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Accelerator Mass Spectrometric Measurement of the In Vivo Kinetics of High and Low Dose Propanil Disposition in Mice

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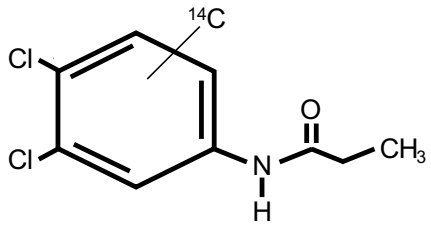
ABSTRACT

We have documented that the herbicide, propanil, is immunotoxic in mice and our in vitro tissue culture experiments largely recapitulate the in vivo studies. Laboratory studies on environmental contaminants are the most meaningful when these studies are conducted using concentrations that approximate levels in the environment. Many techniques to measure the distribution and pharmacokinetics (PK) on compounds rely on techniques, such as liquid scintillation counting (LSC) of radio-labeled starting compound require concentrations higher than environmental levels. To overcome this problem and provide insight into whether environmental levels represent a potential concern for human health, we conducted a study to measure propanil distribution in three immune organs, using ultrasensitive accelerator mass spectrometry (AMS). We used two doses; the lower dose modeled levels expected in the environment or long-term occupational exposure to low doses, while the higher dose was to model the effects of an accidental exposure. The distribution and PK profiles from these two different concentrations was markedly different. The profile of the high dose (concentration) exposure was indicative of saturation of the detoxifying capability of the animal. In contrast, at the lower environmentally-relevant concentration, in vivo concentrations of propanil in spleen, liver and blood dropped to a very low level by 720 minutes. These studies are valuable in determining appropriate concentrations to be used in laboratory studies and the utility of the AMS.



INTRODUCTION

Propanil, 3,4-dichloropropionaniline, is an herbicide primarily used in the United States to control weeds in the production of rice. In vivo immunomodulatory effects of propanil include dose-dependent changes to T-dependent and T-independent antibody responses, as well as the mixed lymphocyte responses and cytokine production from splenocytes [1]. Functionally similar effects were also noted following in vitro propanil exposure of spleen, peritoneal exudate cells (macrophages) or T cell (EL-4) and macrophage cell lines (IC-21) [2-6].

In vitro, as well as in vivo, laboratory studies are aimed at understanding the mechanism of propanil's action on macrophage and lymphocyte function. However, the full utility of these experiments to determine possible effects on humans exposed environmentally are often compromised due to a lack of data to indicate the concentrations of the compound typically found in a particular organ, e.g., in the spleen. Thus, leading to the problem that the laboratory study concentrations do not approximate environmental or occupational levels.

Environmental exposures are often chronic low doses whereas occupational exposures can be both insidious low levels over long periods or acute (accidental) exposure at much higher concentrations. Concentrations used for laboratory studies are often based on estimated organ levels, determined by giving concentrations high enough to cause a measurable toxic effect or in some cases, high acute concentrations are used to mimic possible chronic exposures over long periods when it is known that the compound accumulates in vivo to high residual levels. Previously published experiments on propanil have defined a range of concentrations and doses from a no-observable-adverse-effect level (NOAEL) in both the in vivo and in vitro exposure scenarios [1-6], however, these were determined without regard to an appropriate environmental or occupational dose or concentration within specific organs of interest.

Typical methods of quantifying small molecule metabolism often requires relatively high levels of the starting compound so that the parent compound and/or metabolites can be detected over time. Traditionally, such experiments have been performed using radioactive labeled forms of the compound and measuring radioactivity levels in the cells and organs at various times after exposure. Tissue distribution of higher doses needed for more typical detection methods, like LSC, are likely significantly higher from that seen with environmentally-relevant concentrations. These higher concentrations may provide misleading results, especially for in vivo experiments because the higher dose could saturate the detoxification pathways and allow non-metabolized compound to continue to circulate. Determination of the relevance of in vitro studies also require knowing the actual tissue propanil levels and decay kinetics after in vivo dosing at concentrations likely to be encountered environmentally.

