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Analysis of 1,4-Dimorpholino-7-phenylpyrido[3,4-*d*]pyridazine (DS-511) and Its Metabolites in Biological Specimens. II.[1] Fluorodensitometric Method for Simultaneous Determination of DS-511 and Its Metabolites in Plasma[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 plasma was established. DS-511 and the metabolites were extracted from plasma with ethyl acetate. The organic layer was subjected to thin-layer chromatography with ethyl acetate–benzene (3:2). The air-dried chromatogram was moistened with 1-butanol, then the fluorescent spots were quantitatively determined with a spectro-densitometer in a fluorescence mode. A linear calibration plot for DS-511 was obtained in the concentration range of 0.02—0.50 μg per 1 ml of plasma. The accuracies as the coefficients of variation were 8 and 3% at concentrations of 0.05 and 0.25 μg of DS-511 per 1 ml of plasma, respectively. This method can be used to analyze plasma levels of DS-511 after its administration to rats and dogs, and should also be applicable to human plasma.

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

In the preceding paper,[1] we described the structural determination of some metabolites of 1,4-dimorpholino-7-phenylpyrido[3,4-*d*]pyridazine (DS-511, **1**), a new type of hypotensive diuretic,[4] in dog and rat urine as well as in dog bile juice. It was shown that the following three metabolic pathways operate: (1) hydroxylation at *para* and/or *meta* positions of the 7-phenyl group; (2) hydrolytic removal of the morpholine group at the 1- or 4-position of **1**; (3) cleavage of the morpholine ring by successive oxidation and hydrolysis.

As an aid for the clinical study of DS-511, a method for the quantitative determination of **1** and its metabolites in blood was developed, using rat and dog plasma as test samples. The method involves extraction with organic solvent, separation by thin-layer chromatography (TLC) and fluorometric analysis on the TLC plate with a densitometer.

Experimental

Samples and Standards——Standard samples of DS-511 (**1**), its monodemorpholino derivatives (**2** and **3**) and the *p*-hydroxylated compound (**4**) were kindly supplied by Dr. Oka of this Research Division, as described previously[1] (see Chart 1 for the structures). Male Sprague–Dawley (JCL: SD) rats weighing about 240 g (7 weeks old) were deprived of food but given free access to water for 16—18 hr prior to the experiments, then **1**, 10 mg/kg, was administered orally with saline solution. The rats were decapitated after 0.5, 1, 3, 5, 8 or 24 hr, and plasma samples obtained by centrifuging the blood in the presence of heparin were stored in a freezer (−20°). Dog plasma was obtained in a similar manner using three male beagles (weighing 9.4—10.8 kg); plasma samples were taken from the cephalic vein 0.5, 1, 2, 3, 4, 8 or 24 hr after oral administration of **1** (10 mg/kg).

1) Part I: M. Kuwayama, S. Miyake, K. Omura, and K. Itakura, *Chem. Pharm. Bull.* (Tokyo), **27**, 1544 (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) Y. Inada, Y. Shibouta, H. Shimakawa, K. Nishikawa, and S. Kikuchi, *Arzneim.-Forsch.*, **28**, (II), 1105 (1978).

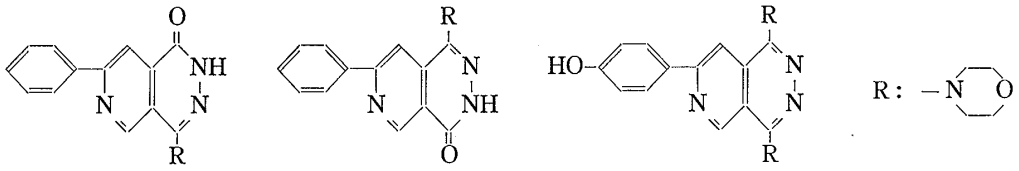

Chart 1. Structures of the Metabolites of DS-511

Apparatus and Conditions of Measurements——A Shimadzu RF-502 spectrofluorometer was used to measure the emission and excitation spectra in various solvents. TLC densitograms were obtained on a Shimadzu CS-900 chromatogram scanner equipped with a fluorometer and double-wavelength spectrophotometer under the following conditions.

excitation: 365 nm (mercury lamp)
emission: 550 nm (filter)
slit: 0.5×15 mm
sensitivity: low-high (fluorescence mode) and 1—10 mV (Shimadzu U-225 MCS recorder)
scanning: linear scan (40 mm/min)

Scans were controlled so as to pass over the center of each TLC spot and the peak areas were calculated as the product of the peak height and the width at half the peak height.

