Comparative Pharmacokinetics of Coumarin Anticoagulants III

Factors Affecting the Distribution and Elimination of Bishydroxycoumarin (BHC) in Isolated Liver Perfusion Studies

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The kinetics of elimination of BHC in man is unusual in that the apparent first-order rate constant for elimination appears to decrease with increasing dose. No such dose dependency is apparent in rats and certain other species of animals. The purpose of this study was to determine if this difference in BHC elimination kinetics is due to a difference in the interaction of BHC with plasma proteins of rats and man. Isolated rat livers were perfused with a mixture of red blood cells from rats and plasma from donor rats or man (two concentrations) which contained various concentrations of BHC. The concentration of BHC was determined periodically in the perfusate and terminally in the liver. It was found that the apparent rate constant for the metabolism of BHC in isolated perfused rat livers is affected by the initial concentration of drug and by the source and concentration of plasma proteins, and that it is a function mainly of the distribution of BHC between liver and plasma. There is a significant difference in the affinity of BHC to plasma proteins from man and rat, respectively, and there appears to be also a difference in the relative importance of the factors which affect BHC elimination in man and rats.

THE PHARMACOKINETIC characteristics of the anticoagulant drug bishydroxycoumarin (BHC) in man are unusual in that drug levels in the plasma decline exponentially following absorption and distribution, but with half-lives which increase with increasing dose (1, 2). Similar dose-dependent characteristics have been noted in the elimination of several other drugs (3) but the basis for this unusual phenomenon has not as yet been elucidated. In addition, there is some indication that the apparent volume of distribution of BHC in man decreases with increasing dose (2). This type of effect has apparently not been observed with any other drug.

The mechanism of the unusual pharmacokinetic characteristics of BHC has been the subject of continuing inquiry in this laboratory. The unique concentration-dependent plasma protein binding characteristics of BHC described in the initial report in this series (4), and the unexpected type of dose dependence of the apparent volume of distribution of this drug in man as reported by O'Reilly *et al.* (2), suggested that the unusual pharmacokinetic characteristics of BHC may be due to protein binding. On the other hand, it has been suggested that a dose dependence in the biologic half-life of a drug may be the result of substrate inhibition of the enzyme responsible for the biotransformation of that drug (3). A recent study in this laboratory (5) has shown that there is no apparent dose dependence in the distribution and elimination kinetics of BHC in rats in the dose range of 2 mg./kg. to 20 mg./kg. (which is equivalent to an initial plasma concentration of about 20 mcg./ml. and 200 mcg./ml.). It was also found in initial experiments that the affinity of BHC to human plasma proteins is much greater than to rat plasma proteins. It was therefore thought that a study of the effect of human plasma and rat plasma, respectively, on the elimination of BHC by isolated perfused livers would establish the role, if any, of plasma protein binding in the elimination of this drug. Furthermore, this technique permits an assessment of the effect of drug "dose" (*i.e.*, concentration) on the elimination kinetics under conditions where distribution factors can be accounted and corrected for.

In the study described here, the kinetics of BHC at various concentrations was investigated in isolated rat liver perfusion systems, using rat blood as well as mixtures of human blood plasma and blood cells from rats. (Plasma containing human blood cells does not flow adequately through rat livers.) The kinetic constants thus obtained were compared with similar constants obtained from studies on intact animals. The results of this investigation demonstrate the relative importance of distribution and concentration in the elimination of BHC in both isolated livers and intact animals. They demonstrate also that

Received May 6, 1968, from the Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication July 24, 1968.
Supported in part by a grant from the Heart Association of Erie County, Inc.
Previous paper: Nagashima, R., Levy, G., and Back, N., *J. Pharm. Sci.*, **57**, 68(1968).
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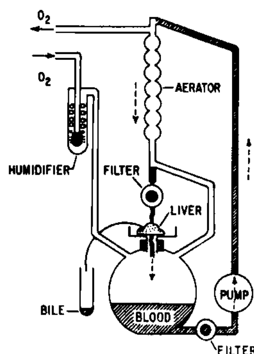

Fig. 1—Schematic representation of isolated rat liver perfusion apparatus. The filter is a sheet of Nylon (100 mesh), and the perfusion pump is of peristaltic type (model T-8, Sigmamotor, Inc., Middleport, N.Y.). The whole system is enclosed in a Plexiglas chamber in which the temperature is thermostatically maintained at 37°. The liver is covered with gauze pads saturated with Ringer solution. The blood is oxygenated with a humidified O_2–CO_2 mixture (95:5 by volume).

a rigorous pharmacokinetic analysis, incorporating all pertinent variables, permits a quantitative correlation of data obtained *in vitro* and *in vivo*.

