UDC 615.752-092.21

98. Hisao Tsukamoto, Keitaro Kato, and Kiyoshi Tatsumi: Metabolism of Drugs. XV.[1] Isolation and Paper Chromatography of Urinary Glucuronide of Salicylic Acid in the Rabbit.

(*Pharmaceutical Institute, Medical Faculty, University of Kyushu**)

Salicylic acid and its derivatives have been widely used in medicine. There is consequently an extensive literature on the metabolism and excretion of salicylates. The occurrence of conjugated glucuronic acid in the urine of man and animals receiving salicylic acid has been known for a considerable time.

Using ^{14}C-labeled salicylic acid, Alpen, *et al.*[2] demonstrated the existence of both ether- and ester-types of glucuronides in human urine, and the ether-type in dog urine. Robinson and Williams[3] isolated the ester glucuronide of salicylic acid as methyl (*o*-acetoxybenzoyl-tri-*O*-acetyl-*β*-D-glucopyranosid)uronate from the urine of a man receiving salicylic acid, and the ether glucuronide of methyl salicylate as methyl (*o*-methoxycarbonylphenyl-tri-*O*-acetyl-*β*-D-glucopyranosid)uronate from the urine of rabbits fed with methyl salicylate. However, they failed to isolate crystalline derivative of glucuronide from the urine of rabbits receiving salicylic acid.

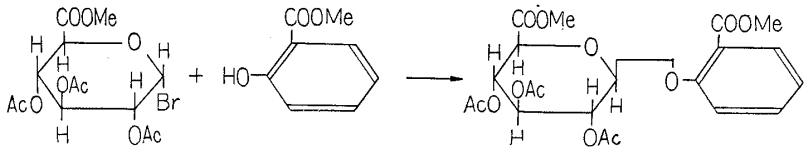
The present investigation is a study on the glucuronide formation in the metabolism of salicylic acid using rabbits. Both types of glucuronides were detected by paper chromatography in the urine of rabbits receiving salicylic acid and the ether type was isolated as methyl (*o*-methoxycarbonylphenyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosid)uronate.

Methods and Results**

Paper Chromatographic Method—Ascending development was employed with Toyo Roshi No. 50. Solvent system used was BuOH-AcOH-H_2O (4 : 1 : 5). Metabolites were detected on paper chromatograms by spraying with the following reagents: (1) 1% $NaIO_4$, then 1% $KMnO_4$, followed by benzidine reagent[4]; (2) 2% $FeCl_3$; and (3) aniline phthalate.[5] Rf values and color reactions are listed in Table I.

Methyl (*o*-Methoxycarbonylphenyl-tri-*O*-acetyl-*β*-D-glucopyranosid)uronate—This was prepared by the method of Lunsford and Murphey,[6] in which quinoline was used instead of isoquinoline. The oily substance obtained from the reaction mixture as described in the above paper was dissolved in *iso*-PrOH, treated activated charcoal, and recrystallized from *iso*-PrOH to needles, m.p. 111~113°; $[\alpha]_D^{29}$ −44.6°(c=1.5, $CHCl_3$). Yield, 0.8 g. (34%). *Anal.* Calcd. for $C_{21}H_{24}O_{12}$: C, 53.85; H, 5.16. Found: C, 53.69; H, 5.46.

The compound gave a negative $FeCl_3$ test and did not reduce the Fehling solution. $FeCl_3$ test was positive after warming in 10% HCl.



* Katakasu, Fukuoka (塚元久雄, 加藤敬太郎, 辰己 淳).

** All melting points are uncorrected.

1) Part XIV. H. Tsukamoto, A. Yamamoto: This Bulletin, **5**, 565(1957).
2) E. L. Alpen, H. G. Mandel, V. W. Rodwell, P. K. Smith: J. Pharmacol., **102**, 105(1951),
3) D. Robinson, R. T. Williams: Biochem. J. (London), **62**, 23(1956).
4) M. L. Wolfrom, J. B. Miller: Anal. Chem., **28**, 1037(1956).
5) S. M. Partrige: Nature, **164**, 443(1949).
6) C. D. Lunsford, R. S. Murphey: J. Org. Chem., **21**, 580(1956).

