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Analysis of 1,4-Dimorpholino-7-phenylpyrido[3,4-*d*]pyridazine (DS-511) and Its Metabolites in Biological Specimens. III.[1] Fluorodensitometric Method for the Simultaneous Determination of DS-511 and Its Metabolites in Urine and Bile[2]

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A simple and rapid fluorodensitometric method for the simultaneous determination of 1,4-dimorpholino-7-phenylpyrido[3,4-*d*]pyridazine (DS-511) and its metabolites in urine and bile was established. The biological specimens containing DS-511 and its metabolites were separated into lipophilic and hydrophilic layers by extraction with ethyl acetate. The hydrophilic layer was incubated at 37° for 24 hr with β-glucuronidase and arylsulfatase, then separated again into two layers by a similar extraction procedure. The lipophilic layers were purified by two-dimensional thin-layer chromatography with chloroform–methanol (10: 1) and ethyl acetate–benzene (3: 2). The hydrophilic layer was passed through a column of Amberlite XAD-2 (100—200 mesh), eluting with ethanol, and the eluate was purified by stepwise thin-layer chromatography with ethyl acetate–benzene (3: 2) and chloroform–methanol (2: 1). After moistening the air-dried chromatogram with 1-butanol, the fluorescent spots were quantitatively determined with a spectrodensitometer in the fluorescence mode. The recoveries and the coefficients of variation (c.v.) with this method were 89—116% (c.v. 6—14%) for lipophilic compounds and 93.0—95.0% (c.v. 5—6%) for hydrophilic compounds at concentrations between 0.1—1.0 μg/ml in biological specimens. This method could be used to analyze biological specimens from rats and dogs after administration of DS-511 and should also be applicable to human specimens.

Keywords——1,4-dimorpholino-7-phenylpyrido[3,4-*d*]pyridazine; sub-micro determination; fluorometry; thin-layer chromatography; biological specimen

Previously, we described the identification of DS-511 metabolites in human, dog and rat urine and bile juice, and presented a method for the quantitative determination of DS-511 (**1**) and its metabolites in the blood of these species.[1,4,5] This paper reports further work on the quantitative analysis of these compounds in urine and bile juice of the rat and dog.

Experimental

Authentic Samples——DS-511 (**1**), 4-morpholino-7-phenylpyrido[3,4-*d*]pyridazin-1(2H)-one[6] (**2**), 1-morpholino-7-phenylpyrido[3,4-*d*]pyridazin-4(3H)-one[6] (**3**), 7-(3-hydroxyphenyl)-1,4-dimorpholinopyrido-[3,4-*d*]pyridazine[7] (**4**), and 7-(4-hydroxyphenyl)-1,4-dimorpholinopyrido[3,4-*d*]pyridazine[7] (**5**) were kindly provided by Dr. Y. Oka of our division. 7-(4-Hydroxy-3-methoxyphenyl)-1,4-dimorpholinopyrido[3,4-*d*]-pyridazine (**6**), 4-[N-carboxymethyl-N-(2-hydroxyethyl)amino]-1-morpholino-7-phenylpyrido[3,4-*d*]pyridazine

1) Part II: M. Kuwayama, K. Itakura, and S. Miyake, *Chem. Pharm. Bull.*, **27**, 2321 (1979).
2) This work was presented at the 97th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1977.
3) Location: *Jusohonmachi, Yodogawa-ku, Osaka 532, Japan.*
4) M. Kuwayama, S. Miyake, K. Omura, and K. Itakura, *Chem. Pharm. Bull.*, **27**, 1544 (1979).
5) M. Kuwayama, S. Miyake, and K. Omura, to be published elsewhere.
6) Y. Oka, K. Omura, A. Miyake, K. Itoh, M. Tomimoto, N. Tada, and S. Yurugi, *Chem. Pharm. Bull.*, **23**, 2239 (1975).
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sodium salt (**7**) and 1-[N-carboxymethyl-N-(2-hydroxyethyl)amino]-4-morpholino-7-phenylpyrido[3,4-*d*]-pyridazine sodium salt (**8**) were prepared as described previously.[4]