EXPERIMENTAL

Liver Perfusion Techniques and Apparatus—The techniques and apparatus for the isolated rat liver perfusion studies (Fig. 1) were similar to those described by Miller *et al.* (6) and Sokal *et al.* (7, 8). Liver donors were normal, fed, male Sprague-Dawley rats weighing 300–500 g. Their livers were excised and perfused with 100 ml. oxygenated perfusing fluid consisting of diluted rat blood or mixtures of diluted human plasma and rat blood cells. The perfusing fluid entered the liver by free flow through a cannula in the portal vein, at a hydrostatic pressure of 13–15 cm. The fluid returned to the reservoir through a cannula in the inferior vena cava. It could then recirculate through the liver. The bile was collected every 0.5 hr. The viability of the livers was monitored by the appearance of the organ, flow rate of the perfusing fluid, bile production, and glucose levels in the perfusing fluid.[1]

Surgical Procedures—Surgical procedures were performed under light ether anesthesia. The abdomen was opened, the gastrohepatic and gastroduodenal ligaments were cut, and the bile duct was cannulated with a PE-10 polyethylene tube (Clay Adams, Inc., New York, N.Y.). The hepatic artery was ligated above the branch running toward the duodenum, and two loose ligatures were placed around the portal vein. The inferior vena cava was ligated and the ligature previously placed around the portal vein distal to the liver was tied. A glass cannula (o.d. 3 mm.) was firmly placed through an incision in the portal vein. One and one-half milliliters sodium heparin solution (250 USP units/ml.) was injected through this cannula. The chest was then opened and reflected, and a cannula was inserted through the inferior vena cava above the diaphragm. One-half milliliter sodium heparin solution was injected into the inferior vena cava through the second cannula. Finally, the liver was carefully dissected out and removed from the animal together with the attached diaphragm, the two cannulas, and the catheter attached to the bile duct. The total time for the procedure from opening the abdomen to starting the perfusion was approximately 15 min.

Perfusion Fluid—Heparinized blood was obtained from normal, fed, male Sprague-Dawley rats by cardiac puncture. Generally, the blood from 10–15 donor rats was pooled for any one perfusion experiment. It was diluted with Ringer solution such that for each part by volume of plasma, there was added 0.7 part by volume of Ringer solution. The actual volume of Ringer solution added to the blood was determined on the basis of hematocrit values, which ranged from 43–46% in the pooled blood. One milliliter of BHC solution (various concentrations),[2] 0.25 ml. sodium heparin solution (1,000 USP units/ml.), and 0.5 ml. antibiotics solution[3] were added to 100 ml. of the diluted rat blood.

Heparinized human plasma was prepared by centrifugation at $1,800 \times g$ for 20 min. of blood obtained by venipuncture from a healthy adult male volunteer. The plasma was then diluted by adding either 0.7 or 1.7 parts of Ringer solution to 1 part by volume of plasma. The rat blood cells were obtained by centrifugation at $1,800 \times g$ for 20 min. of heparinized blood obtained from 10 to 15 donor rats. The cells were washed with Ringer solution. Thirty-two milliliters of packed rat blood cells was mixed with 68 ml. of diluted human plasma, to which 1 ml. of BHC solution (various concentrations), 0.25 ml. sodium heparin solution (1,000 USP units/ml.), and 0.5 ml. antibiotics solution were added. The blood was collected and the perfusing fluid was prepared from it no longer than 3 hr. before a perfusion experiment. During the experiment, 0.4 ml. each of sodium heparin solution and antibiotics solution were added to the perfusing fluid in the reservoir every hour.

Collection of Perfusion Fluid Samples—Two and two-tenths-milliliter aliquots of perfusing fluid were withdrawn from the reservoir every 10 min. for the initial 0.5 hr. and every 0.5 hr. thereafter for a total of 4 hr. Part of each sample was used to determine glucose levels, the remainder was centrifuged to obtain "plasma phase" for the determination of BHC.

Assay Method for BHC—BHC in the plasma phase was determined by a modification of the method of Axelrod *et al.* (10). The assay consists essentially of adjustment of plasma to pH 3.0, extraction with heptane, transfer of the drug into 2.5 *N* NaOH, and spectrophotometry at 314 mμ (4). BHC metabolites and the antibiotics added to the plasma phase did not interfere with the assay.

BHC concentration in the liver was determined by the paper chromatographic method of Christensen (11) except that the liver homogenate was adjusted to pH 4.3 using 1.5 *M* citrate–phosphate buffer prior to the extraction of the drug with ethylene dichloride. The average recovery of BHC from liver

[1] Glucose levels were determined by an AutoAnalyzer (Technicon Instruments Co., Chauncey, N. Y.) using a ferricyanide reduction method (9).

[2] Prepared by dissolving one part by weight of bishydroxycoumarin (Nutritional Biochemicals Co., Cleveland, Ohio) and 10 parts by weight of tris(hydroxymethylamino)methane (Nutritional Biochemicals Co.) in freshly distilled water.

[3] The antibiotics solution contained 2.46 mg. penicillin G potassium, 0.6 mg. polymyxin, and 0.48 mg. streptomycin/ml.

homogenates to which 3.3 to 100 mcg. of the drug/g. wet tissue were added was 87%. Triplicate determinations were made.

Stability of BHC in Aqueous Solution Under Oxygenated Condition—An aqueous solution of 25 mcg. BHC/ml. in 0.05 M phosphate buffer (pH 7.45) was maintained at 38° and gassed continuously with a mixture of 95% oxygen and 5% carbon dioxide. One-half-milliliter aliquots were withdrawn at zero time and after 3 hr. of oxygenation. The concentrations of BHC were determined as described previously for plasma samples.

