Dose-Dependent Pharmacokinetics and Hepatobiliary Transport of Bromophenol Blue in the Beagle

ROBERT J. WILLS *, GERALD J. YAKATAN [§x], and ROBERT S. PEARLMAN

Received September 17, 1982, from the *College of Pharmacy, University of Texas at Austin, Austin, TX 78712.* Accepted for publication May 26, 1983. Present addresses: *Hoffmann-La Roche, Department of Pharmacokinetics and Biopharmaceutics, Nutley, NJ 07110 and [§]Warner-Lambert Co., Pharmaceutical Research Division, Morris Plains, NJ 07950.

Abstract □ The pharmacokinetic profile of bromophenol blue (I) in the plasma, urine, and bile of beagle dogs was determined after intravenous administration of 5-, 20-, and 30-mg/kg doses. In addition, two competitors, probenecid and phenylbutazone, were interacted with I *in vivo* and with I and rat liver cytoplasmic protein fractions Y and Z *in vitro* as a means of elucidating the mechanism of intrahepatic transport of I. Compound I was determined spectrophotometrically at 587 nm. In plasma, I displayed apparent first-order dose-dependent kinetics. The percentage of I bound to plasma proteins was ~92.5% over the dose range studied. Consecutive injections of equal doses of I produced statistically different terminal half-lives ($p < 0.05$), suggesting the possibility of a saturable uptake process. In the presence of each competitor, the disposition of I was altered significantly ($p < 0.05$): phenylbutazone displaced I from plasma protein, while probenecid decreased the binding of I to liver proteins in the Z-fraction. The Z-fraction bound a larger amount of I than the Y-fraction, suggesting a larger binding capacity. Under no circumstances was the binding of I to the Y-fraction altered. Cumulative biliary excretion data showed that the elimination of I in bile accounted for 92–99% of the dose delivered. The biliary excretion σ^- plots displayed no dose dependency, suggesting that the dose-dependent plasma half-life is due to a dose-dependent liver uptake (as opposed to elimination) process.

Keyphrases □ Pharmacokinetics—bromophenol blue, dogs, hepatobiliary transport □ Bromophenol blue—pharmacokinetics, hepatobiliary transport, dogs □ Hepatobiliary transport—bromophenol blue, dogs, pharmacokinetics

An understanding of the complex processes involved in hepatobiliary transport will lead to a more complete understanding of the disposition of the many compounds eliminated by this route. Radiological (1–3) and pharmacological (4–7) investigations have addressed various aspects of these transport processes, but relatively few pharmacokinetic studies have been conducted. The use of blood, urine, and bile data to suggest or support a model of hepatobiliary uptake and elimination appears to be a promising research objective. Takada and coworkers (8–10) proposed the use of bromophenol blue (I) as a model compound for the study of hepatobiliary transport in rats. This was confirmed by Wills *et al.* (11), who noted that I is primarily excreted in bile, is not metabolized, elicits no pharmacological action, and is not intestinally reabsorbed. Although studies conducted in rats have been informative, the pharmacokinetics of the hepatobiliary transport of I have not been addressed in higher animals. Although a few studies using dogs have been conducted (1, 3, 12), the feasibility of obtaining a useful bile concentration profile has yet to be demonstrated.

In rat plasma, I displays a dose dependency which was related to the saturation of proteins involved in the liver uptake process (8). These proteins were separated as cytoplasmic protein fractions Y, Z, and T-binder (9, 13–15), and it was subsequently shown (10) that both Y and Z act as uptake sites, Z and T-binder act as storage sites, but only Y functions as an intracellular carrier to the bile canaliculi.

Takada and co-workers (8–10) used competitors to elucidate the hepatobiliary transport mechanism in rats. Two competitors, probenicid and phenylbutazone, were selected because of their ability to inhibit liver uptake of organic anions (13, 16, 17) and to displace drug from plasma proteins (18–20), respectively.

The nature of the hepatobiliary uptake and elimination processes in higher animals is still the subject of controversy. Shanker (21) hypothesized the existence of three different carrier systems for liver uptake of anionic, cationic, and neutral compounds. However, Schwenk (6) has recently presented conflicting data.

