UDC 591.133.2 : 547.592.1'314.2

27. Toshiro Murata : Metabolic Fate of 1-Ethynylcyclohexyl Carbamate. II.[1] Studies on the Glucuronide excreted in the Urine of Humans receiving 1-Ethynylcyclohexyl Carbamate.

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In the preceding paper, the author suggested the presence of a glucuronide of the metabolite in the urine of man who received 1-ethynylcyclohexyl carbamate (ECC). This paper deals with studies on the glucuronide formation in the metabolism of ECC.

Increased excretion of urinary glucuronic acid in the urine from rabbits administered with ECC was observed and the experiment suggested the formation of a glucuronide in the metabolism of ECC *in vivo*. Two kinds of supposed metabolites of ECC are able to form the glucuronides. One is monohydroxy-ECC which was isolated by the author and McMahon,[2] and the other is 1-ethynylcyclohexanol which can be prepared by hydrolysis of ECC, but its presence in the metabolites of ECC has never been ascertained.

In the present series of work, separation of glucuronide from urine of man who received ECC was attempted and a glucuronide was identified by paper chromatography and was proved to be that of OH-ECC.

Experimental

Excretion of Glucuronic Acid in the Urine of Rabbits after Administration of ECC—Rabbits weighing 2~3 kg. were administered with 0.5 g. of ECC in the form of a suspension in 0.2% sodium alginate solution by means of stomach tube.

Estimation of urinary glucuronic acid was carried out according to the method described by Ishidate and Nambara.[3] The results are shown in Table I. Remarkable increase in the amount of urinary glucuronic acid was observed after administration of ECC.

Table I. Excretion of Glucuronic Acid in the Urine of Rabbit

Rabbit No.	Average excretion of glucuronic acid (mg./day)	Excreted glucuronic acid after administration of ECC (mg.)				
		1~3 hr.	3~6 hr.	6~8 hr.	8~24 hr.	Total
1	20.95	56.63	12.33	37.07	113.10	219.13
2	39.67	61.56	68.43	33.39	174.49	337.87
3	16.51	.....110.30.....			59.08	170.08
4	99.67	60.66	31.76	22.53	69.00	183.95
5	79.73	21.90	80.55	52.08	41.09	195.62

Separation of the Glucuronide from Human Urine after receiving ECC—Urine sample of man who received 1.0~2.0 g. of ECC orally was collected for 8 hr. after administration of ECC. The urine was adjusted to about pH 4.0 with glacial AcOH, treated with saturated $(AcO)_2Pb$ solution until no further precipitation occurred, and the precipitate was discarded by filtration. The filtrate (1) was brought to about pH 8.0 with NH_4OH and saturated basic lead acetate solution was added in excess. The basic lead salt that precipitated was filtered off, washed with water and MeOH, made into a fine suspension in MeOH, and Pb was removed by saturation with H_2S. After removal of PbS by filtration, MeOH was evaporated to dryness at 20~25° under a reduced pressure. The residue was dissolved in a small amount of water, insoluble substances were removed by filtration, and the filtrate (2) was extracted with Et_2O until Et_2O layer no longer colored. The aqueous solution (3) was evaporated to dryness at 20~25° under a reduced pressure and a dark brown, gummy substance was obtained.

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1) Part I. T. Murata : This Bulletin, 8, 629 (1960).
2) R. E. McMahon : J. Am. Chem. Soc., 80, 411 (1958).
3) M. Ishidate, T. Nambara : This Bulletin, 5, 515 (1957).

