Pharmacokinetics of Highly Ionized Drugs II: Methylene Blue—Absorption, Metabolism, and Excretion in Man and Dog after Oral Administration

ANTHONY R. DiSANTO* and JOHN G. WAGNER▲

Abstract □ Methylene blue was administered orally to seven normal human subjects at a dose of 10 mg. in capsule form. Total urinary recovery ranged from 53 to 97% of the dose, with an average of 74%. Of the material recovered, an average of 78% was excreted as leucomethylene blue (stabilized in some salt, complex, or combination form) and the remainder as methylene blue. Some excretion rate–time plots of both methylene blue and leucomethylene blue showed evidence of a circadian rhythm. In a male dog and a female dog, administered 15 mg./kg. methylene blue orally, no drug was detected in blood. The female dog was catheterized and urine was collected for 10 hr. postdosing; recovery was 2.4% of the dose. The female dog was also administered a 10-mg. dose of methylene blue orally, and urine was collected by catheter over 14 hr. Recovery was 3.8% of the dose. It was concluded that methylene blue is well absorbed in man and poorly absorbed in the dog after oral administration.

Keyphrases □ Pharmacokinetics of highly ionized drugs—species-dependent absorption, metabolism, and excretion of methylene blue, man, dog □ Methylene blue—species-dependent absorption, metabolism, and excretion after oral administration, man, dog □ Absorption, species dependent—methylene blue after oral administration, man, dog

The pH-partition hypothesis of Shore *et al.* (1) suggests that highly ionized drugs are either poorly absorbed or not absorbed at all. The pKa of methylene blue[1] is about 0 to −1; hence, this drug is completely ionized in the GI pH range of 1–8. Our studies show that methylene blue is well absorbed in man and poorly absorbed in the dog after oral administration. To our knowledge, this is the first reported case in which a drug, well absorbed in man, is apparently very poorly absorbed in the dog. The potential error in screening new orally administered drugs and/or determining the effect of various variables (*e.g.*, drug excipients and surfactants) on absorption in the dog and then predicting results in man from the dog results is obvious.

EXPERIMENTAL

Human Studies—Seven adult male volunteers, with no known diseases, weighing between 54.5 and 95.3 kg. (120 and 210 lb.) and being between 21 and 40 years of age, were selected. Hand-filled hard gelatin capsules containing 10 mg. of methylene blue USP[2] were used. The night before administration of the drug, the subject fasted overnight and for 4 hr. postdosing on the treatment day. To ensure high urine flow rates and adequate urine collections, each subject ingested 240 ml. (8 fl. oz.) of water at −2, −1.5, −1, −0.5, 0, 1, 2, 3, and 4 hr., where 0 refers to the time of drug administration. Just before dosing at 0 time, the bladder was emptied and the urine was saved. For six of the seven subjects, urine was quantitatively collected in polyethylene bottles in the intervals 0–0.5, 0.5–1, 1–2, 2–3, 3–4, 4–6, 6–9, 9–24, 24–33, 33–48, 48–57, 57–72, 72–81, 81–96, 96–105, and 105–120 hr. All partial urine collections were refrigerated. In one subject (Subject 7) a more extensive sampling schedule was used: 0–0.5, 0.5–1, 1–1.5, 1.5–2, 2–2.5, 2.5–3, 3–4, 4–6, 6–8, and then at various intervals *ad libitum* (*i.e.*, there were no partial urine collections). Samples from all subjects were either assayed or frozen immediately as they were received.

Dog Studies—Two dogs were used in the studies, a male beagle dog and a female mongrel dog. The male beagle was administered 15 mg./kg. (168 mg.) of methylene blue in a hard gelatin capsule, and blood samples were taken after insertion of an intravenous catheter in the right hind leg. Samples were taken at 0, 0.27, 0.50, 1.0, 2.0, 4.0, and 6.9 hr. postadministration. Urine samples were not collected in this study.