In this report, we discuss efforts to determine the pharmacokinetic profile (plasma and urine) of I following intravenous administration in intact beagle dogs, to determine the pharmacokinetic profile (plasma, urine, and bile) of I following intravenous administration in beagle dogs with cannulated common bile ducts and excised gallbladders, to observe the effect of probenecid and phenylbutazone on the pharmacokinetic parameters for I, and to examine the action of these two competitors by *in vitro* rat liver protein binding studies.

EXPERIMENTAL SECTION

Bromophenol blue[1] (I) was purchased from the manufacturer. Purity was established by melting point and TLC. A mixture of propylene glycol–ethanol–sterile water (4:1:5) was found to be a suitable intravenous injection vehicle[2].

One female and two male beagles, weighing 11–18 kg, were used in all studies; all three dogs were 7 years old. Hematology values were determined to be within normal limits. The dogs were fasted 18 h prior to drug administration, and at least 2 weeks were allowed between studies unless otherwise noted.

Bromophenol Blue Intravenous Bolus Studies—Compound I was administered as 5-, 20-, or 30-mg/kg doses. Sample times varied with dose, as indicated in the figures. Blood samples were transferred to stoppered evacuated tubes[3] containing sodium heparin and centrifuged. The plasma was removed and stored at −20°C until assayed. Urine was collected as produced and also stored frozen until assayed.

Competitor Studies—Probenecid[4] and phenylbutazone[5] were supplied by the manufacturers. Each competitor was administered 1 h before the I dose. The doses of probenecid, phenylbutazone, and I were 125, 75, and 20 mg/kg, respectively. The half-life of each competitor was ~8 h (17, 22).

Plasma Protein Binding Studies—The extent of binding of I was determined after dialysis using a rotary five-cell (volumes of 1.36 mL; membrane surface area of 4.52 cm^2) dialysis apparatus[6]. The dialysis apparatus was assembled with equivalent *in vivo* concentrations of I dissolved in pH 7.4 isotonic buffer on one side *versus* pooled dog plasma on the other side, placed in a water bath at 37°C, and incubated for 2 h at 10 rpm. The time required for equilibration was established by dialyzing spiked buffer against unspiked buffer.

The effect of the competitors on the plasma protein binding of I was also determined. To 4 mL of pooled dog plasma, 100 μL of a 16-mg/mL solution of competitor was added to give a final concentration equivalent to an *in vivo* concentration (17, 22) of the competitor at time zero. This solution was dialyzed against spiked buffer, which encompassed the concentrations of I found

[1] Fisher Scientific Co., Fair Lawn, N.J.
[2] Personal communication J. W. McGinity, Drug Dynamics Institute, College of Pharmacy, University of Texas, Austin, Tex.
[3] Vacutainer; Becton, Dickenson and Co., Rutherford, N.J.
[4] Merck Sharp and Dohme, West Point, Pa.
[5] Ciba-Geigy, Summit, N.J.
[6] Spectrum Medical Industries, Inc., Los Angeles, Calif.

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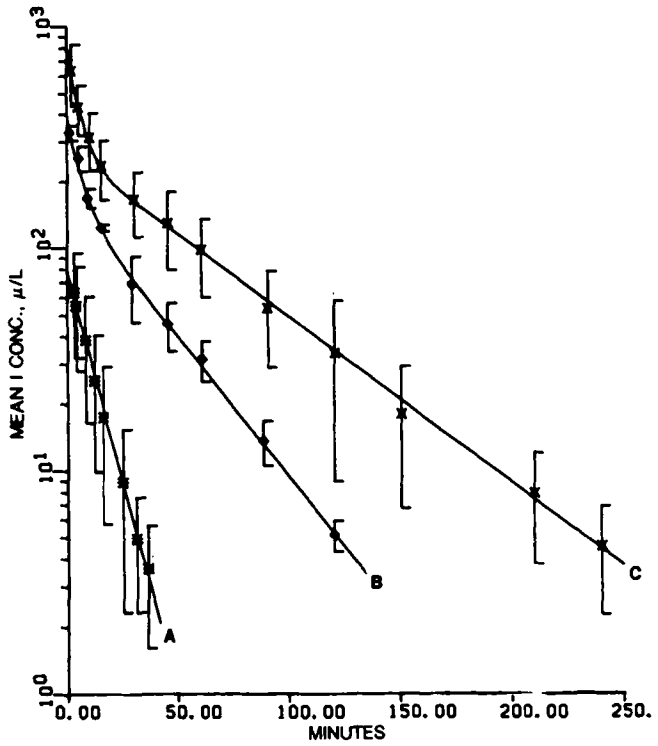

Figure 1—*Mean* ± SD (n = *3) plasma concentrations of I after an intravenous bolus injection to a dog. Key: (A) 5-mg/kg dose; (B) 20-mg/kg dose; (C) 30-mg/kg dose.*

in vivo. All solutions were collected and any cell volume changes were noted. The samples were assayed immediately after removal from the dialysis apparatus.

Bile Duct Cannulation Studies—Under pentobarbital anesthesia, a midline incision was made[7] and the common bile duct was exposed. After excising the gallbladder, a small incision in the common bile duct was made ~1 cm from its entrance to the duodenum, and a T-shaped cannula was inserted and ligated in place. A second T-shaped cannula was placed into the duodenum and both silicone rubber[8] cannulae were attached to a larger stainless steel cannula which was inserted through the abdominal wall. Although the cannula into the duodenum would have allowed the return of bile between experiments, previous experience indicated that this would subject the animals to an increased risk of hepatic infection. Thus, total bile was collected at all times. Bile salt dietary supplements[9] were given to maintain the animals' weights at constant values.

During experiments, bile was collected in pre-tared screw-capped vials[10] and frozen until assayed. In addition, blood and urine were collected. The sampling times varied with dose. Only 1 week was allowed between studies because animal viability was poor.

Liver Protein Binding Studies—Two male Sprague–Dawley rats, 400 g, were anesthetized by a 70-mg/kg ip injection of sodium pentobarbital. A midline laparotomy exposed the internal viscera. The hepatic vein was severed, followed by cannulation of the hepatic portal vein with a 22-gauge needle connected to an infusion pump[11] containing pH 7.4 isotonic phosphate buffer. The liver was perfused until the color appeared pink (after ~200 mL of buffer). The livers were then excised, dried, weighed, and stored frozen.

In preparation for extraction and purification of the liver protein fractions (9), the livers were homogenized[12] to a 25% homogenate with 0.25 M sucrose–0.01 M phosphate buffer (pH 7.4) and then ultracentrifuged[13] at 100,000 × *g* for 2 h at 20°C. After removing the surface lipids, the supernatant was collected. A 1-mL aliquot of supernatant was used for determination of the total protein concentration (23).

A total of nine mixtures containing supernatant, I, and the competitors were prepared as follows. To 5 mL of supernatant, 6.7 mg (10 μmol) of I and 0, 10, 20, 30, or 40 μmol of either probenecid or phenylbutazone were added. Each mixture was incubated for 24 h at 4°C and then eluted using 0.01 M phosphate buffer pH 7.4 on a Sephadex G-75 column (2.5 × 45 cm)[14] previously equilibrated with the phosphate buffer (24). Five-milliliter fractions were collected using a fraction collector[15]. Absorbances of I and protein for a given tube were determined at 587 and 280 nm, respectively, using a double-beam spectrophotometer[16]. The absorbances measured at 280 nm were used as a fingerprint for liver protein fraction identification.

The total amount of I bound to the void volume (X fraction), Y, and Z fractions was quantitated as follows. After determining the amount of I in each tube the amounts were then added together mathematically from tubes 0–7, 8–14, and 29–64 (representing I bound to the void volume, Y, and Z fractions, respectively). The total amount of I bound in the presence of each competitor

Table I—Pharmacokinetic Disposition Parameters Following an Intravenous Bolus Injection of I

Dog	$t_{1/2}$, min	CL_p, mL/min	Vd_{ss}, L	$AUC_{0\to\infty}$, μg/mL min
		30-mg/kg Dose		
A	40.8	22.9	1.40	22,300
B	31.5	23.1	1.01	18,300
C	31.5	32.7	1.43	10,200
Mean	34.1[a]	26.2	1.28	16,900
± *SD*		5.6	0.23	6,160
		20-mg/kg Dose		
A	25.7	51.0	1.73	6480
B	25.8	44.3	1.27	6430
C	23.0	30.1	1.02	7540
Mean	24.8[a]	42.0	1.34	6820
± *SD*		11.0	0.36	628
		5-mg/kg Dose		
A	6.7	154	2.02	570
B	6.5	159	1.81	357
C	7.7	70.8	1.01	1070
Mean	6.9[a]	128	1.61	666
± *SD*		49.4	0.53	370

[a] Harmonic mean half-life.

