ous results are obtained which might well be due to an interaction of PVP with itself.

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Keyphrases

Complex formation
Sodium fluorescein–polyvinylpyrrolidone system
pH effect on complex formation
Colorimetric analysis

Comparative Pharmacokinetics of Coumarin Anticoagulants I

Unusual Interaction of Bishydroxycoumarin with Plasma Proteins—Development of a New Assay

By RENPEI NAGASHIMA, GERHARD LEVY*, and EINO NELSON†

A widely used method for the determination of bishydroxycoumarin (BHC) in plasma, which involves extraction of BHC from acidified plasma (pH <1) into an organic solvent, fails at high concentrations of BHC. Evidence is presented to show that this is not due to a limited solubility of the drug in the organic phase but rather to a concentration- and pH-dependent interaction of plasma protein with BHC. Complete extraction of BHC from the blood plasma of rats, guinea pigs, dogs, monkeys, and man is obtained readily only in the narrow pH range of 3.0 to 3.5. A new method of analysis for BHC in plasma has been developed on the basis of these findings and its specificity is demonstrated. It is shown that the development of analytical methods for drugs in plasma or serum, which involve extraction of the drug into an organic phase, cannot be based solely upon a consideration of the effect of pH on the distribution of the drug between the organic and aqueous phases. It is necessary also to consider the effect of pH (and possibly of other factors, such as the type of organic solvent, the buffer system, and ionic strength) on the physical-chemical properties of plasma proteins as they affect the type and magnitude of interaction of these proteins with the drug.

THE PHARMACOKINETICS of the coumarin anticoagulants has been and is the subject of intensive investigation in many laboratories (1–24). Studies have been concerned with the kinetics of elimination of various coumarin anticoagulants in several animal species (2–11) and in man (12–17), as well as with the interaction of these anticoagulants with various other drugs (*e.g.*, 18–23) and with plasma proteins (24). Of particular interest is the unusual, dose-dependent pharmacokinetics of bishydroxycoumarin (BHC) elimination in man, as well as the biologic fate of this drug which so far has eluded adequate characterization (25, 26).

The most widely used method for the determination of BHC is that of Axelrod and his associates (27), which involves extraction of the drug from plasma acidified with 3 *N* hydrochloric acid to pH <1 into *n*-heptane, followed by re-extraction into 2.5 *N* aqueous sodium hydroxide solution, and spectrophotometric measurement of BHC in the latter phase. In the course of studies in this laboratory on the comparative pharmacokinetics of BHC elimination, which will be described in subsequent reports (28, 29), it was noted that the assay method of Axelrod and his associates fails at relatively high concentrations of BHC. Therefore, a new analytical method for the determination of BHC in plasma or serum has been developed. This method and studies concerning its specificity are described in detail in view of the extensive interest in and continuing research related to the pharmacokinetics of the coumarin anticoagulants.

It will be shown that the recovery of BHC from plasma of various species, including man, is un-

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usually pH- and concentration-dependent. This phenomenon has wider implications in that it might be found also with other highly protein-bound drugs. It is evident on the basis of findings to be described here that the extractability of a drug from plasma is related not only to the degree of ionization and the intrinsic partition coefficient of the drug itself, but also to the physical-chemical state of plasma protein at a given pH and the degree and nature of the interaction of the drug with such protein(s) under given conditions.

EXPERIMENTAL

Materials—Heparinized blood was obtained from rats (Sprague-Dawley) and guinea pigs (Hartley) by cardiac puncture, and from dogs, rhesus monkeys, and humans by venipuncture. Plasma was obtained by centrifugation, pooled within the same species, and stored at −15° (for no longer than 1 month) until used. Bishydroxycoumarin[1] [m.p. 288–290°, uncorrected; lit. (30) m.p. 287–293°] was obtained from Nutritional Biochemicals Co., Cleveland, Ohio.

Preparation of Plasma Samples Containing Known Concentrations of BHC—A series of plasma samples containing various concentrations of BHC was prepared in the following manner. One-half milliliter of BHC solution, prepared by dissolving one part of bishydroxycoumarin and 10 parts of tris(hydroxymethyl)aminomethane (Nutritional Biochemicals Co.) in distilled water, was added to 2 ml. of plasma. The samples were maintained at 36–37° for 2 hr. and then stored at −15° (for no longer than 2 weeks) until used.

Micro-Adaptation of Axelrod's Method for the Determination of BHC (27)—Depending on the concentration of BHC, about 0.06 to 0.25 Gm. of plasma was pipetted into a tared 15-ml. capacity centrifuge tube (with a screw cap lined with polyethylene) which was then weighed. The sample was acidified with 0.03 ml. 3 *N* HCl, and the total volume of the aqueous phase was adjusted to approximately 0.35 ml. with distilled water. At this point the pH of the aqueous phase was found to be less than 1. Three milliliters of heptane was added and the tube was shaken vigorously for 10 min. by hand[2] and centrifuged. Two milliliters of the organic phase was transferred to another centrifuge tube, an exact volume of 2.5 *N* NaOH (0.5 to 2 ml.)[3] was added, and the tube was shaken for 5 min., by hand and centrifuged. The absorbances of the aqueous phase at 314 and 360 mμ were determined in a fused silica microcell, 10-mm. light path, with a Beckman DU spectrophotometer using a 0.3-mm. pinhole slit with 2.5 *N* NaOH in the reference cell. The absorptivity was determined with standard solutions of BHC in 2.5 *N* NaOH. Measurements at two wavelengths were necessary for plasma blank corrections, as described in a subsequent paragraph. Some studies were done using 0.5 ml. of suitably diluted plasma, in which case the following volumes of reagents were used: 0.2 ml. of distilled water, 0.06 ml. of 3 *N* HCl, and 6 ml. of heptane. All glassware was cleaned by immersion in an acid dichromate bath for at least 12 hr.

Extraction Efficiency as a Function of pH—BHC was extracted from plasma into *n*-heptane and assayed as described under *New Assay Method for BHC* except that the plasma was adjusted to various pH values by means of specified types and concentrations of buffering agents.

