan). Blood samples of 7 ml were collected at scheduled time intervals in heparinized tubes and centrifuged at 1000 *g* for 5 min; plasma was immediately separated from the red blood cells. Remainders (red blood cells) were analyzed as the

TABLE I

CONDITIONS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

	Compound analysed	
	Tripamide	Hydroxylated tripamide
Column*	Hitachi gel 3011 (1) [Styrene divinylbenzene (SVB)] Reversed-phase	Nucleosil C_{18} (2) Reversed-phase
Particle size	10 μm	10 μm
Dimensions	500 × 4.6 mm	250 × 4.6 mm
Mobile phase	1% aqueous acetic acid—methanol (10:90, v/v)	water—methanol (60:40, v/v)
Flow-rate	0.7 ml/min	0.7 ml/min
Temperature	32°C	40°C
Injector	Rheodyne Model 7120 (injection loop of 100 μl)	
Detection	254 nm	
Pre-column*	Permaphase ODS (50 × 4.6 mm) (3)	

*The packing materials 1, 2 and 3 were purchased from Hitachi Co. (Tokyo, Japan), Macherey-Nagel Co. (Düren, G.F.R.) and DuPont (Wilmington, DE, U.S.A.), respectively.

concentration of tripamide in the red blood cells. Urine samples were collected at the scheduled time intervals.

Extraction from plasma

To 3 ml of plasma placed in a screw-capped tube (50 ml, pyrex, Iwaki Co.), 50 μl of a methanol solution containing internal standard I (625 ng) and 3 ml of distilled water were added. The extraction was made twice using 20 ml of diethyl ether each time by shaking for 15 min and centrifuging for 5 min at 1000 *g*. The combined ether layers were condensed to 5 ml in a screw-capped tube (20 ml) at 45°C in a stream of nitrogen. Two milliliters of 0.05 *M* borate buffer (pH 9) were added and shaken gently for 15 min. After centrifuging for 5 min at 1000 *g*, the ether layer was taken and evaporated to dryness at 45°C in a stream of nitrogen. The residue was dissolved in 0.25 ml of methanol by sonication at 80°C with a Bransonic 12 (Yamato Kagaku). After centrifuging for 5 min at 1000 *g*, a 50-μl aliquot of the solution was injected into the HPLC column.

Extraction from red blood cells

To red blood cells obtained from 7 ml of blood, 0.8 ml (20 μg) or 0.2 ml (5 μg) of methanol solution of internal standard I was added, then 50 ml of distilled water were added for hemolysis. The hemolyzed red blood cell solution was placed in separating funnel (volume 300 ml) and extracted with 100 ml of diethyl ether by shaking by hand vigorously and then with a mechanical shaker gently for 20 min. After standing for 30 min, the ether layer was separated and evaporated in vacuo using a rotary evaporator. The residue was dissolved in 5 ml of methanol and transferred to test tubes. The methanol was evaporated at 45°C in a stream of nitrogen. The residue was dissolved in 0.4 ml

of methanol and a 40-μl aliquot of the solution was injected into the high-performance liquid chromatograph.

Extraction from urine

Ten milliliters of urine placed in a screw-capped tube (50 ml) were adjusted to pH 7 with 1 *N* sodium hydroxide solution, then 20 μl of methanol solution containing 3.3 μg of internal standard II were added. Extraction was made twice using 20 ml of diethyl ether each time by shaking for 15 min and centrifuging for 5 min at 1000 *g*. The combined layers were condensed to 10 ml in a screw-capped tube (20 ml) at 45°C in a stream of nitrogen. Then, 0.5 ml of 1 *N* sodium hydroxide solution was added to the residual solution and shaken for 5 min. After centrifuging for 5 min at 1000 *g*, the ether layer was discarded. To the aqueous solution, 0.2 ml of 6 *N* hydrochloric acid solution and 5 ml of diethyl ether were added and then shaken for 5 min. After centrifuging for 5 min at 1000 *g*, the ether layer was discarded and the water layer was adjusted to pH 7 with 1 *N* sodium hydroxide solution and 1 ml of 0.05 *M* phosphate buffer (pH 7) was added. To the solution 10 ml of diethyl ether were added and shaken for 15 min. After centrifuging for 5 min at 1000 *g*, the ether layer was transferred to a test tube and evaporated at 45°C in a stream of nitrogen. The residues were dissolved in 0.4 ml of methanol and a 40-μl aliquot of the solution was injected onto the column.

