UDC 615.7[547.581]-092.21

15. Hisao Tsukamoto*1 and Seisuke Terada*2 : Metabolism of Drugs. XXVII.*3 Metabolic Fate of *p*-Hydroxybenzoic Acid and its Derivatives in Rabbit. (3).

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In the previous papers,[1,12] the occurrence of five metabolites in the urine of rabbits receiving methyl *p*-hydroxybenzoate was reported. They were *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid, *p*-carboxyphenyl glucuronide (ether-type glucuronide), *p*-hydroxybenzoyl glucuronide (ester-type glucuronide), and *p*-carboxyphenyl sulfate. Three of these metabolites, the ether-type glucuronide, *p*-hydroxyhippuric acid, and *p*-hydroxybenzoic acid, were isolated as crystalline materials.

p-Hydroxyhippuric acid[2] has been isolated by Quick[3] from the urine of animals receiving *p*-hydroxybenzoic acid, and the unchanged acid and *p*-carboxyphenyl sulfate[4] were detected as the metabolites.

As for the occurrence of conjugated glucuronic acid in the metabolism of salicylic acid in man and animals,[5,6] both glucuronides, the ether and the ester-types, have been demonstrated as the urinary metabolites. For example, they were already isolated as methylacetyl derivative from the urine of animals receiving salicylic acid and its methyl ester, by Williams, *et al.*[6] and by Tsukamoto, Kato, and Tatsumi,[7] respectively. The isolation of both type glucuronide derivatives was also accomplished by Tsukamoto and Yamamoto[8] on feeding rabbits with *p*-aminosalicylic acid. Bray, *et al.*[9] isolated *p*-carbamylphenyl glucuronide as a urinary metabolite in rabbits receiving *p*-hydroxybenzamide. The ester-type glucuronide of anisic acid was isolated as methylacetyl derivative by Williams, *et al.*[10] from the urine of rabbits receiving anisaldehyde. However, the occurrence of ester-type glucuronide in the metabolism of *p*-hydroxybenzoic acid in rabbit has not yet been reported.

The present paper describes the biotransformation of *p*-hydroxybenzoic acid in rabbits. The ester-type glucuronide was isolated from the urine as two derivatives whose structures have been established as methyl (*p*-methoxybenzoyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate and methyl (*p*-acetoxybenzoyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate, and five metabolites were detected in the urine, similar to the case of methyl *p*-hydroxybenzoate, by means of paper chromatography.

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Experimental

Separation of the Metabolite Fraction from the Urine of Rabbits——A total dose of 7.8 g. of *p*-hydroxybenzoic acid (0.8 g./kg. body wt.) was administered by stomach tube as a 12% solution in the form of Na salt to 4 male rabbits, each weighing 2.2~2.8 kg.

The 24-hr. urine was treated with $(AcO)_2Pb$[11] as previously reported.[12] A brownish substance so obtained was mixed with a small amount of H_2O and the mixture was extracted with Et_2O to remove the unchanged acid (3.2 g.) which was excreted with the metabolites. When the aqueous solution was evaporated to dryness in a reduced pressure, the crude gum (3.7 g.) was yielded.

Isolation of *p*-Hydroxyhippuric Acid and the Ether-type Glucuronide——The crude gum was dissolved in about 30 cc. of H_2O and the solution was continuously extracted with Et_2O in a Soxhlet liquid extractor for 40 hr. The prisms that separated from Et_2O solution were collected by filtration. Et_2O filtrate was evaporated to dryness and the residue was treated with activated charcoal in MeOH. After the filtrate had been evaporated to dryness in a reduced pressure, the residual solid was recrystallized from hot H_2O. The crystals collected were combined with the above prisms and recrystallized from hot H_2O to colorless needles, m.p. 236~238°. Yield, 0.8 g. This compound showed no depression in melting point when mixed with *p*-hydroxyhippuric acids which was isolated from the urine of rabbits receiving methyl *p*-hydroxybenzoate,[12] and the chemical properties also were the same.