Determination of Rate of Extraction—Two and one-half milliliters of suitably diluted[4] BHC-containing rat plasma and 1 ml. of buffer solution were placed in a 60-ml. bottle furnished with a polyethylene cap. After a specified time, 20 ml. of heptane was layered over the aqueous phase. The bottle was then capped and agitated in an upright position on a reciprocating shaker (280 strokes per min., 5.9-cm. stroke travel). Three-milliliter aliquots of the organic phase were withdrawn 15, 30, 60, and 120 min. after the beginning of agitation, and an equal volume of *n*-heptane was added after each withdrawal. The BHC concentration in each 3-ml. aliquot of the heptane phase was determined by extraction of the drug into 2.5 *N* NaOH and spectrophotometric measurement at 314 mμ. The pH-adjusting agents used were 1.5 *N* HCl (for pH 0.6), 1.5 *M* citrate–phosphate buffers (for pH 3.0 and 4.9), and 1.5 *M* acetate buffer (for pH 4.9).

New Assay Method for BHC—*Small Scale Assay*—One-tenth milliliter of citrate–phosphate buffer (0.99 and 0.51 *M*, pH 3.0) and an appropriate volume of distilled water were added to 0.06 to 0.25 Gm. of plasma to yield a total volume of 0.35 ml. of sample with a pH of between 3.0 and 3.2. The same procedure as described for the micro-adaptation of Axelrod's method was then followed.

Large Scale Assay—This method was employed in the case of dog, monkey, and human plasma, where it is possible to obtain 0.6 ml. or more of plasma repeatedly from any one animal. Six-tenths milliliter suitably diluted plasma, 0.2 ml. citrate–phosphate buffer (1.5 *M*, pH 3.0), and 6 ml. of heptane were placed in a 15-ml. centrifuge tube, shaken for 10 min., and centrifuged. Four milliliters of the organic phase was transferred to another centrifuge tube and extracted with 1 to 5 ml. of 2.5 *N* NaOH, and the BHC concentration of the aqueous phase was determined at 314 and 360 mμ, using 2.5 *N* NaOH in the reference cell.

The lower limit of the sensitivity of the assay procedure is a function mainly of the contribution of the plasma blank to the total absorbance. One microgram of BHC/ml. of plasma can be determined readily in a plasma sample of 0.5 ml., but the plasma blank will be equivalent to almost one-third of the drug alone. The upper limit of the assay is determined by the solubility of BHC in heptane (approximately 14 mcg./ml. at room temperature). Thus, undiluted plasma containing up to 100 mcg./ml. can be assayed readily by the large scale assay method, although the volume of NaOH phase may

[1] The material showed a single spot when visualized with diazotized *p*-nitroaniline or under ultraviolet light, on a thin-layer chromatogram (Silica Gel G) developed with a solvent mixture of chloroform–heptane–acetic acid (10:15:4).

[2] This resulted in more rapid extraction than mechanical agitation as used by Axelrod *et al.* (27).

[3] One part of 2.5 *N* NaOH can quantitatively extract the drug from 4 parts of heptane.

[4] Dilution of plasma containing high concentration of BHC is necessary prior to extraction with *n*-heptane due to the limited solubility of BHC in the organic solvent.

have to be increased. Higher concentrations of BHC in the plasma require that the plasma sample be diluted prior to extraction. The Beer-Lambert law holds for BHC concentrations as high as 20 mcg./ml. in 2.5 *N* NaOH.

Correction for Plasma Blanks—The contribution of plasma blanks is negligible at BHC concentrations of 50 mcg./ml. and above. The blank contribution is, however, appreciable when BHC concentrations are quite low. In the small scale assay, when the drug is extracted from the organic phase into 0.5 ml. of 2.5 *N* NaOH, blank values ranged from 0.15 mcg./ml. to 0.30 mcg./ml. apparent BHC. This includes the contribution of plasma as well as organic solvent. While plasma blank values obtained from different animals vary appreciably, it was found that the ratio of the absorbances at 314 mμ (A_{314}) and 360 mμ (A_{360}) are quite constant. The average ratios, A_{314} to A_{360}, were 0.9 for the rat, 1.4 for the guinea pig, 1.0 for the dog, 0.6 for the monkey, and 0.9 for man. The plasma blanks for the large scale assay were approximately 0.15 mcg./ml. for the dog and monkey, and 0.35 mcg./ml. for man, in terms of apparent BHC.

For the determination of BHC in plasma, the absorbances of the sodium hydroxide extract are determined at 314 mμ and 360 mμ. The plasma blank value at 314 mμ is calculated from the absorbance of sample at 360 mμ and the known ratio of absorbance of plasma blanks at 314 and 360 mμ (R). The absorbance of BHC at 360 mμ is very small (about 1/110 of its absorbance at 314 mμ) and thus contributes only negligibly to the apparent plasma blank value. It may however be incorporated in the calculations in the following manner:

$$\text{plasma blank } A_{314} = R(A_{360} - 0.009 \times A_{314})$$

when A_{314} due to BHC plus blank $\gg A_{314}$ due to blank alone.

Specificity of the New Assay Method for BHC—The specificity of the new assay method for the determination of BHC, as opposed to metabolites of this drug, was determined by a partitioning method similar to that of Axelrod *et al.* (27). For this purpose, heparinized plasma samples were obtained from animals which had received BHC intraperitoneally at least 7 hr. earlier. Samples were also obtained from BHC-containing blood which had been perfused through an isolated rat liver since this was considered to be an effective means of obtaining plasma which contains both BHC and its metabolites. The procedures used in the partitioning study are outlined schematically in Fig. 1. In essence, the plasma was extracted with *n*-heptane, the organic phase was removed, and an appropriate volume of isoamyl alcohol was added to it.[5] This latter solution was then equilibrated with buffer solutions of pH 4.0 (0.5 *M* citrate-phosphate), 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 [tris-(hydroxymethyl)aminomethane–HCl; ionic strength, 0.1]. The amount of BHC remaining in the organic phase was determined by extracting the latter with 2.5 *N* NaOH, followed by spectrophotometry of that extract.

