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Studies on Trimetoquinol. II.[1] The Metabolic Fate of Trimetoquinol

Chiyoko Satoh, Taku Nagao, Takeo Kono and Akio Kiyomoto

Biological Research Laboratory, Tanabe Seiyaku Co., Ltd.[2]

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Racemic Trimetoquinol [*dl*-1-(3,4,5-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (I)] hydrochloride was administered to rabbits and rats by intravenous injection and the metabolites excreted into the 24 hr urine pools were investigated. Unchanged I and five metabolites, *dl*-1-(3,4,5-trimethoxybenzyl)-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline (II), *dl*-1-(3,4,5-trimethoxybenzyl)-6-hydroxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline (III) and glucuronides of I, II and III were identified. Species differences between rabbit and rat were observed in the capacity of O-methylation and in the ratio of the two O-methylated isomers. In rabbits, a majority of administered I appeared as glucuronide of I, whereas glucuronide of III occupied the highest proportion in rats.

Trimetoquinol [*l*-1-(3,4,5-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline][3] has been reported to be one of the most potent bronchodilators hitherto described in the literature.[4] Its molecular structure has not only a resemblance to papaverine on the whole, but also a similarity to catecholamines in the tetrahydroisoquinoline portion of the molecule. It seemed worth-while to study the metabolic fate of this drug in animals in relation to its pharmacological activity. In part I of this series Meshi, *et al.*[1] reported the glucuronide formation and O-methylation of ^{3}H-trimetoquinol (*l*) and its optical isomer (*d*) in guinea pigs, but no stereospecific difference has been observed except for small quantitative differences. On the other hand, a species difference was observed in the ratio of methylation and glucuronide formation *in vitro* between rat and guinea pig. In the present studies, in order to isolate and identify the metabolites, racemic trimetoquinol hydrochloride (I HCl) was administered to rabbits and rats, and a possible species difference was also examined.

Experimental

Materials——I HCl and its 6-O-methyl- and 7-O-methyl derivatives [*dl*-1-(3,4,5-trimethoxybenzyl)-6-methoxy-7-hydroxy-1,2,3,4-terahydroisoquinoline hydrochloride (II HCl) and *dl*-1-(3,4,5-trimethoxybenzyl)-6-hydroxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (III HCl), respectively were synthesized in the Organic Chemical Research Laboratory of Tanabe Seiyaku Co., Ltd.[5] tritium labeled I HCl (specific activity 126 mCi/m mole), whose structure is shown in Chart 1, was synthesized in the Biological Research Laboratory of Tanabe Seiyaku Co., Ltd.[6] Each of the synthesized samples showed a single spot (or a single radioactive band) on paper chromatogram in butanol–acetic acid–water (4:1:5, v/v) and chloroform–acetic acid–water (2:1:1, v/v).

β-Glucuronidase was prepared from rat liver by the procedure of Kiyomoto, *et al.*[7]; the activity of the enzyme preparation was about 1500 Fishman's phenolphthalein units/ml.

1) Part I: T. Meshi, M. Otsuka and Y. Sato, *Biochem. Pharmacol.*, **19**, 2937 (1970).
2) Location : *Kawagishi, 2-2-50, Toda, Saitama.*
3) E. Yamato, M. Hirakura and S. Sugasawa, *Tetrahedron, Suppl.* **8**, 129 (1966).
4) Y. Iwasawa and A. Kiyomoto, *Jap. J. Pharmacol.*, **17**, 143 (1967); M. Sato, I. Yamaguchi and A. Kiyomoto, *ibid.*, **17**, 153 (1967).
5) Unpublished.
6) T. Takahashi and Y. Sato, *Radioisotopes*, **17**, 374 (1968).
7) A. Kiyomoto, S. Harigaya, S.Ohshima and T. Morita, *Biochem. Pharmacol.*, **12**, 105 (1963).

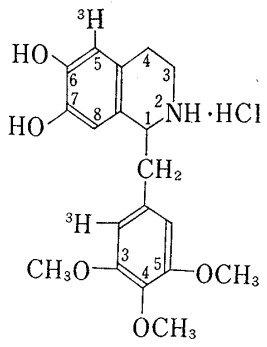


Chart 1. Tritium Labeled Recemic Trimetoquinol Hydrochloride

Methods——The radioactivity of urine samples and metabolite solutions at each purification step was measured in an Aloka Liquid Scintillation Spectrometer LSC-502 equipped with the automatic quenching monitor system. For this purpose 0.1 ml of sample solution was mixed with 15 ml of scintillation fluid consisting of 100 mg of 1,4-bis-(5-phenyloxazolyl)-benzene, 4g of 2,5-diphenyloxazole, 300 ml of ethanol and 700 ml of toluene.

Ascending paper chromatography was carried out on Toyo Roshi filter paper No. 51 in butanol–acetic acid–water (4:1:5, upper phase, solvent A) or chloroform–acetic acid–water (2:1:1, lower phase, solvent B). Distribution of the radioactivity on paper chromatograms was determined using an Aloka Chromatogram Scanner TRM-1B. Each radioactive band was cut into small pieces, transferred to counting vials and eluted with 0.5 ml of distilled water. After adding 0.5 ml of ethanol and the scintillation fluid, the radioactivity was counted in the liquid scintillation counter. The metabolites were stained either by spraying first 20 % aqueous solution of Na_2CO_3 and then the Folin–Ciocalteu's Phenol reagent[8] or by spraying 10% solution of phosphomolybdic acid in ethanol and then exposing the paper chromatogram to ammonia gas.[9]

Qualitative test for the ene-diol group was performed by the method of Weygand.[10]

Column chromatography of I and its O-methylated metabolites was carried out by the procedure of Weil-Malherbe and Bone.[11]

For gas chromatography trifluoroacetylation of the metabolites was performed by stirring with trifluoroacetic anhydride–methylene dichloride (1:3) mixture at room temperature. A Perkin Gas Chromatograph eqquipped with a hydrogen flame ionization detector and a glass column, 1.7 m in length and 1.5 mm in internal diameter was used. The column packing was 1.5 % SE-30 on chromosorb W (Shimazu). The column temperature was 210° and the flow rate of the carrier gas (nitrogen) was 30 ml/min. The ratio of the metabolites was calculated from the height of each peak.

Experimental and Result

A The Metabolic Fate of I in Rabbits——After 24 hr starvation, three male rabbits (2.7—2.8 kg) were injected with I HCl (50 mg/kg) from the ear vein. The rabbits were allowed free access to water, but no food. Urine specimens were collected under toluene for 24 hr. Administration of I HCl and collection of urine were repeated two more times after several days of the intervening feeding period. The total dose of I HCl was 128 mg. One of the three (7.47×10^8 dpm) with unlabeled rabbits received ^{3}H-IHcl I HCl (167 mg) at the last injection. The radioactive 0—24 hr urine (460 ml) was pooled separately from other unlabeled urine. About 68% (5.1×10^8 dpm) of the administered radioactivity was recovered.

Paper Chromatography of Radioactive Metabolites of I——An aliquot of the radioactive urine was applied linearly (1 cm wide) to the filter paper and chromatographed with solvent A. As showin in Fig. 1, two radioactive bands were detected (band 1, *Rf* 0.70; band 2, *Rf* 0.30) and these bands coincided with the colored bands when the paper chromatogram was stained as described in the Methods. This indicates that the radioactive metabolites still possess at least one aromatic hydroxyl group in the molecule. The *Rf* value of band 1 corresponded with that of I. The radioactive compounds were eluted with water and aliquots were analyzed for uronic acid by Dische's calbazole–sulfuric acid reaction[12] and for phenolic hydroxyl by the Folin–Ciocalteu reaction. The substance from band 1 (*Rf* 0.70) showed a negative uronic acid reaction, but that from band 2 (*Rf* 0.30) showed a positive reaction, and both

8) O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 627 (1927).
9) R.F. Riley, *J. Am. Chem. Soc.*, **72**, 5782 (1950).
10) F. Weygand and E. Csendes, *Chem. Ber.*, **85**, 45 (1952).
11) H. Weil-Malherbe and A.D. Bone, *Biochem. J.*, **51**, 311 (1952).
12) Z. Dische, *J. Biol. Chem.*, **167**, 189 (1947).