Urine and Bile——Four male JCL Sprague-Dawley rats weighing 300 to 350 g, 10 to 12 weeks old, were orally fed **1** at 5 mg/kg with physiological saline after fasting overnight. Urine of two rats was collected for 24 hr using metabolic cages. Bile juice of the other two rats, after cannulation to the bile duct under anesthesia with pentobarbital, was collected for 24 hr at scheduled intervals. The experiments on the dog were done using a male dog weighing 6 kg. It was orally fed **1** at a dose of 5 mg/kg and the urine was collected for 24 hr using a metabolic cage. After being kept for a week without any drug, the dog was anesthetized with pentobarbital and ligation of the cystic duct followed by cannulation to the bile duct was performed. The dog was fed **1** again at the same dosage 24 hr after the operation and bile samples were collected. Human bile juices, obtained from subjects with informed consent in a clinical study (dose: **1**, 250 mg/man), were kindly provided by Dr. S. Tominaga at Shinko Hospital in Kobe.

Scanning Spectrofluorodensitometry——A Shimadzu CS-900 thin-layer chromatogram scanner was used. The conditions of measurement were the same as those used in the previous study.[1]

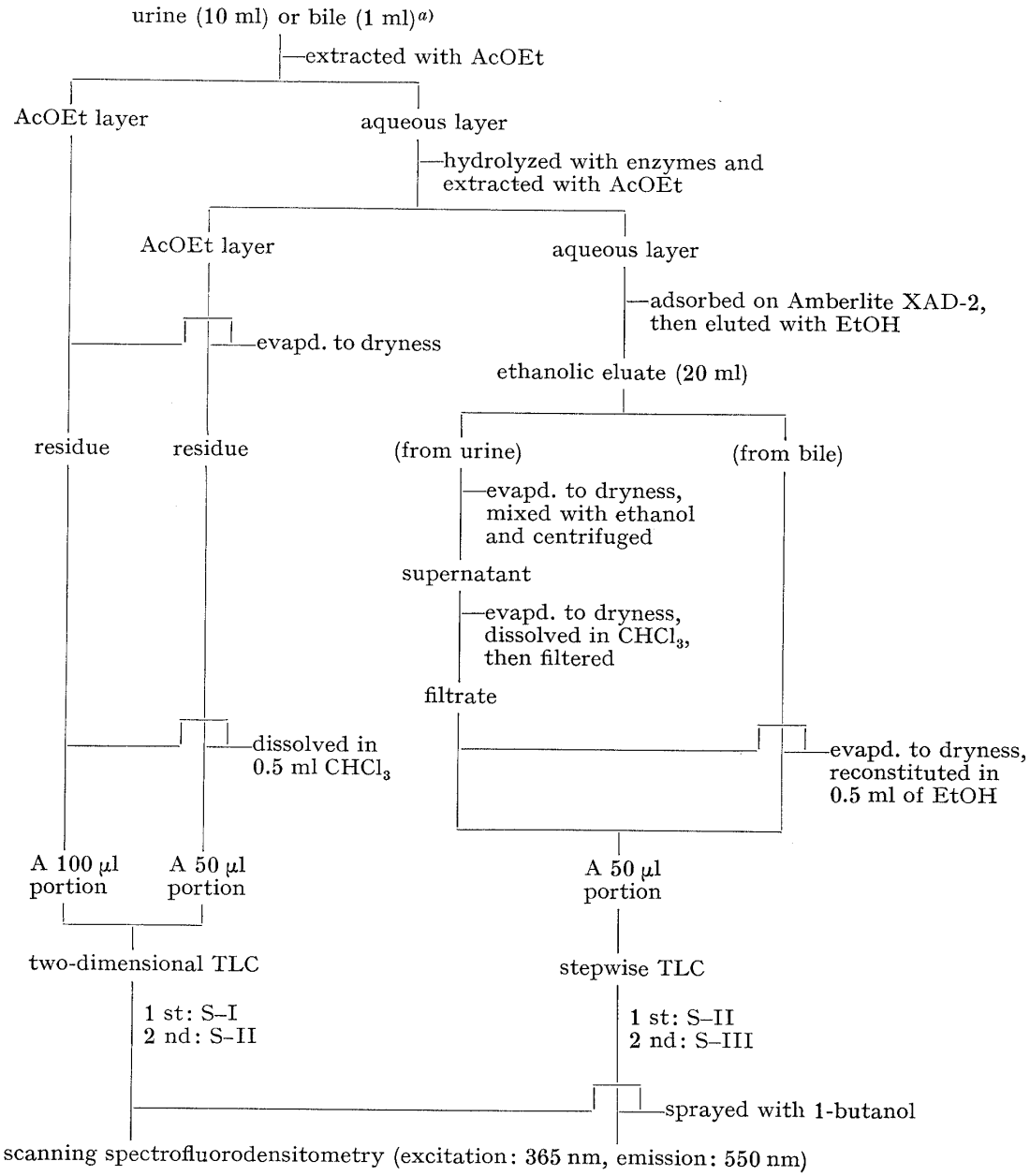

Chart 1. Procedure for the Determination of DS-511 and Its Metabolites in Urine and Bile

a) In the case of bile, 10 ml of water is added.

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Thin-Layer Chromatography (TLC)——As developing solvents for TLC, the following three mixtures were used: S-I, chloroform–methyl alcohol (10: 1); S-II, ethyl acetate–benzene (3:2); S-III, chloroform–methyl alcohol (2: 1).