For purpose of comparison, other plasma samples were extracted with ethyl acetate following addition of solid sodium chloride. This is known to extract not only BHC but also its metabolites (7). The ethyl acetate extract was evaporated under reduced pressure, the residue was dissolved in *n*-heptane–isoamyl alcohol mixture,[6] and this solution was equilibrated with buffers of various pH values as described above. The results of the partitioning experiments are described in terms of the fraction of BHC (or apparent BHC) remaining in the organic phase.

RESULTS

Determination of BHC in Plasma by the Method of Axelrod et al. (27)—Known amounts of BHC were added to plasma samples obtained from rats, guinea pigs, dogs, and man to yield BHC concentrations ranging from 2 to 200 mcg. per ml. or Gm. of plasma. These plasma samples were analyzed by the micro-adaptation of the method of Axelrod *et al.* (27). The results, depicted in Fig. 2, show that all of the assay values were appreciably lower than theory at BHC concentrations above 50 mcg./ml.[7] The logarithmic scales of Fig. 2 tend to de-emphasize the magnitude of the deviation of the analytical results from theory; note that the assay results at BHC concentrations of 100 and 200 mcg./ml. were in some instances only 25 to 50% of theory. It was also found that there was appreciable intersubject variation in the results of an analysis of BHC in plasma of different healthy humans (Table I). Several modifications of the method of Axelrod and associates (27) were tried (Table II), but these did not result in any appreciable improvement in the recovery of BHC. The results listed in Table II rule out the possibility that the incomplete extraction is due to a possible saturation of the organic phase with BHC. It was noted in general that the recovery of BHC was more a function of the ratio of the concentration of BHC to that of plasma than of the concentration of BHC in the plasma *phase*.[8] This becomes apparent upon comparison of the results obtained by the unmodified assay method and the assay method which involves a thirteenfold dilution of the plasma prior to extraction. This degree of dilution produced a substantial decrease in the concentration of plasma proteins in the aqueous phase. Yet, neither the dilution of plasma proteins nor the decrease in the concentration of BHC produced a significant increase in the recovery of the drug.

Extraction Efficiency as a Function of pH—Figure 3 shows the effect of pH on BHC extraction efficiency, using rat plasma containing 200 mcg./ml., which was diluted to 2.5 times its volume with water, and *n*-heptane as the extracting solvent.

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[5] Note that isoamyl alcohol was added *after* the extraction The use of isoamyl alcohol was necessary because it was desired to compare the partitioning profiles of BHC and its metabolites. These metabolites do not dissolve in *n*-heptane alone.

[6] This is where the addition of isoamyl alcohol became necessary in order to dissolve the BHC metabolites.

[7] Note, however, that Axelrod *et al.* (27) made no claim for the usefulness of their method above this concentration. Therefore, nothing in our results or discussion should be interpreted as a criticism of the contribution made by these investigators.

[8] The plasma *phase* consists of plasma and aqueous diluent (*i.e.*, water, buffer, and/or HCl solution) in a ratio appropriate for effective extraction of the drug into the organic solvent; the terms "concentration in plasma phase" and "concentration in plasma" must be distinguished accordingly.

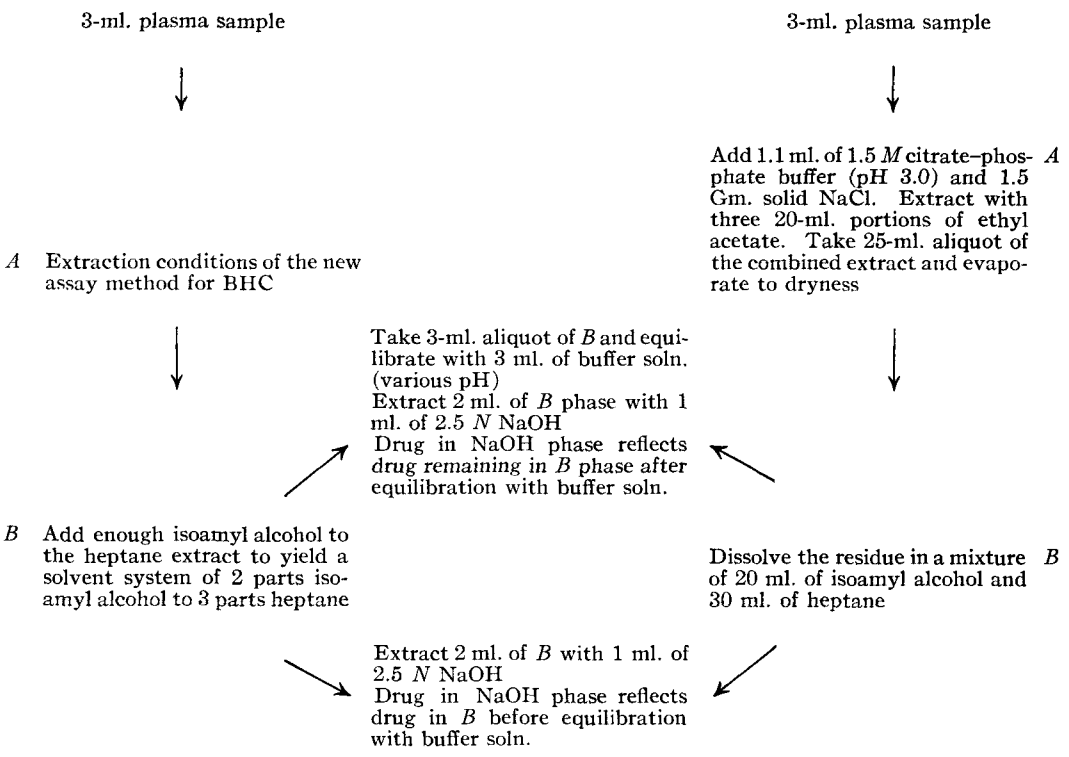

Fig. 1—Schematic diagram of the pH-partitioning procedure used to determine the specificity of the assay method for BHC. For further details see Experimental.

