Environmentally-Relevant Mixtures in Cumulative Assessments: An Acute Study of Toxicokinetics and Effects on Motor Activity in Rats Exposed to a Mixture of Pyrethroids.

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Running Title: **Pyrethroid Kinetics and Effect on Motor Activity.**

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Abstract

Due to extensive use, human exposure to multiple pyrethroid insecticides occurs frequently. Studies of pyrethroid neurotoxicity suggest a common mode of toxicity and that pyrethroids should be considered cumulatively to model risk. The objective of this work was to use a pyrethroid mixture that reflects human exposure to common pyrethroids to develop comparative toxicokinetic profiles in rats, then model the relationship between brain concentration and motor activity. Data from a national survey of child care centers were used to make a mixture reflecting proportions of the most prevalent pyrethroids: permethrin, cypermethrin, β-cyfluthrin, deltamethrin, and esfenvalerate. The mixture was administered orally at one of two concentrations (11.2 and 27.4 $mg_{*}kg^{-1}$) to adult male rats. At intervals from 1-24 hours, motor activity was assessed and the animals sacrificed. Pyrethroid concentrations were measured in blood, liver, fat, and brain. After controlling for dose, there were no differences in any tissue concentrations, except blood at the initial time point. Elimination half-lives for all pyrethroids in all tissues were < 7 hours. Brain concentrations of all pyrethroids (when *cis* and *trans*-permethrin were pooled) at the initial time point were proportional to their relative dose. Decreases in motor activity indicated dose additivity and the relationship between pyrethroid brain concentration and motor activity was described by a four parameter sigmoidal E_{max} model. This study links environmental data with toxicokinetic and neurobehavioral assays to support cumulative risk assessments of pyrethroid pesticides. The results support the additive model of pyrethroid effect on motor activity and suggest that variation in the neurotoxicity of individual pyrethroids is related to toxicodynamic rather than toxicokinetic differences.

Keywords: Pyrethroids, Toxicokinetics, Cumulative Risk, Motor Activity.

Introduction

Pyrethroid pesticides are some of the most commonly applied residential use insecticides in the United States (U.S.), and survey data have repeatedly demonstrated the occurrence and co-occurrence of pyrethroids in residences and child care facilities (Morgan *et al.*, 2004; Stout *et al.*, 2009; Tulve *et al.*, 2006). Their presence in these locations is of concern because children spend the majority of their time indoors (Graham and McCurdy, 2004), may be more susceptible than adults to pyrethroid induced health effects (Tornero-Velez *et al.*, 2010), and non-dietary ingestion of pyrethroids from indoor sources is an important exposure pathway for children (Morgan *et al.*, 2007).

Pyrethroids have 1-3 chiral carbons and are typically divided into two groups, dependent upon the presence or absence of a cyano group at the α-carbon of the alcohol moiety. Both groups are neurotoxicants in mammalian test species but have different high dose acute primary effects. In general, pyrethroids lacking the α-cyano group cause tremors (Type I or T), while α-cyano pyrethroids produce a salivation/choreoathetosis syndrome (Type II or CS). Both types primarily disrupt nervous system function by prolonging the opening of voltage sensitive sodium channels (Narahashi *et al.*, 1998; Soderlund *et al.*, 2002), although kinetic differences between the two types have been noted (Soderlund and Bloomquist, 1989).

Studies of motor activity (Wolansky *et al.*, 2006; Wolansky *et al.*, 2009), functional observational battery (Weiner *et al.*, 2009), and ion channel disruption (Breckenridge *et al.*, 2009), have established that induced symptoms of neurotoxicity vary among the individual

pyrethroids. In the motor activity studies, Wolansky *et al.* (2006) ranked the relative potency (RP) of several pyrethroids and demonstrated their dose-additivity (Wolansky *et al.*, 2009). Citing the importance of the shared effect on sodium channels and the additive effect on motor activity, the U.S. EPA currently proposes that Type I and II pyrethroids share a common mechanism of toxicity and therefore present a cumulative risk (U.S. EPA, 2011) under the Food Quality Protection Act (FQPA, 1996).

Because pyrethroids co-occur and can act additively, it is useful to establish toxicokinetic and neurotoxicity data reflecting the collective nature of the exposure. To date, most pyrethroid toxicokinetic reports are from single analyte studies (Anadón *et al.*, 1991; Anadón *et al.*, 1996) (Anadón *et al.*, 2006; Godin *et al.*, 2010; Hutson and Logan, 1986; Kim *et al.*, 2008; Ohkawa *et al.*, 1979) and comparison of the results is problematic because of differences in doses, vehicles, and routes of administration (Crofton *et al*., 1995). Further, these studies did not include a neurotoxicity assessment. Single analyte toxicokinetic assessments by White *et al.* (1976) and Scollon *et al.* (2011) included neurotoxicity endpoints, but differences in study design and objectives severely limit comparison of the results. Multi-pyrethroid toxicokinetic profiles such as that by Marei *et al.* (1982) did not assess neurotoxicity or incorporate exposure related concepts such as route and relative concentration in the study design. A literature review revealed no multi-pyrethroid toxicokinetic studies that included an effects endpoint.

The objectives of this research were to 1) use a mixture of pyrethroids that are most frequently detected in an indoor environment to develop comparative toxicokinetic profiles of individual pyrethroids in selected rat tissues, 2) evaluate whether pyrethroid toxicokinetics explain differences in RP reported by Wolansky *et al.* (2006; 2009), and 3) model the relationship between pyrethroid brain concentration and acute motor activity. A pyrethroid mixture was constructed using data from a national probabilistic sampling of care centers (Tulve *et al*., 2006) to determine the identity and relative proportions of the pyrethroids in the mixture. The pyrethroids selected were: permethrin, cyfluthrin, cypermethrin, deltamethrin, and esfenvalerate. Rats were divided into two dose groups and dosed orally at one of two levels with the total pyrethroid concentration in the groups equal to 1.5× (low dose) or 3.7× (high dose) the ED30 (Effective Dose30 - dose resulting in a 30% motor activity decrease) assuming dose-addition (Wolansky *et al*., 2009). This research connects the multiple pyrethroids found in child care facilities with dose, toxicokinetics, target organ concentrations, and acute effects. This is a novel approach to the study of chemical mixtures that links exposure science with toxicology.

Materials and Methods

Identification and Formulation of Pyrethroids for Dosing Mixture

The methods used to select the pyrethroids for this study have been described (Tornero-Velez *et al.*, 2011). Selection was based on a national study (Tulve *et al.*, 2006) of a randomly selected set of 168 child care centers from across the U.S. Data for a set of 15 pyrethroids and pyrethrins from indoor surface wipe floor samples were used. For each center, the fractional surface loading (FSL) of each pyrethroid was determined. For each pyrethroid species, its specific FSL

was averaged across the centers. Many samples had non-detectable pyrethroid levels so the analysis was limited to centers with higher pyrethroid surface loadings. To do this, the centers were sorted by total pyrethroid surface load ($ng_{*}cm^{-2}$) and the top 10% of centers (17 centers) identified. In the 17 centers, six pyrethroids accounted for 96.4% of the total pyrethroid surface loaded mass . Normalized by these 6 pyrethroids, the average FSLs were: cypermethrin (0.288), deltamethrin (0.034), esfenvalerate (0.027), *cis*-permethrin (0.198), *trans*-permethrin (0.324), and β−cyfluthrin (0.129).