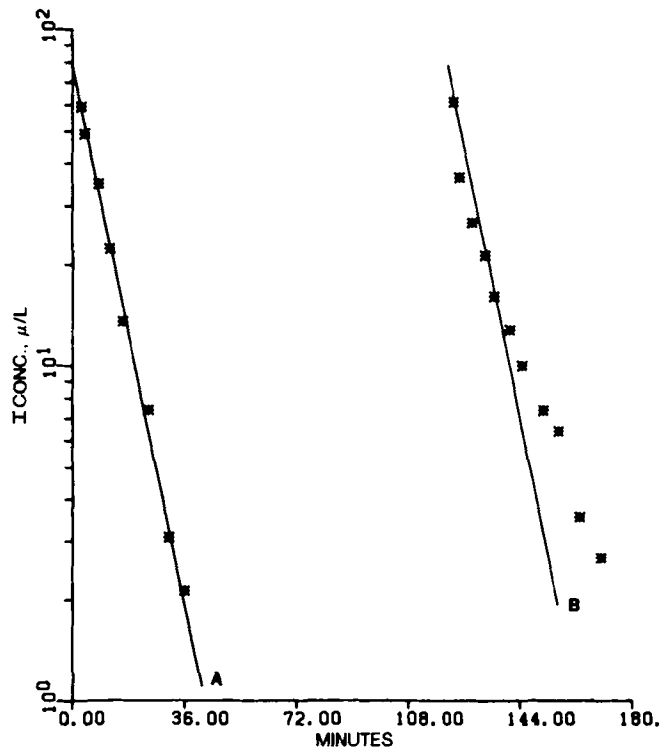

Figure 2—*Plasma concentrations of I following an intravenous bolus injection of 5 mg/kg at time zero (A) and a second injection at 2 h (B). The solid lines represent the fitted line from the first injection data.*

[7] Procedure developed by Dr. R. Shumacher and performed by Mr. J. Wiley and Ms. A. Black at Warner-Lambert, Ann Arbor, Mich.
[8] Silastic.
[9] Reheis Chemical Co., Tarrytown, N.Y.
[10] Kimble, Toledo, Ohio.
[11] Harvard Apparatus, Millis, Mass.
[12] Brinkman Instruments, Westbury, N.Y.
[13] Beckman Instruments, Inc., Palo Alto, Calif.
[14] Fisher and Porter Co., Warminster, Pa.
[15] Instrumentation Specialties Co., Lincoln, Neb.
[16] Perkin-Elmer Corp., Maywood, Ill.