The gummy substance was dissolved in a small volume of EtOH and insoluble substance was filtered off. To the filtrate (4), 50% $(AcO)_2Ba$ solution was added until precipitation was complete. The precipitate was collected by filtration, washed with EtOH, and dried over anhyd. $CaCl_2$ *in vacuo*. The brownish white powder so obtained was dissolved in a small amount of water and filtered. The filtrate was brought to about pH 7.0 and a saturated basic lead acetate solution was added to the solution in excess. The precipitate produced was collected, washed with water and MeOH, and dried over anhyd. $CaCl_2$ *in vacuo*. The slightly yellow powder was made into a fine suspension in MeOH and Pb was removed by saturation with H_2S. After removal of PbS by filtration, yellow glucuronide solution (5) was evaporated to dryness under a reduced pressure at 15° and dried over P_2O_5 *in vacuo*. A pale yellowish, very hygroscopic glucuronide was obtained.

Paper Chromatography of the Glucuronide Solutions—The solutions (1) to (5), obtained in the course of procedure described above, were developed on paper strips for 20 hr. by using the solvent system of BuOH–AcOH–H_2O (4:1:5). The glucuronides of the metabolite were detected with (A) Tollens' reagent[1] and (B) $NaIO_4$ reagent, followed by spraying with benzidine reagent prepared according to the method of Horrocks.[4] Rf values are listed in Table II.

Table II. Paper Chromatography of the Glucuronide Solution from Procedure of Separation

Soln. No.	Reagent	Rf value						
(1)	(A)	0.09	0.15	0.21	0.25	0.36	0.41	0.46
	(B)	0.09	0.15	0.21	0.31		0.41	
(2)	(A)		0.15	0.21	0.25	0.36		0.46
	(B)		0.15	0.21	0.25	0.36	0.41	
(3)	(A)			0.21	0.25	0.36	0.41	0.46
	(B)			0.21	0.25		0.41	
(4)	(A)			0.21		0.36	0.41	0.46
	(B)			0.21		0.36		
(5)	(A)			0.21		0.36		
	(B)			0.21				

Hydrolysis of the Glucuronide with β-Glucuronidase—Partially purified glucuronide was dissolved in a small amount of water and the aqueous solution was incubated with β-glucuronidase and 0.1*M* acetate buffer (pH 4.5) at 38° for 2 hr. After incubation, the mixture was boiled in a water bath for 2 min., protein that precipitated was removed by centrifugation, the supernatant was decanted, and evaporated to dryness *in vacuo* at 30°. The residue was extracted with a few drops of water and the extract was developed on paper along with known samples. The spot at Rf 0.21 became indistinguishable and appearance of a new spot (Rf 0.12~0.14) was observed by spraying both reagents (A) and (B). The Rf value of the spot was identical with that of glucuronic acid.

The centrifuged protein precipitate was extracted with CH_2Cl_2, the extract was evaporated, the residue was dissolved in a small amount of EtOH, and developed on paper. One yellow spot at Rf 0.81 was detected by spraying with reagent (A) and the color and Rf value of this spot well agreed with those of OH-ECC.

Discussion

The result of estimation of the urinary glucuronic acid from rabbits administered with ECC strongly suggested glucuronide excretion. Thus, separation of the glucuronide from urine of man who received ECC orally was carried out by formation of basic lead or barium salt of the glucuronide. Paper chromatography of each fraction in the fractionation steps showed the presence of one common substance whose Rf value was 0.21. This compound was purified by the succeeding separation procedure and the substance obtained was strongly reactive with naphthoresorcinol reagent and both reagents (A) and (B). This substance was hydrolyzed by treatment with β-glucuronidase and the glucuronogenin was identified as OH-ECC by paper chromatography.

4) R. H. Horrocks : Nature, **164**, 444 (1949).

These findings showed the substance of Rf 0.21 to be the glucuronide of OH-ECC.

The author expresses his deep gratitude to Prof. H. Tsukamoto of the University of Kyushu for his kind guidance and to Prof. T. Ukita of the University of Tokyo for his encouragement throughout the course of this work.

Summary

Marked increase of urinary glucuronic acid was observed in rabbits administered with ECC. A glucuronide of the metabolite of ECC was separated from urine of man who received ECC orally and it was identified as the glucuronide of OH-ECC by the reaction of β-glucuronidase and subsequent paper chromatography.

(Received November 25, 1960)