After removal of *p*-hydroxyhippuric acid, the aqueous mother liquor was evaporated to dryness in a reduced pressure. The dried residue was dissolved in a minimum amount of MeOH and $(iso\text{-}Pr)_2O$ was cautiously added dropwise to the solution until a slight turbidity appeared. After the filtrate had been allowed to stand overnight, the crystalline mass produced was collected and recrystallized repeatedly from MeOH-$(iso\text{-}Pr)_2O$ to colorless needles, m.p. 193~195°(decomp.). Yield, 0.05 g. The chemical properties and melting point were identical with those of the ether-type glucuronide which was isolated as a urinary metabolite of methyl *p*-hydroxybenzoate.[12]

Isolation of the Ester-type Glucuronide as Derivatives——The aqueous solution, from which *p*-hydroxyhippuric acid and the ether-type glucuronide were removed by continual extraction with Et_2O, was evaporated to dryness in a reduced pressure and the residual gum was allowed to stand for 2 days in a vacuum desiccator. About 20 cc. of MeOH was added to the light brown solid and, after removal of insoluble substance, the filtrate was treated with activated charcoal. This filtrate was evaporated to dryness in a reduced pressure to leave a yellowish gum (2.8 g.).

1) ***p*-Methoxyl Derivative**——To 1 g. of the above gum in 20 cc. of MeOH an Et_2O solution of CH_2N_2, freshly prepared from 5 g. of nitrosomethylurea, was added with cooling in ice and the mixture was allowed to stand overnight in a refrigerator. The solvent was removed by evaporation, the residue was dissolved in 5 cc. of pyridine and 3.5 cc. of Ac_2O, and the mixture was allowed to stand overnight at room temperature. The reaction mixture was poured into 50 cc. of ice-water with stirring. After the mixture had been allowed to stand overnight, the precipitate was collected and dissolved in Et_2O. Et_2O solution was washed successively with 2% H_2SO_4, H_2O, 1% $NaHCO_3$, and H_2O, and dried over anhyd. Na_2SO_4. This solution was concentrated to about 10 cc. by evaporation, petr. ether was carefully added dropwise until a slight turbidity appeared, and its filtrate was allowed to stand overnight at room temperature. The crystalline material that separated was collected and recrystallized from a small amount of EtOH to colorless fine needles, m.p. 155~157°; $[\alpha]_D^{13}$ −18.8°(c=5.0, $CHCl_3$). Yield, 0.12 g. *Anal.* Calcd. for $C_{21}H_{24}O_{12}$: C, 53.84; H, 5.16. Found: C, 54.11; H, 5.19.

When mixed with the synthesized methyl (*p*-methoxybenzoyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (IV), this compound showed no depression in melting point. On the other hand, though melting point of this compound was very similar to that of the derivative of ether-type glucuronide, methyl (*p*-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate, m.p. 157~158°, already isolated[1] as a urinary metabolite of methyl *p*-hydroxybenzoate, melting point was certainly depressed on admixture. This compound was also identified with the synthetic sample by infrared absorption spectrum and chemical properties.

2) ***p*-Acetoxyl Derivative**——To a suspension of 1 g. of the above gum in 5 cc. of Ac_2O, 1 cc. of 47% BF_3-Et_2O solution was added dropwise with stirring. After standing for 1 hr. at room temperature, the mixture was poured into 50 cc. of ice-water and allowed to stand overnight in a refrigerator. The aqueous solution was extracted with AcOEt-Et_2O(2:1), AcOEt-Et_2O layer was washed successively with 2% H_2SO_4 and H_2O, dried over anhyd. Na_2SO_4, and evaporated to dryness in a reduced pressure. A brownish gum obtained was dissolved in 30 cc. of MeOH, the solution was mixed

11) I. A. Kamil, J. N. Smith, R. T. Williams: Biochem. J., **50**, 235 (1951).
12) H. Tsukamoto, S. Terada: This Bulletin, **10**, 86 (1962).

with an Et_2O solution of CH_2N_2, freshly prepared from 10 g. of nitrosomethylurea, with cooling in ice and the mixture was allowed to stand overnight in a refrigerator. After removal of an excess of CH_2N_2 in a reduced pressure, Et_2O solution was washed with 2% $NaHCO_3$ and H_2O, and dried over anhyd. Na_2SO_4. The dried solution was concentrated to about 10 cc. by evaporation, petr. ether was added dropwise until a slight turbidity appeared and the mixture was left overnight. The crystalline material so obtained was collected and recrystallized from EtOH to colorless silky needles, m.p. 182~184°; $[\alpha]_D^{13}$ −16.0°(c=3.0, $CHCl_3$). Yield, 0.07 g. *Anal.* Calcd. for $C_{22}H_{24}O_{13}$: C, 53.23; H, 4.87. Found: C, 53.58; H, 4.90.

