Environmentally relevant pyrethroid mixtures: A study on the correlation of blood and brain concentrations of a mixture of pyrethroid insecticides to motor activity in the rat

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ABSTRACT

Human exposure to multiple pyrethroid insecticides may occur because of their broad use on crops and for residential pest control. To address the potential health risk from co-exposure to pyrethroids, it is important to understand their disposition and toxicity in target organs such as the brain, and surrogates such as the blood when administered as a mixture. The objective of this study was to assess the correlation between blood and brain concentrations of pyrethroids and neurobehavioral effects in the rat following an acute oral administration of the pyrethroids as a mixture. Male Long-Evans rats were administered a mixture of β-cyfluthrin, cypermethrin, deltamethrin, esfenvalerate and cis- and trans-permethrin in corn oil at seven dose levels. The pyrethroid with the highest percentage in the dosing solution was trans-permethrin (31% of total mixture dose) while deltamethrin and esfenvalerate had the lowest percentage (3%). Motor activity of the rats was then monitored for 1 h. At 3.5 h post-dosing, the animals were euthanized and blood and brain were collected. These tissues were extracted and analyzed for parent pyrethroid using HPLC-tandem mass spectrometry. Cypermethrin and cis-permethrin were the predominate pyrethroids detected in blood and brain, respectively, at all dosage levels. The relationship of total pyrethroid concentration between blood and brain was linear (r = 0.93). The pyrethroids with the lowest fraction in blood were trans-permethrin and β-cyfluthrin and in brain were deltamethrin and esfenvalerate. The relationship between motor activity of the treated rats and summed pyrethroid blood and brain concentration was described using a sigmoidal E_{max} model with the Effective Concentration$_{50}$ being more sensitive for brain than blood. The data suggests summed pyrethroid rat blood concentration could be used as a surrogate for brain concentration as an aid to study the neurotoxic effects of pyrethroids administered as a mixture under the conditions used in this study.

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1. Introduction

Pyrethroids are a class of synthetic insecticides with a structure based on the botanical pyrethrins. The structural commonality of pyrethrins and pyrethroids are the acid and alcohol moieties that are linked by an ester group. Another common feature of pyrethrins and pyrethroids is that they may have 1–3 chiral carbons and are isomeric (Soderlund et al., 2002). Consequently, there may be differences in the metabolism of the isomers (hydrolysis vs. oxidation) as well as insecticidal potency (Miyamoto, 1990; Soderlund et al., 2002). In general, pyrethroids tend to have greater insecticidal activity and are less susceptible to

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environmental degradation than pyrethrins (Bradberry et al., 2005).

The many uses of pyrethroids including agricultural, commercial and residential pest control, and veterinary and medical practices (Amweg et al., 2005; Bradberry et al., 2005) may lead to human exposure. Residues of multiple pyrethroids are detected in surface wipe samples collected from child care centers (Tulve et al., 2006) and residential homes (Stout et al., 2009), indoor air and dust samples (Rudel et al., 2003), and on fruits and vegetables (USDA, 2014). A biological monitoring study in Canada of a metropolitan populace exposed to pyrethroids in the diet found that the study population was mainly exposed to permethrin and cypermethrin (Fortin et al., 2008). From their use in agriculture and pest management, humans can be exposed to multiple pyrethroids (Fortin et al., 2008; Heudorf and Angerer, 2001; Stout et al., 2009; Tulve et al., 2006; Tornero-Velez et al., 2012).

Most pyrethroids are commonly classified into two groups, termed Type I and II, based on chemical structure and neurotoxic effects in rodents (Soderlund et al., 2002; Soderlund, 2012). Type I pyrethroids (e.g., permethrin, bifenthrin) contain either a primary or secondary alcohol, and their neurotoxic syndrome hallmark is tremor. Type II pyrethroids (e.g., cypermethrin and deltamethrin) are primary alcohols with a cyano group on the alpha-carbon of the alcohol. Oral administration of exposure to Type II pyrethroids results in choreoathetosis and salivation.

The mode of action of Type I and II pyrethroids appears to be binding to and disruption of voltage-gated sodium channels in targeted neurons (Soderlund et al., 2002; Soderlund, 2012). Wolansky et al. (2006) conducted extensive dose-response assays using motor activity as a neurobehavioral endpoint in rats exposed to eleven pyrethroids administered by oral gavage individually; all compounds produced a dose-related decrease in motor activity, but the potency was variable, dependent upon the pyrethroid administered. In a later study, Wolansky et al. (2009) reported dose-additive effects in rats exposed to an eleven chemical mixture of Type I and II pyrethroids for a decrease in motor activity. An in vitro study by Cao et al. (2011) reported dose-additive effects in inducing sodium influx in primary cultures of murine cerebro-cortical neurons with this same pyrethroid mixture. However, based on several other in vivo and in vitro studies, Breckenridge et al. (2009) proposed that Type I and Type II compounds have separate mechanisms of neurotoxicity. Nevertheless, the U.S. Environmental Protection Agency in 2011 determined the pyrethroids share a common mechanism of action for a cumulative risk assessment (US EPA, 2011).

The nervous system is the primary target tissue for the neurotoxicity produced soon after acute exposure to pyrethroids in laboratory animals. Mice and rats administered very low doses of pyrethroids by direct infusion into the brain display pyrethroid poisoning signs, including tremors (Lawrence and Casida, 1982; Gray and Rickard, 1982a). Neurotoxicological endpoints such as tremors and decreased motor activity in rats administered deltamethrin (i.v.) (Gray and Rickard, 1982b) and bifenthrin (p. o.) (Scollon et al., 2011), respectively, are correlated with brain concentrations of these pyrethroids. As humans are exposed to multiple pyrethroids (Fortin et al., 2008; Heudorf and Angerer, 2001; Stout et al., 2009; Tulve et al., 2006; Tornero-Velez et al., 2012), it is important to understand the disposition of these pesticides, particularly to target organs such as the brain. In the present work we examined the distribution to blood and brain of an environmentally-relevant mixture of five pyrethroid compounds after an acute oral gavage in adult rats (Table 1). The objectives of this study were to: (1) determine the blood and brain concentrations of the test chemicals to characterize the relationships between administered mixture dose and target tissue level; (2) assess the correlation of blood and brain concentrations of the administered pyrethroids to motor activity, a behavioral end point. Only the concentrations of parent pyrethroids were determined as metabolism is thought to be a principal detoxication mechanism in pyrethroid intoxications in mammals (Soderlund et al., 2002). Information on dose to target tissue of a mixture of pyrethroids and relating it to a behavioral effect may reduce uncertainties in the cumulative health risk assessment of this class of insecticides.

2. Materials and methods

2.1. Chemicals

The selection process for the five pyrethroids used in this study has been described by Tornero-Velez et al. (2012). The choice of pyrethroids used in this study was based on a national study of a randomly selected set of 168 child-care centers from across the United States (Tulve et al., 2006). Basically, the five pyrethroids had a greater frequency of occurrence and made up roughly 95% of the pyrethroid load found in the Tulve et al. (2006) study.