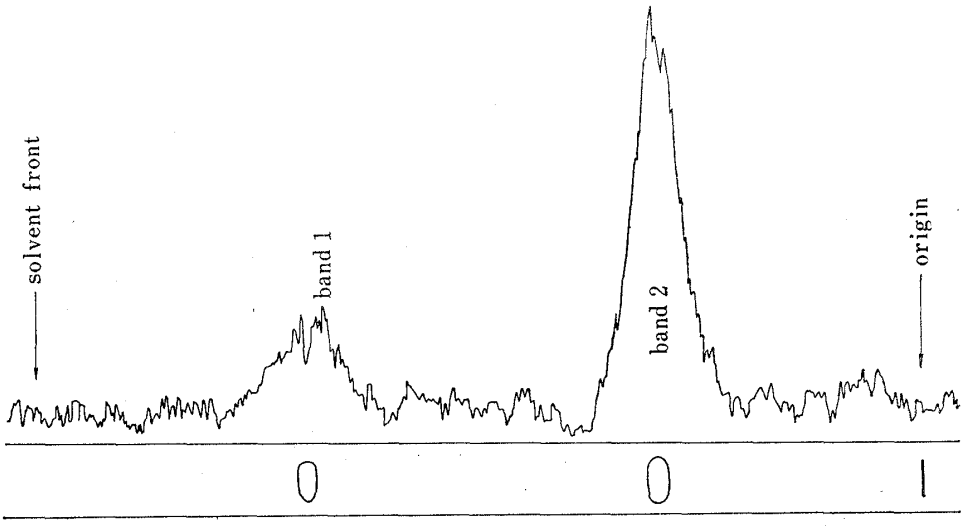


Fig. 1. Radiogram of a Paper Chromatogram of 24 hr Urine of the Rabbit Intravenously injected with Racemic ^{3}H-Trimetoquinol Hydrochloride

The Chromatogram was developed in *n*-butanol–acetic acid–water (4:1:5).

substances showed positive reactions for phenolic compounds. These results suggested that the *Rf* 0.7-substance was unchanged I and/or non-conjugated metabolite of I and the *Rf* 0.3-substance was glucuronide of I and/or its derivative.

***Rf* 0.7-Compounds (Non-conjugated Metabolites)**——When the radioactive urine was extracted with chloroform after adjusting pH to 9.2 with NH_4OH and the chloroform extract was re-extracted immediately with dilute HCl of pH 2, the *Rf* 0.7-substance was completely transferred to the acidic aqueous layer. This aqueous layer contained about 9% of the ^{3}H excreted in the urine. It was confirmed that remaining 91% of the ^{3}H were in the aqueous alkaline solution after chloroform extraction and recovered as *Rf* 0.3-substance.

The non-radioactive urine pool was treated separately as described above. The acidic extracts from both radioactive and non-radioactive urine specimens were combined and concentrated *in vacuo*. A portion was applied on a column of alumina and washed with 0.2M acetate buffer (pH 8.4). A part of the radioactivity appeared in the effluent and washing buffer and the rest of the radioactivity was adsorbed on the column and eluted with 0.2N HCl. This indicated that the *Rf* 0.7-substance consisted of at least two compounds. The total recovery of the radioactivity from the alumina column was 96% in duplicate experiments. The ratio of the radioactivity in the effluent to that in eluate fraction was 34:66. The radioactivity in the effluent fraction was extracted with chloroform at pH 9.2 and then transferred to acidic water. Aliquots of this fraction were paper chromatographed and a single radioactive band of 0.78 (solvent A) or 0.94 (solvent B) was detected. These values were identical with those of II and III but higher than those of authentic I (*Rf* 0.70 in solvent A and 0.20 in solvent B), and suggested the existence in this fraction of a metabolite or metabolites that were more lipophilic than I. The eluate from the column with. 0.2N HCl gave a single radioactive band of *Rf* 0.66 (solvent A) or 0.16 (solvent B). Eluates of the all radioactive bands from paper showed a positive Folin–Ciocalteu reaction. These results suggested that the effluent fraction contained mono-O-methylated metabolites of I (MAQ) ,and the eluate fraction contained unchanged I; a lowering of the *Rf* values of the eluate fraction may be due to contaminating $AlCl_3$ from the column.

