Pharmacokinetics and Metabolism of Hexachlorobenzene in the Rat and the Rhesus Monkey

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The metabolism of hexachlorobenzene (HCB) has been studied with respect to its tissue distribution, disposition, pharmacokinetics, and biotransformation in rats and rhesus monkeys. Preliminary results using the rat for a short-term (48 h) study indicate that a very small portion (<2%) of the intravenously administered dose was excreted via various routes. Radioactivity was widely distributed in the tissues, with fat containing the highest level. Long-term studies were conducted in three rhesus monkeys for 100 days, 6 months, and 1 year, respectively, following intravenous dosing of [^{14}C]HCB. At the end of 1 year, cumulative fecal and urinary excretion accounted for 28.2 and 1.6% of the administered dose, respectively. HCB was widely distributed in the tissues. Among over 30 tissues analyzed, fat and bone marrow contained the highest level of radioactivity. A three-compartment, mammillary, open-system model was constructed to study the pharmacokinetics of HCB in the rhesus monkey. Based on computer-derived values using this model, theoretical curves representing the time-course behavior of plasma, fecal, and metabolized HCB in the rhesus monkey were found to match experimental data satisfactorily. Long-term storage of HCB in fat is the basic reason for the slow elimination rate of this compound from the animal. In the rat the amount of polar metabolites formed was estimated to be less than 0.2% of the administered dose. The very slow rate of metabolism of HCB was even more evident in the rhesus monkey. Thus, 1 year after an intravenous injection, the cumulative fecal and urinary metabolites were estimated to be only 2.8 and 1.6% of the administered dose, respectively. A major fecal metabolite was identified as pentachlorophenol (PCP) by various chromatographic means. There was also traces of pentachlorobenzene (QCB) in the feces of the treated rhesus monkeys. All of the urinary radioactivity was in the form of polar metabolites of HCB; the indentities of these metabolites were unknown, although they were neither QCB nor PCP.

Hexachlorobenzene (HCB), a fungicide and an environmental contaminant, has attracted a considerable amount of interest in the scientific community in recent years. Many studies have been conducted concerning various aspects of biochemistry of this chemical, and the information generated has been reviewed by several sources (Stijve, 1971; Fletcher, 1973; National Academy of Sciences, 1975).

Since 1972 there have been a number of studies concerning the absorption, tissue distribution, and excretion of HCB in various species of animals following single doses or chronic treatment (Avrahami and Steele, 1972a,b,c; Iatropoulos et al., 1975; Koss and Koransky, 1975; Mehendale et al., 1975; Morita and Oishi, 1975; Rozman et al., 1975). The intestinal absorption of HCB in the rat was found to be dependent on the vehicle used for oral administration. Thus, the rates of absorption were 80 and 6% of the dose, respectively, when HCB was given in olive oil or in water suspension (Koss and Koransky, 1975). The absorption and transport of HCB have also been reported to be predominantly via the lymphatic system with only minor involvement of the portal venous system (Iatropoulos et al., 1975). In general, the tissue distribution of HCB appears to be directly related to the lipid contents of the tissue, with fat containing the highest levels (Avrahami and Steele, 1972a,b,c; Villeneuve et al., 1974; Iatropoulos et al., 1975; Koss and Koransky, 1975; Mehendale et al., 1975; Morita and Oishi, 1975; Rozman et al., 1975). The excretion of HCB from all animal species so far studied is slow. After oral dosing, the excretion rate was approximately 17% in the rat in 7 days (Mehendale et al., 1975) and 36 and 30% in the rat and the rhesus monkey, respectively, in 40 days (Rozman et al., 1975). When a single dose of HCB was given intraperitoneally to the rat, the excretion rate was approximately 39% of the administered dose in 2 weeks (Koss and Koransky, 1975). In all cases, fecal excretion was the predominant route. Since there is a realistic probability of constant exposure of HCB at residual levels to the human population, the fate of the chemical in the animal, particularly with regard to its pharmacokinetic behavior, is of great importance. Unfortunately, comparatively little information is presently available in this regard.

The metabolism of hexachlorobenzene (HCB) in different animals has been studied by a number of laboratories all over the world since 1960 (Parke and Williams, 1960; Stijve, 1971; Mehendale and Matthews, 1973; Metcalf et al., 1973; Koss and Koransky, 1975; Lui and Sweeney, 1975; Mehendale et al., 1975; Morita and Oishi, 1975; Rozman et al., 1975; Yang et al., 1975a; Yang and Pittman, 1975; Koss et al., 1976). The development of the knowledge in this area has followed a typical pattern for the biologically and chemically persistent chlorinated hydrocarbons. HCB was initially considered as not being metabolized in the animal (Parke and Williams, 1960). As analytical techniques advanced greatly, the biotransformation of HCB was first suggested and subsequently confirmed (Stijve, 1971; Mehendale and Matthews, 1973; Metcalf et al., 1973). With the aid of sophisticated instrumental analysis, recent work (Lui and Sweeney, 1975; Koss et al., 1976) has positively established the presence of pentachlorophenol (PCP), pentachlorothiophenol, tetrachlorohydroquinone, and tetrachlorothiophenol as urinary and/or fecal metabolites of HCB.

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This investigation was undertaken to study the pharmacokinetics and metabolism of [^{14}C]HCB in the rat and rhesus monkey following an intravenous injection for varying experimental periods. Since an intravenous bolus injection offers the advantage of by-passing the absorption phase encountered in the oral dosing, information concerning the kinetic behavior and metabolic fate of HCB in the animal would offer additional knowledge to this area.