Standard Procedure for Quantitative Analysis of DS-511 and Its Metabolites in Urine and Bile——Urine (10 ml) or a mixture of bile juice (1 ml) and water (10 ml) was used as the sample solution. The flow sheet of the analytical procedure is given in Chart 1. The sample solution is first extracted twice with 30 ml each of AcOEt, followed by evaporation and redissolving in 0.5 ml of $CHCl_3$. A 0.1 ml portion of the solution is applied to one corner, 20 mm from both edges, of a TLC plate (E. Merck, silica gel $60F_{254}$, Art. 5715). The plate is developed with S-I to about 12 cm and, after drying, 20 μl of a solution containing 0.0005% each of **1**, **2**, **3**, **5** and **6** is spotted at the corner towards which the sample has been developed with S-I. The second development is then conducted normal to the first with S-II to about 12 cm. Spots due to **1**, **2**, **3**, **5** and **6** are detected under UV light (365 nm) and the fluorescence intensity of each spot is measured with a chromatogram scanner as shown in Fig. 1. Second, the aqueous layer is buffered with 15 ml of 0.1 M phosphate buffer (pH 5.8±0.2) and hydrolyzed with 30 mg each of β-glucuronidase and arylsulfatase at 37° for 24 hr. The hydrolysate is readjusted to pH 7 and extracted with AcOEt as described above. The AcOEt layer is worked up as described above except that a 50 μl sample is spotted in this case. The remaining aqueous solution is passed through an Amberlite XAD-2 column, 10 mm i.d. and 5 cm long, with a resin of particle size from 100 to 200 mesh. The metabolites **7** and **8** are quantitatively adsorbed on the column, which, after washing with water, is eluted with 20 ml of EtOH. When the sample is bile juice, the eluate is concentrated under reduced pressure and the residue is redissolved in 0.5 ml of EtOH, one-tenth of which is then subjected to

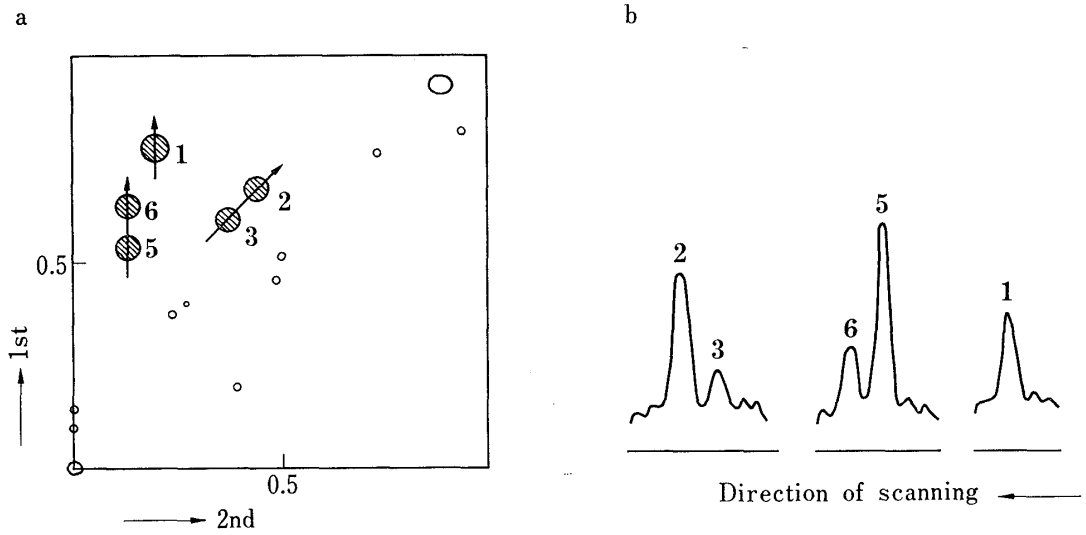

Fig. 1. Thin-Layer Chromatogram and Corresponding Spectrofluorodensitograms of DS-511 and Its Metabolites Extracted from Spiked Urine[a)]

a) Processed sample of urine containing 1 μg of each of the authentic compounds in 10 ml. Arrows indicate the scanning directions.

stepwise TLC as described later. When the sample is urine, the ethanolic eluate is concentrated as described above, then 5 ml of EtOH is added to the residue followed by mixing and centrifugation at 3000 rpm for 5 min. The supernatant is then evaporated to dryness under reduced pressure and the residue is extracted four times with 5 ml each of $CHCl_3$ under vigorous shaking. The extract is filtered through Toyo Roshi No. 5 filter paper and the filtrate, after evaporation to dryness, is redissolved in 0.5 ml of EtOH. One-tenth of the solution is treated in the same manner as the bile juice sample, that is, the solution is spotted in a short line 20 mm from the bottom of a TLC plate and 20 μl of a solution of **7** at a concentration of 0.0005% in EtOH is applied to both sides of the line about 2 cm from each end of the line. The plate is developed with S-II to about 15 cm, followed by drying and subsequent development with S-III in the same direction for the same distance. After detecting the metabolites under UV light (365 nm), they are measured with the chromatogram scanner.

Results

Determination of DS-511 and Its Lipophilic Metabolites

DS-511 and its lipophilic metabolites, **2**, **3**, **4**, **5** and **6**, were extracted with ethyl acetate from neutral or weak alkaline solution with a recovery of more than 98%. The *Rf* values of

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the compounds for the various solvent mixtures are given in Table I. Compounds **1**, **2**, **3** and **5** were separated well with S–II as reported previously.[1] Compound **4** overlapped with **5** in every solvent system. To separate **6** from **4** and **5**, another solvent, S–I, had to be used. Thus, two-dimensional TLC with S–I and S–II sufficiently separated **1**, **2**, **3**, **4** and **5**, and **6** for measurement with the chromatogram scanner. Figure 1a shows a chromatogram of a blank urine sample to which 1 μg each of **1**, **2**, **3**, **5** and **6**, had been added, followed by processing according to Chart 1. Scanning was done in the direction of the arrows in the figure in the fluorescence mode, and the recorded curves are shown in Fig. 1b. Clearly, the metabolites could be measured without any overlapping with each other or with any fluorescent components of urine under these conditions. The sample of bile juice also gave a similar result.

Table I. *Rf* Values of DS-511 and Its Metabolites on Thin-Layer Chromatograms

Compounds	*Rf* value on TLC (Kieselgel HF_{254})				
	S–I	S–II	S–III	S–IV[a]	S–V[a]
1	0.76	0.20	0.88	0.85	0.73
2	0.65	0.45	0.85	0.84	0.76
3	0.59	0.39	0.84	0.83	0.76
4	0.53	0.13	0.88	0.79	0.69
5	0.52	0.13	0.88	0.79	0.69
6	0.62	0.14	0.87	0.80	0.70
7	0.01	0	0.44	0.45	0.51
8	0.01	0	0.50	0.52	0.48

a) S-IV: *n*-BuOH–2.8% NH_4OH–EtOH (5: 2: 1).
S-V: *n*-BuOAc–AcOH–$(Me)_2CO$–H_2O (10: 6: 3: 4).