Each pyrethroid used in the dosing solution was provided by its respective manufacturer as follows: permethrin and cypermethrin (FMC Corporation, Philadelphia, PA); deltamethrin and β-cyfluthrin (Bayer CropScience, Research Triangle Park, NC); and esfenvalerate (DuPont Crop Protection, Wilmington, DE). The physical and chemical properties of the pyrethroids used in this study have been described previously (Wolansky et al., 2006).

The solvents used for processing samples included acetone, hexanes, ethyl acetate, methanol (Fisher Scientific, Pittsburgh, PA), cyclopentane and acetonitrile (Honeywell Burdick & Jackson, Muskegon, MI). These solvents were pesticide grade or better. The water used for all sample analysis had a resistance of 18 MΩ. Corn oil was purchased from Sigma (St. Louis, MO; Product Number C8267).

Primary calibration standards including cis-permethrin (99% purity), trans-permethrin (94%), deltamethrin (99%), cypermethrin (98%), β-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). These labeled standards included cis-permethrin, trans-permethrin, β-cyfluthrin and cypermethrin.

2.2. Animals

Male Long-Evans rats (Charles River Laboratories, Wilmington, MA) were obtained at 55–58 days of age, and housed two per cage

Table 1
Pyrethroid type, percentage of dose in mixture and potency of pyrethroids administered as a mixture to rats.

	β-Cyfluthrin	Cypermethrin	Deltamethrin	Esfenvalerate	Permethrin[b]
Type	II	II	II	I	I
% of total mixture dose	12.9	28.8	3.4	2.7	52.2
Relative potency[a]	1.136	0.235	1.000	2.092	0.059

[a] Relative potency based on a pyrethroid ED$_{30}$ for effect on motor activity relative to deltamethrin as the index pyrethroid (Wolansky et al., 2006).
[b] Relative potency for permethrin determined from 40:60 cis:trans-permethrin.

in standard polycarbonate hanging cages (45 cm × 24 cm × 20 cm) containing heat-sterilized pine shavings (Northeastern Products, Inc., Warrensburg, NY). All animals were given a 1–2 week acclimation period and were maintained on a 12:12 h photoperiod (0600:1800). Feed (Purina 5001 Lab Chow) and tap water were provided *ad libitum*. Tap water (Durham, NC, city water) was filtered through sand and activated charcoal filters, and then re-chlorinated to 4–5 ppm Cl⁻ before use. Colony rooms were maintained at $22 \pm 2\,°C$ and relative humidity at $55 \pm 20\%$. The facility is approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC). All experimental protocols were approved in advance by the National Health and Environmental Effects Research Laboratory's Institutional Animal Care and Use Committee.

2.3. Experimental

The treatment groups consisted of a corn oil control and seven doses of the pyrethroid mixture prepared in corn oil. Concentrations of the pyrethroids for the highest or 1X dose in mg/kg were: permethrin (40% *cis*, 60% *trans*), 14.3; cypermethrin, 7.89; β-cyfluthrin, 3.54; deltamethrin, 0.93; esfenvalerate, 0.74. The total pyrethroid dose in the 1X stock mixture was 27.4 mg/kg. As a frame of reference, it is estimated that the daily exposure to permethrin in a human adult male (70 kg) is 3.2 μg/day (ATSDR, 2003). The percentage of each pyrethroid in the dose and their% potency for reduction in motor activity within the mixture are shown in Table 1. In addition to the 1X stock solution, dilutions of this stock included in the scheme were 0.01X, 0.04X, 0.1X, 0.33X, 0.5X and 0.66X (see Supplemental Table 1 for dose of pyrethroid at each dilution). Each individual pyrethroid in the 1X stock solution contributed low levels ranging from sub-threshold to low-effective doses based on previously reported Effective Dose₃₀ (ED₃₀) levels for motor activity in the rat computed by Wolansky et al. (2006). Except for β-cyfluthrin at 0.66X (5% higher) and 1X (60% higher), the doses of the individual pyrethroids examined in this study were less than the individual ED₃₀s determined by Wolansky et al. (2006). Fresh stock mixtures were prepared by dissolving the pyrethroids in corn oil before dosing. Mixture solutions were stirred with intermittent heating (max. 40–45 °C) for at least 15 min before administration. All doses were delivered at a dose volume rate of 1 mL/kg at room temperature. There were twelve animals in each dose group.

Beginning at two hours post-dosing, the motor activity of each animal was assessed for one hour (Wolansky et al., 2006). Previous studies showed that the time of peak effect for cypermethrin and permethrin was 1.5 h and 2 h for β-cyfluthrin, deltamethrin and esfenvalerate (Wolansky et al., 2006; Crofton and Reiter, 1984, 1988). The animals were euthanized at 3.5 h post-dosing by exsanguination (cardiac puncture) under CO₂-induced anesthesia. Whole blood was collected in 2 mL aliquots and frozen in a methanol/dry ice bath. The brain was then removed from the expired animals and placed in liquid nitrogen. Blood and brain samples were stored at −80 °C until processed for extraction.

The method of Godin et al. (2010) was used to process and extract the tissues for parent pyrethroids. Frozen brain was pulverized in a Spex CertiPrep 6850 freezer/mill (Metuchen, NJ) to form a fine homogeneous tissue powder. A portion of the powdered brain was weighed (350–400 mg) before extraction. For blood, two 2 mL aliquots were extracted. Prior to extraction, both brain and blood samples were spiked with ¹³C₆-*trans*-permethrin that served as a surrogate standard. Samples were extracted with acetone:hexane and the extracts were cleaned as described by Starr et al. (2012). Cleaned extracts were dried under nitrogen and reconstituted in 1 mL methanol:water (9:1, v:v). Internal standards (¹³C₆-*cis*-permethrin, ¹³C₆-cyfluthrin and ¹³C₆-

cypermethrin) were added and the samples were transferred to autosampler vials. All samples were stored at −20 °C until analysis.

2.4. Instrumental analysis

Sample analysis was performed using an AB SCIEX (Framingham, MA) model API 4000™ Liquid Chromatography-Tandem Mass Spectrometry (LC–MS/MS) system configured with a Turbo Ion Spray. Conditions and settings were the same as those described by Starr et al. (2012). The mobile phase consisted of methanol:5 mM ammonium acetate in water (98:2) and flowed at a rate of 400 μL/min. An analytical column (C18, 3.5 μm, 150 × 3 mm) from Agilent Technologies (Santa Clara, CA) was used. Under the conditions used, *cis*- and *trans*-permethrin were separated, but the isomers for cypermethrin and β-cyfluthrin were not resolved. Deltamethrin and esfenvalerate were essentially one isomer each in the dosing solutions, so there were no isomers to resolve for these two pyrethroids. The method of validation, limits of detection and quantitation and quality control and assurance procedures were the same as presented by Starr et al. (2012).\

2.5. Data analysis

Surrogate corrections were made to the calculated pyrethroid blood and brain concentrations by dividing the detected pyrethroid concentration by the recovered surrogate fraction. Several pyrethroid tissue concentration values were less than the level of quantitation (LOQ) (See Supplemental Table 2 for pyrethroid LOQs in blood and brain). These samples were not included in the data analysis.