MATERIALS AND METHODS

Chemicals. [U-^{14}C]HCB (3.80 mCi/mmol) was purchased from Mallinckrodt Chemical Works, St. Louis, Mo. Its radiochemical purity was demonstrated to be greater than 99% by TLC and high-pressure liquid chromtography (LC). Gas chromatographic (GC) analyses (electron-capture detector) of this sample showed that it was free from contamination. Nonlabeled HCB was obtained from Eastman Kodak Co., Rochester, N.Y. Before use, it was recrystallized twice from hot benzene. Soluene 100 and 350, Dimilume-30, and [^{14}C]toluene standards were obtained from Packard Instrument Co., Downers Grove, Ill. Liquifluor was purchased from New England Nuclear, Boston, Mass. All other reagents and chemicals were of the highest grade commercially available.

Animals and Dosing. Two male Sprague-Dawley rats (194 and 348 g) and three female rhesus monkeys, *Macaca mulatta* (no. 1079, 4.9 kg; no. 1036, 6.1 kg; no. 1111, 4.4 kg), were selected from our animal colonies for this study. The [^{14}C]HCB samples for intravenous (iv) injection were prepared according to a slight modification of the method described by Wiener et al. (1976). [^{14}C]HCB in chloroform was mixed with 1,2-propanediol and the chloroform was evaporated by a stream of N_2 in a warm bath (approximately 50 °C). In this manner the extremely nonpolar behavior of HCB may be overcome and a maximal concentration of 1 mg of HCB/mL of 1,2-propanediol may be obtained. After cooling the HCB–1,2-propanediol solution briefly, 5 to 8 volumes of plasma from corresponding animals was added, and the resulting mixture was used for iv injection. In the case of the rat, plasma was prepared from a blood sample taken from another animal and the HCB sample was injected via the tail vein. With the monkey, plasma was obtained from the same animal and the HCB sample was injected via the saphenous vein. Since HCB was found to be adsorbed onto the plastic wall of disposable Plastipak syringes (Yang, unpublished results), glass syringes and stainless steel needles were used throughout the study. In this study monkey no. 1111 was dosed first; only 0.22 mg/kg or 12.9 μCi of [^{14}C]HCB was administered due to the limited solubility of HCB in 1,2-propanediol. The subsequent studies, the volumes of the 1,2-propanediol in the doses was increased to allow a higher concentration of [^{14}C]HCB. Consequently, monkeys no. 1079 and no. 1036 received 0.38 mg/kg or 24.7 μCi and 0.32 mg/kg or 26.2 μCi [^{14}C]HCB, respectively. In all cases the HCB level was below the no-ill effect level.

Short-Term Rat Studies. Following the administration of an iv dose of 1.3 μCi [^{14}C]HCB (approximately 0.1 mg), the rat was immediately placed in a closed metabolic system of glass and stainless steel construction (Delmar Scientific Co., Maywood, Ill., and water and food were provided. By means of a vacuum pump, air was drawn into the animal chamber after it has passed through Drierite and lithium hydroxide to remove moisture and CO_2, respectively. The air in the animal chamber was then drawn through a series of four traps to remove moisture (dry-ice trap), volatile chemicals (toluene trap), and CO_2 (ethanolamine traps). The animal was maintained in this system for 48 h prior to sacrifice. Radioactivity in urine, feces, various tissues, and the four traps was measured.

Long-Term Studies in Rhesus Monkeys. Each of the three monkeys was treated as described below: The animal was restrained on a board and an appropriate dose of [^{14}C]HCB was administered intravenously. Depending on the individual animal, five to eight blood samples (5 mL each) were taken from the animal in the first 6 h after the treatment. The monkey was then placed in a stainless steel metabolism cage with water and food provided. Subsequently, blood samples, urine, and feces were collected daily for the first week and weekly or twice a week (urine) thereafter. Fecal samples were kept in a freezer until processed; the urine from each monkey was pooled and a small amount of toluene was added. The urine samples were kept at 4 °C in a cold room. The monkeys were sacrificed at 100 days (no. 1079), 6 months (no. 1036), and 1 year (no. 1111), respectively.

In the course of this investigation, monkey no. 1036 suffered a tongue injury in an escape attempt. Consequently, forced feeding was necessary for a period of 5 weeks. During this period, the stress experienced by this monkey was clearly reflected in irregular levels of plasma radioactivity. Although the animal eventually recovered and behaved normally, none of the data from this monkey was used for any kinetic studies.

Measurement of Radioactivity. Plasma, urine, whole blood, and the other liquid samples were mixed directly with a Triton liquid scintillator (Patterson and Green, 1965) at a sample/scintillator ratio of less than 1/10. Tissues were dissolved in Soluene 100 or 350 at 50 °C in the proportions of approximately 100 mg/mL of Soluene. In some instances, 0.2-mL aliquots of 30% H_2O_2 were added to the digested samples to bleach the dark-brown color. The samples were then mixed with 10-mL aliquots of Dimilume-30 to inhibit chemiluminescence. In the case of feces, each weekly sample was ground with a hand-operated mechanical grinder to insure uniformity. Counting samples (approximately 100 mg each) were then taken from this homogenized feces. These counting samples were either treated with Soluene as described above or they were air-dried and combusted in a Packard Model 306 sample oxidizer. Parallel tests using the same batches of monkey feces revealed that the results from either method were comparable (Yang, unpublished results). All samples were counted in a Packard Tri-Carb Model 3380 liquid scintillation spectrometer. In the case of the blood and plasma samples and the Soluene-treated samples, an equilibration time of 24 h was allowed for optimal counting results. Whenever possible, each tissue and every collection of feces and urine was sampled at least in triplicate. Each sample was counted for at least 50 min or to a gross count of 10 000. The internal standard method was used to calculate absolute activity.