Determination of Hydrophilic Metabolites

Compounds **7** and **8** are amphoteric electrolytes and even at their isoelectric point, can hardly be extracted with ethyl acetate. However, they were found to be adsorbed quantitatively on Amberlite XAD-2, which is frequently used for the adsorption of drugs from urine samples.[8] Elution with more than 10 ml of ethanol recovered **7** and **8** in yields of more than 95%. These metabolites were separable by TLC with polar solvent systems. Among them, S–III was the most suitable, since the solvent system containing ammonia water required more than 4 hr for development, and the system containing acetic acid quenched the fluorescence considerably.

In the case of bile, a recovery test using 1 μg of **7** showed that the ethanolic eluate was soluble in 0.5 ml of ethanol after evaporation to dryness, and TLC with S–III gave a well-separated spot. In contrast, the ethanolic eluate of a urine sample contained a certain amount of material insoluble in 0.5 ml of ethanol, so 5 ml of ethanol was used to extract metabolites from the evaporated residue of the ethanolic eluate. Furthermore, TLC of the supernatant from this ethanol extract showed that some fluorescent urine component(s) interfered with the determination of **7**. After several attempts to eliminate this interference, chloroform extraction of the evaporated residue was found to be an efficient method. In addition, stepwise TLC using S–III after development with S–II resulted in better separation of **7** and **8** from urine components than when S–III alone was used. Figures 2 and 3 show examples of stepwise thin-layer chromatograms of urine and bile extracts, respectively.

8) J.M. Fujimoto and V.B. Haarstad, *J. Pharmacol. Exp. Therap.*, **165**, 45 (1969).

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Table II. Effects of pH and Incubation Time for Enzymolysis of Conjugated Metabolites

Enzyme[a] pH Incubation time (hr)[a]	G and S 6.8 to G 5.0 to S 24 each	G plus S							
		5.0	5.5			6.0			6.8
		24	19	24	48	19	24	48	24
Found (μg/ml)									
3	9.0	8.8	8.7	8.7	8.7	7.9	9.1	9.1	8.9
5	10.8	10.4	10.4	10.4	10.9	10.7	10.4	10.7	10.5
6	72	56	62	65	69	64	68	65	53

a) Human bile (1 ml), 0.1 M phosphate buffer (10 ml), β-glucuronidase (G: 30 mg) and arylsulfatase (S: 30 mg) were incubated at 37°.

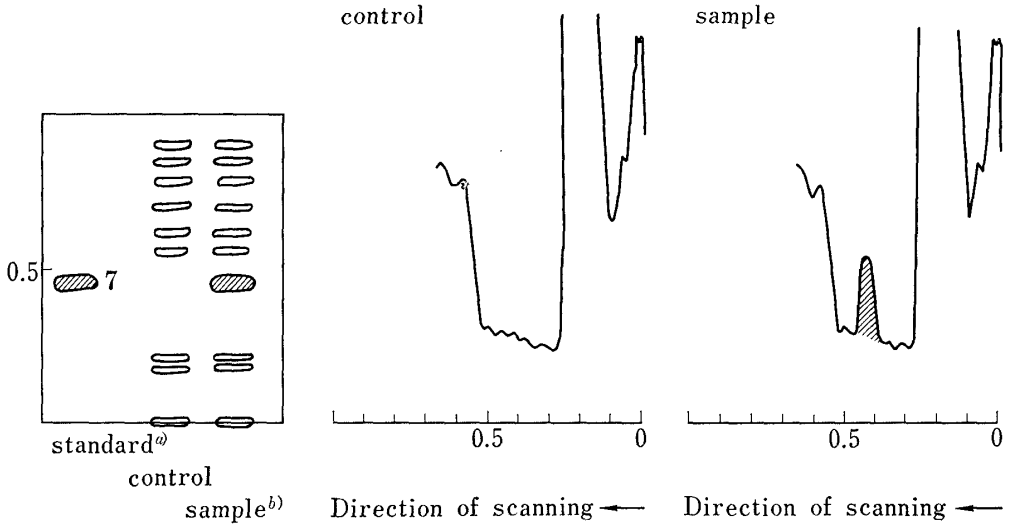

Fig. 2. Thin-Layer Chromatogram and Corresponding Spectrofluorodensitograms of a DS-511 Metabolite extracted from Spiked Urine

