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Studies on Metabolism of Drugs. XI.[1] A New Metabolite of Sulfaphenazole in Man. (2)

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In order to elucidate the unknown metabolite found in the human urine after ingestion of sulfaphenazole, the urine was treated with Dowex 2 and 50, purified by basic lead acetate treatment, and finally separated by paper chromatography. The product was obtained as colorless prisms of mp 185—188° (decomp.). The ring-N-glucuronide of sulfaphenazole was acetylated with acetic anhydride and pyridine, and purified through paper and column chromatography over Dowex 50W-X4. The colorless crystals thereby obtained were identical with the extracted product in mp, elemental analysis, paper chromatographic behavior, and ultraviolet and infrared spectra. Therefore, this metabolite was found to be ammonium 1-deoxy-[1-phenyl-5-(N^4-acetylsulfanilamino)-3-pyrazoline-2-yl]-D-glucopyranosiduronate with three moles of water of crystallization. It was assumed that sulfaphenazole is first acetylated at N^4-position and then the ring-N-glucuronide is formed *in vivo*.

Sulfaphenazole is one of long-duration sulfa drugs now being used widely, was discovered by Schmidt and Druey,[3] and has been examined in clinical application.[4] It has a characteristic structure, with a phenylpyrazole ring in N^1-position of sulfanilamide. Because of this characteristic structure and the absence of unchanged sulfaphenazole and its N^4-acetylated compound in its metabolites in human ruine after ingestion of 2 g of sulfaphenazole, as evidenced by paper chromatography, this substance was considered to undergo metabolism different from other sulfa drugs. Previous paper of this series[5] reported the presence of ring-N-glucuronide in the metabolites of sulfaphenazole.

It was found through paper chromatography of the human urine developed with a solvent system of butanol–acetic acid–water (4: 1: 5) that an unknown metabolite was present at *Rf* 0.17, besides a spot of sulfaphenazole N-glucuronide at *Rf* 0.11, and this product colored with Ehrlich reagent after hydrolysis. This metabolite was therefore extracted and various examinations were made. As a result of these examinations and comparison with synthesized sample, this product was found to be a compound formed by acetylation of the amino group in the glucuronide.

Experimental

Detection of the Metabolite——One-dimensional Paper Chromatography: Toyo Roshi No. 51 filter-paper (2×40 cm). Developing solvents: BuOH–AcOH–H_2O (4:1:5), BuOH–AcOH–H_2O (4:1:2), BuOH–pyridine–H_2O (6:4:3), C_6H_5OH–H_2O–1N NH_4OH (79:20:1), BuOH–MeOH–H_2O (3:1:1), BuOH saturated with 3N NH_4OH.

1) Part X: M. Ueda, I. Takegoshi, and T. Koizumi, *Chem. Pharm. Bull.* (Tokyo), **19**, 2041 (1971).
2) Location: *3190 Gofuku, Toyama.*
3) P. Schmidt and J. Druey, *Helv. Chim. Acta*, **41**, 306 (1958).
4) E. Pasargikian, G. Ibba, and G. Pinna, *Merva Med.*, **1958**, 4699 [*C.A.*, **53**, 8410 (1959)[; N. Gargano and S. Grazi, *ibid.*, **1958**, 4707 [*C.A.*, **53**, 8410 (1959)]; G. Zangaglia, A. Ferrata, and F. Cambieri, *ibid.*, **1958**, 4713 [*C.A.*, **53**, 8410 (1959)]; G. Fontana, *ibid.*, **1958**, 4726 [*C.A.*, **53**, 8411 (1959)].
5) M. Ueda, N. Murakami, and Y. Nakagawa, *Chem. Pharm. Bull.* (Tokyo), **16**, 345 (1968).

Detecting Reagent: Ehrlich reagent[6] (for detection of amino group). Tsuda reagent[7] (for detection of aromatic amino group). Aniline phthalate reagent[8] (for detection of reducing sugar).

Preparation of Standard Sample——Sulfaphenazole (I): Commercial pure powder was used.

N^4-Acetylsulfaphenazole (II): Prepared by the method of Enoki.[9] Five grams of I was reduced to a fine powder, 10 g of Ac_2O was added to it, and the mixture was allowed to stand at room temperature with exclusion of moisture, with occasional stirring, by which the powder dissolved with evolution of heat and crystals precipitated out. After standing for 3 hr, the crystals were collected and recrystallized from MeOH to 4 g of crystals, mp 203—204°. II does not color with the Ehrlich reagent, and dissolves in 1N NH_4OH.

Sulfaphenazole 2′-Glucuronide (III): The product was extracted from human urine by the method previously described.[5]

Ammonium Glucuronate: A solution of 176 mg of special grade commercial glucuronic acid lactone, added with equimolar amount of NH_4OH, and made up to 100 ml with H_2O.

