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Biodisposition and Biochemical Effects of a New Phosphoramidate Series in Rat Tissues

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Organophosphorus compounds have been widely used for a few decades in agriculture for crop protection. Thousands of these compounds have been screened and over one hundred of them have been marketed for this purpose (Hassall 1990). In addition to being economical and efficient in pest control (Chirions and Geraud-Pouey 1996; Geraud-Pouey et al. 1997), organophosphorus pesticides are generally characterized by low persistence in the environment and in tissues of the living organisms. However, poisoning studies indicated that moblization of toxicants from tissues to blood was observed in intoxicated patients (Anonymous 1985). Therefore, there is a need for new organophosphorus pesticides that are more selective and less persistent in body tissues (Garcia-Repetto et al. 1997; Garcia-Repetto et al. 1995). New series of *O*-aryl phosphoramidates and benzo-1,3,2-dioxaphospholenes were recently synthesized in our laboratory and found to have good insecticidal (Ali 1999; Ali and Zidan 2000) and potent fungicidal activities relative to some commercial pesticides (Ali and Ali 2000a; 2000b). In addition, they are expected to give non-toxic metabolites e.g. catechol, phenol and amino acid esters.

The present work deals with the persistence of four members of these groups in different tissues in comparison with that of a commercial pesticide, sumithion, known to have a relatively low acute toxicity to mammals (Hollingworth et al. 1967; Hayes and Laws 1990) with a reported LD_{50} ~ 250 mg / kg body weight (Worthing 1987). Alkaline phosphatase is a metalloenzyme that catalyzes the nonspecific hydrolysis of inorganic (Yoza et al. 1997) and organic (Natchev 1988) phosphates. It is used to detoxify toxic organophosphorus compounds (Vayron et al. 2000; Renard et al. 1999). The *in vivo* effects of the tested compounds on this enzyme are also described.

MATERIALS AND METHODS

The phosphoramidates **1**, **2**, *N*-ethoxyphenoxyphosphinyl) glycine ethyl ester and L-glutamic acid diethyl ester respectively, and **3**, **4**, *N*-(2-oxido-1,3,2-benzodioxaphosphol-2-yl) glycine ethyl ester and L-glutamic acid diethyl ester respectively (Fig. 1) were synthesized as previously published (Ali 1999; Ali and Zidan 2000). Sumithion (95%), *O*, *O*-dimethyl-*O*-(3-methyl-4- nitrophenyl) phosphorothioate, was obtained from Sumitomo Chemical Co.

Adult male albino Wistar rats (4 - 6 months old) weighting 100 ± 15 g were housed in six groups of 20 - 22 animals; one group served as the control, one group for each

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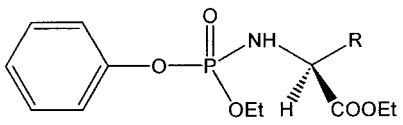
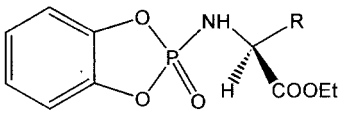


R = H **(1)**
R = CH_2CH_2COOEt **(2)**



R = H **(3)**
R = CH_2CH_2COOEt **(4)**

Figure 1. Phosphoramidate structures.

of the four new compounds, and one group for sumithion. Animals fed on standard laboratory diet for a week for acclimation. Compounds **1** - **4** and the commercial pesticide sumithion were administrated orally by a gavage with olive oil as a vehicle. