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Trimetazidine in Blood, Bile, Organs and Urine[1]

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Plasma levels of trimetazidine in rabbits, rats, and mice were determined. Plasma levels in animals showed unusual curve about 1 hr after oral administration and this reason was investigated by determining distribution of the drug in animal tissues and excretion of the drug in bile.

Metabolic pathways of trimetazidine in rabbits were investigated qualitatively and it was ascertained by thin-layer chromatography that the methylene group connecting the benzene and piperazine rings is not severed by metabolism in rabbits.

Trimetazidine dihydrochloride, 1-(2,3,4-trimethoxybenzyl)piperazine dihydrochloride, is a remedy for ischemic heart diseases with less toxicity. Pharmacological properties of trimetazidine dihydrochloride were reported by Yamada.[3] and its toxicity was studied by Shimamoto, *et al.*[4]

Present work was carried out for determination of trimetazidine in blood and bile, its distribution in rat organs, and metabolic pathway of trimetazidine in rabbits.

Experimental

Detection of Metabolites of Trimetazidine in Plasma——A mixture of 0.5 ml of plasma, taken 0.5 hr after oral ingestion of 700 mg/kg of trimetazidine dihydrochloride, and 0.5 ml of 5N HCl was kept in an incubator at 37±2° for 1 hr. The mixture was neutralized with 0.5 ml of 5N sodium carbonate solution and 1.5 ml of water, and 1 g of sodium chloride and 7 ml of methylene chloride were added. This mixture was shaken vigorously for 1 hr and centrifuged. After evaporation of the methylene chloride layer, the residue was dissolved in 50 μl of ethanol, and 10 μl of this solution was spotted for thin-layer chromatography (TLC) (A), the result of which is shown is Table I.

Determination of Trimetazidine in Plasma (Methyl Orange Method[5])——A mixture of 1 ml of plasma, 1 ml of water, and 1 ml of methyl orange solution was stirred thoroughly. After standing for 15 min, 4 ml of methylene chloride was added and shaken 20 times up and down. Absorbance of a clear lower layer obtained by centrifugation was determined at 410 mμ. A mixture of 0.9 ml of normal animal plasma and 0.1 ml of trimetazidine solution of known concentration was treated as above to prepare a calibration curve.

Methyl orange solution was prepared by mixing equal volumes of 0.5% methyl orange (Merck) solution and 2.5% boric acid solution, at the time of use.

Collection of Rabbit and Rat Bile——Rabbits varying in weight from 3.0 to 3.5 kg were anesthesized with urethan, 1 g per kg subcutaneously, after fasting for approximately 24 hr. Blood pressure was recorded by a mercury manometer connected to a cannula in a carotid artery.

After a midline incision, the common bile duct was cannulated as close to the duodenum as possible without injuring pancreatic tissue. The cannula consisted of polyethylene tubing (Igarashi Ika Kogyo Co., Ltd., No. 30). The cystic duct was clamped at the base of the gall bladder. Bile collection was begun after the cannula was free of gall bladder bile that may have been expressed during the cannulation. Bile was collected for succesive 30 min periods into 5 ml graduated cylinders located so that the opening of the cannula was at or below the level of the common bile duct.

1) This constitutes Part XLII of a series entitled "Studies on Absorption and Excretion of Drugs" by S. Naito. Part XLI: S. Naito and K. Umetsu, *J. Pharm. Sci.*, under contributing.
2) Location: *Yamashina Misasagi, Higashiyama-ku, Kyoto.*
3) H. Yamada, unpublished; Y. Fujita, *Japan. J. Pharmacol.*, **17**, 19 (1967).
4) K. Shimamoto, S. Takaori, Y. Fujita and H. Usui, "Gendai no Rinsho," (Japan), Vol. 1, 1967, p. 226.
5) B.B. Brodie and S. Udenfriend, *J. Biol. Chem.*, **158**, 705 (1945).

At the end of the first (control) hr, trimetazidine (5% solution), when used, was administered orally by a stomach tube. Bile was collected in 5 ml fractions for 7.5 hr following administration of the compound.