The high sensitivity of accelerator mass spectrometry (AMS) provides a solution to the aforementioned problems. AMS can perform tissue distribution and PK measurements using an input dose that is hypothesized to closely approximate environmental levels. In the case of the higher dose used herein, this is a concentration that more likely approximates the exposure level of an acute exposure accident, e.g., during mixing or application of the product. AMS lowers concentration detection limits by 12-fold and dramatically lowers mass detection limits by 10^5-fold compared to liquid scintillation counting (LSC), which requires relatively high concentrations of the labeled compound in order to reproducibly detect the isotope [7]. AMS is capable of reproducibly measuring levels as low as 1.0 attomole (≈60 attocurie) of ^{14}C per mg of sample level. In contrast. LSC-compatible doses are far higher than is likely to occur from an environmental or occupational exposure, and thus, may provide data that does not represent a scenario that accurately reflects the disposition of the chemical under normal conditions. This report details the differences in the

disposition curves when occupational- versus environmentally-relevant doses of propanil are administered. In addition, as propanil is a heavily used herbicide, it provides information on the disposition of the compound in key organs. Finally, it highlights the utility of using the highly sensitive technique of AMS to measure sample concentrations believed to be environmentally relevant.

MATERIALS AND METHODS

Chemicals.

[^{13}C]-propanil (3,4-dichloropropionaniline), 99% pure, was purchased from ChemService, Inc. (West Chester, PA). Appropriate concentrations of propanil were obtained first by dilution in ethanol (99%) and finally by dissolving in corn oil (Mazola). ^{14}C-labeled propanil (97.2% purity verified by high pressure liquid chromatography) was synthesized by Moravek Biochemicals (Brea, CA) as 3',4'-dichloropropionanilide, [ring-^{14}C] (Lot number 123-188-0123). The specific activity of the [^{14}C]-propanil was 12.3 mCi/mmol. Stock [^{14}C]-propanil was diluted to a concentration compatible with the AMS in absolute ethanol (99%)(AAPER Alcohol and Chemical Co., Shelbyville, KY).

Animals.

Female C57Bl/6 mice weighing 18 to 20g were purchased from Charles River (Wilmington, DE). All animals were allowed to acclimate to our vivarium facilities at least 7 days before their inclusion in an experiment and were provided food and water ad libitum. All animals were assayed between 7 and 12 weeks of age. All animal experiments were approved by the WVU Institutional Animal Care and Use Committee.

In vivo exposure to propanil.

C57Bl/6 mice were injected i.p. with either 200 μg/g of [^{12}C]-propanil plus 854 pg/g of [^{14}C]-propanil or 854 pg/g of [^{14}C]-propanil. Five mice per dose level at each time point were used. These are designated as the high and low dose, respectively. The high dose was determined by the concentration historically administered in previously published studies on the immunotoxic effects of in vivo propanil exposure plus a nominal increase to provide the isotope (reviewed in Salazar et al. [1]). The low dose is a concentration of [^{14}C]-propanil that is compatible with AMS. Higher concentrations of [^{14}C]-compound would overwhelm the counting electronics of the AMS instrument. As there is limited data on environmental levels, this low dose is included to approximate the effects of a dose similar to what is presumed to be anthropogenically relevant.

Experimental Design.

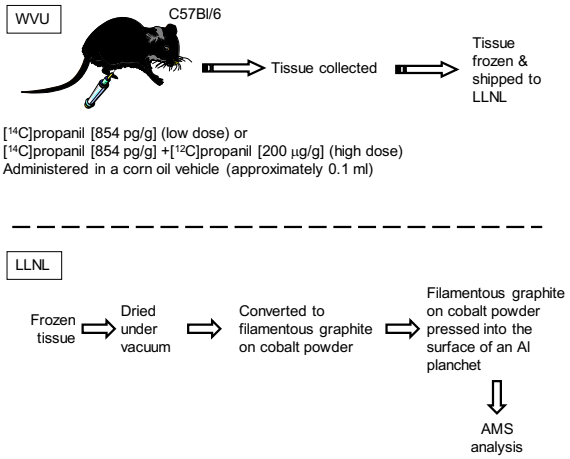


Figure 1. Outline of the experimental design. C57Bl/6 mice were injected with propanil at time zero. At 1, 10, 20, 40, 80, 120, 240, 360, 720 minutes after propanil injection, blood was collected into a capillary tube and then the animal was euthanized and the liver and spleen removed by surgical ablation. All tissue was then snap frozen in liquid nitrogen for shipment to the AMS facility at LLNL. New surgical instruments were used on each animal to avoid cross contamination. At the AMS facility, frozen tissue was processed as outlined. Detailed methodology found in Vogel et al.[8]

