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Original article

Effect of chlorpyrifos and enrofloxacin on selected enzymes in rats

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Abstract

This study examined the effect of chlorpyrifos and/or enrofloxacin on the activity of acetylcholinesterase (AChE) in the blood and brain, and the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum. The experiment was conducted on Wistar strain rats. Chlorpyrifos was administered with a stomach tube at a dose of 0.04 LD_{50} for 28 days and enrofloxacin at a dose of 5 mg/kg bw for 5 consecutive days. The experiment found that enrofloxacin changed the activity of the enzymes under study only to a small extent. At the dose applied in the experiment, chlorpyrifos decreased the activity of AChE significantly, both in blood and in the brain, and increased the activity of ALT and AST in rat serum. The administration of chlorpyrifos in combination with enrofloxacin changed the activity of the enzymes under study only slightly. A weaker, but longer, inhibition of AChE activity in both blood and the brain was observed in this group compared to the animals exposed only to chlorpyrifos. However, although enrofloxacin, like chlorpyrifos, increases the activity of ALT and AST in serum, their combined administration did not increase the hepatotoxic effect.

Key words: chlorpyrifos, enrofloxacin, AChE, ALT, AST, rats

Introduction

Intensification of plant and animal production and the need to reduce economic losses caused by pests and parasites necessitate using a range of chemicals whose introduction to the environment can result in adverse health reactions, both in humans and in animals. Among the potentially hazardous chemical compounds, bioactive substances, e.g. pesticides, are of particular importance (Maroni et al. 2000, Androutsopoulos et al. 2012).

Organophosphorus insecticides are one of the main groups of chemicals used to control plant and animal parasites (Eddleston and Phillips 2004, DPR 2009). Chlorpyrifos (*O,O*-diethyl-*O*-[3,5,6-trichloro-2-pyridyl]-phosphorothioate) is a noteworthy compound among these chemicals. It is a synthetic organophosphorus insecticide with a broad spectrum insecticide and acaricide action, commonly used around the world in agriculture and public health care (Solomon et al. 2014). In a process catalysed by the cytochrome P450-dependent monooxygenase system, chlorpyrifos is metabolically converted into its oxygen analogue – chlorpyrifos-oxon, which is a strong inhibitor of acetylcholinesterase (AChE), which results in cholinergic symptoms of poisoning (Busby-Hjerpe et al. 2010, Flaskos 2012).

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Enrofloxacin (1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxoquinoline-3-carboxylic acid) is an antibacterial chemotherapeutic agent – one of the second generation quinolones (Skyrianou et al. 2011) which are highly active towards Gram-negative and some Gram-positive bacteria, as well as intracellular microorganisms, e.g. *Mycoplasma spp.* and *Chlamydia spp*. (Brown 1996, Schink et al. 2013). Their mechanism of action is based on blocking two types of topoisomerases (DNA gyrase/topoisomerase II and topoisomerase IV) which results in disrupting the replication of genetic material in microorganisms and inhibition of protein synthesis in bacteria (Vancutsem et al. 1990, Onodera et al. 2002). Owing to enrofloxacin's potential antibacterial and favourable pharmacokinetic properties, including its high bioavailability (compared to other quinolone analogues) and excellent tissue penetration, it is widely used in veterinary medicine (Gonzalez et al. 2010, Abutarbush et al. 2012).

Due to the common use of pesticides, their potential interaction with various chemical compounds (including drugs) is a serious issue in experimental and clinical toxicology; in practice, mixed intoxications usually occur, i.e. simultaneous exposure of an organism to several potentially toxic compounds.

The compounds used in the experiment (chlorpyrifos, enrofloxacin) are typical substances used in agriculture and in veterinary medicine. The literature contains only scarce reports on interactions of organophosphorus insecticides and fluoroquinolones.

The aim of this study was to determine the effect of chlorpyrifos and enrofloxacin, administered individually or in combination, on the activity of acetylcholinesterase (AChE) in the blood and brain – as a biomarker of exposure to organophosphorus insecticides – and the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as an effect of hepatotoxic action. The model of subacute exposure was selected for the experiment since poisonings of this type occur relatively frequently.

