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158. Toyozo Uno,*1 Teishiro Kushima, and Takashi Hiraoka*2 : Studies on the Metabolism of Sulfadimethoxine. II.*3 Determinations of Metabolites in Human and Rabbit Urine after Oral Administration of Sulfadimethoxine.

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The concentration and the amounts of sulfadimethoxine metabolites in both human and rabbit urine were determined separately using paper chromatography for 168 hours after administration.

In human urine the main metabolite was sulfadimethoxine-N^1-glucuronide, and more than 70% of the whole metabolites was excreted in this form.

In the case of rabbit, the main metabolite was N^4-acetyl sulfadimethoxine.

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In the previous paper,*3 we reported that three metabolites of sulfadimethoxine excreted in human urine were separated, namely they were unchanged sulfadimethoxine, N^4-acetylsulfadimethoxine, and sulfadimethoxine-N^1-glucuronide or its lactone form.

In this report, rabbit metabolites were investigated using the same method described in the previous paper.*3

J. W. Bridges, *et al.*[1,2] found sulfadimethoxine-N^4-glucuronide as ammonium salt in addition to the above three metabolites in human and rabbit urine, therefore, the presence of the N^4-glucuronide was also investigated.

There have been many reports[3~10] regarding the determination of sulfadimethoxine metabolites. In large number of them, the concentration was determined only by modified Bratton-Marshall method, which included N^1-glucuronide as well as unchanged sulfadimethoxine.

B. A. Koechlin[3] and S. Okamoto[6] measured unchanged sulfadimethoxine and glucuronide separately by extracting unchanged sulfadimethoxine and N^4-acetylsulfadimethoxine using organic solvents. However there has been no report of systematic investigation on the metabolism of sulfadimethoxine.

We determined the concentration of each metabolite in urine using the modified Bratton-Marshall method after paper chromatografic separation.

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Experimental

Paper Chromatography and Electrophoresis——See the previous paper.*3 Aniline hydrogene phthalate reagent (1.66 g. of phthalic acid and 0.93 g. of aniline were dissolved in 100 ml. of *n*-BuOH saturated with H_2O) was used instead of naphthoresorcinol reagent.

Standard for the Paper Chromatography of Sulfadimethoxine-N^4-glucuronide——To the mixture of 3.1 g. of sulfadimethoxine (0.01 mol.) and 1*N* KOH 20 ml., 1.8 g. of glucuronolactone (0.01 mol.) was added. The crystallized white needles which seemed unchanged sulfadimethoxine were filtered off. Various attempts to obtain the pure N^4-glucuronide crystals from the mother liquid was unsuccessful.

The mother liquid was subjected to paper chromatography using the same solvents described in the previous paper.*3 Three spots were found on the chromatograms. The main spot was colored with Ehrlich's reagent and with Tsuda's reagent, but it was not colored with aniline hydrogen phthalate reagent. Its Rf values coincide with the Rf values of sulfadimethoxine. The other two spots, though they were both far smaller compared with the former, were colored with any one of the above three reagents, and one of them coincided with the Rf values of sulfadimethoxine-N^1-glucuronide. The other one had the Rf values shown in Table I. The latter was also detected from the mixture of glucuronolactone and sulfadimethoxine in aqueous NH_4OH solution. This spot was thought to be N^4-glucuronide.

Preliminary Test for Detection of Sulfadimethoxine Metabolites in Human and Rabbit Urine——Uno and Ueda's method[11] was reexamined for sulfadimethoxine. Small variations of the amount of reagents and the reaction time were found not to give any affect to measurement within the difinite limits. The color was stable between 0.5～1.5 hours after color development.

The determination of sulfadimethoxine was little influenced by the presence of N^4-acetylsulfadimethoxine.

The hydrolysis condition of N^4-acetylsulfadimethoxine was set to heat for 30 minutes at 80° in about 2*N* HCl from the results of preliminary acidic and basic hydrolysis.

Water was selected as the solvent of elution, because it gave the best recovery and minimum errors in H_2O, 0.1*N* HCl, and 0.1*N* NH_4OH.

