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Elimination of Methylene Blue in Dogs after Oral or Intravenous Administration[1]

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In order to estimate the extent of gastrointestinal absorption of methylene blue (MB) in dogs and to search for more contributory route of excretion than urine, elimination of MB was investigated after oral and intravenous administration at a dose of 15 mg/kg in mongrel dogs.

After oral administration, total MB, which was the sum of unchanged MB and leucomethylene blue (LMB), was recovered from urine and feces with averages of 3.9 and 44.3% of the dose, respectively. When MB was intravenously injected at the same dose, total urinary and fecal recoveries were 6.6 and 19.9%, respectively. Therefore, it was assumed that about 60—70% of orally administered MB should have been absorbed from the gastrointestinal tracts of dogs. In anesthetized dogs, total biliary recovery was about 7 times greater than that from urine after intravenous administration of MB to dogs.

On the basis of these results, it was concluded that total urinary recovery should not be taken as an index of gastrointestinal absorption after oral administration of MB to dogs, and that there would not be a great species difference in gastrointestinal absorption of MB between man and the dog.

A few metabolites other than LMB were found in urine and feces of dogs, though quantitative analysis was not carried out.

Keywords——methylene blue; drug elimination; urinary excretion; fecal excretion; biliary excretion; drug metabolism; gastrointestinal absorption; pharmacokinetics; dog

In the previous paper[3] it was reported that methylene blue was absorbed from the small intestinal tracts of rats, guinea pigs, and rabbits *in situ* and that such a great species difference in the absorption characteristics as had been found between man and the dog[4] was not observed in these experimental animals.

In the present paper, urinary, fecal, and biliary excretion was investigated after oral or intravenous administration of methylene blue to dogs. The primary purpose of this work was to estimate the extent of gastrointestinal absorption of methylene blue in dogs by using the results of the urinary and fecal excretion and to search for more contributory route of excretion than urine.

After oral administration of methylene blue very small fraction of the dose was excreted into urine, and larger fraction of it into feces. Furthermore, the similar excretion pattern was observed after intravenous administration. Therefore, it was assumed that poor recovery of methylene blue from urine after oral administration was not due to poor gastrointestinal absorption of the drug but due to large excretion of it into feces through bile. A few metabolites other than leucomethylene blue were found in urine and feces, though quantitative analysis was not carried out.

Experimental

Materials——Methylene blue (MB) was purchased from Merck and Co., Inc., and purity was verified by JP VIII test. MB content in the product was 99.8% after drying. Azure B (Wako Pure Chemical Industries,

1) This work was read at the 96th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, Apr., 1976.
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3) J. Watanabe and K. Mori, *Chem. Pharm. Bull.* (Tokyo), **25**, 1194 (1977).
4) A.R. DiSanto and J.G. Wagner, *J. Pharm. Sci.*, **61**, 1086 (1972).

Ltd.) and azure A (Chroma-Gesellschaft Schimd and Co.) were used as authentic samples in the metabolic study. All other chemicals and solvents were commercially available and of analytical grade.

Methylene blue was administered orally in a hard gelatin capsule (No. 00) to the dog. In all of the intravenous administration 2 w/v % methylene blue was dissolved in sterile water for injection every time and 0.75 ml/kg of the solution was given to the dog.

Animals——Male mongrel dogs weighing 10.0—11.0 kg were fed with dog food CD-5 (Japan Clea Co.), and used after treatment with a few types of anthelmintics. The animals were fasted for 24 hr prior to administration of methylene blue and for 10 hr after dosing, but were allowed free access to water. The interval of drug administration was at least 4 weeks.