Another portion of the combined concentrated extracts of the urine was trifluoroacetylated and submitted to gas chromatography. Three peaks were detected with retention times as shown in table II and were assigned to I, III and II.

Data from alumina column chromatography and gas chromatography indicated that I, II and III amounted to about 64%, 20% and 16%, respectively, of the total non-conjugated

Table I. Separation of *Rf* 0.7-Compounds into I and Its O-Methylated Derivatives by Column Chromatography on Alumina

	Ratio of radioactivity	*Rf* in paper chromatography Solvent A	Solvent B
Fraction from *Rf* 0.7-compounds			
Effluent	34%	0.78	0.94
Eluate	66%	0.66	0.16
Authentic sample			
I HCl		0.70	0.20
II HCl		0.78	0.94
III HCl		0.78	0.94

A portion of the acidic solution of *Rf* 0.7-compounds from rabbit urine was concentrated *in vacuo* and the residue was dissolved in acetate buffer pH 8.4 (1 ml) and passed through a column of alumina (0.5 g). The column was washed succesively with 3 ml each of acetate buffer and water (effluent) and then eluted with 0.2N HCl (eluate). The ratio of the radioactivity was corrected for a recovery of 96%.

Table II. Gas Chromatography of Trifluoroacetates of *Rf* 0.7-Compounds and Reference Compounds

	Retention times (min)	Ratio of radioactivity %
Authentic sample		
I HCl	8.1	
III HCl	11.9	
II HCl	14.7	
Rf 0.7-compounds	8.0	64
	12.5	16
	14.3	20

metabolite fraction. Since the non-conjugated metabolite fraction represented 9% of the total metabolites excreted into the 24 hr urine, the percentages of I, II and III of the total metabolites in the urine were 5.8%, 1.8% and 1.4%, respectively.

***Rf* 0.3-Compounds [Glucuronides of I Derivatives]**——After extraction of the *Rf* 0.7-compounds, the *Rf* 0.3-compounds which remained in the alkaline aqueous layer were purified by two steps; 1) precipitation as lead salts and 2) isolation with ion exchange resins (Chart2). The first step of purification was performed separately for the radioactive urine and the non-radioactive urine pool by the procedure of Williams.[13] After elimination of the lead as PbS, the radioactive and non-radioactive solutions were combined and the second step of the purification followed. Recoveries in the elution of the *Rf* 0.3-compounds from Dowex 50 and Dowex 1 were 90%, judged from radioactivity. An aliquot of the radioactive eluate from Dowex 1 was paper chromatographed with solvent A and a single radioactive band of *Rf* 0.3 was detected. Another aliquot of the eluate was proved by Dische's carbazole-sulfuric acid reaction to contain an equimolecular amount of uronic acid to the amount of I which was calculated from the radioactivity on an assumption that I was metabolized and excreted by all three rabbits in the same manner.[14] Cinchonidine (500 mg) in ethanol (100 ml) was added to the concentrated eluate (20 ml, 1.47×10^8 dpm) from Dowex 1 and the clear mixture was kept at room temperature for 40 hr. After concentrating the mixture to 2 ml, the precipitated free cinchonidine was

13) I.A. Kamil, J.N. Smith and R.T. Williams, *Biochem. J.*, **50**, 235 (1951).
14) We confirmed that *Rf* 0.3-compounds consisted of equivalent amounts of I and uronic acid in a separate experiment, in which radioactive *Rf* 0.3-compounds were purified from the urine of one rabbit after injection ^{3}H-HCl of the same specific activity.

