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93. Toyozo Uno and Yutaka Sekine: Studies on the Metabolism of Sulfadiazine. II.*1 Quantitative Separation of Excrements in the Human Urine after Administration of Sulfadiazine.

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In the first paper of this series, four kinds of metabolites except unchanged sulfadiazine, namely N^4-acetylsulfadiazine, sulfadiazine-N^4-glucuronide, sulfadiazine-N^4-sulfonate and sulfanilamide, were reported to be excreted in the human urine after administration of sulfadiazine. In an attempt to elucidate the metabolic fate of sulfadiazine in detail quantitative investigations concerning above mentioned metabolites were carried out.

The determination of the excrements in urine after administration of sulfadiazine has been carried out[1~4] only by Bratton-marshall's method and therefore little attention has been paid to the metabolites except unchanged sulfadiazine and N^4-acetylsulfadiazine. However, in the course of elucidating the metabolic fate of sulfadiazine in detail, other metabolites should not be neglected. From this point of view, the following method of determination was devised to determine each metabolite. In order to separate the metabolites in urine, paper chromatography was employed and after development, portions corresponding to the products were cut out, and eluted. Each of these substances was hydrolyzed and determined by the colorimetric method as sulfadiazine using the Ehrlich's reagent. The optimum conditions of hydrolysis were checked for each metabolite.

Experimental and Results

Reagents, Solvents and Apparatus

Ehrlich's reagent*3: 2% *p*-dimethylaminobenzaldehyde ethanol solution

Solvent A: BuOH-AcOH-H_2O (5:1:4)
Solvent B: BuOH saturated with 3% NH_4OH
Paper: Toyo-Roshi No. 51 40×40 cm.
Shimadzu Spectrophotometer Model QR 50

*1 Part I. T. Uno, H. Yasuda, Y. Sekine: This Bulletin, **11**, 872 (1963).
*2 Yoshida-konoe-cho, Sakyo-ku, Kyoto (宇野豊三, 関根 豊).
*3 For detection of the spots after development, a volume of 1/50 of conc. HCl was added to 2% *p*-dimethylaminobenzaldehyde ethanol solution.

1) R.H. Silber, I. Clark: Arch. Biochem., **10**, 9 (1946).
2) J.N. Smith, R.T. Williams: Biochem. J., **42**, 351 (1948).
3) M. Allinne: Ann. Pharm. franc., **4**, 56 (1946).
4) G.A. Ponomarev: Formakal i Toksikol, **9**, No. 1, 13 (1946); Chem. Abstr., **41**, 1333 (1947).

Method of Separation

One tenth ml. of sample urine which was collected at regular intervals after administration of sulfadiazine was spotted lineary between point P and Q as shown in Fig. 1 and also spotted on R and O in order to compare the position of the spots. Development was carried out ascendingly up to almost upper end of the paper with solvent A and solvent B, and after drying the paper in air, part T and T′ were cut out and the positions of the spots were confirmed with the Ehrlich's reagent. Refering to the Rf values of each metabolite on part T and T′, part S was devided into three parts as shown in Fig. 1. In Fig. 1 the spot of N^4-acetylsulfadiazine with solvent B was found to overlap with that of urea and hence in the case of this solvent the position corresponding to N^4-acetylsulfadiazine was not cut off.