Distribution of BHC in the Red Blood Cells (RBC)—Exactly 1 ml. of an aqueous solution containing 0.2 mcg. BHC/ml. was added to a 10-ml. mixture consisting of 2.75 ml. rat RBC and 7.25 ml. heparinized diluted rat plasma (1.7-fold dilution). This was mixed thoroughly and maintained at 37°. One-milliliter aliquots of the fluid were withdrawn at appropriate intervals and centrifuged to obtain plasma phase for the determination of BHC. The fluid was mixed before each withdrawal by repeated inversion of the tube to prevent sedimentation of the cells.

Equilibration of BHC Between Human Plasma and Rat Plasma—Cellophane tubing [2.54 cm. (1 in.) width when flat, Visking Co., Chicago, Ill.] was soaked in $^1/_{15}$ M phosphate buffer (pH 7.4) for 4 days. The tubing was then sleeved onto a glass rod and half-everted to make a double cellophane wall with only one end open. Three milliliters of heparinized plasma (either rat or human), 0.5 ml. BHC solution of specified concentration, and 1.5 ml. $^1/_{15}$ M phosphate buffer (pH 7.4) were pipeted into the space between the two cellophane walls. The tubing was then placed in a 13-ml. round vial containing 3 ml. plasma from the other source, 0.5 ml. BHC solution, and 1.5 ml. of $^1/_{15}$ M phosphate buffer (pH 7.4). The tubing in the vial was adjusted so that the two fluids were at the same level. This was done by placing one end of the tubing between the screw cap and the thread of the vial. The vials were agitated in an upright position on a reciprocating shaker in a cold room maintained at 2°. One-half-milliliter aliquots were withdrawn from both plasma phases at 4-day intervals and were assayed for BHC as described previously.

The same experiment was carried out also with more diluted plasmas (10-fold dilution). Here, each of the fluids consisted of 0.5 ml. plasma, 0.5 ml. BHC solution of specified concentration, and 4 ml. $^1/_{15}$ M phosphate buffer.

Determination of Elimination Rate Constants and Distribution Ratios—The apparent rate constant for drug elimination in a liver perfusion system is inversely proportional to the volume of the perfusing fluid (12). The drug concentrations in aliquots of the perfusing fluid obtained during a liver perfusion experiment had to be corrected therefore for the decreasing volume of this fluid with time due to repetitive withdrawal of samples. This correction was made by means of the relationship:

$$C_2^c = C_1^c - \frac{V}{V_0}(C_1 - C_2) \qquad \text{(Eq. 1)}$$

where C_2^c is the corrected plasma concentration of BHC in a given sample of perfusing fluid, C_2 is the actual concentration of BHC in that sample, C_1 is the actual concentration of BHC in the preceding sample,

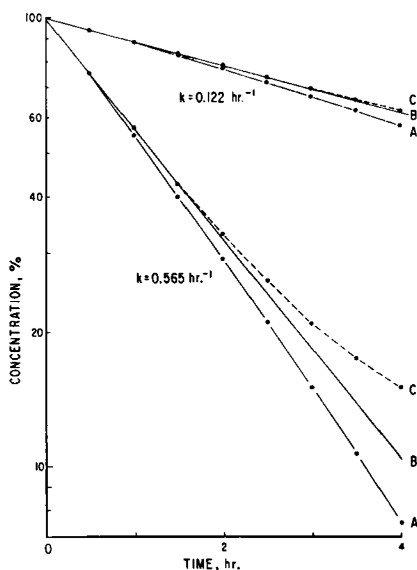

Fig. 2—The effect of a decrease in the volume of the perfusing fluid (due to repeated withdrawal of samples) on the time course of the drug concentration decline in an isolated perfused liver system. Shown are the expected concentration changes for a rapid and a slow apparent first-order elimination process if the volume of the perfusing fluid remains constant with time (B); results which would be obtained if the volume of the perfusing fluid (initially 100 ml.) decreases with time due to removal of 2.2-ml. samples every 10 min. for the first half hour and every 30 min. thereafter (A); results obtained by correcting the data in A by the procedure described in the text (C).

C_1^c is the corrected plasma concentration of BHC in the preceding sample (or the actual concentration if the preceding sample is the first sample), V is the volume of perfusing fluid prior to withdrawal of the sample containing BHC = C_2, and V_0 is the initial volume of the perfusing fluid. Equation 1 applies exactly if drug elimination proceeds at a constant rate and can be used also if the rate of drug elimination is proportional to concentration, provided that the concentration change in the sampling interval is small. Figure 2 shows the effect of a continuous decrease in the volume of perfusing fluid, due to withdrawal of 2.2-ml. samples every 30 min. from an original volume of 100 ml., on the observed concentrations of a drug. The examples in the figure refer to a rapid and a slow hypothetical first-order process, respectively. The line labeled B shows the concentration decline if the volume of the perfusing fluid had remained constant; Line A shows the expected actual concentrations when samples are withdrawn at intervals (note that this line curves downward and may suggest a saturation phenomenon if the need for data correction is not recognized); Line C shows the expected data obtained by applying the correction procedure described in Eq. 1. The deviation from the "true" line becomes significant only after about two half-lives; no such deviation occurs if the drug is eliminated at a constant rate. The rate constants of the hypothetical examples presented in Fig. 2 are identical with the lowest and the highest rate constant, respectively, encountered in this study.

