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Isolation and Characterization of Metabolites derived from 1-*tert*-Butylamino-3-(2,3-dimethylphenoxy)-2-propanol (D-32), a New β-Blocker[1,2]

Seijiro Honma and Akira Kambekawa

Research Division, Teikoku Hormone Mfg. Co.[3]

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The metabolic fate of D-32, which is a new adrenergic β-blocking agent, has been investigated with rabbit, rat and monkey. Eleven unconjugated metabolites and four conjugated metabolites were separated from urine after oral administration of D-32. The structure of these metabolites were deduced from physico-chemical data and definitely characterized by direct comparison with the authentic samples (see Chart 1). The biochemical significance of the transformation observed and the pharmacological activity of metabolites have been discussed.

A new adrenergic β-blocking agent, 1-*tert*-butylamino-3-(2,3-dimethylphenoxy)-2-propanol (D-32), which was first synthesized by Suzuki, *et al.*,[4] exhibits the much more potent β-blocking activity than propranol.[5] The recent finding that oral administration is several times more effective as compared with intravenous injection[5] prompted us to explore the metabolic fate of this drug. In addition it appeared to be of particular interest to examine the species difference in the metabolism and pharmacological activity of the biotransformation products. The present paper deals with the isolation and characterization of the metabolites in the rat, rabbit and monkey.

The tritium labeled substrate was orally given to these animals. Approximately 80% of the administered radioactivity was excreted in urine of the rabbit and monkey, while in the rat the recovery rates in feces and urine were 46 and 31%, respectively (Table I). The collected urine was hydrolyzed with β-glucuronidase and the hydrolyzate was then submitted to solvolysis in the usual manner. The deconjugated metabolites were divided into the acidic and basic fractions by the solvent extraction at appropritate pH.

1) In this paper the following abbreviation were used: 4′-hydroxy D-32=1-*tert*-butylamino-3-(2,3-dimethyl-4-hydroxyphenoxy)-2-propanol, 3′-hydroxymethyl D-32=1-*tert*-butylamino-3-(2-methyl-3-hydroxymethylphenoxy)-2-propanol, 2′-hydroxymethyl D-32=1-*tert*-butylamino-3-(2-hydroxymethyl-3-methylphenoxy)-2-propanol, 4′-hydroxybutanol D-32=1-*tert*-hydroxybutylamino-3-(2,3-dimethyl-4-hydroxyphenoxy)-2-propanol, 3′-hydroxymethyl-4′-hydroxy D-32=1-*tert*-butylamino-3-(2-methyl-3-hydroxymethyl-4-hydroxyphenoxy)-2-propanol, xylenol=2,3-dimethylphenol, 4′-hydroxy D-32 4′-sulfate=1-*tert*-butylamino-3-(2,3-dimethyl-4-hydroxyphenoxy)-2-propanol 4-sulfate, 4′-hydroxy D-32 2-glucuronide=1-*tert*-butylaminomethyl-2-(2,3-dimethyl-4-phenoxy) ethyl-β-D-glucopyranosiduronic acid, D-32 2-glucuronide=1-*tert*-butylaminomethyl-2-(2,3-dimethylphenoxy) ethyl-β-D-glucopyranosiduricacid, 3′-hydroxymethyl D-32 2-glucuronide=1-*tert*-butylaminomethyl-2-(2-methyl-3-hydroxymethylphenoxy)ethyl-β-D-glucopyranosiduronic acid, 3′-hydroxymethyl D-32 3′-glucuronide=1-*tert*-butylamino-2-hydroxy-3-(2-methyl-3-hydroxymethylphenoxy)propan-3-yl-β-D-glucopyranosiduronicacid.

2) A part of this work was presented at the 93rd Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April 1973.

3) Location: *1604, Shimosakunobe, Takatsu-ku, Kawasaki.*

4) Y. Suzuki, K. Tsukamoto, Y. Hiramatsu, and A. Izumi, Japan Patent 641951 (1972) [*C.A.*, 74,22544*k* (1971)].

5) Y. Hiramatsu, A. Izumi, and S. Nishida, Proceedings 46th Meeting Japanese Pharmacological Society, Kumamoto, April, 1973.

Table I. Excretion of Radioactivity in Urine and Feces after Administration of ^3H_3C-D-32 to Several Animals

Time	Rat				Rabbit	Monkey
	p.o.		*i.v.*		*p.o.*	*p.o.*
	Urine (%)	Feces (%)	Urine (%)	Feces (%)	Urine (%)	Uurine (%)
0—24 hr	37.7	30.1	28.4	44.5	57.0	71.0
24—48	1.8	1.0	2.0	1.7	11.2	8.6
48—72			0.7		7.2	9.1
Total	39.5	32.0	31.1	46.2	75.4	88.7

Table II. The Urinary Excretion of Various Metabolites of D-32 in Various Species

Metabolites found in urine	Animal[a]		
	Rat (%)	Rabbit (%)	Monkey (%)
I	20.0	60.0	40.0
II	60.0	15.0	36.0
III	3.0	3.0	1.0
IV	—	—	4.0
V	3.0	4.0	—
VI	1.0	2.0	1.0
VII	0.5	3.0	0.3
VIII	1.0	1.0	1.2
IX	1.0	1.0	1.0
X	1.0	1.0	0.5
XI	0.5	1.0	1.0
Unkown	9.0	9.0	15.0

a) Analyses were carried out on urine collected for 2 days after dosing in the animals.

Basic Metabolites

A gummy extract was submitted to gel filtration on Sephadex LH-20 for removal of the nonradioactive substances. The radioactive metabolites were then separated into four fractions by partition chromatography on Celite[6] as illustrated in Fig. 1. The excreted amounts of the metabolites in urine are listed in Table II.

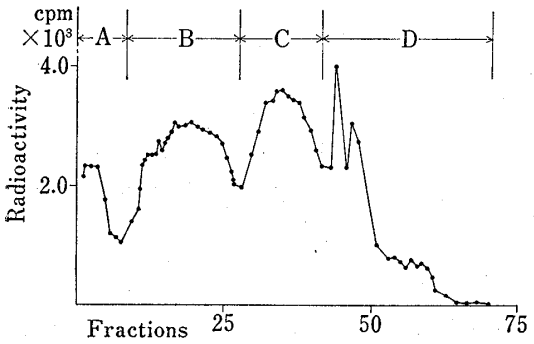
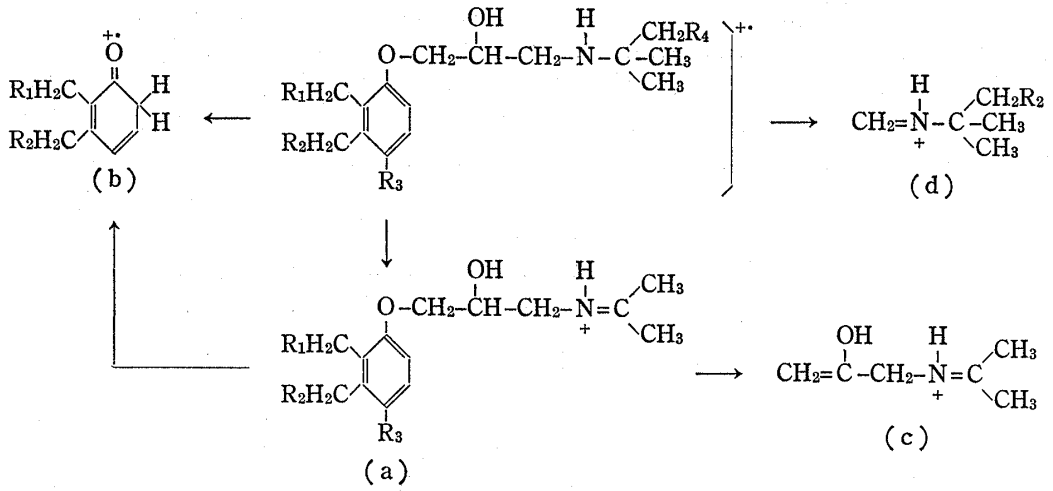