Recovery

The addition of 200 ng of tripamide to plasma and red blood cells resulted in overall recoveries of 90% for plasma and 97% for red blood cells. The recovery of tripamide from urine was 80% at a concentration of 2.5 μg/ml and that of 3-hydroxy- and 8-hydroxytripamide was 37.2 ± 0.6% at 0.19 μg/ml and 37.3 ± 2.3% at 0.17 μg/ml, respectively.

RESULTS AND DISCUSSION

Determination of tripamide in plasma and red blood cells

A chromatogram of plasma extract is shown in Fig. 2A. Two peaks, originating from endogenous compounds, are observed at retention times of 18.1 and 19.3 min. Fig. 2B is a chromatogram of extract to which 100 ng of tripamide (retention time 17.2 min) and 125 ng of the internal standard I (22.2 min) were added. They are sufficiently separated from both tripamide and internal standard I and their peaks did not interfere with the peaks of either tripamide or internal standard I.

A standard curve was prepared by adding known amounts of drug to blank plasma samples and determining the peak height ratios (tripamide/internal standard). The tripamide standard curve was linear in the range 16.7—166.7 ng/ml plasma, and extrapolated through the origin.

The sensitivity limit was 16.7 ng/ml.

A similar result was obtained when known concentrations of drug were added to red blood cells. A typical chromatogram of extracted red blood cells is shown in Fig. 3. To facilitate analysis of red blood cells of high concentra-

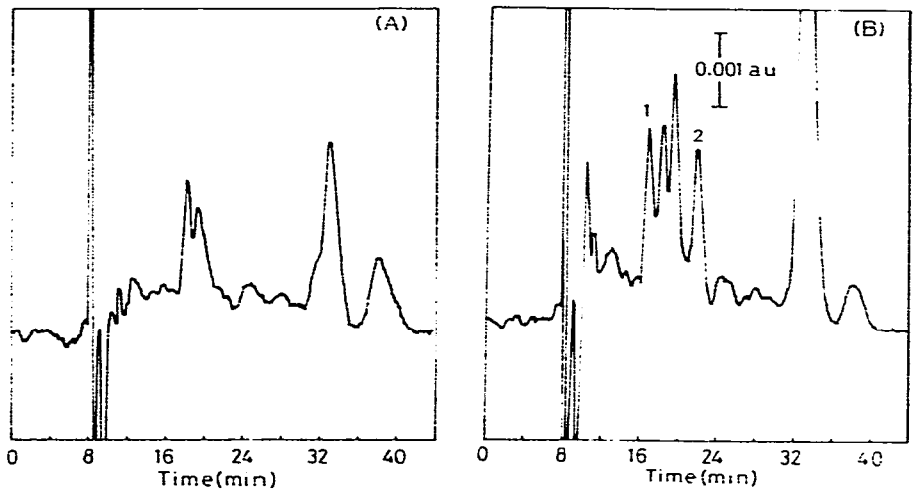

Fig. 2. Chromatograms of (A) plasma extract and (B) plasma extract containing 100 ng of tripamide (1) and 125 ng of internal standard I (2). Three peaks eluting at 18.1, 19.3 and 33.0 min were endogenous plasma constituents. HPLC conditions as in Table I. The detector range for both chromatograms was 0.001 a.u.