Pharmacokinetic Treatment. As a working hypothesis, all distribution and elimination rates of HCB in the rhesus monkey are assumed to be proportional to the concentration of HCB or to differences in concentrations of HCB. This hypothesis permits the description of HCB pharmacokinetics as a mammillary, compartmental system. In this system the concentration of HCB in the central compartment and in any compartment exchanging HCB with the central compartment can be described by polynomial, exponential equations of the form:

$$C = \sum_{i=1}^{n} C_i e^{-\lambda_i t}$$

where C represents concentration at time t, C_i is the ith coefficient, and λ_i the exponent of the ith exponential term (Wagner, 1975). In the case of a drug administered as an

intravenous bolus, n represents the number of compartments, assuming instant mixing of the drug in the central compartment. To determine the number of compartments and to obtain estimates of the C_is and λ_is, a log-liner plot of plasma concentration vs. time was resolved by graphical techniques or by regression analysis. Successful application of this procedure depends upon the use of data obtained at times that are long relative to the terminal half-live ($0.693/\lambda_n$) of the plasma concentration–time curve. In our study we could not satisfy the above constraint due to the very long times involved. Our strategy was: (1) to derive a model (or alternative models) and crude estimates of the C_is and λ_is from the plasma concentration–time curve; (2) to calculate the associated rate constants for distribution, metabolism, and excretion using standard mathematical approaches (Rescigno and Segre, 1966; Portmann, 1970); (3) to modify the model arbitrarily to account for metabolism and excretion, as indicated by our analysis of urine and feces; (4) to optimize the crude estimates for the various rate constants by a computer program utilizing subroutines for numerical integration of the differential mass-balance equations and for parameter optimization.

Optimization was carried out on a DEC-10 (Digital Equipment Corporation) computer. The differential rate equations describing a given model, the starting parameter estimates for the rate constants, and for the volume of distribution of the central compartment and the data file were entered into the BASIC language program FITKIN (Pittman et al., 1976). This program consisted of two subroutines for numerical integration of the differential equations. The first one uses a first-order predictor–corrector method with variable step length (Dorn and McCracken, 1972). The second one uses the Simplex method of Nelder and Mead (1974) to adjust parameter values by function minimization. The function minimized was the sum of the normalized squared deviations between the observed data and the values predicted by the integrated equations, i.e.

$$\sum_{i=1}^{n}\left(\frac{X_i - \hat{X}_i}{X_i + \hat{X}_i}\right)^2$$

(Ottaway, 1973), where X_i is an experimental observation and X_i the associated predicted value. It must be recognized that these solutions are not unique. Though the same final estimates are usually reached when the starting crude estimates are varied, they are not constrained to do so.

Isolation of Monkey Fecal and Urinary Metabolites. A detailed description of the extraction and clean-up procedures for the fecal samples and some of the chromatographic techniques has been given elsewhere (Yang et al., 1975a).

Urine samples, upon acidification with HCl to pH 1 to 2, were extracted three times with an equal volume of ether. The combined ether layer was washed once with distilled water to neutralize the acidity and it was subsequently concentrated to an oily residue under reduced pressure in a rotary evaporator. The residue was dissolved in methanol and was subjected to various chromatographic analyses.

RESULTS AND DISCUSSION

Fate of [^{14}C]HCB in the Rat. Forty-eight hours after iv doses of [^{14}C]HCB, rats excreted approximately 0.2 and 1% of the administered dose in the urine and feces, respectively. No radioactivity was exhaled from the animals nor was there any significant amount of radioactivity in the form of a volatile chemical. A small amount (<0.04% of the dose) of radioactivity was obtained in the moisture trap; whether it was [^{14}C]HCB codistilled with water from the animal chamber was unknown.

Since most of the administered [^{14}C]HCB was retained in the rat, levels of radioactivity in various tissues from each rat was measured. Over 20 tissues were analyzed and all contained radioactivity. The highest levels of radioactivity were found in fat (approximately 3 μg of HCB equivalents/g of tissue), as might be expected for a highly lipophilic compound. The adrenal gland also contained a relatively high level of radioactivity, presumably due to its high lipid content. The levels of radioactivity found in other tissues were much lower, generally in the range of $^1/_{12}$ to $^1/_{300}$ of those in the fat. One interesting observation was that the level of radioactivity in the whole blood was several times higher than that in the plasma. This was due to the binding of HCB to the rat erythrocytes as reported previously by this laboratory (Yang et al., 1975b).

The liver, fat, and feces from the rats were individually homogenized in benzene and extracted in the Soxhlet apparatus for 10 h. After cleanup on a Florisil column and appropriate adjustment of volume, each sample was analyzed by GC and TLC. On the basis of retention time, only HCB was detected in each of these samples. The urine was not analyzed further due to its relatively low radioactivity and the small quantity left after measurement of the radioactivity.

Tissue Distribution and Excretion of Radiochemicals in the Rhesus Monkey. Over 30 tissues from each monkey were analyzed for radioactivity. As expected, the radioactivity was widely distributed (Table I). Fat and bone marrow contained the highest levels of radioactivity among all tissues analyzed (Table I). Adrenal gland which contained approximately $^1/_6$ to $^1/_8$ of that of the fat was the second highest. The levels of radioactivity in other tissues were lower, ranging between $^1/_{10}$ to less than $^1/_{800}$ of those in fat. Liver and the tissues of central nervous system were relatively high in ^{14}C content. The data in Table I are indicative of a general pattern of inverse relationships between the tissue levels of radioactivity and the length of the experimental periods.