Fig. 1. Separation of Basic Metabolites from Rabbit Urine after Administration of ^3H_3C-D-32 by Column Chromatography on Celite

Recrystallization of the radioactive substance in fraction B gave metabolite I as colorless needles. The positive Folin-Ciocalteu's test and the chromatographic behaviors implied the existence of an additional hydroxyl group on the aromatic ring. On the mass spectrum the molecular ion peak appeared at *m/e* 267 indicating an introduction of oxygen atom and the fragment peaks derivable from the side chain at *m/e* 114 and 86. The occurrence of a fragment at *m/e* 138 may be interpreted in such a way that a proton would migrate from the side chain

6) P.K. Siitri, *Steroids*, 2, 687 (1963).



Compounds	R_1	R_2	R_3	R_4	Ion				
					M	a	b	c	d
D-32	H	H	H	H	251	236	122	114	86
I	H	H	OH	H	267	252	138	114	86
II	H	OH	H	H	267	252	138	114	86
III	OH	H	H	H	267	252	138	114	86
IV	H	OH	OH	H	283	268		114	86
V	H	H	OH	OH	283	252	138	114	86

Fig. 2. Comparison of the Mass Spectrum of D-32 and Its Metabolites

to the 4′-hydroxylated xylenol ring (Fig. 2). In addition the nuclear magnetic resonance (NMR) spectrum of metabolite I exhibited a broad signal at 6.60 ppm assigned to two aromatic protons. The acetylated product, however, showed a well defined AB-type doublet (J=8 Hz) supporting the assignment of a newly introduced hydroxy group to 4′-position. These results led to the assumption that metabolite I should be 4′-hydroxy D-32. Indeed, the identity of metabolite I with the synthetic sample was justified by the usual criteria.

Metabolite II from fraction C, isolated as a salt of acetic acid, gave the pink coloration with conc. sulfuric acid. The mass spectrum showed a parent peak at m/e 267 corresponding to an increment of 16 mass units. In the lower mass range the fragment peaks characteristic to the hydroxylated xylenol and side chain appeared at m/e 138, 114 and 86. The proton signal assignable to C-3 methyl group disappeared and instead the signal corresponding to hydroxymethyl appeared newly at 4.60 ppm. It was evident from these data that the structure 3-hydroxymethyl D-32 should be assignable to metabolite II. In actuality the synthetic sample proved to be identical with the metabolite by direct comparison.

From the mother liquor of II, metabolite III was separated by preparative thin-layer chromatography (TLC). Unfortunately this substance could not be obtained in the crystallin state, although it was substantially homogeneous. Metabolite III exhibited the red coloration with conc. sulfuric acid. With respect to the mass spectra II and III were indistinguishable each other in the fragmentation pattern. On the NMR spectrum a signal due to methyl proton in the high field was lost, while that of the hydroxymethyl proton was observed at 4.70 ppm. These results strongly suggested that metabolite III would be a positional isomer of II, namely 2′-hydroxymethyl D-32. The proposed structure was definitely established by direct comparison with the authentic sample.

Further purification of the eluate in fraction D by preparative TLC and subsequent gel

filtration on Sephadex LH-20 yielded metabolite IV. This substance showed a positive result with Folin-Ciocalteu's reagent and afforded the tetraacetate by usual acetylation. The mass spectrum of IV exhibited the molecular ion peak at *m/e* 283 indicating an introduction of two oxygen atoms into D-32. In the lower mass range a new fragment peak, derivable from the oxygenated side chain, appeared at *m/e* 102. The occurrence of a peak at *m/e* 252 could be explained in terms of a fission hydroxymethyl derived from oxidation of one of three methyls of *tert*-butyl group. The structural assignment was further supported by the NMR spectrum exhibiting signals at 1.30 (6H, s, isobutyl), 2.10—2.20 (6H, s, aromatic methyl) and 6.60 ppm (2H, s, aromatic proton). The aromatic ring proton signals assignable to 5 and 6 positions were identical with those of metabolite I. These results permitted us to assign the structure 4′-hydroxybutanol D-32 to metabolite IV. This metabolite was formed only in the monkey, but not in the rat and rabbit.

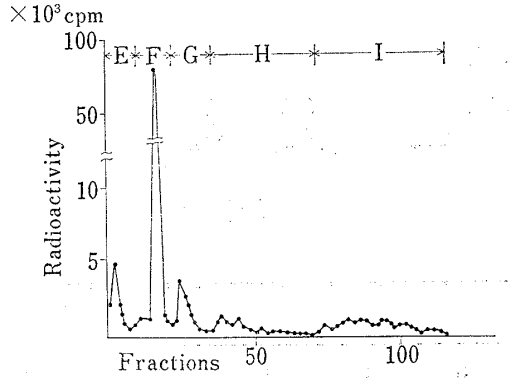


Fig. 3. Separation of Acidic Metabolites from Rabbit after Administration of $^{3}H_{3}C$-D-32 by Column Chromatography on Celite

Metabolite V gave the yellow coloration with conc. sulfuric acid characteristic to the 4′-hydroxy derivative. Existence of two hydroxy groups in the xylenol ring was suggested from the main peaks at *m/e* 283 (M^+), 114 and 86 in the mass spectrum. The methyl proton signal in the low field was absent, while a signal assignable to the 3′-hydroxymethyl appeared at 4.75 ppm. The presence of three hydroxylic group susceptable to usual acetylation was deduced from the mass and NMR spectra. On the basis these data metabolite V was assigned to be 3′-hydroxymethyl-4′-hydroxy D-32.