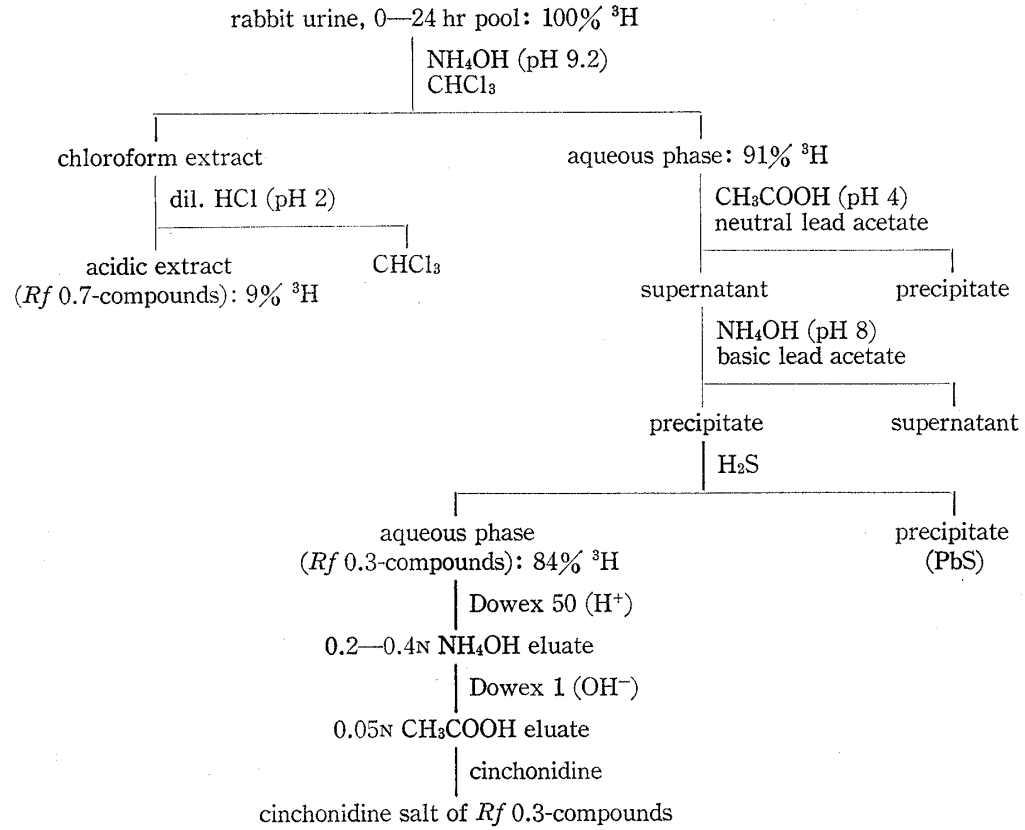


Chart 2. Flow Sheet for Purification of *Rf* 0.3-Compounds

filtered off and the filtrate and washings were combined and concentrated to a syrup. Cinchonidine salts (688 mg) of the *Rf* 0.3-compounds were obtained in an amorphous powder after repeated concentration of the syrup with added ethanol to remove water and then by extraction of free cinchonidine with cold ethanol.

Hydrolysis of *Rf* 0.3-Compounds——The eluate (2 ml) which was assumed by calculation to contain about 3 μmoles of the *Rf* 0.3-compounds were mixed with 2N HCl (2 ml) and kept at 37° for 48 hr. The hydrolysate was shaken with chloroform at pH 9.2 (NaOH), but no radioactivity was extracted into chloroform. It has been reported that metanephrine sulfate is hydrolyzed but metanephrine glucuronide is not under these conditons.[15] A slight amount of inorganic sulfate was detected in the solution of the *Rf* 0.3-compounds by the method of Egami,[16] but no significant difference was observed between before and after acid treatment.

The *Rf* 0.3-compounds were also subjected to enzymatic hydrolysis. After incubation of about 2 μmoles of the *Rf* 0.3-comopunds with β-glucuronidase at 37° for 3 hr in acetate buffer pH 5.2, an aliquot of the incubation mixture was chromatographed with solvent A. In addition to the original band of *Rf* 0.3, a second radioactive band representing 52% of the initial radioactivity appeared at *Rf* 0.7. The eluate of this new band was negative for the uronic acid test, but positive for the phenolic hydroxyl group test. When the incubation was carried out in the presense of 0.46 μg of glucosaccharo-1,4-lactone [SL–⟨1,4⟩], a specific β-glucuronidase inhibitor, the appearance of aglycons decreased to 20% of the initial radioactivity, that is, the enzymatic reaction was inhibited by 62% (Table III). From the data described above, it could be concluded that the *Rf* 0.3-compounds were glucuronides of I and/or its metabolites.