Standard Procedure for the Quantitative Analysis of DS-511 and Its Metabolites in Plasma——The procedure is outlined in Chart 2. First, 3 ml of water, 10 ml of 1% $NaHCO_3$ aqueous solution and 30 ml of AcOEt were added to 1 ml of plasma and the mixture was shaken for 5 min. The AcOEt layer was then separated and the aqueous layer was further extracted with 20 ml of AcOEt. The combined AcOEt layers were washed twice with 10 ml each of water. The AcOEt fraction was evaporated to dryness at about 40° under reduced pressure and the residue was redissolved in 0.5 ml of $CHCl_3$. A portion of the $CHCl_3$ solution, 0.1 ml, was applied in an area of 5×8 mm at the bottom of the TLC plate (E. Merck, silica gel $60F_{254}$, art 5715) and 20 μl aliquots of 0.0005% $CHCl_3$ solutions of each of the standard compounds **1**, **2**, **3** and **4** were applied on both sides of the sample. After developing with AcOEt–C_6H_6 (3: 2) to about 15 cm, the plate was illuminated with a UV lamp and marked at about 2 cm above the spot of **2**. The plate was dried, sprayed with 1-butanol, and then covered with a glass plate after the margin, about 5 mm wide, of the TLC plate had turned white due to the evaporation of 1-butanol. The plate was placed on the TLC densitometer and scanned from the origin to the mark. The amount of each component, **1**, **2**, **3** or **4**, was calculated from standard curves prepared fluorodensitometrically by running through the same procedure using corresponding authentic samples.

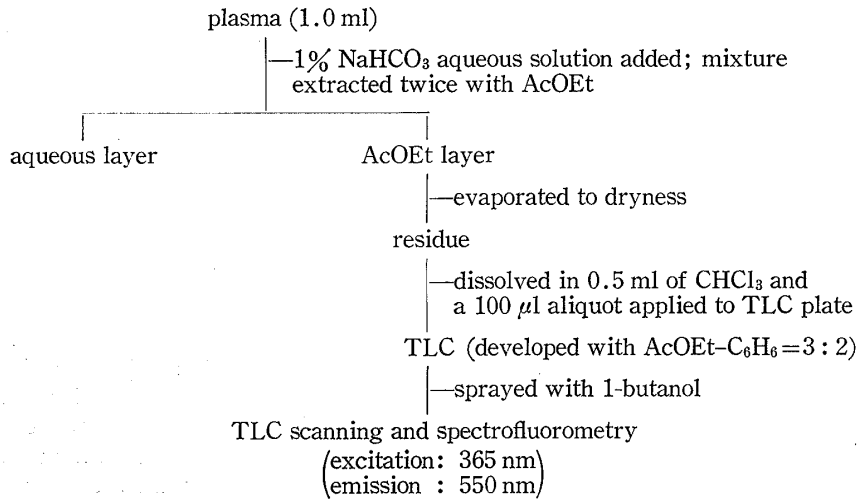

Chart 2. Procedure for the Determination of DS-511 and Its Metabolites in Plasma

Results

Selection of Development Solvent

DS-511 (**1**), **2**, **3** and **4** could be separated well on the TLC plate with a mixture of chloroform and methanol (10: 1) or of ethyl acetate and benzene (3: 2) as the developing solvent. The *Rf* values of these compounds on chromatograms developed with these solvent

Table I. *Rf* Values of DS-511 and Its Metabolites

Compound	*Rf* value	
	Solvent I[a)]	Solvent II[b)]
1	0.76	0.20
2	0.65	0.45
3	0.59	0.39
4	0.52	0.13

a) Chloroform–methanol (10: 1).
b) Ethyl acetate–benzene (3: 2).