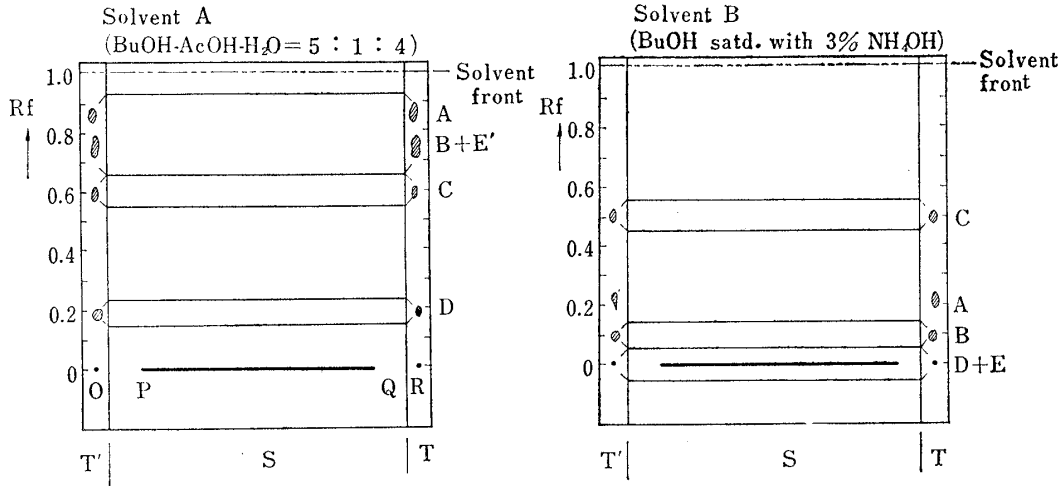

Fig. 1. Paper Chromatogram for Quantitative Separation

—— Cut off line after ascending paper chromatography
▬▬ Sample (start line)
● Reference sample
A N^4-Acetylsulfadiazine
B Sulfadiazine
C Sulfanilamide
D Sulfadiazine-N^4-sulfonate
E Sulfadiazine-N^4-glucuronide
E′ Sulfadiazine derived from the decomposition of sulfadiazine-N^4-glucuronide

Each part cut off was set in the elution vessel and eluted descendingly with 8 ml. of $N/4$ NH_4OH, which could completely elute each metabolic product from paper in any case. These eluates were diluted to 10 ml. with $N/4$ NH_4OH and these solution were used as the sample solution.

Discussion of the Conditions of the Separation of Metabolites

The following experiments were carried out to find out the optimum condition.

It was found that solvents A and B were siutable for avoiding the tailing of sulfadiazine. Rf values for N^4-acetylsulfadiazine, sulfadiazine, sulfanilamide, sulfadiazine-N^4-sulfonate with solvent A were 0.84, 0.76, 0.60, 0.21, and for sulfanilamide, N^4-acetylsulfadiazine, sulfadiazine, sulfadiazine-N^4-sulfonate, and sulfadiazine-N^4-glucuronide with solvent B were 0.49, 0.18, 0.10, 0, 0, respectively. All of the spots were well separated each other with both solvents except in the following cases. Overlap was observed between sulfadiazine-N^4-sulfonate and sulfadiazine-N^4-glucuronide with solvent B because of their high solubilities in water, and a little overlap between the spots of sulfadiazine and N^4-acetylsulfadiazine with the solvent A in the case of too high concentration.

Method of Determination

Sulfadiazine : Five ml. of the sample solution of sulfadiazine (eluate from part B after development with solvent B as shown in Fig. 1) was concentrated to almost dryness and dissolved in about 4 ml. of ethanol. To this 0.3 ml. of $2N$ HCl and 5 ml. of the Ehrlich's reagent were added and allowed to stand for 10 min. at 20~30°, and it was diluted up to 10 ml. accurately. Absorbance at 450 mμ of this solution was measured, and the concentration of sulfadiazine was determined with calibration curve prepared beforehand.

N^4-Acetylsulfadiazine : The sample solution of N^4-acetylsulfadiazine (eluate after development with solvent A) was concentrated to almost dryness and hydrolyzed by heating on a boiling water bath for 30 min. with 15 ml. of N HCl. After cooling, this solution was concentrated and treated as described in the determination of sulfadiazine. The measurement was carried out and the content of N^4-acetylsulfadiazine was calculated by subtracting the value of sulfadiazine and sulfadiazine-N^4-glucuronide*4 from the value obtained.

*4 This substance was decomposed into sulfadiazine and glucuronic acid in the course of development with solvent A.

