DOI:10.2478/10004-1254-61-2010-1986

Scientific Paper

BRAIN TOXICOKINETICS OF PROMETRYNE IN MICE

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Received in July 2009
Accepted in November 2009

Prometryne is a methylthio-*s*-triazine herbicide. Significant trace amounts are found in the environment, mainly in water, soil, and food plants. The aim of this study was to establish brain and blood prometryne levels after single oral dose (1 g kg^{-1}) in adult male and female mice. Prometryne was measured using the GC/MS assay at 1, 2, 4, 8, and 24 h after prometryne administration. Peak brain and blood prometryne values were observed 1 h after administration and they decreased in a time-dependent manner. Male mice had consistently higher brain and blood prometryne levels than female mice. The observed prometryne kinetics was similar to that reported for the structurally related herbicide atrazine.

KEY WORDS: *blood, herbicide, nervous system, toxicity, triazine*

Prometryne (*N,N'*-bis(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine) is used to control annual broadleaf and grass weeds in corn, carrots, parsley, peanuts, cotton, pigeon peas, alfalfa, and many other cultivated plants (1-4). Its use has been associated with increased levels in soil, water, and air, and a risk to the environment and human health (5). Prometryne is relatively persistent in water (6-8) and soil (9, 10). It can also be found in ambient air near production or application sites (11). Significant traces were documented in plants consumed by humans and domestic animals (9, 12-14) and medicinal plants treated with this herbicide (15). Trace levels of prometryne have also been reported in cow and human milk (16, 17). Only minimal information on the toxicokinetics of triazine herbicides in mammals has been published and the knowledge about prometryne kinetics in various organs is restricted to rats (18, 19). Little is known about prometryne presence in the central nervous system (CNS), but the partition coefficient log K_{ow}=3.5 (2) and the polarity of prometryne suggest that it might enter the CNS. The objective of this study was to see the kinetics of a single oral dose (1 g kg^{-1}) of prometryne in the brain and blood of CBA mice and to compare it with previously reported kinetics of the structurally related and widely used herbicide atrazine. We also wanted to see if there were any sex-related differences in prometryne kinetics.

MATERIALS AND METHODS

Animals

For the experiment we used inbred CBA mice of both sexes [(65±3) days old; male=(23.37±1.99) g; female=(22.25±1.14) g] from a mouse colony of the Zagreb University Faculty of Science. The animals were fed on a pellet diet (Pliva, Zagreb, Croatia) and had free access to tap water. Light and dark exchanged

every 12 hours. The experiment observed the Croatian animal welfare regulations (20) and the Guide for the Care and Use of Laboratory Animals (21).

Treatment of experimental animals with prometryne

The mice were randomly distributed to five experimental groups of 12 animals each, six male and six female, that received a single prometryne ($C_{10}H_{19}N_5S$; CAS No. 7287-19-6, EPA Reg. No. 9779-297; technical grade 95 %; Herbos, Sisak, Croatia) oral dose of 1 g kg^{-1} body mass in a 0.2 mL corn oil suspension. The control group was randomly selected from among sibling littermates and treated with the same volume of corn oil without prometryne. The control group also served to verify that the animals had not been exposed to prometryne through food or water.

The selected prometryne dose was the closest to the 1/3 of mice LD_{50} of 3.75 g kg^{-1} that did not show lethal toxicity (LD_{10}) over 48 consecutive hours after administration, as established by pilot tests reported by EPA (1). Dosing was based previous toxicokinetic studies (22-26) that used doses higher than normally environmentally available to ensure detectable residue levels for our purposes.

Mice were weighed before oral administration of prometryne. Corn oil suspension with prometryne was given in the volume of 0.2 mL per animal with small adjustments to individual body mass to keep the same dose of prometryne per kilogram of body mass.

The mice were monitored for any visible clinical or behavioural signs of poisoning. They were killed by cervical dislocation one, two, four, eight, and 24 hours after prometryne administration.