In the case of rats (Wistar strain), the animals, 160 to 190 g in body weight, were treated in the same way as rabbits. The cannula consisted of polyethylene tubing (Igarashi Ika Kogyo Co. Ltd., No. 10). Bile was collected continuously for 7.5 hr following oral administration of the compound without dividing into fractions.

Determination of Trimetazidine in Bile——One milliliter of bile instead of plasma was taken to determine the concentration of trimetazidine, as in the case of determination of trimetazidine in plasma. A mixture of 0.9 ml of normal animal bile and 0.1 ml of trimetazidine solution of known concentration was treated as above, to prepare a calibration curve.

Each group consisted of 4 male rabbits (average weight, 3.3 kg) or male rats (Wistar strain, average weight, 190 g).

Before determination of trimetazidine, metabolites of the drug in bile was investigated by the same procedure described in "Detection of Metabolites of Trimetazidine in Plasma" and no metabolites were observed in bile by TLC (A) as in Table I.

Distribution of Trimetazidine in Rat Organs——To male rats (Wistar strain) weighing about 180—190 g, 700 mg/kg of trimetazidine was administered orally and the animals were killed 1, 7, and 24 hr after the administration. Each group consisted of 3 rats, and six organs, that is, heart, lung, spleen, kidney, brain, and liver, were used for the determination of trimetazidine. The sample of each organ was prepared from a pooled mixture of the same organs in one group and the total weight of a sample used was 2 g for heart, 3 g for lung, 1 g for spleen, 4 g for kidney and brain, and 15 g for liver. These samples were homo-

Table I. Thin-Layer Chromatography
Adsorbent: Diatomite (Kieselgel G), 0.25 mm in thickness

TLC	Solvent	Color developer	Material	*Rf*	Color of spot[a]
A	*n*-butanol–ethylene dichloride–28% ammonia (8: 1: 2)	diazotated *p*-nitroanilin[b]	control urine	nil	
			CH_2Cl_2–urine[c]	0.25	Y
				0.37	Br
				0.42	O
				0.61	L-Y
			A-2[d]	0.42	O
			trimetazidine	0.61	L-Y
			control plasma	nil	
			treated plasma[e]	0.61	L-Y
B	*n*-butanol–ethylene dichloride–28% ammonia (8: 1: 2)	D-P reagent[f]	control urine	nil	
			HCl–urine[g]	0.28	B
				0.34	L-V
				0.43	B
				0.60	B
				0.65	B
				0.75	B
			B-1	0.22	Y
			B-2	tail off	L-Br
			B-3	0.86	L-Y
			B-4	0.63	L-Br
			C-1	0.15	B
			trimetazidine	0.65	B
C	benzene–ethanol (5: 1)	D-P reagent	control urine	nil	
			HCl–urine	0.03	V
				0.05	B
				0.15	V
				0.20	V
				0.33	V
				0.50	L-V
			B-1	0.63	Y
			B-2	0.47	Y-Br
			B-3	0.90	Y
			B-4	0.62	Y
			C-1	0.03	B
			trimetazidine	0.05	B

TLC	Solvent	Color developer	Material	*Rf*	Color of spot[a)]
D	ether–ethanol–28% ammonia (4: 1: 1)	D-P reagent	control urine	nil	
			HCl–urine	0.32	R-Br
				0.40	V
				0.70	B
				0.82	V
				0.93	B-V
			B-1	0.07	Y
			B-2	0.63	Br
			B-3	0.89	Y
			B-4	0.86	Br
			C-1	0.11	B
E	ethyl acetate–28% ammonia (4: 1)	D-P reagent	control urine	nil	
			CH_2Cl_2–urine	0.10	Y-B
				0.16	B
				0.20	B
				0.50	B
				0.55	V
				0.65	Y-Br
				0.71	V
				0.79	V
			A–1	0.65	Y-Br
F	acetone–benzene (2: 1)	D-P reagent	control urine	nil	
			CH_2Cl_2–urine	0	B
				0.55	Br
				0.63	Br
				0.70	Br
				0.75	B
				0.83	V
			A-1	0.70	Br
			trimetazidine	0	B
G	28% ammonia–ethanol–ether–ethylene dichloride (2: 3: 6: 1)	diazotated *p*-nitroaniline	control urine	nil	
			CH_2Cl_2–urine	0.25	Br
				0.34	Y
				0.55	O
				0.71	L-Y
			A-2	0.55	O
			trimetazidine	0.71	L-Y