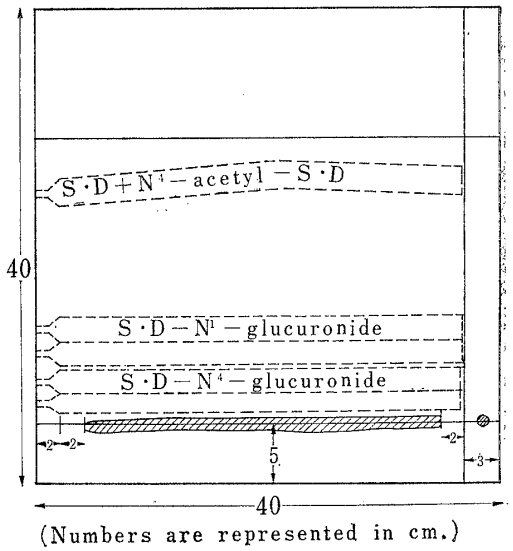
The quantity of eluate to be used for determination was decided to be 25 ml. from the result of preliminary test. The quantity of 20 ml. of H_2O was enough to elute 100 μg. of sulfadimethoxine, 100 μg. of N^4-acetylsulfadimethoxine, and 400 μg. of the N^1-glucuronide.

Calibration curves for determination of sulfadimethoxine and N^4-acetylsulfadimethoxine in urine were linear between the range of treated amounts.

Paper Chromatography for Determination——After administration of 1 g. of sulfadimethoxine for men (a man and a woman : bodyweight 56 kg. and 42 kg. respectively) after a meal, and 40 mg. for male rabbits (body weight 1.9, 2.3, and 2.4 kg.), the urine was pooled in each period, its volume was measured, centrifuged, and 1 ml. (or 0.5 ml. if necessary) of its supernatant was painted on the Toyo Roshi 40×40 cm. along the line 5 cm. upper from under side of the paper as shown in Fig. 1.

One ml. of the standard solution (100 mg. of sulfadimethoxine was dissolved in 1 ml. of 1*N* NaOH and diluted to 1000 ml. with H_2O) was painted as the standard, and 1 ml. of normal urine was also painted as the blank.

The samples, the standard and the blank were developed with *n*-BuOH·iso-PrOH·H_2O (2:1:1) for 16 hours, using Uno and Ueda's two dimensional developing box.[12] The paper was cut off along the line from the right side as shown in Fig. 1, and 10% HCl was sprayed, warmed a few minutes, 0.1% $NaNO_2$, 0.5% $NH_4SO_3NH_2$, 0.1% Tsuda's reagent were sprayed in order.



(Numbers are represented in cm.)

Fig. 1. An Example of Chromatogram

Both sides of the paper in Fig. 1 were illuminated by ultraviolet ray, and distribution of each metabolite in the left side were searched, and cut out along the dotted line shown in Fig. 1.

As unchanged sulfadimethoxine and N^4-acetylsulfadimethoxine have the same Rf value, they were cut out as one piece of the paper.

Because sulfadimethoxine-N^1-glucuronide had a little broad distribution, it was cut out as two pieces of paper of the same shape.

11) T. Uno, M. Ueda : Yakugaku Zasshi, **82**, 759 (1962).
12) *Idem* : *Ibid.*, **80**, 1785 (1960).

It was impossible to find the spot of sulfadimethoxine-N^4-glucuronide in the case of human urine, but the 0.5~3.5 cm. upper portion from the original line was cut off under the ultraviolet examination since the compound was known to have small Rf value by the previous research.

Each piece of the paper was eluted with H_2O by the usual method, and 25 ml. of the eluate was collected in a volumetric flask.

The Determination of Unchanged Sulfadimethoxine——A portion of 6 ml. of the eluate was transferred into a 10 ml. volumetric flask, maintained at 15° and one ml. of 1*N* HCl and 0.5 ml. of 0.1% $NaNO_2$ were added. One half ml. of 0.5% $NH_4SO_3NH_2$ 10 minutes after, and 0.5 ml. of 0.1% Tsuda's reagent 5 minutes after, were added successively.

Finally, the solution was diluted to 10 ml. with water, and mixed well.

After one hour standing, the absorption of the solution was measured at 560 mμ.

$$C=\frac{E-B}{S}\times 100\ (\mu g./ml.)$$

C : Concentration of Sulfadimethoxine in urine.

E : Absorbance of the Sample.

B : Absorbance of the Blank.

S : Absorbance of the Standard.

The Determination of N^4-Acetylsulfadimethoxine——Another portion of 6 ml. of the eluate was transferred into a 10 ml. volumetric flask, 2 ml. of conc. HCl was added and heated at 80° for 30 minutes. The samples were cooled and treated similarly as mentioned above.

The standard solution which was eluted from the paper painted with 100 μg. of sulfadimethoxine was treated similarly. The concentration of N^4-acetylsulfadimethoxine was calculated as follows.

$$C'=\frac{E'-B'}{S'}\times 100-C\ (\mu g./ml.)$$

C' : Concentration of N^4-Acetylsulfadimethoxine in urine represented as μg. of corresponding unchanged sulfadimethoxine.