Materials and Methods

Chlorpyrifos (O,O-diethyl-O-3,5,6-trichloropyridyl phosphorothionate, purity value min. 99.8%) was obtained from the Institute of Industrial Organic Chemistry (Warsaw, Poland). Commercial formulation of enrofloxacin (ENFLOCYNA® SOL) was purchased from Biowet Puławy Ltd. (Puławy, Poland) and contained 50 mg/ml of the active substance.

The studies were conducted on 120 male Wistar rats (from certificate Laboratory Animal House, Brwinów, Poland) of initial body weight 180±10g. During the acclimation and experiment, the animals were kept under standard laboratory conditions (12h light/dark cycle, temperature 22±1°C, humidity 70±10%) with free access to water and a standard laboratory chow. The rats were randomly divided into three experimental (I-III) groups and one control (C) group of 30 animals each.

Chlorpyrifos was given to rats in group I at a dose of 0.04 LD_{50} (6 mg/kg bw) for 28 days and enrofloxacin was applied to rats in group II (as Enflocyna®SOL) at a dose of 5 mg/kg bw for 5 consecutive days. Animals in group III were treated with both of the compounds in the same manner as in groups I and II, with enrofloxacin being given during the last five days of exposure to chlorpyrifos. Chlorpyrifos and enrofloxacin were administered intragastrically via a gastric tube. The remaining animals were untreated and used as the control. After 3, 6, 24, 72 and 168 hours following the last applied dose of the compounds under study, samples of heart-blood were collected for biochemical analyses.

The activity of acetylcholinesterase (AChE) in the blood and the brain was measured according to the method of Y.M. Ruckebush (Ruckebusch and Ruckebusch 1961). The activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum was measured applying the kinetic method with the use of an ALT-alanine aminotransferase and AST-aspartate aminotransferase analytical kit (Pointe Scientific Polska, Poland).

The experimental design and procedures has been approved by the Local Ethics Committee for Animal Experiments at the University of Warmia and Mazury in Olsztyn, Poland.

The results were expressed as arithmetic means (x) and standard errors of the mean (±SEM). The data were analysed statistically using one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. The differences were considered significant at $p<0.05$ and $p<0.01$. Statistical calculations were performed using Statistica 10 PL software (StatSoft, Poland).

Results

The activity of AChE in the blood and the brain and the activity of ALT and AST in serum of rats in particular groups and at specific time intervals are presented in Tables 1-4.

Chlorpyrifos (Group I) statistically ($p<0.01$) decreased the activity of AChE both in the blood and in the brains of the rats, which persisted for up to 72 hours (Tables 1-2). The maximum decrease in the

Table 1. Acetylcholinesterase (AChE) activity in the blood of rats after administration of chlorpyrifos, enrofloxacin and chlorpyrifos and enrofloxacin (expressed as µmol of substrate (acetylcholine bromide) 30 min/ml whole blood).

Group of animals (n = 6)	Time after intoxication				
	3 h	6 h	24 h	72 h	168 h
C (Control)	29.11 ± 1.31	26.03 ± 1.25	29.07 ± 0.69	28.38 ± 0.70	28.15 ± 0.83
I (Chlorpyrifos)	15.93 ± 0.83	16.91 ± 1.09	17.15 ± 0.91	21.01 ± 1.16	27.22 ± 0.89
II (Enrofloxacin)	24.70 ± 0.86	23.66 ± 1.13	27.30 ± 1.76	25.24 ± 1.29	26.24 ± 0.86
III (Chlorpyrifos + Enrofloxacin)	16.19 ± 1.16	16.20 ± 1.99	19.99 ± 1.05	17.98 ± 0.91	23.75 ± 0.89
Statistical differences	$P_{C\text{-}I,III}<0.01$ $P_{C\text{-}II}<0.05$ $P_{II\text{-}I,III}<0.01$	$P_{C\text{-}I,III}<0.01$ $P_{II\text{-}I,III}<0.05$	$P_{C\text{-}I,III}<0.01$ $P_{II\text{-}I,III}<0.01$	$P_{C\text{-}I,III}<0.01$ $P_{II\text{-}I}<0.05$ $P_{II\text{-}III}<0.01$	$P_{C\text{-}III}<0.01$ $P_{I\text{-}III}<0.05$