TABLE I—PHYSIOLOGIC STATUS OF ISOLATED RAT LIVERS DURING PERFUSION[a]

Plasma Source	Amount of BHC in System,[b] mg.	No. of Experiments	Flow Rate of Perfusing Fluid, ml./min.[c]	Volume of Bile Excreted, ml.[c]	Glucose Concn. in Perfusing Fluid, mg./100 ml.[d]
Rat	None	1	15	1.8	225
Rat	1–6	8	19 ± 4	1.6 ± 0.2	220 ± 46
Man	1–6	6	19 ± 3	1.3 ± 0.3	246 ± 51
Man, more diluted[e]	2	2	18, 19	1.5, 1.9	298, —

[a] The data are presented as mean values ± one standard deviation, or as individual values. [b] The system consisted of 32 ml. (packed volume) rat blood cells and 68 ml. diluted plasma (58% unless indicated otherwise). BHC = bishydroxycoumarin. [c] Time, 4 hr. [d] Time, 2 hr. [e] The more diluted plasma consisted of 1 part human plasma and 1.7 parts Ringer solution, *i.e.* dialysis plasma.

The reported apparent first-order elimination constants ($k_{app.}$) were not obtained from the slopes of the log plasma phase concentration *versus* time plots, but rather by successive calculations based on the relationship:

$$k_{app.} = \frac{\Delta C}{C_m} \cdot \frac{V}{V_0} \qquad \text{(Eq. 2)}$$

where ΔC is equal to the observed drug concentration change in a sampling interval, C_m is the observed drug concentration at the midpoint of that sampling interval, V is the volume of the perfusing fluid during the sampling interval, and V_0 is the original volume of the perfusing fluid. Thus, each 4-hr. experiment yielded seven values of $k_{app.}$ (data for the first 30-min. sampling interval were affected by the initial distribution of the drug and were not used in the calculations). These values were quite constant with time and the average $k_{app.}$ for any one experiment usually did not differ by more than 5% from the $k_{app.}$ obtained graphically.

The initial distribution ratio of BHC between liver and plasma phase (C_L^0/C_P^0) was calculated from the extrapolated zero time concentration of BHC in the plasma phase (C_P^0) and add the initial concentration of BHC in the liver (C_L^0). The latter was determined on the basis of the relationship:

$$C_L^0 = (A - V_P C_P^0)/W \qquad \text{(Eq. 3)}$$

where A is the amount of drug introduced into the system, V_P is the volume of the plasma phase,[4] and W is the wet weight of the liver in grams. Equation 3 is based on the observation by Christensen (13), which was verified in this laboratory, that no measurable amounts of BHC are present in or on red blood cells.

RESULTS

Viability of the Isolated Liver Preparation—The flow rate of perfusing fluid at the end of the experiments, the cumulative volume of bile produced during the experiments, and the glucose concentration in the perfusing fluid at the midpoint of the experiments are listed in Table I. These data are representative only since the listed functions were monitored continuously throughout the experiments. No discontinuities were observed. The continuing viability of the liver preparations during the experimental period is evident also from the drug elimination data described in subsequent paragraphs. The flow rates of perfusing fluid observed in this study were similar to those reported for a similar perfusion system (6) and were in the range of hepatic blood flow rates in intact rats (14). They were considerably in excess of the minimal flow rate necesssary to supply fully the oxygen requirement of the liver (15). The time course and rates of bile production observed in this study compare well with those described by other investigators (16, 17), the rates being also in the range obtained with intact rats (18). Glucose levels were also similar to those reported by others (8, 17, 19, 20). The single experiment without drug reported in Table I was not for the purpose of establishing physiologic controls; the listed data were obtained incidentally to a determination of BHC blank values in the isolated perfused liver system.

Stability of BHC—The concentration of BHC in 0.05 M phosphate buffer (pH 7.45) at 38° decreased only by 2% after 3 hr. of oxygenation with a mixture of 95% oxygen and 5% carbon dioxide. The concentration of BHC in perfusing fluid containing human plasma, when circulated under oxygenated conditions at 37° in the perfusion apparatus without liver, did not change measurably during 4 hr. Similar control experiments with rat plasma were complicated by destruction of some of the red blood cells due to the mechanical action of the peristaltic pump. For unknown reasons, this did not occur in experiments with livers. The destruction of the red blood cells in the rat plasma control experiments was accompanied by a decrease in BHC concentration in the plasma, apparently due to adsorption of this drug on the cell debris. This decrease, which was about 15%, occurred suddenly after some time and seemed to coincide with red cell destruction. Since no such destruction occurred in experiments with livers, it was assumed that this effect could be neglected. This assumption is justified also by the fact that in none of the actual experiments did there occur a sudden decrease in BHC concentration in the plasma phase.

Distribution of BHC in the Blood—Addition of BHC to diluted rat blood (the same composition as used in the perfusion experiments) yielded constant plasma levels of BHC during half-hourly sampling for 4 hr. The observed concentration of BHC in the plasma phase was identical to the expected concentration if it is assumed that BHC does not enter or bind on blood cells. These results are consistent with those reported by Christensen (13). They show also that BHC is stable in rat plasma.

Relative Affinity of BHC for Plasma Proteins from Rat and Man—Equilibration of human blood plasma containing BHC against rat blood plasma containing BHC, with the two plasma phases separated by a dialysis membrane, showed that BHC concentration in the human plasma increased with time (Table II).