Enzymatically Liberated Aglycons——For the determination of the aglycon moiety, the glucuronides (1.24×10^7 dpm, 56 μmoles) were recovered from an aqueous solution (4 ml) of

15) E.H. Labrosse, J. Axelrod, I. J. Kopin and S.S Kety, *J. Clin. Invest.*, **40**, 253 (1961).
16) F. Egami and N. Takahashi, *Bull. Chem. Soc. Japan*, **30**, 442 (1957).

Table III. Hydrolysis of *Rf* 0.3-Compounds with β-Glucuronidase and Inhibition by Glucosaccharo-1,4-lactone

Experiments	Incubation mixture			Percentage of metabolites	
	β-Glucuronidase	*Rf* 0.3-compounds	SL<1.4>	*Rf* 0.30 %	*Rf* 0.70 %
a	+	+	−	48	52
b	+	+	+	80	20
c	−	+	−	100	0
d	−	+	+	100	0
e	+	−	+	0	0

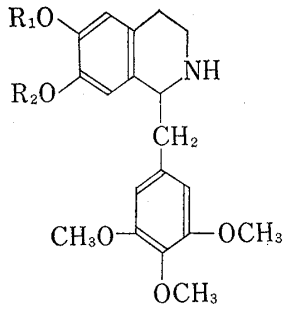
The complete incubation mixture contained: concentrated solution of *Rf* 0.3-compounds (0.1 ml, 2 μmoles), water (0.2 ml), 0.2M acetate buffer pH 5.2 (0.3 ml) and β-glucuronidase preparation (0.1 ml, 150 units). Water was replaced with a solution of glucosaccharo-1,4-lactone [SL <1,4>, 0.46 μg] in the case of b, d and e.

cinchonidine glucuronates (100 mg) by treatment with 2N NaOH (2 ml). The precipitated cinchonidine was centrifuged off and washed. The pH of the pooled filtrate and washing was adjusted to 5 with 2N HCl. After adding 0.2M acetate buffer pH 5.2 (20 ml) and β-glucuronidase solution (8 ml), the mixture was incubated at 37° for 48 hr. Acetate buffer (3 ml) and the enzyme solution (3 ml) were again added at 24 hr. The reaction mixture was then extracted with butanol (20 ml×4). The butanol extract contained 83% of the ^3H of the incubation mixture. Paper chromatography of the aqueous layer and butanol extract with solvent A showed that generated aglycons were completely extracted with butanol and the unreacted glucuronides remained in the aqueous layer. After addition of a few drops of 0.2N HCl, the butanol extract was concentrated, and a portion was used for column chromatography on alumina. The radioactivities in the effluent and in the eluate were in the ratio of 17:83. Duplicate analysis showed a good agreement and the recovery was 97%. Gas chromatographic analysis of the concentrated butanol extract showed that MAQ and I represented about 16% and 84% of the aglycon moiety, respectively. However, the ratio of II and III could not be estimated acurately, since the existence of a large amount of I interfered with the estimation of II and III. Purification of the I fraction and the MAQ fraction was then attempted.

Concentration of the butanol extract (which contained 74% of the ^3H of the initial glucuronide solution) gave crystalline I HCl (13.7 mg, 4.5×10^5 dpm/mg) slightly contaminated with MAQ. The crystals were dissolved in water, chromatographed on paper and eluted with solvent C (ethanol–water–acetic acid, 70: 70: 5). Recrystallization from dil. HCl gave 6 mg (5.4×10^5 dpm/mg) of I HCl. It was positive in the tests for phenolic hydroxyl and ene-diol groups. Its chromatographic properties and infrared spectrum were indistinguishable from those of authentic I HCl. The mother liquor (3×10^6 dpm), from which the first crystalline I HCl had been removed, was concentrated and submitted to paper chromatography with solvent B. The radioactive band of MAQ (*Rf* 0.94) were cut into pieces and eluted with solvent C. After addition of a few drops of 0.2N HCl, the eluate was concentrated to a small volume *in vacuo* and lyophilized. Gas chromatographic analysis showed that the MAQ fraction consisted of 60% of III and 40% of II. These data were expressed as percentages of the ^3H excreted in the urine as showin in Table IV.