This compound showed no depression of melting point when mixed with the synthesized methyl (*p*-acetoxybenzoyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (V) and the chemical properties and infrared absorption spectrum were also identical with those of an authentic sample. The course of separation of the metabolites is shown in Chart 1.

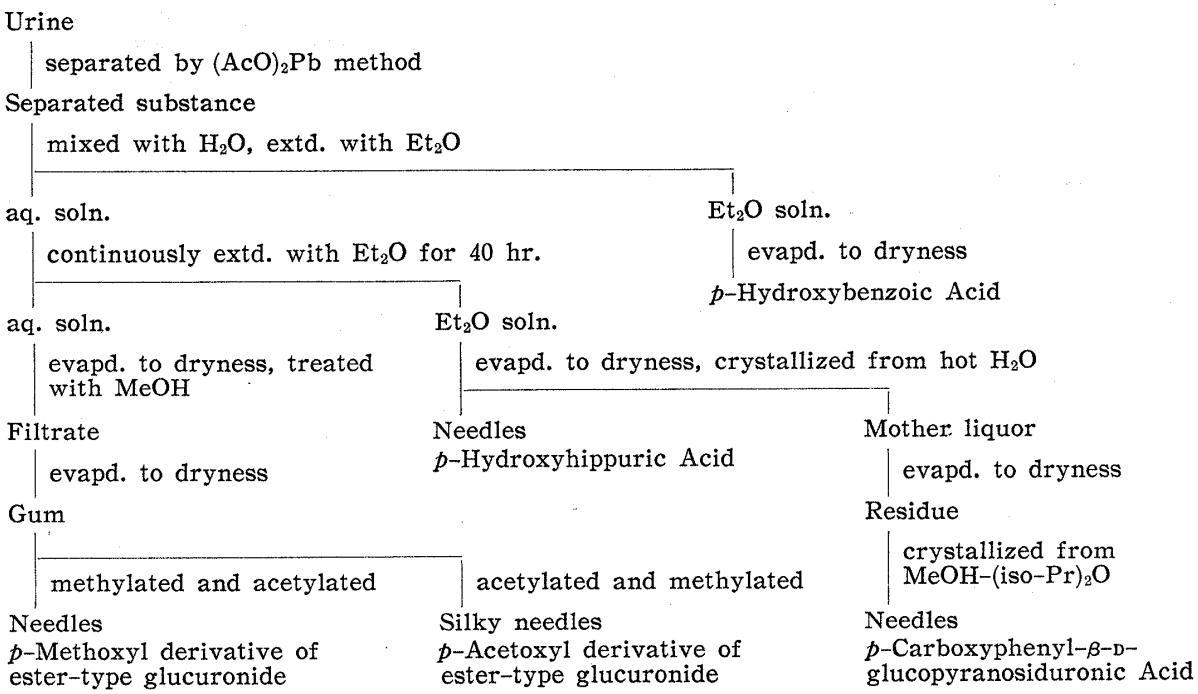


Chart 1. Separation of Metabolites from Urine

Methyl (*p*-Methoxybenzoyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (IV)——To 4.0 cc. of an aqueous solution containing 0.2 g. of NaOH, 0.7 g. of *p*-hydroxybenzoic acid (II) was added, followed by a solution of 1.9 g. of methyl (2,3,4-tri-O-acetyl-α-D-glucopyranosyl-1-bromid)uronate (I) in 10 cc. of Me_2CO. On shaking for a short time, the precipitate formed, which was dissolved in a minimum amount of Me_2CO and the mixture was allowed to stand overnight at room temperature. The reaction mixture was evaporated in a reduced pressure to remove Me_2CO and the resultant solution was extracted with AcOEt-Et_2O(2:1). AcOEt-Et_2O layer was washed with 2% H_2SO_4 and H_2O, dried over anhyd. Na_2SO_4, and the solvent was removed by evaporation in a reduced pressure. Methyl (*p*-hydroxybenzoyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (III) was obtained (1.5 g.) as a white solid. Since (III) was difficult to crystallize, the solid was dissolved in 40 cc. of MeOH, the solution was mixed with an Et_2O solution of CH_2N_2, freshly prepared from 10 g. of nitrosomethylurea, with cooling in ice, and the mixture was allowed to stand overnight in a refrigerator. The solvent was removed by evaporation and the residual crystalline powder was recrystallized twice from EtOH to colorless fine needles, m.p. 156~157°, $[\alpha]_D^{13}$ −19.2°(c=5.0, $CHCl_3$). Yield, 0.6 g. *Anal.* Calcd. for $C_{21}H_{24}O_{12}$: C, 53.84; H, 5.16. Found: C, 53.82; H, 5.16.

