Notes

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Studies on Metabolism of Drugs. XII.[1] Quantitative Separation of Metabolites in Human and Rabbit Urine after Oral Administration of Sulfamonomethoxine and Sulfamethomidine

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Sulfamonomethoxine, N^1-(4-methoxy-6-pyrimidinyl)sulfanilamide, developed and widely used as the long-lasting sulfa drug, was synthesized by Okuda and others,[3] and Oshima and others[4] examined its antibacterial activity and toxity, reporting that it is broken down into the free compound and N^4-acetylated compound *in vivo*. Nakazawa and others[5] also reported that 60% of the drug ingested in the human body is in free form and that N^4-actylated compound is also found. We[6] carried out paper chromatography of human urine after ingestion of sulfamonomethoxine and found three spots which were found to be the unchanged drug, N^4-acetylsulfamonomethoxine, and a newly discovered sulfamonomethoxine N^1-glucosiduronate.

Sulfamethomidine, N^1-(6-methoxy-2-methyl-4-pyrimidinyl)sulfanilamide was synthesized by Loop, *et al.*,[7] which having the same pyrimidine ring, is also one of the long-lasting sulfas used widely. Its metabolites are also reported to be the free and acetylated compounds,[8] and their concentration in blood and urine has been measured. Our examination of the metabolites of sulfamethomidine[9] showed the presence of unchanged drug, N^4-acetylsulfamethomidine, and newly found sulfamethomidine N^1-glucosiduronate.

Metabolites of sulfamonomethoxine and sulfamethomidine in blood and urine have been measured as the free and N^4-acetylated compounds, but now that three substances, including unchanged drug, are known to be present, separatory determination of these metabolites was carried out in order to clarify the quantitative relation with passage of time, both in humans and in rabbits.

Experimental

Reagents——BuOH saturated with 0.5 N NH_4OH, 0.02 and 0.2 N Na_2CO_3, 1,2, and 4 N NaOH, 0.2% $NaNO_2$, 0.5% ammonium sulfamate solution, 0.1% Tsuda reagent (90% EtOH solution of 0.1% β-diethylaminoethyl-α-naphthylamine oxalate), Ehrlich reagent (EtOH solution containing 2% of *p*-dimethylaminobenzaldehyde and 1/50 volume of conc. HCl).

1) Part XI: M. Ueda, K. Orita, and T. Koizumi, *Chem. Pharm. Bull.* (Tokyo), **19**, 2046 (1971).
2) Location: *Gofuku-3190, Toyama.*
3) N. Okuda and I. Kuniyoshi, *Yakugaku Zasshi*, **82**, 1035 (1962); N. Okuda, I. Kuniyoshi, Y. Oshima, and S. Nagasaki, *ibid.*, **82**, 1039 (1962).
4) Y. Oshima, S. Nagasaki, and H. Tachi, *Nippon Yakurigaku Zasshi*, **58**, 50 (1962); Y. Oshima, A. Kasahara, and M. Shibata, *ibid.*, **58**, 59 (1962).
5) S. Nakazawa, G. Ogawa, and H. Oka, *Chiryo*, **44**, 171 (1962).
6) M. Ueda, N. Murakami, H. Atsumura, and K. Furuki, *Yakugaku Zasshi*, **87**, 455 (1967).
7) W. Loop and E. Luhrs, *Ann. Chem.*, **580**, 225 (1953).
8) Y. Kowa, H. Kobayashi, and K. Yasuda, *Chemotherapia*, **10**, 245 (1962); T. Maeda and H. Ishibiki, *ibid.*, **9**, 26 (1961); K. Kurokawa and T. Yamamoto, *ibid.*, **9**, 409 (1961).
9) M. Ueda, N. Murakami, and K. Furuki, *Chem. Pharm. Bull.* (Tokyo), **16**, 352 (1968).