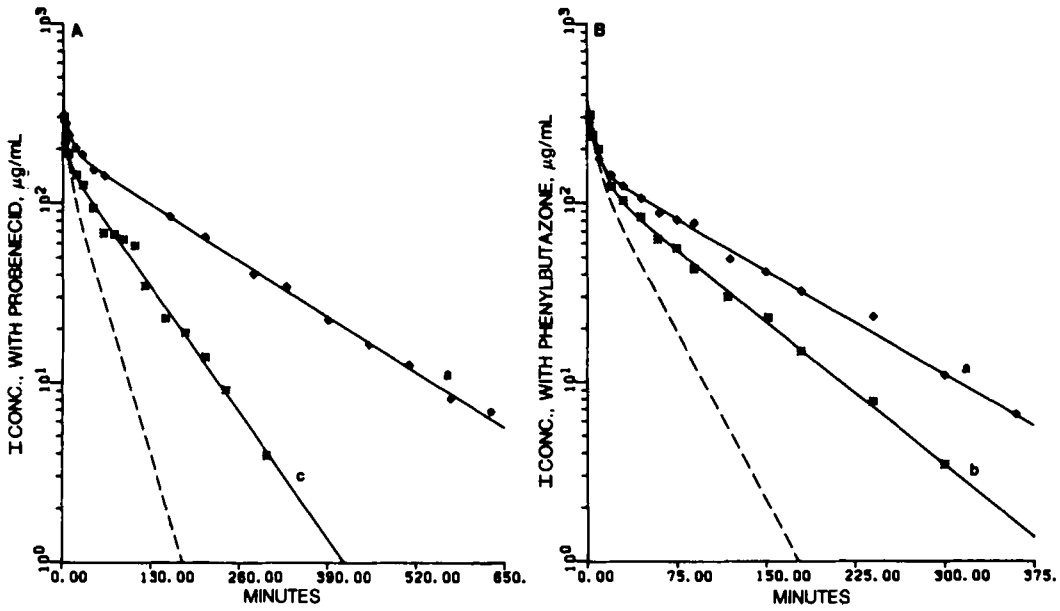

Figure 3—*Plasma concentrations of I after a 20-mg/kg dose of I with a 125-mg/kg dose of probenecid (A) or a 75-mg/kg dose of phenylbutazone (B). Key: (a) dog A; (b) dog B; (c) dog C; (- - -) 20-mg/kg dose of I without the competitor.*

at different competitor concentrations was divided by the control to give the percent reduction in I binding to either the Y or Z fraction. The amount found in the void volume was used for mass balance calculations.

Assay—In all procedures, only I was assayed. Plasma, urine, bile, and the plasma and liver protein binding samples were diluted with phosphate buffer (pH 8), and the absorbance was measured directly at 587 nm. Standards were prepared and run daily. The standard curves were linear (r = 0.9997); the sensitivity was 0.6 μg/mL.

RESULTS

Plasma Concentrations of Bromophenol Blue—The mean plasma concentrations of I *versus* time are shown in Fig. 1. The low dose (5 mg/kg) declined monoexponentially, while the intermediate dose (20 mg/kg) and the high dose (30 mg/kg) declined biexponentially (Fig. 1). The data were fit to a sum of exponentials using NONLIN (25). The relevant pharmacokinetic parameters are given in Table I (26, 27).

The biological half-life ($t_{1/2}$) and plasma clearance (CL_p) were not constant over the range of doses studied (Table I). These results indicate that the elimination of I from plasma is a dose-dependent process in this dose range. The area under the plasma concentration–time curve ($AUC_{0\to\infty}$) increased at a greater than linear rate with increasing dose (Table I).

The percentage of I bound to plasma proteins was 92.5 ± 1.5% at 37°C and was essentially constant over the range of I concentrations examined (100–875 μg/mL). A slight increase in the free fraction of I with increasing I concentration occurred, but was found not to be statistically significant using the Student's t test.

The graph in Fig. 2 indicates the plasma concentrations following two identical 5-mg/kg doses of I administered 2 h apart. The first dose was no longer detectable in the plasma just prior to the second dose, yet the second dose was eliminated at a significantly ($p < 0.005$) slower rate. The half-lives were 6.7 and 12.7 min, respectively.

Drug Competition With Bromophenol Blue—The concentration–time profiles of I following prior administration of probenecid and phenylbutazone are shown in Fig. 3a and b. The data was fit to a sum of exponentials using NONLIN (25).

In the presence of each competitor, the half-life increased, the area under the plasma concentration–time curve increased, and the clearance decreased (Table II). The volume of distribution at steady state (Vd_{ss}) was essentially unchanged, indicating that the competitors had no effect on the overall distribution of I.

Phenylbutazone produced a significant change ($p < 0.05$) in the plasma protein binding of I. The percent of I bound was 86.1 ± 2.9%, corresponding to nearly a twofold increase in the free I concentration over that found in the absence of phenylbutazone. Probenecid had no effect on the plasma protein binding of I (92.2 ± 1.2%).

Liver Protein Binding Studies—The results of the *in vitro* studies of I binding to rat liver protein fractions are presented in Table III. Figure 4a and b shows the protein and I elution profiles. From left to right the protein peaks correspond to protein in the void (unidentified protein) volume, proteins in the Y-fraction, and proteins in the Z-fraction (13–15), while the I peaks indicate binding to the respective protein peaks. Probenecid competed with I for Z-fraction binding sites, resulting in a significant decrease in the amount of I bound and a corresponding increase in the amount of free I (Table III). In Fig. 4a the profile representing I bound to Z steadily decreased with an increase in probenecid concentration. Phenylbutazone did not affect I binding to Z-fraction (Fig. 4b, Table III). Neither competitor affected the amount bound to the Y-fraction; because the amount of I bound to the Y-fraction was ~1–3% of that bound to the Z-fraction, the results were not presented.