Sulfadiazine-N^4-sulfonate : The sample solution of sulfadiazine-N^4-sulfonate (eluate after development with solvent A) was concentrated to almost dryness and hydrolyzed by heating on a boiling water bath for 15 min. with 10 ml. of *N* HCl. After cooling, it was treated as described in the determination of sulfadiazine.

Sulfadiazine-N^4-glucuronide : The sample solution of sulfadiazine-N^4-glucuronide which was eluted from part D and part E after development with solvent B as shown in Fig. 1 was concentrated and treated as described in the determination of sulfadiazine-N^4-sulfonate. The content of sulfadiazine-N^4-glucuronide was calculated by subtracting the value of sulfadiazine-N^4-sulfonate from the total value determined on this solution.

Sulfanilamide : The sample solution (eluate after development with solvent A and B) was treated by the same method as that of sulfadiazine.

After the absorbance of each solution was measured, the determination was carried out. The calibration curve of sulfadiazine was substituted for that of each metabolite. Accordingly, the content of the each metabolite was caluculated as the amount of sulfadiazine.

Discussion of the Conditions of the Determination of Metabolites

The following experiments were carried out in order to find out the above mentioned optimum condition.

a) Condition of Color-development

Absorption spectrum : Absorption spectrum of a solution of sulfadiazine reacted with the Ehrlich's reagent has a maximum at 450 mμ.

2% *p*-Dimethylaminobenzaldehyde-ethanol solution : The volume of this solution was varied from 1 to 7 ml. with the interval of 1 ml. to examine the effect on the absorbance. As the absorbance became constant when more than 5 ml. of the reagent was added, the volume to be added was decided as 5 ml.

Effect of temperature and reaction time on absorbance : To examine the effect of temperature and reaction time, the absorbances were measured after 1, 3, 5, 7, 10, 15, 20, and 30 min. at the room temperature. The absorbance was constant when the reaction time was beyond 5 min. When the reaction temperature was changed from 20° to 30° with the interval of 5° and the reaction time was fixed to 10 min., the absorbance did not changed. Therefore the determination was carried out at 20°~30°.

Stability on the colored solution : The change of the absorbance was checked after coloration in order to examine the stability of the colored solution. Absorbance was constant in 60 min. after coloration.

b) Hydrolysis

The effect of the time of the hydrolysis was investigated using hydrochloric acid in order to find out the optimum condition for each metabolite.

N^4-Acetylsulfadiazine : The sample solution (the *N*/4 ammonium hydroxide solution containing 0.2 mg. % N^4-acetylsulfadiazine) was concentrated to almost dryness and dissolved in 15 ml. of *N* hydrochloric acid and heated for 5, 10, 20, 25, or 30 min. on a boiling water bath. The absorbance became constant after hydrolysis for 25 min. and spot of N^4-acetylsulfadiazine could not be found on paper after the hydrolysis for longer than 25 min. Accordingly, 30 min. was found to be suitable for the hydrolysis of N^4-acetylsulfadiazine.

Sulfadiazine-N^4-sulfonate and sulfadiazine-N^4-glucuronide : After concentration of the sample solution (the *N*/4 ammonium hydroxide solution containing 0.1 mg. % sulfadiazine-N^4-sulfonate or sulfadiazine-N^4-glucuronide), the residue was dissolved in 10 ml. of *N* hydrochloric acid and was heated for 3, 5, 8, 10, or 15 min. and treated as described above. In this case, 10 min. was found to be suitable for the hydrolysis of both sulfadiazine-N^4-sulfonate and sulfadiazine-N^4-glucuronide.

Calibration Curve

One gram of sulfadiazine was dissolved in equimolecular *N* NaOH, and this solution was diluted up to volume of 100 ml. with distilled water. This solution was diluted with distilled water and the following standard sulfadiazine solutions were prepared; sulfadiazine 10, 20, 30, 50, 70, 100 mg. %. These standard solutions were subjected to the above mentioned separation method and the calibration curve was obtained by the same treatment as that described in determination method.