The whole experiment was repeated three times.

Collection of blood and brains

Blood was collected from the heart according to the standard protocol (27). Samples of whole blood were immediately frozen (-80 °C) until further use, which followed within three days.

After blood collection, animals were perfused with PBS to make sure that the brain prometryne referred to brain parenchyma only. Dissected brains were placed on ice pads, weighed and immediately homogenised with constant cooling. To collect brain and blood samples we used new and thoroughly cleaned sets of instruments for each mouse to avoid any contamination with traces of prometryne from other animals.

Brain and blood prometryne extraction and the GC/MS assay

Blood and brain prometryne was analysed using the standard GC/MS assay as described earlier (22, 28). Briefly, to extract prometryne from blood and brain, we mixed 0.2 mL of water atrazine solution (3.5 μg mL^{-1}) as internal standard and 0.15 g of sodium wolframate (Na_2WO_4, Kemika, Zagreb, Croatia) with 0.5 mL of homogenised sample. After the salt was diluted, we added 2 mL of ethyl acetate/dichloromethane mixture (1:3; Sigma-Aldrich, Taufkirchen, Germany). After 5 min of vortexing and 10 min of centrifugation (6000 rpm), 1 mL of supernatant was transferred and desiccated. After desiccation, 0.1 mL of *n*-hexane (Sigma-Aldrich) was added to the sample for injection into a chromatograph Shimadzu GCMS-QP2010S with a5970 MSD model. We used a HP-5ms GC column (30 m, 0.25 mm i.d.; J&W Agilent Technologies, USA). The injection temperature was 275 °C , with an oven temperature of 90 °C increased to 280 °C in 11 min. A blank sample of blood and brains from control animals was measured in parallel to make sure that the animals were not exposed to prometryne or atrazine (used as an internal standard) through food or water. To establish the calibration curve, we used 20 known concentrations of prometryne and atrazine (range: 4 ng mL^{-1} to 2000 ng mL^{-1}) which were added to the brain and blood samples collected from control mice. Each time before measurement, we added 0.2 mL of internal standard for every 0.5 mL of real samples from animals that received prometryne, and recovery was 80 % to 85 %. No traces of metabolites were seen.

Statistical analysis

For statistical analyses we used Statistica 5.0 software (StatSoft, Tulsa, USA). We analysed group means (± standard deviation of the mean) and made multiple comparisons between groups and sexes with ANOVA. For post-hoc analysis of differences between groups we employed Scheffé's and Duncan's test. We also determined the correlation and regression coefficients between brain and blood concentrations. The level of statistical significance was set at $P \leq 0.01$ and $P \leq 0.05$.

RESULTS

Tables 1 and 2 show blood and brain prometryne contents, respectively. The first two rows in both tables

Table 1 *Blood prometryne concentration in mice at different time points after administration of a single oral dose (1 g kg^{-1})*

Sampling time	Sex	Blood prometryne concentration / ng mL^{-1}				
		Mean	S.D.	Median	Min.	Max.
1 h	m	2765.22[a]	671.83	2911.83	1811.67	3486.67
	f	1740.50	789.18	1484.00	1077.67	3246.33
	m+f	2252.86[A]	880.13	1990.50	1077.67	3486.67
2 h	m	1144.74	327.25	1172.96	645.81	1516.88
	f	779.72	436.16	675.31	384.55	1624.33
	m+f	962.23[B]	414.11	850.46	384.55	1624.33
4 h	m	622.43[a]	179.86	663.85	281.35	799.24
	f	403.96	179.54	363.83	200.81	737.73
	m+f	513.19[C]	205.85	519.36	200.81	799.24
8 h	m	151.41[a]	53.97	152.46	59.27	216.53
	f	83.40	33.84	73.67	42.66	142.99
	m+f	117.41[D]	55.73	116.66	42.66	216.53
24 h	m	31.54[a]	12.24	30.26	14.41	51.78
	f	17.91	6.54	16.55	11.57	30.19
	m+f	24.73[E]	11.76	22.95	11.57	51.78