Biliary Excretion Studies—Although all three dogs were cannulated as described above, only one animal provided complete data. Dog A developed peritonitis despite intensive postoperative maintenance, and dog B developed a colateral bile duct which bypassed the cannula 2 weeks after the surgery.

Table II—Comparison of Pharmacokinetic Disposition Parameters of I (20 mg/kg) in the Presence of a Competitor

Dog	$t_{1/2}$, min[a]	CL_p, mL/min[a]	Vd_{ss}, L	$AUC_{0\to\infty}$, μg/mL·min[a]
		Probenecid, 125 mg/kg		
A	127	9.5	1.60	37,400
C	56.0	15.1	1.08	14,300
		Phenylbutazone, 75 mg/kg		
A	77.9	18.9	1.73	18,500
B	56.3	26.3	1.27	12,200

[a] Significantly different from the no competitor case ($p < 0.005$).

Table III—Effect on the Binding of I to the Z-Fraction in the Presence of a Competitor

Competitor Conc., μmol/mL	I Conc., μmol/mL	Total Amount I Bound, μg	Reduction in I Binding, %	t test
		Phenylbutazone		
0.0	2.0	755	—	—
2.0	2.0	748	1.0	$p > 0.05$
4.0	2.0	754	0.1	$p > 0.05$
6.0	2.0	748	0.9	$p > 0.05$
8.0	2.0	753	0.3	$p > 0.05$
		Probenecid		
0.0	2.0	755	—	—
2.0	2.0	704	6.9	$p < 0.05$[a]
4.0	2.0	699	7.3	$p < 0.05$[a]
6.0	2.0	693	8.2	$p < 0.05$[a]
8.0	2.0	687	9.0	$p < 0.05$[a]

[a] Paired t test performed on duplicate data sets.

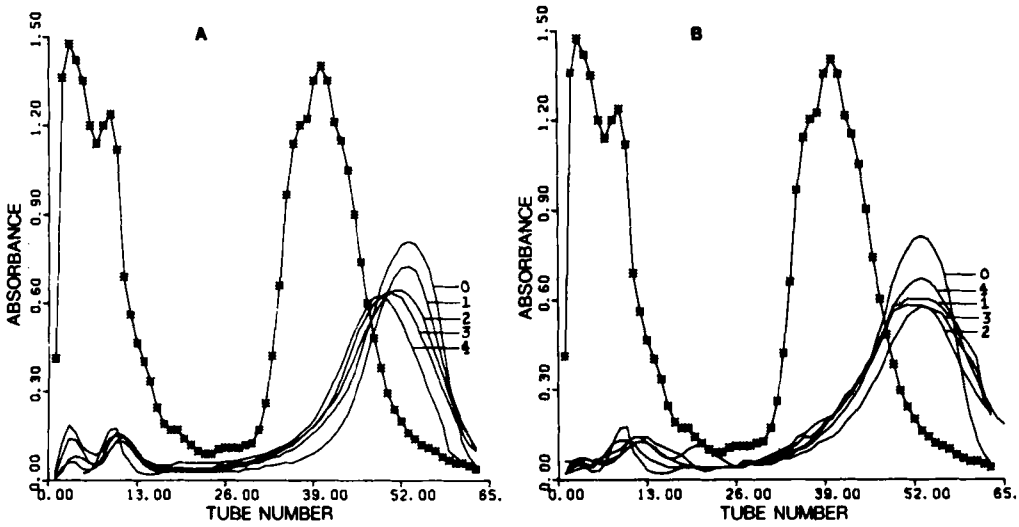

Figure 4—*Elution patterns of I, measured at 587 nm, in the presence of probenecid (A) and phenylbutazone (B) in the rat liver cytoplasmic fraction. 0, 1, 2, 3, and 4 indicate the amount of competitor added as a multiple of the amount of I present. The asterisks represent the protein fraction elution pattern measured at 280 nm. Each tube number represents a 5-mL collection.*

The pharmacokinetic parameters derived from I plasma concentrations following intravenous administration to the cannulated dogs showed an increase in half-life over the values observed in the same dogs before surgery (40.6 *versus* 24.8 min at 20 mg/kg and 11.7 *versus* 6.9 min at 5 mg/kg, respectively). Liver dysfunction did not appear to be the explanation, since hematology values were still within normal limits. The remaining parameters (clearance, volume of distribution, and area under the plasma concentration-time curve) were unchanged.