Recovery Test

A known amount of each standard substance which was added to human urine was measured using the calibration curve obtained by the determination procedure. The values, found and calculated for each substance, are given in Table I and the results are satisfactory. Accordingly, this separatory determination method has been found to be able to apply for the metabolites in urine.

Table I. Recovery Test

	Calcd. (mg.)	Found (mg.)	(%)
Sulfadiazine	50	49.1	(98.2)
N^4-Acetylsulfadiazine	50	48.4	(96.8)
Sulfadiazine-N^4-glucuronide	4.74	4.52	(95.3)
Sulfadiazine-N^4-sulfonate	3.55	3.35	(94.5)
Sulfanilamide	5.00	4.87	(97.4)

Results of Determination

The excrements in urine were separately determined by the above mentioned method after 2 g. of sulfadiazine was administrated orally. The results are shown in Table II and Figs. 2 and 3. As shown in Table II during 48 hr. after administration

Table II. Determination of the Excrements in the Human Urine after Administration of 2 g. of Sulfadiazine (Calculated as sulfadiazine (mg.))

Substance	Time after administration (hr.)									
	0~2	2~4	4~6	6~9	9~12	12~24	24~36	36~48	Total	%[a]
Sulfadiazine	7.0	22.0	74.4	149.6	126.0	286.0	173.2	23.6	862.0	(43.10)
N^4-Acetyl-sulfadiazine	0.6	3.7	37.8	80.8	89.0	311.4	157.6	38.6	729.5	(36.48)
Sulfadiazine-N^4-glucuronide	0.2	0.1	1.2	3.2	2.2	3.6	0.1	0	10.6	(0.53)
Sulfadiazine-N^4-sulfonate	1.3	1.3	3.3	2.6	2.3	1.5	1.3	0.2	13.8	(0.69)
Sulfanilamide	0.2	0.2	0.4	0.5	0.3	2.3	0.7	0.3	4.9	(0.25)
Total	9.3	27.3	117.1	236.5	219.8	616.3	331.8	62.5	1620.8	(81.04)

a) These values are percentage of administered sulfadiazine.

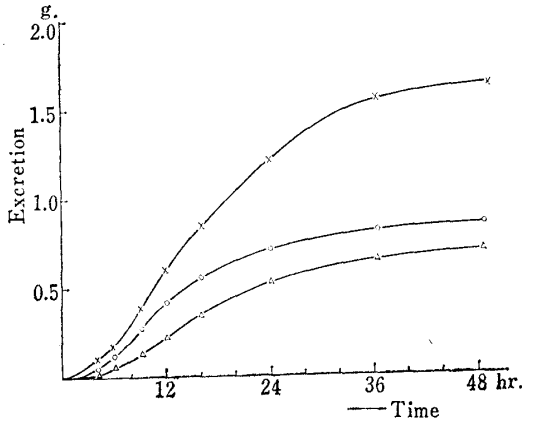

Fig. 2. Cumulative Excretion Curve of Sulfadiazine, N^4-Acetylsulfadiazine and Total Excrement (Administ. 2 g.)

×—× Total excrement
○—○ Sulfadiazine
△—△ N^4-Acetylsulfadiazine (calcd. as sulfadiazine)

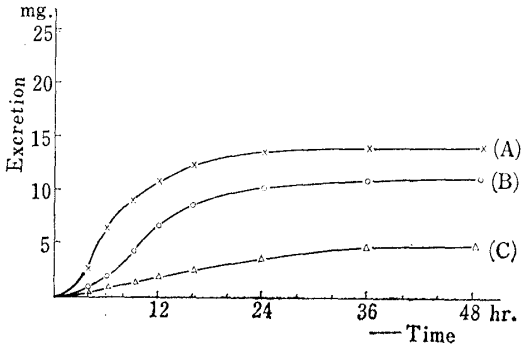

Fig. 3. Cumulative Excretion Curve of Sulfadiazine-N^4-glucuronide, Sulfadiazine-N^4-sulfonate and Sulfanilamide (Administ. 2 g.)