[4] The volume of the plasma phase was corrected for the volume of the liquid trapped between the packed cells (4% of packed cell volume) according to Chien *et al.* (21).

TABLE II—THE RATIO OF BHC CONCENTRATIONS AND AMOUNTS IN HUMAN PLASMA AND IN RAT PLASMA SEPARATED BY A DIALYSIS MEMBRANE

Dilution of Plasma[a]	Initial BHC Concentration, mcg./ml.		BHC Concentration Ratio, Human:Rat[b] Time, days					Ratio of Amounts of BHC in Plasma, Human:Rat, 16 days[b]
	Human Plasma	Rat Plasma	0	4	8	12	16	
1.7-fold	20	20	1.0	1.1	1.1	1.4	1.7	2.5
1.7-fold	20	16.7	1.2	1.1	1.2	1.6	2.0	3.0
1.7-fold	20	13.3	1.5	1.4	1.4	1.6	1.9	2.6
10-fold	3.3	3.3	1.0	1.2	1.8	2.5	2.9	3.1

[a] Diluted with $^1/_{15}$ M phosphate buffer (pH 7.45). [b] Average of two experiments each.

The initially observed decrease was due to water movement to the human plasma phase, resulting from the higher colloid osmotic pressure of human plasma as compared with rat plasma.[5] The very slow rate of equilibration of BHC is due to its extensive plasma protein binding and the resulting low concentration gradient of free drug across the dialysis membrane. Regardless of the criterion used (concentration ratio or ratio of amounts), and despite the fact that equilibration was not complete after 16 days, it is evident that the affinity of BHC to human plasma proteins is much greater than to rat plasma proteins.

Distribution of BHC Between Liver and Plasma Phase—The distribution of BHC between liver and plasma phase was determined by direct assay in five experiments. Despite the difficult and time-consuming assay procedure, the results thus obtained agreed well with distribution ratios obtained by calculations based on liver weight, plasma volume, amount of drug introduced into the system, and extrapolated zero time concentration in the plasma phase (Table III). All other distribution ratios were therefore determined by these calculations.

TABLE III—COMPARISON OF CALCULATED AND DIRECTLY DETERMINED LIVER:PLASMA CONCENTRATION RATIOS OF BISHYDROXYCOUMARIN (BHC) IN RAT LIVER PERFUSION SYSTEMS

Expt. No.	Concentration Ratio, ml./g. By Calculation[a]	By Assay[b]
6	2.2	2.7
12	0.8	0.9
13	1.8	1.8
16	0.6	1.0
17	1.7	1.8

[a] Based on the amount of BHC added to the perfusion system and the extrapolated zero time concentration of BHC in the plasma phase of the perfusing fluid. [b] Determined by assay of BHC in the plasma phase of the perfusing fluid and in rat livers after 4 hr. of perfusion.

Pharmacokinetics of BHC Elimination in Livers Perfused with Rat Plasma—The elimination of BHC proceeded by apparent first-order kinetics in the dose range of 1–6 mg. Figure 3 shows the data obtained in the experiment which yielded the lowest $k_{app.}$ value at each dose level. The other $k_{app.}$ values as well as distribution data are listed in Table IV. It is evident that $k_{app.}$ decreased with increasing dose and that the concentration of BHC in the liver was higher than in the plasma.

[5] The colloid osmotic pressure of undiluted plasma is 330 (280–480) mm. H_2O in man and 260 (220–290) mm. H_2O in rats (22). This difference could cause a movement of 1.1 ml. water from the rat plasma to the human plasma phase under the conditions of this study. The observed value was about 0.8 ml. at 16 days.

Pharmacokinetics of BHC Elimination in Livers Perfused with Human Plasma—The kinetics of BHC elimination from the human plasma phase also followed apparent first-order kinetics. The values of $k_{app.}$ were generally lower than with rat plasma (Table IV). There was no distinct dose dependence of $k_{app.}$ Two experiments with more diluted plasma (2.7-fold rather than 1.7-fold) yielded higher $k_{app.}$ values than the other experiments. Five out of the eight experiments showed liver:plasma concentration ratios of BHC less than unity and therefore considerably lower than those obtained with rat plasma.

DISCUSSION

The apparent first-order rate constant for BHC elimination in an isolated perfused rat liver system should reasonably be a function of the concentration of BHC at the site of the biotransformation (the liver) and of the size of the liver. Thus, it is evident that distribution factors should profoundly affect the elimination of BHC. The "true" rate constant for elimination may be determined by correcting for variable drug distribution between liver and plasma, and for the weight of the liver. This may be done by calculating the fraction, F, of the total amount of drug in the system which is actually in the liver. Thus,

$$k_{app.} = k \cdot F \qquad \text{(Eq. 4)}$$

where k is the "true" rate constant for BHC elimination. F and $k_{app.}/F$ values for the experiments with rat plasma are shown in Table V. Listed also are comparable data obtained from intact rats. It may be noted that $k_{app.}$ values determined *in vitro* were

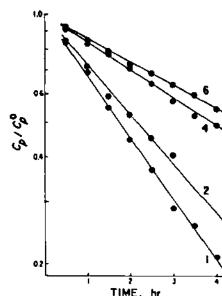

Fig. 3—Concentration of bishydroxycoumarin (BHC) in plasma phase of an isolated rat liver perfusion system as a function of time. The perfusing fluid consisted of diluted rat blood and the amount of BHC in the system ranged from 1–6 mg. (indicated by figures in the graph). The BHC concentrations are expressed as a fraction of the extrapolated zero time concentration.