a) B: blue, B-V: bluish violet, Y-B: yellowish blue, Y: yellow, L-Y: light yellow, Y-Br: yellowish brown, L-Br: light brown, Br: brown, R-Br: reddish brown, O: orange, V: violet, L-V: light violet.
b) Diazotated *p*-nitroanilin reagent was prepared by the method of Demole.[6)]
c) Methylene chloride extracts from HCl–urine (foot-note g).
d) Compound (A-2) is listed in Table III.
e) Plasma was obtained from animals after oral administration of trimetazidine.
f) D-P reagent: Dragendorff's reagent[7)] after spraying platinic chloride-potassium iodide reagent.[8)]
g) Lyophilized residue neutralized with Na_2CO_3 solution after treating with hydrochloric acid. This procedure was taken to cleave unknown conjugated form of the metabolites.

genized with ethanol using 6 ml for heart and lung, 5 ml for spleen, 7 ml for kidney and brain, and 18 ml for liver. The supernatant of the centrifuged homogenate was diluted with ethanol to make 1: 30 for heart and lung, 1: 15 for spleen, 1: 50 for kidney and brain, and 1: 100 for liver. Absorbance of the diluted homogenate solution thus obtained was determined at 272 mμ.

Mean concentration of trimetazidine in each organ was obtained from three groups at each sampling time.

Each organ of rat receiving no drug was treated as above to prepare a control sample. The mixture of control homogenate and trimetazidine solution of known concentration was treated as above to prepare a calibration curve.

6) E. Demole, *Chromatographic Rev.*, **1**, 8 (1959).
7) R. Munier, *Bull. Soc. Chim. Biol.*, **35**, 1225 (1953).
8) D. Waldi, K. Schnackerz and F. Munter, *J. Chromatog.*, **6**, 61 (1961).

Separation of Metabolites of Trimetazidine in Rabbit Urine——About 350 ml of urine collected for 48 hr from two rabbits, each ingesting 600 mg/kg of trimetazidine, was submitted to freeze-drying. Yield of the residue (A) was about 22 g. The residue (B) of urine, obtaid from two rabbits which did not receive the drug, served as a control.

A mixture of 500 mg of the residue (A) or (B) and 1 ml of 5N HCl was kept in an incubator at 37±2° for 1 hr. After the addition of 1 ml of 5N sodium carbonate solution, the residue A′ from (A) or B′ from (B) was submitted to TLC. No difference was observed in the number of spots between the residue (A) and A′ or (B) and B′.

On the other hand, methylene chloride extract A″ from the residue A′ or B″ from the residue B′ was also submitted to TLC as in Table I. The methylene chloride extract A″ or B″ was prepared by shaking the residue A′ or B′ vigorously with 4 ml of methylene chloride, and the separated methylene chloride layer was evaporated to dryness on a steam bath.

Result and Discussion

Before quantitative determination of trimetazidine in plasma, it was ascertained that no metabolites of the chemical was observed in TLC (A) as shown in Table I. Therefore, plasma level of trimetazidine in rabbits, rats, and mice shown in Fig. 1—3, was determined without any consideration with existences of its metabolites. Methyl organge reaction used in the present work for determination of trimetazidine in plasma has been carried out principally by the method reported by Brodie, *et al.*[5] Brodie, *et al.* indicated that this color reaction can be applied for assay of a large number of alkaloids and synthetic alkaline organic compounds, applying to the development of an analytical procedure for the estimation of the cinchona alkaloids, particularly cinchonidine. Afterwards, the methyl orange reaction has been used for the determination of various alkaloids,[9] meperidine (piperidine derivative),[10] meclizine (piperazine derivative),[11] and cyclizine and chlorcyclizine (piperazine derivatives).[12] Trimetazidine forms highly soluble complex with methyl orange in methylene chloride and this complex was very stable at room temperature (20±2°) at least for 2 hr. No difference of absorbance was found at 18° and 22°. A calibration curve in plasma was made in concent-