The major route of excretion of radiochemicals from the treated monkeys was via the feces. At the end of the various experimental periods, monkey no. 1079, 1036, and 1111 excreted 17.1, 8.8, and 28.2% of the administered dose, respectively. The reason for the lower fecal excretion in monkey no. 1036 was probably the lower output of feces during the period of 5 weeks following the accident described above. The time-course data on fecal excretion of HCB and metabolites, as presented in the 1-year study, are shown in Figure 1. It is typified by an initial phase of relatively rapid excretion, followed by a second phase of very slow excretion.

Urinary excretion of radiochemicals in the treated monkeys amounts to a very small portion of the administered dose. The radioactivity in urine was only about three to four times background after the first 24 h, and it fell to less than twice background within 3 months. In the case of monkey no. 1079, the collection of urine was for the entire 100-day experimental period; the total excretion amounted to only 1.8% of the administered dose. In monkey no. 1036, the urine collection was terminated at the end of 3 months and that for no. 1111 was terminated at the end of 6 months. The total amounts excreted by these two monkeys were 1.1 and 1.6% of the dose,

Table I. Distribution of Radioactivity in Various Tissues of Monkeys Treated with [^{14}C]HCB[a]

Tissues	HCB equivalents (ng/g or mL of tissues)		
	No. 1079 (100 day)	No. 1036 (0.5 year)	No. 1111 (1 year)
Liver	307.5	105.0	35.7
Spleen	39.9	23.1	9.8
Kidney	83.0	30.1	9.1
Adrenal gland	367.5	328.7	72.5
Heart	53.4	38.7	6.7
Lung	47.8	23.8	4.8
Bile	145.4	36.6	14.8
Pancreas	152.9	30.3	7.3
Diaphragm	39.6		
Fat (subcutaneous)	3170.3	1829.8	430.5
(omental)	2899.1	1789.9	397.8
Muscle	18.8	14.4	2.1
Skin	303.6		16.1
Hair	12.8	11.3	1.9
Bladder	25.2	29.4	2.6
Ovary	76.9	78.7	35.1
Fallopian tube	119.0	57.8	10.8
Uterus	28.9	14.8	4.0
Cervix	22.9	15.0	4.1
Vagina	38.7	53.9	8.8
Spinal cord	128.7	73.2	17.8
Thalamus and hypothalamus	113.8	52.9	19.1
Cerebellum	72.0	41.8	11.2
Cerebrum	100.3	44.1	12.9
Pons	138.5		
Medulla	127.4		
Pituitary gland	50.6	48.6	6.6
Thyroid		38.2	5.3
Esophagus		18.3	8.4
Salivary glands		70.2	11.7
Bone marrow		1637.8	372.6
Sciatic nerve		105.5	24.1
Lymph nodes			26.3
Stomach	38.1	23.8	4.7
Small intestine	49.5	78.5	7.9
Caecum	68.1	207.0	19.4
Large intestine	68.3	233.0	10.8
Whole blood	22.8	15.1	0.5
Plasma	25.8	19.4	1.8

[a] The iv doses of [^{14}C]HCB for the monkeys were 0.38, 0.32, and 0.22 mg/kg for no.'s 1079, 1036, and 1111, respectively.

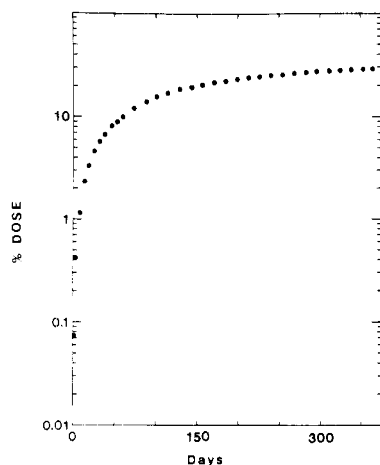

Figure 1. Cumulative fecal excretion of radioactivity from a rhesus monkey (no. 1111) treated with [^{14}C]HCB.

respectively. Figure 2 presents the time-course data for the urinary excretion or radiochemicals by monkey no. 1111.

As indicated in a later section of this paper, none of the urinary radioactivity was present in the form of unchanged HCB. In contrast, approximately 90% of the radioactivity in the feces was present as [^{14}C]HCB. In view of the fact that fecal radioactivity accounted for a major portion of the excreted radiochemicals, and the fact that a significant amount of radioactivity was present in the bile (Table I), it seems evident that biliary excretion is an important mechanism for the disposition of HCB in the rhesus monkey.

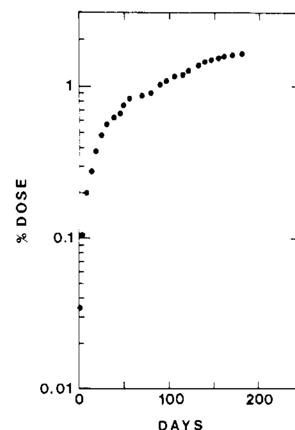

Figure 2. Cumulative urinary excretion of radioactivity from a rhesus monkey (no. 1111) treated with [^{14}C]HCB.

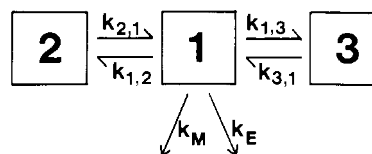

Figure 3. Pharmacokinetic model for HCB in the rhesus monkey. Compartment no. 1 is the central compartment, containing plasma and any tissue for which the distribution phases are very rapid. Compartments no. 2 and no. 3 are, respectively, a slow and a fast tissue compartment. Exchange between the central and peripheral compartments is controlled by the rate constants $k_{1,2}$, $k_{2,1}$, $k_{1,3}$, and $k_{3,1}$. Excretion and metabolism are controlled by constants k_E and k_M, respectively. All units are days^{-1}.