B Isolation of II and III from the Rat Urine——One hundred male Wistar rats (150—200 g) were injected with I HCl (50 mg/kg) from the tail vein. The total dose of I HCl was 853 mg. Two of the rats received 0.682 mg (5.0×10^8 dpm) and 0.648 mg (4.75×10^8 dpm) of ^3H-I HCl, of which 68 and 71% radioactivity were recovered in the 0—24 hr urine, respectively. The radioactive urine containing 5.84×10^8 dpm was mixed with the unlabeled urine pool and extracted with butanol. The butanol extract contained about 23% of the ^3H in the urine. The paper chromatography showed that the butanol extract contained non-conjugated meta-

TABLE IV. The Metabolites excreted into the 24-hr-Urine of Rabbits and Rats Intravenously Injected with Racemic Trimetoquinol Hydrochloride



R_1	R_2		Rabbits %	Rats %
H	H	I	5.8	16.1
CH_3	H	II	1.8	6.9[a]
H	CH_3	III	1.4	
GU[b]		I-G[c]	75.5	27.7
CH_3	GU	II-G[c]	6.2	12.3
GU	CH_3	III-G[c]	9.3	37.0

a) Further fractionation of the MAQ fraction was not attempted.
b) Abbreviation of glucuronic acid. The position of substitution with glucuronic acid is not known.
c) I-G, II-G and III-G are abbreviation of the glucuronides of I, II and III, respectively.

olites (*Rf* 0.70 in solvent A), which consisted of 70% of I (*Rf* 0.20 in solvent B) and 30% of MAQ (*Rf* 0.94 in solvent B). About 77% of the radioactivity remained in the aqueous fraction as glucuronides (*Rf* 0.33 in solvent A). Purification of the glucuronides was carried out twice by precipitation as lead salts. Determination of the aglycone moiety was carried out as described in part A. The aglycone moiety of the glucuronides from the rat urine consisted of 36% of I, 48% of III and 16% of II. These data were expressed as percentages of the ^3H excreted in the urine as shown in Table IV (column 5).

Discussion

When ^3H-I was administered intravenously to rabbits, sixcompounds, *i.e.*, unchanged I, II, III and their respective glucuronides were found to be excreted into the 0—24 hr urine pool in a total recovery of 68%. The percentage of each compound to the total excreted ^3H is summarized in Table IV (column 4). Since 91% of the excreted compounds consisted of glucuronides of I and its methylated derivatives, of which the glucuronide of I was 75.5%, the glucuronide formation plays the major role in the metabolism of I in rabbits. When the data shown in Table IV are arranged from the viewpoint of O-methylation, the excreted compounds consist of 81.3% of I and its glucuronide, 10.7% of 7-methyl I and its glucuronide and 8.0% of 6-methyl I and its glucuronide. Although mono-O-methylated metabolites represented only 18.7% of the total excreted radioactivity, it is interesting that the methylation occurred at both 7- and 6-hydroxy groups to almost the same extent.

In the metabolism of I in rats, not only the glucuronide formation but also the mono-O-methylation is important. As the total glucuronides represent 77% of the radioactivity excreted in the 24 hr urine pool and 64% of the glucuronides are methylated compounds, the methylated aglycons represent 49.3% of the ^3H in the urine. When MAQ in the "non-conjugated metabolites" are included, the total methylated compounds amount to 56.2% of ^3H in the rat urine, about three times the percentage (18.7%) of methylated metabolites in the rabbit urine. Thus, the methylation is a major metabolic pathway of I in rats. Another

species difference in the metabolic fate of I between rabbit and rat was observed in the ratio of the total III to the total II. Gas chromatographic analysis showed that the methylation occurred at 7- and 6- hydroxyl in the ratio of 5:4 in rabbit and 3:1 in rat.

Since Meshi, *et al.*[1] observed that the ratio of the methylated to the unmethylated compounds in urine decreased when guinea pigs were pretreated with pyrogallol, a catechol-O-methyltransferase (COMT) inhibitor, the methylation of I is presumably catalyzed by COMT. Catecholamines are known to be methylated by COMT *in vivo* preferentially at the *meta* hydroxyl group,[17] whereas considerable degrees of simultaneous *para*-O-methylation of catechols (3,4-dihydroxy-acetophenone, 3,4-dihydroxy-phenylmethyl carbinol) and dopamine by COMT have been observed *in vitro*.[18] The catechol moiety of 2-hydroxyestrone, a major metabolite of estrone in man is randomly O-methylated by COMT giving rise to both 2- and 3-methylether derivatives in approximately equal quantities.[19] 7,8-Dihydroxychlorpromazine has been reported to form mainly 7-hydroxy-8-methoxy-chlorpromazine and lesser amounts of 8-hydroxy-7-methoxy-chlorpromazine on incubation with COMT and S-adenosyl methionine.[20]

