Notes

[Chem. Pharm. Bull. 12 (6) 731 ~ 734]

UDC 612.015.34 : 612.396.21 : 615.778.475

Hisao Tsukamoto, Keitaro Kato, and Kazuo Yoshida : Metabolism of Drugs. XLII.*1 Isolation of Ester Glucuronides of *p*-Aminosalicylic Acid and Salicylic Acid from the Urine of Rabbits.

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It has been well known that aromatic carboxylic acids can be conjugated with glucuronic acid in the animal body to form ester glucuronides, which have labile β-glucosiduronic linkages in their molecules. It is conceivable that this type of glucuronide may play a broad role in metabolism due to its lability. For the purpose to use ester glucuronides as donor substrates in the glucuronyl transfer reaction of β-glucuronidase, isolation of ester glucuronides of *p*-aminosalicylic acid and salicylic acid has been undertaken. Since both glucuronides have phenolic hydroxyl groups which might serve as glucuronic acid acceptors, it would be of interest to serve these glucuronides as glucuronyl donors without other acceptor phenols.

The ester glucuronides of *p*-aminosalicylic acid was isolated as a methyl acetyl derivative from the urine of rabbits administered with *p*-aminosalicylic acid by Tsukamoto, Yamamoto, and Kamata.[1] The ester glucuronide of salicylic acid was isolated as a methyl acetyl derivative from the urine of a man by Robinson and Williams,[2] but the same authors failed to isolate this glucuronide from the urine of rabbits administered with salicylic acid. The present investigation was undertaken to isolate the free forms of ester glucuronides of *p*-aminosalicylic acid and salicylic acid from the urine of rabbits administered the aglycons. Isolation of the former was successful but not of the latter. In the case of *p*-aminosalicylic acid, glucuronides fraction (this fraction contained the ester and ether glucuronides of *p*-aminosalicylic acid and *m*-aminophenyl glucuronide) was separated as the lead salts and regenerated with hydrogen sulfide according to a modification of the method of Kamil, Smith, and Williams.[3] The separated glucuronides fraction was chromatographed on a cellulose powder column using ethyl acetate–acetic acid–water (5:2:2) as an eluent. The free ester glucuronide of *p*-aminosalicylic acid was at first obtained in crystalline form by this method. Since the above isolation procedure was tedious and the free ester glucuronide was not obtained in good yield, the extraction technique with ethyl acetate was adopted. The free ester glucuronide was extracted from the syrupy solution of the separated glucuronides fraction in a minimum of water by several extraction with a large volume of ethyl acetate. The combined extracts were evaporated to dryness and the residue was crystallized from water. The free ester glucuronide melted at 145° (decomp.). The isolated free ester glucuronide of *p*-aminosalicylic acid was methylated with diazomethane and acetylated with boron trifluoride–acetic anhydride, and the methyl acetyl derivative was identified with authentic methyl (2-acetoxybenzoyl-4-acetamido-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate by mixed fusion and comparison of infrared spectra.

The glucuronides fraction of salicylic acid was also separated as the lead salt and regenerated with hydrogen sulfide. Isolation of the ester glucuronide of salicylic acid

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*1 Part XLI. H. Tsukamoto, K. Oguri, T. Watabe, H. Yoshimura : J. Biochem., **55**, 394 (1964).

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1) H. Tsukamoto, A. Yamamoto, O. Kamata : This Bulletin, **5**, 565 (1957).

2) D. Robinson, R. T. Williams : Biochem. J., **62**, 23 (1956).

3) I. A. Kamil, J. N. Smith, R. T. Williams : *Ibid.*, **50**, 235 (1951).

from the separated glucuronides fraction by cellulose powder column chromatography using isopropylalcohol–acetic acid–water (5:2:1) and isopropylalcohol–ethanol–water (4:1:2) as eluent was unsuccessful, and other attempts to crystallize this glucuronide also failed. Therefore, it was isolated as a methyl acetyl derivative. The separated glucuronides fraction was submitted to successive extraction with ether. The ether extract contained the ester glucuronide predominantly. This partially purified ester glucuronide was methylated with diazomethane and acetylated with pyridine–acetic anhydride. The resulting gum of the methyl acetyl derivative was chromatographed on an acid treated alumina column using benzene as an eluent. The effluent was evaporated to dryness and the residue was crystallized from ethanol. This compound melted at 139~140°, and was identified with synthetic methyl (2-acetoxybenzoyl-2, 3, 4-tri-O-acetyl-β-D-glucopyranosid)uronate by mixed fusion and comparison of infrared spectra.