Separation of Glucuronide from the Urine of Rabbits—The animals used were male rabbits weighing 2.5~3.5 kg. They were housed in metabolism cages and fed "Okara" (soybean curd residue) only. Sodium salicylate (0.4 g./kg. body wt. as salicylic acid) was administered by stomach tube as a 9% (w/v) solution. To separate glucuronides from the urine, the method of Kamil, Smith, and Williams[7] was used. The 24-hr. urine of 5 rabbits was filtered through cotton wool and brought to about pH 4 with glacial AcOH and then treated with saturated aqueous lead acetate until precipitation was complete. The precipitate was discarded by filtration. The filtrate was brought to about pH 8 with a little NH_4OH and saturated aqueous basic lead acetate added in excess. The basic lead precipitate was filtered off, washed with water, made into a fine suspension in MeOH, and the lead removed by saturation with H_2S. After removal of PbS by filtration, MeOH solution of glucuronides was evaporated to dryness at 20~25° under reduced pressure. The residue was dissolved in a small volume of water and extracted with Et_2O to remove salicylic and gentisic acids. The aqueous solution was evaporated to dryness at 25° under reduced pressure and a red colored gum was obtained.

Paper Chromatography of the Gum of Glucuronides—When the gum was developed on paper strips for 16 hrs., the chromatogram indicated 4 blue spots (Rf 0.69, 0.28, 0.24, and 0.11) and 3 violet spots (Rf 0.69, 0.28, and 0.24) on spraying the reagent (1) and (2), respectively. The spot of Rf 0.11 was also obtained from the gum of normal urine.

To detect the glucuronide, the gum was developed on a large filter paper (40×40 cm.) using the same solvent. The zone of Rf 0.69, which was previously tested by color reagents, was cut out, eluted with H_2O, and evaporated to dryness under reduced pressure (material A). The zone of Rf 0.28 and 0.24 was cut out together, because these Rf values were very near, and treated as above (material B).

(A) and (B) again indicated the same spots by paper chromatography as before the elution and both gave a positive naphthoresorcinol test. When (A) was treated with 0.1% NaOH at 55° for 1 hr.,[8] salicylic acid was obtained as crystals and by paper chromatography. The spot corresponding to salicylic acid (Rf 0.88) was detected using the reagent (2) and the spot corresponding to glucuronic acid (Rf 0.12) was detected by the reagent (3). Both spots were detected by the reagent (1).

(B) was resistant to alkali hydrolysis. After hydrolysis with 5% HCl at 80° for 1 hr., the spot of unchanged (A) still remained and (A) was partially decomposed into salicylic acid and glucuronolactone (Rf 0.32), which were identified by paper chromatography. After acid hydrolysis of (B) under the same conditions as above, the paper chromatogram indicated 2 spots corresponding to salicylic acid and gentisic acid (Rf 0.79) when sprayed with the reagent (2), and one brown spot corresponding to glucuronolactone when sprayed with the reagent (3). These 3 spots were detected together by the reagent (1).

Table I. Rf Values and Color Reactions of the Metabolites contained in the Glucuronide Fraction

Reagent / Rf	(1) $NaIO_4$, $KMnO_4$, benzidine	(2) $FeCl_3$	(3) Aniline phthalate	(4) conc. HCl-EtOH, $FeCl_3$
0.69	blue	violet	pink	—
0.56	blue	colorless	colorless	violet
0.28	blue	violet	colorless	—
0.24	blue	violet	colorless	—
0.11	blue	colorless	colorless	—

Purification of the Gum of Glucuronides—The gum of glucuronides obtained from 5 rabbits was dissolved in a minimum of EtOH and Et_2O added until precipitation no longer occurred. The precipitate was removed by filtration and the filtrate was evaporated to dryness under reduced pressure. The residue was a reddish gum. Paper chromatography of this gum showed the presence of a new substance whose Rf value was 0.56. In this case, the spot of Rf 0.69 still remained and the spots of Rf 0.28, 0.24, and 0.11 all disappeared. The spot of Rf 0.56 was detected with the reagent (1) and gave a negative $FeCl_3$ test.

This gum was dissolved in a small volume of EtOH and 50% aqueous $Ba(AcO)_2$ was added until precipitation was complete. The precipitate was collected by filtration, washed repeatedly with EtOH, and dried over anhyd. $CaCl_2$ *in vacuo*, giving a brownish white powder of Ba salt of glucuronides (1.4 g.). It was dissolved in water and the solution was filtered. The filtrate was brought to about pH 7 and saturated aqueous basic lead acetate added in excess. The basic lead precipitate was washed by centrifugation, made into a fine suspension in MeOH, and Pb removed by satura-

7) I. A. Kamil, J. N. Smith, R. T. Williams: Biochem. J. (London), **50**, 235(1951).
8) Y. Shirai, T. Ohkubo: J. Biochem. Japan, **41**, 337(1954).

tion with H_2S. After removal of PbS by filtration, MeOH solution of glucuronides was evaporated to dryness under reduced pressure at 15° and dried over P_2O_5 *in vacuo*. A pale yellowish glucuronide (0.9 g.) was obtained.