[A-E] *A>B>C>D>E at a significant level (P≤0.01)*
[a] *significantly higher (P≤0.05) than in female mice at the same time point*

Table 2 *Brain prometryne mass fraction in mice at different time points after administration of a single oral dose (1 g kg^{-1})*

Sampling time	Sex	Brain prometryne mass fraction / ng mg^{-1}				
		Mean	S.D.	Median	Min.	Max.
1 h	m	9.502[a]	2.123	9.767	6.751	12.416
	f	6.256	2.687	5.115	3.890	9.887
	m+f	7.879[A]	2.864	8.384	3.890	12.416
2 h	m	2.560	0.728	2.384	1.857	3.975
	f	2.006	0.881	1.708	1.279	3.656
	m+f	2.283[B]	0.823	2.254	1.279	3.975
4 h	m	1.188	0.268	1.142	0.882	1.577
	f	1.015	0.391	0.826	0.653	1.577
	m+f	1.102[C]	0.332	1.045	0.653	1.577
8 h	m	0.441	0.166	0.394	0.276	0.667
	f	0.338	0.091	0.328	0.236	0.448
	m+f	0.389[D]	0.139	0.346	0.236	0.620
24 h	m	0.102	0.031	0.101	0.051	0.138
	f	0.090	0.024	0.085	0.065	0.133
	m+f	0.096[E]	0.027	0.094	0.065	0.138

[A-E] *A>B>C>D>E at a significant level (P≤0.01)*
[a] *significantly higher (P≤0.05) than in female mice at the same time point* [a] *significantly increased (p ≤ 0.05) compared to the value recorded in female mice at the same time point*

bring separate results by the sexes and the third row combines these results. Table 3 is structured like the first two tables and shows correlation and regression coefficients. Figures 1 and 2 show brain-blood correlations in either sex, and Figure 3 combines them.

Blood prometryne concentration

Prometryne (in ng mL^{-1}) was detected in all blood samples collected over 24 hours from administration (Table 1). During that time blood concentration declined steadily. One hour after dosing, blood prometryne significantly differed (P≤0.01) between

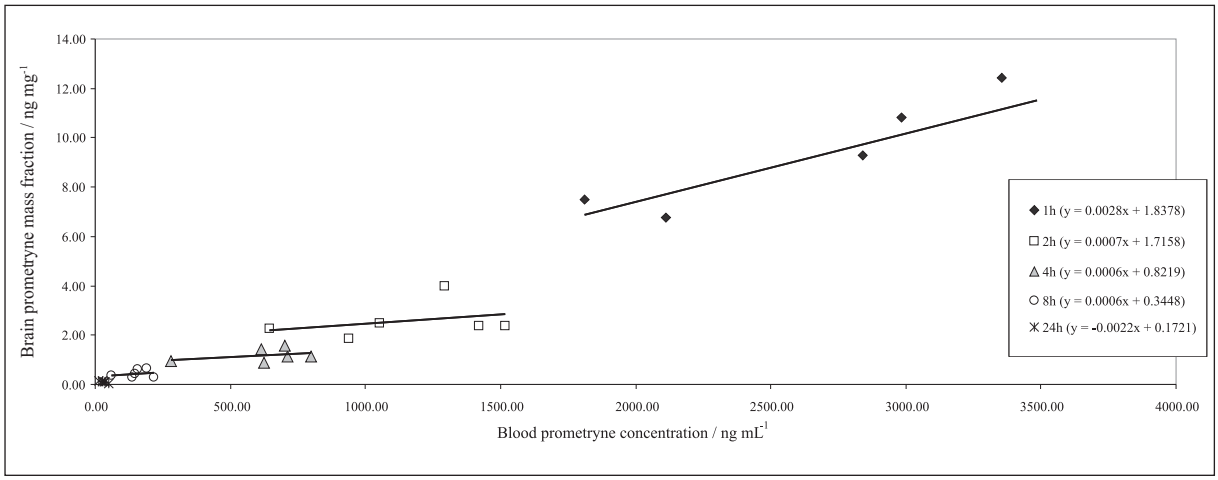

Figure 1 *The relationship between brain and blood prometryne levels measured at different time points after administration of a single dose in male CBA mice*