Table I. Rf Values of Glucuronides

Compound	Solvent system			
	A	B	C	D
p-Aminosalicylic acid				
Ester glucuronide	0.28	0.58	—	—
Ether glucuronide	0.17	0.33	—	—
m-Aminophenyl glucuronide	0.14	0.21	—	—
Salicylic acid				
Ester glucuronide	0.69	—	0.70	0.81
Ether glucuronide	0.56	—	0.55	0.67

Experimental

Paper Chromatographic Method——Ascending development was employed with Toyo Roshi No. 50. Solvent system employed were (A) BuOH–AcOH–H_2O (4:1:5), (B) AcOEt–AcOH–H_2O (5:2:2), (C) iso-PrOH–AcOH–H_2O (5:2:1) and (D) iso-PrOH–EtOH–H_2O (4:1:2). Metabolites were detected by spraying the following reagents on the chromatogram : (1) Ehrlich reagent, 2% *p*-dimethylaminobenzaldehyde in MeOH–conc. HCl (3:1); (2) 2% $FeCl_3$; (3) aniline phthalate; (4) $NaIO_4$–benzidine.[4]

The ester and ether glucuronides of *p*-aminosalicylic acid and *m*-aminophenyl glucuronide were detected with the reagent (1). The ester glucuronide of salicylic acid was detected with the reagents (2), (3), and (4). The ether glucuronide of salicylic acid was detected with the reagent (4).

Isolation of the Ester Glucuronide of *p*-Aminosalicylic Acid——The animal used were male rabbits weighing 2.6~3.0 kg. A solution of sodium *p*-aminosalicylate (4.5 g.) in H_2O (20 ml.) was administered *via* stomach tube to each rabbit. The 24 hr. urine of 9 rabbits was collected and the decomposition of the ester glucuronide was prevented by the addition of glacial AcOH. The collected urine was filtered through cotton wool, brought to pH 4 with glacial AcOH, and then treated with satd. $Pb(OAc)_2$ solution until precipitation was complete. The precipitate was removed by filtration. The filtrate was brought to pH 7.0 with NH_4OH to which satd. $Pb(OAc)_2 \cdot Pb(OH)_2$ solution was added in excess. The basic lead precipitate was filtered off, washed with H_2O, made into a fine suspension in MeOH, and Pb was removed by saturation with H_2S. After removal of PbS, MeOH solution was evaporated to dryness under reduced pressure at 20~25°. The residue was dissolved in 70 ml. of H_2O and insoluble material was filtered off. The filtrate was extracted with Et_2O to remove *p*-aminosalicylic acid and *p*-acetamidosalicylic acid and evaporated to dryness under reduced pressure at 25~30°. The resulting red colored gum was treated with 20 ml. of EtOH and filtered to remove an insoluble material. To this solution 50% $Ba(AcO)_2$ solution was added until precipitation was complete. The precipitate was collected by filtration and washed repeatedly with EtOH. The Ba salt of glucuronide was dissolved in H_2O and the solution was filtered. The filtrate was brought to pH 7.0 to which satd. $Pb(OAc)_2 \cdot Pb(OH)_2$ was added in excess. The basic lead precipitate was collected by filtration, washed with H_2O, made into a fine suspension in MeOH, and Pb was removed by saturation with H_2S. After removal of PbS, MeOH solution was evaporated to

4) D. F. Mowery : Anal. Chem., 29, 1560 (1957).