TABLE IV—DISTRIBUTION AND ELIMINATION OF BHC IN ISOLATED PERFUSED RAT LIVER SYSTEMS

Expt. No.[a]	Liver Wt.,[b] g.	Plasma Source[c]	Amt. of BHC in System, mg.[c]	Calculated Concn. Ratio[d] (Liver:Plasma), ml./g.	$k_{app.}$, hr.$^{-1}$	$t_{0.5}$, hr.
3	11.5	Rat	1	2.5	0.45	1.5
4	11.4	Rat	1	2.9	0.57	1.2
5[e]	11.1	Rat	2	2.5	0.31	2.2
6	14.3	Rat	2	2.2	0.38	1.8
20	15.6	Rat	4	1.5	0.31	2.3
21	17.7	Rat	4	1.8	0.19	3.7
7	14.9	Rat	6	2.0	0.16	4.4
8	13.2	Rat	6	1.8	0.25	2.8
10	12.9	Man	1	0.8	0.21	3.2
11	12.6	Man	1	1.7	0.17	4.1
12	12.2	Man	2	0.8	0.18	3.8
13	15.7	Man	2	1.8	0.19	3.7
14	13.6	Man	6	0.7	0.19	3.6
15	13.1	Man	6	0.8	0.12	5.7
16	13.0	Man, more diluted	2	0.6	0.23	3.0
17	14.9	Man, more diluted	2	1.7	0.29	2.4

[a] Experiments 1, 2, 9, 18, and 19 were control experiments (no drug or no liver) and therefore are not listed. [b] Wet weight. [c] See footnotes *b* and *e* in Table I. [d] See footnote *a* in Table III. [e] This experiment was the first one in the series and was terminated at 3 hr.

TABLE V—EFFECT OF DISTRIBUTION AND DOSE ON THE ELIMINATION KINETICS OF BHC IN PERFUSED RAT LIVER SYSTEMS AND INTACT RATS

Expt. No.	Dose, mg./kg. Body Wt.[a]	C_P^0, mcg./ml.	Initial Concn. in Liver,[b] mg./g. Liver	Fraction of Dose in Liver Total F	Fraction of Dose in Liver Per g.	$k_{app.}$, hr.$^{-1}$	$k_{app.}/F$, hr.$^{-1}$
			Isolated Liver Perfusion Systems				
3	2.9	10.2	0.026	0.30	0.026	0.45	1.5
4	2.9	9.8	0.028	0.32	0.028	0.57	1.8
6	4.7	19.8	0.044	0.32	0.022	0.38	1.2
5	6.0	20.7	0.051	0.29	0.026	0.31	1.1
20	8.6	43.5	0.063	0.25	0.016	0.31	1.2
21	7.6	39.6	0.071	0.32	0.018	0.19	0.6
8	15.2	64.1	0.118	0.26	0.020	0.25	1.0
7	13.5	61.0	0.119	0.30	0.020	0.16	0.5
			Intact Animals				
From Ref. 5	2	23	0.006[c]	0.11	0.0031	0.12	1.1
From Ref. 23	5	47	0.011[c]	0.079	0.0025	0.11	1.4
From Ref. 13	27	160	0.070[c]	0.087	0.0026	0.075	0.9
From Ref. 5	20	200	0.088[c]	0.15	0.0044	0.14	1.0

[a] In the case of perfused livers, the weight of the donor rat. [b] C_P^0·(concn. ratio, liver:plasma)/1,000. [c] Based on 33.5 g. liver/kg. body weight (26). Concentration ratios, liver:plasma, in intact rats from *Reference 23*.

generally two to three times higher than those obtained *in vivo*, but that $k_{app.}/F$ values were comparable. This demonstrates the marked effect of distribution on the elimination kinetics of BHC and

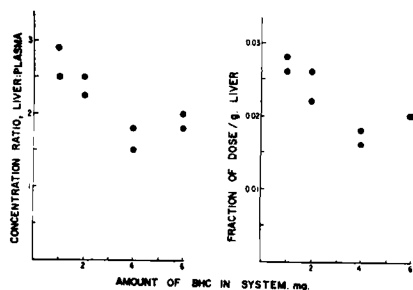

Fig. 4—Effect of the amount of BHC on the distribution of this drug between liver and plasma phase in an isolated rat liver perfusion system with diluted rat blood as the perfusing fluid.

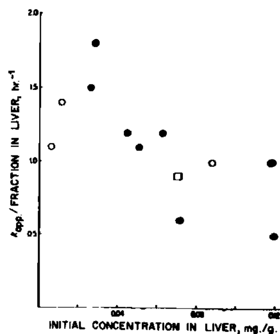

Fig. 5—The relationship between the rate constant for BHC elimination (corrected for variable distribution of BHC between liver and plasma due to variable liver weight, drug binding, etc.) and the initial concentration of this drug in the liver. Key: ●, isolated perfused livers; ○, intact rats; □, intact rats based on data from Reference 13.

TABLE VI—EFFECT OF DISTRIBUTION AND DOSE ON ELIMINATION KINETICS OF BHC IN ISOLATED RAT LIVERS PERFUSED WITH A MIXTURE OF HUMAN PLASMA AND RAT BLOOD CELLS

Expt. No.	Dose mg./Kg., Body Wt.[a]	C_P^0, mcg./ml.	Initial Concn. in Liver,[a] mg./g. Liver	Fraction of Dose in Liver Total F	Per g.	$k_{app.}$, hr.$^{-1}$	$k_{app.}/F$, hr.$^{-1}$
10	2.6	12.5	0.010	0.13	0.010	0.21	1.6
11	2.7	11.0	0.019	0.24	0.019	0.17	0.71
12	5.5	25.2	0.019	0.11	0.009	0.18	1.6
13	4.3	20.4	0.037	0.29	0.018	0.19	0.66
14	14.8	75.6	0.056	0.13	0.010	0.19	1.5
15	15.3	74.9	0.055	0.12	0.009	0.12	1.0

[a] As in Table V.

explains why the half-life of BHC was so much shorter in the perfused liver system than in the intact animals. The dilution of the plasma, which is necessary to obtain adequate flow rates in the *in vitro* system, results in greater partitioning of BHC into the liver than is the case in intact rats, and therefore results in greater $k_{app.}$ values.

The results of the perfusion studies with rat plasma show two dose (concentration)-dependent effects. One of these is the liver:plasma concentration ratio of BHC. It first decreases with an increasing amount of BHC in the system and then increases (Fig. 4). This effect is evident also in a plot of the fraction of the total dose/g. of liver *versus* the amount of BHC in the system (Fig. 4). The same phenomenon has been found in intact rats and in *in vitro* extraction studies (23). It is due apparently to the unusual concentration dependency in the plasma protein binding of BHC as described and discussed in a previous report (4). The other dose-dependent effect shown by the results of the perfusion experiments with rat plasma is the decrease in the "true" rate constant for BHC elimination with increasing "dose." In this context, "dose" or "concentration" is more appropriately the concentration of BHC in the liver rather than in the plasma phase. A plot of $k_{app.}/F$ *versus* BHC concentration in the liver is shown in Fig. 5. It is readily apparent that the *in vitro* $k_{app.}/F$ value decreases with increasing BHC concentration in the liver and that the *in vivo* data fit very well into the pattern. Therefore, there are two dose-dependent factors which affect the kinetics of BHC elimination, often in opposite directions: drug distribution between liver and plasma, and the apparent self-inhibition in the metabolism of BHC at higher doses. The mechanism of this latter effect is not yet understood, but there are several other drugs which exhibit the same phenomenon (3).

The liver:plasma concentration ratio of BHC in isolated rat liver systems perfused with human plasma was generally lower than in systems perfused with rat plasma, and the $k_{app.}$ values were consequently generally lower (Table IV). No such difference between the data obtained with rat plasma and human plasma, respectively, is evident in $k_{app.}/F$ values, *i.e.*, in the rate constants corrected for distribution effects (Tables V and VI). The differences in the distribution of BHC are consistent with the greater affinity of this drug to human plasma proteins (Table II). The distribution data obtained with human plasma were much more variable than those obtained with rat plasma. The rat plasma used in any one perfusion experiment was pooled from 10 to 15 animals while the human plasma was always obtained from one and the same individual. The latter procedure was expected to yield a plasma with relatively constant characteristics but it is apparent retrospectively that the use of pooled human plasma would have been more appropriate. Differences from one day to the other in the fatty acid content of the plasma obtained from a single human subject, related to the time and type of meals prior to collection of blood, are likely to have resulted in differences in the BHC binding characteristics of each plasma sample (24).

It is evident from the results of this study that the pharmacokinetics of BHC distribution and elimination are quite complex. There are indications that the relative importance of the dose-dependent distribution and apparent self-inhibition effects differs with each animal species. The biologic half-life of very high doses of BHC in rats is actually much shorter than the half-life of small doses (23), suggesting that the contribution of the distribution effect is greater than that of the apparent self-inhibition effect. On the other hand, the biologic half-life of BHC increases with increasing dose in man (1, 2, 25), indicating that the apparent self-inhibitory effect is more important than the distribution effect in the elimination of BHC in man in the dose range studied.