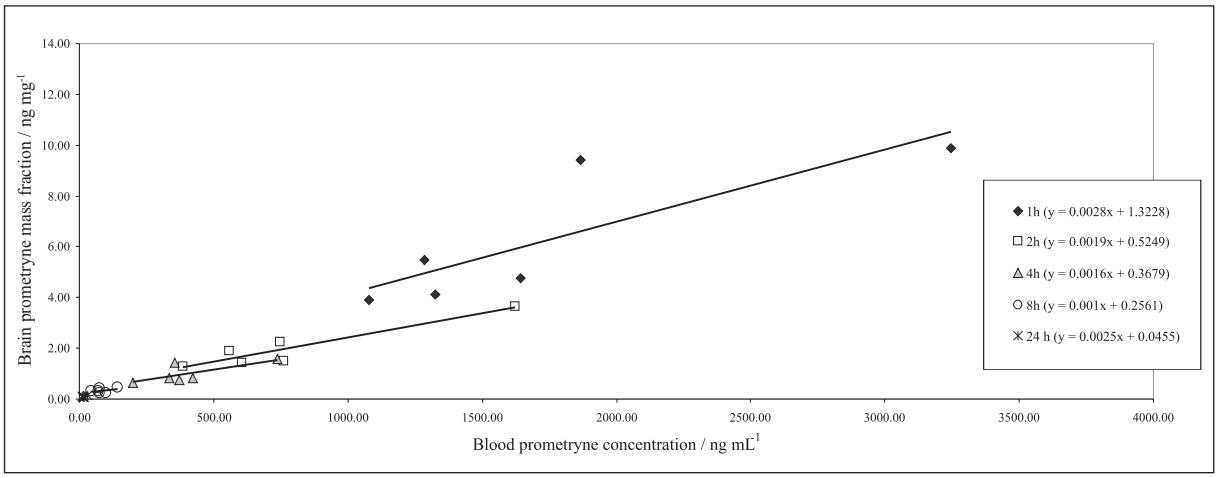

Figure 2 *The relationship between brain and blood prometryne levels of prometryne measured at different time points after administration of a single dose in female CBA mice*

male and female mice, as males had almost double (1.6 fold) the prometryne concentration in females. Two hours after prometryne administration, females had 1.5 times lower blood prometryne than males, but the difference was not significant. Viewed combined (m+f), prometryne concentration dropped 2.3 times from hour one to hour two post dosing ($P \leq 0.01$).

Four hours after administration, blood prometryne dropped further still (1.9 times in respect to hour two, $P \leq 0.01$ and 4.4 times in respect to hour one, $P \leq 0.01$). Again, the difference between males and females was significant (1.5 times lower in female mice, $P \leq 0.01$). On hours eight and 24 blood prometryne rapidly dropped in all treated animals; it dropped 4.4 times between hour four and eight and 4.7 times between hours 8 and 24. The difference between the sexes was still significant ($P \leq 0.01$, 1.8 times lower in female mice).