Paper Chromatography of Purified Glucuronides—Paper chromatographic examination of the purified glucuronides showed 2 spots at Rf 0.69 and 0.56. The spot of Rf 0.69, which gave a positive $FeCl_3$ test and was hydrolyzed to salicylic acid and glucuronic acid with alkaline solution as mentioned above, seemed to be ester glucuronide of salicylic acid. The spot of Rf 0.56 was detected by the reagent (1) and gave a positive $FeCl_3$ test after spraying a mixture of conc. HCl-EtOH (1 : 2) and heating at 70° for 5~10 mins. The purified glucuronides were developed on a large filter paper; the zone of Rf 0.56 was cut out similarly and treated as above (material C). When (C) was treated with 5% HCl at 80° for 1 hr., salicylic acid was obtained as crystals and by paper chromatography 2 spots corresponding to salicylic acid and glucuronolactone were detected. On the other hand, the spot of Rf 0.56 was resistant to alkali hydrolysis. From these experimental facts, this spot seemed to be ether glucuronide of salicylic acid.

Isolation of Methyl (*o*-Methoxycarbonylphenyl-tri-*O*-acetyl-β-D-glucopyranosid)uronate—The purified glucuronide was dissolved in 20 cc. of MeOH and an ether solution of CH_2N_2, freshly prepared from 5 g. of nitrosomethylurea, was added. The mixture was allowed to stand overnight in a refrigerator and a small amount of precipitate which formed was removed by filtration. The filtrate was evaporated to dryness under reduced pressure and yielded pale orange material. It was dissolved in 5 cc. of pyridine, 3.5 cc. of Ac_2O was added, the mixture was allowed to stand overnight at room temp., and then stirred into 50 cc. of ice water. After the mixture had been allowed to stand overnight, the pale brown precipitate was collected and dissolved in Et_2O. Et_2O solution was washed with 2% HCl and water, dried over anhyd. Na_2SO_4, and dried under reduced pressure. The residue was crystallized from EtOH after standing overnight in a refrigerator. Yield, 0.23 g. Colorless needles, m.p. 100~105°. After recrystallization from *iso*-PrOH, m.p. 111~113°; $[\alpha]_D^{29}$ −43.3°(c=1.5, $CHCl_3$). *Anal.* Calcd. for $C_{21}H_{24}O_{12}$: C, 53.85; H, 5.16. Found: C, 53.96; H, 5.44.

When mixed with authentic methyl (*o*-methoxycarbonylphenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid) uronate, the melting point was not depressed. This compound is non-reducing and gives a positive $FeCl_3$ test after acid hydrolysis.

Discussion

The spots of Rf 0.28 and 0.24 contained salicylic acid, gentisic acid, and glucuronic acid after acid hydrolysis and were resistant against alkali hydrolysis. Thus, if a glucuronide was contained in these two spots, it would be the ether type, and the ether glucuronide of salicylic acid ought to give a negative ferric chloride test.

For above reasons the glucuronic acid seems to be conjugated with gentisic acid in an ether-type glucuronide. However, it is not possible to decide which of the spots corresponds to the ether glucuronide of gentisic acid.

Robinson and Williams[3] had failed to isolate a crystalline derivative of glucuronide from the urine of rabbits receiving salicylic acid. In our laboratory, in spite of repeating the experiment, no crystalline material was isolated from the gum obtained by the method of Kamil, Smith, and Williams,[7] and the crystalline material was not isolated until the gum was purified as barium salt. From the pure glucuronide fraction, the isolation of ether glucuronide as methyl (*o*-methoxycarbonylphenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)uronate was successful, and the structure was confirmed with the synthetic compound, but the isolation of ester glucuronide have so far been unsuccessful and now further investigation is proceeding.

The authors are indebted to Miss S. Tada for the elemental analysis.

Summary

Both ether and ester glucuronides of salicylic acid were detected by paper chromatography in the urine of rabbits receiving salicylic acid. The ether glucuronide was isolated as methyl (*o*-methoxycarbonylphenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)uronate and the structure was confirmed with the synthetic compound.

(Received August 12, 1957)