E' : Absorbance of the Sample.

B' : Absorbance of the Blank.

S' : Absorbance of the Standard.

The Determination of Sulfadimethoxine-N^1-glucuronide——Sulfadimethoxine-N^1-glucuronide was determined by the same method as that of unchanged sulfadimethoxine.

The Determination of Sulfadimethoxine-N^4-glucuronide——Sulfadimethoxine-N^4-glucuronide was determined by the same method as that of N^4-acetylsulfadimethoxine. The concentration was calculated as follows.

$$C''=\frac{E''-B''}{S'}\times 100\ (\mu g./ml.)$$

C'' : Concentration of Sulfadimethoxine-N^4-glucuronide in urine represented as μg. of corresponding unchanged sulfadimethoxine.

E'' : Absorbance of the Sample.

B'' : Absorbance of the Blank.

Results and Discussion

As described in experimental, pure sulfadimethoxine-N^4-glucuronide was not obtained, but the paper chromatograms of its mother liquid showed a possibility of sulfadimethoxine-N^4-glucuronide presence, whose Rf values were shown in Table I.

Table I. The Rf Values of a Compound which may be Sulfadimethoxine-N^4-glucuronide

	n-BuOH satd. with H_2O	*n*-BuOH· iso-PrOH·H_2O (2:1:1)	*n*-BuOH· iso-PrOH·0.1*N* NH_4OH (2:1:1)	*n*-BuOH satd. with 3% NH_4OH	*n*-BuOH· H_2O·AcOH (5:1:4)	*n*-BuOH satd. with 0.1*N* HCl
Rf Values	0.03	0.05	0.06	0.03	—	—

Table II. The Concentration of Sulfadimethoxine Metabolites in Human Urine

Time	Unchanged Sulfadimethoxine[a]			N^4-Acetylsulfadimethoxine			Sulfadimethoxine-N^1-glucuronide[a]			Sulfadimethoxine-N^4-glucuronide[a]			Urine Volume[b]	
hours	a	b	mean	a	b	mean	a	b	mean	a	b	mean	a	b
4	0	1.9	1.0	4.3	5.5	4.9	30.5	12.4	21.5	0	0	0	108	215
8	15.5	14.5	15.0	23.0	58.5	40.8	198.7	257.9	228.3	3.7	12.8	8.3	103	98
24	15.0	31.7	23.4	54.6	59.2	56.9	378.6	363.4	371.0	3.7	2.2	3.0	505	670
48	19.3	27.6	23.5	57.8	53.9	55.9	312.1	298.6	305.4	5.8	0.9	3.4	510	680
98	3.1	7.5	5.3	21.6	32.4	27.0	105.9	84.9	95.4	0	0.9	0.5	1560	1900
168	0	0.6	0.3	4.3	20.7	12.5	21.8	30.7	26.3	0	2.8	1.4	2590	1720

a: man b: woman a) γ/ml. as sulfadimethoxine b) ml.

Table III. The Concentration of Sulfadimethoxine Metabolites in Rabbit Urine

Time	Unchanged Sulfadimethoxine[a]				N^4-Acetylsulfadimethoxine[b]				Sulfadimethoxine-N^1-glucuronide[a]				Sulfadimethoxine-N^4-glucuronide[a]				Urine Volume[b]		
hours	a	b	c	mean	a	b	c	mean	a	b	c	mean	a	b	c	mean	a	b	c
4	0.2	0	12.1	4.1	4.2	4.6	13.1	7.3	0	0	0.2	0.1	0	4.6	2.0	2.2	115	38	71
8	17.7	9.8	23.4	17.0	14.1	27.3	26.1	22.5	2.3	0	1.9	1.4	7.5	4.6	3.0	5.0	100	86	65
24	34.5	19.6	23.5	25.9	67.4	75.8	96.0	79.7	8.2	0.6	7.4	5.4	6.6	0.7	5.6	4.3	99	67	70
48	8.0	10.9	13.8	10.9	31.1	57.2	20.6	36.3	1.2	0	7.9	3.0	6.6	3.7	2.7	4.3	188	138	170
96	8.0	0	2.0	3.3	9.4	18.0	0.3	9.2	3.3	0	7.4	3.6	6.6	1.5	4.0	4.0	365	320	350
168	8.0	0	3.3	3.8	4.4	2.2	1.6	2.7	3.3	0	5.7	3.0	6.6	2.2	4.6	4.5	176	350	280

a) γ/ml. as sulfadimethoxine b) ml.