Experimental Procedures——(i) Urinary and Fecal Excretion after Oral Administration: Before oral administration of a 15 mg/kg dose of methylene blue to a dog, a catheter (Argyle, Cat. No. 108, Size 8FG) was inserted into the bladder, and it was emptied and washed two times with each 20 ml of warmed 0.9% NaCl. The animal was maintained for two or three days in a metabolic cage, where urine and feces were collected separately. Urine samples were taken every hour for 10 hr, and at 24, 48, and 72 hr by washing the bladder two times with each 20 ml of warmed 0.9% NaCl. The washings and urine were mixed, diluted with appropriate volume of 0.9% NaCl and analyzed for methylene blue (MB) and leucomethylene blue (LMB). Feces samples were collected at 24 and 48 hr after oral administration of MB. Feces samples were homogenized with appropriate volume of 0.9% NaCl and analyzed for MB and LMB.

(ii) Urinary and Fecal Excretion, and Blood Levels of MB after Intravenous Administration: MB solution was injected through the right front leg vein or the right hind leg vein of the dog. The dose was 15 mg/kg. The dog was maintained in a metabolic cage, and urine and feces were collected at the almost same sampling times as those in oral administration experiments. Blood samples were withdrawn through the left front leg vein and the sampling times were recorded.

(iii) Urinary and Biliary Excretion after Intravenous Administration: The dogs were anesthetized with intravenous administration of 30 mg/kg sodium pentobarbital through the left front vein. The front leg or the left hind leg vein was infused with a buffered solution of mannitol and pentobarbital at the rate of 2 ml/min. The solution, which was similar to that used by Dreyfuss, *et al.*,[5] contained: mannitol, 100 g; K_2HPO_4, 0.9 g; KH_2PO_4, 0.2 g; sodium pentobarbital, 25.5 mg/kg of body weight; and sufficient sterile water to make a volume of 2 l. After a midline incision was made, the bile duct was cannulated with polyethylene tubing (Hibiki No. 5) near its entrance into the duodenum. Two % MB solution (0.75 ml/kg: 15 mg/kg BW) was intravenously administered through the right hind leg vein. Urine was collected in a similar manner to that described for oral administration experiments. Urine and bile samples were taken every hour for 8 hr, and at the end of the experiment the gallbladder was excised to collect the bile in it. Urine and bile were diluted with 0.9% NaCl appropriately, and analyzed for MB and LMB.

(iv) Isolation of Metabolites of MB: Urine and feces samples obtained after intraveous administration of MB were extracted with 1,2-dichloroethane (DCE). The DCE layer was evaporated to dryness *in vacuo* at 40°. The residue was dissolved in a small amount of ethanol, and the solution was submitted to thin-layer chromatography (TLC) separation. Thin-layer chromatograms for separatory purpose were run on 20×20 cm glass pre-coated with 250 μ of Silica Gel GF_{254} (Merck), and *n*-butanol–acetic acid–water (4: 1: 5) was used for development. Ethanol was used as solvent for extraction of metabolites from the adsorbent. Each extract was submitted to comparative TLC and to visible absorption analysis. The visible absorption spectra were recorded with a Shimazu Spectrophotometer Model UV-210.

Determination of MB and Total MB——The concentration of LMB was calculated by substracting MB from total MB concentration in the same sample solution. Determination of MB or total MB concentration was carried out according to the method of DiSanto and Wagner,[6] though the method for total MB was partially modified to prevent degradation of MB.

(i) MB and Total MB in Urine and Feces: For determination of MB in urine, 5 ml of the diluted urine was added to 1.2 g NaCl and 7 ml of DCE in a centrifuge tube, which was covered with aluminum foil. The mixture was shaken for 30 min, and centrifuged for 10 min at 3000 rpm. After removing the upper water layer, the optical density of MB in DCE layer was measured at 657 nm. To determine total MB (MB+LMB), another 5 ml portion of the diluted urine was added to 1.2 g NaCl and 0.05 ml of 0.5 N HCl, heated for 3 min on a boiling water bath, and 7 ml of DCE was added to the mixture after cooling. Succeeding procedure was just the same as described for MB determination. As for the analysis of MB or total MB in feces of the dog, feces samples were homogenized with 0.9% NaCl, and 5 ml of the homogenate was used according to the same method described for urine.