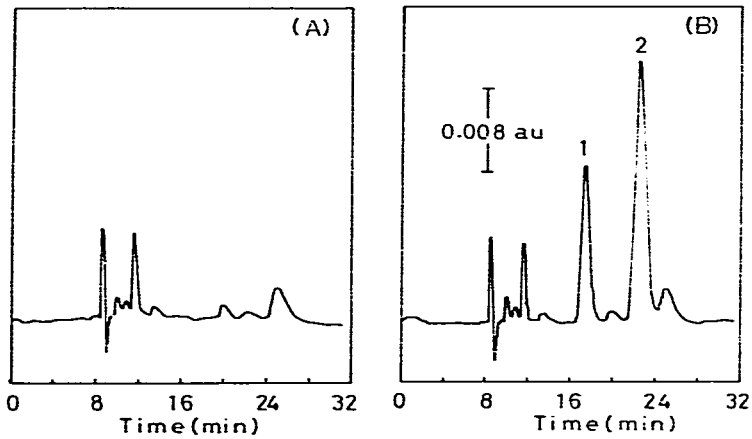

Fig. 3. Chromatograms of (A) red blood cell extract and (B) red blood cell extract containing 1 μg of internal standard and 400 ng of tripamide. HPLC conditions as in Table I. The detector range for both chromatograms was 0.008 a.u.

tion, a high-range standard curve was also prepared by adding known high concentration of the drug and 5 μg of internal standard I to red blood cell samples.

Determination of tripamide and its metabolites in urine

Fig. 4B is a chromatogram of an extracted urine sample to which 200 ng of tripamide and 330 ng of the internal standard II were added. As shown in the blank sample (Fig. 4A), no interfering peak originating from an endogenous compound was found. The calibration curve was obtained by adding known amounts of tripamide to urine samples. The ratio of the peak height of tripamide to that of the internal standard was linear in the range 50—800 ng/ml.

Fig. 5B shows a chromatogram of a urine sample obtained from a volun-

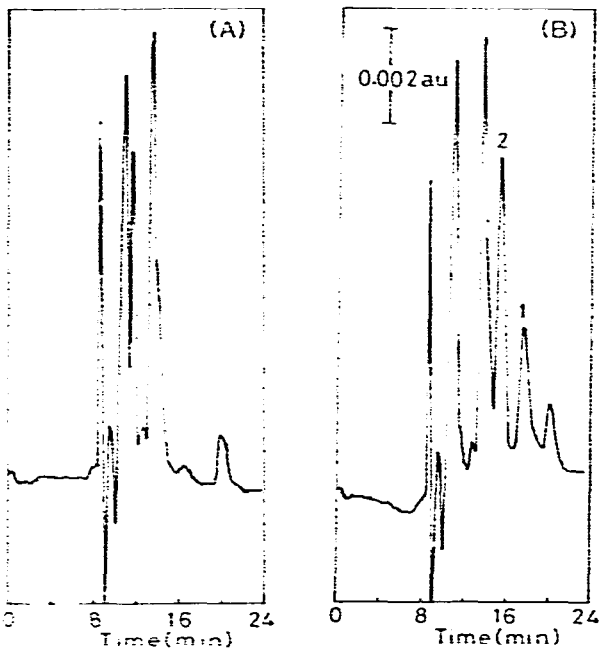

Fig. 4. Chromatograms of (A) blank urine and (B) urine extract containing 200 ng of tripamide (1) and 330 ng of internal standard II (2). Solvent: 1% aqueous acetic acid—methanol (10:90, v/v). Solvent flow-rate: 0.7 ml/min. Column: Hitachi gel 3011, 10-μm particle size, 500 × 4.6 mm. Column temperature: 32°C.