Pharmacokinetics of HCB in the Rhesus Monkey. Since the purpose of this study was to elucidate the long-term behavior of HCB in animals, as a basis to predicate its behavior in man, pharmacokinetic analysis was an integral part of this process. Initially, an attempt was made to describe HCB behavior with a two-compartment model. The deviation of predicted values of plasma concentrations of HCB from corresponding experimental values were so great that this model was rejected. Subsequently, 2 three-compartment models were constructed, one with excretion of HCB from a peripheral compartment and one with excretion of HCB from the central compartment. Since the predicted values for plasma, fecal, and metabolized HCB from the former model were not demonstrably better than those from the latter, the latter model was chosen as being the simplest case. A schematic of this three-compartment model is shown in Figure 3. The mass balance equations for this model are:

$$d[HCB]_1/dt = [k_{2,1}\cdot HCB_2 + k_{3,1}\cdot HCB_3 - (k_{1,2} + k_{1,3} + k_M + k_E)[HCB]_1\cdot V_1]/V_1$$

$$dHCB_2/dt = k_{1,2}[HCB]_1\cdot V_1 - k_{2,1}\cdot HCB_2$$

$$dHCB_3/dt = k_{1,3}[HCB]_1\cdot V_1 - k_{3,1}\cdot HCB_3$$

$$dHCB_M/dt = k_M[HCB]_1\cdot V_1$$

$$dHCB_E/dt = k_E[HCB]_1\cdot V_1$$

Table II. Parameter Values for HCB Pharmacokinetic Model

Parameter	Monkey no. 1079 Starting[a] estimates	Monkey no. 1079 Final[b] estimates	Monkey no. 1111 Starting[a] estimates	Monkey no. 1111 Final[b] estimates
$k_{1,2}$, day^{-1}	241	9.444×10^{-3}	13.8	9.342×10^{-3}
$k_{2,1}$, day^{-1}	2.27	1.659×10^{-5}	2.40	5.664×10^{-5}
$k_{1,3}$, day^{-1}	714	2.598	15.8	2.568
$k_{3,1}$, day^{-1}	45.3	1.405	22.4	1.864
$k_{1,0}$, day^{-1}	6.77×10^{-1}	7.841×10^{-3}	4.57×10^{-2}	5.129×10^{-3}
k_M, day^{-1}		1.855×10^{-3}		0.988×10^{-3}
k_E, day^{-1}		5.986×10^{-3}		4.141×10^{-3}
V_1, L	0.353	14.854	5.40	19.152
Dose, mg	1.83		0.965	

[a] Calculated from the initial values (shown in Table III) for the coefficients of the equation describing the plasma-concentration–time curve. [b] Obtained from the starting estimates shown above by a computer-based modeling program.

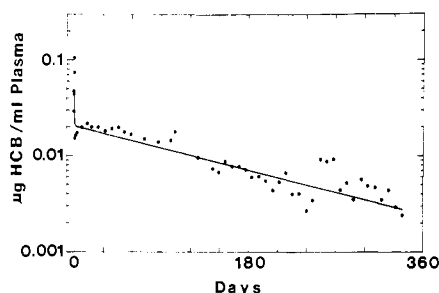

Figure 4. HCB pharmacokinetics, plasma concentration time curve: solid line, computer derived values; solid circles, experimental values.

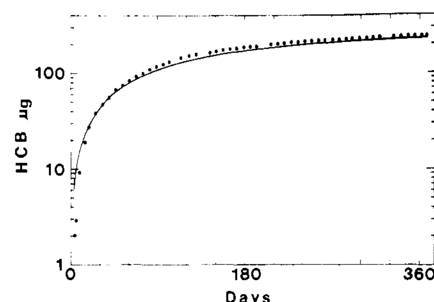

Figure 5. HCB pharmacokinetics, fecal excretion time curve: solid line, computer-derived values; solid circles, experimental values.

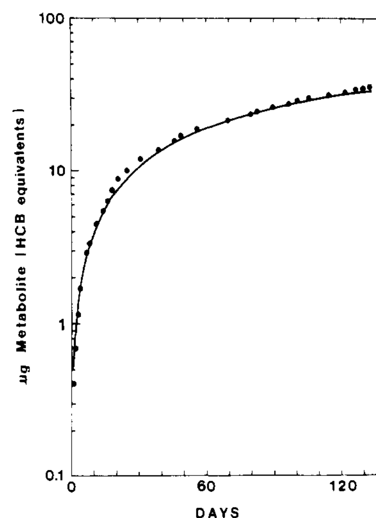

Figure 6. HCB pharmacokinetics, excretion of metabolized HCB: solid line, computer-derived values; solid circles, experimental values.

where $[HCB]_1$ represents the concentration of HCB in compartment 1 (the central compartment), $HCB_{2,3,M \text{ or } E}$ represents the amount of HCB in compartment 2 or 3 or the amount metabolized or the amount excreted, $k_{i,j}$ (i,j = 1, 2, 3) represents the first-order rate constants (days^{-1}) for the exchange of HCB between compartments, $k_{M \text{ or } E}$ represents the first-order rate constant (days^{-1}) for metabolism or for excretion of HCB, and V_1 represents the volume of distribution of HCB in the central compartment. Crude estimates for V_1, $k_{i,j}$, and $k_{1,0}$ (the elimination rate constant for HCB from the plasma) were obtained, as described in the Materials and Methods section, by graphical analysis of the plasma-concentration time curve. The elimination rate constant ($k_{1,0}$) was arbitrarily divided into k_M and k_E, based on our observations of the relative amounts of HCB metabolized and excreted.