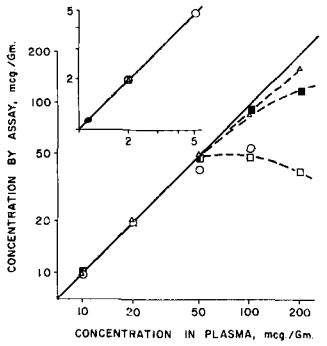

Fig. 2—Apparent concentration of BHC, as determined by the method of Axelrod et al., *plotted against the actual concentration of BHC in the plasma of rats* (■), *guinea pigs* (○), *dogs* (□), *and man* (△). *Average of at least 3 determinations for plasma from rat and guinea pig, and at least 2 determinations for plasma from dog and man. The inset represents the results obtained at concentrations lower than 10 mcg./Gm. In plasma from dog and man, the concentrations shown are expressed as mcg./ml. rather than mcg./Gm.*

TABLE I—RECOVERY OF BISHYDROXYCOUMARIN (BHC) FROM BLOOD PLASMA[a] OF SEVERAL HEALTHY HUMANS BY AXELROD'S METHOD

Subject[b]	Age, Yr.	Recovery, %[c]
A	25	63, 50
N	30	69, 64
K	35	86, 83
M	26	44. 49
R	26	57, 49
F	26	58, 44

[a] 200 mcg. of BHC/ml. of plasma. [b] All subjects were males. [c] Results of two separate analyses.

It can be seen that complete extraction of BHC is obtained only in a narrow pH range of pH 3.0 to 3.5. At pH <3 and >4.5 there is a precipitous decrease in the extractability of BHC from plasma. These effects are not due to specific buffers or differences in ionic strength. Complete extraction at pH 3 was obtained when the pH adjustment was made with either citrate–phosphate buffer or monochloroacetate buffer; a pronounced decrease in extraction efficiency was observed at pH 2.5 and below with monochloroacetate buffer as well as when hydrochloric acid was used to adjust pH. Similarly, though the ionic strength increased from 0.047 at pH 2.4 to 0.32 at pH 0.8 when HCl was used to adjust pH, the ionic strength decreased from 0.25 at pH 3.1 to 0.03 at pH 1.9 when monochloroacetate served as the buffer system. Nevertheless, the decrease in extraction efficiency at the lower pH was observed with both systems. It is possible that the magnitude of this decrease is affected by ionic strength. Variability of extraction efficiency in duplicate experiments at a given pH was very small

TABLE II—RECOVERY OF BHC FROM RAT PLASMA BY VARIOUS MODIFICATIONS OF AXELROD'S METHOD

Conditions	BHC Concn., mcg./ml. →	Recovery, mcg./ml.[a] 100	150	200
Axelrod's method, unmodified[b]		93	110	111
Thirteenfold dilution of plasma before extraction		100	121	118
Five repetitive extractions each with 3 ml. heptane		89	...	150
Single extraction with 3 ml. ethylene dichloride		85	...	106

[a] Average of 3 determinations except for the repetitive extractions where 2 determinations were made. [b] Additional data shown in Fig. 2.

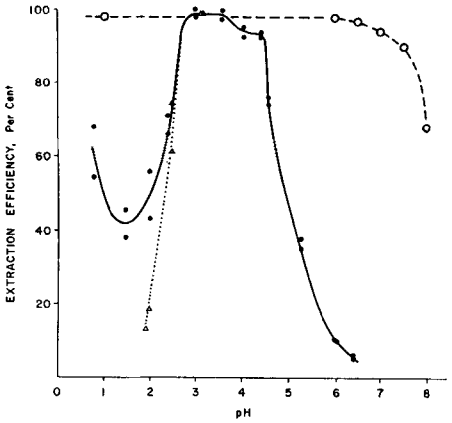

Fig. 3—Effect of pH on the efficiency of BHC extraction from rat plasma containing 200 mcg. of BHC/ml. Five-tenths milliliter of 2.5-fold diluted plasma was adjusted with 0.25–3 N HCl to pH <2.5 (●), with 1.5 M monochloroacetate buffers to pH ≤3.0 (△), and with 1.5 M citrate-phosphate buffers to pH ≥3.0 (●) prior to extraction with 7.5 volumes of n-*heptane. The concentration of buffers in the plasma phase was approximately 0.4 M. The data points represent individual values. Key: ---○---, BHC extraction efficiency from simple aqueous BHC solution as calculated from the partitioning data of Axelrod* et al. *(27).*

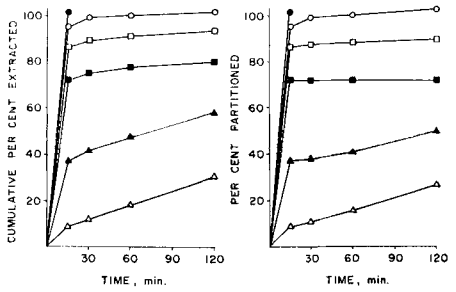

Fig. 4—Effect of pH and BHC concentration on kinetics of BHC extraction from rat plasma. Symbols are defined in Table III. (Further details under Experimental.)

in the pH range of 3.0 to 6.5, and rather pronounced at pH <2.5. This can be related to the kinetics of BHC extraction, as will be shown subsequently. The extraction efficiency *versus* pH profile of BHC from plasma differs appreciably from that of BHC from water (Fig. 3). The extraction of BHC from simple aqueous solution is practically complete at pH 6 and below.[9]