Prometryne in brain

Prometryne (in ng mg^{-1}) was detected in the brains of mice at all time points over the 24 hours after administration (Table 2). Brain prometryne mass fractions steadily declined throughout the experiment. On post-dose hour one, it was 1.5 times higher in male than in female mice ($P \leq 0.01$), which is similar to blood concentration differences between the sexes. On hour two post dose (m+f), the mass fraction dropped 3.5 times ($P \leq 0.01$). Female mice now had 1.3 times lower prometryne brain mass fraction than male mice, but the difference was not significant. Again even though female mice had 1.3 times lower brain prometryne, the

difference was not statistically significant. On hours eight and 24, brain prometryne was very low, but still detectable. It dropped 2.8 times from hour four to eight and 4.05 times from hour eight to 24. Female mice had 1.1 times lower brain prometryne than male mice on hour eight and 1.5 times on hour 24 post dose, but again, the difference was not statistically significant.

Correlation and regression analysis

Table 3 and Figures 1-3 show the correlation between blood and brain prometryne levels and regression coefficients. The highest significant positive correlation between brain and blood prometryne was determined in samples analysed one hour after administration. Two hours after administration, the correlation was still significant and positive in females, but not in males. When analysed together, however, the correlation was positive and almost significant (P=0.0508). The same was observed for samples collected four hours post dose. Again, the correlation was positive in both sexes, but significant (P≤0.05) only in female mice. The correlation for combined data was positive and significant (P≤0.05), although lower than on hour two. On hours eight and 24 post dose the correlation between brain and blood prometryne levels was no longer significant in any group.

DISCUSSION

This study was designed to simulate a normal oral exposure to prometryne from the environment (29) as previously shown by Maynard et al. (22) in rats. One of the goals was to compare the allometric differences (30-32) between rats used in the study of Maynard et al. (22) and mice which we regularly used in earlier prometryne toxicity studies (33, 34). One of the key documents that directed the experiment was the OECD 407 guideline for 28-day subchronic toxicity studies in rodents (21). According to this guideline, it is safe to give tested chemicals at intervals of every 24 h to avoid overdosing or bioaccumulation. However, Maynard et al. (22) established prometryne presence in rats even after 48 h. While no such findings have been available for mice our results suggest that even though OECD 407 guideline recommends 24-h intervals for test chemicals, this recommendation should be taken with caution. After 24 h, prometryne was not completely eliminated from the blood of mice (Table 1), which suggests that the remaining prometryne concentrations, however low, might build up with subchronic dosing and raise the bioavailable concentration (29).

Although prometryne was present in the blood of exposed animals, its percentage in respect to the

Table 3 *Correlation and regression coefficients between brain and blood prometryne level at different sampling times following a single oral dose (1 g kg^{-1})*

Sampling time	Sex	r	b	R^2	Significance
1 h	m	0.87707	0.00280	0.76930	P≤0.01
	f	0.83252	0.00280	0.69310	P≤0.01
	m+f	0.90510	0.00294	0.81940	P≤0.01
2 h	m	0.33125	0.00070	0.10970	
	f	0.93988	0.00190	0.88340	P≤0.01
	m+f	0.74860	0.00149	0.56050	P≤0.05
4 h	m	0.39374	0.00060	0.15500	
	f	0.73704	0.00160	0.54320	P≤0.05
	m+f	0.61870	0.00098	0.38230	
8 h	m	0.20636	0.00060	0.04260	
	f	0.36176	0.00100	0.13090	
	m+f	0.42400	0.00105	0.18000	
24 h	m	-0.87960	-0.00220	0.77370	P≤0.01
	f	0.68227	0.00250	0.46550	
	m+f	-0.24070	-0.00054	0.57900	