The values resulting from the computer-based minimization of starting parameter estimates for the model are presented in Table II. Corresponding values are similar for each monkey. The values k_E and k_M represent the overall rate constants for excretion of HCB and of metabolized HCB, respectively. Their sum is equal to the value for $k_{1,0}$, the elimination rate constant of HCB from the central compartment. The use of k_M in this manner assumes either the rapid conversion of HCB to metabolite(s) in the central compartment or the rapid excretion of metabolite(s) once the relatively slow conversion of HCB occurs. The absence of detectable metabolite(s) in the plasma and the fact that the metabolite(s) are considerably more polar than HCB argue for the second case, i.e., rapid excretion after slow metabolism.

Figures 4–6 demonstrate the comparison between the experimental values and the computer-derived values for plasma, fecal, and metabolized HCB, respectively. In each of these figures, the computer-derived values are presented as a solid line. Only the results from monkey no. 1111 were presented here since the 100-day study (no. 1079) provided similar results. As shown in Figure 6, the excretion of HCB metabolites was expressed in terms of HCB equivalents due to the fact that not all metabolites were characterized.

It is of interest to note that the described process of minimization of the parameters resulted in values which were lower than the starting parameter estimates by one to several orders of magnitude, except for the volume of distribution of the central compartment, which was correspondingly higher (Table II). These changes in parameter values suggesting that the early events recorded in the plasma curve were being subjugated to later events not delineated by the plasma curve, a result rather surprising in view of the fact that the original estimates were derived from equations which fit the plasma concentration curves of each monkey rather well. The cause of the apparent discrepancy was the inclusion of values for excreted and metabolized HCB into the data file used by the minimization program. The explanation for the observed changes might be conceptualized as follows: The starting

Table III. Coefficients of the Equation[a] Defining HCB Concentration in Monkey Plasma

Coefficient	Monkey no. 1079		Monkey no. 1111	
	Initial[b] values	Computed[c] values	Initial[b] values	Computed[c] values
λ_1, day^{-1}	990	4.014	45.6	4.440
λ_2, day^{-1}	12.8	6.059×10^{-3}	8.81	6.128×10^{-3}
λ_3, day^{-1}	7.6×10^{-3}	7.478×10^{-6}	6.1×10^{-3}	2.033×10^{-6}
C_1, mg/L	5	8.028×10^{-2}	1.1×10^{-1}	2.927×10^{-2}
C_2, mg/L	1.4×10^{-1}	4.298×10^{-2}	4.8×10^{-2}	2.092×10^{-2}
C_3, mg/L	4.2×10^{-2}	6.497×10^{-5}	2.4×10^{-2}	1.885×10^{-4}

[a] $[HCB] = C_1e^{-\lambda_1 t} + C_2e^{-\lambda_3 t} + C_3e^{-\lambda_3 t}$. [b] Estimated from the plasma-concentration-time curve by graphical analysis (see Materials and Methods). [c] Calculated from the final parameter estimates listed in Table II.

parameter estimates were derived from a plasma curve which included data from early times after HCB administration. This early data represented rapid processes of equilibration of HCB between the plasma and parts of the body, which would delineate exchange between the central and one or more peripheral compartments. Relatively slow processes such as metabolism, excretion, or passage of HCB into poorly perfused tissues would be represented by data acquired at later times and might constitute one or more additional compartments. Very slow processes such as the passage of HCB out of poorly perfused tissues, such as fat, into which it partitions very favorably, might not be discerned as a separate event in the plasma-concentration time curve, especially in a case in which the data tended to scatter widely. The latter process, however, would have an enormous effect on the appearance of HCB and metabolites(s) in the excreta. Therefore, the plasma curves would tend to delineate rapid events strongly and slow events weakly, whereas the cumulative excretion curves would have the opposite effect.

To visualize the extent of the changes discussed above upon the projected plasma curves, new equations describing them were derived in the following manner: The general equation, defined by a three-compartment model for expressing the fraction in the central compartment of any dose of a compound at various times after administration takes the form: $C_t = C_1e^{-\lambda_1 t} + C_2e^{-\lambda_2 t} + C_3e^{-\lambda_3 t}$. By rearrangement and substitution in the appropriate equations (Rescigno and Segre, 1966), a cubic equation containing λ_1, λ_2, and λ_3 as roots and constants obtained from the first five parameter values ($k_{i,j}$) in Table II was derived. Solution of this equation yielded values for λ_1, λ_2, and λ_3 (Table III). Subsequent use of these values and of $k_{i,j}$ (Table II) in equations for C_i (Rescigno and Segre, 1966) yielded values for C_1, C_2, and C_3, as specific coefficients for equations for the plasma concentration of HCB in each monkey for the doses administered. These derived coefficients are listed in Table III along with the initial estimated coefficients obtained by graphical reduction of the plasma-concentration vs. time curves. It is immediately apparent that the effect of including excretion data in the computer program for parameter estimation was to eliminate the term describing very early events and introduce a term describing very late events, shifting the elimination half-life in the plasma ($0.693/\lambda_3$) from hundreds of days to hundreds of years. Similar results were obtained in a study of mirex pharmacokinetics (Pittman et al., 1976).