a) 0.1 μg of authentic compound.
b) Processed sample of urine containing 1 μg of authentic compound **7** in 10 ml.

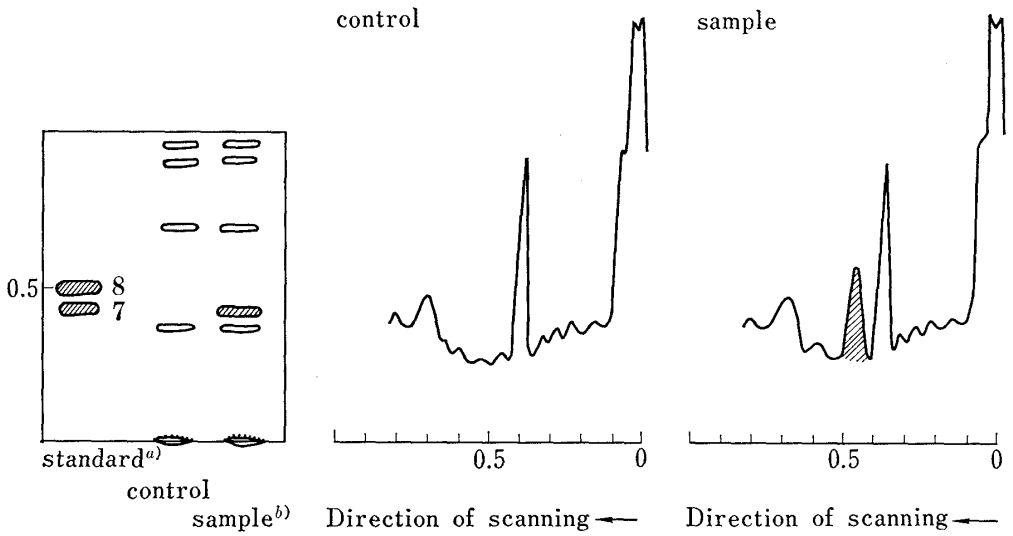

Fig. 3. Thin-Layer Chromatogram and Corresponding Spectrofluorodensitograms of DS-511 Metabolites extracted from Spiked Bile

a) 0.1 μg of each authentic compound.
b) Processed sample of bile containing 1 μg of authentic compound **7** in 1 ml.

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Hydrolysis of Conjugated Metabolites

Conjugated metabolites were much more abundant in humans than in rats or dogs in comparison with the amounts of non-conjugated metabolites.[5] Thus, we used human bile juice to seek suitable conditions for hydrolysis with enzymes. The results are shown in Table II. Conjugates of **3** and **5** were hydrolyzed equally under all the conditions tested in this study. As for conjugates of **6**, the hydrolysis yields when β-glucuronidase and arylsulfatase were allowed to react simultaneously at pH 5.5 and 6.0 were comparable to those upon successive treatment with both enzymes at their individual optimal pHs. We therefore adopted the simultaneous treatment at pH 5.8 as a timesaving method. The incubation time was set at 24 hr at 37° on the basis of the results shown in Table II.