Materials and Apparatus——Toyo Roshi No. 50 filter paper (40×26 cm). Paper chromatography (Toyo Kagaku Type B, with stainless steel spiral), Toshiba ultraviolet lamp Model FL-20 BLB, incubator (width×height×depth: 100×70×60 cm), Hitachi spectrophotometer Model 139.

Descending Elution Apparatus for PPC (Fig. 2): A wooden box (35×38×33 cm in inner dimensions) with a window coverd with a transparent plastic sheet on one side and a wooden board placed on the top. The box is lined with thick, absorbent cotton cloth, which is sprayed with deionized water at the time of elution so as to saturate the box with water vapor. For elution liquid, four trough made of hard vinyl plastic of 0.3 cm thickness (5×33.5×1.5 cm in inner dimensions) were placed in parallel on a wooden board, 29.3 cm in height from the bottom of the box. Two glass plates (6×32.5 cm) of 0.2 cm in thickness were placed together in one trough, to one edge (6 cm) of the glass plates in the liquid, about 1 cm of the paper to be eluted was placed between the glass plates on further end, and the paper was eluted by the descending method with the eluting solvent in the trough. The receiver was a 10-ml of conical graduate with round signed exactly at 3 ml, or a 10-ml measuring flask round graduated exactly at 1.5 ml.

Separation——This was refered to the method reported earlier.[10] As shown in Fig. 1, 0.3 ml of the sample urine was streaked at one end of the filter paper using a pointed tip measuring pipette between P and Q, and one spot at R in order to find the position of spots. Toyo Kagaku Type B chromatography apparatus was used, BuOH saturated with 0.5 N NH_4OH as the developing solvent, and developed for 16 hr in an incubator at 28°. After the paper was dried in air, the end strip (T) was cut off and sprayed with the Ehrlich reagent to know the approximate position of the spots. With this strip as a guide and with reference to the *Rf* of spots from ordinary urine components that fluoresce under irradiation from an ultraviolet lamp, the eluted paper was cut into two parts, AB and C, as shwon in Fig. 1.

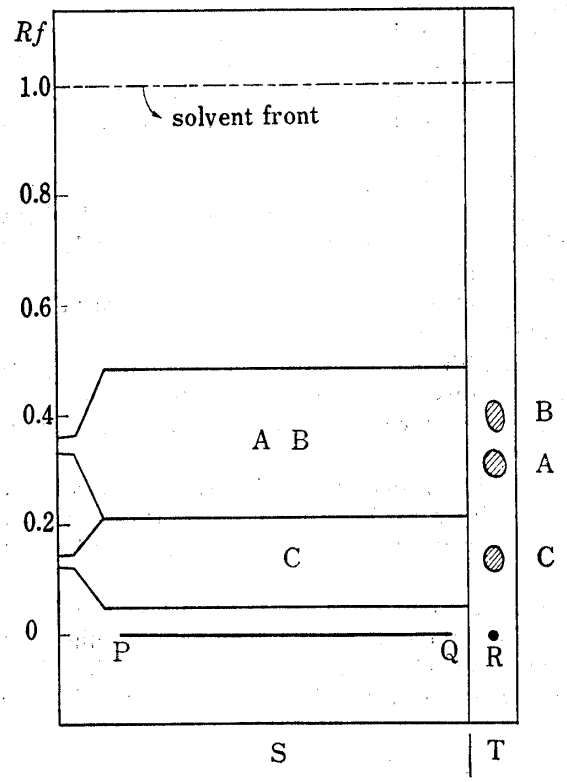

Fig. 1. Paper Chromatogram for Quantitative Separation

solvent: BuOH satd.with 0.5N NH_4OH
Tōyō filter paper No. 50 40×26.5cm 28°, 16 hr
—: sample (start line)
●: reference sample
—: Cut off line after ascending paper chromatography
A: sulfamonomethoxine or sulfamethomidine
B: N^4-acetylsulfamonomethoxine or N^4-acetylsulfamethomidine
C: sulfamonomethoxine N^1-glucuronide or sulfamethomidine N^1-glucuronide