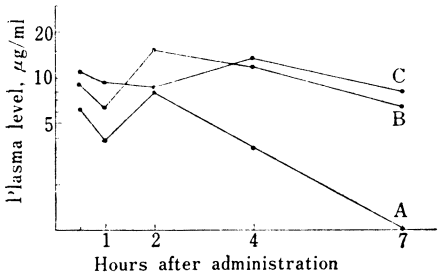


Fig. 1. Mean Plasma Level of Trimetazidine after Oral Administration of Trimetazidine Dihydrochloride to Rabbits at Different Doses

Each group consisted of 4 male rabbits (average weight, 2.3 kg for 400 mg/kg dose and 3.0 kg for 500 and 700 mg/kg doses)
keys: A, 400 mg/kg; B, 500 mg/kg; C, 700 mg/kg

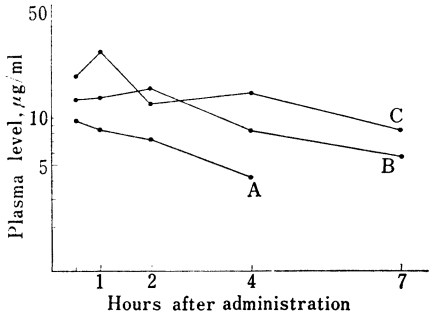


Fig. 2. Mean Plasma Level of Trimetazidine after Oral Administration to Rats in Different Dose

Each group consisted of 5 male rats (Wistar strain, average weight, 170 g). Each group of animals was killed at the sampling time. At the time of sacrifice, each animal was bled completely, and the blood reserved for analysis.
keys: A, 300 mg/kg; B, 500 mg/kg; C, 700 mg/kg

9) A.O. Gettler and I. Sunshine, *Anal. Chem.*, **23**, 779 (1951).
10) J.J. Burns, B.L. Berger, P.A. Lief, A. Wollack, E.M. Papper, and B.B. Brodie, *J. Pharmacol. Exptl. Therap.*, **114**, 289 (1955).
11) S.A. Narrod, A.L. Wilk and C.T.G. King, *J. Pharmacol. Exptl. Therap.*, **147**, 380 (1965).
12) R. Kuntzman, A. Klutch, I. Tsai, and J.J. Burns, *J. Pharmacol. Exptl. Therap.*, **149**, 29 (1965).

rations of 10, 20, 30, and 40 μg/ml of trimetazidine. A calibration curve (with standard error) obtained by the least square method from ten repeated experiments at 20° was $y=68.0x\pm1.23$, where y is trimetazidine content (μg/ml) and x shows absorbance at 410 mμ. Therefore, it can be said that the methyl orange reaction is suitable for determination of trimetazidine.

Plasma level following intravenous administration of trimetazidine in rabbits as shown in Fig. 4, was also determined to find a relationship if any, between plasma level and pharmacological activities.

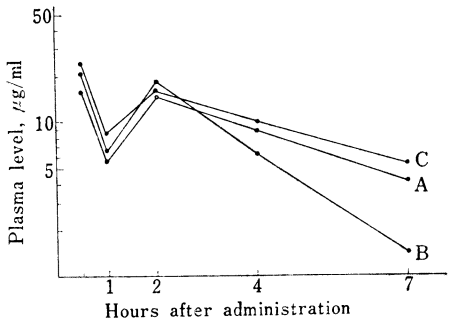


Fig. 3. Mean Plasma Level of Trimetazidine after Oral Administration of Trimetazidine Dihydrochloride to Mice in Different Doses

Each group consisted of 10 male mice (dd strain, average weight, 13 g). Each group of animals was killed at the sampling time. At the time of sacrifice, each animal was bled completely, and the blood reserved for analysis.

keys: A, 500 mg/kg; B: 700 mg/kg; C: 900 mg/kg.