Biliary excretion σ^- is plotted in Fig. 5. The abnormalities in the curve for the 30-mg/kg dose were due to the temporary clogging of the cannula. Combined with the urine output, the amount recovered ranged from 95.7 to 101.1% of the dose. The amount recovered from the bile ranged from 92.3 to 98.9% of the dose. The σ^- plot appeared to be linear for all doses. There was no statistical difference ($p < 0.05$) between the half-lives at all doses (mean harmonic half-life = 163 min). Rate plots were also constructed, but were difficult to interpret. The biliary excretion rate data will be discussed briefly in another report (28).

Urinary Excretion Data—The amount of I recovered in urine from the intravenous and biliary excretion studies ranged from 1.5 to 7.2%. These data were considered significant in the pharmacokinetic interpretation of I and were used only for mass balance recovery purposes.

DISCUSSION

The plasma data presented in Fig. 1 and Table I clearly indicate that the rate of I elimination from plasma is dose dependent, and the rapid increase in AUC with increasing dose seems to suggest some saturable process. Since 95-101% of the dose was recovered as unchanged drug, it appears that the dose dependency is not due to I metabolism. Takada *et al.* (8) found that metabolic inhibitors did not affect the liver uptake of I in rats, suggesting that the dose dependence is not due to saturation of an active transport system. The fact that the extent of plasma protein binding of I remains essentially constant over the dose range indicates that the dose-dependent elimination kinetics are not due to changes in plasma protein binding. The data shown in Fig. 2 further suggest that, since the first dose was essentially eliminated from the plasma, the observed dose dependency may be due to the binding of I in other tissues. Accordingly, the binding of I to liver protein fractions was investigated.

The peak assignments (as discussed in the *Results*) in Fig. 4a and b are supported by earlier studies (13-15), but differ from those reported by Takada *et al.* (9). In the present study, each I peak corresponds to a protein peak while in the work of Takada *et al.* (9) the I supposedly bound to the Z-fraction did not have a corresponding protein peak; the I peak we have identified with the Z-fraction was labeled free I in their work (9). We observed free I eluting at much later times than are plotted (Fig. 4). Takada and co-workers also identified a fraction, T-binder, which elutes at a time similar to the Z-fraction. It is possible that this peak was masked by the large Z-fraction peaks in Fig. 4a and b. Since the only difference in methodology between our study and that of Takada *et al.* appeared to be the strain of rat (Sprague-Dawley in this study, Wistar in their work), further investigation may be warranted, because it is unlikely that strain differences would account for these discrepancies.

The data summarized in Table III indicate that neither competitor interfered with I binding to the Y-fraction and that probenecid competed with I for Z-fraction binding sites while phenylbutazone did not. Since both probenecid and I are anionic at physiological pH while phenylbutazone is neutral, these findings tend to support the hypothesis (21) of the existence of separate pathways for the biliary uptake and elimination of charged and neutral species.

We found that the amount of I bound to the Y-fraction is far smaller than the amount bound to the Z-fraction. This tends to support the earlier hypothesis (8, 9) that organic anions are taken up by the liver *via* two pathways (binding to the Y- and Z-fractions), one of which (Y-fraction) is of limited capacity and easily saturable. Once the Y-fraction is saturated, I is still eliminated from the plasma at a reduced, but apparently first-order, rate through uptake by the large-capacity Z-fraction.

The increased I plasma half-life following administration of probenecid appears to be due to competition for the Z-binding sites involved in the uptake process. The increased I plasma half-life following administration of phenylbutazone appears to be due to the displacement of I from plasma proteins