Acidic Metabolites

The extract was chromatographed on Celite and the radioactive metabolites were separated five fraction as shown in Fig. 3. Each of them, if necessary, was further purified by the preparative thin-layer chromatography (TLC), First, metabolite VI which was isolated from fraction E and F by further purification proved to be xylenol judged from mixed melting point measurement and TLC comparison with the authentic sample. The structure was unequivocally characterized by the reverse isotope dilution method. The preparative TLC of the eluated from fraction G gave metabolite VII. According to the physical and chemical properties it seemed very likely to be 4-hydroxyxylenol. The structure of VII was determined by direct comparison with the synthetic sample.

Metabolite VIII which was isolated from fraction H was obtained as colorless needles. The infrared (IR) spectrum exhibited an absorption band due to the hydroxyl function at 3400 cm^{-1}. Acetylation of VIII in the usual manner afforded the diacetate. As for the mass spectra of metabolite VIII and its acetate the parent ion peaks appeared at *m/e* 196 and 280, respectively. These results strongly suggested that metabolite VIII would be 2, 3-dimethylphenoxy propane-2,3-diol. The structure was definitely established by direct comparison with the authentic sample.

Purification of the nonpolar fraction by preparative TLC gave neutral metabolite IX. Based upon the physico-chemical data IX was assumed to be 2-methyl-3-hydroxymethylphenol and identity with the authentic sample was confirmed by mixed melting point measurement and TLC comparision.

From the fraction H metabolite X was isolated as colorless plates. The IR spectrum showed the characteristic band at 1710 and 1740 cm^{-1} indicating the present of carboxylic acid. On the mass spectrum the molecular ion and fragment peaks appeared at *m/e* 180 (M^+)

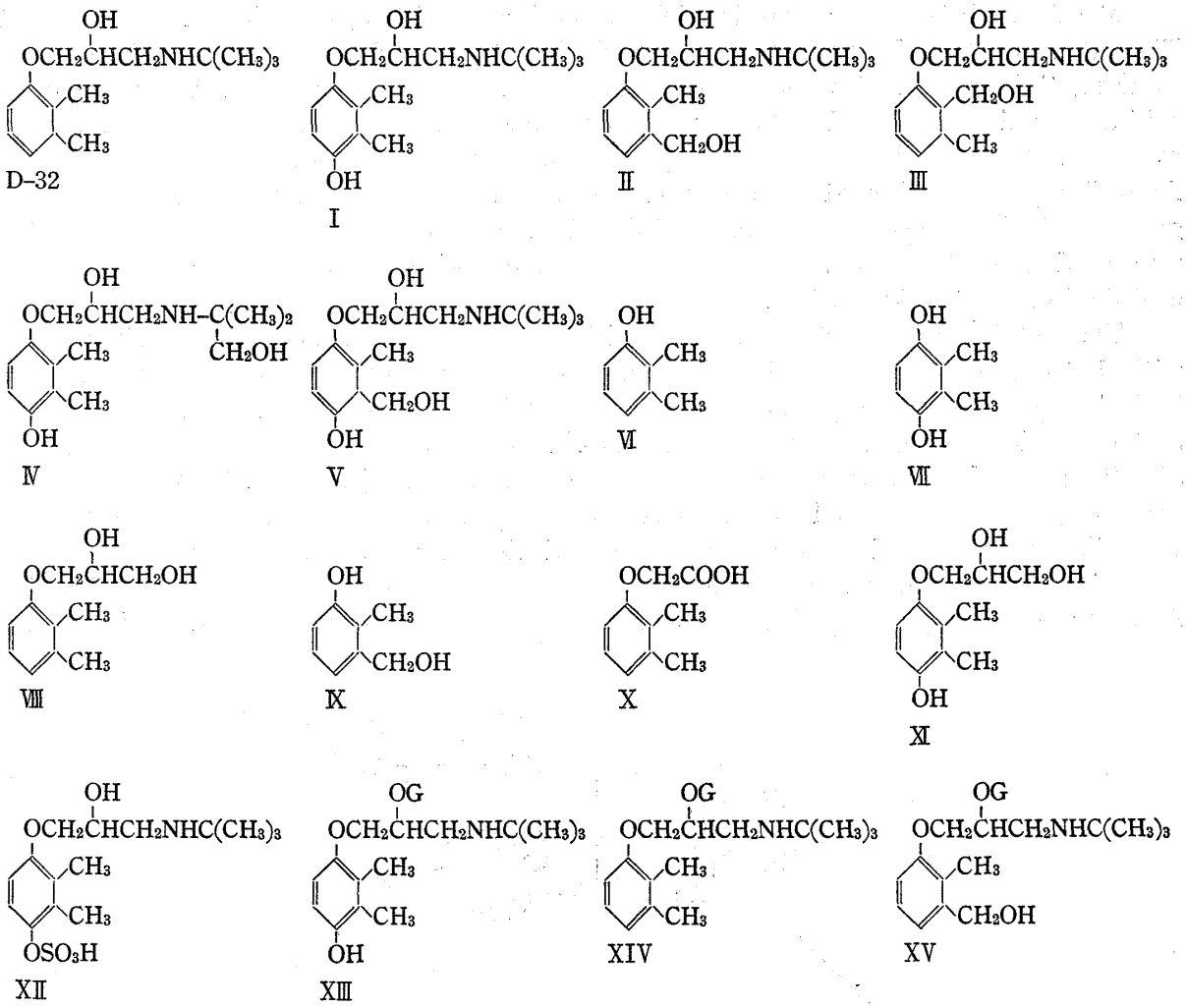


Chart 1

and 135 (M^+–COOH), respectively. These data led to the assignment of the 2,3-dimethylphenoxyacetic acid to metabolite X. The assigned structure was establitshed by direct comparison with the authentic sample.

Conjugated Metabolites

The recent studies revealed that the conjugate of physiologically active substance is not always the end product in certain case it possesses more potency than the free.[7,8] A particular interest in these respects prompted us to explore the conjugated metabolites formed from D-32. A suspended solution of D-32 in milk was orally administered to an adult rhesus monkey for 7 days. The urine collected for following 10 days was extracted with *n*-butanol–ethyl acetate to remove the free metabolites, and the aqueous layer was then separated and evaporated. The gummy substance obtained was then submitted to gel-filtration on Sephadex G-25 for elimination of the nonradioactive substance. The eluate thus obtained was then chromatographed on Sephadex LH-20. Further purification of the main radioactive metabolites was efficiently achieved by column chromatography on Amberlite XAD-2[9] employing aq. methanol as eluent followed by gel filtration on Sephadex LH-20.

7) H. Yoshimura, S. Ida, K. Oguri, and H. Tsukamoto, Proc. 1st Symp. on Drug Metabolism and Action, Chiba, 1970, p. 107.
8) F. Herr, C. Revesz, A.J. Manson, and J.B. Jewell, "Chemical and Biological Aspect of Steroid Conjugation," ed. by S. Bernstein and S. Solomon, Springer-Verlag, Berlin, 1970, p. 368.
9) H.L. Bradlow, *Steroids*, 11, 265 (1968).