values expressed as means ± SEM
n – number of rats in the group

Table 2. Acetylcholinesterase (AChE) activity in the brain of rats after administration of chlorpyrifos, enrofloxacin and chlorpyrifos and enrofloxacin (expressed as µmol of substrate (acetylcholine bromide) 30 min/ml of homogenate of brain tissue).

Group of animals (n = 6)	Time after intoxication				
	3 h	6 h	24 h	72 h	168 h
C (Control)	219.13 ± 1.31	224.92 ± 4.28	226.68 ± 4.08	224.77 ± 1.46	219.98 ± 3.48
I (Chlorpyrifos)	140.60 ± 4.14	147.89 ± 6.98	152.62 ± 5.62	185.05 ± 5.98	200.08 ± 4.57
II (Enrofloxacin)	194.69 ± 5.34	197.33 ± 5.71	211.28 ± 6.05	217.00 ± 6.52	212.11 ± 3.37
III (Chlorpyrifos + Enrofloxacin)	146.51 ± 3.22	148.02 ± 5.33	157.48 ± 4.28	177.79 ± 4.68	182.79 ± 5.06
Statistical differences	$P_{C\text{-}II}<0.05$ $P_{C\text{-}I,III}<0.01$ $P_{II\text{-}I,III}<0.01$	$P_{C\text{-}II}<0.05$ $P_{C\text{-}I,III}<0.01$ $P_{II\text{-}I,III}<0.01$	$P_{C\text{-}I,III}<0.01$ $P_{II\text{-}I,III}<0.01$	$P_{C\text{-}I,III}<0.01$ $P_{II\text{-}I,III}<0.01$	$P_{C\text{-}III}<0.01$ $P_{I\text{-}III}<0.05$ $P_{II,III}<0.05$

values expressed as means ± SEM
n – number of rats in the group

Table 3. Alanine aminotransferase (ALT) activity in serum of rats after administration of chlorpyrifos, enrofloxacin and chlorpyrifos and enrofloxacin (expressed as IU/l).

Group of animals (n = 6)	Time after intoxication				
	3 h	6 h	24 h	72 h	168 h
C (Control)	47.74 ± 2.45	41.25 ± 2.13	41.20 ± 1.82	44.20 ± 1.02	48.33 ± 1.92
I (Chlorpyrifos)	66.60 ± 5.24	58.35 ± 3.61	53.48 ± 3.34	50.83 ± 4.04	50.21 ± 2.77
II (Enrofloxacin)	54.28 ± 3.38	45.02 ± 2.60	43.62 ± 3.03	42.79 ± 3.89	48.44 ± 3.24
III (Chlorpyrifos + Enrofloxacin)	61.88 ± 2.47	53.04 ± 2.29	51.27 ± 3.77	49.06 ± 3.77	50.92 ± 3.28
Statistical differences	$P_{C\text{-}I}<0.01$ $P_{C\text{-}III}<0.05$	$P_{C\text{-}I}<0.01$ $P_{I\text{-}II}<0.05$	$P_{C\text{-}I,III}<0.05$		

values expressed as means ± SEM
n – number of rats in the group

Table 4. Aspartate aminotransferase (AST) activity in serum of rats after administration of chlorpyrifos, enrofloxacin and chlorpyrifos and enrofloxacin (expressed as IU/l).