Using these values to apportion the pyrethroids, two dose mixture groups were constructed so that the total pyrethroid dose administered to each group was equal to 1.5× (low dose) or 3.7× (high dose) the ED_{30}. These levels were chosen because both doses were expected to result in measurable concentrations of the pyrethroids in all tissues for at least eight hours, and also be disparate enough to result in tissue concentrations that were significantly different (between dose levels) to establish whether the toxicokinetics were dose dependent or independent. In addition, both dose groups were expected to have measurable loss of motor activity but not exhibit the high dose acute primary effects of pyrethroid toxicity. The concentrations of the pyrethroids used in this study and their proportions in the dose mixtures are listed in Table 1. The RP (Wolansky *et al.*, 2006) and the group identity of each pyrethroid (Type I or II) are also listed in Table 1. The relative toxicity of each pyrethroid in the mixtures was calculated by multiplying the percent of total dose for each pyrethroid in the dosing mixtures by its RP. The

resulting order expressed as toxicity equivalents was: permethrin < deltamethrin ≈ esfenvalerate < cypermethrin < β-cyfluthrin.

Chemicals and Standards

All chemicals used in this study were screened for pyrethroid contamination. Acetone, hexanes, ethyl acetate, methanol (Fisher Scientific, Pittsburgh, PA), cyclopentane and acetonitrile (Honeywell Burdick & Jackson, Muskegon, MI) were pesticide grade or better. All water used for sample analysis was 18 MΩ resistance. Primary calibration standards including c*is*-permethrin (99 %), *trans*-permethrin (94 %), deltamethrin (99 %), cypermethrin (98 %), cyfluthrin (98 %) and esfenvalerate (98 %), were purchased from Absolute Standards (Hamden, CT). Ring-labeled (phenoxy-$^{13}C_6$) pyrethroids used as internal standards or surrogates were purchased from Cambridge Isotope Laboratories (Andover, MA) and included: *cis*-permethrin, *trans*-permethrin, cyfluthrin, and cypermethrin. The physical and chemical properties of the pyrethroids used in the dosing solutions have been described previously (Wolansky et al. 2006). Each was provided by its respective manufacturers as follows: permethrin and cypermethrin (FMC Corporation, Philadelphia, PA), deltamethrin and ß-cyfluthrin (Bayer CropScience, Research Triangle Park, NC), and esfenvalerate (Dupont Crop Protection, Wilmington, DE). Corn oil was purchased from Fisher Scientific.

Calibration standards were prepared in reconstituted cleaned extracts of blank tissues and were 1, 10, 25, 50, 75 and 100 $ng_{*}mL^{-1}$. Two additional sets of calibration standards were prepared for samples where the calculated concentration of one or more of the pyrethroids extended beyond

the original calibration curve. These standards were also prepared in extracted and cleaned tissues and ranged from to 0.25 ng/ml to ng$*$mL^{-1}, and from 25 ng$*$mL^{-1} to 1500 ng$*$mL^{-1}. The surrogate standard ($^{13}C_6$ *trans*-permethrin) was added to all tissues prior to extraction. Internal standards ($^{13}C_6$ *cis*-permethrin, $^{13}C_6$ cyfluthrin, and $^{13}C_6$ cypermethrin) were added immediately prior to analysis.

Animals

Male 60 day-old Long Evans rats were purchased from Charles River Laboratories (Raleigh, NC) and allowed to acclimate for a minimum of 4 days in an American Association for the Accreditation of Laboratory Animal Care (AAALAC) approved facility. Rats were housed in pairs in cages (45 cm × 24 cm × 20 cm) lined with heat-treated pine shavings bedding. Temperature, humidity, and light:dark photoperiod were maintained at 21 ± 2^{o}C, 50 ± 10% and 12L:12D, respectively. Feed (Purina Rodent Chow 5001, Barnes Supply Co., Durham, NC) and tap water were provided *ad libitum*.

Experimental

The treatment groups in this study consisted of a corn oil control group and two dose mixture levels. Stock mixtures of the pyrethroids at appropriate concentrations were dissolved in corn oil immediately before administration. The preparation was stirred with intermittent heating (max. 40-45^{o}C) for at least 15 minutes. All doses (Table 1) were delivered orally in corn oil at 1 mL$*$kg^{-1}. Control animals received corn oil (1 mL$*$kg^{-1}) only. At 1, 2, 4, 8, or 24 hours post-

dosing, the motor activity of each animal was assessed over a one hour period, then the animals were sacrificed (2.5, 3.5, 5.5, 9.5, or 25.5 hours post-dosing) and tissue samples were taken. The number of animals in each group at 1, 2, 4, and 8 hours was: 4 (control), 6 (low dose) and 4 (high dose). At 24 hours there were: 2 controls, 4 low dose, and 4 high dose animals). All animal procedures were approved by the U.S. EPA's National Health and Environmental Effects Research Laboratory's Institutional Animal Care and Use Committee.

Motor Activity

The test used for motor activity was conducted as described by Wolansky *et al.* (2006). Briefly, animals were placed in a series of 16 figure-eight mazes, each with 12 photo-transistor/photodiode pairs. Each beam interruption was recorded as an activity count and captured both horizontal and vertical movement. Testing lasted for 1 hour and total motor activity was the sum of horizontal and vertical counts. Total activity of each test animal was calculated as a percentage of the mean activity of the control animals at the relevant time point.

Tissue Collection and Processing

Anesthesia was induced by CO_2 and cardiac blood was taken via heart puncture during exsanguination. Whole blood was collected in 2 mL aliquots and frozen in a methanol/dry ice bath. Whole brain (seperated at the level of the *foramen magnum*), abdominal subcutaneous fat, and liver tissues were collected from each animal, post-mortem. These samples were flash frozen in liquid nitrogen and homogenized. All tissue samples were stored at -80^{o}C.

The procedures used to extract and purify the pyrethroids were similar to that developed for analysis of deltamethrin in rat tissues (Godin *et al.*, 2010). The mass of brain used for each sample was 350-400 mg and was weighed while frozen. Frozen brain, liver, and fat were pulverized in a Spex CertiPrep 6850 freezer/mill (Metuchen, NJ) to form a fine homogenous tissue powder. Blood samples were prepared using the 2 mL aliquots. Prior to extraction, both brain and blood were placed in a glass culture tube and spiked with $^{13}C_6$-*trans*-permethrin that served as a surrogate standard. Samples were vortex extracted for 10 minutes with 5 mL acetone:hexane (2:8, V:V) and then centrifuged at 3000 rpm for 10 min. The organic layer was transferred to another culture tube. The extraction was repeated two additional times with 3 mL acetone:hexane (2:8, V:V). The combined organic extract was dried under nitrogen and redissolved in 1 mL hexane. The extracts were loaded onto 500 mg silica Solid Phase Extraction (SPE) columns (Waters, Inc., Milford, MA), rinsed with 5 mL hexane, and eluted with 5 mL of 6% ethyl acetate in hexane. Eluants were dried under nitrogen, then dissolved in 1 mL methanol:water (9:1, V:V). Internal standards were added and the samples transferred to autosampler vials. All samples were stored at -20^{o}C until analysis.

Fat and liver concentrations were both determined using 250-300 mg samples that were weighed while still frozen. Prior to extraction, both tissue types were placed in culture tubes and spiked with $^{13}C_6$-*trans*-permethrin that served as a surrogate standard. Samples were extracted once as described above and the organic layers were collected. The process was repeated two additional times for the liver with 3 mL acetone:hexane (2:8, V:V) and once with 3 mL acetone:hexane

(2:8, V:V) for the fat. The extracts were filtered through polytetrafluoroethylene filters (0.45 μm) which were then washed with 3.5 mL acetone:hexane (2:8, V:V). The organic phase was dried under nitrogen and dissolved in 3 mL cyclopentane:ethyl acetate (3:7, V:V). Pyrethroids were separated from lipids via Gel Permeation Chromatography (GPC) using an OI Analytical Biobead Prep Column J2 Scientific (College Station, TX) and a (3:7, V:V) cyclopentane:ethyl acetate isocratic mobile phase (5 mL/min). Purified extracts were dried under nitrogen, and dissolved in 2 mL hexane. Remaining lipids were removed by thrice partitioning the extracts with an equal volume of acetonitrile saturated with hexane. The acetonitrile fractions were combined, dried under nitrogen, and dissolved in 1 mL methanol:water (9:1, V:V). Internal standards were added and the samples transferred to autosampler vials. All samples were stored at -20^{o}C until analysis.

Instrument Analysis

Sample analysis was performed using an AB SCIEX model API 4000^{TM} Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS) system configured with a Turbo Ion Spray (TIS). Tables 1 and 2 of the Supplemental Material list the LC conditions and MS/MS settings. Under the conditions used, the *cis-trans* isomers of permethrin were separated, but those isomers were not resolved for cypermethrin or cyfluthrin.

Method Validation and Limits of Detection (LOD), and Quantitation (LOQ)

The mean method recoveries of the pyrethroids from each type of tissue were determined using four replicates each of 10 and 75 ng tissue spikes. The LOD and LOQ for each pesticide were

calculated using sixteen replicates of each tissue type spiked with mixture that contained either: 250, 500, 750, or 1250 pg of each pyrethroid. The samples were processed and analyzed four separate times over a one-week period. Analyte concentrations were pooled to calculate group standard deviations of the estimated concentrations. Using the standard deviations as the dependent variable and the theoretical concentrations as the independent, least squares regression was used to calculate the intercept. The intercept of the equation was defined as S_0 with the LOD approximated by $3 \times S_0$, and the LOQ approximated by $10 \times S_0$ (Taylor, 1987).

Quality Control and Quality Assurance (QC/QA)

A set of matrix-based calibration standards was analyzed immediately before and after each sample set. Quality control procedures included remaking standards when the initial calibration curve data did not fit a first order equation with $r^2 \geq 0.99$. When the slope of the post-run calibration curve differed substantially from the initial, the LC and/or mass spectrometer was cleaned and the samples were re-analyzed. A tissue blank and a mid-level tissue spike sample were each analyzed after each 6 samples in every set.

The acceptable range for surrogate recovery in the samples was set at 80 - 120%. No surrogate corrections were made to calculated concentrations. When sample surrogate recoveries were outside the acceptance criteria, additional tissue was processed and analyzed. If no tissue was available then the result for that sample was not included in any further analyses. Data below the method LOQ were not used.

Data Analysis

All data were processed and analyzed using SAS/STAT software, version 9.2 (SAS Institute, Cary, NC). Prior to analysis the data were stratified by pyrethroid, tissue, time, and dose (and replicate averaged within rat) and tested to determine data distribution type. The assumption of normality was supported by Shapiro-Wilk statistics for a majority (>85%) of the distributions, therefore statistical tests for normally distributed data were used.

Analysis of variance (ANOVA) was used to evaluate method precision and the effect of rats, replicates, and their interaction on the variability in measured residues within each of the tissue, time, and dose groupings. In addition, paired Students t-tests were used to compare replicate measurements, also within tissue, time, and dose groupings.

Based on the report by Wolansky *et al.* (2006), peak tissue concentration (brain, blood liver) was presumed to occur at or before the 2.5 hour time point. The assumption was verified by t-test comparisons of concentrations at 2.5 and 3.5 hours and the 2.5 hour time point was used to compare tissue uptake by pyrethroid and dose group, and, as the first time point in calculation of the elimination constants. Predicted tissue-blood partition coefficients for all pyrethroids were calculated using the octanol-water partition coefficient based algorithm developed by Poulin and Krishnan (1995).

Elimination constants were estimated using data from hours 2.5 through 9.5 inclusive. Data from the 25.5 hour time point were excluded due to a high percentage of samples with concentrations below the LOQ. First order elimination was assumed and times of sample collection were regressed against transformed residue concentrations (natural logarithmic) grouped by dose and tissue. A generalized linear model (PROC GLM) was used to test each pyrethroid in each tissue for heterogeneity between dose groups and to determine whether each elimination slope was statistically different than zero. Where the slope was not different than zero, that dose group was not used in calculating the pyrethroid half-life. Where the regression slopes of the dose groups were homogeneous and both different than zero, half-lives ($t_{1/2}$) and corresponding confidence bounds of individual pyrethroids in each tissue were estimated using dose-adjusted and dose-pooled data. Then, the heterogeneity of half-lives between individual pyrethroids was evaluated by tissue, also using PROC GLM.

Relative proportions were calculated for individual pyrethroid concentrations at 2.5 hours. To do this, individual pyrethroids were normalized to equal a percentage of the total pyrethroid load for that tissue and animal. The expected contribution of each pyrethroid equaled the percent pyrethroid of the total pyrethroid dose as listed in Table 1. Tissue-to-blood concentrations were calculated by dividing each tissue concentration (within an animal) by the corresponding blood concentration.

Means and standard deviations of motor activity of both dose were calculated at each time point and a four parameter logistic, or sigmoidal E_{max} model (Dmitrienko *et al.*, 2007) was used to relate variability in motor activity to total pyrethroid brain concentration:

$$Y = E_{max} + \frac{(E_{min} - E_{max})}{1 + \left(X/EC_{50}\right)^{h}}$$

Where Y is the control-normalized response, E_{max} and E_{min} represent the upper and lower bounds of the response, X is the total pesticide concentration, EC_{50} represents the concentration at which the median response is attained (or inflection point of the curve), and h is the hill coefficient (slope). Pyrethroids were modeled individually, then by Type (I and II), and finally as a group. Only data from rats with brain concentrations above the LOQ were included and data from the final time point were not used. Statistical significance was assigned at $p \leq 0.05$.