The female mongrel dog was fasted overnight, and a 15-mg./kg. (132-mg.) dose and a 10-mg. dose of methylene blue were administered (3 weeks apart) in a gelatin capsule. An intravenous catheter was inserted into the left hind leg, and 250 mg. of sodium pentobarbital[3] was administered. A catheter[4] was inserted and the bladder emptied. An intravenous infusion of 5% dextrose was then started to ensure adequate hydration. From one-tenth to two-tenths of a milliliter of sodium pentobarbital (40 mg./ml.) was given periodically to maintain anesthesia. Urine samples were then collected and assayed immediately. In the case of the 15-mg./kg. dose of methylene blue administered to the female dog, urine samples were taken every hour for 10 hr. The samples taken at 11 and 12 hr. contained blood and were discarded. Sampling was then stopped. Sampling was stopped 14 hr. after the 10-mg. dose since the assay sensitivity was reached. Blood samples were taken at 2.5 and 3.5 hr. after administration.

ASSAY

Urines and bloods were assayed by the extraction–spectrophotometric method of DiSanto and Wagner (2).

The pH of each urine sample was also taken.

RESULTS AND DISCUSSION

Tables I and II give the results of the oral administration of methylene blue to man and show that an average of 74% (range 53–97%) of the administered dose of methylene blue was recovered in the urine. These data clearly show that although methylene blue is completely ionized in the GI pH range, it is well absorbed in man. Interesting, however, is the fact that methylene blue was apparently very poorly absorbed in the dog after oral administration (Table III). Following the oral administration of a 132-mg. dose (15-mg./kg.) and a 10-mg. dose of methylene blue (given 3 weeks apart) to the female dog, only 2.4 and 3.8% of the administered dose was recovered in urine, respectively. Blood samples taken at 2.5 and 3.5 hr. after administration gave zero concentration values for methylene blue. The male dog, administered a 15-mg./kg. (168-mg.) dose of methylene blue orally, gave blood concentration values of methylene blue, 0–6.9 hr. after dosing, below the assay sensitivity of 0.02 mg./ml., which also indicated rather poor absorption of methylene blue in the dog (Table IV). The reason(s) for this difference in the absorption of methylene blue in the dog and man is not known and probably first requires a knowledge of how methylene blue is absorbed in man. This study will be undertaken in the near future.

The color of the urine is not indicative of the amount of methylene blue absorbed, because most of the dye is excreted as leucomethylene

[1] See *Appendix*.
[2] Fisher Scientific.
[3] Nembutal Sodium, Abbott Laboratories, Chicago, Ill.
[4] No. 16 Bard Foley.

Table I—Oral Administration of 10 mg. of Methylene Blue to Man (Subjects 1–6)