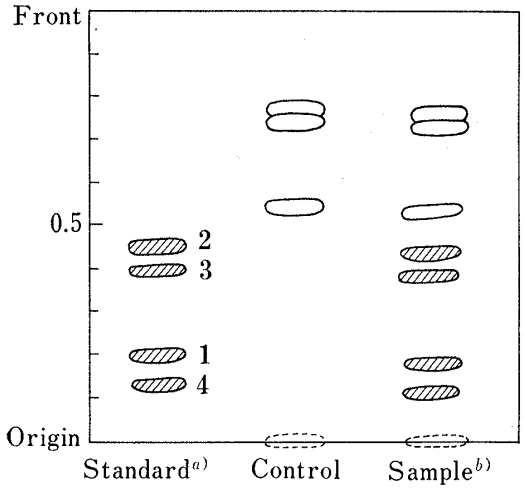

Fig. 1. Thin-layer Chromatogram of DS-511 and Its Metabolites in Plasma

a) Samples of 0.05 μg each of authentic compounds were used.
b) Processed sample of plasma containing 0.25 μg of each authentic compound.

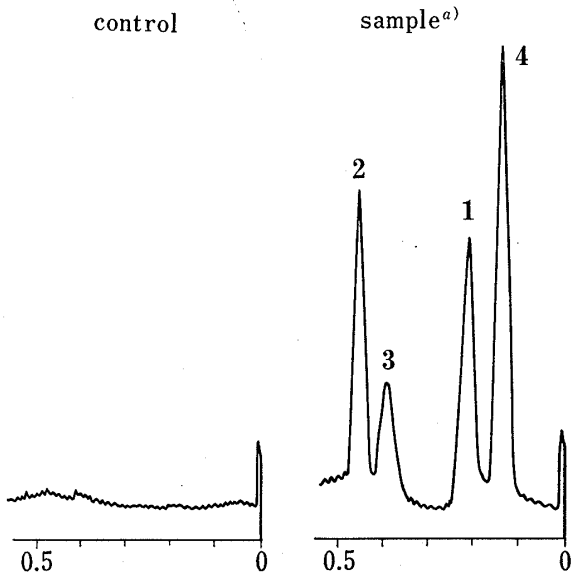

Fig. 2. TLC Fluorodensitogram of DS-511 and Its Metabolites extracted from Spiked Plasma

a) Processed sample of plasma containing 0.25 μg of each authentic compound in 1 ml of plasma.

systems are shown in Table I. When the former solvent system was used with plasma samples, however, some peaks due to plasma components overlapped with those of **1** or its metabolites thus decreasing the accuracy of the analysis. As shown in Fig. 1 and 2, such interactions could be avoided by using the ethyl acetate and benzene system.

Fluorescence

DS-511 (**1**) and its metabolites, **2**, **3** and **4**, showed fluorescence in methanol and other organic solvents. The excitation and emission spectra in methanol are shown in Fig. 3. The fluorescence was weakest in **1**, while the polar metabolites, **2**, **3** and **4**, gave fluorescence 1.5—2 times stronger than that of **1**. Among them, the fluorescence of the 4-demorpholino derivative, **3**, was the strongest, though the maxima of both the emission and excitation spectra were located at shorter wavelengths than those of the other compounds. The maxima of the emission and excitation spectra of the four compounds in methanol and 1-butanol are listed in Table II. The maxima of the emission spectra in 1-butanol were at shorter wavelength than those in methanol in every case, but the patterns of the spectra were similar in both solvents. There was little difference between the excitation spectra in the two solvents.