This gave a positive naphthoresorcinol test, and reduced Benedict reagent. An alkaline hydrolysate of this compound was acidified and then extracted with Et_2O. Et_2O layer was evaporated and the residue gave a red color with the Millon reagent.

Methyl (*p*-Acetoxybenzoyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (V)——To (III), prepared from 0.7 g. of (II) as in the case of (IV), 5 cc. of pyridine and 3.5 cc. of Ac_2O were added and the mixture was allowed to stand overnight at room temperature. The reaction mixture was poured into 50 cc. of ice-water and allowed to stand overnight in a refrigerator. The crystalline material thus formed was collected, washed thoroughly with H_2O, and dried in a desiccator. The dried

crystals were recrystallized from EtOH to colorless silky needles, m.p. 183~184°, $[\alpha]_D^{13}$ −16.6°(c=3.0, $CHCl_3$). Yield, 0.7 g. *Anal.* Calcd. for $C_{22}H_{24}O_{13}$: C, 53.23; H, 4.87. Found : C, 53.54; H, 4.85.

This reduced Benedict reagent and gave a positive naphthoresorcinol test. An alkaline hydrolysate of this compound was acidified and then extracted with Et_2O. Et_2O layer was evaporated and the residue gave a red color with the Millon reagent. Infrared absorption spectra of (IV) and (V) are shown in Fig. 1 and the route of their preparation in Chart 2.

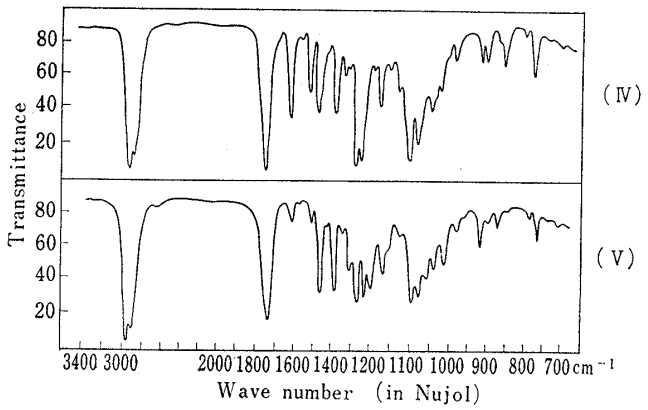


Fig. 1. Infrared Absorption Spectra
(IV) Methyl (*p*-methoxybenzoyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate
(V) Methyl (*p*-acetoxybenzoyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate

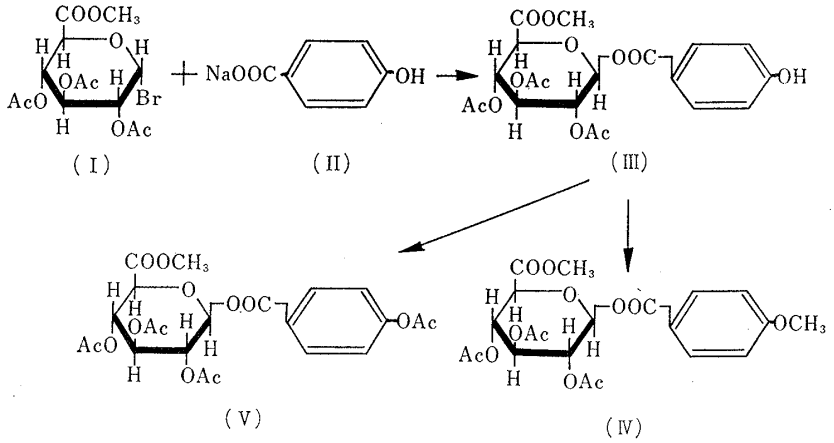
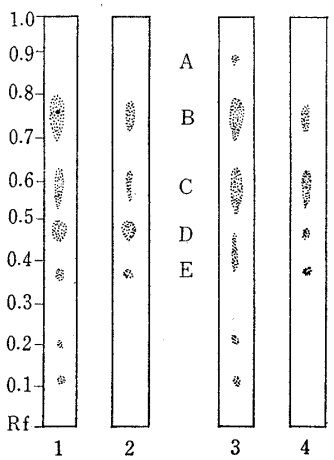