Extraction of Acetylated III from Human Urine——Urine (12 liters) was collected from 3 persons who had ingested 1 g of I every 12 hr for 3 days. This urine was passed through a column (diameter × length: 11×18 cm) of Dowex 2-X4 (50—100 mesh, OH form) and the column was washed with water. The resin was collected in a beaker, washed with H_2O, and returned to the original column. The resin column was then washed with 3% AcOH until the effluent became almost colorless. A column (3×12 cm) of Dowex 50W-X4 (100—200 mesh, H form) was connected below the column of Dowex 2. Elution of the columns with 10% AcOH resulted in desorption of the objective metabolite because it is not adsorbed by Dowex 50, and impurities were removed to a certain extent. The effluent was added with NH_4OH to near neutralization and solution was concentrated under a reduced pressure. This and all subsequent concentration procedures were carried out in N_2 stream at a temperature below 40°. The solution was adjusted to pH 4.5, 30% $(AcO)_2Pb$ was added and the precipitate formed was filtered off. The filtrate was brought to pH 7 with NH_4OH and 30% basic lead acetate was added. The precipitate formed was collected by filtration washed with H_2O, and dried. The powdered filter cake was triturated with 5% NH_4OH to a sludgy state and H_2S was blown into this sludge. PbS formed was removed by filtration and the filtrate was passed through a column (0.9×7 cm) of Dowex 50W-X4 (100—200 mesh, H form). The effluent was neutralized with NH_4OH and concentrated under a reduced pressure. The solution was streaked on eight pieces of H_2O-washed filter paper (40×40 cm) of Toyo Roshi No. 50, developed with BuOH–AcOH–H_2O (4: 1: 5), and dried in air. The parts of the filter papers with the metabolite (*Rf* 0.42) were cut out and extracted three times with H_2O. The extracted solution was passed through a column (0.7×2.5 cm) of Dowex 50W-X4 (100—200 mesh, H form), the effluent was neutralized with NH_4OH, and the solution was evaporated to dryness under a reduced pressure, affording 170 mg of colorless prisms, mp 185—188° (decomp.). *Anal.* Calcd. for $C_{23}H_{27}O_9N_5S\cdot 3H_2O$: C, 45.77; H, 5.51; N, 11.60. Found: C, 45.89; H, 5.47; N, 11.53. UV $\lambda_{max}^{H_2O}$ mμ (log ε): 272 (4.33). IR ν_{max}^{KBr} cm^{-1}: 1142 (SO_2), 1253 (C-N), 1313 (SO_2), 1670 (C=O), 3450 (OH).

Hydrolysis of Acetylated III——Acetylated III of 10 mg extracted as above was dissolved in 0.3 ml of 1N HCl. The solution was heated at 60° for 15 hr to effect hydrolysis and was neutralized with 2N NH_4OH. This neutralized solution was submitted to paper chromatography, with I and ammonium glucuronate solution as standards, and the chromatograms were compared by using the Ehrlich reagent for the detection of I and aniline phthalate reagent for ammonium glucuronate. The spots for I and ammonium glucuronate appeared at *Rf* 0.95 and 0.17, respectively when developed with BuOH–pyridine–H_2O (6: 4: 3), at *Rf* 0.91 and 0.26 with BuOH–AcOH–H_2O (4: 1: 2), and at *Rf* 0.95 and 0.14 with C_6H_5OH–H_2O–1N NH_4OH (79: 20: 1), and the *Rf* values obtained with the test sample and standard solution agreed completely.

Formation of Acetylated III——A solution of 140 mg of III dissolved in 1.4 ml of pyridine, added with 0.06 ml of Ac_2O, was allowed to stand in a water bath of 45° for 36 hr. This solution was dissolved in 20 ml of H_2O and passed through a column (1.0×6 cm) of Dowex 50W-X4 (100—200 mesh, H form). The effluent was concentrated under a reduced pressure to *ca.* 2.5 ml and this was streaked on five filter papers (40×40 cm) of Toyo Roshi No. 50, preliminarily washed with H_2O. The papers were developed with BuOH–AcOH–H_2O (4: 1: 5) until the solvent front reached the end of the filter paper. The papers of the filter paper containing the desired product (detected by Ehrlich reagent after hydrolysis with 10% HCl) were cut out, extracted three times with H_2O, and the aqueous extract was evaporated under a reduced pressure. This solution was again submitted to paper chromatography was above, developed with BuOH–MeOH–H_2O (3: 1: 1). The aqueous extract obtained from the developed chromatograms was passed through a column (0.5×4 cm) of Dowex 50W-X4 (100—200 mesh, H form) and the effluent was neutralized with NH_4OH. This solution was evaporated to dryness under a reduced pressure and 36 mg of colorless prisms, mp 185—188° (decomp.), was obtained. *Anal.* Calcd. for $C_{23}H_{27}O_9N_5S\cdot 3H_2O$: C, 45.77; H, 5.51; N, 11.60. Found: C, 45.59; H, 5.43; N, 11.74. UV $\lambda_{max}^{H_2O}$ mμ (log ε): 272 (4.33). IR ν_{max}^{KBr} cm^{-1}: 1142 (SO_2), 1253 (C-N), 1313 (SO_2), 1670 (C=O), 3450 (OH).