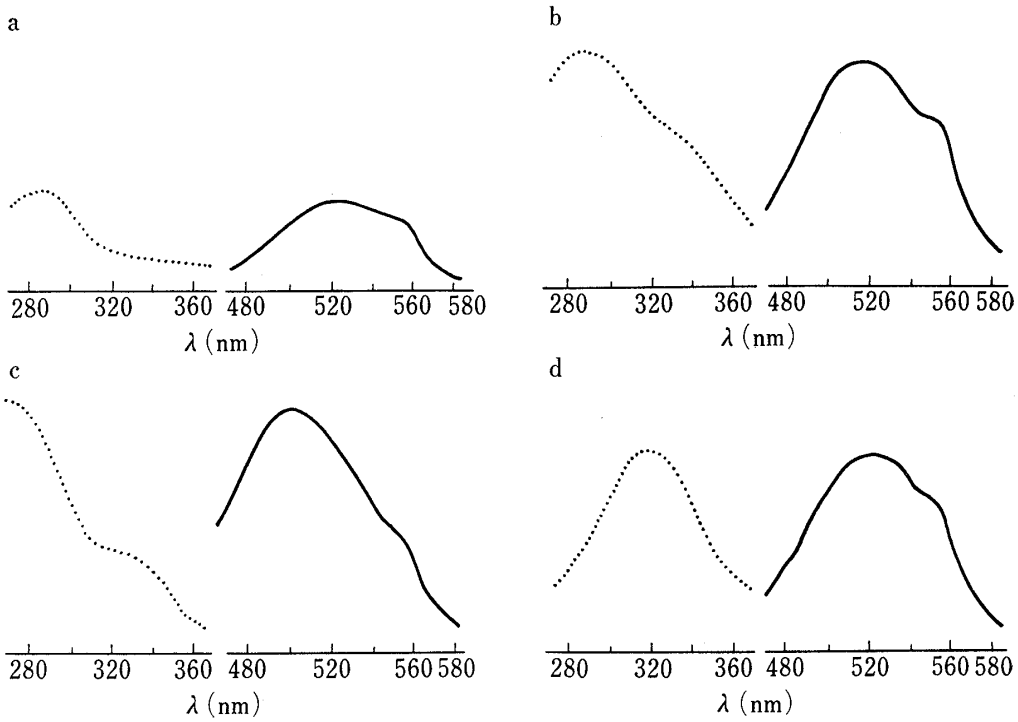

Fig. 3. Excitation and Emission Spectra of DS-511 and Its Metaboiites in Methanol

a: compound **1**; The excitation spectrum with emission at 520 nm and the emission spectrum with excitation at 290 nm are shown.
b: compound **2**; The spectra measured as described above are shown.
c: compound **3**; The excitation spectrum with emission at 500 nm and the emission spectrum with excitation at 275 nm are shown.
d: compound **4**; The excitation spectrum with emission at 520 nm and the emission spectrum with excitation at 320 nm are shown.

TABLE II. Fluorescence Spectral Characteristics of DS-511 and Its Metabolites

Compound	Maximum wavelength (nm)			
	Methanol		Butanol	
	Excitation	Emission	Excitation	Emission
1	290	520	290	510
2	290	520	290	505
3	275	500	275	490
4	320	520	320	510

TABLE III. Relative Fluorescence Intensities of DS-511 and Its Metabolites in Several Solvents

Solvent	Compound[a]			
	1	**2**	**3**	**4**
Methanol	100[b]	156	178	137
Ethanol	114	207	254	162
1-Propanol	120	221	284	167
1-Butanol	123	228	291	177
1-Pentanol	120	224	294	171
Chloroform	120	215	254	137
Ethyl acetate	76	173	220	92
Acetic acid	7	7	18	11

a) The concentration of each compound was 2 μg per ml.
b) The intensity in methanol was taken as 100.

Solvent Effect on the Fluorescence Intensity

With excitation at 290, 290, 275 and 320 nm and emission at 520, 520, 500 and 520 nm, respectively, the intensities of the fluorescence due to **1**, **2**, **3** and **4** were measured in various solvents (Table III). All of the compounds showed strong fluorescence in alcoholic solvents and chloroform. The fluorescence intensities of **1**, **2** and **4** were strongest in 1-butanol, whereas that of **3** was a little stronger in 1-pentanol than in 1-butanol.

Maintenance of the Fluorescence Intensity on TLC Plates

When a TLC plate was taken from the developing chamber and the fluorescence was observed without any cover on the plate, the intensity decreased gradually as the developing solvent evaporated. To follow the intensity change, a low-volatility solvent, 1-butanol, was applied to a dried TLC plate and the fluorescence intensity of **1** was measured at intervals. The fluorescence slowly increased for the first 30 or 40 min, then gradually faded. If the TLC plate was covered with a glass plate of the same size when the fluorescence intensity became maximum, the intensity was retained for more than 30 min.

Calibration Curve and Recovery

The calibration curve was obtained by applying various amounts (10—100 ng) of **1** or its metabolites to TLC plates and measuring the fluorescence intensity after development. For each compound, a linear relationship existed between the amount of sample applied and the observed fluorescence intensity (Fig. 4, sensitivity: 10 mV). A similar relationship was also found in the sample range of 0.1 to 0.5 μg (sensitivity: 2 mV), indicating that the range of linearity is satisfactory for quantitative analysis. To determine the recovery of **1** and its metabolites through the present analytical procedure, each compound was added to blank plasma to give 0.05 or 0.25 μg/ml and the procedure was followed. The results are given in Table IV. The recoveries were 93—107% and the variation coefficients were calculated to be 3—12%. The detection limits were 0.02 and 0.05 μg/ml for **4** and **3**, respectively, when 250 μl of the chloroform solution was spotted on the TLC plate.