(ii) MB in Whole Blood: Two or 5 ml of whole blood sample was added to 0.8 or 1.0 g NaCl and 7 or 20 ml of DCE, respectively. The contents were shaken for 30 min, and centrifuged for 10 min at 3000 rpm. The absorbance of the DCE extract was determined at 657 nm.

5) J. Dreyfuss, J.J. Ross, Jr., and E.C. Schreiber, *J. Pharm. Sci.*, **60**, 821 (1971).
6) A.R. DiSanto and J.G. Wagner, *J. Pharm. Sci.*, **61**, 598 (1972).

(iii) MB and Total MB in Bile: Bile samples were diluted from 1: 200 to 1: 1000 with 0.9% NaCl solution. Five ml of the diluted bile was used for quantitative analysis of MB or total MB according to the same procedure described for urine.

Calibration curves for MB in urine, feces, bile, and whole blood samples were made respectively by adding known amount of MB ethanol solution to those biological materials, which were taken prior to the MB administration experiments, and by employing the same analytical procedures described above.

Results and Discussion

Urinary and Fecal Excretion after Oral Administration of Methylene Blue (MB)

When 15 mg/kg body weight dose of MB was orally administered to the dog, very small fraction of the dose was recovered from the urine as shown in Fig. 1 and Table I. The average percentage of total MB recovered from the urine in 72 hr was only 3.87% to the dose, and 95.6% of the total MB was excreted as leucomethylene blue (LMB). These results are similar to those obtained by DiSanto and Wagner,[4] in which 2.4% of the dose was recovered as total MB from 10 hr urine and LMB in the total MB reached a percentage of 95.8% after oral ad ministraion of MB at a dose of 15 mg/kg in a dog.

Table I. Cumulative Amounts Excreted in Feces and Urine after Oral or Intravenous Administration of Methylene Blue (15 mg/kg) to Dogs[a]

Sample	Time (hr)	% excreted (*p.o.*)[b]			% excreted (*i.v.*)[b]		
		MB[c]	LMB[d]	Total MB[e]	MB[c]	LMB[d]	Total MB[e]
Feces	0—24	33.3 ±8.8[f]	7.8±2.3[f]	42.8±5.6	—	—	—
	0—48	34.4 ±8.5[f]	8.2±2.3[f]	44.3±5.2	13.1 ±6.0	3.6±2.0	16.7±4.3
	0—72	—	—	—	15.2 ±4.2	4.7±2.7	19.9±1.6
Urine	0— 8	0.03±0.02	2.1±0.5	2.1±0.5	0.15±0.05	2.7±1.2	2.8±1.2
	0—10	0.03±0.02	2.3±0.6	2.3±0.6	0.17±0.05	3.0±1.2	3.2±1.2
	0—24	0.08±0.02	3.1±0.8	3.2±0.8	0.36±0.14	4.7±1.3	5.1±1.2
	0—48	0.16±0.15	3.5±0.9	3.7±1.1	0.44±0.17	5.6±1.5	6.0±1.5
	0—72	0.16±0.15	3.7±0.8	3.9±1.0	0.46±0.18	6.2±1.6	6.6±1.6

a) Dog No. 1, 10.0 kg; No. 2, 11.0 kg; No. 3, 11.0 kg.
b) Mean value±standard deviation (n=3).
c) MB, methylene blue.
d) LMB, leucomethylene blue.
e) Total MB, MB+LMB.
f) The value indicates a mean of two dogs.

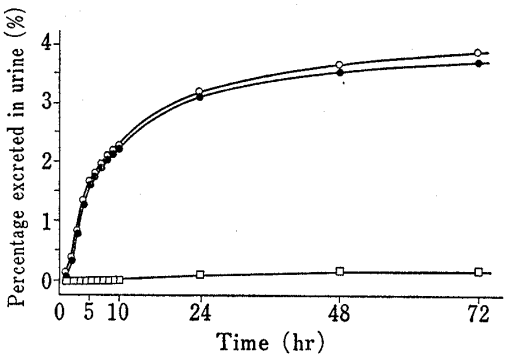

Fig. 1. Cumulative Amounts Excreted in Urine after Oral Administration of Methylene Blue (15 mg/kg) to Dogs

—○—, total MB (total methylene blue, MB+LMB).
—●—, LMB (leucomethylene blue).
—□—, MB (methylene blue).
Each point indicates a mean value of three dogs.