The overall experimental design is outlined in Figure 1. At each indicated time point after propanil administration, 50 µl of whole blood was collected directly into a capillary tube via the retroorbital sinus and then the animal was euthanized for spleen and liver collection. These tissues were snap frozen in liquid nitrogen and then shipped on dry ice to LLNL for AMS processing. The details of AMS preparation are as described previously [8]. Calculations of tissue concentrations from the AMS data is described in the Supporting Information.

Pharmacokinetics and Statistical Analysis.

The pharmacokinetic parameters of propanil were calculated by non-compartmental analysis using PK Solutions Software (Summit Research Services, Montrose, CO). The half-life ($T_{1/2}$), time of maximum propanil concentration (T_{max}), and initial propanil concentration (C_{max}) were determined by observations from the concentration versus time data. Area under the curve (AUC) was calculated for intervals 0 to *t* where *t* is the time of the last measurable concentration (12 h) using the linear trapezoidal method.

Error bars in all figures represent the standard deviation of triplicate samples at each time point. These experiments have been repeated at least three times for each time point.

RESULTS

Baseline ^{14}C-Measurements.

Non-treated tissues are used to determine the natural level of ^{14}C. An average of three non-treated tissue samples was obtained for the calculations. These determinations showed an average of 1.095 ± 0.014 Modern. This figure is used as the 'undosed ^{14}C' value in Equation A1-1 (Supporting Information) to calculate the fmol of [^{14}C]-propanil per mg of tissue. This value also provides a check of the laboratory background levels of ^{14}C. Many laboratories that have previously used

[^{14}C]-compounds have background levels sufficiently high to swamp the highly sensitive AMS instrument. The value determined indicates that our laboratory ^{14}C background levels are at contemporary environmental levels, e.g., in normal rodent chow, atmospheric, etc.[8].

Disposition of propanil in the liver.

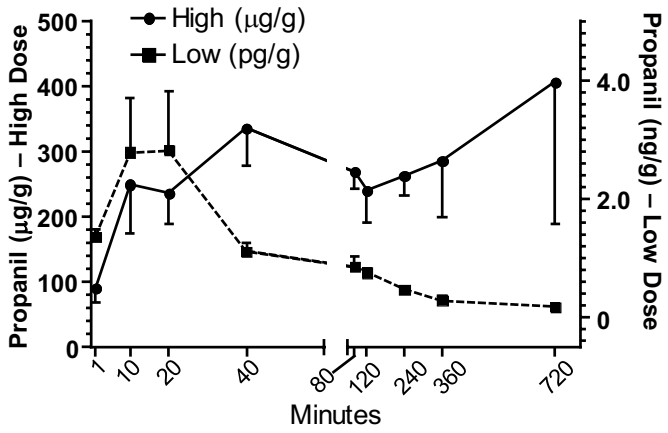


Figure 2. Time course of [^{14}C]-propanil degradation in liver. Liver samples were collected and analyzed as described. Values on the ordinate reflect only the actual concentration of [^{14}C]-propanil in the liver. Total propanil in the tissue of high dose animals was 2.342×10^5 times the level of the [^{14}C]-propanil. Since the ring structure was labeled, these ^{14}C levels could reflect either [^{14}C]-propanil or its metabolite. Data are the mean for five replicate samples (±SD) for one representative experiment. This experiment was repeated three times. Note that the level of error was calculated for all data points on both curves, however, the level of error was often so small that the bar was covered by the data point symbol.