REFERENCES

(1) Weiner, M., Shapiro, S., Axelrod, J., Cooper, J. R., and Brodie, B. B., *J. Pharmacol. Exptl. Therap.*, **99**, 409 (1950).
(2) O'Reilly, R. A., Aggeler, P. M., and Leong, L. S., *Thromb. Diath. Haemorrhag.*, **11**, 1(1964).
(3) Dayton, P. G., Cucinell, S. A., Weiss, M., and Perel, J. M., *J. Pharmacol. Exptl. Therap.*, **158**, 305(1967).
(4) Nagashima, R., Levy, G., and Nelson, E., *J. Pharm. Sci.*, **57**, 58(1968).
(5) Nagashima, R., Levy, G., and Back, N., *ibid.*, **57**, 68 (1968).
(6) Miller, L. L., Bly, C. G., Watson, M. L., and Bale, W. F., *J. Exptl. Med.*, **94**, 431(1951).
(7) Sokal, J. E., Sarcione, E. J., and Henderson, A. M., *Endocrinology*, **74**, 930(1964).
(8) Sokal, J. E., and Weintraub, B., *Am. J. Physiol.*, **210**, 63(1966).
(9) Ferrari, A., Kessler, G., Russo-Alesi, F. M., and Kelly, J. M., *Ann. N. Y. Acad. Sci.*, **87**, 729(1960).
(10) Axelrod, J., Cooper, J. R., and Brodie, B. B., *Proc. Soc. Exptl. Biol. Med.*, **70**, 693(1949).
(11) Christensen, F., *Acta Pharmacol. Toxicol.*, **21**, 299 (1964).
(12) Nagashima, R., and Levy, G., *J. Pharm. Sci.*, **57**, 1991(1968).
(13) Christensen, F., *Acta Pharmacol. Toxicol.*, **21**, 307 (1964).
(14) Grayson, J., and Mendel, D., "Physiology of Splanchnic Circulation," Williams and Wilkins, Baltimore, Md., 1965, p. 74.
(15) Mayes, P. A., and Felts, J. M., *Excerpta Medica International Congress Series*, No. 115, p. 16(1966).
(16) Williamson, J. R., Garcia, A., Renold, A. E., and Cahill, G. F., *Diabetes*, **15**, 183(1966).
(17) Brauer, R. W., Pessotti, R. L., and Pizzolato, P., *Proc. Soc. Exptl. Biol. Med.*, **78**, 174(1951).
(18) Byers, S. O., and Friedman, M., *Am. J. Physiol.*, **168**, 297(1952).
(19) Burton, S. D., George, S. S., and Ishida, T., *J. Appl. Physiol.*, **15**, 128(1960).

(20) Meyers, H. G., Flammann, J., Albaum, G., and Staib, W., *Biochem. Zeitsch.*, 344, 514(1966).
(21) Chien, S., Usami, S., Dellenback, R. J., and Gregersen, M. I., *Science*, 157, 827(1967).
(22) "Blood and Other Body Fluids," Altman, P. L., and Dittmer, D. S., Eds., Fed. Am. Soc. Exptl. Biol., Washington, D. C., 1961, pp. 12,15.
(23) Nagashima, R., and Levy, G., to be published.
(24) Foster, J. F., "The Plasma Proteins," Vol. 1, Putnam, F. W., Ed., Academic, New York, N. Y., 1960, p. 179.
(25) Nagashima, R., Levy, G., and O'Reilly, R. A., to be published.
(26) "Handbook of Biological Data," Spector, W. S., Ed., W. B. Saunders, Philadelphia, Pa., 1956, p. 163.

Keyphrases

Coumarin anticoagulants
Bishydroxycoumarin (BHC)—distribution, elimination
Biologic half-life—dose dependency
Liver perfusion, isolated—experimental technique
Pharmacokinetics—BHC distribution, elimination

Comparative Pharmacokinetics of Coumarin Anticoagulants IV

Application of a Three-Compartmental Model to the Analysis of the Dose-Dependent Kinetics of Bishydroxycoumarin Elimination

By R. NAGASHIMA, G. LEVY*, and R. A. O'REILLY†

It has been reported that the elimination of bishydroxycoumarin (BHC) in man shows unusual dose-dependent characteristics. Recent studies with isolated perfused rat livers have shown that the liver:plasma distribution ratio of BHC increases at high doses (resulting in more rapid elimination of the drug) and that drug-metabolizing activity is apparently inhibited at high BHC levels (resulting in decreased elimination of the drug). A mathematical model based on a three-compartmental open system consisting of a plasma compartment, a rapidly accessible drug-metabolizing compartment, and a more slowly accessible compartment has been developed for a detailed pharmacokinetic analysis of BHC elimination in man. This analysis shows that the increase in the plasma half-life of BHC in man with increasing dose is due primarily to a decrease in the activity of the drug-elimination process and not to dose-dependent distribution effects. The three-compartmental model presented here has two unique attributes; the first compartment (following intravenous administration) is equated to the plasma volume rather than combining plasma and the so-called well perfused tissues into a single compartment, and an apparent volume of distribution which remains constant throughout the terminal elimination phase (β-phase) has been defined.

There are now several examples of drugs which are eliminated exponentially from the plasma (following absorption and initial distribution) but where the apparent first-order rate constant for drug elimination decreases with increasing dose (1). One of the earliest and best documented examples is the elimination of bishydroxycoumarin (BHC) in man (2, 3) and monkeys (4). Recent investigations with isolated perfused rat livers have shown that the distribution of BHC into the liver (*i.e.*, the site of biotransformation) increases with increasing drug concentration but that BHC apparently inhibits its own biotransformation when high concentrations of the drug are present in the liver (5). Physical-chemical studies of the interaction between BHC and plasma proteins

Received May 22, 1968 from the Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication July 3, 1968.
Supported in part by U. S. Public Health Service grant HE 8058-06.
The authors express their appreciation to Dr. M. Gibaldi, and Mr. Wm. T. Tusko, Department of Pharmaceutics, and Dr. S. Ohki, Department of Biophysics, State University of New York at Buffalo, for stimulating discussions and helpful review of the paper.
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Previous paper: Nagashima, R., Levy, G., and Sarcione, E. J., *J. Pharm. Sci.*, 57, 1881(1968).