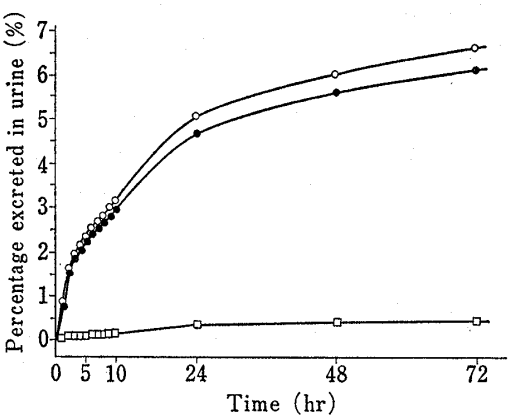

Fig. 2. Cumulative Amounts Excreted in Urine after Intravenous Administration of Methylene Blue (15 mg/kg) to Dogs

—○—, total MB; —●—, LMB; —□—, MB.
Each point indicates a mean value of three dogs.

Since fecal excretion had not been investigated for MB in dogs, MB and total MB in feces of dogs were determined after oral administration. The results are shown in Table I. MB and total MB recovered from 48 hr feces were 34.4 and 44.3%, respectively. The percentage of LMB was about 20% to the fecally excreted total MB. Though the fecal recovery was as much as 44.3% to the oral dose, it should not be concluded immediately that the recovery is only due to the unabsorbed MB. Fecal excretion after oral administration may be sometimes modified by biliary excretion and the other route of excretion. Therefore, MB was intravenously administered to the dog to determine the blood level, urinary and fecal excretion.

Urinary and Fecal Excretion, and Blood Level after Intravenous Administration of Methylene Blue (MB)

In order to investigate the fate of MB in dogs without the effect of gastrointestinal absorption process, the parenteral administration experiments were carried out, and the cumulative amounts excreted in urine were depicted in Fig.2 and a part of the data was listed in Table I. The average of total MB in 72 hr urine was only 6.6%. and the most part of it, 93.0%, was excreted as leucomethylene blue (LMB). Though the urinary excreted amount of total MB after intravenous dosing was larger than that after oral administration, it was still much smaller than the dose which had been injected into the blood srteam. It was indicated that urinary recovery by itself after oral administration should not be used as an index for gastrointestinal absorption of MB. If it is assumed that the urinary excretion characteristics of MB is not affected by the route of administration, the estimate of absorbed amount from gastrointestinal tracts after oral administration of MB would be about 60% of the dose by the following equation: $(3.87/6.61)\times 100=58.5$ (%).

As for the fecal excretion after intravenous administration of MB, the considerable amount compared with the excreted amount in urine was found in 72 hr as shown in Table I. MB and total MB in feces reached 15.2% and 19.9%, respectively. The percentage of LMB was 23.6% to the total MB. In this case the total MB in feces might have been excreted through the bile or the other route of excretion in the alimentary tract of the dog.

Therefore, if the total MB in feces after oral administration consists of the unabsorbed MB and the excreted MB from the blood stream in the alimentary tract, *i.e.*, 19.9% to the absorbed MB is excreted into feces, the absorbed amount of MB after oral administration would be estimated according to the following equation: $(100-X)+0.199X=44.3$(%), where X is the percentage of the gastrointestinally absorbed amount of MB, and the fecal recovery in 48 hr is used instead of that in 72 hr, because 72 hr feces sample could not be collected in the experiment. Then, X is 69.5 (%). This value of about 70% for absorption ratio from fecal excretion data is comparable to the value of about 60% from urinary excretion data. Although there is no elaborate report on the absorption mechanism of MB from the gastrointestinal tracts in dogs such as have been reported for the mechanism in rats.[7] it is assumed in this paper that about 60—70% of orally administered MB should have been absorbed from the gastrointesinal tracts of dogs. Since the average percentage of the gastrointestinally absorbed amount of MB was described to reach 74% in man,[4] it is also assumed that there would not be a great species difference in gastrointestinal absorption of MB between man and the dog.