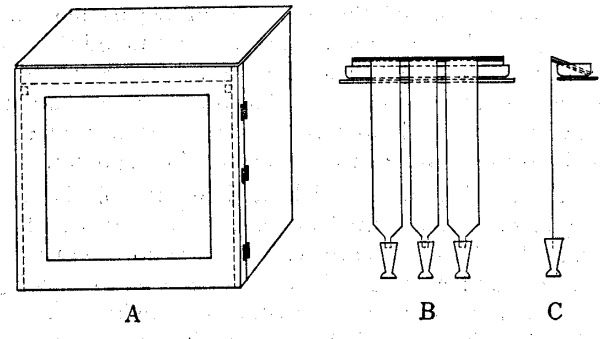

Fig. 2. Outline Views for Elution from Paper Strips

A: front view of elution box (width×height×depth: 38×33×35 cm)
B: front view for elution from paper strips
C: side view for elution from paper strips

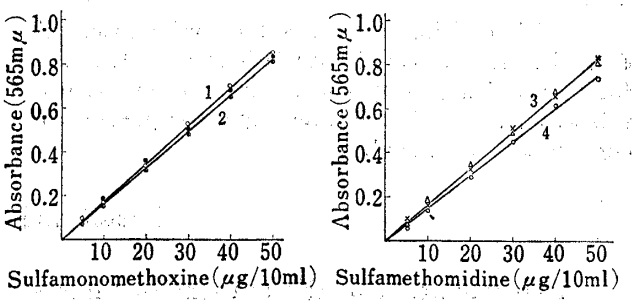

Fig. 3. Calibration Curves for Metabolites of Sulfamonomethoxine and Sulfamethomidine

1: —○—: N^4-acetylsulfamonomethoxine
—●—: sulfamonomethoxine N^1-glucuronide
2: —◐—: sulfamonomethoxine
3: —×—: N^4-acetylsulfamethomidine
—△—: sulfamethomidine N^1-glucuronide
4: —⊙—: sulfamethomidine

10) T. Uno and M. Ueda, *Yakugaku Zasshi*, **82**, 759 (1962).

Right-hand side of the paper strip AB was placed between the two glass plates, placed in the trough, and developed and eluted with 0.2 N Na_2CO_3 solution, by the descending method, as shown in Fig. 2. If about 2 ml of the liquid is eluted into the 10-ml conical graduate, the substances would have been eluted completely. This eluted liquid is made up exactly to 3 ml with 0.2 N Na_2CO_3 solution, and each 1 ml of this solution is used to determine the amount of unchanged drug and its N^4-acetylated compound.

The paper strip C is also eluted in the same manner with 0.02 N Na_2CO_3 solution and the liquid is allowed to collect in a 10-ml measuring flask. If the liquid is allowed to collect to *ca.* 1 ml, the substance would have been completely eluted. If the spot has been eluted with less liquid, the collected solution is made up to 1.5 ml with 0.02 N Na_2CO_3 and used for determination of N^1-glucuronide.

Method of Determination——This was effected by the diazotized method reported earlier[10] and is measured with spectrophotometer at 565 mμ.

Calibration Curve——A solution of 100 mg of standard sample of sulfamonomethoxine and sulfamethomidine or 108.5 mg of acetylsulfamonomethoxine and 107.6 mg of acetylsulfamethomidine to correspond to 100 mg of sulfamonomethoxine and sulfamethomidine respectively dissolved in 8 ml of 0.2 N NH_4OH is diluted to 200 ml with normal urine. This solution is diluted with normal urine to make standard solution containing 0, 50, 100, 200, 300, 400, or 500 μg/ml of the each drugs. This solution is submitted to the foregoing separation and determination procedures to prepare a calibration curve.