Kinetics of BHC Extraction from Plasma—The rate of BHC extraction from rat plasma was determined as a function of BHC concentration, pH of the plasma phase, and time interval between pH adjustment of the plasma and extraction with heptane. The various experimental conditions are listed in Table III and the results are shown in Fig. 4. In the case of plasma samples containing a low concentration (10 mcg./ml.) of BHC, complete extraction was obtained within 15 min., regardless of pH (<1 to 3.0) and time between pH adjustment and extraction. On the other hand, complete extraction of BHC from plasma containing 200 mcg./ml. was obtained within 15 min. only when the plasma pH was 3.0 and extraction was carried out immediately after pH adjustment. The increase in extraction of BHC with time, as shown in the lefthand graph of Fig. 4 for all other experiments involving 200 mcg. BHC/ml. plasma, could reflect either a slow rate of extraction of BHC or could be due to the fresh solvent which was added to replace that portion of organic phase which had been removed for assay (see under *Experimental*). A distinction between these two mechanisms can be made by expressing the amount of BHC in the heptane phase at any given time as a percent of the total amount of the drug in the system at that time (*i.e.*, sum of BHC in heptane phase and in plasma phase). This is shown in the righthand graph of Fig. 4 in terms of "percent partitioned" as a function of time.[10] At pH 4.9, the extraction of BHC from plasma containing 200 mcg./ml. was at its maximum within 15 min. and subsequent increases in the cumulative percent extraction as a function of time were due to addition of fresh organic solvent. The constancy of "percent partitioned" with time at a value of about 70% reflects the effect of plasma protein binding on the apparent partition coefficient of BHC at pH 4.9 since, in the absence of plasma proteins, extraction of BHC is complete within 15 min.

At low pH's (0.6 and 3.0) it is evident that the incomplete extraction of BHC at any given time

[9] The pKa_1 of BHC appears to be about 6.5 on the basis of Axelrod's partitioning data which have been verified in this laboratory. Other workers have reported a pKa_1 value of 5.7 based on potentiometric titration (31).

[10] The reader is cautioned to distinguish between "partition coefficient" (which is an equilibrium constant) and "percent partitioned." No assumption is made as to whether the latter reflects an equilibrium or a kinetic situation.

TABLE III—EXPERIMENTAL CONDITIONS FOR THE DETERMINATION OF THE KINETICS OF BHC EXTRACTION FROM PLASMA

Symbol in Fig. 4	BHC Concn., mcg./ml.	pH	Buffer System	Time Between pH Adjustment and Extraction, hr.
●	10	0.6	HCl 0.4 *N*	0 and 1
●	10	3.0	Citrate–phosphate, 0.4 *M*	0 and 1
●	200[a]	3.0	Citrate–phosphate, 0.4 *M*	0
○	200[a]	3.0	Citrate–phosphate, 0.4 *M*	1
□	200[a]	3.0	Citrate–phosphate, 0.4 *M*	3
■	200[a]	4.9	Citrate–phosphate, 0.4 *M*	0 and 1
■	200[a]	4.9	Acetate, 0.4 *M*	0 and 1
▲	200[a]	0.6	HCl, 0.4 *N*	0
△	200[a]	0.6	HCl, 0.4 *N*	0.5 and 1

[a] Diluted fourfold with distilled water (1 part plasma and 3 parts water) prior to pH adjustment.

is due to a kinetic effect. The rate of extraction decreased with decreasing pH, and decreased also at a given pH as the time interval between pH adjustment and extraction was increased. A detailed consideration of factors affecting the rate of extraction will not be undertaken here, but it should be pointed out that these factors include the time between pH adjustment and extraction, sampling interval, agitation intensity, volume of organic phase, and volumes of the aliquots removed for assay. In general, the time course of extraction consisted of an initial rapid phase followed by a slower exponential phase. Representative rate constants of the latter are 0.33 hr.$^{-1}$ at pH 3.0 after 3 hr. standing, and 0.14 hr.$^{-1}$ at pH 0.6 after 1 hr. standing. Thus the extraction efficiency *versus* pH profile shown in Fig. 3 reflects an equilibrium phenomenon at pH's above the optimum, and kinetic phenomena at pH's below the optimum pH for extraction. In fact, complete extraction of BHC may be attained at pH <3.0 by prolonged agitation. However, this would, for example, require more than 20 hr. in the case of plasma adjusted to pH 0.6 when the interval between pH adjustment and extraction is 1 hr. The adverse effect of low pH on BHC extraction from plasma is reversible as shown in Table IV.

New Assay Method for BHC in Plasma—The new assay method for BHC in plasma, which involves extraction of the drug with heptane from plasma adjusted to about pH 3.2, was used to determine the concentration of BHC in blood plasma obtained from rats, guinea pigs, dogs, monkeys, and man. These plasma samples were prepared by adding appropriate amounts of BHC to plasma obtained from unmedicated animals to yield BHC concentrations from 0.8 to 400 mcg./ml. The results, listed in Table V, show essentially complete recovery of the drug in all instances. The standard deviation of the analytical data was 1 to 2% except in the case of monkey plasma, where the possibility of a small and practically insignificant concentration-dependency of BHC recovery precluded the use of the combined data (*i.e.*, all concentrations of BHC) for the determination of a percent standard deviation.

TABLE IV—REVERSIBILITY OF ADVERSE pH EFFECT ON BHC EXTRACTION FROM RAT PLASMA

Conditions[a]	Extraction Efficiency,[b] %
Plasma adjusted to pH 3.0 and extracted immediately	99,99
Plasma adjusted to pH <1 and extracted after 30 min.	14,14
Plasma adjusted to pH <1 and extracted after 90 min.	10,12
Plasma at pH <1 for 30 min. then at pH 3.0 for 60 min.	61,70
Plasma at pH <1 for 30 min. and at pH ≃7 for 30 min. then adjusted to pH 3.0 and extracted immediately	99,101

[a] Plasma samples contained 200 mcg. BHC/ml. and were diluted 2.5-fold with distilled water prior to pH adjustment. [b] One ml. plasma phase was extracted for 10 min. with 8 ml. *n*-heptane. Results of two separate analyses.