6) A.E.A. Werner, *Lancet*, **236**, 18 (1939); T. Uno and M. Ueda, *Yakugaku Zasshi*, **80**, 1785 (1960).
7) K. Tsuda and S. Matsunaga, *Yakugaku Zasshi*, **62**, 362 (1942).
8) S.M. Partridge, *Nature*, **164**, 443 (1949).
9) K. Enoki, *Yakugaku Zasshi*, **80**, 1735 (1960).

Result

The new metabolite of sulfaphenazole was extracted from urine of persons ingesting this sulfa drug, after treatment of the urine by column chromatography through Dowex 2-X4 and Dowex 50-X4, treatment of its effluent with lead acetate, and finally by paper chromatography. The product was obtained as colorless prisms of mp 185—188° (decomp.), which failed to color to the Ehrlich or Tsuda reagent but did color to the Ehrlich reagent after the paper chromatogram was sprayed with 10% hydrochloric acid and warmed at 60° for 1 hr. Hydrolysis of the metabolite with hydrochloric acid and neutralization of the reaction solution with ammonia gave positive reaction to aniline phthalate reagent. This solution was found to contain sulfaphenazole and glucuronic acid as a result of paper chromatography. Mild hydrolysis of the metabolite with hydrochloric acid or ammonia at *ca.* 40° resulted in gradual decomposition to sulfaphenazole 2′-glucuronide and then to sulfaphenazole. This evidence suggested that this metabolite is a derivative of sulfaphenazole 2′-glucuronide in which some group is bonded to its amino group. Acetylation of the extracted sulfaphenazole 2′-glucuronide and separation by paper chromatography gave a compound in which an acetyl group is bonded to N^4 position in sulfaphenazole 2′-glucuronide. This product showed no depression of mp on mixed fusion with the synthesized product and elemental analytical values of the two were identical. The *Rf* values of the product in paper chromatogram developed with BuOH–MeOH–H_2O, BuOH–AcOH–H_2O, and BuOH saturated with 3N NH_4OH were 0.23, 0.25, and 0.08, respectively, in the metabolic product and synthesized sample, and ultraviolet and infrared spectra of the two were also in good agreement (Fig. 1). Therefore, this new metabolite is ammonium 1-deoxy-[1-phenyl-5-(N^4-acetylsulfanilamino)-3-pyrazolin-2-yl]-D-glucopyranosiduronate with 3 moles of water of crystallization, and will be represented by the following formula.

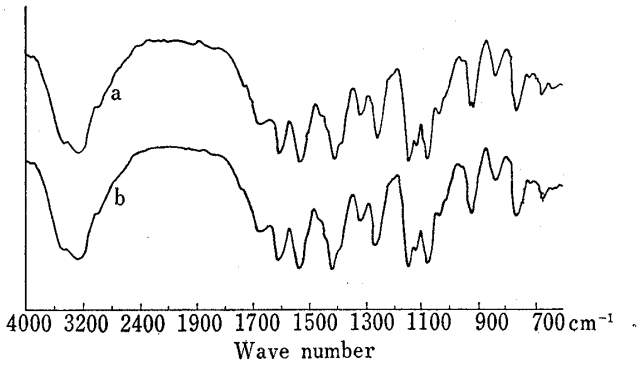
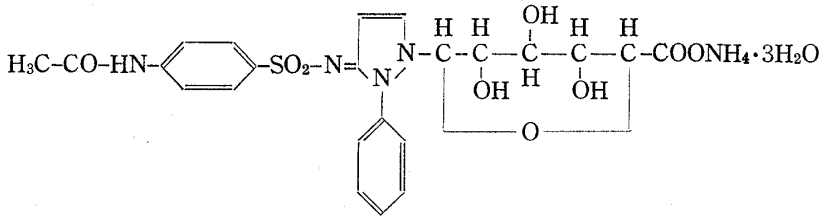


Fig. 1. Infrared Absorption Spectra (KBr)
a: synthetic sample b: extracted sample



Discussion

Because of its large molecule, it is adsorbed by X8 of Dowex 50 and 2, and can not be eluted out completely, so that X4 was used for its isolation. Metabolites of sulfa drugs obtained were usually a primary metabolite and this is the first example of a secondary metabolite of sulfas obtained. In order to see which of the metabolites, the acetylated compound or glucuronide, is formed first *in vivo*, 0.5 g of N^4-acetylsulfaphenazole was administered and examination of the metabolites showed the presence of a large amount of this new metabolite, without detection of the unchanged drug or sulfaphenazole. Ingestion of 0.5 g of sulfaphenazole 2′-glucuronide resulted in excretion of unchanged glucuronide alone and not

its acetylated derivative. This may be due to the fact that the glucuronide is soluble in water and is excreted rapidly, but it may be assumed that the ingested sulfaphenazole is first acetylated *in vivo* and then conjugates with glucuronic acid. It is interesting that ingestion of a usual dose of sulfaphenazole results in the excretion of the water-soluble 2′-glucuronide and its acetylated derivative, and not the unchanged sulfaphenazole or its acetylated compound, as would ordinarily be expected in the case of other sulfa drugs.

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