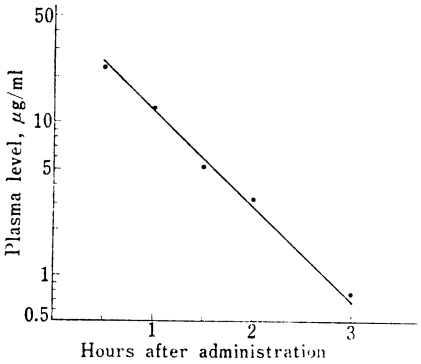


Fig. 4. Mean Plasma Level of Trimetazidine after Intravenous Administration of the Drug (80 mg/kg) to 3 Male Rabbits (Average Weight, 2.3 kg)

Some of mean plasma levels in animals showed unusual curve (hollow) about 1 hr after oral ingestion of trimetazidine as shown in Fig. 1—3. To find the reason for the formation of unusual curve, the amount of trimetazidine excreted in bile of rabbits and rats was investigated. Logically, some metabolites of trimetazidine should exist in animal bile, but no metabolites of the drug except unchanged drug were detected in TLC (A) as shown in Table I by the same method as in the case of metabolites of trimetazidine in plasma. It might be that the biliary metabolites of trimetazidine were little compared to unchanged trimetazidine in bile. Therefore, trimetazidine in bile was roughly determined without pre-separation of unchanged trimetazidine from its metabolites.

While the bile of rabbit was being collected under continuous anesthesia using urethan, arterial blood pressure and respiration were also recorded on a kymograph. It was thereby ascertained that trimetazidine has no effect on arterial blood pressure, respiration, bile flow, or total bile volume excreted during 7.5 hr.

It became clear that trimetazidine is excreted in bile but the amount excreted is extremely small. Bile volume and trimetazidine in bile, excreted during 7.5 hr, were 12.8 ± 2.7 ml/kg and 116 ± 23 μg/kg, respectively, for rabbits at 300 mg/kg dose, and 22.6 ± 5.4 ml/kg and 288 ± 47 μg/kg for rats at 700 mg/kg dose.

Still some question remains where another portion of trimetazidine ingested is distributed in when abnormality appears on a curve of plasma level 1 hr after the administration as shown in Fig. 1—3. Therefore, distribution of trimetazidine in rat organs was determined, as shown in Table II. Trimetazidine in tissues was assayed in the ultraviolet (UV)-region. Logically,

tissue homogenate shows high absorbance, therefore, it was ascertained whether UV-method can be used for determination of trimetazidine in various tissue homogenates. Each group consisted of three rats received no drug. Mean absorbance (control) of heart, lung, spleen, kidney, brain, and liver at 272 mμ showed 0.386±0.022, 0.398±0.020, 0.428±0.027, 0.334±0.018, 0.465±0.025, and 0.424±0.038, respectively. On the other hand, mean absorbance of 50 μg/ml of trimetazidine in aqueous solution was 0.230±0.005 at 272 mμ, in five experiments. Determination of trimetazidine in tissue homogenates by the UV-method should be valuable in rough estimation, because even the largest standard error, 0.038 (liver), corresponds less than 10 μg/ml of trimetazidine in aqueous solution. The fact that some amount of trimetazidine was found in rat organs 1 hr after the administration shows good interpretation for the abnormality appearing on a curve of plasma level as shown in Fig.s 1—3, together with execrtion of the chemical in bile. Therefore, it may be assumed that trimetazidine is first absorbed and distributed to some organs, and then the drug re-appears in blood, combining the drug from organs with that from enterohepatic circulation. It is also interesting that the chemical is distributed in heart, spleen, brain, and kidney, since the drug is widely being used as a remedy for ischemic heart diseases. Trimetazidine in rat organs disappears from lung and kidney 7 hr after the administration, and the drug in other organs also vanished 24 hr after the administration. These facts show that trimetazidine does not accumulate in organs for long time and the drug must be termed safe in the point of accumulation or deposition of the chemical in animal tissues.