Metabolite XII which was isolated from fraction L and M was obtained as colorless plates. This substance showed the negative result with Folin-Ciocalteu's test and possessed an empirical formula, $C_{15}H_{25}O_6NS$. On the IR spectrum the intense absorption bands due to sulfonic acid appeared at 1275 and 1040 cm^{-1}. Upon treatment with hydrochloric acid XII underwent hydrolysis yielding a phenolic aglycone, which proved to be identical with metabolite I. Accordingly this compound was assumed to be a sulfate conjugated with a phenolic hydroxyl group. The NMR spectrum of XII exhibited the signal of aromatic protons 6.68 and 7.21 ppm as doublet (J=9 Hz). On the basis of these evidences metabolite XII was identifiep as 4'-hydroxy D-32-4'-O-sulfate.

The metabolite XIII which was isolated from fraction L and M exhibited positive reaction with both naphthoresorcinol[10] and Folin-Ciocalteu's reagent, and also underwent facile hydrolysis with beef-liver β-glucuronidase to provide metabolite I and glucuronic acid. The β-glucuronidase linkage was also verified by NMR spectrum where an anomeric proton appeared at 4.45 ppm as doublet with coupling constant of 6 Hz.[11] In order to determine the attached position XIII was coverted into the acetate-methyl ester derivative (XIII') by treatment with diazomethane and then with acetic anhydride and pyridine. The mass spectrum exhibited a parent peak at *m/e* 667 together with the fragment peaks at *m/e* 488, 432 and 317. A strong peak at *m/e* 488 would be assignable to a fragment derived from the side chain pos-

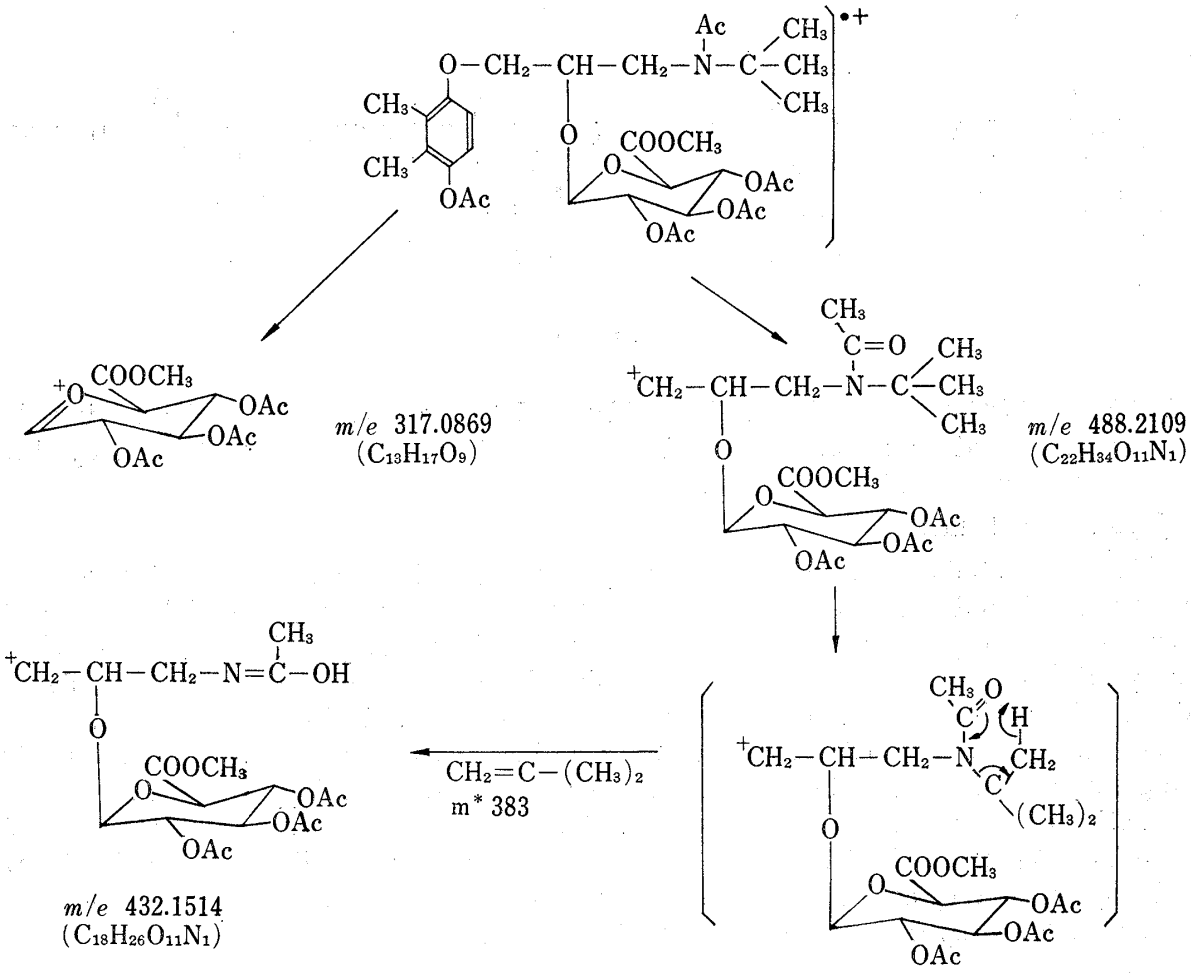


Chart 2

10) M. Ueda, N. Murakami, K. Furuki, and H. Atsumura, *Chem. Pharm. Bull.* (Tokyo), **16**, 352 (1968).
11) M. Neeman and Y. Hashimoto, *J. Am. Chem. Soc.*, **84**, 2972 (1962).

sessing the glucuronyl moiety. A fragment at *m/e* 432 would be formed by proton migration from the tertiary butyl group to positively charged carbonyl oxygen of the N-acetyl group with simultaneous loss of isobutene. This assumption was supported by the appearance of metastable ion at *m/e* 383. It was further confirmed by the high resolution mass spectrum, where strong peaks appeared at *m/e* 488.2109 ($C_{22}H_{34}O_{11}N$) and 432.1514 ($C_{18}H_{26}O_{11}N$), respectively. On the basis of these evidences the attached position of a glucuronyl residue was deduced to be a hydroxyl group of the propanol amine moiety. These evidences together led us to conclude that metabolite XIII should be 4′-hydroxy D-32 2-glucuronide.

Metabolite XIV which was isolated from fraction J, K and L exhibiting a positive naphthoresorcinol test was similarly converted into the acetate-methyl ester derivative (XIV′). The mass spectrum of XIV′ showed characteristic peaks at *m/e* 609 (M^+), 488, 432 and 276, although sufficient amount of the specimen was unavailable. From these results metabolite XIV would be assigned to the structure D-32 2-glucuronide.