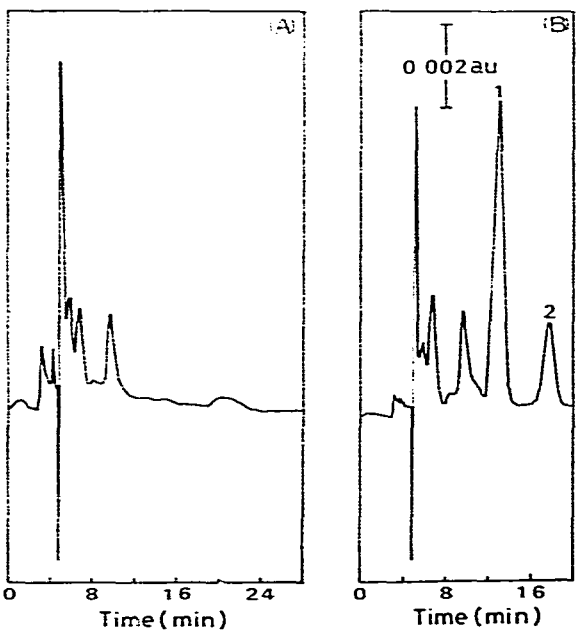

Fig. 5. Chromatograms of (A) blank urine and (B) the hydroxylated metabolites as a single medication in a human urine sample. 1 and 2 on the chromatogram show 8-hydroxy- and 3-hydroxytripamide, respectively. Solvent: water—methanol (60:40, v/v). Solvent flow-rate: 0.7 ml/min. Column: Nucleosil C_{18}, 10-μm particle size, 250 × 4.6 mm. Column temperature: 40°C.

teer after oral administration of tripamide at the dose of 90 mg. As shown in the blank urine sample (Fig. 5A), no interfering peaks were found. Retention times were 12.4 min for 8-hydroxytripamide and 16.8 min for 3-hydroxytripamide.

The calibration curves of 3-hydroxy- and 8-hydroxytripamide were obtained as follows. [^{14}C]Tripamide was incubated with rat liver microsomes under NADPH and O_2, and the two hydroxylated metabolites formed were purified by thin-layer chromatography [6]. Known amounts of 3-hydroxy- and 8-hydroxytripamide, which were calculated on the basis of specific radioactivity of [^{14}C]tripamide, were injected onto the column. The peak area of the compounds was linear in the range from 0.17—1.09 μg per injection for 8-hydroxytripamide and 0.25—1.28 μg for 3-hydroxytripamide.

Preliminary pharmacokinetic study

The analytical method described above has sufficient sensitivity for pharmacokinetic studies in human subjects administered orally 90 mg of tripamide. Table II shows the levels of tripamide in plasma and red blood cells in three volunteers. The plasma concentration reached a maximum of 117.6 ± 17.3 ng/ml at 4 h after administration and could not be detected at 24 h. The red blood cell concentration also attained a peak of 4.98 ± 0.33 μg/ml 4 h after administration.

The time—course of the red blood cell concentration showed a monophasic decline and the half-life was 9.5 h. The concentration of tripamide in red blood cells was markedly higher than that in plasma, showing that 95—98% of tripamide in total blood is present mainly in red blood cells.

Table III shows the renal excretion rate of tripamide in volunteers after oral administration. The unchanged drug excreted in the urine was 0.8 ± 0.1% of the dose. The rate of renal excretion with monophasic phase ($t_{½}$ = 8.9 h) was similar to that of disappearance from red blood cells ($t_{½}$ = 9.5 h). Table IV shows the renal excretion of 3-hydroxy- and 8-hydroxytripamide follow-

TABLE II

CONCENTRATIONS OF TRIPAMIDE IN PLASMA AND RED BLOOD CELLS

Each value is the mean ± S.E. (n = 3). n.d. = not detectable.

Time (h)	Concentration	
	Plasma (ng/ml)	Red blood cells (μg/ml)
1	22.2 ± 2.2	1.94 ± 0.70
2	101.2 ± 17.2	4.18 ± 0.76
3	103.1 ± 6.0	4.81 ± 0.41
4	117.6 ± 17.3	4.98 ± 0.33
6	49.0 ± 16.5	3.90 ± 0.35
8	51.9 ± 20.5	3.35 ± 0.47
24	n.d.	1.09 ± 0.26
48	n.d.	0.21 ± 0.08
72	n.d.	n.d.