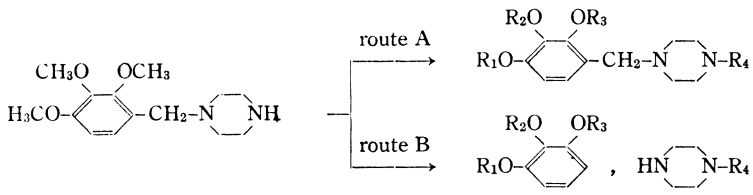
Table II. Distribution of Trimetazidine (μg/g) in Rat Organs After Oral Administration

Hours after administration	Heart mean±SE	Lung mean±SE	Spleen mean±SE	Kidney mean±SE	Brain mean±SE	Liver mean±SE
1	1722±28	1078±33	1448±125	788±51	1205±82	570±46
7	623±57	0	1430± 82	0	261±38	302±37
24	140±70	0	0	0	0	0

Each group consisted of 3 male rats. Mean trimetazidine concentration in each organ was obtained from three groups.

Metabolites of trimetazidine in rabbit urine were studied on a lyophilized residue of the urine within 48 hr after oral administration of 600 mg/kg of the drug to rabbits. Since many spots were observed from the freeze-dried residue in TLC, quantitative determination of the metabolites in urine could not be made.

Two main routes can be presumed for the metabolism of trimetazidine as shown below.

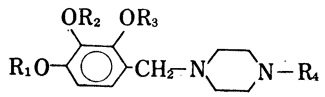


In accordance with these pathways, several compounds shown in Table III were synthesized for comparison in TLC with the metabolites in rabbit urine. The syntheses of these compounds were reported separately.[13]

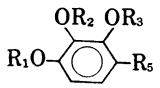
If trimetazidine were to be severed at the methylene, benzene and piperazine derivatives should be detected in TLC. Urine samples were the lyophilized residue neutralized with

13) S. Naito, S. Osumi, K. Kitao, M. Tetsuo, M. Kitaura, and T. Fujita, *J. Pharm. Sci.*, **60**, 1257 (1971).

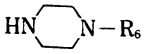
TABLE III. Presumed Metabolites of Trimetazidine and the Compounds Synthesized



A



B



C

Compound	Substituents						Chemical name
	R_1	R_2	R_3	R_4	R_5	R_6	
A-1	CH_3	CH_3	CH_3	$COCH_3$	—	—	1-(2,3,4-trimethoxybenzyl)-N^4-acetylpiperazine
A-2	CH_3	H	CH_3	H	—	—	3-hydroxy-2,4-dimethoxybenzylpiperazine
B-1	CH_3	CH_3	CH_3	—	COOH	—	2,3,4-trimethoxybenzoic acid
B-2	H	H	H	—	COOH	—	2,3,4-trihydroxybenzoic acid
B-3	CH_3	CH_3	CH_3	—	CH_3	—	2,3,4-trimethoxy-1-methylbenzene
B-4	H	H	H	—	CH_3	—	2,3,4-trihydroxy-1-methylbenzene
C-1	—	—	—	—	—	H	piperazine
C-2	—	—	—	—	—	CH_3	N-methylpiperazine

sodium carbonate after treatment with hydrochloric acid (hereafter abbreviated as HCl-urine) and methylene chloride extract from HCl–urine (hereafter abbreviated as CH_2Cl_2–urine).

TLC systems used for detection of metabolites of trimetazidine are shown in Table I. Presence of unchanged trimetazidine in rabbit urine metabolites was ascertained by TLC (A) to (E) and (G). The compounds (B–1), (B–2), (B–3), (B–4), and (C–1) were not detected through TLC (B), (C), and (D), and the compound (C–2) was also not observed by TLC (B). These facts positively show that there is no possibility for the route B in metabolic pathways of trimetazidine in rabbits.

The compound (A–1) was detected in rabbit urine by TLC (E) and (F), and the compound (A–2) by TLC (A) and (G). A micro-melting point determination of the mixture of evaporated residue of ethanolic extract from 5—10 spots having the same *Rf* value in TLC (A) or (G), and an authentic sample was used for the identification of compound (A–2).

It is very interesting that the unchanged trimetazidine, and the compound (A–1) and (A–2) were found in urinary metabolites of rabbits and no derivatives of simple benzene and piperazine could be recognized by thin-layer chromatography.

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