Analysis of Radiochemicals in Monkey Fecal Extracts. Our previous report on the analysis of the pentane-CH_2Cl_2 (4:1) eluate from Florisil column chromatography of the benzene extract (Yang et al., 1975a) was confirmed with additional analytical work. The conclusion that HCB represents more than 98% of the radiochemical in the eluate was further substantiated by results from GC–MS. In addition, traces of QCB were also present based on analyses from LC and GC. Since the [^{14}C]HCB sample used in this study was free from contamination, these traces of QCB should be regarded as a result of the metabolic transformation of HCB.

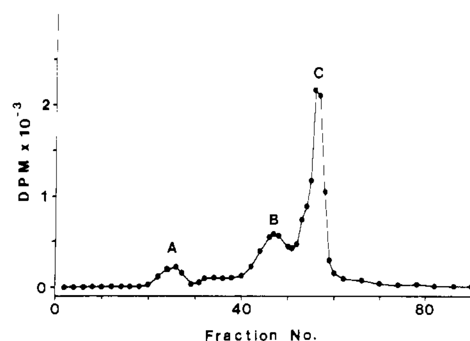

Figure 7. Sephadex LH-20 column chromatography of radiochemials in monkey fecal extract (methanol eluate): column dimension, 50 cm × 2.7 cm i.d.; solvent, methanol; flow rate, 60 drops/min.

The first methanol eluate from the Florisil column contained, in addition to a small portion of the radioactivity (5% of the benzene extract), a large quantity of monkey fecal contaminants. The pooled, concentrated extract, in general, was a dark-brown solution containing a large amount of white crystals. A portion (~150 mL) of this dark-brown supernatant containing approximately 25 000 dpm was concentrated in a rotary evaporator under reduced pressure to about 10 mL. This sample was stored at −20 °C overnight to induce further crystallization of the monkey fecal contaminants. Subsequently, it was centrifuged to remove the crystals. The resulting supernatant was dried over anhydrous Na_2SO_4 and was chromatographed on a Sephadex LH-20 column. As shown in Figure 7, the elution profile indicated the presence of three radioactive peaks. Most of the highly pigmented fecal contaminants were eluted before or with peaks A and B. The identities of the radiochemicals in these two peaks are not known due to the relatively low radioactivity and the presence of a large quantity of contaminants. The fractions in peak C were pooled and, after appropriate concentration, were analyzed by TLC, LC, and GC. The results from TLC and LC analyses revealed that most of the radioactivity cochromatographed with PCP (Figures 8A, 8B, and 9). There also appeared to be some traces of relatively nonpolar radiochemicls (Figure 8); the identities of these compounds are unknown. The presence of PCP in peak C was also confirmed by GC following chemical derivatization. When the radiochemical in peak C was methylated, the resulting compound had a retention time identical with that of synthetic pentachloroanisole.

The methanolic Soxhlet extract also contained a large quantity of pigmented fecal materials in conjunction with

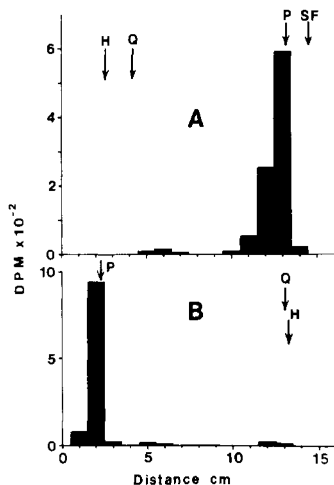

Figure 8. Cochromatography of HCB (H), QCB (Q), and PCP (P) standards and radiochemicals in monkey fecal extract (peak C, Figure 7) by TLC: (A) reverse phase, mobile phase: acetonitrile–methanol–water–acetone (4:3:2:1, v/v); immobile phase: 5% paraffin oil in ether; (B) normal phase, solvent: acetone–methanol-*n*-heptane (1:1:8, v/v). SF = solvent front.

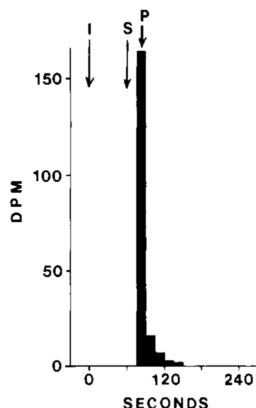

Figure 9. Cochromatography of PCP (P) standard and the radiochemical in monkey fecal extract (peak C, Figure 7) by LC: column, Permaphase ODS reverse phase (1 m × 2.1 mm i.d.); solvent, acetonitrile–water (3:7, v/v); operating conditions: temperature, 50 °C; pressure, 1500 psi. I = injection, S = solvent front.

a small amount of radioactivity (6% of the fecal ^{14}C). On the basis of GC analysis following Florisil column chromatography, the pentane–CH_2Cl_2 eluate contained neither HCB nor QCB. Attempts to isolate and characterize the radiochemicals present in this extract were unsuccessful due to the many interfering materials. Chromatography of this extract on a Sephadex LH-20 column using methanol as a solvent resulted in excessive streaking of the radiochemicals and of the pigmented materials. When Darco G-60 activated carbon was added to this extract and the mixture stirred overnight, a portion of the pigmented materials as well as some radioactivity was absorbed in the carbon. It is possible that the polar metabolites were bound to the pigmented monkey fecal contaminants.

The results described above indicated that unchanged HCB was present only in the pentane–CH_2Cl_2 eluate from the Florisil column and that the remaining radioactivity in other fecal extracts and column eluates was very likely derived from metabolites of HCB. Based on this conclusion, the fecal excretion of HCB metabolites appeared to the approximately 10% of the total fecal radiochemicals; about one-third of the metabolites was present as PCP but the remaining two-thirds could not be identified. Since in a 1-year study (no. 1111) 28.2% of the administered intravenous dose was excreted by the monkey, the cumulative fecal metabolites were therefore estimated to account for only 2.8% of the administered [^{14}C]HCB.