In the intravenous administration experiments mentioned above, the whole blood levels of MB were determined simultaneously. The results were indicated in Table II, and the average blood levels were analyzed according to the two-compartment open model. Parameters were estimated by least-squares fitting[8] of the whole blood concentrations. The most probable curve calculated and the estimated parameters were given in Fig. 3. The

7) *a*) J. Nakamura, Y. Yoshizaki, M. Yasuhara, T. Kimura, S. Muranishi, and H. Sezaki, *Chem. Pharm. Bull.* (Tokyo), **24**, 683 (1976); *b*) *Idem, ibid.*, **24**, 691 (1976).
8) W.E. Deming, "Statistical Adjustment of Data," John Wiley and Sons, Inc., New York, 1946.

Table II. Whole Blood Concentration of Methylene Blue after Intravenous Administration (15 mg/kg) in Dogs[a]

Time hr (min)	Whole blood concentration (μg/ml)			
	Dog No. 1	Dog No. 2	Dog No. 3	Mean $\pm$ S.D.[b]
0.167(10)	11.9	15.6	23.2	16.9
0.333(20)	5.39	9.33	10.0	8.25
0.500(30)	3.64	6.04	4.71	4.80
1.500(90)	1.73	0.48	0.62	0.96
3.500(210)	1.49	0.11	0.20	0.60
6.500(390)	0.84	0.10	0.14	0.36

a) Dog No. 1, 10.0 kg; No. 2, 10.0 kg; No. 3, 11.0 kg.
b) S.D., Standard deviation.

whole blood levels of MB were reported by DiSanto and Wagner,[9] administering five different single doses of MB to the dog. These levels after a 15 mg/kg *i.v.* dose were plotted in Fig. 3 to be compared with the result of the corresponding experiment in this paper. Though the whole blood levels in this paper, *i.e.* open circles in Fig. 3, took rather higher values than those in ref. (9) 20 min after intravenous administration, the differences seemed to be not essential and due to individual difference of dogs.

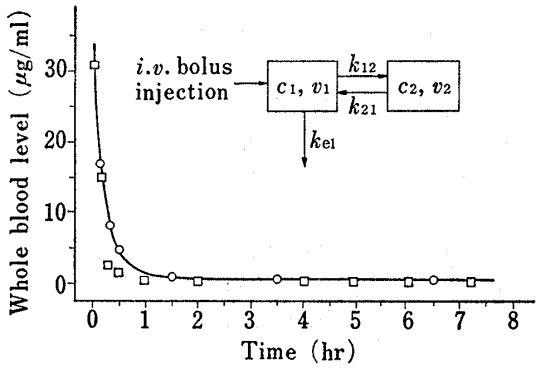

Fig. 3. Mean Whole Blood Concentration of Methylene Blue after Intravenous Administration (15 mg/kg) in Dogs

○, observed (mean, $n=3$); □, data from ref. (9)[a]; ——, calculated according to two-compartment open model illustrated below.[b]
a) Ref. (9): See text.
b) Estimated parameters with standard errors (Weight (i)$=1/C_{i1}$, where C_i is the whole blood concentration of MB.): $k_{12}=1.83\pm0.146(hr^{-1})$, $k_{21}=0.348\pm0.060(hr^{-1})$, $k_{el}=2.48\pm0.173(hr^{-1})$, $V_1=2.32\pm0.458(1/kg)$, $V_2=0.442\pm0.0211(1/kg)$.