(A) Sulfadiazine-N^4-sulfonate (calcd. as sulfadiazine)
(B) Sulfadiazine-N^4-glucuronide (calcd. as sulfadiazine)
(C) Sulfanilamide (calcd. as sulfadiazine)

of 2 g. of sulfadiazine, about 80% of administered drug was excreted and percentages of the excreted to administered were 43.1%, 36.5%, 0.5%, 0.7%, 0.3% for sulfadiazine, N^4-acetylsulfadiazine, sulfadiazine-N^4-glucuronide, sulfadiazine-N^4-sulfonate, and sulfanilamide.

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Summary

Excretion of sulfadiazine, N^4-acetylsulfadiazine, sulfadiazine-N^4-glucuronide, sulfadiazine-N^4-sulfonate, and sulfanilamide into human urine was separately determined by paper chromatography by the following method.

One tenth ml. of urine obtained after oral administration of sulfadiazine was spotted lineary on a filter paper (Toyo Roshi No. 51 40×40 cm.) and developed ascendingly with a solvent system of butanol saturated with water and a solvent of

BuOH–AcOH–H_2O (5:1:4) for about 20 hours. The paper was dried in air, portions corresponding to the products were cut out, and eluted descendingly with $N/4$ ammonium hydroxide. Each of these metabolites was hydrolyzed with hydrochloric acid and determined by the colorimetric method using the Ehrlich's reagent as sulfadiazine. The content was calculated from a calibration curve prepared beforehand.

The amount excreted 48 hours after oral administration of 2 g. of sulfadiazine was 1620 mg., calculated as sulfadiazine, including 862 mg. of sulfadiazine, 730 mg. of N^4-acetylsulfadiazine, 11 mg. of sulfadiazine-N^4-glucuronide, 14 mg. of sulfadiazine-N^4-sulfonate, and 5 mg. of sulfanilamide, calculated as sulfadiazine respectively.

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94. Ichiro Matsuo, Kazuro Sugimoto,*1 and Sadao Ohki*2 : Synthesis of Quinolizine Derivatives. XVI.*3 Synthesis of 3-(Subst.-benzyl)quinolizidine.

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The sulfate of sparteine, one of the alkaloids isolated from *Cytisus scoparius* and *Lupinus luteus* has been widely used as an uterus contracting agent, bnt its action was considered to be not strong enough for the practical use. Therefore, in order to find out more active substances than sparteine with stronger activity, the authors attempted to synthesize scores of quinolizidine derivatives and their pharmacological actions were discussed.[1)]

In this paper since sparteine was considered to be composed of two quinolizidine nucleus, syntheses of simple quinolizidine derivatives such as 3-(*p*-subst.-benzyl)quinolizidine were described to examine the contracting activity.

Among the compounds synthesized, 3-phenyl-, 3-butyl- and 3-amyl-quinolizidines were found to show several folds of activity to sparteine sulfate, when tested by the Magnus method with the extracted intestinal canal from guinea pigs. However, the test results of these compounds *in vivo* was not always parallel to that by the Magnus method and a big question was raised upon the practical efficacy. Based upon these facts, preparation of quinolizidine series of stronger activity with less accessory reactions, were attempted with great expectation.

This report deals with the discovery of one promissing compound with stronger activity, even by the test *in vivo*.

In a previous paper,[1)] 3-subst.-quinolizidine was found to show the probable chemical structures to cause the action. Among the 3-subst. compounds synthesized, 3-(subst.-benzyl)-derivative (I) was found to show a remarkable action, and especially

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*3 Part XIV : This Bulletin, **14**, 147 (1966). Part XV : Yakugaku Zasshi, submitted for publication.
1) Part XIII : This Bulletin, **10**, 1250 (1962).