Specificity of the New Assay Method for BHC—The specificity of the new assay method for BHC, particularly with respect to the lack of interference by biotransformation products of that drug, was investigated by means of the pH-partition profile (27). For this purpose, 20 mg. of BHC/Kg. of body weight was administered intraperitoneally to rats and guinea pigs, blood was withdrawn 7 to 8 hr. later [equivalent to about 1.5 biologic half-lives of BHC (28)] and the plasma thus obtained from animals of each species was pooled. The pH-partition profile for apparent BHC from rat plasma is shown in Fig. 5 and from guinea pig plasma in Fig. 6. There is excellent agreement between these profiles and that obtained by use of pure BHC in water. On the other hand, the use of an initial extraction procedure which is known to extract not only BHC but also some of its metabolites yields a quite different partition profile (stippled line in Figs. 5 and 6) and shows also that BHC metabolites are more polar than the parent drug. An even more rigorous determination of assay specificity was made by using plasma containing BHC which had been perfused for 3 hr. through an isolated rat liver (29). During the period of perfusion, the BHC concentration in the plasma decreased to less than one-third of its initial concentration. Thus, this sample is likely to have contained relatively high concentrations of BHC metabolites (except for the unlikely possibility that these metabolites were trapped totally in the liver and/or excreted completely in the bile).[11] The results of the partitioning experiment are shown in

[11] Christensen (7) has shown that the plasma of rats contains appreciable concentrations of BHC metabolites 6 and 24 hr. after intravenous administration of BHC.

TABLE V—RECOVERY OF BHC FROM PLASMA OF VARIOUS ANIMAL SPECIES BY THE NEW ASSAY METHOD

BHC Concn., mcg./ml.	Recovery, % Rat[a]	Guinea Pig[a]	Dog[b]	Monkey[a]	Man[a]
0.8	...	...	106	...	...
2	103	100	...	101	103
4.1	...	...	98	...	...
5	...	...	...	...	99
8	97	...	...	...	...
10	...	102	...	98	98
25	100	...	...	...	...
31.2	...	...	97	...	...
50	98	101	...	...	100
56.3	...	...	98	...	...
100	101	100	...	97	99
121.5	...	...	96	...	...
150	...	...	...	96	...
200	99	...	...	...	98
400	...	100	...	...	...
Mean ± S.D.[c]	99 ± 2[d]	101 ± 1[d]	97 ± 1[e]	[f]	99 ± 2
Total No. of samples	19	8	4	8	12

[a] Average of 2–4 determinations. [b] Single determinations. [c] Mean and S.D. of individual values irrespective of concentration. [d] Data from the 2 mcg./ml. samples were excluded due to pronounced variations within group (3 samples). [e] The value for the 0.8 mcg./ml. sample was excluded due to pronounced difference from those for the other concentrations. [f] Not calculated because relative recovery appears to be concentration dependent.

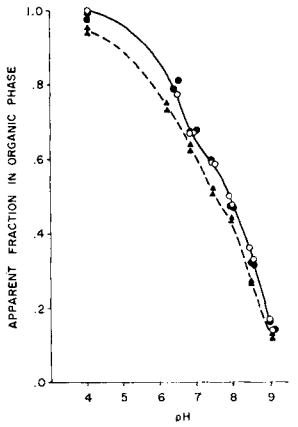

Fig. 5—Specificity of the new assay method for BHC in rat plasma, as determined by pH-partitioning. The plasma obtained from two rats 7 hr. after an i.p. dose of 20 mg. of BHC/Kg. was subjected to the new assay method (heptane extraction at pH 3) and to ethyl acetate extraction, and the pH-partition profiles of the substance(s) in each extract were determined. (See under Experimental *for details.) Pure BHC added directly to the organic phase used in the partitioning experiment* (○), *heptane extract (new assay method)* (●), *ethyl acetate extract* (▲).

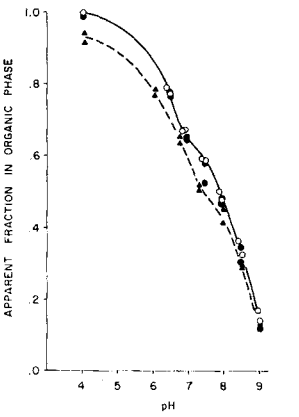

Fig. 6—Specificity of the new assay method for BHC in guinea pig plasma, as determined by pH-partitioning. The plasma was obtained from two guinea pigs 8 hr after an i.p. dose of 20 mg. BHC/Kg. (See Fig. 5 for other details.)

Table VI and indicate a very good agreement between results obtained with the plasma and with an aqueous solution of BHC. There was no noticeable interference by BHC metabolites in the assay.

DISCUSSION

Evaluation of the Method of Axelrod et al. (27) for the Determination of BHC in Plasma—The recovery of BHC from the plasma of rats, dogs, and man by the method of Axelrod *et al.* (27) is essentially quantitative at BHC concentrations up to 50 mcg./ml., but becomes incomplete and markedly concentration-dependent at BHC concentrations exceeding 50 mcg./ml. (Fig. 2). In guinea pig plasma, the recovery of added BHC was quantitative at 10 mcg./ml. but already incomplete at 50 mcg./ml. These results are not due to a limited solubility of BHC in *n*-heptane, nor to the precipitation of BHC in the acidified aqueous phase, since extraction of BHC from aqueous solution does not show the concentration dependence found with plasma. In addition to its incompleteness, the recovery of BHC in high concentrations from plasma shows poor reproducibility and appears to vary in

TABLE VI—SPECIFICITY OF THE NEW ASSAY METHOD FOR BHC AS TESTED BY pH-PARTITIONING USING PLASMA CONTAINING BHC AND METABOLITES DERIVED FROM PERFUSION OF ISOLATED RAT LIVER

	Fraction in Organic Phase	
pH	From Aqueous Soln. of BHC	From Plasma Containing BHC and Metabolites
4.2	0.99	0.99
6.4	0.79	0.76
6.8	0.68	0.70
7.4	0.58	0.56

the plasma samples obtained from different subjects (Table I).