dryness under reduced pressure at 20°. Paper chromatography indicated that the yielded red gum mostly consisted of the ester glucuronide of *p*-aminosalicylic acid. This gum was dissolved in a minimum volume of H_2O to which 150 ml. of AcOEt was added. The stoppered flask was shaken vigorously and, after standing for some time, the AcOEt layer was separated from the glucuronides syrup by decantation. This extraction procedure was repeated 4 times with 150 ml. of AcOEt. When the syrup lost the liquidity, a minimum volume of H_2O was added again after decantation of AcOEt layer. The combined extracts were evaporated to dryness under reduced pressure and the pale brown powder was obtained. This powder was crystallized from H_2O. Yield, 0.58 g. After recrystallization from H_2O, it melted at 145° (decomp.), $[\alpha]_D^{14}$ +14.0 (c=1.0, EtOH). *Anal.* Calcd. for $C_{13}H_{15}O_9N$: C, 47.41; H, 4.56; N, 4.25. Found: C, 47.14; H, 4.81; N, 4.15. After hydrolysis of this compound with 5% NH_4OH at 60° for 30 min., *p*-aminosalicylic acid and glucuronic acid were detected by paper chromatography. This compound was completely hydrolyzed by β-glucuronidase.

Methylation and Acetylation of the Ester Glucuronide of *p*-Aminosalicylic Acid——In a small volume of MeOH was dissolved 0.2 g. of the ester glucuronide of *p*-aminosalicylic acid to which 15 ml. of Et_2O solution of CH_2N_2 was added. The mixture, after standing at room temperature for 1 hr., was evaporated to dryness under reduced pressure. The crystalline residue was dried over P_2O_5 *in vacuo*. To the suspension of this residue in 1 ml. of Ac_2O was added 0.2 ml. of BF_3 in Et_2O (47%). The mixture, after standing at room temperature for 1 hr., was poured into ice H_2O with stirring. The precipitate was collected by filtration and triturated with a small volume of MeOH. The resulting powder was filtered off and crystallized from MeOH-H_2O. Recrystallization from MeOH-H_2O yielded 0.07 g. of white crystals, melting at 190~191°, $[\alpha]_D^{14}$ −38° (c=1.0, $CHCl_3$). *Anal.* Calcd. for $C_{24}H_{27}O_{14}N$: C, 52.06; H, 4.91; N, 2.53. Found: C, 51.83; H, 5.14; N, 2.61. There was no depression of the melting point when this compound was mixed with authentic methyl (2-acetoxybenzoyl-4-acetamido-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate and its IR spectrum was identical with that of the authentic specimen.

Methyl (2-Acetoxybenzoyl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate——To a solution of 1 g. of methyl (1-bromo-2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate in 10 ml. of dehyd. MeCN, 1.5 g. of silver salicylate was added. The mixture was shaken for 30 min. After removal of the precipitate, the filtrate was concentrated under reduced pressure. The residue was extracted with hot EtOH. The EtOH solution was filtered, treated with carbon, and then concentrated to a small volume. The concentrated solution was filtered and, after cool, a crystalline product was obtained therefrom (0.8 g.). An additional yield of 0.2 g. was obtained from the mother liquor. The combined crops were recrystallized from EtOH to needles, m.p. 140~141°. Yield, 0.8 g. After recrystallization from EtOH, the product melted at 142~143°. *Anal.* Calcd. for $C_{22}H_{24}O_{13}$: C, 53.23; H, 4.84. Found: C, 53.36; H, 4.89.