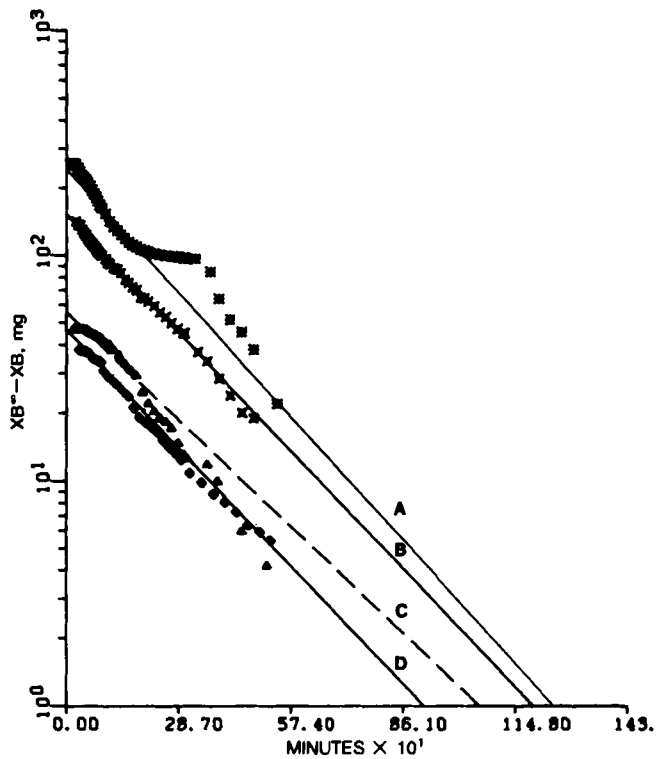

Figure 5—σ^- *Plot of I elimination in bile after doses of 30 mg/kg (A), 20 mg/kg (B), and 5 mg/kg (C) to dog C and a 5-mg/kg to dog B (D).*

and the subsequent increase in free I plasma concentrations, which saturate the uptake process in a manner similar to large doses of I.

Biliary excretion data obtained from rats (8–11) have often been of sufficient quality to enable the pharmacokinetic support of intrahepatic models such as that suggested above. Although our data was quite limited, it seems to suggest that data of sufficient quality may be difficult to obtain from dogs. The fact that the biliary excretion half-life was found not to vary with dose supports our contention that the changing plasma half-life reflects a saturable hepatic uptake rather than an elimination process.

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Effect of Nonuniform Bile Flow Rate on the Rate of Biliary Excretion of Bromophenol Blue in the Beagle

ROBERT S. PEARLMAN ˣ, GERALD J. YAKATAN §, and ROBERT J. WILLS *

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Present addresses: *Hoffmann-La Roche Inc., Department of Pharmacokinetics and Biopharmaceutics, Nutley, New Jersey 07110 and §Warner-Lambert Co., Pharmaceutical Research Division, Morris Plains, NJ 07950.

Abstract □ Following the intravenous administration of bromophenol blue to beagle dogs, graphs of the biliary excretion rate *versus* time displayed drastic fluctuations which render them of little value for standard pharmacokinetic modeling purposes. It was shown that these fluctuations in excretion rate are highly correlated with corresponding fluctuations in the bile flow rate. An expression was derived which accounts for the primary effect of nonuniform bile flow rate on the biliary excretion rate. This treatment would enable the use of such biliary excretion rate data for pharmacokinetic modeling. Secondary effects of nonuniform bile flow on the biliary excretion rate are also discussed. It is suggested that the modeling of other flow rate-dependent elimination processes could benefit from such a treatment.

Keyphrases □ Bromophenol blue—biliary excretion, nonuniform bile flow rate, dogs □ Biliary excretion—bromophenol blue, dogs, nonuniform bile flow rate □ Bile flow rate—nonuniform, biliary excretion of bromophenol blue, dogs

Biliary excretion has long been a subject of intensive investigation (1). Within the past decade, there has been an increase in the number of studies in which the bile duct has been cannulated to permit the determination of drugs and metabolites in the bile. These studies typically involve collecting bile samples at relatively long intervals and, usually, the cumulative amounts of drug excreted are reported as a function of time (2–5). These studies have been particularly useful in assessing the magnitude of the first-pass effect and the role of hepatobiliary elimination in the overall elimination of a wide variety of compounds (1). More recently, it has been shown that high-quality bile data can provide pharmacokinetic support for an intrahepatic model for hepatobiliary elimination in rats (6, 7). Takada *et al.* (7) obtained biliary excretion rate data (mg/h *versus* time) of sufficient quality to enable a statistically significant fit to a tetraexponential function, which they identified with a five-compartment model for hepatobiliary transport.

The possibility of obtaining similar data in higher animals is appealing, yet the problem must be viewed somewhat pessimistically due to the fact that the bile flow rate fluctuates

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