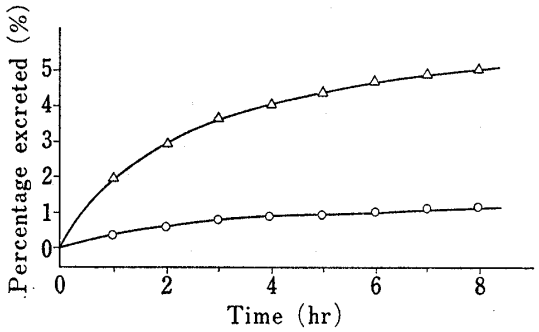

Fig. 4. Cumulative Amounts Excreted in Bile and Urine after Intravenous administration of Methylene Blue (15 mg/kg) to Dogs

—△—, total MB(methylene blue) in bile;
—○—, total MB in urine.
Each point indicates a mean value of two dogs.

Urinary and Biliary Excretion after Intravenous Administration of Methylene Blue (MB)

Even in the intravenous administration experiment fecal excretion of total MB was observed and the recovery reached 19.9%, which was considerably larger than that in urine. To search for the main origin of the total MB in feces the biliary excretion was investigated following intravenous administration of MB to dogs under anesthetization, and simultaneously the urinary excretion was determined. The cumulative amounts of total MB excreted both in urine and bile increased with time as shown in Fig. 4. The total MB in bile, which was collected through the cannula, reached 5.05% of the dose 8 hr after administration, and total

9) A.R. DiSanto and J.G. Wagner, *J. Pharm. Sci.*, **61**, 1090 (1972).

Table III. Cumulative Amounts Excreted in Bile and Urine after Intravenous Administration of Methylene Blue (15 mg/kg) to Dogs[a]

Sample		Time	% excreted (*i.v.*)[b]		
			MB[c]	LMB[d]	Total MB[e]
Bile	1)	0—1	0.340±0.054	1.59±1.23	1.94±1.28
	2)	0—4	0.742±0.110	3.32±2.17	4.06±2.27
	3)	0—8	0.943±0.206	4.11±2.39	5.05±2.55
	4)	Bile[f]	2.17 ±0.120	0.56±0.28	2.73±0.40
	5)	3)+4)	3.11 ±0.078	4.67±2.09	7.77±2.16
Urine	1)	0—1	0.054±0.001	0.30±0.02	0.35±0.03
	2)	0—4	0.104±0.016	0.75±0.07	0.85±0.09
	3)	0—8	0.149±0.007	1.02±0.16	1.17±0.15

a) Dog No. 1, 9.0 kg; No. 3, 10.5 kg.
b) Mean vale±standard deviation(n=2).
c) MB, methylene blue.
d) LMB, leucomethylene blue.
e) Total MB, MB+LMB.
f) Bile in gallbladder at the end of the experiment.

MB of 2.73% was recovered from bile in gallbladder at the end of the experiment, while the amount excreted in urine was only 1.17%. These data, including the excreted amounts of MB and LMB, were given in Table III. The result indicated that total MB in bile was 6.6 times larger than that in urine.

On the other hand total MB was detected from the gastric contents at the end of the experiment, but the amount was less than 0.3% of the dose. As it is already known that gastric emptying rate is decreased by anesthetization[10] or by biliary fistulation,[11] it is hardly considered that large amount of total MB is excreted into the stomach under the condition of this experiment. Furthermore, total MB found in intestinal tracts was only 0.4% in this experiment, which indicated net exorption in the intestine was negligibly small.

Therefore, it is assumed that the large fraction of total MB in feces is due to the biliary excreted MB in the intravenous administration experiment. As for the ratios of LMB to total BM excreted in bile and feces, the considerable difference was observed after intravenous administration of MB. The ratio of LMB to total MB in feces was about 24%, while that in bile was about 81%. This difference might result from oxidation in analytical procedure for LMB in feces, since it was noticed in the experiment that feces sample became gradually bluish in the air. The same sort of oxidation might occur in the procedure of collecting and washing out bile in gallbladder at the end of experiment, though in this case it was not visually confirmed because of the concentration of MB itself.