r – correlation coefficient
b, R^2 – regression coefficients

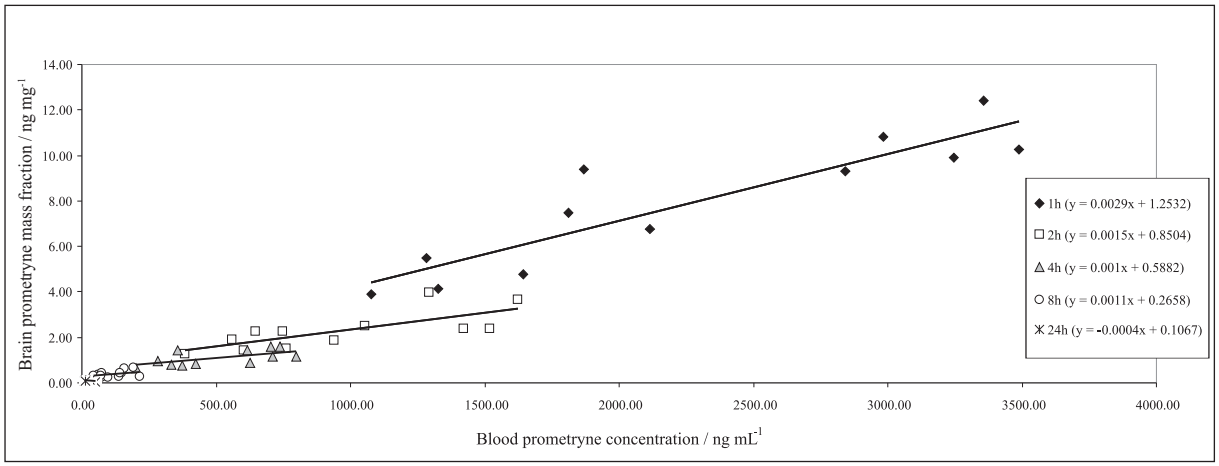

Figure 3 *The relationship between brain and blood levels of prometryne measured at different time points after administration of single acute dose in male and female CBA mice combined*

administered dose was relatively low. Maynard et al. (22) found that 1.2 % to 1.9 % of the original oral dose of prometryne was absorbed and distributed in the blood of rats. All this suggests that blood absorption of orally administered prometryne is relatively low, at least in laboratory rodents.

Data about prometryne toxicokinetics in the CNS of mammals are scarce, and our study gives an elementary view of prometryne's potential fraction and retention in the brain of mice (Table 2). Prometryne in mice brain was in even lower levels than in blood (Table 2). We selected the brain as the study organ for prometryne toxicokinetics (35) because it is well protected against xenobiotics and the hardest of organs to penetrate (36-38).

Prometryne's unpolar molecular structure and its octanol/water partition coefficient make prometryne likely to reach the brain, because brain structures are rich in lipids and have a tendency to uptake or concentrate lipofilic substances (37, 39). Having in mind that prometryne has low blood absorption and yet it has been traced in so distant a compartment as the brain over 24 h, then this means that prometryne has a potential to pass through all compartments of the mammalian organism, including placenta and put embryo at risk.

To the best of our knowledge, our study is the first to show the potential of prometryne to enter mouse brain, and it is the first to analyse prometryne absorption, bioaccumulation, and elimination in neural tissue over a short time. The only records available in literature refer to the neurotoxic kinetics of atrazine, a pesticide structurally similar to prometryne (23, 24, 26). Due to similar structure, the two compounds may share some toxicological properties (33, 34). Our results confirm this assumption, as they correspond to the findings of Stoker and Cooper (40), who examined the toxicokinetics of atrazine in rat brain and other organs. Similar to our results for prometryne, about 0.003 % of the administered dose of atrazine was found in the brain (40).

Atrazine seems to directly affect the neuroendrocrine function (40, 41). As our results confirm the affinity of prometryne for neural tissue, similar effects on the neuroendocrine functions are possible, but they have to be verified in future studies.

Brain and blood prometryne levels in our study decreased over 24 h (Tables 1, 2, and 3) and correlated well, but not over the full range of data. This finding is expected, given the rapid distribution and/or elimination in the first two hours. Slower elimination is expected from hours 2 to 8, with elimination half-time ($t_{1/2}$) of approximately 2 h, and even slower terminal elimination with a half life of about 8 h. Similar decline was demonstrated for atrazine in rats (23). It could be concluded that most of prometryne absorption in mouse blood occurs within the first hour, followed by a rapid decline in concentration. This drop in the prometryne level is highly correlated between the brain and blood (Table 3 and Figure 1). The decrease rate from hour one to hour two is higher in the brain (Δc_{m+f}=3.45x h^{-1}) than in blood (Δc_{m+f}=2.34x h^{-1}). This points to an active process of rapid prometryne elimination from the brain, since the prometryne has to move from brain against the higher concentration in blood (36, 44, 45).