TABLE III

RENAL EXCRETION OF TRIPAMIDE IN HUMANS

Subject	Excretion			
	μg in 72 h	%	K (h^{-1})	$t_{½}$ (h)
T. I.	749	0.8	0.063	11.0
A. K.	557	0.6	0.092	7.5
T. K.	813	0.9	0.086	8.1
Mean ± S.E.		0.8 ± 0.1		8.9 ± 1.1

TABLE IV

RENAL EXCRETION OF THE HYDROXYLATED METABOLITES IN HUMANS

Subject	Metabolite	Excretion		Total (%)	3-Hydroxy / 8-Hydroxy
		mg in 72 h	%		
T. I.	3-Hydroxytripamide	1.95	2.2	8.1	0.37
	8-Hydroxytripamide	5.29	5.9		
A. K.	3-Hydroxytripamide	6.21	6.9	11.0	1.68
	8-Hydroxytripamide	3.70	4.1		
T. K.	3-Hydroxytripamide	5.62	6.2	11.8	1.11
	8-Hydroxytripamide	5.05	5.6		

ing administration of tripamide at the dose of 90 mg. The excretion of total hydroxylated metabolites ranged from 8.1 to 11.8%, about ten times that of the unchanged drug. Preliminary pharmacokinetic results in humans as shown in this paper indicate that tripamide is metabolized extensively and excreted as hydroxylated metabolites in urine. None of the hydroxylated metabolites could be detected in the blood. The hydroxylated metabolites do not appear in the blood, since the metabolites formed in the liver are mainly excreted into the bile [6] and the hydroxylated metabolites excreted in the urine seem to be hydroxylated by renal microsomes.

The finding of individual variations in the excretion of 3-hydroxy- and 8-hydroxytripamide may be attributed to inherent variations in its metabolism in the volunteers.

In conclusion, the specificity and sensitivity of this procedure appear to be satisfactory for pharmacokinetic studies on tripamide.

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REFERENCES

1 T. Igarashi, Y. Nakajima and M. Kobayashi, Shinryo To Shinyaku, 16 (1979) 1253 (in Japanese).
2 M. Kobayashi, N. Mori, T. Tomiuga and T. Igarashi, Shinryo To Shinyaku, 16 (1979) 1001 (in Japanese).
3 E. Takahashi, M. Ozawa, K. Honda, S. Ushiyama, M. Nara, K. Sugawara, K. Doiziri, K. Watanabe, K. So, H. Honma, S. Obara, T. Kimura and F. Fusa, Yakuri To Chiryo, 6 (1978) 2849 (in Japanese).
4 Z. Saito, T. Hashiba, S. Miyamoto, K. Uchida, S. Morimoto, R. Takeda, M. Minami, Y. Tomari and T. Seo, Shindan To Chiryo, 64 (1979) 2158 (in Japanese).
5 K. Kawasaki, K. Fukiyama, M. Ueno, Y. Noda and T. Omae, Rinsho To Kenkyu, 56 (1979) 566 (in Japanese).
6 T. Horie, T. Ohno and K. Kinoshita, Xenobiotica, 11 (1981) 197.
7 M.J. Cooper, A.A. Sinaiko, M.W. Anders and B.L. Mirkin, Anal. Chem., 48 (1976) 1110.
8 I.L. Honigberg, J.T. Steward, A.P. Smith, R.D. Plunkett and D.W. Hester, J. Pharm. Sci., 63 (1974) 1762.
9 R.E. Moskalyk, R.A. Locock, L.G. Chatten, A.M. Veltman and M.F. Bielech, J. Pharm. Sci., 64 (1975) 1406.
10 C.J. Little, A.D. Dale, D.A. Ord and T.R. Marten, Anal. Chem., 49 (1977) 1311.
11 B. Lindström, J. Chromatogr., 100 (1974) 189.
12 H. Hamano, T. Nakamura, S. Kuriyama and M. Yamanaka, Jap. Pat., 7305,585 (Cl.07d, A61K); C.A., 78 (1973) 136070r.