The remaining conjugated metabolite XV which was isolated from fraction J exhibited a positive result with naphthoresorcinol test and a carbonyl absorption at 1600 cm^{-1} on the IR spectrum. Being treated with beef-liver β-glucuronidase, XV liberated readily an aglycone which proved to be identical with metabolite II by comparison with the authentic sample. On the NMR spectrum of XV the proton signals of *tert*-butyl, methyl and hydroxymethyl groups appeared at 1.42, 2.22 and 4.70 ppm, respectively. The attached position of glucuronic acid was deduced by comparison with the NMR spectrum of 3′-hydroxymethyl D-32 3′-glucuronide (XVII) which was isolated from the rat bile. The proton signal of hydroxymethyl group in XVII shifted down field with 0.3 ppm due to the sugar moiety, whereas the chemical shift of hydroxymethyl proton in XV was not affected. These results indicated that metabolite XV would be 3′-hydroxymethyl D-32 2-glucuronide.

Discussion

It is evident from the data that the absorption of D-32 was substantially complete after oral and intravenous administration. There can be seen a marked species difference in the excretion pattern. The rabbit and monkey excreted 75—90% of the dose into urine, whereas the rat did 31—46% of the dose both into urine and feces. In accordance with this result approximately 50% of the dose was excreted into the bile in the rat. The species difference in the excretion pattern involving biliary and renal clearance may be dependent upon the nature of metabolites. The amphoteric and basic metabolites, namely aromatic ring and methyl hydroxylation products, constituted about 70—90% of the urinary metabolite, whereas the acidic metabolites formed by oxidation of the side chain were about 30—10%. Almost the metabolites were excreted in urine as the conjugated form and the free metabolites were detectable in very small amount. Metabolite I, 4′-hydroxy D-32, was excreted as two conjugate forms, namely a sulfate attached to C-4′ position and a glucuronide linked to hydroxyl group of the side chain. On the other hand the 4′-hydroxylated metabolite in the rat bile was exclusively conjugated with glucuronic acid at C-4′. Another main metabolites was 3′-hydroxymethyl derivative and its positional isomer was formed to much smaller extent. Metabolite II found in the monkey was almost conjugated with glucuronic acid through the hydroxyl group of the side chain, while the glucuronide isolated from the rat bile was conjugated with the 3′-hydroxymethyl group. It is to be noted that glucuronic acid is exclusively bound to the hydroxyl function of the side chain in the monkey. However, the plausible explanation for the species difference between the monkey and rat in the conjugate form is not unavailable.

The occurrence of metabolite IV, the hydroxybutyl derivative, was found only in the monkey and not in other animals. In the rat, rabbit and dog the methyl group on the benzene

12) S. Honma and A. Kambekawa, "In preparation."

ring was susceptible to *in vivo* and *in vitro*[12] oxidation resulting in formation of the aromatic hydroxymethyl derivative, but the *tert*-butyl group of the side chain was not disturbed. These findings imply that the different enzyme system may be involved in the oxidation of the methyl group.

The main metabolite formed by the cleavage of the side chain was the glycol and accounted for 2—3% of the metabolites. As to the metabolic fate involving the side chain D-32 may possibly be first transformed into the primary amine by N-dealkylation, which in turn was led to the aldehyde and then to the glycol. The mode of this biotransformation appears to be close to that of catecholamine.[13] The xylenol analogs may possibly be formed by the successive oxidation.

The metabolic fate of propranolol[14–18] and adrenaline is different each other. In the former case these can be seen the occurrence of the side chain oxidation and/or aromatic ring hydroylation. The most principal metabolite in urine is naphthoxyacetic acid which accounts for 40% of the oral dose. In contrast adrenaline is metabolized into the N-demethylation compound and to catechol 3-monomethyl ether, which are then in part transformed into the conjugate or mandelic acid. These findings indicate that N-deisopropylation is somewhat more difficult than N-demethylation.[19] In addition D-32 is metabolized into the side chain cleavage product whose amount corresponds to 10% of the dose. Thus, elimination of N-*tert*-butyl group appears to be much more difficult than that of N-isopropyl group.[20,21] It is to be noted that D-32 is exclusively converted into the 4′-hydroxy derivatives as phenolic metabolite in the rabbit, dog and monkey, while in the rat the D-32 is mainly metabolized to the 3′-hydroxy methyl D-32. No evidence for the formation of 5′-hydroxylated metabolite by *in vitro* and *in vivo* experiment could be hitherto obtained though the extensive efforts were made.

Finally, it should be emphasized that 4′-hydroxy and 3′-hydroxymethyl derivatives are two or three times more potent than D-32 itself in the activity to antagonize isoprenaline induced tachycardia in the rat, while 2′-hydroxy (III) and 4′-hydroxy 4′-O-sulfate (XII) exhibit very weak potency.[22] The blood level of the active metabolites, I and II, is much higher than those of unchanged D-32 in the case of oral administration. On the other hand the blood concentration of these metabolites corresponds to 5—10% of the dose, when D-32 is administered intravenously.[23] It is evident from these data that the pharmacological activity is principally attributable to these active metabolites rather than the unchanged D-32.

Experimental

General Procedure——All melting point were taken on a micro hot-stage apparatus and uncorrected. Ultraviolet (UV) and infrared (IR) spectra were run on Hitachi Model ESP-3 and EPI-G2 spectrometers, respectively. Nuclear magnetic resonance (NMR) spectra were recorded on Hitachi Model R-20A spectrometer at 60 MHz using tetramethylsilane as an internal standard. Abbreviation using s=singlet, d=doublet, t=triplet, q=quartet and m=multiplet. Mass spectral measurement was run by direct insertion technique Hitachi Model RMU-6E spectrometer.

Thin-Layer Chromatography (TLC)——TLC was carried out on Silica gel HF254 plate employing the following solvent systems: system I=benzene/ether (5: 1), system II=$CHCl_3$/MeOH (9: 1), system III=

13) E.H. LaBrosse, J. Axelrod, I.J. Kopin, and S. Kety, *J. Clin. Invest.*, **40**, 253 (1961).
14) A. Hayes and R.G. Cooper, *J. Pharmacol. Exptl. Therap.*, **176**, 302 (1971).
15) J.W. Paterson, N.M. Conolly, C. Tdollery, A. Hayes, and R.G. Cooper, *Pharm. Clin.*, **2**, 127 (1970).
16) T. Walle and T.E. Gaffney, *J. Pharmacol. Exptl. Therap.*, **182**, 83 (1972).
17) G.L. Tindell, T. Walle, and T.E. Gaffney, *Life Sciences*, **11**, 1029 (1972).
18) O.M. Bakke, D.S. Davies, L. Davies, and C.T. Dollery, *Life Sciences*, **13**, 1665 (1973).
19) S.E. Mayer, *J. Pharmacol. Exptl. Therap.*, **135**, 204 (1962).
20) J. Kamm, A. Szuna, and R. Kuntzman, *J. Pharmacol. Exptl. Therap.*, **182**, 507 (1972).
21) J. Burns, R.A. Salvador, and L. Lembergee, *Ann. N.Y. Acad. Sci.*, **139**, 843 (1967).
22) Y. Hiramatsu, A. Izumi, and S. Honma, "In preparation."
23) S. Honma and A. Kambekawa, *Yakugaku Zasshi*, "in press."

benzene/ether/AcOH (250: 50: 1), system IV=AcOEt/MeOH/conc. NH_4OH (24: 6: 1), and system V= AcOEt/MeOH/conc. NH_4OH (10: 10: 1).