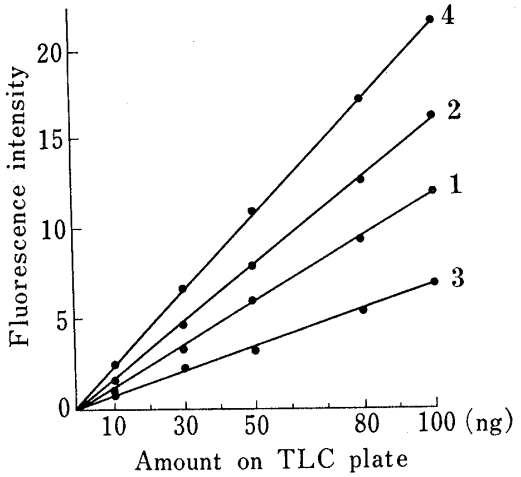

Fig. 4. Calibration Plots for DS-511 and Its Metabolites

Blood Level after Administration

DS-511 (**1**) was administered to dogs and rats orally at a dose of 10 mg/kg. The blood levels of **1** and its metabolites were followed for 24 hr. The metabolites were not present in

Table IV. Recovery and Its Reproducibility of DS-511 and Its Metabolites from Spiked Plasma

Concentration in plasma		Compound 1	2	3	4
0.05 μg/ml	Mean (n=3, μg/ml)	0.047	0.051	0.053	0.050
	Recovery (%)	94	102	106	100
	c.v. (%)[a]	8	3	4	12
0.25 μg/ml	Mean (n=4, μg/ml)	0.233	0.234	0.253	0.268
	Recovery (%)	93	94	102	107
	c.v. (%)[a]	3	6	9	7

a) c.v.: coefficient of variation.

Table V. Plasma Concentrations in Rats and Dogs after Oral Administration of DS-511 (Dose: 10 mg/kg)

			Concentration of DS-511 in plasma (μg/ml)						
Male JCL: SD rat ($n=3$)	Time (hr)	0	0.5	1	3	5	8	24	
	Mean ±S.E.	0	0.15± 0.03	0.38± 0.06	0.30± 0.01	0.15± 0.07	0.03± 0.01	≦0.02[a]	
Male beagle dog ($n=3$)	Time (hr)	0	0.5	1	2	3	4	8	24
	Mean ±S.E.	0	0.10± 0.02	0.31± 0.05	0.20± 0.02	0.14± 0.03[b]	0.09± 0.01	0.04± 0.01	0.02± 0.0

a) Less than 0.02 μg/ml in two specimens.
b) Obtained from two specimens.

detectable amounts in any of the blood samples. Table V lists the concentrations of **1** in the plasma. The maximum level was reached one hr after administration in both dogs and rats. After 24 hr, the level had fallen nearly to the detection limit.

Discussion

The measurement of blood levels of drugs and their metabolites after administration is important in clinical studies. However, when the drug activity is high, the measurement of blood levels becomes more difficult. Highly sensitive gas chromatography, *i.e.*, ECD–GC or FPD–GC, and high performance liquid chromatography have been used for such analysis in a wide variety of samples. TLC is now being reconsidered as an alternative method because of the marked improvement in densitometric detection, together with the simplicity of the method. As regards detection, fluorometry is one of the most sensitive techniques, comparable, in some cases, to radiometry. TLC, however, is unsuitable for fluorometry since silica gel may cause quenching and/or scattering of the fluorescent light. In this study we found that by moistening the gel appropriately with a rather non-volatile solvent and preventing its further evaporation, fluorometry could be used as a powerful detection method in combination with TLC. As shown in Table III, in 1-butanol solution **3** gave the strongest fluorescence among the four compounds tested. However, the detection limit in the present analytical procedure was highest (0.05 μg/ml) for **3** (Fig. 4). This resulted from the use of a glass plate as a cover after TLC to prevent solvent evaporation, which ruled out excitation at short wavelength due to light absorption by the glass. When the cover glass was removed and excitation at 313 nm was used, the fluorescence of **3** increased more than three times, but the intensity values were not sufficiently reproducible to allow quantitative analysis.

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