The tissue response data were first analyzed by linear regression using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Analysis of dose effects on tissue concentrations was done after normalizing tissue levels by dividing the tissue concentration by the administered dose of pyrethroid. Normalized tissue concentration levels were analyzed using linear regression with a significance level of $p < 0.05$. Data were used in the dose effect analysis only if the $N \geq 3$.

In assessing the correlation between blood and brain concentrations of pyrethroid, the measured concentrations of the pyrethroids in each animal were summed to obtain a pyrethroid load in blood and brain. Linear regression analysis was used with a significance level of $p < 0.05$.

The effect of administered dose of total pyrethroid concentration on motor activity was assessed using a one-way analysis of variance (ANOVA) (GraphPad Prism 6) with a significance level of $p < 0.05$. The post-hoc test was a Dunnett's multiple comparison test with a significance level of $p < 0.05$.

Brain to blood concentration ratios for each pyrethroid were calculated by dividing each individual pyrethroid brain concentration by the respective blood concentration of each animal. The data were then averaged within each dose group.

Non-linear regression analysis (GraphPad Prism) was used to assess the relationship between blood or brain concentrations of the summed pyrethroid concentration (i.e., load) and motor activity. A sigmoidal E_{max} model was used for this analysis and is given as:

$$Y = E_0 - [E_{max} \times X^n]/[b^n + X^n].$$

For this analysis, X is the total pyrethroid concentration in the tissue, E_0 is the estimated motor activity when X is 0, E_{max} is the estimated maximum possible decrease in motor activity, b is the estimated Effective Concentration₅₀ (EC_{50}) and n is a shape parameter. When n equals 1, the dose-response relationship is linear. When n is greater than 1, the dose-response relationship

becomes threshold-like. The biologically important characteristics of this function are that it has lower and upper extremes that are determined by the biological response independent of the concentration. For this study, E_0 was constrained to be less than 130 and E_{max} was constrained to be less than 100 (Scollon et al., 2011).

The theoretical relative percent contributions (RPC) of individual pyrethroids in brain that reduces motor activity were estimated by the following equation:

$$RPC_i = (C_i \times RP_i)/(\sum_{i=1 \text{ to } n} C_i \times RP_i) \times 100$$

Where RPC_i is the relative percent contribution of pyrethroid i to motor activity response, C_i is the brain concentration of

pyrethroid i, and RP_i represents relative potency of pyrethroid i (Table 1).

3. Results

The concentrations of pyrethroids in blood and brain increased with administered dose (Fig. 1). Other than β-cyfluthrin in blood (r = 0.85), the correlation coefficient values were ≥ 0.95 for all pyrethroids in both tissues (Fig. 1 and Table 2). The slopes of the regression lines of all pyrethroids in both tissues were significantly greater than zero (Table 2). While the slopes were all positive, in blood, they varied from a low of 1.8 for *trans*-permethrin to a high of 67.3 for deltamethrin; in brain, the slopes varied from a low of 2.4 for *trans*-permethrin to a high of 34.6 for *cis*-permethrin. In
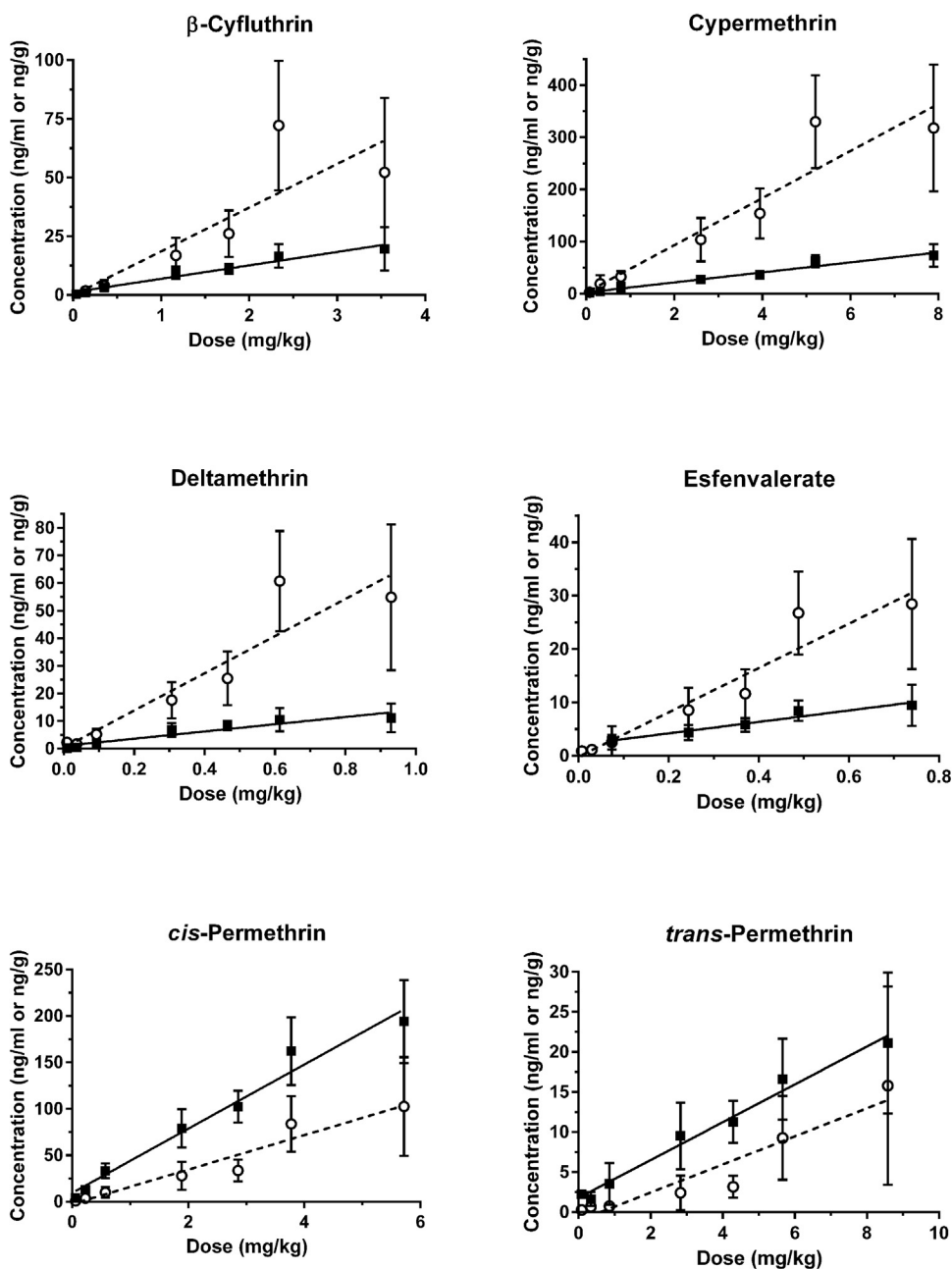

Fig. 1. Relationship between administered dose of pyrethroid and concentration in blood (○) and brain (■). Data represents mean ± SD, N = 1-12 (the N for each line can be found in Tables 3 and 4). The solid and dashed lines represent the linear regression of the blood and brain data, respectively.