The hypothetical relative percent contributions of individual pyrethroids in brain tissue in reducing motor activity were estimated as follows:

$$RPC_{ij} = \frac{x_{ij} \times RP_i}{\sum_{i=1}^{n} x_{ij} \times RP_i} \times 100$$

Where RPC_{ij} is the relative percent contribution of pyrethroid i to measured response at hour j, x_{ij} is the brain tissue concentration of pyrethroid i at hour j, and RP_i represents the RP of pyrethroid i (Table 1), assumed to be constant across time. PROC REG was used to calculate the rate of change (in percent contribution) over time for each pesticide. Statistical significance was assigned at $p \leq 0.05$.

Results

Method Validation; LOD / LOQ

The pooled percent recoveries (± standard deviation) of all pyrethroids from each tissue type were as follows: blood, 82 ± 7; brain, 92 ± 7; fat, 84 ± 7; and liver, 85 ± 6. The calculated LOQ for all pyrethroids in brain, liver, and fat were less than 3 $ng_{*}g^{-1}$ and less than 300 $pg_{*}mL^{-1}$ in blood.

All samples from pyrethroid dosed animals had quantifiable concentrations of all pyrethroids, at both 2.5 and 3.5 hour time points. Measurable concentrations of all pyrethroids were present in all fat samples at all time points. At 5.5 hours, more than 50% of the liver samples (low dose group) had concentrations of *trans*-permethrin (67%) and β-cyfluthrin (83%) that were below the LOQ. At 9.5 hours, all liver concentrations of *trans*-permethrin and β-cyfluthrin, and 66% of deltamethrin concentrations, were below the LOQ. Esfenvalerate in blood (low dose group; 83% ≤ LOQ) at 9.5 hours was the only other time point/analyte where more than 50% of the concentrations were below the LOQ. As stated earlier, data from the 25.5 hour collection were not used due to a lack of quantifiable data from tissues other than fat.

Tissue Uptake

At the initial time point (hour 2.5), higher doses generally resulted in higher mean tissue concentrations for each pyrethroid (Table 2). The dose group differences were statistically

significant for all pyrethroids in all tissues except: esfenvalerate in brain and cypermethrin, deltamethrin, trans-permethrin and β-cyfluthrin in fat. Comparison of dose-adjusted tissue concentrations (tissue concentration / dose) at hours 2.5 and 3.5 indicated maximum uptake occurred at or before the 2.5 hour time point.

After adjusting for dose, concentrations were not statistically different between the two dose levels at 2.5 hours for any tissue except blood, where the mean concentrations of all pyrethroids in the high dose group remained higher than the low dose group. These differences were significant for cypermethrin ($p=0.014$), deltamethrin ($p=0.019$), *trans*-permethrin ($p=0.002$), and β-cyfluthrin ($p=0.025$), but not for esfenvalerate ($p=0.083$), or *cis*-permethrin ($p= 0.080$). At the 3.5 hour time point, t-tests of dose adjusted concentrations showed that these differences in blood had disappeared.

At 2.5 hours the most notable difference in relative tissue concentration vs. relative dose concentration was *trans*-permethrin (31% of total administered) at ≤ 13% of the total pyrethroid load in all tissues. Using Tables 1 and 2, no consistent relationships appeared between relative tissue concentrations and relative dose concentration, except in the brain. All pyrethroids in brain, except *cis*-permethrin (48%) and *trans*-permethrin (5%), were consistent with their relative dosing proportions. However, when *cis* and *trans* isomers are summed, the relative proportion of permethrin is also close to its percentage of 52% in the dosing solution.

The theoretical partition coefficients (Poulin and Krishnan, 1995) predicted high tissue-to-blood ratios and little variation for all pyrethroids with coefficients ranging from 21 - 22 (liver-to-blood), 434 – 438 (fat-to-blood), and 28.1 – 28.4 (brain-to-blood). As seen in Table 3, the tissue-to-blood ratios for all tissues at the initial time point of this study indicate the partitioning was much lower and more varied than predicted. Mean brain-to-blood ratios in the high dose group were all less than those of the low dose group and the ratios for cypermethrin, deltamethrin, and esfenvalerate were all less than 1. Similar dose dependent differences were observed for all pyrethroids for the fat-to-blood ratios and, as in the brain, the fat-to-blood ratios of cypermethrin, deltamethrin, and esfenvalerate were all lower than the permethrins and β-cyfluthrin. The dose related dependency was repeated in liver-to-blood ratios (initial time point), with the exception of cypermethrin. As with the other tissues, the liver-to-blood ratio of β-cyfluthrin was nearer the permethrins than to cypermethrin and deltamethrin, the two most similar type II pyrethroids. Excepting low dose deltamethrin, the liver-to-blood ratios were all ≥ 1.

Tissue Elimination

The calculated elimination constants and relevant statistics for each pyrethroid (by dose group and tissue) are provided in the Supplemental Material. The half lives, estimated using pooled high and low group data (where applicable) for blood, brain and liver are located in Table 4. Half-lives in fat were not calculated because only one of the rate constants (deltamethrin; low dose, $p= 0.04$) was statistically different from zero. The half-lives were less than 2 hours in blood, from 3 to 6.2 hours in brain and 2.3 hours or less in liver. The within-tissue half-lives of

the pyrethroids were not statistically different in blood or liver. In brain, the half-life of β-cyfluthrin was statistically less than all other pyrethroids except *trans*-permethrin. The half-life of cypermethrin was statistically different than the two pyrethroids with the longest half lives; *cis*-permethrin and esfenvalerate, as well as β-cyfluthrin. Finally, the half-life of *trans*-permethrin was statistically different from esfenvalerate.

Motor Activity and Brain Concentration of Pyrethroids

Both mixture doses evoked mild clinical signs of pyrethroid toxicity. Restlessness and episodes of non-locomotor behaviors such as scratching, pawing, burrowing and body shakes were observed along the initial 2-3 hours after dosing but there were no signs of high-dose pyrethroid symptoms such as hyper-salivation, whole body tremors and choreoathetosis in any animals. The lack of a full expression of type-specific signs of pyrethroid toxicity was consistent with the mild pyrethroid-specific signs of neurotoxicity observed in a prior pyrethroid mixture study where total doses were higher (Wolansky et al., 2009).

The results of motor activity (as a percentage of control), for each time period are presented in Figure 1. The time period of peak effect (largest mean decrease) for the low dose group was 1-2 hours post dosing. The peak effect for the high dose group occurred during the 2-3 hour interval. After the peak effect, motor activity of both groups increased over the next two time periods, after which no additional increase was apparent.

Excepting esfenvalerate, brain concentrations of individual pyrethroids fit the sigmoidal E_{max} model to predict decreases in motor activity. Similarly, models of Type I and II groups were both statistically significant. Therefore, the final model (Figure 2) used the summed concentration of all pyrethroids. The F-test statistic for the model was significant ($p<0.0001$) with estimated high and low motor activity thresholds of 113 % ($p<0.0001$) and 34 % ($p=0.16$), respectively, of controls. The slope of -2 (between the threshold values) indicated a 2 ng/gm increase in total pyrethroid brain concentration resulted in a 1% decrease in motor activity and the estimated EC_{50} was 217 $ng_{*}g^{-1}$ ($p= 0.0006$).

Excluding *trans*-permethrin, all regression slopes of estimated relative percent contribution (to total pyrethroid concentration) were statistically significant ($p<0.05$). The slopes for cypermethrin and cyfluthrin were negative while the slopes of esfenvalerate, deltamethrin, and *cis*-permethrin where positive. At 2.5 hours the estimated relative percent contributions were: β-cyfluthrin, 42%; cypermethrin, 21%; deltamethrin, 13%; esfenvalerate, 13%; *cis*-permethrin, 8%; *trans*-permethrin 1%. At 9.5 hours these values had changed to: β-cyfluthrin, 23%; cypermethrin, 17%; deltamethrin, 17%; esfenvalerate, 26%; *cis*-permethrin, 18%; *trans*-permethrin 1%.

Discussion

Implications of Study Design

The use of low dose, empirically based, pyrethroid mixtures to estimate toxicokinetic parameters is a significant advance in connecting exposure science with toxicology. Although simplified in its use of a single exposure pathway and single dose design, this study incorporated the concept of environmentally-relevant cumulative dose in evaluating tissue uptake and elimination. Use of a mixture provided direct comparative data for uptake and elimination of each pyrethroid in each tissue. Simultaneous dosing with five pyrethroids that included representatives of Types I and II helped to address whether non target site kinetic differences were important correlates of motor activity. Although not assessed directly, potential interactions between the pyrethroids were inherent in this study design and reflect interactions expected from actual exposures.

Kinetics

Liver

Relative to dose, liver concentrations of *trans*-permethrin at 2.5 hours were much lower than *cis*-permethrin and the other pyrethroids (Tables 1 and 2). This result was unexpected due to the similarity of all pyrethroid half lives in both blood and liver (Table 4), and the narrow range of predicted pyrethroid liver-to-blood partition coefficients (21 – 22). It is possible that the *trans*-permethrin concentrations resulted from increased binding of *trans*-permethrin by hepatic or circulatory proteins, but literature reports suggest that covalent binding rates of pyrethroids by hepatic proteins are generally low. An *in vivo* study of (Hoellinger *et al*., 1983) found that less than 6% each of the type I pyrethroids cismethrin and bioresmethrin were covalently bound to rat liver proteins. In addition, Catinot *et al*. (1989) found the covalent binding in homogenates of rat liver to be less than 10% for the type II pyrethroids deltamethrin and cypermethrin.

Unfortunately, a literature review revealed no reports of *cis* or *trans*-permethrin hepatic protein binding.

The lower *trans*-permethrin concentrations may have resulted from significant levels of intestinal metabolism, or less absorption. Crow *et al.* (2007) and Nakamura *et al.* (2007) also reported that intestinal and hepatic cytosol carboxylesterases could cleave the ester linkage of *trans*-permethrin but not deltamethrin or *cis*-permethrin. However, Nakamura *et al.* (2007) reported rat intestinal hydrolysis rates to be only 33% of the liver and Crow *et al*. (2007) estimated that intestinal hydrolysis accounted for only 2.5% of the total hydrolytic activity for *trans*-permethrin in the rat. The dose adjusted concentration (dose groups pooled) of β-cyfluthrin in the liver at 2.5 hours was lower than that of *cis*-permethrin and cypermethrin. This was unexpected because β-cyfluthrin is a type II pyrethroid, which differs from cypermethrin by addition of fluorine at the 4 position of the alcohol moiety and enrichment of 2 pairs of diasteromers, specifically, (S)α, 1(R)-*cis*- + (R)α, 1(S)-*cis*-; (S)α, 1(R)-*trans*- + (R)α, 1(S)-*trans*-. Unfortunately, there appear to be no published reports describing cyfluthrin toxicokinetics.

Single pyrethroid *in vitro* studies have shown pyrethroid specific differences in rates of metabolism and the importance of different pathways. In general, the predicted order of clearance rates for pyrethroids (Soderlund *et al*., 1977) in mixed esterase/oxidase systems is: *trans* esters (unsubstituted at primary alcohol) > *cis* esters (unsubstituted at primary alcohol) > *trans* esters (substituted at α carbonyl) > *cis* esters (substituted at α carbonyl) and it is not clear

why the liver half lives of all pyrethroids in the current study were approximately equal. As Soderlund used mouse microsomal preparations it is possible that the disparity is due to *in vivo* vs. *in vitro* or species related differences. Literature reports of *in vivo* or *in vitro* metabolic rates determined using a pyrethroid mixture are largely absent. However, there is some evidence of interaction between the *cis*/*trans* isomers of permethrin. Scollon *et al.* (2009) demonstrated an *in vitro cis*/*trans* interaction between permethrin isomers whereby the clearance of the *trans* isomer was slowed significantly in the presence of the *cis* isomer while elimination of the *cis* isomer was slowed slightly.

Clearly, more in vitro work evaluating interactions of pyrethroids and their *cis*/*trans* isomerism in esterase/oxidative mediated metabolic systems would be useful. These data suggest that intestinal metabolism may explain some of the differences between *cis*- and *trans*-permethrin toxicokinetics, but other mechanisms, such as differences in absorption, may also play a role. Further research needs to be performed to determine whether the loss of the *trans*-isomer extends to cypermethrin and β-cyfluthrin as both are also *cis*/*trans* mixtures.

Blood

The transient dose-dependent concentration of the pyrethroids in the blood at 2.5 hour indicates non-linear absorption kinetics. The non-linearity may be attributed to absorption rather than metabolism because of the consistency between the estimated elimination half-lives of the pyrethroids (Table 4) in blood. The pyrethroids in the high dose group may have been absorbed more efficiently because they were more concentrated in the corn oil vehicle. For example,

Wolansky *et al*. (2007) found that reducing the volume of the corn oil vehicle five-fold, increased the potency of orally administered bifenthrin by a factor of two. Further, in the current study, all pyrethroids showed this dose related difference regardless of their relative concentration in the mixture, suggesting the effect was related to the total pyrethroid concentration, rather than the concentration of the individual pesticides. Although Godin *et al.* (2010) examined the bioavailability of deltamethrin and found it to be dose independent (0.3 and 3 mg/kg), the total pyrethroid doses in this study (Table 3) were higher than the doses of deltamethrin in the cited study.

When the two dose groups were pooled at 2.5 hours, the liver-to-blood ratio of deltamethrin (0.88 ± 0.24) was similar to the 1:1 ratio reported by Mirfazaelian *et al*. (2006), but higher than the 2:7 ratio seen by Godin *et al*. (2010). Published reports with this type of *in vivo* data for the other pyrethroids are lacking. In the current study, the liver-to-blood ratios of all pyrethroids were 1:1 or greater and t ½'s of all pyrethroids in blood were not different than in liver. In addition, the liver-to-blood ratio of deltamethrin was significantly lower ($p \leq 0.05$) than all other pyrethroids except cypermethrin. As stated earlier, differences in octanol-water based partition coefficients estimations are very small and do not explain differences in liver-to-blood ratios. Alternatively, differences in blood protein binding may provide an explanation, but studies of the capacity of serum proteins to bind permethrin (Abu-Qare and Abou-Donia 2002), cismethrin, and bioresmethrin (Hoellinger *et al*. 1985) suggests that this pathway is not significant.

Fat

Fat functioned effectively as a sink for the pyrethroids in this study as all were more concentrated in fat than other tissues. The rapid uptake and lack of elimination in fat throughout the study time course was expected since pyrethroids are lipophilic and lipases are not thought to be important in the metabolism of pyrethroids (Crow *et al.*, 2007). The results are consistent with observed slow elimination rates *in vivo* studies of deltamethrin (Godin *et al.*, 2010; Mirfazaelian *et al.*, 2006). Assuming fat comprises 7% of total rat body mass (Schoeffner *et al.*, 1999), the percent of each pyrethroid in fat was less than 2% of its administered dose. The uptake by adipose tissue is low compared to other lipophillic chemicals such as dioxin (Diliberto *et al.*, 1996). Because the octanol-water partition coefficients of pyrethroids and dioxin are comparable, the difference in adipose tissue distribution is likely due to a higher rate of pyrethroid metabolism in other tissues.

Excepting low dose esfenvalerate, concentrations in fat did not increase during the study (Supplemental Table 3). Therefore, the data did not support significant redistribution of the pyrethroids from other tissues to the fat. In addition, the long half-life of the pyrethroids in the fat and rapid metabolism in liver and blood precluded adipose tissue from being a secondary source for measurable redistribution to other tissues.

Brain Kinetics and Motor Activity

High-low dose related differences in blood concentrations at 2.5 hours were not reflected in the brain. In addition, all brain-to-blood ratios were much lower than predicted by their partition

coefficients, suggesting a limitation in crossing the blood brain barrier that affected all study pyrethroids. Brain-to-blood ratios did not segregate into Type I and Type II pyrethroids (Table 3), nor did they appear to be a function of the absolute concentration in the blood or differences in brain elimination rates. Ultimately crossing the blood-brain barrier by each pyrethroid is likely determined by its tertiary structure and a more sophisticated analysis of pyrethroid structure activity relationships would be useful.

The observed differences in $t_{1/2}$ of the pyrethroids in the brain pyrethroid did not sort according to the rates predicted from oxidative and hydrolytic activity in liver or serum. Interestingly, Ghiasuddin and Soderlund (1984) found the specificity of mouse brain esterases were different than the mouse liver esterases and the brain esterases demonstrated activity toward *trans*-permethrin and fenvalerate, but were relatively inactive in the hydrolysis of *cis*-permethrin or deltamethrin. Potentially, this may also occur in the rat.

An important finding of this study was the similarity between the relative proportion of each pyrethroid in the brain and its percentage in the dosing mixture (after summing *cis*- and *trans*-permethrin isomers) at the 2.5 hour time point. Therefore, administered dose predicted relative brain concentrations at a time point near the peak effects of individual pyrethroids on motor activity as noted by Wolansky *et al.* (2009). This may simplify efforts to model low dose cumulative risk from these pyrethroids since it appears metabolic variations in other tissues were offset by differences in partitioning from blood to brain. The similarity of the relative

proportions of the pyrethroids in the brain and dosing solution implies that differences in RP of each pyrethroid are not a function of toxicokinetic differences. Rather, their relative impact on motor activity is likely dependent on specific interactions with ion channels.

The decreases in motor activity of 34% (low dose, 1.5x ED_{30}) and 67% (high dose, 3.7x ED_{30}) at the times of peak effect (1-2 hours low dose group, 2-3 hours high dose group) agree with the decrease of approximately 40% and 60% predicted by Wolansky *et al.* (2009) and therefore support those researchers pyrethroid dose additive effects model. The occurrence of greatest mean decrease in motor activity at 1-3 hours is consistent with the time to peak effect for the individual pyrethroids of 1.5 to 2.0 hours also reported by Wolansky *et al.* (2009). That studies' finding of a return to normal motor activity function several hours post dosing, also occurred in this study (Figure 2). In addition, the minimum dose threshold, linear dose response range, and asymptotic nature of the maximum response in the Wolansky model were apparent in this studies 4 parameter model of motor activity and pyrethroid brain concentration (Figure 2). The brain concentrations of the pyrethroids in this study provide the toxicokinetic data underlying the dose additive pyrethroid effects model and assist the interpretation of that model by showing the relationship between response and the concentration of this mixture of pyrethroids in the brain. Further, the fit of the 4 parameter model ($p < 0.001$) and the overlap of the data from the two dose groups at 4 time points indicates that the pyrethroid brain concentration is the important determinate of motor activity and that dose and time predict brain concentration and therefore effect. This supports research by Scollon *et al.* (2011) who found a similar relationship between bifenthrin dose, brain concentration, and motor activity.

Although the contribution of individual pyrethroids to loss of motor activity could not be directly measured, the hypothetical relative contributions suggest that β-cyfluthrin would be the greatest contributor to loss of motor activity at 2.5 hours, and *cis*/*trans*-permethrin the least. At 9.5 hours individual pyrethroids would contribute similarly to total toxicity. It is important to note that these estimates should be interpreted cautiously as Wolansky *et al.* (2006) determined RP's in single chemical studies using multiple doses at the time of peak effect. Additional data on RP from single chemical, time-series studies would provide insight into the validity of combining RP and tissue concentration across time in this model.

Conclusions

Environmentally relevant dosing schemes may be used as a conceptual model in future studies of chemical mixtures. In this study it added a practical component to a toxicokinetic study that would be useful in cumulative risk assessments.

Distribution of the pyrethroids to all tissues was rapid with maximum concentrations likely at, or before, the 2.5 hour time point. Excepting blood at 2.5 hours, relative tissue concentrations were dose independent. In liver, blood, and fat there was no apparent relationship between uptake and pyrethroid relative dose concentrations, lipid solubility, or structural groupings. The most notable results were the very low proportionate tissue concentrations of *trans*-permethrin.

Initial concentrations of cypermethrin, deltamethrin, β-cyfluthrin, and esfenvalerate in brain reflected their relative proportion in the dosing solution. When concentrations *cis* and *trans*-permethrin were summed, the relative brain permethrin concentration also reflected dose.

The similarity of the elimination half-lives of individual pyrethroids in blood and liver was unexpected considering *in vitro* differences of previous studies. In brain, half-life differences between the slowest and most rapidly cleared pyrethroids were unrelated to relative dose concentration or obvious differences in chemical structure.