In our study, we also observed sex-related differences in brain and blood prometryne kinetics. Blood prometryne was significantly higher in male than in female mice at all measured times, save for hour two when it was higher, but not significantly. Brain prometryne levels were similar between males and females, except on hour one measurement when males had a significantly higher prometryne mass fraction. The differences between the sexes are particularly clear if one looks at medians (Tables 1 and 2). Higher prometryne levels (especially the peak values of the hour one) in males suggest higher absorption than in females. We also observed a different dynamic of change between the sexes. In both brain and blood, males had 1.5 times higher values on hour one. In the brain this difference was dropping towards hour 24 (1.1 times), while in blood it rose (1.76 times on hour 24). This might be explained by different body mass between the sexes [male=(23.37±1.99) g; female=(22.25±1.14) g] or by different fat and muscle ratio. Another explanation might be that active brain elimination occurs at the same rate regardless of the sex, while elimination mechanisms (detoxification enzymes) in blood may slightly differ between males and females (see the correlation curves in Figures 2 and 3). Therefore, neurotoxicity studies of prometryne with mice as a model organism should take into account sex differences in prometryne brain bioavailability.

Log-normal plots reveal a possible three-step elimination that could be modelled with classical pharmacokinetic models, which could greatly help researchers in designing prometryne neurotoxicity experiments. In our study, the correlation between brain and blood prometryne was stronger in female mice. Regression coefficients show slower prometryne elimination rate in male mice than in female. Regression analysis (as in Figures 1, 2, and 3) makes it possible to calculate time-course changes in blood and brain prometryne levels.

Acknowledgements

We would like to thank Mr Gordan Mršić, head of the Centre for Forensic and Toxicological Investigation "Ivan Vučetić" of the Ministry of Interior for his support and help. This study was supported by the Ministry of Science, Education and Sports of the Republic Croatia (grant no. 110-0000000-1255). Our deep thanks go to Mrs Marija Potočić and Mr Nino Vukmanić for technical support and to retired Professor Oscar P. Springer for moral support and advice.

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Sažetak

TOKSIKOKINETIKA PROMETRINA U MOZGU MIŠEVA

Prometrin je metiltio-*s*-triazinski herbicid. Značajne količine prometrina zaostaju u tragovima u okolišu, poglavito u vodi, tlu i biljkama koje rabimo za prehranu. Cilj je rada izmjeriti količinu prometrina koja se apsorbira u mozgu i krvi nakon primijenjene akutne oralne doze (1 g kg^{-1} tjelesne mase) u odraslih miševa obaju spolova. Razine prometrina u mozgu i krvi izmjerene su GC/MS-om tijekom 1., 2., 4., 8. i 24. sata nakon izlaganja. Utvrđeno je da je udio prometrina koji se zadržava u živčanom tkivu relativno nizak ali detektabilan u odnosu na koncentraciju u krvi i koncentraciju primijenjene doze. Najviše koncentracije u krvi i maseni udjeli u mozgu zabilježeni su tijekom 1. sata nakon izlaganja, a s vremenom izmjerene vrijednosti značajno opadaju. Uočena je značajna razlika između mužjaka i ženki pri čemu mužjaci imaju značajno više razine prometrina u mozgu i krvi nego ženke. Opisana toksikokinetika prometrina pokazuje sličnosti s otprije opisanom i poznatom toksikokinetikom strukturalno sličnog herbicida atrazina.

KLJUČNE RIJEČI: *herbicidi, krv, otrovnost, triazini, živčani sustav*

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