Group of animals (n = 6)	Time after intoxication				
	3 h	6 h	24 h	72 h	168 h
C (Control)	76.02 ± 3.75	80.74 ± 3.24	83.10 ± 3.02	77.20 ± 3.12	80.74 ± 3.81
I (Chlorpyrifos)	94.88 ± 6.64	106.08 ± 3.06	99.89 ± 2.73	87.07 ± 2.43	88.84 ± 5.11
II (Enrofloxacin)	83.28 ± 4.55	91.02 ± 4.11	89.68 ± 3.71	74.96 ± 3.47	82.74 ± 4.62
III (Chlorpyrifos + Enrofloxacin)	91.35 ± 5.98	98.66 ± 5.76	99.01 ± 6.25	82.39 ± 4.35	77.79 ± 5.44
Statistical differences	$P_{C\text{-}I,III}<0.01$	$P_{C\text{-}I,III}<0.01$	$P_{C\text{-}I,III}<0.01$		

values expressed as means ± SEM
n – number of rats in the group

activity of AChE compared to the control (45.3% in blood and 35.8% in the brain) was observed after 3 hours. Inhibition of AChE activity remained at a similar level for up to 24 hours, which was followed by a steady increase in enzyme activity, with the process being faster in the blood. After 168 hours following exposure, the activity of AChE in the blood was lower by 3.3%, and in the brain by 9.1% than in the control group. After enrofloxacin was administered (Group II), a slight decrease in the activity of AChE was observed. The highest, statistically significant ($p<0.05$), decrease in this group was observed after 3 hours in the blood and after 6 hours in the brain (15.1% and 12.3%, respectively) (Tables 1-2). The profile of changes of AChE activity in the group of rats subjected to combined intoxication (Group III) was similar to that observed in the group of rats exposed only to the organophosphorus insecticide; however, slower reactivation of AChE was observed in both the blood and the brain.

The activity of ALT and AST in rat serum following the administration of chlorpyrifos (Group I) was elevated compared to the control throughout the experiment (i.e. until the 168th hour). The increase was statistically significant ($p<0.01$) until the 24th hour (Tables 3-4). The highest increase in the activity of the enzymes (ALT – 41.4%, AST – 31.4%) was observed after 6 hours. Enrofloxacin (Group II) at the dose applied increased the activity of ALT and AST in serum, especially during the initial period of the experiment, i.e. within the first 24 hours. The largest increase in the activity of ALT (13.7%), compared to the control, was observed after 3 hours, whereas for AST it was after 6 hours (12.7%). The activity of enzymes during the other time intervals fluctuated within a similar range as for the control. The changes in the activity of the enzymes under analysis which occurred in group III of the animals (which received both chlorpyrifos and enrofloxacin) were similar to those observed in group I, but their intensity was lower (Tables 3-4).

Discussion

Determination of the activity of AChE in different tissues is a good biomarker of intoxication with organophosphorus insecticides. A decrease in the enzyme activity is a consequence of the main mechanism of toxic action of organophosphorus compounds, which consists of inhibition of the activity of cholinesterase enzymes (Farahat et al. 2011, Roszczenko et al. 2013). For chlorpyrifos, its oxidised form – chlorpiryfos-oxon, formed in reactions of desulphuration and oxidation in the liver is responsible for its action (Sams et al. 2004). This results in inhibition of AChE activity, accumulation of synaptic acetylcholine, excessive stimulation of neurons and cholinergic symptoms of poisoning (Vale and Lotti 2015).

In the present study, subacute poisoning with chlorpyrifos resulted in the inhibition of AChE activity in the blood and the brain of rats, which depended on the duration and type of tissue. The strongest inhibition of AChE occurred during the first hours after exposure and the physiological level was restored after several days. Inhibition of AChE was more pronounced in the blood of rats and the enzyme reactivation was much slower in the brain. These findings are consistent with those of other authors, who have also observed a decrease in the activity of cholinesterases following exposure to chlorpyrifos, both to a single dose and to repeated doses (Marty et al. 2012, Reiss et. al. 2012). Yan et al. (2012) applied various doses of chlorpyrifos to rats for four weeks and found AChE in

the blood to decrease; doses of 1, 5 and 10 mg/kg bw resulted in a decrease of 46.7%, 64.6% and 68.8%, respectively, compared to the control. Similarly, Goel et al. (2000) observed a significant decrease in the activity of AChE in the serum and liver of rats following administration of chlorpyrifos for eight weeks (every other day) at a dose of 13.5 mg/kg bw.