Table 2
Level of significance of the slope (p value), F value, degrees of freedom (DF), correlation coefficient (r), and equation of the regression line of pyrethroid concentrations in blood and brain following oral administration of a mixture of pyrethroids.[a]

	β-Cyfluthrin	Cypermethrin	Deltamethrin	Esfenvalerate	*cis*-Permethrin	*trans*-Permethrin
Blood						
p value	0.0321	<0.0001	0.0023	0.0006	0.0003	0.001
F value	10.4	46.0	32.7	59.8	77.5	47.6
DF	1, 4	1, 5	1, 5	1, 5	1, 5	1, 5
r	0.85	0.95	0.98	0.96	0.97	0.95
equation	y = 18.6x − 0.03	y = 45.2x + 2.6	y = 67.3x + 0.4	y = 41.5x − 0.2	y = 18.6x − 2.5	y = 1.8x − 1.1
Brain						
p value	<0.0001	<0.0001	0.0011	0.0058	<0.0001	<0.0001
F value	133.8	160.7	46.2	50.0	195.8	306.1
DF	1, 5	1, 5	1, 5	1, 3	1, 5	1, 5
r	0.98	0.98	0.95	0.97	0.99	0.99
equation	y = 5.7x + 1.2	y = 9.6x + 2.5	y = 13.1x + 1.0	y = 10.7x + 2.1	y = 34x.6 + 9.5	y = 2.4x + 1.8

[a] Data from graphs in Fig. 1.

addition, the slopes for β-cyfluthrin, cypermethrin, deltamethrin and esfenvalerate were higher in blood than brain. For *trans*-permethrin, the slopes were similar in both tissues. In contrast, the slope was about 2-fold higher in brain than in blood for *cis*-permethrin. Adjusting the tissue concentrations by administered dose, showed that there were no significant dose effects on pyrethroid blood concentrations (Table 3). For brain, significant dose effects were detected for β-cyfluthrin, cypermethrin, and *cis*- and *trans*-permethrin (Table 4). The general trend was that the dose-adjusted pyrethroid brain concentration decreased with increased administered dose.

The slope of the line describing the relationship of summed pyrethroid concentration between blood and brain was significantly different from zero (p < 0.0001; F value = 536.7; degrees of freedom: 1, 82) and positively correlated (r = 0.93) (Fig. 2).

The fractional composition of the pyrethroid load (based on weight) in blood and brain relative to administered dose is shown in Fig. 3. There were differences between the pyrethroid fractions in blood and brain. Within each tissue, the general trend was the pyrethroid fractions were similar over the dose range administered. For blood, the rank order from highest fraction to lowest tended to be cypermethrin (approximate fraction of 0.6–0.8), *cis*-permethrin (0.09–0.18), β-cyfluthrin and deltamethrin (approximately 0.05–0.12), esfenvalerate (0.02–0.05) and *trans*-permethrin (0.01–0.02). The only exception was for β-cyfluthrin at the lowest dose, which was not quantifiable. For brain, the fraction was highest for *cis*-permethrin (0.6–0.7), followed by cypermethrin (0.2), β-cyfluthrin and *trans*-permethrin (0.02–0.07), deltamethrin (0.004–0.05) and esfenvalerate (0.03). Esfenvalerate was not quantifiable in brain at the two lowest administered doses.

The brain:blood ratios of the pyrethroids are shown in Table 5. There was preferential distribution of *cis*- and *trans*-permethrin in

brain, with ratios exceeding 1 at all administered doses. For deltamethrin, the ratios were less than 1 for all doses, indicating preferential distribution in the blood except at the lowest dose, where the levels were below the LOQ. For β-cyfluthrin, cypermethrin and esfenvalerate, the lower doses had ratios of approximately 1 and decreased to less than 1 with increased dose, showing preferential distribution to the blood.

Motor activity of the rats was similar within the 0- (i.e., control)-to-0.33X mixture dose range, but declined at higher doses (Fig. 4). The motor activity of the rats administered the 1X dose decreased approximately 60%. The one-way ANOVA revealed a significant dose-related decrease (p < 0.0001; F value = 9.915; degrees of freedom: 7, 88) in motor activity with increased total pyrethroid administered dose. The motor activity of the 0.66X and 1X groups were significantly less than control (p < 0.01 and p < 0.0001, respectively). The relative percent contributions (RPC, see above for equation) of individual pyrethroids to decrease motor activity were determined by multiplying the relative potency of the specific pyrethroid by the brain concentration of that pyrethroid divided by the sum of the relative potency multiplied by the brain concentration for all pyrethroids. In the 0.66X group, the RPCs were: β-cyfluthrin, 27%; esfenvalerate, 22%; cypermethrin 21%; deltamethrin, 15%; *cis*-permethrin, 14%; and *trans*-permethrin, 1%. At the 1X dose, the RPCs were β-cyfluthrin, 26%; esfenvalerate, 24%; cypermethrin, 21%; *cis*-permethrin, 14%; deltamethrin, 14%; and *trans*-permethrin, 1%.

The effect of the pyrethroids on motor activity was assessed versus summed pyrethroid concentration in blood and brain (Fig. 5, Table 6). For concentrations less than approximately 100 ng/mL or ng/g in blood or brain, respectively, the sigmoidal E_{max} model computed a nearly 100% (i.e., control-like) level of motor activity, although there was variability among the animals at

Table 3
Dose normalized blood concentrations (ng/mL/dose) at 3.5 h post-administration of a mixture of pyrethroids by oral gavage in rats.