The liver is both a major organ involved in detoxification of xenobiotics and an immune organ. Thus, the propanil levels within this organ are important to the understanding of the disposition of propanil from the body. Figure 2 shows the disposition curves for [^{14}C]-propanil from the liver at the two doses utilized (also see Table 1). The liver concentration in animals administered the high dose increased markedly over the first 10 minutes after which the levels plateaued (Figure 2; Table

1). At this concentration, the disposition of propanil appears to follow a saturation kinetic profile with the highest dose reached at 20-40 minutes after propanil administration and does not decrease appreciably over the time course of the experiment.

In contrast, animals injected with the low dose demonstrate an early peak at 10-20 minutes (Figure 2; Table 1). Levels quickly dropped during the next 20 minutes; however, there appears to be at least one additional disposition pattern from a period beginning at 40 minutes (Figure 2). Beginning at 40 minutes, the disposition showed a protracted kinetic pattern.

Disposition of propanil in the spleen.

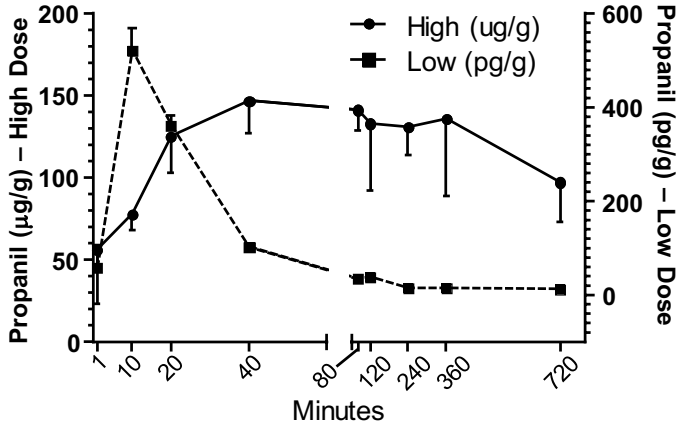


Figure 3. Time course of [^{14}C]-propanil degradation in spleen. Spleen samples were collected and analyzed as described. Values on the ordinate reflect only the actual concentration of [^{14}C]-propanil in the spleen. Total propanil in the tissue of high dose animals was 2.342×10^5 times the level of the [^{14}C]-propanil. Since the ring structure was labeled, these ^{14}C levels could reflect either [^{14}C]-propanil or its metabolite. Data are the mean for five replicate samples (±SD) for one representative experiment. This experiment was repeated three times. Note that the level of error was calculated for all data points on both curves, however, the level of error was often so small that the bar was covered by the data point symbol.

The spleen is a key immune organ and several of our previous studies [1-5] have reported the effects of propanil on the function of immune cells from this organ. Thus, a measurement of the disposition of propanil in this organ will allow the correlation of in vitro dose-related effects with actual propanil concentrations in the spleen over time. As shown in Figure 3 (Table 1), disposition of the high dose of propanil from the spleen over time shows a similar pattern to that noted in the liver. Propanil accumulated in the spleen until approximately 40 minutes after injection, plateaued at this time and was only decaying slightly after 720 minutes. This profile appears to be a saturation kinetic pattern.

Measurement of propanil levels after the low dose, again demonstrated a peak at ≤10 minutes (Figure 3; Table 1). These levels also dropped during the next 30 minutes. After this precipitous drop, the kinetics of propanil disposition followed a protracted decay pattern very similar to the liver.

Disposition of propanil in the blood.