The liver is the organ where activation and detoxification of chemical substances, including pesticides and drugs, takes place. It has been demonstrated in many studies that organophosphorus insecticides (e.g. chlorpyrifos) cause liver damage by changing the activity of liver enzymes, such as ALT, ALP, AST and LDH (Gokcimen et al. 2007, Flehi-Slim et al. 2015).

An increase in ALT and AST activity in rat serum, observed in our studies, confirms the hepatotoxic effect of chlorpyrifos. This insecticide has been shown to increase the activity of ALT at the dose applied in the study, which was observed mainly until 24 hours after administration of the compound. The difference in the activity of the aminotransferases may be a consequence of organ specificity and subcellular location of the enzymes. ALT is more specific in liver damage (Giannini et al. 2005); it is present mainly in the cytoplasm of liver cells and it is released to the blood following even small damage to the cytoplasmic membranes. On the other hand, 80% of AST is present in mitochondria and 20% in the cytosole of hepatocytes, so an increase in its activity in serum may be a sign of a disorder in mitochondrial membrane function and an increase in its permeability, as well as more profound damage to cell structures (Rej 1989). Moreover, AST has high extrahepatic activity in skeletal muscles, heart and kidneys, which makes it less specific for liver examination (Wolf 1999). This may be the reason for a slightly lower activity of the enzyme compared to ALT, which was observed in our study.

Despite the hepatotoxic effect of organophosphorus insecticides, the mechanisms of liver cell damage have not yet been fully elucidated. Moreover, organophosphorus compounds demonstrate oxidative properties both in acute and chronic poisonings by changing the activity and content of enzymatic and non-enzymatic antioxidants (Spodniewska et al. 2014, Deng et al. 2016). The free-radical process of oxidation of unsaturated fatty acids and other lipids (which results in the formation of their peroxides), disturbs cell function, which manifests itself in rupturing cell membranes, changing receptor functions and loss of integrity of cell membranes, e.g. in liver cells. It cannot be ruled out that the increase in the activity of aminotransferases observed in studies conducted by the authors may have been caused by liver disfunctions, defects in enzyme synthesis or damage to the cell membrane of hepatocytes as well as hypoxia and disorder of the energy balance of the body. Both prevention of the effects of oxidative stress and such symptoms as convulsions and tremors, which occur in poisoning, increase the body's demand for energy (Lukaszewicz-Hussain 2011).

Fluoroquinolones are a group of antibacterial drugs used mainly against bacteria which cause diseases of the respiratory tract and skin, and infections of the urogenital system. Since there are no reports of studies on the effect of enrofloxacin (and other fluoroquinolones) on the activity of cholinoestrases, it is difficult to provide a clear interpretation of the findings of the authors' research, which demonstrates that the administration of enrofloxacin resulted in slight inhibition of AChE activity in rat blood and brain. A decrease in the activity of AChE can be attributed to a direct effect on AChE or, which is more probable, to damage to neuronal membranes caused by peroxidation of lipids, since both enrofloxacin and its metabolites are known to cause oxidative stress (Gurbay et al. 2007, Wang et al. 2009).

Although used widely and regarded as safe antibiotics, fluoroquinolones can cause damage to liver parenchyma. There have been reports in the literature which describe a temporary increase in the activity of liver enzymes, such as AST, ALT and ALP, following the application of gemifloxacin or levofloxacin (Roy et al. 2010, Figueira-Coelho et al. 2010). Our own studies showed that the hepatotoxic effect after administration of enrofloxacin is temporary and reversible. Enrofloxacin has been shown to cause a slight increase in the activity of ALT and AST in rat serum. Enzyme activity reached the normal level within several days of discontinuation of administration of the compound administration. Similarly, Tras et al. (2001) observed an increase in AST activity in serum of dogs which were given enrofloxacin intramuscularly at a dose of 5 mg/kg bw for 14 days. The enzyme activity returned to the physiological values after the exposure was discontinued. However, Elkholy et al. (2009) did not observe any changes in the activity of liver enzymes after giving enrofloxacin to chickens for 5 days at a dose of 10 mg/kg bw (as two preparations: Baytril® and Enrotry®).