t, hr., Interval	Amount Excreted in Interval, mcg.											
	Subject 1		Subject 2		Subject 3		Subject 4		Subject 5		Subject 6	
	Free	Leuco	Free	Leuco	Free	Leuco	Free	Leuco	Free	Leuco	Free	Leuco
0–0.5	0.295	6.53	1.83	27.4	0.712	3.10	0.406	1.39	0.410	5.51	4.10	0.630
0.5–1	1.78	120.	15.1	459.	20.5	110.	4.94	74.7	15.0	166.	7.03	173.
1–2	24.2	959.	56.8	931.	32.7	192.	62.6	733.	53.1	396.	7.29	681.
2–3	59.5	818.	71.9	588.	49.4	310.	117.	712.	42.2	295.	8.90	506.
3–4	41.1	615.	55.4	402.	26.2	297.	76.3	486.	36.3	224.	2.34	333.
4–6	66.8	424.	132.	703.	94.7	600.	204.	560.	67.5	283.	12.1	415.
6–9	109.	133.	69.5	628.	393.	294.	238.	243.	11.8	251.	63.1	764.
9–24	781.	133.	590.	1372.	390.	881.	728.	1184.	253.	1476.	925.6	1374.
24–33	21.9	116.	147.	638.	523.	291.	367.	431.	72.6	198.	121.4	224.
33–48	201.6	37.4	283.	687.	221.	376.	254.	436.	58.1	580.	74.8	337.
48–57	7.82	40.1	72.2	368.	46.1	53.1	68.5	90.8	16.0	51.7	30.0	33.
57–72	113.	8.31	74.1	194.	15.3	29.2	153.	143.	108.	186.	24.1	38.
72–81	5.31	13.1	49.7	141.	9.90	9.50	38.0	84.3	20.1	84.5	10.3	17.4
81–96	26.7	2.89	18.1	31.2	10.4	1.89	63.3	56.9	37.8	177.	18.1	35.5
96–105	1.38	3.61	5.94	19.3	2.82	7.46	31.8	14.8	12.3	112.	8.38	23.0
105–120	8.68	1.89	8.09	25.5	4.00	9.80	26.4	18.4	31.6	101.	8.17	19.6
Total amount	1470.	8206.	1651.	7212.	1840.	3465.	2436.	5267.	836.	4585.	1326.	4971.
Percent dose recovered in urine	96.8		88.6		53.0		77.0		54.2		63.0	

blue, stabilized in some salt, complex, or combination form. Of the dye recovered in the urine of man, an average of 78% (range 65–85%) was excreted as stabilized leucomethylene blue.

Figures 1*a* and 2*a* show the semilogarithmic excretion rate–time plots of methylene blue and stabilized leucomethylene blue for Subject 1 and give evidence of a circadian rhythm characterized by unchanged methylene blue being excreted more rapidly during the nighttime than daytime, with the converse for stabilized leucomethylene blue. This diurnal cycling in the excretion rates could be detected in four of the seven human subjects administered methylene blue. There was no observable relationship between excretion rate and urine pH in any subject. The exact reason for this apparent rhythmic variation in the excretion rates of methylene blue and stabilized leucomethylene blue is not known. Rhythmic variations of this nature have usually been ascribed to metabolic processes. However, where a circadian rhythm in metabolism has been reported, it usually only occurred with an oxidative process of metabolism (3, 4) and it appears unlikely that the diurnal cycling in the excretion rates of methylene blue and stabilized leucomethylene blue is due to its reductive metabolism. It is quite feasible that the compound stabilizing leucomethylene blue is a metabolic by-product, which possesses a circadian rhythm such that its rate of metabolism is greater during the day than night.

Levy (5) noted a pronounced difference in the values for the rate of metabolism for benzylpenicillin during the ambulatory state and during bed rest, with the rate of metabolism being greater during the ambulatory state. He stated that this difference in the values for rate of metabolism "is probably only an apparent one which is not necessarily indicative of an intrinsically impaired capacity for drug biotransformation during bed rest," since: "Pharmacokinetic models in current use do not provide for variations in plasma volume, variable blood flow rates through organs, and other region- or organ-specific circulatory changes" which occur during bed rest. It is reasonable, therefore, to propose that the hypothesized

Table II—Oral Administration of 10 mg. of Methylene Blue to Man (Subject 7)

t, hr., Interval	Amount Excreted in Interval, mcg.	
	Free	Leuco
0–0.5	0.328	4.47
0.5–1	7.71	245.
1–1.5	17.4	338.
1.5–2	16.5	384.
2–2.5	26.1	220.
2.5–3	64.9	222.
3–4	47.0	334.
4–5	107.	619.
5–6	58.0	463.
6–8	159.	725.
8–9.25	54.1	482.
9.25–12	13.0	663.
12–21.5	274.	982.
21.5–24	34.4	201.
24–29	120.	227.
29–36	126.	312.
36–45	60.1	337.
45–46	5.00	20.3
46–51.66	49.6	144.
51.66–54.25	17.14	51.9
54.25–57	7.55	56.2
57–60.25	10.3	90.9
60.25–63.75	3.85	47.2
63.75–69.33	15.6	60.7
69.33–72	6.00	30.0
72–76.5	9.80	28.0
76.5–84	16.1	25.0
84–93	8.20	18.7
93–104	14.8	10.3
104–108.5	4.55	9.34
108.5–110.33	0.812	4.62
110.33–117.75	0.544	9.91
Total amount	1353.	7365.
Percent dose recovered in urine	87.2	

Table III—Oral Administration of Methylene Blue to the Female Dog

t, hr., Interval	15-mg./kg. Dose (132 mg.), Cumulative Amount Excreted, mcg.		10-mg. Dose, Cumulative Amount Excreted, mcg.	
	Unchanged	Leuco	Unchanged	Leuco
0–1	0.410	0.820	0.002	0.330
1–2	3.54	1.03	0.016	1.33
2–3	15.5	86.9	0.206	9.79
3–4	25.0	421.	1.07	30.3
4–5	32.1	816.	2.57	63.6
5–6	41.3	1152.	3.53	108.
6–7	66.2	1782.	6.05	159.
7–8	94.6	2450.	9.35	198.
8–9	135.0	2784.	12.0	242.
9–10	190.	3101.	14.9	288.
10–11	—	—	18.0	314.
11–12	—	—	19.7	343.
12–13	—	—	20.1	353.
13–14	—	—	26.3	363.
Recovery, percent of dose:				
Total	2.4		3.8	
As leucomethylene blue	2.3		3.6	
As unchanged methylene blue	0.1		0.2	

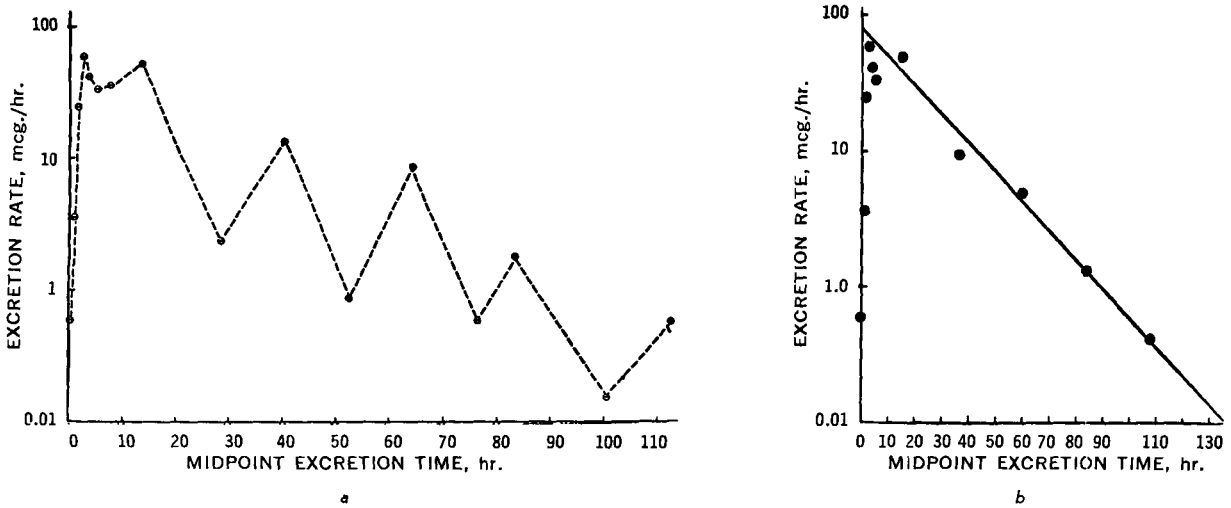

Figure 1—(a) *Plot of the logarithm of the excretion rate of unchanged methylene blue* versus *the midpoint excretion interval for Subject 1. Key:* ⊖, *day; and* ●, *night.* (b) *Plot of the logarithm of the excretion rate of unchanged methylene blue* versus *the midpoint excretion interval for Subject 1 when day and night samples were pooled.*

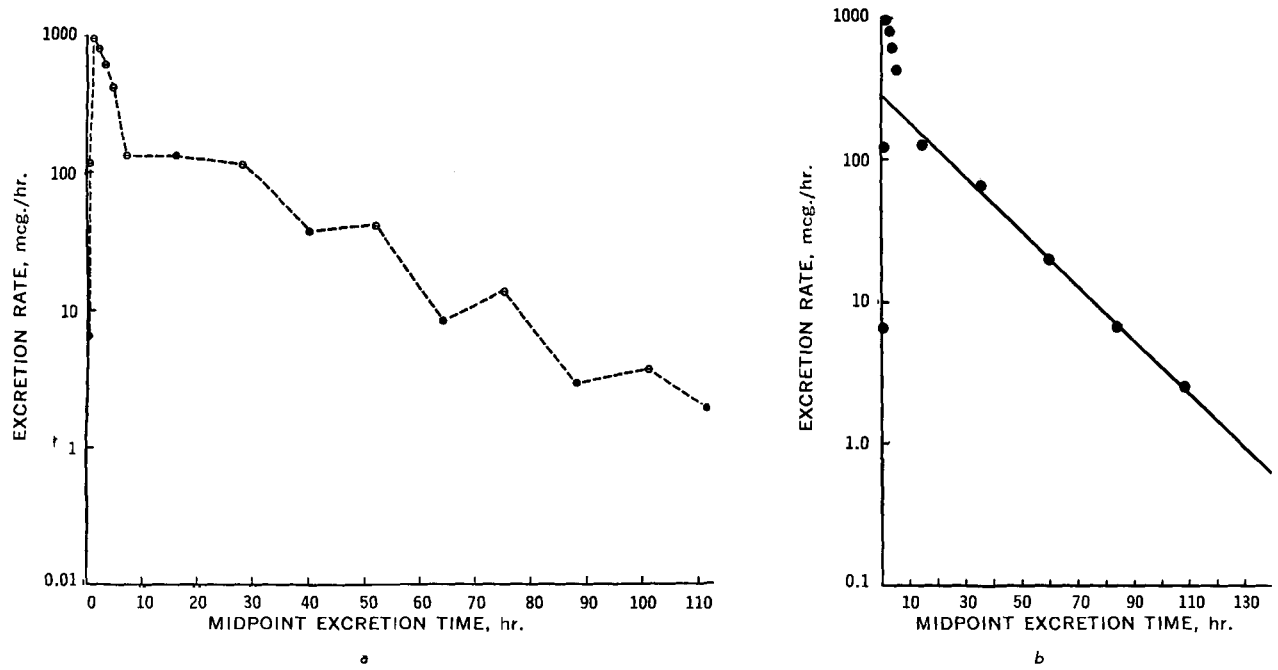

Figure 2—(a) *Plot of the logarithm of the excretion rate of stabilized leucomethylene blue* versus *the midpoint excretion interval for Subject 1. Key:* ⊖, *day; and* ●, *night.* (b) *Plot of the logarithm of the excretion rate of stabilized leucomethylene blue* versus *the midpoint excretion interval for Subject 1 when day and night samples were pooled.*

metabolic by-product which stabilizes leucomethylene blue could be explained similarly.

If the urinary data were pooled such that 24-hr. urine aliquots were taken, all evidence of a circadian rhythm in excretion rate disappeared. This calculation was done with the data of Subject 1 and is shown as Figs. 1*b* and 2*b*.

In most of the subjects, apparent erratic absorption was indicated by the excretion rate–time plots; hence absorption could not adequately be described by a smooth continuous function (*e.g.*, by a one or two exponential function). Due to this apparent erratic absorption and apparent rhythmic variation of the excretion rates in man, a quantitative kinetic analysis of the urinary data was not attempted. Figures 3 and 4 show the semilogarithmic excretion rate–time plots for methylene blue and stabilized leucomethylene blue obtained from Subject 7, when extensive urine samples were obtained. It is difficult to ascribe a pharmacokinetic model to these data; however, for illustrative purposes the two-compartment open model with first-order absorption was assumed to apply and was fit to a cumulative urinary excretion–time equation (Eq. 1) for this model using the program NONLIN[6]:

$$A_u = A_u^{\infty}\left[1 - k_a k_{el}\left\{\left(\frac{k_{21} - \alpha}{\alpha(k_a - \alpha)(\beta - \alpha)}\right)e^{-\alpha(t-t_0)} + \left(\frac{k_{21} - \beta}{\beta(k_a - \beta)(\alpha - \beta)}\right)e^{-\beta(t-t_0)} + \left(\frac{k_{21} - k_a}{k(\alpha - k_a)(\beta - k_a)}\right)e^{-k_a(t-t_0)}\right\}\right] \quad \text{(Eq. 1)}$$

The parameters k_{12}, k_{21}, k_{el}, k_a, A_u^{∞}, and t_0 (where k_a is the first-order rate constant for absorption, A_u^{∞} is the cumulative amount of drug excreted at infinite time, t_0 is the lag time before absorption

[6] Kindly supplied by Dr. C. M. Metzler, The Upjohn Co., Kalamazoo, Mich.

Table IV—Blood Level of Methylene Blue after Oral Administration of 15-mg./kg. Dose to the Male Dog

Time, hr.	Concentration, mcg./ml.
0.27	0
0.50	0
1.00	0.003
2.01	0.019
4.00	0.012
6.87	0

Table V—Two-Compartment Open Model with First-Order Absorption Analysis of Urinary Data of Subject 7

	Unchanged Drug	Stabilized Leucomethylene Blue
k_a, hr.$^{-1}$	0.129	0.917
k_{12}, hr.$^{-1}$	1.28	0.049
k_{21}, hr.$^{-1}$	0.121	0.064
k_{el}, hr.$^{-1}$	0.847	0.116
A_u^∞	1354.	7438.
t_0, hr.	0.016	0.040
r^2	0.999	1.000
Corr.	0.998	1.000

begins, k_{12} and k_{21} are the first-order rate constants between Compartments 1 and 2, and k_{el} is the first-order rate constant for loss of drug from Compartment 1) were estimated. Table V shows that both methylene blue and stabilized leucomethylene blue data obtained from Subject 7 were fitted excellently to Eq. 1. These excellent fits to Eq. 1 show that with extensive urine sampling (as was done with Subject 7), the variations observed in the excretion rate–time data are "smoothed out" by fitting to the cumulative amount excreted–time equation. When fitting urinary data to this equation, one must exert caution. Simply looking at the log excretion rate *versus* time plot qualitatively indicates that absorption is not a first-order process, and similarly for elimination.

SUMMARY

1. The major metabolite of methylene blue following oral administration to man and dog was shown to be leucomethylene blue, stabilized in some salt, complex, or combination form in urine but not in blood.

2. The color of the urine following oral administration of methylene blue to man was shown to be unrelated to the amount absorbed, since in seven subjects an average of 78% of the drug recovered in the urine was excreted as stabilized leucomethylene blue.

3. Apparent diurnal cycling of the excretion rates of methylene blue and stabilized leucomethylene blue was observed in man following oral administration.

4. Absorption of methylene blue was shown to be species dependent. Methylene blue was well absorbed in man and poorly absorbed in the dog.