Analysis of Radiochemicals in Monkey Urine Extracts. In order to determine the amounts of [^{14}C]HCB and [^{14}C]QCB present in the urine, aliquots of pooled samples (300 mL) were partitioned successively with three equal volumes of hexane. Approximately 3.8% of the radioactivity could be extracted in this manner. GC analysis of the combined hexane extracts revealed that neither HCB nor QCB was present. A peak with a longer retention time than either HCB or QCB was present on the chromatogram. Its identity was not established.

The urine samples were also subjected to extraction with more polar solvents. Simple repeated partition in a separatory funnel was found to be more effective than that achieved by using a liquid–liquid extractor. When the urine was not acidified, three successive extractions with equal volumes of ether or ethyl acetate resulted in the removal of 12–21% of the radioactivity. On the other hand, 73–91% of the radioactivity could be recovered in ether or ethyl acetate extracts when the urine was acidified to pH 1–2. In general, ether was found to be more satisfactory for this purpose because the contaminants appeared to be less soluble in ether than these metabolites.

Ether extracts of acidified monkey urine were chromatographed on a Sephadex LH-20 column after appropriate treatment as outlined in the Materials and Methods section. The resulting elution profile showed two major radioactive peaks. Further treatment including TLC and GC analysis failed to identify these radiochemicals. However, the following conclusion may be drawn: (1) these polar, acidic radiochemicals are not HCB, QCB, or PCP; (2) they might be hydroxylated compounds because the methylation process converted them to hexane-extractable compounds. These compounds, however, were neither pentachloroanisole nor the methylation product of tetrachlorohydroquinone.

The above findings indicate that all the urinary radioactivity was derived from HCB metabolites. However, it represented only a small portion of total ^{14}C as the recovery of urinary radioactivity never exceeded 2% of the administered dose.

Analysis of Radiochemicals in Monkey Fat and Liver. Fat was ground in a mortar with anhydrous Na_2SO_4 and then subjected to the three-stage Soxhlet extraction using benzene, methanol, and H_2O as successive solvents. This process failed to remove all the radioactivity from the tissue. However, subsequent extractions with benzene and methanol in an Omni-mixer did remove all radioactivity from the tissues. In all the extracts so obtained, HCB was the only labeled compound.

Liver samples were homogenized in benzene with an Omni-mixer prior to the Soxhlet extractions. The results of the three-stage extraction and subsequent Florisil column chromatography of the benzene extract are shown in Table IV. The overall recovery of radioactivity ranged between 98.9 and 103.9%. The benzene extract, which contained the bulk of the radioactivity, was chromatographed on a Florisil column. As shown in Table IV, most of the radioactivity eluted with pentane–CH_2Cl_2, and this radiochemical was identified as HCB. The first methanol eluates from the Florisil column contained small amounts of radioactivity (Table IV) which were pooled and analyzed by various chromatographic methods. The results indicated that approximately 60% of the radioactivity was present as HCB and that the remainder was derived from polar metabolite(s). Chromatographic analyses of the

Table IV. Recovery of Radioactivity in Monkey Livers following Administration of [14C]HCB

Step	Sample	Relative radioactivity		
		No. 1079	No. 1036	No. 1111
Soxhlet extraction	Original liver sample	100	100	100
	Benzene extract	83.2	75.4	95.8
	CH_3OH extract	7.5	4.6	3.8
	H_2O extract	0.2	0.6	0
	Dried liver residue	13.0	18.3	4.4
	(Total recovery)	103.9	98.9	103.9
Florisil column chromatography	Benzene extract	100	100	100
	Pentane-CH_2Cl_2 eluate	96.3	89.4	90.5
	First CH_3OH eluate	0.8	6.2	11.6
	Second CH_3OH eluate	0	0	0.7

methanolic Soxhlet extract indicated that the radiochemical was [14C]HCB. It is interesting to note that the dried liver residue contains a fairly large portion of the radioactivity. The significance of this observation is unknown at the present time.

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Excretion and Metabolism of 3,4,5-Tribromo-*N*,*N*,α-trimethyl-1*H*-pyrazole-1-acetamide in the Rat

Rex E. Hornish* and John L. Nappier

The title compound, a potential preemergence herbicide, labeled in the C-3(5) position with carbon-14, was administered orally to rats to determine the excretion pattern and the metabolism scheme. The herbicide was rapidly absorbed and metabolized, and the metabolites were readily excreted; 90% of the dose was excreted within 72 h. No significant residue levels were found in any tissue. The metabolism was characterized by two successive side chain N-demethylations, followed by hydrolysis to 3,4,5-tribromo-α-methyl-1*H*-pyrazole-1-acetic acid. This tribromo acid was the major metabolite, but was not the end product of metabolism. A reductive debromination of the tribromo acid occurred selectively at the C-5 position to produce the second most abundant metabolite. Debromination at either C-3 or C-4 was not detected.

3,4,5-Tribromo-*N*,*N*,α-trimethyl-1*H*-pyrazole-1-acetamide (I) has shown exceptional preemergence herbicidal activity against sedges, grasses, and broadleafs in various crops including peanuts and sugar beets. The by-products of these crops, peanut hay and sugar beet tops, are often incorporated into animal fodder. Thus, the herbicide and its residues become potential feed ingredients in the diet of food-producing animals. We were, therefore, interested in establishing the mammalian excretion patterns and

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