Dose group	β-Cyfluthrin	Cypermethrin	Deltamethrin	Esfenvalerate	*cis*-Permethrin	*trans*-Permethrin
0.01X	N.D.[a]	36.2 ± 26.3 (12)	251.0 ± 146.6 (4)	115.8, 126.8 (2)	14.6 ± 5.1 (7)	2.7, 3.2 (2)
0.04X	12.7 ± 8.7 (11)[b]	59.6 ± 52.6 (12)	46.9 ± 27.1 (10)	38.2 ± 12.5 (5)	17.1 ± 10.1 (12)	2.0 (1)
0.1X	11.2 ± 6.8 (12)	41.2 ± 14.5 (12)	56.3 ± 21.0 (12)	34.5 ± 19.0 (12)	17.6 ± 10.0 (12)	0.9 ± 0.4 (5)
0.33X	14.4 ± 6.4 (12)	39.8 ± 16.0 (12)	57.4 ± 21.5 (12)	34.9 ± 17.5 (12)	14.9 ± 7.9 (12)	0.9 ± 0.8 (9)
0.5X	14.8 ± 5.6 (12)	39.0 ± 12.2 (12)	54.7 ± 20.9 (12)	31.4 ± 12.4 (12)	11.7 ± 4.2 (12)	0.7 ± 0.4 (8)
0.66X	30.9 ± 11.8 (12)	63.3 ± 17.2 (12)	99.0 ± 29.6 (12)	54.8 ± 15.9 (12)	22.2 ± 7.9 (12)	1.6 ± 0.9 (11)
1X	14.7 ± 9.0 (12)	40.3 ± 15.4 (12)	59.0 ± 28.5 (12)	38.5 ± 16.5 (12)	18.0 ± 9.3 (12)	1.8 ± 1.4 (12)
Deviation from zero[c]	N.S.[d]	N.S.	N.S.	N.S.	N.S.	N.S.

[a] Not Determined (all values below LOQ).
[b] mean ± SD (N).
[c] Linear regression analysis on dose-normalized data for N ≥ 3.
[d] Not Significant (p > 0.05).

Table 4
Dose normalized pyrethroid brain concentrations (ng/g/dose) at 3.5 h post-administration of a mixture of pyrethroids by oral gavage in rats.

| Dose group	β-Cyfluthrin	Cypermethrin	Deltamethrin	Esfenvalerate	cis-Permethrin	trans-Permethrin
0.01X	11.6 ± 2.3 (4)[a]	18.2 ± 11.7 (12)	28.4, 30.7 (2)	N.D.[b]	74.7 ± 30.9 (12)	17.3, 34.4 (2)
0.04X	7.8 ± 2.7 (12)	15.4 ± 4.7 (12)	13.3 ± 4.3 (11)	18.6 ± 9.0 (12)	57.0 ± 17.9 (12)	4.8 ± 2.6 (10)
0.1X	9.3 ± 2.3 (12)	14.3 ± 3.0 (12)	18.4 ± 7.5 (12)	37.7 ± 38.1 (9)	58.6 ± 13.9 (12)	4.1 ± 3.0 (12)
0.33X	8.2 ± 2.2 (12)	10.4 ± 3.2 (12)	22.2 ± 8.1 (12)	17.8 ± 5.9 (10)	41.9 ± 10.9 (12)	3.4 ± 1.5 (12)
0.5X	6.3 ± 1.2 (12)	9.2 ± 2.0 (12)	17.8 ± 3.7 (12)	14.3 ± 5.7 (10)	35.8 ± 6.0 (12)	2.6 ± 0.6 (12)
0.66X	7.1 ± 2.2 (12)	12.0 ± 2.2 (12)	17.1 ± 6.9 (12)	17.3 ± 3.9 (11)	43.0 ± 9.7 (12)	2.9 ± 0.9 (12)
1X	5.5 ± 2.6 (12)	9.3 ± 2.8 (12)	12.0 ± 5.6 (12)	12.8 ± 5.2 (12)	33.9 ± 7.8 (12)	2.5 ± 1.0 (12)
Deviation from zero[c]	p = 0.0344	p = 0.0306	N.S.[d]	N.S.	p = 0.019	p = 0.0163

[a] Mean ± SD (N).
[b] Not determined (all values below LOQ).
[c] Linear regression analysis on dose-normalized data for N ≥ 3.
[d] Not Significant (p > 0.05).
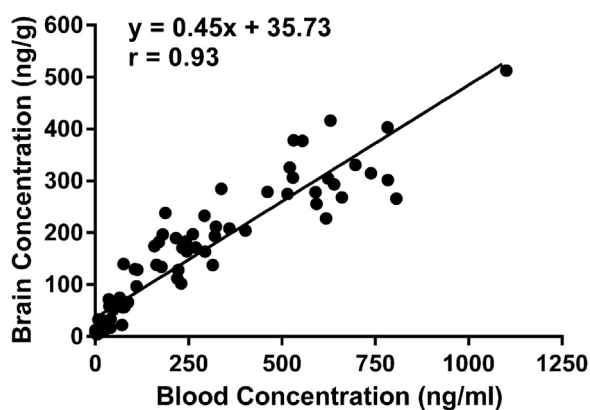
Fig. 2. The relationship between blood (ng/mL) and brain (ng/g) summed pyrethroid concentration following oral administration of an environmental mixture of pyrethroids in the rat. The closed circles represent individual data points and the solid line the best fit line using a linear equation.

concentrations below this level. With increasing tissue concentrations greater than 100 ng/mL or ng/g in blood or brain, respectively, the motor activity of the animals decreased. The shape parameters of the model for both blood (3.7 ± 2.2, mean ± SE) and brain (5.4 ± 2.9) were greater than 1, indicating a threshold-like effect of the pyrethroids on motor activity. The estimated EC_{50} for decrease in motor activity was slightly lower in brain (207 ± 25 ng/g) than in blood (246 ± 39 ng/g).

4. Discussion

Assessing the concentration of a toxicant in a target organ such as the brain or a surrogate biological matrix such as the blood may provide more valuable information for a toxicity assessment than solely considering administered dose (Meador et al., 2008). In the present paper the blood and brain concentrations of a mixture of pyrethroid insecticides administered by oral gavage to rats were determined. The concentrations of β-cyfluthrin, cypermethrin, deltamethrin, esfenvalerate, and cis- and trans-permethrin in rat blood and brain increased linearly with increased dose. However, the rates of these increases were not equivalent. The differences in slopes between the pyrethroids and tissues indicate that although the chemical structures are similar, being based on the natural pyrethrins, there are dispositional (absorption, distribution, metabolism and excretion) differences among them. The uptake into blood and brain of the pyrethroids did not correspond with the Type I and Type II groupings or relative potencies, as previously reported by Starr et al. (2012), who conducted a time course study with the same proportional mixture of pyrethroids. Dispositional differences may have an important influence on the neurotoxicity

of the test chemicals of this work as $ED_{30}s$ for decreased motor activity have been found to vary from 1.2 to 42.7 mg/kg in the rat (Wolansky et al., 2006).