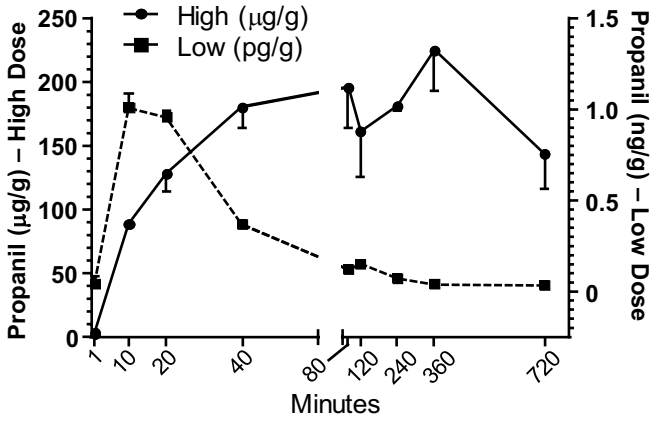


Figure 4. Time course of [^{14}C]-propanil degradation in whole blood. Blood samples were collected and analyzed as described. Values on the ordinate reflect only the actual concentration of [^{14}C]-propanil in the blood. Total propanil in the tissue of high dose animals was 2.342×10^5 times the level of the [^{14}C]-propanil. Since the ring structure was labeled, these ^{14}C levels could reflect either

[^{14}C]-propanil or its metabolite. Data are the mean for five replicate samples (±SD) for one representative experiment. This experiment was repeated three times. Note that the level of error was calculated for all data points on both curves, however, the level of error was often so small that the bar was covered by the data point symbol.

Distribution of propanil throughout the body would occur via the circulatory system. Therefore, important information is obtained by assaying blood levels of [^{14}C]-propanil during the course of the experiment. Animals dosed with the low dose showed a sharp peak of blood activity 10 minutes after injection (Figure 4; Table 1). There was a substantial decrease by 40 minutes, however, they did not drop to an initial plateau until 80 minutes and showed a slower, but still decreasing kinetics between 80 and 240 minutes. After 240 minutes, the blood levels essentially plateaued (Figure 4; Table 1). These data are suggestive of three distinct kinetic patterns; the early peak and then degradation or tissue disposition between 0 and 40-80 minutes, a tissue deposition kinetic curve rise between 40 to 80 minutes and then decaying over the next 160 to 200 minutes, and finally a relatively steady-state circulating level from 360 minutes to the termination of the experiment.

The high dose levels demonstrated a peak much later, at 80 minutes and the decay or tissue deposition was very shallow over the remainder of the experiment. This would be consistent with saturation kinetics, i.e., all tissue stores are saturated, and the bulk of the material therefore simply circulates as it decays, is permanently sequestered, or is eliminated through the urinary and/or fetal routes.

Pharmacokinetics of propanil in each organ assayed

In the liver the mean apparent distribution half-life ($t_{1/2\alpha}$) was similar in both the high and low dose animals (Table 2). The terminal half-life ($t_{1/2\beta}$) in the high dose animals was negative due to

the increasing concentration of propanil in the liver at the later time points. In the spleen the $t_{1/2\,\beta}$ was twice as long in the high dose group compared to the animals receiving the lower dose indicating a slower elimination from the spleen in the high dose group. This was also the case for the blood. In all three tissues, the T_{max} was achieved much sooner in the low dose group compared to the high dose group. Overall the low dose of propanil was absorbed more quickly in all three tissues and was eliminated faster compared to the high dose group suggesting saturation of elimination mechanisms at the higher dose.

DISCUSSION

The primary objective of this study was to determine the elimination and PK profiles of propanil when administered at doses that approximate an environmental exposure concentration. Propanil is an herbicide commonly used on rice to control grassy-weeds. We reported numerous immunotoxic effects of propanil using dose-response assays in mice and concentration-dependent assays in tissue culture [1-6], however, the concentrations used are based on levels needed to show a measurable effect. Critical to our ability to extrapolate our laboratory findings to human exposures, we needed to measure the tissue levels of propanil for a given dose. We utilized two dose levels. The low doses was chosen to model a very low exposure level to propanil, similar to that expected in some environmental exposures. The high dose was identical to a frankly immunotoxic dose we have previously reported [1].