An increasing number of reports have been published in recent years on the interaction of organophosphorus insecticides with various compounds. However, the majority of the studies concern the protective effect of some elements, e.g. Zn, Se and vitamins, e.g. A, C and E, with respect to the

oxidative effect of organophosphorus compounds, including chlorpyrifos (Goel et al. 2005, Aly ed al. 2010, Ben Amara et al. 2011). There have been some investigations on mixtures of pesticides and their cholinergic interaction. In their study of rats intoxicated with chlorpyrifos and/or diazinone, Tichmalk et al. (2005) observed the strongest inhibition of ChE in serum following the administration of the largest dose of chlorpyrifos (i.e. 60 mg/kg bw) administered individually and together with diazinone. Wang et al. (2014) administered chlorpyrifos and/or carbaryl in three doses, i.e. in a small ($^1/_{125}$ LD_{50}), medium ($^1/_{50}$ LD_{50}) and large dose ($^1/_{20}$ LD_{50}) and found the activity of AChE in the brain following administration of a medium and a large dose to be similar to that observed in a group of animals exposed only to chlorpyrifos, which indicates that their administration in combination does not intensify the inhibition of the enzyme under study. No intensification of inhibition of ChE activity in plasma or in the brain following administration of chlorpyrifos and α-cypermethrin was also noted by Wielgomas and Krechniak (2007). Similar findings have been observed in the authors' own research, when the activity of AChE in blood and in the brain in a group of rats exposed to chlorpyrifos and enrofloxacin given together changed according to the same trend as in a group of animals intoxicated only with chlorpyrifos, with reactivation of AChE slower both in the blood and in the brain.

As yet, there have been no reports in available literature on the interaction of chlorpyrifos and enrofloxacin (or fluoroquinolones) on the activity of cholinesterases and liver enzymes.

It has been shown in the authors' own research that enrofloxacin extended the process of inhibition of AChE activity caused by chlorpyrifos and delayed reactivation of the enzyme, whereas combined administration of both compounds did not intensify the hepatotoxic effect. The profile of changes was similar to that observed in a group of animals exposed only to chlorpyrifos. A much weaker decrease in AChE activity and a smaller increase in liver parameters compared to the rats which were given chlorpyrifos were also observed by Goel et al. (2000) and by Mansour and Mossa (2010) after oral administration of chlorpyrifos and zinc to rats. The absence of a synergistic action of chlorpyrifos and enrofloxacin was also observed in our previous studies concerning the effect of these compounds on the activity of CAT, SOD and GPx (Barski et al. 2011) and vitamins A and E in rats (Spodniewska et al. 2015).

This study found that no intensification of changes in the activity of the enzymes under analysis can be attributed to inhibition by fluoroquinolones, including enrofloxacin, of the activity of cytochrome P450 isoforms, i.e. CYP1A2 and CYP3A4 (Regmi et al. 2005, Zhang et al. 2011), since isoforms of the CYP1 and CYP3 family are known to be responsible for biotransformation of organophosphorus insecticides to toxic metabolites – oxones (Buratti et al. 2003, Foxenberg et al. 2011).

Conclusions

The present study found that chlorpyrifos and enrofloxacin used in the experiment decreased the activity of AChE in both the blood and brain and increased the activity of ALT and AST in the serum of rats. Changes in the activity of the enzymes were more pronounced following the administration of chlorpyrifos. Enrofloxacin extended the process of inhibition of AChE activity caused by chlorpyrifos and delayed reactivation of the enzyme. However, the hepatotoxic effect of chlorpyrifos was not found to be intensified by the administration of enrofloxacin (the activity of ALT and AST in serum was slightly lower compared to intoxication only with chlorpyrifos).

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