In the case of the glucuronides, 5.547 mg of sulfamonomethoxine N^1-glucuronide and 5.447 mg of sulfamethomidine N^1-glucuronide to correspond to 3.333 mg as sulfamonomethoxine or sulfamethomidine is diluted to 20 ml with normal urine, and these solution are prepared into standard solution containing 0, 16.66, 33.33, 66.66, 100, 133.3, or 166.6 μg/ml. These solution are submitted to separation and determination as described above to prepare the calibration curve (Fig. 3).

Examination of the Methods of Separation and Determination——1) Sulfamonomethoxine and its N^4-acetylated compound, and sulfamethomidine and its N^4-acetylated compound cannot be separated completely by paper chromatography and they were eluted together. The spots which color immediately to the Tsuda reagent after diazotization are sulfamonomethoxine and sulfamethomidine. The amount of these substacted from the amount eluted from the spot that colors after hydrolysis with NaOH corresponds to the amount of the acetylated compound.

2) If HCl is used and heated for hydrolysis of the acetylated compound, a part of the filter paper component that is eluted undergoes hydrolysis and an aldehyde-like substance seems to be formed which is considered to condense with the amino group in the sulfa drug. The solution obtained by hydrolysis with HCl and heating, when diazotized and colored with the Tsuda reagent, gives lower content and the calibration curve does not become a straight line. If the test solution is heated in NaOH alkalinity, then acidified with HCl and diazotized, the calibration curve becomes a straight line.

3) The acetylated compound dissolves well in 0.2 N Na_2CO_3 without decomposition. Sulfamonomethoxine and sulfamethomidine also dissolve well in Na_2CO_3. For this reason, the components collect at *Rf* 0.9 by descending elution and the substances are eluted completely with a small quantity of the elution liquid.

4) For oral administration to rabbits, the sulfa drugs were reduced to a fine powder, suspended in 0.4% carboxymethylcellulose paste, and infused into the rabbit stomach by a rubber tubing fitted with a funnel. Male rabbits of about 3 kg in body weight are placed in a rabbit urine cage and urine was collected every 24 hr.

5) Direct Dilution Method: In order to calculate the total amount of the sulfa drug, the urine was diluted suitably, heated with HCl to hydrolyze the acetylated compound, and the amount determined as total sulfa drug, using normal urine as blank, instead of the above-described separation method. Since this avoid the mixing of filter paper component, calibration curve was prepared separately from which the content was calculated.

Result and Discussion

Determination of the amount of metabolites periodically after ingestion of 1 or 2 g of the sulfa drug gave results summarized in Tables I and II, and cumulative values are shown in Fig. 4 and 5.

In order to calculate the recovery of the metabolites, authentic metabolites dissolved in normal human urine as shown ingredients (with nearly above measured amount) in Table III, and above separation and determination were carried out for calculate the mean values and standard errors (Table III).

In both cases, the drug was given after early breakfast. The values at 24, 48, 72, and 96 hr mean that they were measured after an overnight sleep, and these values show clearly

TABLE I. Amount of Substances excreted in Human Urine after Ingestion of Sulfamonomethoxine (Calculated as Sulfamonomethoxine (mg))

Subject and dose (g)	Sub-stance[a]	Time of urine collection (hr after ingestion) 2	4	8	12	24	36	48	60	72	84	96	% of total excreted	% of dose excreted
A 1	SM	3.4	15.0	41.4	45.5	39.9	30.4	28.8	18.9	16.1	10.0	3.5	27.8	91
	ASM	1.4	6.2	33.0	39.6	104.3	91.2	70.2	48.1	37.1	23.5	13.0	51.3	
	SMG	0.8	4.5	20.7	21.6	43.4	35.2	23.4	19.5	12.6	6.0	3.0	20.9	
B 1	SM	0.5	15.2	52.8	62.7	77.6	75.8	35.7	29.6	11.7	15.2	3.9	39.7	91
	ASM	0.8	3.2	26.4	26.6	91.3	72.3	46.2	49.6	18.9	18.4	9.9	42.3	
	SMG	0.0	2.4	21.0	15.2	26.4	36.0	21.7	19.3	7.8	10.4	3.6	18.0	
C 2	SM	3.8	6.9	41.5	34.0	96.5	127.0	65.7	53.0	26.7	21.6	21.3	33.3	75
	ASM	1.0	10.9	45.6	65.1	142.7	106.7	105.0	89.3	50.1	42.8	20.2	45.4	
	SMG	1.5	6.3	26.3	31.1	74.6	61.8	40.6	36.7	11.7	14.1	13.5	21.3	
D 2	SM	3.0	7.1	36.8	28.4	93.0	81.7	42.8	27.6	18.0	13.4	12.5	23.8	76
	ASM	1.0	5.8	51.2	89.6	206.1	143.8	112.2	48.3	49.9	32.8	19.5	50.6	
	SMG	0.0	4.0	38.7	44.3	104.7	56.4	49.1	34.5	22.6	18.2	14.0	25.6	