Column Chromatography on Celite——Celite 545 (Johns-Manville) was washed with 6N HCl twice at 40—60° and then with H_2O, MeOH and ether, successively and dried before use. The stationary phase-impregnated Celite was prepared according to Siiteri method[24] and packed in a column. The following systems were used for the separation of basic and acidic metabolites, respectively: system A=isooctane/*tert*-BuOH/H_2O/conc. NH_4OH (8: 20: 19: 1) and system B=AcOEt/isooctane (9: 13).

Gas-Liquid Chromatography (GLC)——The apparatus used was a Shimadzu Model GC-4BM gas chromatograph equipped with a hydrogen flame ionization detector and a U-shaped glass tube (3 mm i.d.) packed with 3% SE-30 on Gas Chrom P (60—80 mesh). The temperatures of column, detector, and injection chamber were kept at 170°, 200°, and 200° respectively. N_2 was used as the carrier gas at a flow rate of 80 ml/min.

Gel Filtration——Sephadex LH-20 and G-25 (medium) pharmacia Fine Chem., Inc.) were previously equilibrated with eluent for 18 hr and then used.

Radioactive Counting——Counting was carried out on Kobe Kogyo Model EA-118 liquid scintillation spectrometer. For toluene-soluble samples toluene containing 2,5-diphenyloxazole (4 g/liter) and 1,4-bis(5-phenyl-2-oxazolyl)benzene (100 mg/liter) was used as a scintillator. Aqueous samples were counted in a scintillator, composed of dioxane (1 liter), naphthalene (100 g), 2,5-diphenyloxazole (8 g) and 1,4-bis (4-methyl-5-phenyl-2-oxazolyl)benzene (250 mg). For quench correction the channel ratio and external standards were employed.

Preparation of Labeled D-32——^3H_3C-Xylenol was synthesized by shaking a solution of xylenol (500 mg) dissolved in ^3H_2O (5 ml) with 10% Pd/C (250 mg) at 100° for 1 hr.[25] ^3H_3C-D-32 (specific activity 32 μCi/mg) was prepared from purified ^3H_3C-xylenol in the manner as reported by Suzuki, *et al.*[4]

Animals——The adult male rabbits weighing about 3 kg, the adult Wistar rats weighing about 350 g and a male rhesus monkey weighing 10 kg were housed in the cage, respectively that designed to minimize fecal contamination of the urine.

Administration of D-32 and Collection of Urine——A single dose of a solution of ^3H_3C-D-32 (500 mg, 10 μCi) as HCl salt was injected into a stomach through a catheter in five rabbits and the urine was collected in bottle for 72 hr after administration. A solution of ^3H_3C-D-32 (50 mg, 1 μCi) as HCl salt was orally given to each of 20 rats and the urine was pooled in bottle for 72 hr. In a monkey, a solution of ^3H_3C-D-32 (800 mg, 5 μCi) in milk was orally administered for 7 days and the urine collected for 10 days.

Hydrolysis of Conjugates——The collected urine was adjusted to approximately pH 5 with 50% H_2SO_4 and then to pH 4.5 with 0.1M acetate buffer and incubated with beef-liver β-glucuronidase at 37° for 72 hr. The incubated fluid was adjusted to pH 1.0 with 50% H_2SO_4 and deconjugated acidic metabolites were extracted with two-fold volume of ether. The organic layer was washed with water and dried over anhydrous Na_2SO_4. Evaporation of solvent gave the acidic metabolites as gummy substance. The aqueous layer was adjusted to pH 10 with NaOH solution and the basic metabolites were extracted with two-fold volume of *n*-BuOH–AcOEt (7: 5) and the organic phase was washed with H_2O. Evaporation of solvent afforded the basic metabolites as an oil. The remaining aqueous layer was then brought to pH 1.0 with 50% H_2SO_4, saturated with NaCl (20 g/100 ml) and extracted with AcOEt. The organic phase was separated and allowed to stand at 37° for 48 hr. Separation of acidic and basic metabolites was carried out in the manner as described below.

Separation of Basic Metabolites——The gummy substance was passed through a column (4×65 cm) packed with Sephadex LH-20 using *n*-BuOH–H_2O (99: 1) as eluent for removal of the nonradioactive substance. Eluate was dissolved in a minimum volume of system A, mixed with the appropriate amount of Celite and packed into a column (2×45 cm) which was packed with Celite (76 g) impregnated with the stationary phase. Each 15 ml of effluent with system A was fractionally collected as shown in Fig. 1.

Separation of Acidic Metabolites——The gummy substance (1.34 g) was chromatographed on a column (2.5×50 cm) which was packed with Celite (70 g) as described above. Each 10 ml of effluent with system B was fractionally collected as shown in Fig. 3.

Identification of the Metabolites

1-*tert*-Butylamino-3-(2,3-dimethyl-4-hydroxyphenoxy)-2-propanol (4′-Hydroxy D-32, Metabolite I)——Separation was accomplished as acetic acid salt. Subsequent recrystallization from AcOEt gave metabolite I as colorless needles. mp 120—125°. Analytical sample was obtained recrystallization from isopropanol–ether containing HCl. mp 181—183°. The metabolite showed staining with conc. H_2SO_4. NMR (CD_3OD solution) δ: 1.14 (s, 9H, *tert*-butyl), 2.15 (d, 6H, aromatic methyl), 6.60 (s, 2H, aromatic 5,6-H). Mass Spectrum *m/e*: 267 (M^+), 252 (M^+-CH_3), 138 (4-hydroxyxylenol), 114 and 86 (base). The metabolite proved to be entirely identical with the synthetic sample by mixed melting point measurement, IR and NMR spectral comparison.

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1-*tert*-Butylamino-3-(2-methyl-3-hydroxymethylphenoxy)-2-propanol(3′-Hydroxymethyl D-32, Metabolite II)——Fraction C was purified by preparative TLC using solvent system IV and recrystallized from acetone containing AcOH to give II (113 mg). mp 149—153°. The metabolite showed pink staining with conc. H_2SO_4. NMR (CD_3OD solution) δ: 1.40 (s, 9H, *tert*-butyl), 2.25 (s, 3H, aromatic-methyl), 6.8—7.2 (m, 3H, aromatic H). Mass Spectrum *m/e*: 267 (M^+), 252 (M^+–CH_3), 138 (3′-hydroxymethylxylenol), 114, and 86. Mixed melting point on admixture with the authentic sample and IR spectrum comparison showed identity of two samples.