The results presented here have several consistencies with two studies by Starr et al. (2012, 2014). Starr et al. (2012) examined the tissue (blood, brain, fat and liver) time course (2.5–24.5 h post-administration) of the same mixture of pyrethroids at doses of 0.4X and 1X. More recently, Starr et al. (2014) reported the time course of this mixture in blood and brain at doses of 0.4X and 1.3X. In both studies (Starr et al., 2012, 2014) no differences in the dose normalized blood concentrations of the pyrethroids at 3.5 h were reported as observed in the present study. In brain, no significant differences in the dose normalized pyrethroid brain concentrations at 2.5 h (3.5 h not reported) were observed by Starr et al. (2012) and at 3.5 h by Starr et al. (2014). For deltamethrin and esfenvalerate in the present study at 3.5 h, no significant differences in dose normalized brain concentrations were found. However, significant differences were observed for β-cyfluthrin, cypermethrin and cis- and trans-permethrin. The trend was for higher dose normalized concentrations at the low doses, which decreased as dose increased. Although significantly different (p < 0.05), the decreases in dose normalized brain concentrations were two-fold or less. Experimental variability may explain the differences between the studies. Starr et al. (2012, 2014) also reported that the relative concentrations of pyrethroids in brain reflected that in the administered dose if the permethrin isomer brain concentrations are summed. A similar finding was observed in the present study.

In the administered pyrethroid mixture, trans-permethrin changed the most with regard to fractional composition between the dosing solution and the tissues, as reported by Starr et al. (2012, 2014). The fraction of trans-permethrin decreased greatly in blood, but slightly less so in brain, relative to the dosing solution. One explanation for this considerable decrease of trans-permethrin in rat blood is that a serum carboxylesterase hydrolyzes trans-permethrin much faster than cis-permethrin, deltamethrin and esfenvalerate (Crow et al., 2007). Hydrolysis of pyrethroids by carboxylesterases is an important elimination pathway for this class of insecticides (Godin et al., 2006; Ross et al., 2006; Crow et al., 2007; Scollon et al., 2009). In addition, the trans configuration of pyrethroids with a primary alcohol, such as trans-permethrin, are in general more readily hydrolyzed by carboxylesterases than the trans isomers with a secondary alcohol (e.g., cypermethrin) and pyrethroids in the cis configuration (e.g., cis-permethrin) (Abernathy et al., 1973; Ross et al., 2006). While rat serum has carboxylesterase activity towards pyrethroids, human serum is devoid of such activity (Crow et al., 2007; Godin et al., 2007). This species difference in the metabolism of pyrethroids in serum has implications in species extrapolation of pyrethroid toxicity from rat to human. The rat serum carboxylesterase appears to be a single isozyme with differential pyrethroid hydrolyzing
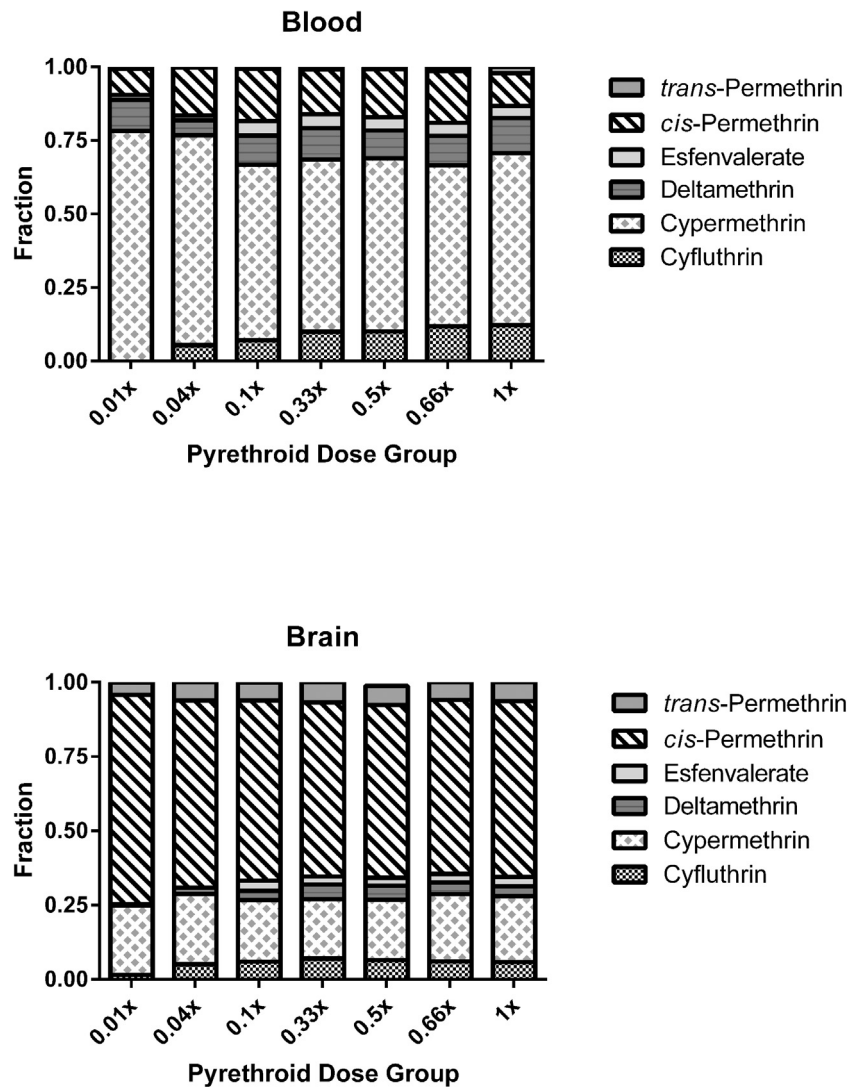
Blood

Brain

Fig. 3. Fractional composition of pyrethroids in blood and brain of rats administered a mixture of pyrethroids by oral gavage. Data extracted from Fig. 1. At 3.5 h post-administration, the animals were sacrificed and blood and brain were collected. The tissue were extracted and analyzed for parent pyrethroid. The dose bar in Figs. 4A and 4 B represents the fractional pyrethroid composition of the administered dose.

Table 5
Brain to blood ratio of pyrethroids in rats 3.5 h after administration of a mixture of pyrethroids by oral gavage.

Dose group	β-Cyfluthrin	Cypermethrin	Deltamethrin	Esfenvalerate	*cis*-Permethrin	*trans*-Permethrin
0.01X	N.D.[a]	1.1 ± 1.6 (12)	N.D.	N.D.	5.2 ± 2.8 (7)	N.D.
0.04X	1.0 ± 1.1 (8)[b]	0.4 ± 0.2 (12)	0.4 ± 0.3 (10)	N.D.	4.8 ± 3.3 (11)	N.D.
0.1X	1.1 ± 0.6 (12)	0.4 ± 0.1 (12)	0.4 ± 0.4 (12)	1.3 ± 1.2 (9)	4.1 ± 1.6 (12)	4.0 ± 2.6 (5)
0.33X	0.7 ± 0.4 (12)	0.3 ± 0.1 (12)	0.4 ± 0.2 (12)	0.6 ± 0.3 (10)	3.8 ± 2.4 (12)	8.9 ± 11.4 (9)
0.5X	0.5 ± 0.2 (12)	0.3 ± 0.1 (12)	0.4 ± 0.2 (12)	0.5 ± 0.1 (10)	3.5 ± 1.4 (12)	4.4 ± 1.9 (8)
0.66X	0.3 ± 0.2 (12)	0.2 ± 0.1 (12)	0.2 ± 0.1 (12)	0.4 ± 0.2 (11)	2.1 ± 0.7 (12)	2.2 ± 0.9 (11)
1X	0.5 ± 0.3 (12)	0.3 ± 0.1 (12)	0.2 ± 0.1 (12)	0.4 ± 0.3 (11)	2.3 ± 0.9 (12)	2.0 ± 1.2 (12)