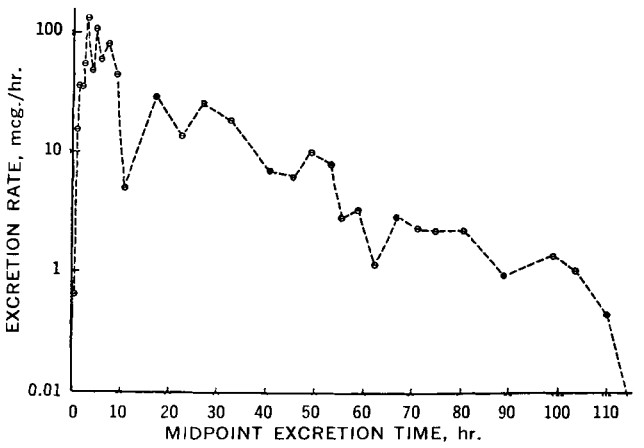

Figure 3—*Plot of the logarithm of the excretion rate of unchanged methylene blue* versus *the midpoint excretion interval for Subject 7 where extensive sampling was conducted. Key: ⊖, day; and ●, night.*

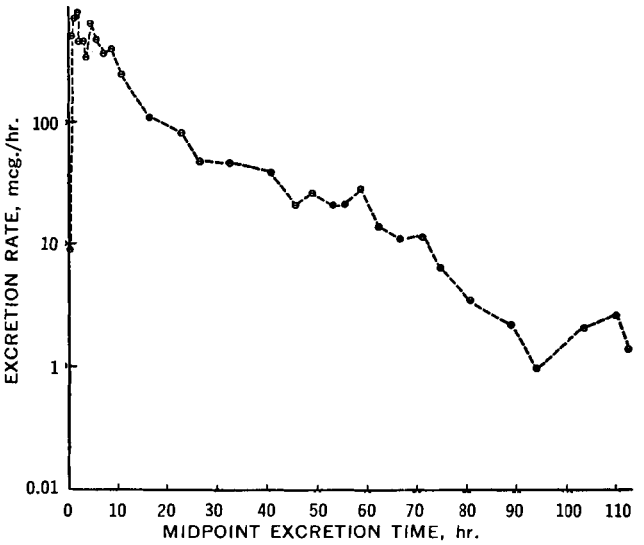

Figure 4—*Plot of the logarithm of the excretion rate of stabilized leucomethylene blue* versus *the midpoint excretion interval for Subject 7 where extensive sampling was conducted. Key: ⊖, day; and ●, night.*

APPENDIX

A first attempt to determine the pKa of methylene blue titrimetrically with standard hydrochloric acid failed since the methylene blue solution responded like pure water. This, conservatively, indicated that the pKa was less than 2.

The authors determined the pKa to be −1 using a spectrophotometric method and Hammett's acidity function (6). Independently, Vandenbelt[6] determined the pKa as 0.04 by a spectrophotometric procedure in which 1 *N* HCl was assumed to have a pH of 0; hence the activity coefficient was neglected. This pKa was based on absorbances read at 665 nm. in water and 0.5, 1, and 5 *N* HCl. There was also evidence of a second pKa of −1.3 based on a decrease in bands at about 680 and 755 nm. in strong acid (5 *N* HCl, 6–21 *N* H_2SO_4); a plot of band height *versus* normality of sulfuric acid indicated a half-ionization at 21 *N* H_2SO_4, corresponding to a pKa of −1.3. However, this change in band height occurred over a smaller range of acidity (12–24 *N*) than is usually necessary. This could have been due to a peculiarity of the sample or sulfuric acid at these high normalities. An ionization change or aggregation or something else occurred, but the true situation would require further investigation.