1-*tert*-Butylamino-3-(2-hydroxymethyl-3-methylphenoxy)-2-propanol (2′-Hydroxymethyl D-32, Metabolite III)——The mother liquor of metabolite I and II was submitted to the preparative TLC using solvent system IV. Elution of the adsorbent corresponding to the spot with acetone and recrystallization of the eluate from aq. MeOH gave metabolite III as colorless needles. mp 98—100°. The metabolite showed red color with conc. H_2SO_4. NMR (CD_3OD solution) δ: 1.40 (9H, s, *tert*-butyl), 2.40 (3H, s, aromatic-CH_3), 6.70—7.20 (3H, m, aromatic-H). Mass Spectrum *m/e*: 267 (M^+), 252 (M^+–CH_3), 138 (2-hydroxyxylenol), 114, and 86. The metabolite proved to be identical with the authentic sample in respect with NMR and mass spectra, and distinctly different from 3′-hydroxymethyl isomer in TLC and GLC.

1-*tert*-Hydroxybutylamino-3-(2,3-dimethyl-4-hydroxyphenoxy)-2-propanol (4′-Hydroxybutanol D-32, Metabolite IV)——The fraction D was submitted to the preparative TLC, and the adsorbent corresponding to the spot was eluted with acetone to give metabolite IV as an oily substance. The eluate was further chromatographed on Sephadex LH-20 column (1×60 cm) using iso-PrOH as eluent. Recrystallization from MeOH afforded metabolite IV as colorless plates. mp 112—116°. This compound showed a positive reaction with Folin-Ciocalteu's reagent and yellow staining with conc. H_2SO_4. NMR (CD_3OD solution) δ: 1.30 (6H, s, aliphatic CH_3), 2.10—2.20 (6H, d, aromatic-CH_3), 3.50 (2H, s, C<u>H</u>$_2$OH) 6.60 (2H, s, aromatic-5,6-H). Mass Spectrum *m/e*: 283 (M^+), 252 (M^+–CH_2OH), 117, 114 and 102.

1-*tert*-Butylamino-3-(2-methyl-3-hydroxymethyl-4-hydroxyphenoxy)-2-propanol (4′-Hydroxy 3′-Hydroxymethyl D-32, Metabolite V)——In the case of rabbit and rat fraction E was chromatographed on Sephadex LH-20 column (2×45 cm) using iso-PrOH as eluent. The eluate was further purified by the preparative TLC using solvent system IV. Elution of the adsorbent corresponding to the spot with acetone and recrystallization from acetone–AcOH gave metabolite V as colorless plates. mp 184—186°. This substance showed a positive reaction with Folin-Ciocalteu's reagent and yellow staining with conc. H_2SO_4. NMR (CD_3OD solution): 1.40 (9H, s, *tert*-butyl), 2.28 (3H, s, aromatic-CH_3), 4.75 (2H, s, C<u>H</u>$_2$OH), 6.72 (2H, s, aromatic-4,5-H). Mass Spectrum *m/e*: 283 (M^+), 268 (M^+–CH_3), 250, 154, 138, and 86. Usual acetylation with pyridine–Ac_2O furnished the tetraacetate as an oily product. NMR ($CDCl_3$ solution): 1.45 (9H, s, *tert*-butyl), 2.0—2.35 (15H, m, N- and O-Ac, aromatic-CH_3), 5.20 (2H, s, C<u>H</u>$_2$OAc), 6.7—7.12 (2H, m, aromatic-5,6-H). Mass Spectrum *m/e*: 451 (M^+), 436 (M^+–CH_3), 174 and 158.

Xylenol (Metabolite VI)——Fraction E and F were combined and submitted to preparative TLC using solvent system 1. Elution of the adsorbent corresponding to the spot with acetone and recrystallization of the eluate from hexane gave metabolite VI as colorless needles. mp 76—78°. The metabolite showed a positive reaction with Folin-Ciocalteu's reagent. The structure of metabolite was characterized by the reverse isotope dilution method. To the metabolite was added xylenol (50 mg) as a carrier and was recrystallized repeatedly to constant specific activity.

No.	Crystallized from	Specific activity (dpm/mg)
1	hexane	4.76×10^4
2	hexane	4.25×10^4
3	hexane	4.75×10^4

4-Hydroxyxylenol (Metabolite VII)——Fraction G was submitted to preparative TLC using solvent system II. Elution of the adsorbent corresponding to the spot with acetone and recrystallization of the eluate from benzene–hexane gave metabolite VII as colorless needles. mp 197—200°. The metabolite showed a positive reaction with Folin-Ciocalteu's reagent. Mass Spectrum *m/e*: 138 (M^+). Mixed melting point on admixture with synthetic sample and IR spectra comparison showed identity of two samples.

1-Xylenoxy-2,3-propanediol (Metabolite VIII)——Fraction H was purified by preparative TLC and the eluate was recrystallized from hexane to give metabolite VIII (1 mg) as colorless needles. Mass Spectrum *m/e*: 196 (M^+), 178 (M^+–H_2O), 165, 122. The metabolite proved to be entirely identical with synthetic sample by mixed melting point measurement, IR and NMR spectral comparison. Usual acetylation with pyridine–Ac_2O furnished the diacetate as an oily product. Mass Spectrum *m/e*: 280 (M^+), 159, 122.

3-Hydroxymethylxylenol (Metabolite IX)——Fraction F was submitted to column chromatography (2.2×60 cm) on Sephadex LH-20 using 99% *n*-BuOH as eluent. Eluate was recrystallized from benzene–hexane to give metabolite IX as colorless needles. mp 142—145°. NMR (C_5D_5N solution) δ: 2.55 (3H, s, aromatic-CH_3), 5.0 (2H, s, C<u>H</u>$_2$OH), 7.10—7.50 (3H, m, aromatic-H). Mass Spectrum *m/e*: 138 (M^+), 120,

91 (benztropylium ion). The metabolite proved to be entirely identical with the synthetic sample by mixed meltingpoint measurement, IR and NMR spectral comparison.

1-Xylenoxyacetic Acid (Metabolite X)——Fraction A was submitted to column chromatography (2.2×60 cm) on Sephadex LH-20 using 99% *n*-BuOH as eluent. The eluate obtained was purified by the preparative TLC using solvent system VI. Elution of the adsorbent corresponding to the spot with acetone and recrystallization of the eluate from benzene–hexane gave metabolite X as colorless needles. mp 180—182°. NMR (C_5D_5N solution) δ: 2.18 (3H, s, aromatic-CH_3), 2.30 (3H, s, aromatic-CH_3), 4.96 (2H, s, CH_2), 6.76, 7.50 (3H, m, aromatic-H). Mass Spectrum *m/e*: 180 (M^+), 163 (M^+–18), 135 (M^+–CO_2H), 121. IR ν_{max}^{KBr} cm^{-1}: 1710, 1740 (carboxylic acid).