Table IV. The Amounts of Sulfadimethoxine Metabolites excreted in Human Urine (cumulative)

Time	Unchanged Sulfadimethoxine			N^4-Acetylsulfadimethoxine[a]			Sulfadimethoxine-N^1-glucuronide[a]			Sulfadimethoxine-N^4-glucuronide[a]		
hours	a	b	mean	a	b	mean	a	b	mean	a	b	mean
4	0	0.4	0.2	0.5	1.2	0.9	3.3	2.7	3.0	0	0	0
8	1.6	1.8	1.7	2.9	6.9	4.9	23.8	27.9	25.9	0.4	1.3	0.9
24	9.9	23.1	16.5	30.1	46.6	38.4	215.0	271.4	243.2	2.3	2.7	2.5
48	19.7	41.9	30.8	59.6	83.2	71.4	374.2	474.4	424.3	5.3	3.3	4.3
96	24.5	56.1	40.3	93.3	144.8	119.1	539.4	635.8	587.6	5.3	5.0	5.2
168	24.5	57.1	40.8	104.4	180.4	142.4	595.9	688.6	642.3	5.3	9.8	7.6

a) mg. Sulfadimethoxine

Table V. The Amounts of Sulfadimethoxine Metabolites excreted in Rabbit Urine (cumulative)

Time	Unchanged Sulfadimethoxine				N^4-Acetylsulfadimethoxine[a]				Sulfadimethoxine-N^1-glucuronide[a]				Sulfadimethoxine-N^4-glucuronide[a]			
hours	a	b	c	mean	a	b	c	mean	a	b	c	mean	a	b	c	mean
4	0	0.9	0	0.3	0.2	0.9	0.5	0.5	0	0	0	0	0	0.2	0.1	0.1
8	0.8	2.4	1.8	1.7	2.6	2.6	1.9	2.4	0.1	0	0.2	0.1	0.8	0.6	0.3	0.6
24	2.1	4.0	5.2	3.8	7.7	9.3	8.6	8.5	0.3	0	1.0	0.4	1.5	0.7	0.7	1.0
48	3.6	6.4	6.7	5.6	15.6	12.8	14.4	14.3	1.6	0	1.2	0.9	2.7	1.2	1.2	1.7
96	3.6	7.1	9.6	6.8	21.4	12.9	17.8	17.4	4.2	0	2.4	2.2	5.1	1.7	2.6	3.1
168	3.6	8.0	11.0	7.5	22.2	13.4	18.5	18.0	5.8	0	3.0	2.9	6.3	2.5	3.9	4.2

a) mg. Sulfadimethoxine

The chromatogram of human urine after oral administration of 1 g. of sulfadimethoxine did not show any spot other than the three spots reported in the previous paper.

In the case of rabbit, the position of the spot shown in the Table I was often masked with the spot of normal urine, which interfered detection. The existence of sulfadimethoxine-N^4-glucuronide in rabbit urine was highly obscure.

The concentration of each metabolite in human and rabbit urine is shown in Tables II and III. The cumulative amounts of each metabolite were calculated from the measured values and urine volumes. Tables IV and V show the cumulative amounts of the metabolites excreted in human and rabbit urine respectively.

The comparison of the amounts of the excreted metabolites in man and in rabbit were given in Table VI. As can be seen in this Table, the main metabolite in man was sulfadimethoxine-N^1-glucuronide which was 77% of total metabolites, and N^4-acetylsulfadimethoxine, unchanged sulfadimethoxine followed. In the case of rabbit, the main metabolite was N^4-acetylsulfadimethoxine which was 55% of total metabolites, and unchanged sulfadimethoxine followed.

Table VI. The Comparison of the Amounts of the Sulfadimethoxine metabolites excreted in Human and Rabbit Urine

	Man	Rabbit
Unchanged Sulfadimethoxine[a)]	4.9	22.9
N^4-Acetylsulfadimethoxine[a)]	17.1	55.1
Sulfadimethoxine-N^1-glucuronide[a)]	77.1	8.9
Sulfadimethoxine-N^4-glucuronide[a)]	0.9	12.8
The Rates of the total excreted Amounts to the administered Amount	83.3	81.8

a) Shown in percentages for the Amounts of the total metabolites.

In human urine, 0.9% of sulfadimethoxine-N^4-glucuronide was found, but this is within the range of error, and consequently the conclusion for the existence of the N^4-glucuronide is that there are no or very little N^4-glucuronide in human urine, and some in rabbit urine.

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