The incomplete recovery of BHC from plasma at high concentrations can easily be overlooked. While it is standard practice to ascertain the adequacy of an analytical method for a drug in plasma at concentrations in the range suitable for instrumental analysis, it is usually taken for granted that plasma samples containing higher concentrations of drug may be diluted appropriately and that they can then be analyzed as readily as plasma samples containing lower concentrations of drug. Such is not the case with BHC (Table II). Apparently the extractability of this drug from plasma depends more on the BHC:plasma protein concentration ratio than on the absolute concentration of BHC. This phenomenon has some interesting implications and can lead to unusual artifacts resulting in the possibility of serious errors in pharmacokinetic interpretations. This is illustrated in the results obtained after intravenous administration of BHC in doses of 2 mg./Kg. and 20 mg./Kg. to guinea pigs, where the plasma concentrations of BHC were determined either by the method of Axelrod *et al.* (27) or by the new method which assures complete recovery of the drug (Figs. 7 and 8). The plasma concentrations determined by the method of Axelrod *et al.*, when plotted semilogarithmically, show a downward curvature with time. When plotted on linear coordinates, the data obtained with the 20 mg./Kg. dose yield essentially a straight line. It might be concluded, therefore, that the elimination of BHC is an apparent zero-order process, as had been suggested previously (12). [Note, however, that this hypothesis was rejected upon further investigation (17).] However, it is clearly evident from the results obtained with the new assay method (Fig. 8) that such conclusions are the result of an artifact due to the decreasing extraction efficiency of the method of Axelrod *et al.* at higher BHC concentrations.[12] In fact, BHC elimination follows apparent first-order kinetics in the guinea pig at doses of 2 and 20 mg./Kg. (Fig. 8). These observations led to a re-examination of published data on BHC elimination with the purpose of determining if pharmacokinetic analyses of others,

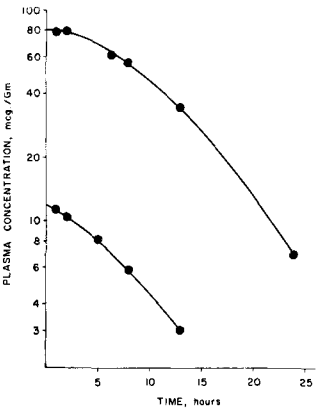

Fig. 7—Apparent plasma concentrations of BHC, as determined by the method of Axelrod et al., *in a guinea pig receiving intravenous doses of 2 mg./Kg. (lower curve) and 20 mg./Kg. (upper curve) of the drug. One-week interval between tests.*

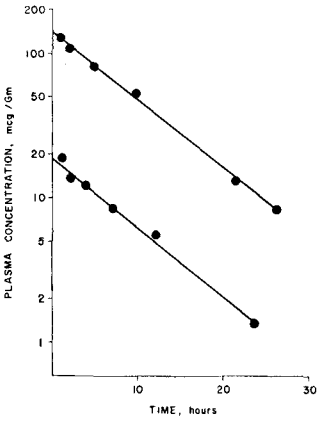

Fig. 8—Plasma concentrations of BHC, as determined by the new assay method, in a guinea pig receiving intravenous doses of 2 mg./Kg. (lower curve) and 20 mg./Kg. (upper curve) of the drug. One-week interval between tests.

and particularly the reported dose dependence of BHC elimination in man (12, 15), could be explained by the assay problems described in this report. It appears that this possibility can be excluded since published data are in a concentration range where assay problems are unlikely. Such problems will be encountered mainly in pharmacokinetic investigations with rather high doses of BHC (28, 29).

The Effect of pH on BHC Extraction from Plasma—The problems encountered in the determination of BHC in plasma by the method of Axelrod *et al.* led to an examination of the effect of pH on the extraction of BHC from plasma. It was found that complete extraction of the drug from plasma containing high concentrations of BHC was achieved only in a relatively narrow pH range of 3.0 to 3.5 (Fig. 3). The lower recovery in the pH range of 4 to 6 appears to be primarily due to a decreased apparent partition coefficient of BHC as a consequence of protein binding (Fig. 4). At higher pH

[12] The apparently incomplete recovery of BHC from plasma by the method of Axelrod *et al.* following the 2 mg./Kg. dose (Fig. 7) might appear to be quantitatively inconsistent with the recovery data shown in Fig. 2. However, this is due to the pronounced variability of BHC recovery at low pH as shown in Fig. 3. When, in another animal, BHC plasma levels were determined simultaneously by the method of Axelrod *et al.* and by our new method, similar differences in results were obtained, but the data derived from the Axelrod assay were not describable by either first- or zero-order kinetics.

the decrease in extraction efficiency is due to ionization of BHC (Fig. 3). The decreased extraction efficiency at pH's below 3 is a rate, rather than an equilibrium, phenomenon (Fig. 4 and Table IV). Quantitative extraction of BHC at pH <1 from plasma containing high concentrations of this drug would require almost 1 day. The slow rate of extraction at the low pH leads to poor reproducibility of results obtained by procedures involving a short extraction period and yielding incomplete recoveries (Fig. 3). This is probably why the data listed in Table I show poor reproducibility. While there apparently has not been a systematic study of the effect of pH on the extraction of BHC from plasma and other biologic tissues to date, it is of interest that Christensen (40) reported recently that the recovery of BHC from rat liver and muscle tissue was more complete at pH 4 and 5 than at pH <2 and >7. This is reasonably consistent with the findings of this study. The results described here may also have some relation to observations of others that the gastrointestinal absorption of BHC is very slow and prolonged in man, particularly at higher doses (12, 15). For example, O'Reilly *et al.* (15) noted that absorption of orally administered BHC in man occurred over about 65 hr. with a dose of 300 mg., 95 hr. with 600 mg., and 210 hr. after administration of 1200 mg. While the dissolution rate of BHC in gastrointestinal fluid will be very low due to the poor water solubility of the drug, it appears unlikely that undissolved particles could be retained in the gastrointestinal tract for a week or longer. What appears to be an unusually slow gastrointestinal absorption of BHC, when calculated by means of a single-compartment pharmacokinetic model, could actually be an unusual distribution phenomenon requiring the use of a multiple-compartment model. However, the pronounced affinity of BHC at high concentrations and low pH to plasma proteins, as observed in this study, suggests the possibility of a similar phenomenon in the gastrointestinal tract. BHC might be bound in the gastrointestinal mucosa for a prolonged period of time, as has been suggested by O'Reilly *et al.* (15). This could be the case particularly if the pH optimum for binding of BHC to mucosal protein is in the physiologic range.