Chart 2. Preparation of Derivatives of Ester-type Glucuronide



Solvent System : BuOH-AcOH-H_2O(4:1:5)
1, 2) In case of *p*-hydroxybenzoic acid
3, 4) In case of methyl *p*-hydroxybenzoate
1, 3) Gum before continuous extraction with Et_2O
2, 4) Gum after continuous extraction with Et_2O
A, *p*-Hydroxybenzoic acid
B, *p*-Hydroxyhippuric acid
C, *p*-Carboxyphenyl glucuronide
D, *p*-Hydroxybenzoyl glucuronide
E, *p*-Carboxyphenylsulfate

Fig. 2. Paper Chromatograms of Gum obtained from the Urine of Rabbits receiving *p*-Hydroxybenzoic Acid and its Methyl Ester.

Paper Chromatography of Metabolites——Ascending development was employed with Toyo Roshi No. 50. The metabolites were visualized by spraying the reagents as described previously.[12] When the crude gum obtained by $(AcO)_2Pb$ separation was developed with the solvent system of

BuOH-AcOH-H_2O(4:1:5), the spots appeared mostly at Rf 0.74, slightly at Rf 0.57, clearly at Rf 0.48, and a small one at Rf 0.38, on the paper chromatogram on spraying with coloring reagents. However, with the gum obtained after continuous extraction with Et_2O, the spots became small at Rf 0.74 and 0.57, the section of Rf 0.48 made a large spot, and that of Rf 0.38 remained the same.

After this gum had been developed on a large filter paper, each zone, whose position was tested with color reagents, was cut out and eluted by the method already described.[12] Behavior of each of these eluates to alkali, acid, and color reagents was examined and *p*-hydroxyhippuric acid, the ether-type glucuronide, the ester-type glucuronide, and *p*-carboxyphenyl sulfate were detected from higher Rf value spots.

In Fig. 2, the chromatographic data have been compared with the urinary metabolites of methyl *p*-hydroxybenzoate.

Discussion

After feeding rabbits with *p*-hydroxybenzoic acid, similarly in the case of using methyl *p*-hydroxybenzoate, *p*-hydroxyhippuric acid and ether-type glucuronide were isolated on continuous extraction with Et_2O from the metabolite fraction obtained by the lead acetate separation from the urine. Moreover, unchanged acid was excreted in a large amount in the urine and *p*-carboxyphenyl sulfate was detected only in a small quantity.

Williams, *et al.*[6] have isolated ester-type of salicylic acid as a derivative from the urine of a dog receiving salicylic acid and the ether-type of that on feeding rabbits with methyl salicylate.

Although the ether-type glucuronide of *p*-hydroxybenzoic acid had been only isolated from the urine of rabbits in the metabolism of methyl *p*-hydroxybenzoate, the isolation of ester-type glucuronide as derivative was successful together with that of ether-type, using rabbits, in the case of *p*-hydroxybenzoic acid, in the present series of experiments. This fact may explain the observed difference on the glucuronide formation in the metabolism of *p*-hydroxybenzoic acid and its methyl ester.

In the present work, two isolated derivatives of the ester-type glucuronide were structurally established as methyl (*p*-methoxybenzoyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate and methyl (*p*-acetoxybenzoyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate with the synthesized samples.

The authors are indebted to Mrs. H. Mazume of Pharmaceutical Faculty, University of Nagasaki, for the determination of infrared spectra and the elemental analyses.

Summary

The ester-type glucuronide of *p*-hydroxybenzoic acid was isolated as two derivatives from the urine of rabbits receiving *p*-hydroxybenzoic acid and their structures were established as methyl (*p*-methoxybenzoyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate and methyl (*p*-acetoxybenzoyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate.

When feeding *p*-hydroxybenzoic acid to rabbits, similarly as in using the methyl ester, the unchanged acid, *p*-hydroxyhippuric acid, the ether-type glucuronide, the ester-type glucuronide, and *p*-carboxyphenyl sulfate were excreted as five metabolites in the urine.

(Received January 25, 1961)