Isolation of the Ester Glucuronide of Salicylic Acid as the Methyl Acetyl Derivative——The animals used were 20 male rabbits weighing 2.6~3.5 kg. A solution of sodium salicylate (1.2 g.) in H_2O (20 ml.) was administered *via* stomach tube to each rabbit. The separation of glucuronides was performed by the method described in an earlier paper from this laboratory.[5] The yielded glucuronides gum was dissolved in a minimum of H_2O, neutralized with $NaHCO_3$ to which EtOH was added until precipitation was complete. The precipitate was collected by filtration and washed with EtOH. The Na salt of glucuronides was dissolved in H_2O and the solution was filtered. The filtrate was brought to pH 7.0 to which satd. $Pb(OAc)_2$ solution was added in excess. The basic lead precipitate was collected by centrifugation, made into a fine suspension in MeOH, and Pb was removed by saturated with H_2S. After removal of PbS by filtration, MeOH solution was evaporated to dryness under reduced pressure at 15°. The resulting pale yellowish gum (2.5 g.) was dissolved in 50 ml. of H_2O and the solution was extracted with Et_2O successively for 12 hr. The Et_2O extract was evaporated to dryness under reduced pressure, and this residue mostly consisted of the ester glucuronide of salicylic acid (0.7 g.). To the solution of this residue in a small volume of MeOH 50 ml. of Et_2O solution of CH_2N_2 was added. The mixture, after a standing overnight in a refrigerator, was evaporated to dryness under reduced pressure. The residue, after drying over P_2O_5 *in vacuo*, was dissolved in 5 ml. of pyridine and to the resulting solution 3.5 ml. of Ac_2O was added. After the mixture was allowed to stand at room temperature for 3 days, it was poured into ice H_2O with stirring and extracted 3 times with Et_2O. The combined extracts were washed successively with 1% HCl, $NaHCO_3$ solution, and H_2O, dried over anhyd. $CaCl_2$ and then evaporated to dryness under reduced pressure. The yielded gum was passed through a column of acid treated Al_2O_3 using benzene as an eluent. 2.5 g. of Al_2O_3 which was treated with 10% HCl, washed with H_2O until pH 4.0, and dried at 110°, was packed in this column. The effluent was evaporated to dryness under reduced pressure. The residue was crystallized from a small volume of EtOH after standing in a refrigerator. The crystalline compound melted at 138~140°. This compound when mixed with authentic methyl (2-acetoxybenzoyl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate showed no depression of melting point and its IR spectrum was identical with that of the authentic specimen.

5) H. Tsukamoto, K. Kato, K. Tatsumi: This Bulletin, **5**, 570 (1957).

The authors are indebted to Miss Kishi and Miss Tominaga for their assistance in the experimental work. Thanks are also due to Mr. Matsui for spectral analysis, and to Miss Indo for microanalysis.

Summary

1. The ester glucuronide of *p*-aminosalicylic acid was isolated from the urine of rabbits administered with *p*-aminosalicylic acid. The methyl acetyl derivative of this compound was identified with synthetic methyl (2-acetoxybenzoyl-4-acetamido-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate.

2. The ester glucuronide of salicylic acid was isolated as methyl (2-acetoxybenzoyl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate from the urine of rabbits administered with salicylic acid, and the structure was confirmed with the synthetic compound.

(Received December 18, 1963)

[Chem. Pharm. Bull. 12 (6) 734 ~ 737]

UDC 612.015.34 : 577.154

Hisao Tsukamoto, Keitaro Kato, Kazuo Yoshida, and Kiyoshi Tatsumi : Metabolism of Drugs. XLIII.*1 Comparison of Glucuronyl Transfer Activity between β-Glucuronidase and Uridine Diphosphate Transglucuronylase.

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Glucuronide formation is one of the most important detoxication mechanism in animal body and has been understood to be catalyzed by uridine diphosphate transglucuronylase which was first described by Dutton and Storey.[1] On the other hand, Fishman and Green have provided another conjugation mechanism, in which β-glucuronidase catalyzes the transfer of the glucuronyl group from ethereal glucuronides to acceptor alcohols.[2]

While the former mechanism is acceptable for formation of all kinds of glucuronides, the latter involves certain limitation for its general application, since β-glucuronidase have been shown to catalyze the transfer to alcohols, but not to phenolic acceptor when ethereal glucuronides were used as the donor substrate.

The present investigation has been undertaken to recheck the previous results described above and at the same time, to decide which is a preferential mechanism for glucuronide formation in animal body by comparing the activity of both systems, in which the same acceptors were used. For this purpose, the several phenolic compounds, such as 2-naphthol, *p*-cresol, *p*-nitrophenol, *m*-aminophenol, and *p*-aminosalicylic acid were employed as an acceptor and shown to be transfered glucuronyl group from uridine diphosphate glucuronic acid by the former mechanism, but not from phenolphthalein β-D-glucuronide or *p*-nitrophenyl β-D-glucuronide by β-glucuronidase system.

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*1 Part XLII. H. Tsukamoto, K. Kato, K. Yoshida : This Bulletin, 12, 731 (1964).

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1) *a*) G. J. Dutton, I. D. E. Storey : Biochem. J., **57**, 275 (1954); *b*) G. J. Dutton : *Ibid.*, **64**, 693 (1956).

2) W. H. Fishman, S. Green : J. Biol. Chem., **225**, 435 (1957).