[a] Not Determined (insufficient data to calculate a ratio).
[b] mean ± SD (N).

activity; *trans*-permethrin is hydrolyzed efficiently by this enzyme, whereas deltamethrin, esfenvalerate, cypermethrin (alpha isomer) and *cis*-permethrin are hydrolyzed to a lesser extent (Crow et al., 2007). The higher concentrations of the pyrethroids relative to that of *trans*-permethrin in blood in the present study may be explained by the higher activity of this enzyme towards *trans*-permethrin.

Pyrethroid-metabolizing carboxylesterases are also found in the liver and intestines (Nishi et al., 2006; Crow et al., 2007). In rat liver, hydrolase A and B are highly expressed carboxylesterase isozymes and are part of the carboxylesterase 1 (CES1) family (Hosokawa, 2008; Okura et al., 2014). These two isozymes have differential hydrolytic activity towards deltamethrin and esfenvalerate (Godin et al., 2006) and *cis*- and *trans*-permethrin (Ross et al., 2006). The hydrolysis of *trans*-permethrin measured as catalytic activity (k_{cat}) is 5- and 10-fold greater than *cis*-permethrin for hydrolase B and A, respectively (Ross et al., 2006). In human liver, two predominant and highly expressed carboxylesterases have been identified and are termed hepatic carboxylesterase-1
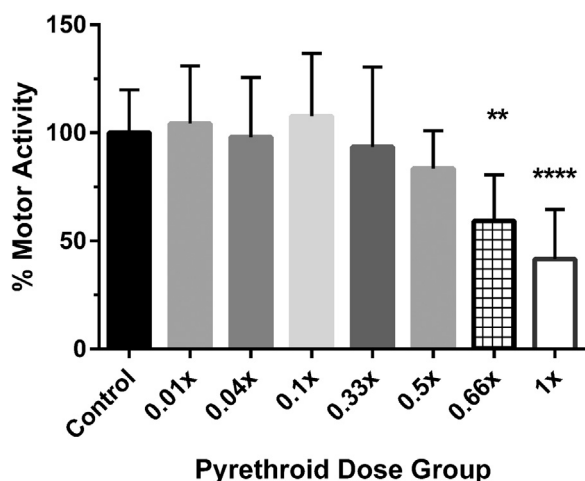
Fig. 4. % Motor activity in rats versus pyrethroid mixture dose group administered by oral gavage. Motor activity of the rats was monitored from 2 to 3 h post-administration. Data represents mean ± SD, N = 12 per dose group. **, p < 0.01; ****, p < 0.0001.
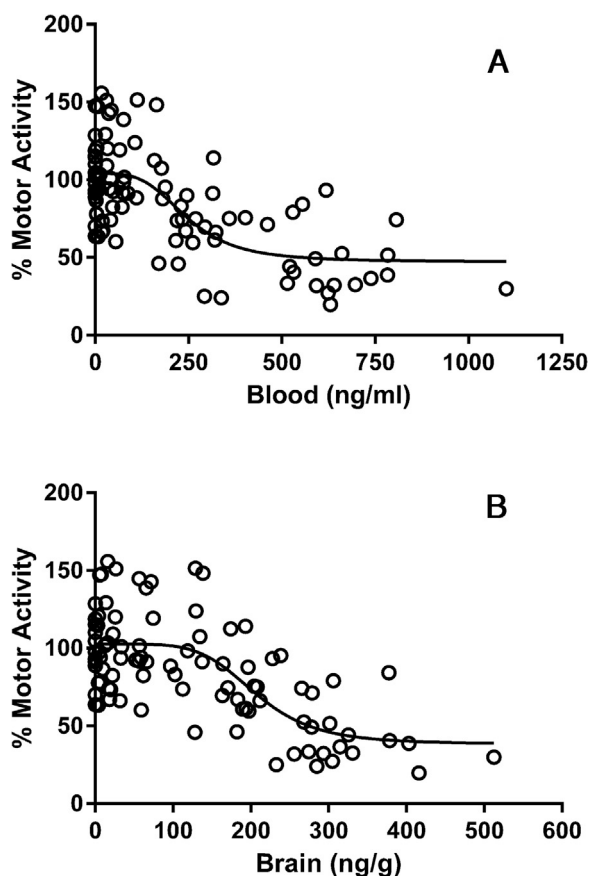

Fig. 5. Summed pyrethroid concentration in blood (A) and brain (B) with respect to % motor activity in rats. Animals were administered a mixture of pyrethroids at different doses and assessed for motor activity from 2 to 3 h post-administration. At 3.5 h, the animals were sacrificed and blood and brain were removed. Tissues were extracted and analyzed for parent pyrethroids by LC–MS/MS. Data points represent individual animals. The solid line represents the results from the sigmoidal E_{max} model analysis.

(hCE-1) and hepatic carboxylesterase-2 (hCE-2) (Brzezinski et al., 1994; Pindel et al., 1997). Differential activity between these two isozymes have been reported for deltamethrin and esfenvalerate,

with hCE1 having a higher hydrolytic specific activity towards these two pyrethroids (Godin et al., 2006). Ross et al. (2006) observed both isozymes have a higher activity toward *trans*-permethrin than *cis*-permethrin.

Intestinal metabolism by rat carboxylesterases may also explain the lower fraction of *trans*-permethrin relative to the other pyrethroids in blood. Crow et al. (2007) incubated a pool of rat intestinal microsomes with deltamethrin and *trans*-permethrin. The specific activity for hydrolysis of *trans*-permethrin was ∼0.2 nmole/min/mg protein and deltamethrin was not hydrolyzed. Nakamura et al. (2007) reported that the hydrolysis rate of *trans*-permethrin was 5-fold greater than that for *cis*-permethrin in rat intestinal microsomes. Rat intestinal carboxylesterases appear to contribute to the metabolic clearance of *trans*-permethrin to a greater extent than *cis*-permethrin and deltamethrin, and may explain in part for its low levels in blood relative to the other pyrethroids.