Factors Affecting Drug–Albumin Interactions—BHC in the plasma is bound mainly, if not exclusively, to albumin (12, 15). An interpretation of the results obtained in this study requires, therefore, a consideration of factors affecting the interaction of drugs with plasma albumin. This in turn requires a consideration of the effect of pH on the conformation and physical-chemical properties of albumin. Plasma albumin undergoes a largely reversible structural alteration at pH $\simeq$4 (32). This involves a structural expansion at the low pH which is driven by electrostatic forces and which leads to formation of a species with greater electrophoretic mobility and higher viscosity (33). This expanded form of albumin is known as the F form as opposed to the more compact N form at neutral pH. A similar electrostatic expansion of albumin occurs above pH 9 (32). The N to F transition of albumin is accompanied by a pronounced decrease in the ability for hydrophobic binding of substances such as alkanes (33). It is believed that apolar binding sites are located in the interior of the albumin molecule and that they are therefore not in contact with polar solvent. N to F transition is thought to cause disruption of apolar clusters in the albumin molecule, resulting in the exposure of these regions to the aqueous solvent. This is accompanied by a drastic decrease in the capacity to bind alkanes such as butane and pentane (33) and drugs such as the xanthines (34). The N–F equilibrium is affected by ionic strength and specific counter ions and by interacting substances such as detergents (32, 35) and organic solvents used in extractions, including heptane (36). Detailed physical-chemical studies of bovine and human plasma albumin suggest that the N to F change actually involves a double transition, one at pH 3.6 and the other at pH 3.0 (39). Significantly, this coincides with the region of optimum pH for extraction of BHC from plasma (Fig. 3). It is evident, therefore, that the high extraction efficiency of BHC at pH's slightly below 4 as compared to higher pH's is due to the N to F transition of albumin at pH $\simeq$ 4 and the decreased binding of neutral molecules such as BHC by the F form of albumin. At pH 2.5, the positive charge on the protein is at its maximum and further lowering of pH appears to reduce the expansion of albumin molecules due to the increased ionic strength of the environment (37). There is evidence for slow aggregation of albumin below pH 3.2 (32), reflected among others by a slow increase in the viscosity of albumin solutions (38). The viscosity increase proceeds more rapidly at lower pH (38). The reversibility of this aggregation decreases with increased duration of exposure of albumin to low pH (32). The decreased extraction efficiency below pH 3 is apparently due to the conformational change of albumin at the low pH involving partial refolding of the expanded molecule (37) and/or aggregation (32). The decreased extractability of BHC with time at low pH (Fig. 4) is consistent with the relatively slow structural change of albumin at low pH. Specifically, the more rapid decrease in extractability of BHC at pH <1 as compared to pH 3 (Fig. 4) is paralleled by the more rapid increase in viscosity of albumin solutions at the lower as compared to the higher pH's (38). These effects are reversible by pH adjustment, as is evident from the data in Table IV.

Last, an explanation must be sought for the unusual concentration dependence of BHC extraction at low pH. O'Reilly (24), who studied the interaction of warfarin with human plasma albumin, concluded that the albumin molecule undergoes a reversible configurational alteration in the process of binding the drug, thereby yielding additional binding sites. It is likely that the same is true for BHC in view of its structural similarity to warfarin. One may then conceive of a series of events whereby BHC in low concentrations is bound to readily accessible peripheral binding sites on the albumin molecule, with increasing concentrations of BHC bringing about a partial unfolding of the albumin molecule and thereby causing the exposure of additional binding sites in the internal region of the macromolecule. If, during extraction at low pH, the peripherally bound BHC dissociates first, it is conceivable that the albumin molecules will contract and aggregate, and thus make it difficult for BHC molecules bound to internal sites in the albumin molecule to be extracted. Foster has recognized

the possibility of such a mechanism by suggesting that "A variety of substances, both ionic and nonionic, but particularly substances with considerable hydrophobic character, could become trapped within the postulated hydrophobic interfaces and be released only when the protein reaches an ionic environment favoring conversion to the F form" (32). In summary, BHC in high concentrations can be extracted readily from plasma only in a pH range where the interaction between albumin molecules (*i.e.*, aggregation) is minimal and where these molecules are maximally expanded.

New Assay Method for BHC in Plasma—The method of Axelrod *et al.* has been modified by changing the pH adjustment of the plasma from less than 1 to about 3.2 to assure maximum extraction efficiency. Essentially quantitative recovery of BHC in concentrations ranging from 2 to 400 mcg./ml. was obtained with plasma from rats, guinea pigs, dogs, monkeys, and man (Table V), and this assay method has been used successfully in a comparative pharmacokinetic study of BHC elimination in these species (28). The specificity of the new method, particularly in terms of distinguishing between BHC and its biotransformation products, has been demonstrated (Figs. 5 and 6; Table VI).

Conclusions—The findings of this study go clearly beyond the discovery of an unusual concentration dependence of a widely used method for the determination of BHC in plasma, the identification of the consequent possibility of interesting pharmacokinetic artifacts, and the development of a new assay method which is not subject to these shortcomings. Guttman and Gadzala (34), who studied the interaction of xanthine derivatives with bovine serum albumin, considered as their most interesting observation the sensitivity of the binding behavior of albumin to pH-dependent changes in its structure. This observation can now be extended to include the sensitivity in the extractability of a strongly albumin-bound drug to pH-dependent conformational changes of the albumin molecule. It is not unlikely that similar effects will be encountered with other strongly protein-bound drugs. The optimum pH for extraction of such drugs from plasma or other biologic materials may depend not solely on the pKa of the drug (as it affects the distribution of the drug between an organic phase and an aqueous phase of given pH), but also on the effect of pH on the physical-chemical properties of plasma proteins as they affect the type and magnitude of interaction of these proteins with the drug. The pH-extraction efficiency profile may be affected in some instances also by ionic strength and by the nature of the buffer system and organic solvent used in the extraction.

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Keyphrases

Coumarin anticoagulants
Bishydroxycoumarin in plasma—analysis
n-Heptane extraction
UV spectrophotometry—analysis
pH effect on extraction efficiency
Kinetics of extraction of bishydroxycoumarin from plasma
Albumin—bishydroxycoumarin interaction