Isolation of Metabolites of Methylene Blue (MB)

The sum of the excreted amounts of total MB in urine and feces was only 26.5% of the dose 72 hr after intravenous administration of MB as shown in Table I. The rest of the injected MB, about 70% of the dose, might have been excreted as metabolites other than LMB, or retained in the deep tissue compartment of the dog. In this paper metabolites in urine and feces were investigated. In order to determine the number of metabolites and their properties, they were purified as much as possible by successive TLC separation, and R_f values on TLC and visible absorption spectra were examined.

Results on metabolites in urine were shown in Table IV. Three spots were observed from dog urine as DCE-extracted metabolites (D-1, D-2, D-3) by TLC. Metabolite D-1 in

10) E.J. Van Liere, *Gastroenterology*, **8**, 82 (1947).
11) J. Watanabe, H. Okabe, Y. Nakajima, K. Mizojiri, and R. Yamamoto, *Xenobiotica*, "Submitted (1977).

Table IV was considered as unchanged MB by *Rf* values and λ_{max}. Metabolite D-2 was assumed to be azure B or azure A. Further identification could not be done from the data in Table IV

TABLE IV. *Rf* Values and λ_{max} of Metabolites of Methylene Blue in the Urine of Dogs

Metabolite or authentic sample	*Rf* in solvent system[a] (1)	(2)	(3)	(4)	(5)	λ_{max} (DCE)[b] nm
Metabolite						
D-1	0.20	0.19	0.41	0.04	0.10	657, 292
D-2	0.26	0.28	0.44	0.15	0.15	642, 289
D-3	0.34	0.44	0.49	0.29	0.28	620
Authentic sample						
MB[c]	0.21	0.18	0.41	0.04	0.12	657, 292
Azure B[d]	0.25	0.24	0.42	0.15	0.14	642, 290
Azure A[e]	0.26	0.27	0.45	0.15	0.14	642, 290

a) Developing solvent systems are (1) *n*-butanol-acetic acid-water (4:1:5), (2) *n*-propanol-formic acid (80:20), (3) ethylacetate-glacial acetic acid-water(5:2:2), (4) ethanol-conc. HCl (99:1), and (5) methanol-2N HCl (93:7).
b) DCE, 1,2-dichloroethane.
c) MB, methylene blue: 3,7-bis(dimethylamino)-phenothiazinnium chloride.
d) Azure B, 7-(dimethylamino)-3-(methylamino)-3H-phenothiazine hydrochloride.
e) Azure A, 7-(dimethylamino)-3-imino-3H-phenothiazine hydrochloride.

in this paper, though M. Watanabe, *et al.*[12] reported that azure B was assumed as one of the metabolites in rat urine by TLC and visible absorption spectra. There is no other experimental evidence about metabolite D-3, but D-3 may be an oxidation product of MB, a sulfone or a sulfoxide, because Underhill and Closson[13] found that when injected into rabbits, cats and dogs, MB and its oxidation product, a sulfone, are excreted in the urine.

From the feces metabolites D-1, D-2, D-3, and besides D-4 were found on TLC. Metabolite D-4 was a purple spot and had a *Rf* value of about 0.7 on TLC with developing solvent system (1), which is described on a foot-note in Table IV.

Though attempts to identify these metabolites in urine and feces by GC–MS was unsuccessful probably because of insufficient amounts and instability of the isolated metabolites, further investigation on metabolites should be necessary in order to obtain a more detailed feature of the fate of MB in dogs.

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12) M. Watanabe, T. Hamano, and T. Matsumoto, *Shinyaku To Rinsho*, **23**, 691 (1974).
13) F.P. Underhill and O.E. Closson, *Am. J. Physiol.*, **13**, 358 (1905); through R.T. Willams, "Detoxication Mechanisms," Chapman and Hall Ltd., 1959, p. 666.