1-(4-Hydroxyxylenoxy)-2,3-propanediol (Metabolite XI)——After removal of metabolite X by gel filtration on Sephadex LH-20 the remaining radioactive fraction was submitted to preparative TLC using solvent system VI. Elution of the adsorbent corresponding to the spot with acetone and recrystallization of the eluate from acetone gave metabolite XI as colorless needles. mp 109—113°. This substance showed a positive reaction with Folin-Ciocatteu's reagent. Mass Spectrum *m/e*: 212 (M^+), 194 (M^+–H_2O), 181, 151, 138. IR ν_{max}^{KBr} cm^{-1}: 3300 (OH).

Separation of Conjugated Metabolites——The pooled urine (1250 ml) was adjusted to pH 7.0 with dil. H_2SO_4 and free metabolites were removed by extraction with 30% *n*-BuOH–AcOEt. The aq. layer was concentrated below 40° *in vacuo* to give gummy substance. This substance was dissolved in H_2O and passed through a column of Sephadex G-25 for removalof nonradioactive substances using H_2O as eluent. The effluent (fraction 24—100) was concentrated and then chromatographed on Sephadex LH-20 (4×50 cm) twice using 20% methanol–H_2O as eluent. After evaporation of the radioactive main fraction an oily residue (1.2 g) was rechromatographed on AmberliteXAD-2 column (4×58 cm) using methanol-H_2O as eluent. The effluent (fraction 17—33) was evaporated in vacuo. The eluate was submitted to column chromatography (2.2×55 cm) on Sephadex LH-20 and fraction of 5 ml was collected as follows:

Fraction	No.	Weight	Metabolite
J	33—36		XIV, XV
K	37—42	252 mg	XIV, XIII
L	43—55	193 mg	XII, XIII, XIV
M	54—65	106 mg	XII, XIII

1-*tert*-Butylamino-3-(4-hydroxy-2,3-dimethylphenoxy)-2-propanol 4-sulfate (4′-Hydroxy D-32 4′-Sulfate, Metabolite XII)——Recrystallization of the eluate from MeOH gave metabolite XII (62 mg) as colorless needles. mp 230—233° (decomp.). The metabolite showed negative reaction of Folin-Ciocalteu's reagent. *Anal.* Calcd. for $C_{15}H_{25}O_6NS$: C, 51.80; H, 7.20; N, 4.22. Found: C, 52.03; H, 7.18; 4.23. IR ν_{max}^{KBr} cm^{-1}: 1275, 1040 (SO_3). NMR (CD_3OD solution) δ: 1.40 (9H, s, *tert*-butyl), 2.15, 2.25 (6H, s, aromatic-CH_3), 6.68, 7.21 (2H, d (J=9 Hz), aromatic-5,6-H). A portion of metabolite XII (17 mg) was dissolved in MeOH (30 ml) containing conc. HCl (0.3 ml) and then incubated at 35° for 48 hr. The incubation mixture was adjusted to pH 10 with NaOH and extracted with AcOEt, washed with H_2O and dried over anhydrous Na_2SO_4. After evaporation of solvent the crude product was recrystallized from AcOEt–AcOH to give metabolite 1 (4 mg). mp 118—122°. The hydrolyzate showed a positive reaction with Folin-Ciocalteu's reagent and proved to be identical with 4′-hydroxy D-32 by mixed melting point measurement and TLC comparison with the authentic sample. From these evidences metabolite XII was assigned the structure 4-hydroxy D-32 4′-sulfate.

1-*tert*-Butylaminomethyl-2-(4-hydroxy-2,3-dimethylphenoxy)ethyl-β-D-glucopyranosiduronic Acid (4′-Hydroxy D-32 2-Glucuronide, Metabolite XIII)——The mother liquor of metabolite XII was submitted to the preparative TLC using system V and the eluate was further purified by column chromatography (3×55 cm) on Sephadex LH-20 using H_2O as eluent. The oily product obtained was recrystallized from MeOH–ether to give XIII as colorless powder. mp 245—252° (decomp.). This substance exhibited positive reaction with both naphthoresorcine and Folin-Ciocalteu's reagents. NMR (CD_3OD solution) δ: 1.42 (9H, s, *tert*-butyl), 2.1, 2.18 (6H, s, aromatic-CH_3), 4.45 (1H, d, (J=6 Hz), anomeric-H), 6.60 (2H, s, aromatic-5,6-H). The conjugate XIII (2 mg) was dissolved in acetate buffer (0.1M, pH 4.5) and incubated with beef-liver β-glucuronidase (100000 Fishman units) at 37° for 48 hr. The incubation mixture was extracted with AcOEt and then extract was submitted to TLC using system I. The hydrolyzate proved to be identical with 4′-hydroxy D-32 by TLC comparison with the authentic sample. To a solution of the conjugate (8 mg) in MeOH (10 ml) was added an ether solution of CH_2N_2 and then allowed to stand for 30 min at room temperature. After decomposition of the excess reagent with AcOH the resulting solution was evaporated to give an oily product, which in turn was treated with Ac_2O (2 ml)–pyridine (2 ml) in the usual manner. The reaction mixture was diluted with ether, washed with H_2O and dried over anhydrous Na_2SO_4. After evaporation of solvent an oily residue (5 mg) obtained was submitted to preparative TLC using system I. Elution

of the adsorbent corresponding to the spot with acetone gave metabolite XIII as a colorless oil. Mass Spectrum *m/e*: 667 (M^+), 488, 432, 334, 317 (G′).

1-*tert*-Butylaminomethyl-2-(2,3-dimethylphenoxy) ethyl-β-D-glucopyranosiduronic Acid (D-32 2-Glucuronide, Metabolite XIV)——The mother liquor of metabolite XII was submitted to the preparative TLC using system V and the eluate was further purified by column chromatography (3.0×55 cm) on Sephadex LH-20 using H_2O as eluent. The eluate showed a positive reaction with naphthoresorcine reagent. This substance was treated with CH_2N_2 and then with Ac_2O–pyridine in the manner as described above to give the acetate-methyl ester as an oily substance. Mass Spectrum *m/e*: 609 (M^+), 488, 317, 276.

1-*tert*-Butylaminomethyl-2-(2-methyl-3-hydroxymethylphenoxy) ethyl-β-D-glucopyranosiduronic Acid (3′-Hydroxymethyl D-32 2-Glucuronide, Metabolite XV)——A portion of fraction J was submitted to preparative TLC using system V. The eluate (18 mg) was further purified by column chromatography (2×55 cm) on Sephadex LH-20 to give metabolite XV as an oily substance, which showed positive reaction with naphthoresorcine reagent. NMR (CD_3OD solution) δ: 1.42 (9H, s, *tert*-butyl), 2.22 (3H, s, aromatic-CH_3), 4.70 (2H, s, CH_2OH), 6.8—7.1 (3H, s, aromatic-H). Metabolite XV (2.0 mg) was treated with beef-liver β-glucuronidase (100000 units) in the manner as described in XIII. The incubation mixture was extracted with AcOEt and the extract was submitted to TLC using system IV. The hydrolyzate proved to be identical with 3-hydroxymethyl D-32 by TLC comparison with authentic sample.

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