Calibration Curves and Recovery

Various amounts (10—100 ng) of DS-511 and its metabolites were developed by two-dimensional or stepwise TLC and the fluorescence intensity of each spot was measured to obtain a calibration curve for each compound. Since each compound has a different fluorescence activity, the sensitivities naturally differ, but linear plots were obtained for all the compounds. Recovery studies were performed on 10 ml of urine or 1 ml of bile to which **1**, **2**, **3**, **5**, **6**, **7** and **8** were added at a concentration of 0.1 or 1 μg/ml. The results obtained with urine samples are shown in Table III. At the concentration of 1 μg/ml, the recoveries of **1** and its lipophilic metabolites, **2**, **3**, **5** and **6**, were 89 to 104%, the variation coefficients being 6 to 11%. The minimum detectable levels of **3** and **5** were estimated to be 0.1 and 0.03 μg in 10 ml of urine, respectively. Hydrophilic metabolites **7** and **8** were separated from interfering substances of urine origin by transfer to chloroform, as described above. Recoveries of these metabolites through the whole analytical process were kept at levels similar to those of lipophilic metabolites by four extractions with 5 ml each of chloroform and paper filtration of the extract (Table III). The detection limit was somewhat higher, about 0.3 μg in 10 ml of urine, as a result of background fluorescence.

Table III. Recovery and Reproducibility in the Quantitation of DS-511 and Its Metabolites from Spiked Urine[a]

Concentration in urine		Compound						
		1	**2**	**3**	**5**	**6**	**7**	**8**
0.1 μg/ml	Mean (n=5, μg/ml)	0.10	0.11	0.12	0.11	0.09	Not examined	
	Recovery (%)	100	114	116	106	88		
	c.v. (%)[b]	14	10	14	11	10		
1.0 μg/ml	Mean (n=5, μg/ml)	0.96	1.01	1.04	1.00	0.89	0.93	0.95
	Recovery (%)	96.2	101	104	100	88.8	93.0	95.0
	c.v. (%)	11	10	7	8	6	6	5

a) Similar results were also obtained in the case of bile juice.
b) c.v.: coefficient of variation.

In the case of bile juice, the analytical precision and recoveries of these metabolites were comparable to those obtained with urine, and the minimum detectable levels of **3**, **5** and **7** were estimated to be 0.1, 0.03 and 0.3 μg/ml, respectively, in bile.

Urinary and Biliary Levels after Administration

Urine and bile specimens of rats and dogs orally administered **1** at 5 mg/kg were analyzed by the present method. As shown in Table IV, lipophilic metabolites **2**, **3**, **5** coexisting with **4**, and **6** were present more in conjugated form than in the free form, except for **2** and **3** in some samples. Metabolite **5** was detected in rat bile only in conjugated form but was found

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TABLE IV. Determination of DS-511 and Its Metabolites in Bile and Urine after Oral Administration of DS-511 to Rats and a Dog (dose: 5 mg/kg)

			JCL-SD rat[a]											Beagle dog						
			Bile							Urine				Bile					Urine	
			(A+B)/2 Concentration (μg/ml)					A Mass (μg)	B Mass (μg)	C		D		Concentration (μg/ml)				Mass (μg)	Conc. (μg/ml)	Mass (μg)
Time (hr)			0—2	2—4	4—6	6—8	8—24	0—24	0—24	0—24		0—24		0—2	2—4	4—6	6—8	0—8	0—24	
Volume (ml)			3.2	2.5	2.1	1.9	13.9	20.9	26.0	16.5		25.5		2.5	7.5	4.1	4.0	18.1	45.5	
Fraction		Compound								Conc. (μg/ml)	Mass (μg)	Conc. (μg/ml)	Mass (μg)							
Lipophilic (L)	Free	**1**	0.2	0.2	0.2	0.2	0.2	4.2	3.5	0.09	1.5	0.07	1.8	1.2	0.9	0.7	0.7	15	0.14	6.4
		2	n.d.	n.d.	0.1	0.1	0.1	1.6	0.5	0.07	1.2	0.06	1.5	0.3	0.2	0.3	0.2	4	0.02	0.9
		3	n.d.	n.d.	0.1	0.1	0.1	1.6	0.5	0.04	0.7	0.05	1.3	0.4	0.4	0.4	0.3	7	0.02	0.9
		5[b]	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	0.17	2.8	0.35	8.9	0.3	0.6	0.5	0.6	10	0.07	3.2
		6	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	n.d.	0	n.d.	0	n.d.	n.d.	n.d.	n.d.	0	n.d.	0
Hydrophilic	L-Conjugate	**2**	n.d.	n.d.	0.1	n.d.	n.d.	0.2	0.3	0.10	1.7	0.04	1.0	0.3	0.1	0.3	n.d.	3	0.03	1.4
		3	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	0.11	1.8	0.02	0.5	0.4	0.2	0.3	0.3	5	0.08	3.6
		5[b]	1.2	1.7	3.0	2.8	3.1	57.6	65.1	1.35	22.3	0.50	12.8	5.0	12.0	9.9	8.0	175	0.40	18.2
		6	0.1	0.1	0.2	0.1	0.1	2.3	2.6	0.03	0.5	0.02	0.5	2.3	6.0	5.3	4.1	89	0.32	14.6
	Free	**7**	7.3	10.0	11.3	8.7	8.6	156	267	1.97	32.5	1.25	31.9	159	172	113	81.9	2478	3.87	176