a) SM: sulfamonomethoxine ASM: N^4-acetylsulfamonomethoxine SMG: sulfamonomethoxine N^1-glucosiduronate

TABLE II. Amount of Substances excreted in Human Urine after Ingestion of Sulfamethomidine (Calculated as Sulfamethomidine (mg))

Subject and dose (g)	Sub-stance[a]	Time of Urine collection (hr after ingestion) 2	4	8	12	24	36	48	60	72	84	96	% of total excreted	% of dose excreted
B 1	SD	0.5	5.5	11.5	9.0	19.2	14.4	7.0	6.4	2.9	1.0	0.3	10.1	77
	ASD	0.2	3.7	13.1	15.0	42.7	49.2	23.1	19.3	12.0	9.4	6.9	25.3	
	SDG	0.6	14.6	52.5	57.0	138.2	90.0	55.3	37.7	23.5	17.7	9.3	64.5	
E 1	SD	0.0	3.6	12.6	10.8	15.0	16.4	6.3	6.0	5.0	6.5	0.0	11.4	72
	ASD	0.6	2.8	19.6	20.0	25.0	39.7	21.0	16.0	8.0	11.7	4.0	23.4	
	SDG	0.4	11.8	56.0	59.2	123.5	82.2	46.2	41.0	22.0	18.2	8.0	65.2	
D 1	SD	1.8	3.9	9.1	6.5	20.5	10.6	5.0	4.1	1.2	2.0	0.0	7.7	84
	ASD	1.0	5.1	15.6	16.3	56.2	60.0	39.6	34.8	33.4	16.9	8.3	34.2	
	SDG	4.3	17.5	60.4	51.9	135.5	93.5	52.4	34.8	19.6	12.9	6.2	58.2	
B 2	SD	2.1	11.5	37.8	20.2	39.4	41.0	18.2	14.0	6.6	7.3	5.0	12.4	82
	ASD	3.1	8.0	23.8	40.3	99.2	100.0	47.8	46.2	23.1	24.6	18.5	26.6	
	SDG	5.6	28.4	111.8	97.8	252.4	176.0	122.7	85.4	47.3	41.9	22.4	60.8	
C 2	SD	1.5	7.3	25.4	31.5	49.0	28.7	20.3	18.9	6.3	16.7	5.4	13.3	79
	ASD	1.1	10.9	21.2	27.0	108.5	95.7	72.8	54.3	26.6	42.2	24.6	30.7	
	SDG	2.5	25.3	82.7	75.0	215.6	149.6	119.0	81.1	37.8	67.8	29.3	56.0	
D 2	SD	1.0	3.2	15.7	14.2	39.2	28.5	12.5	11.0	3.5	6.8	1.2	7.6	90
	ASD	0.2	4.0	24.5	42.6	140.4	93.3	43.2	40.9	27.1	12.4	8.6	24.2	
	SDG	5.7	20.9	91.6	403.6	305.5	165.9	107.5	58.8	39.7	24.8	6.3	68.2	

a) SD: sulfamethomidine, ASD: N^4-acetylsulfamethomidine, SDG: sulfamethomidine N^1-glucosiduronate