In the molecule of I, the 6-hydroxyl group is situated *meta* to C_4 and *para* to C_1 and the 7-hydroxyl group is situated in the opposite manner. Methylation at both 6-hydroxyl and 7-hydroxyl groups of I seemed to be reasonable when the positional relationship of the hydroxyl groups of I and the evidence of methylation at both hydroxyl groups of catechols are taken into consideration. It is interesting that 7-methylation predominates in our *in vivo* experiments and that species differences between rabbit and rat were observed in the capacity as well as in the positional ratio of the methylation.

The gas chromatographic analysis of the butanol extract of aglycons indicated that N-methylation and O-demethylation in the trimethoxybenzyl moiety did not occur.

Some additional evidence that the *Rf* 0.3-compounds are O-glucuronides is as follows. As I is very labile to oxidation by air, an aqueous solution of I is apt to turn brown and finally an amorphous precipitate appears in the solution. This phenomenon occurs very rapidly in alkaline medium. But the *Rf* 0.3-compounds were stable when they were eluted with 0.2—0.4N NH_4OH from the column of Dowex 50 in the purification procedure. This stability against alkaline medium suggests the substitution of at least one of the two hydroxyl groups. The stabilizing substituent could be the methyl group of MAQ, but since as much as four fifths of the aglycon moiety was I in rabbit, glucuronic acid must have occupied one of the hydroxyl groups to stabilize the molecule against oxidation. The stability in 1N HCl at 37° for 48 hr, the equimolecular existence of uronic acid and the aglycon, and hydrolysis by β-glucuronidase and its inhibition by glucosaccharo-1,4-lactone indicate that the *Rf* 0.3-compounds are O-glucuronides but not sulfoconjugates nor N-glucuronides.

In some radioactive urine specimens from rabbits in a separate experiment, a third radioactive band (*Rf* 0.56) was observed on the paper chromatogram in solvent A. The radioactive compounds in band 3 were eluted with water and chromatographed in solvent A after treatment with NH_4OH for 1 hr. A single radioactive band of *Rf* 0.30 appeared instead of the original band of *Rf* 0.56. On the contrary, when treated with HCl (pH 2) for 24 hr, half of the band 2-compounds (*Rf* 0.30) were converted to the band 3-compounds (*Rf* 0.56). This phenomenon of mutual exchange of band 2 and band 3 suggested the lactone formation from the band 2- to the band 3- compounds. The lactonized glucuronides amounted to 3 to 20% of the glucuronide fraction. As the pH of the urine specimens containing lactonized glucuronides was near 7 and no change was observed in pH of the usual glucuronide containing urine, it is not likely that lactonization occurred during urine collection. However, it is not clear whether lactonization occurred *in vivo* or in the process of paper chromatography.

17) J. Axelrod, S. Senoh and B. Witkop, *J. Biol. Chem.*, **233**, 697 (1958).
18) S. Senoh, J. Daly, J. Axelrod and B. Witkop, *J. Am. Chem. Soc.*, **81**, 6240 (1959).
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Meshi, *et al.*[1] observed four radioactive spots on the paper chromatogram in solvent A of a urine sample from the guinea pig which received ^{3}H-Trimetoquinol (*l*) intravenously. But, in the present studies with rabbit and rat, we observed only two somewhat broad radioactive bands, indicating overlapping of the bands of non-methylated and methylated compounds. This difference might be due to the fact that we had to apply large amounts of the urine sample on paper because we injected large quantities of non-labeled I along with ^{3}H-labeled I into the animal so that our metabolites had much lower specific radioactivities than those described by Meshi, *et al.* Large amounts of other substances present in the urine might have prevented sharp separation of the closely related metabolites. However, since the pattern of distribution of the metabolites was qualitatively common among guinea pig, rabbit and rat, a species difference is not likely to account for this paper chromatographic difference.

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