The major advantage of AMS for toxicokinetic measurements is its ability to measure compounds at or below what would be expected from an environmental or occupational exposure. In this study, we not surprisingly demonstrated that propanil exposure at environmentally relevant doses provided a substantially different toxicokinetic profile than the higher dose that more closely parallels an acute poisoning. The relationship of our doses is illustrated when they are compared

to the potential propanil concentrations obtainable during agricultural application. Barnes et al. [9] measured field concentrations by assaying propanil levels in 10 cm^2 denim patches placed in the swath of aerially applied propanil during routine agricultural applications. Measured propanil concentrations in five separate applications ranged from 0.532 to 5.233 × 10^3 µg (mean=2.771±0.96 × 10^3) of propanil in this 10 cm^2 denim patch after aerial application of 3.4-4.5 kg propanil per hectare to a field [9]. Using the value stated in this report [9], i.e., a 150-pound (≈68 kg) individual would have approximately 1620 cm^2 of exposed skin; and assuming that all the propanil was absorbed, an individual's mean potential dermal exposure level would be approximately 0.66 mg propanil per kg of body weight (45 mg per 68 kg). This value is ≈77 times greater than the low dose used for this study. However, actual systemic levels of agents after dermal exposure varies over a wide range depending on the chemical structure, lipophilicity, etc., and is also likely to be influenced by the nature of the "inert" ingredients in the commercial product. Photodegradation would further reduce the environmental concentrations [10]. Respiratory absorption would likely provide a greater likelihood of systemic levels. Barnes et al. [9] also performed air sampling of propanil levels in the swath of the aerial application from 0 to 60 m after spraying and up to 100 meters from the field. They measured propanil levels of 1.6 to 17.7 µg per liter of sampled air per hour. Since we did not expose our animals via the respiratory tract, we cannot make a comparison between our low dose concentration and that measured in the air by Barnes et al. [9]

What is apparent from the comparison of the two dose levels is that a very low dose appears to be metabolized rather quickly. The lower $t_{1/2\beta}$ in liver than spleen and blood possibly reflects active detoxification by that organ, whereas, blood and spleen levels represent the circulating levels. In contrast, the high, frankly toxic dose show what appears to be a saturation state where the

detoxification mechanism cannot completely metabolize the compound over the 720 minutes measured. Another caveat is that we chose to use propanil with the ring structure labeled and thus, the labeled compound measured by the AMS could be a metabolite of propanil, and this is likely the case at the later time points. However, the two major metabolites of propanil, N-hydroxy-3,4-dichloroaniline and 6-hydroxy-3,4-dichloroaniline are toxic [11-13] and knowledge of their toxicokinetic profile is also valuable information. Given the reduction in labeled compound at the low dose, it appears that in vivo detoxification or urinary/fetal elimination has removed a majority of the compound within 720 m. Tissue levels after the high dose continued to be present at 720m indicating that both detoxification and urinary/fetal elimination was not capable of eliminating the compound by this time.

In summary, we demonstrate the utility of AMS to follow the degradation of propanil, an herbicide used extensively in rice agriculture. The lowest (environmental) dose used showed a biphasic elimination curve and tissue levels dropped to near undetectable levels by 720m. In contrast, higher frankly immunotoxic levels increased to a plateau by 40m and continued to be present in the tissue at approximately the same levels by 720m indicating a saturation of the animal's detoxification capability as well as its inability to eliminate the compound via the urinary/fetal routes.

ASSOCIATED CONTENT

Supporting Information.

The calculations necessary to convert AMS values (modern) to tissue concentrations

The following file is available free of charge. AMS_value_conversion_calculation.pdf

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. §These authors contributed equally. J.B.B., K.T., R.S. and J.V. designed the experiments; J.B.B., R.S. and A.R. performed the biological experiments; J.V. performed the AMS assays; T.O. performed the calculations; and M.M. performed the PK calculations.

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Notes

The authors declare no competing financial interests.

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ABBREVIATIONS

LLNL, Lawrence Livermore National Laboratory; NOAEL, no-observable-adverse-effect level; AMS, Accelerator Mass Spectrometry; LSC, liquid scintillation counting.