a) Rats A, B, C and D weighed 334, 304, 360 and 319 g, respectively.
b) One metabolite, **5**, coexisted with **4**.

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in both forms in rat urine and in dog bile and urine. Metabolite **6** seemed to exist only in conjugated form in every case. Among these metabolites, the conjugated form of **5** was most abundant. The amount of hydrophilic metabolite **7** was always much more than the sum of **5** and its conjugate. The ratios of the **7** to **5**-conjugate in rats and dogs were from 1.5 to 4 and from 10 to 14, respectively.

Discussion

In the present analytical procedure, the structural isomers **4** and **5** were not separately determined. These metabolites may be separated by reversed phase TLC, but the method lacks reproducibility and its unsuitable for routine analysis. The ratio of these two metabolites can be determined by gas chromatography after trimethylsilylation.[4] Metabolites **7** and **8** could be separated better by TLC with S–IV or S–V. However, as stated above, the development with S–IV was time-consuming and one of the components of S–V, acetic acid, quenched the fluorescence of the metabolites. Two-dimensional TLC deformed the spots, making them unsuitable for densitometric analysis with the chromatogram scanner. On the basis of these considerations, single-dimensional stepwise TLC was adopted for the analysis. In urine, a fluorescent substance overlapped with the spots of **7** and **8**. Although the sodium salts of **7** and **8** were highly hydrophilic, they had low solubility in chloroform (8 μg/ml). Thus, **7** and **8** could be separated from the overlapping substance which was completely insoluble in chloroform. Separation of **7** and **8** on stepwise TLC was not complete. However, their ratio could be estimated from the calibration curve of the peak height ratio obtained with standard samples containing 0.1, 0.2 and 0.5 of **8** against 1.0 of 7. When the ratio of **8** to **7** was lower than 0.1, the presence of **8** was not detectable, being hidden under the background. Table IV clearly shows that hydrolytic dissociation of the morpholino groups from positions 1 or 4 took place without any noticeable regional selectivity in both rats and dogs. On the other hand, the morpholino group at position 4 was much more susceptible to oxidative cleavage than that at position 1 and the amount of metabolite **8** was usually less than one-twentieth of that of **7**, which is one of the most important metabolites excreted. Among the lipophilic metabolites, **6**, which is abundant in humans, was present in small amounts in rats. The conjugated form of this metabolite was one of the important metabolites after conjugated **5** in dogs. This suggests that the dog has a metabolic pathway similar to that of humans. 7-(3,4-Dihydroxyphenyl)-1,4-dimorpholinopyrido[3,4-*d*]pyridazine, which has been identified among human metabolites, was very labile to oxidation due to its catechol structure[9] and could not be measured by the present method on TLC. Some modifications may be necessary for the quantitation of this metabolite. The present procedure provides a precise analytical method with a high recovery at least at the drug concentration level of 1 μg/ml. It should be useful for urine and bile juice analysis in metabolic and toxicity studies on laboratory animals, especially for comparative metabolic studies for selecting animal species to be used as surrogates for humans.

Acknowledgement The authors are grateful to Drs. E. Ohmura, H. Morimoto and M. Nishikawa of this division for their encouragement throughout this work.

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