Brain concentration was predicted by dose and time, and pyrethroid brain concentration predicted motor activity. Relative uptake and elimination of the pyrethroids in brain did not correspond with the Type I and Type II groupings or the RP's noted in previous studies. Therefore, differences in pyrethroid kinetics are insufficient to explain behavioral responses observed in previous studies.

Supplementary Data: The supplementary material consists of four tables. Table 1 lists the chromatograph and mass spectrometer settings used for all analysis while analyte specific mass spectrometer settings are provided in Table 2. Estimated pyrethroid elimination constants and associated test statistics for each dose group and each tissue are presented in Table 3.

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Table 1. Concentration of pyrethroids in low and high dose mixtures.

Pyrethroid	Type[a]	Low Dose (mg·kg^{-1})	High Dose (mg/·kg^{-1})	% of Total Dose	Relative Potency[b]
cypermethrin	II	3.2	7.9	29	0.235
deltamethrin	II	0.4	0.9	3	1.000
esfenvalerate	II	0.3	0.7	3	2.092
cis-permethrin	I	2.3	5.7	21	0.059[c]
trans-permethrin	I	3.5	8.6	31	
β-cyfluthrin	II	1.5	3.5	13	1.136
Total Dose		11.2	27.4		

[a] Based on presence or absence of a cyano group at the α-carbon of the alcohol moiety, and the high dose acute physiological effects.

[b] Potency based on ED_{30} for effect on motor activity relative to deltamethrin as the index pyrethroid (Wolansky *et al.*, 2006).

[c] Relative potencies of *cis*/*trans*-permethrin isomers were not determined independently.

Table 2. Tissue concentration by dose group at 2.5 hours after dosing.

			Mean tissue concentration ± standard error (blood $ng_{*}mL^{-1}$; brain, fat, liver $ng_{*}g^{-1}$)					
tissue	**dose group**	**n**	**cypermethrin**	**deltamethrin**	**esfenvalerate**	***cis*-permethrin**	***trans*-permethrin**	**β-cyfluthrin**
blood	Low	4	141[a] ± 21	25[a] ± 4	12[a] ± 2	52[a] ± 9	5[a] ± 2	15[a] ± 3
	High	4	647 ± 77	115 ± 14	52 ± 12	196 ± 19	46 ± 4	87 ± 19
brain	Low	6	68[a] ± 9	10[a] ± 1	7 ± 1	122[a] ± 16	11[a] ± 2	28[a] ± 4
	High	3	143 ± 15	19 ± 4	10 ± 3	230 ± 31	32 ± 6	58 ± 9
fat	Low	6	565 ± 112	83 ± 11	72[a] ± 9	512[a] ± 77	216 ± 37	233 ± 48
	High	3	1076 ± 239	158 ± 51	146 ± 34	908 ± 165	406 ± 93	466 ± 111
liver	Low	6	140[a] ± 27	26[a] ± 4	25[a] ± 4	95[a] ± 15	18[a] ± 6	38[a] ± 9
	High	3	400 ± 26	71 ± 5	65 ± 5	219 ± 10	64 ± 18	139 ± 14

[a] Mean concentration statistically different ($p \leq 0.05$) than high dose concentration

Table 3. Tissue-to-blood pesticide concentration ratios

Dose Group	hour	cypermethrin	deltamethrin	esfenvalerate	*cis*-permethrin	*trans*-permethrin	β-cyfluthrin
Mean Brain-to-Blood Concentration Ratio (± standard error of the mean)							
Low	2.5	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	2.6 ± 0.4	3.2 ± 1.1	2.1 ± 0.4
High	2.5	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	1.2 ± 0.6	0.7 ± 1	0.7 ± 0.4
All	3.5	0.5 ± 0.1	0.5 ± 0.1	0.8 ± 0.4	3.3 ± 1.3	3 ± 1.4	2.4 ± 0.9
	5.5	1.7 ± 0.2	3.4 ± 1.0	4.6 ± 0.9	16.3 ± 5	19.5 ± 12.3	7.4 ± 1.5
	9.5	2.2 ± 0.6	4.3 ± 1.6	3.4 ± 1.2	49.2 ± 15.2	15.8 ± 2.7	7.4 ± 1.9
	25.5	3.4 ± 1.5	NA[1]	NA	26.5 ± 12.3	NA	NA
Mean Fat-to-blood Concentration Ratio (± standard error of the mean)							
Low	2.5	4 ± 1	4 ± 0	6 ± 1	11 ± 2	58 ± 20	17 ± 4
High	2.5	2 ± 0	1 ± 0	3 ± 0	5 ± 1	9 ± 2	6 ± 1
All	3.5	6 ± 2	8 ± 4	16 ± 9	9 ± 1	38 ± 11	36 ± 18
	5.5	33 ± 7	44 ± 10	69 ± 13	136 ± 58	372 ± 120	187 ± 36
	9.5	134 ± 41	142 ± 30	119 ± 42	1056 ± 424	1476 ± 507	901 ± 232
	25.5	1963 ± 868	462 ± 63	NA	3153 ± 975	1895 ± 729	6512 ± 2683
Mean Liver-to-Blood Concentration Ratio (± standard error of the mean)							
Low	2.5	1.0 ± 0.2	1.0 ± 0.1	2.0 ± 0.2	1.8 ± 0.2	2.8 ± 0.7	2.1 ± 0.3
High	2.5	1.6 ± 0.1	0.6 ± 0	1.3 ± 0.2	1.1 ± 0.1	1.4 ± 0.3	1.7 ± 0.2
All	3.5	1.4 ± 0.1	0.7 ± 0	2.3 ± 0.7	1.3 ± 0.1	1.9 ± 0.4	7.6 ± 5.6
	5.5	1.2 ± 0.1	1 ± 0.1	3.4 ± 1.5	2.6 ± 0.7	9.4 ± 5	2.2 ± 0.8
	9.5	2.2 ± 0.1	4.3 ± 0.3	3.4 ± 0.7	49.2 ± 0.7	15.8 ± 2	7.4 ± 0.8
	25.5	0.4 ± 0.1	NA	NA	NA	NA	NA

1 insufficient data were available to calculate a ratio.

Table 4. Estimated pyrethroid half-lives ($t_{1/2}$) in rat tissues.

Tissue	Pyrethroid	$t_{1/2}$ (hours)	LCL[a] (hours)	UCL[a] (hours)
Blood	cypermethrin	1.5	1.2	2.1
	deltamethrin	1.5	1.2	2.0
	esfenvalerate	1.6	1.2	2.5
	cis-permethrin	1.3	1.1	1.8
	trans-permethrin	1.2	0.9	1.8
	β-cyfluthrin	1.3	1.0	1.8
Brain	cypermethrin	2.7[b]	2.3	3.4
	Deltamethrin	3.9[b]	2.8	6.3
	esfenvalerate	6.2[bcd]	3.4	41.1
	cis-permethrin	5.5[bcd]	4.3	7.7
	trans-permethrin	2.7	2.1	3.9
	β-cyfluthrin	2.0[c]	1.7	2.4
Liver	cypermethrin	1.6	1.3	2.2
	deltamethrin	1.9	1.4	2.8
	esfenvalerate	1.8	1.4	2.5
	cis-permethrin	1.6	1.3	2.1
	trans-permethrin	2.3	1.4	6.1
	β-cyfluthrin	1.3	1.0	2.1

[a] Estimated lower (LCL) and upper (UCL) 95% confidence limits of the mean $t_{1/2}$.