TABLE 1. MEASURED PROPANIL TISSUE LEVELS FOLLOWING A SINGLE INTRAPERITONEAL ADMINISTRATION OF 14C-PROPANIL IN C57BL/6 MICE

LIVER LEVELS		
TIME	**High (μg/g)[1]**	**Low (pg/g)[2]**
1	90.83 ± 22.47	1362.07 ± 135.57
10	249.99 ± 74.36	2792.07 ± 917.84
20	237.01 ± 46.70	2825.80 ± 989.37
40	335.91 ± 57.57	1121.53 ± 140.67
80	268.85 ± 26.34	854.87 ± 175.38
120	240.83 ± 50.05	763.14 ± 14.88
240	262.55 ± 28.81	476.81 ± 73.93
360	286.30 ± 85.90	284.44 ± 82.88
720	407.49 ± 262.17	188.00 ± 34.20

SPLEEN LEVELS		
TIME	**High (μg/g)**	**Low (pg/g)**
1	56.10 ± 32.76	57.39 ± 48.61
10	77.68 ± 10.18	520.28 ± 47.00
20	125.23 ± 22.08	361.40 ± 22.17
40	146.72 ± 19.62	102.26 ± 4.98
80	141.02 ± 12.19	34.24 ± 5.57
120	132.77 ± 40.28	37.82 ± 4.97
240	130.71 ± 16.82	13.96 ± 2.78
360	135.68 ± 46.79	14.23 ± 0.57

720	97.11 ± 23.94	12.09 ± 1.60

BLOOD LEVELS

TIME	High (*μ*g/g)	Low (pg/g)
1	2.86 ± 0.92	40.71 ± 40.79
10	88.41 ± 1.41	1009.66 ± 77.95
20	127.84 ± 13.51	958.68 ± 36.91
40	180.29 ± 16.15	367.95 ± 8.13
80	195.70 ± 31.98	122.16 ± 17.72
120	161.44 ± 36.13	153.56 ± 11.49
240	181.45 ± 3.98	73.69 ± 0.86
360	224.85 ± 31.59	40.50 ± 1.74
720	143.54 ± 27.55	34.50 ± 3.30

[1]High Dose: [^{12}C]propanil [200 μg/g] + [^{14}C]propanil [854 pg/g];

[2]Low dose: [^{14}C]propanil [854 pg/g].

TABLE 2 PHARMACOKINETICS PARAMETERS OF PROPANIL FOLLOWING A SINGLE INTRAPERITONEAL ADMINISTRATION OF ^{14}C-PROPANIL IN C57BL/6 MICE

LIVER LEVELS				
PARAMETER	High Dose[1]		Low Dose[2]	
$\mathbf{C_{MAX}}$	407.5	μg/g	2825.8	pg/g
$\mathbf{T_{MAX}}$	12.0	hr	0.3	hr
$\mathbf{T_{1/2\alpha}}$	0.4	hr	0.3	hr
$\mathbf{T_{1/2\beta}}$	-12.4	hr	5.2	hr
$\mathbf{AUC_{0\text{-}T}}$	3667.5	μg•hr/g	6065.2	pg•hr/g

SPLEEN LEVELS				
PARAMETER	High Dose		Low Dose	
$\mathbf{C_{MAX}}$	146.7	μg/g	520.3	pg/g
$\mathbf{T_{MAX}}$	0.7	hr	0.2	hr
$\mathbf{T_{1/2\alpha}}$	-2.3	hr	0.2	hr
$\mathbf{T_{1/2\beta}}$	16.7	hr	8.0	hr
$\mathbf{AUC_{0\text{-}T}}$	1488.2	μg•hr/g	422.9	pg•hr/g

BLOOD LEVELS				
PARAMETER	High Dose		Low Dose	
$\mathbf{C_{MAX}}$	224.9	μg/g	1009.7	pg/g
$\mathbf{T_{MAX}}$	6.0	hr	0.2	hr
$\mathbf{T_{1/2\alpha}}$	-8.0	hr	0.13	hr
$\mathbf{T_{1/2\beta}}$	17.4	hr	5.2	hr
$\mathbf{AUC_{0\text{-}T}}$	2175	μg•hr/g	1285.9	pg•hr/g

[1]HIGH DOSE: [^{12}C]PROPANIL [200 µG/G] + [^{14}C]PROPANIL [854 PG/G];

LOW DOSE: [^{14}C]PROPANIL [854 PG/G].

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