In our laboratories, methylene blue was shown *not* to partition into methylene chloride (CH_2Cl_2) or chloroform from pure water (resistance >18 megohms) solution (pH 6.95), acetate buffer (pH 4.3), or phosphate buffer (pH 7.25). In partitioning studies with methylene blue in tap water (pH 8.8), borate buffer (pH 10), and pure water plus a drop of 0.5 *N* NaOH (pH 10.4), the methylene chloride and chloroform layers became pink to red in color. We have shown by TLC that this is due to degradations of methylene blue to dimethylthionin, trimethylthionin, and thionin. If water or the buffer contains a compound which ion-pairs with methylene blue, such as sodium chloride, potassium chloride, sodium bromide, potassium iodide, sodium fluoride, trichloroacetic acid, or sodium taurocholate, then transfer of methylene blue to the methylene chloride or chloroform occurs readily. In this case the organic layer becomes blue, and we have shown by TLC that the organic phase contains methylene blue. In such cases the partition coefficient is a function of concentration of the ion-pairing agent, and the parti-

[6] Research Laboratories, Parke, Davis and Co., Ann Arbor, MI 48105

tion coefficient becomes asymptotic at high concentrations of the ion-pairing agent. The assay for methylene blue in urine and blood (2) depends upon such partitioning in the presence of high concentrations of sodium chloride.

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Pharmacokinetics of Highly Ionized Drugs III: Methylene Blue—Blood Levels in the Dog and Tissue Levels in the Rat following Intravenous Administration

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Abstract □ Plasma concentrations following rapid intravenous injection of 2-, 5-, 7.5-, 10-, and 15-mg./kg. doses of methylene blue in a dog were obtained. The single-dose data were fit to both a nonlinear heterogeneous one-compartment open model with binding to one type of tissue and the classical linear two-compartment open model. Fitting to the nonlinear model showed *no* systematic trends in the estimated parameters in relation to dose. Fitting of the concentration data to the classical linear two-compartment open model *did show* systematic trends in the estimated parameters in relation to dose. Rats were injected intravenously with 2-, 5-, 7.5-, 10-, 15-, and 25-mg./kg. doses of methylene blue and were then decapitated 3 min. later. The lungs, liver, kidneys, and heart were removed and assayed. An average of 29.8% of the dose (range 25.2–35.8%) was recovered in those four tissues, indicating very rapid uptake and extensive uptake of methylene blue by tissues. A plot of the average amount taken up by those tissues against the milligrams per kilogram dose was fit by the equation appropriate to the nonlinear model.

Keyphrases □ Methylene blue—dog plasma and rat tissue concentrations after intravenous administration, pharmacokinetics □ Pharmacokinetics of highly ionized drugs—methylene blue, dogs, rats □ Plasma levels, methylene blue—intravenous administration, dogs □ Tissue levels, methylene blue—intravenous administration, rats

A generalized nonlinear pharmacokinetic model was elaborated by DiSanto and Wagner and published by Wagner (1). The relationship of this model to nonlinear models of other investigators was discussed by DiSanto and Wagner (2). The simplest specific case of the generalized model is the heterogeneous one-compartment open model with binding to one type of tissue. All of the equations appropriate to this model were given by Wagner (1) and some of them were given by DiSanto and Wagner (2). The integrated equation for the plasma concentration as a function of time, Eq. 4 of this report, is analogous to, but different than, equations derived by Krüger-Thiemer (3, 4) for nonlinear plasma protein binding of drugs. The purposes of this report are:

1. To list plasma concentrations measured after the intravenous administration of 2-, 5-, 7.5-, 10-, and 15-mg./kg. doses of methylene blue to a male beagle dog.
2. To list tissue levels in heart, lungs, liver, and kidneys measured at 3 min. after the rapid intravenous injection of 2-, 5-, 7.5-, 10-, 15-, and 25-mg./kg. doses of methylene blue to the rat.
3. To evaluate these data by both the classical linear two-compartment open model and the nonlinear heterogeneous one-compartment open model with binding to one type of tissue.

EXPERIMENTAL

Materials—A solution of methylene blue[1] in sterile water for injection was prepared, transferred to 10-ml. vials, and autoclaved. Two vials were assayed and the concentration was calculated from the Beer's law slope of methylene blue in water. The intravenous solution assayed 2% w/v methylene blue. This preparation was used in all of the intravenous administrations of methylene blue to the dog.

[1] Fisher Scientific.