[b] Half-life is statistically different ($p \leq 0.05$) than β-cyfluthrin

[c] Half-life is statistically different ($p \leq 0.05$) than cypermethrin

[d] Half-life is statistically different ($p \leq 0.05$) than *trans*-permethrin

Figure 1. Time course of changes motor activity after an oral dose of a pyrethroid mixture.

Figure 2. Four parameter model of brain pyrethroid concentration and motor activity.

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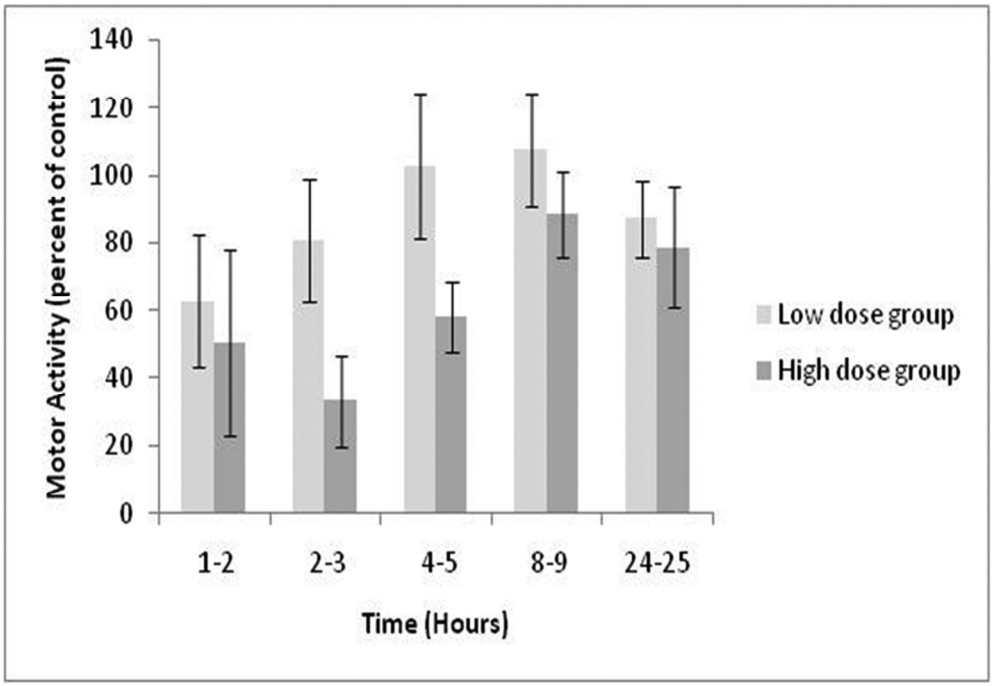

Figure 1. Time course of changes motor activity after an oral dose of a pyrethroid mixture.
88x61mm (300 x 300 DPI)

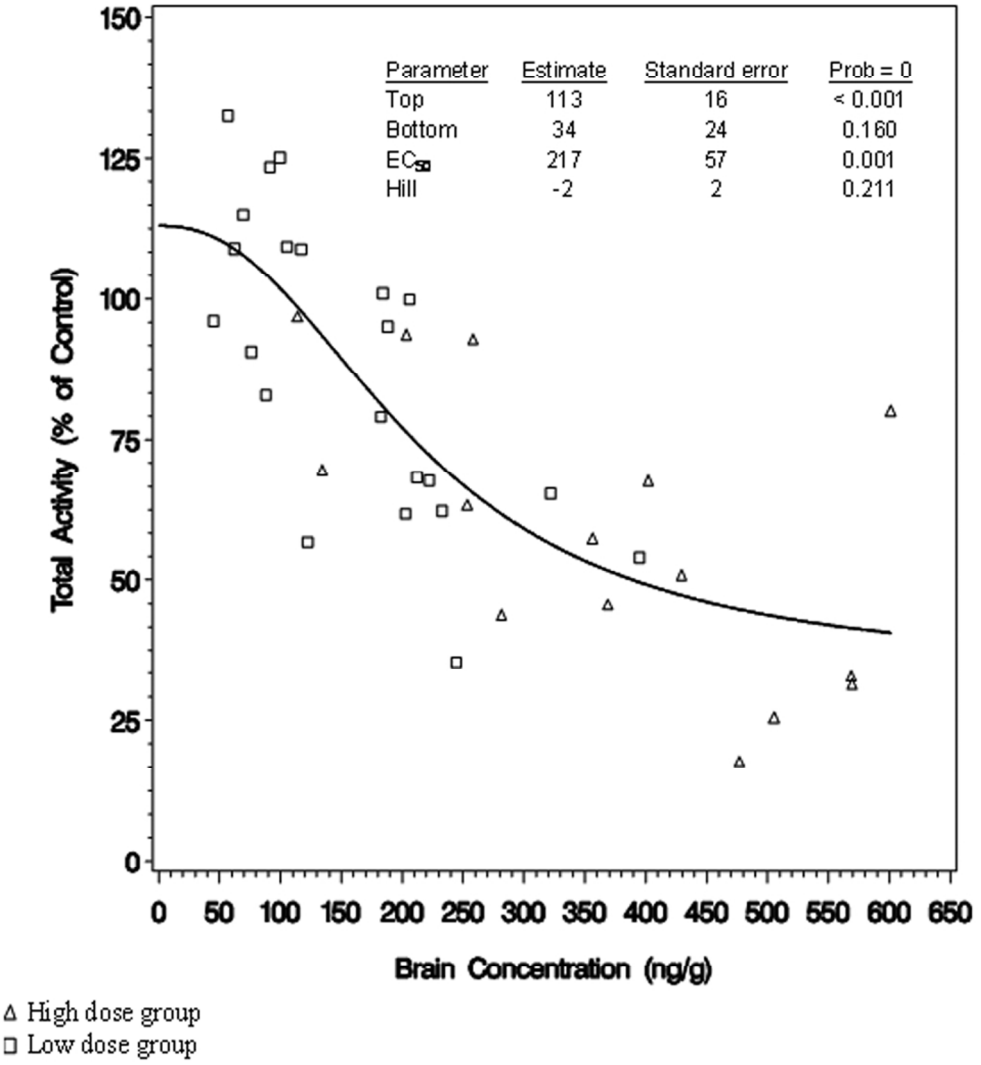

Figure 2. Four parameter model of brain pyrethroid concentration and motor activity.
88x96mm (300 x 300 DPI)