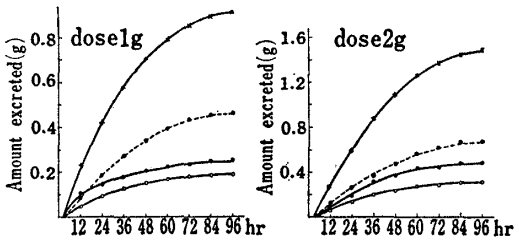

Fig. 4. Cumulative Excretion Curves of Sulfamonomethoxine Metabolites in Human Urine

—●—: sulfamonomethoxine
---●---: N^4-acetylsulfamonomethoxine
—○—: sulfamonomethoxine N^1-glucosiduronate
—×—: total sulfamonomethoxine (calculated as sulfamonomethoxine)

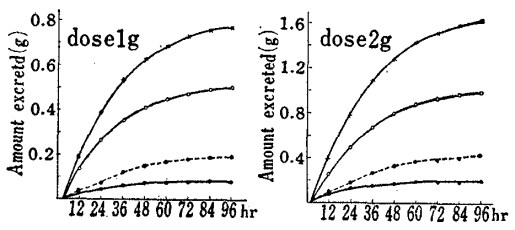

Fig. 5. Cumulative Excretion Curves of Sulfamethomidine Metabolites in Human Urine

—●—: sulfamethomidine
---●---: N^4-acetylsulfamethomidine
—○—: sulfamethomidine N^1-glucosiduronate
—×—: total sulfamethomidine (calculated as sulfamethomidine)

Table III. Recovery Test of Metabolites on Sulfamonomethoxine and Sulfamethomidine from the known Human Urine

Substance	Recovery (%)			$n^{a)}$	Standard Error (%)
	Mean	Max.	Min.		
Sulfamonomethoxine	100.3	102.1	98.2	5	0.69
N^4-Acetylsulfamonomethoxine	100.8	103.7	98.6	5	0.91
Sulfamonomethoxine N^1-glucuronide	100.4	102.3	98.9	5	0.66
Sulfamethomidine	100.1	102.5	97.6	5	0.98
N^4-Acetylsulfamethomidine	99.7	102.1	96.4	5	1.05
Sulfamethomidine N^1-glucuronide	100.5	102.6	98.6	5	0.75

a) number of determinations

ingredients				
	SM	1.632mg	SD	0.612 mg
	ASM	2.325	ASD	1.307
	SMN^1G	1.127	SMN^1G	3.134
	normal urine ad 10 ml		normal urine ad 10 ml	

Table IV. Amount of Substances excreted in Rabbit Urine after Ingestion of Sulfamonomethoxine (SM) and Sulfamethomidine (SD) (Calculated as SM or SD (mg))

Subject and dose (g)	Substance[a)]	Time of urine collection (hr after ingestion)		% of total excreted	% of dose excreted
		24	48		
SM	SM	30	22	25	83
0.25	ASM	125	31	75	
SM	SM	138	23	37	86
0.50	ASM	229	39	63	
SD	SD	22	2	14	68
0.25	ASD	114	13	74	
	SDG	18	2	12	
SD	SD	25	4	10	57
0.5	ASD	207	23	81	
	SDG	23	3	9	

a) ASM: N^4-acetylsulfamonomethoxine ASD: N^4-acetylsulfamethomidine
SDG: sulfamethomidine N^1-glucosiduronate

the difference in the amount of metabolism from those found during the daytime activity of up to 12 hr and at 36, 60, and 84 hr, indicating larger amount excreted during the day.

In rabbits, as shown in Table IV, the amount of the acetylated compound was much more than that found in the humans while the amount of the glucuronide was relatively smaller. In the case of human subjects, glucuronide was the most abundant among the metabolites of sulfamethomidine, and this was true in the case of rabbits, but the amount of glucuronide was small in the metabolites of sulfamonomethoxine, and the amount was not sufficient for determination in the case of rabbits.