While all of the pyrethroids in the mixture were absorbed systemically into blood and brain, the role of uptake and efflux transporters is not known. For the gastrointestinal tract, Mirfazaelian et al. (2006) proposed intestinal efflux transporters for deltamethrin, based on modeling of fecal excretion data in the rat. However, Godin et al. (2010) did not observe dose-dependent bioavailability of deltamethrin in rats, suggesting that transporters are not involved in intestinal transport of this pyrethroid. In *in vitro* studies with Caco-2 cells, efflux of deltamethrin or *cis/trans*-permethrin by multidrug resistant protein 1 was not observed (Zastre et al., 2013). Exposure of resistant and sensitive ticks to acaricides showed no difference in the LC_{50} for cypermethrin in the presence and absence of ABC-transporter inhibitors (Pohl et al., 2012). Furthermore, within the administered dose range used, the fraction of each pyrethroid detected in the brain remained fairly constant. The finding of an overall constant fraction suggests that there was no interaction between the pyrethroids influencing absorption into the brain. The constant fraction also does not give any indication if transporters move pyrethroids into or out of the brain. Presently, it appears that transporters are not involved in intestinal and tissue uptake or efflux of pyrethroids. If transporters are involved, an effect may become apparent at higher doses of pyrethroids administered because of saturation of the transport process.

There was a difference in the distribution of the pyrethroids between blood and brain. The two permethrin isomers preferentially partitioned into brain, whereas cypermethrin, β-cyfluthrin, deltamethrin and esfenvalerate partitioned to a greater extent in blood. These partitioning results are similar to those reported by Starr et al. (2012, 2014), with one exception. The β-cyfluthrin brain to blood ratio was 2.4 in Starr et al. (2012) and approximately 1 or less (depending on the dose) in the present study. The test pyrethroids have a fairly high lipid solubility (Log P > 4.5) and contain no ionizable groups that could retard absorption. Brain to blood partition coefficients of the pyrethroids were calculated by Starr et al. (2012), with values ranging from 28.1 to 28.4. Thus high partitioning of the pyrethroids into brain could be predicted. However, the brain to blood partition values reported by Starr et al. (2012, 2014) and observed in the present study were >3-fold lower than those predicted. A potential reason for greater partitioning of a chemical into blood over a tissue is blood protein binding. Abu-Qare and Abou-Donia (2002) reported low binding of permethrin (isomer not specified) to human serum albumin. Sethi et al. (2014) more recently measured plasma protein and lipoprotein binding of deltamethrin and *cis*- and *trans*-permethrin in rats and humans. With increasing pyrethroid concentration (0.25–100 μM), they observed an increased unbound fraction of pyrethroid. The lowest concentration (0.25 μM) used by Sethi et al. (2014) corresponds approximately to the highest concentrations of deltamethrin

Table 6
Parameter estimates of the pyrethroid load in blood and brain of rats vs. motor activity (% control).[a]

Parameter	Blood	Brain
E_o	104.2 ± 3.6[b]	102.7 ± 3.5
E_{max}	56.9 ± 9.1	64.2 ± 13.3
Shape (n)	3.7 ± 2.2	5.4 ± 2.9
Effective Concentration$_{50}$ (b)	246.2 ± 39.2	207.2 ± 24.8
r^2	0.46	0.47
N[c]	96	96

[a] Data were fit with a sigmoidal E_{max} model. E_0 is the motor activity of the rats when the pyrethroid load is zero and E_{max} is the maximal change from control. E_0 was bound at <130 and E_{max} was bound at <100 in this analysis.
[b] Mean $\pm$ SE.
[c] Number of animals.

(0.16 μM) and *cis*- (0.26 μM) and *trans*-permethrin (0.04 μM) detected in blood in the present study. At 0.25 μM, Sethi et al. (2014) reported approximately 20% of deltamethrin and *cis*- and *trans*-permethrin were not bound to protein. If the observations of Sethi et al. (2014) are consistent with pyrethroid concentrations lower than 0.25 μM, the unbound fraction would be lower. This suggests that at the pyrethroid blood concentrations determined in this study, there would be less pyrethroid available for partitioning into the brain. However, Starr et al. (2012) reported a trend of higher partitioning of the pyrethroids into the brain for the lower dose as observed in this study; partitioning of the pyrethroids into brain decreased as dose increased. The reason for this partitioning observation is not known, but perhaps the physicochemical properties of the pyrethroids including their tertiary structure have an important role here or there is an unknown transporter involved in the uptake of the pyrethroids into the brain.

The correlation between summed pyrethroid tissue concentration and effect on motor activity in this study is consistent with the results of previous five-pyrethroid mixture studies (Starr et al., 2012, 2014). Similarly, β-cyfluthrin in brain had the highest estimated relative percent contribution to decrease in motor activity as reported by Starr et al. (2012, 2014). This study, those by Starr et al. (2012, 2014) and that of Wolansky et al. (2009), who studied an eleven pyrethroid mixture which contained the five pyrethroids studied here, produced comparable results in that the mixtures contained low effective concentrations of individual pyrethroids, yet significant dose-response decreases in motor activity were observed in rats administered the mixture. Furthermore, the present study shows that both blood and brain concentrations of the pyrethroid load are predictive of motor activity to a similar extent, although the results from brain appear to be more sensitive. The EC_{50} for the pyrethroid concentration in brain was lower than in blood, which supports the hypothesis that brain is a target organ for the neurotoxic effects of pyrethroids. The blood and brain concentrations of the pyrethroids were highly correlated at the 3.5 h time point, so it stands to reason that blood concentrations, as a surrogate for brain concentrations, could be an acceptable predictor of motor activity in the rat. However, over time, the concentration of pyrethroids in blood decrease and the ability for blood concentrations to predict motor activity most likely will decrease. For the pyrethroid bifenthrin, administered singly, there was a good correlation between bifenthrin blood concentration and motor activity at 4 h post-dosing (Scollon et al., 2011). However, by 7 h the correlation of bifenthrin blood concentration to motor activity decreased considerably. In the brain, the correlations were similar at 4 and 7 h, suggesting there was little efflux of bifenthrin out of the brain, minimal metabolism in this organ during this time frame or both. This is supported by the observation by Starr et al. (2012) that the pyrethroids have a longer half-life in brain relative to that in blood and liver.

In summary, pyrethroids administered as a mixture to rats by oral gavage were absorbed into blood and brain. With increased dose, there was an increase in the concentration of pyrethroid in blood and brain. There were differences between the fractions of each pyrethroid in the dosing solution with those detected in blood; the fractions in brain were very similar to the dosing solution provided the permethrin isomer concentrations were summed. The pyrethroids β-cyfluthrin, cypermethrin, deltamethrin and esfenvalerate primarily partitioned into blood, whereas *cis*- and *trans*-permethrin primarily partitioned into brain. There was a significant dose-dependent decrease in motor activity of the rats administered the pyrethroid mixture. Further research is clearly needed to explain this differential partitioning of the pyrethroids between blood and brain and its relationship to neurotoxicity and how it relates to the more than 35-fold range in relative potencies among these five pyrethroids.

Conflict of interest

The authors declare to have no competing conflicts of interest.

Disclaimer

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2016.06.013.

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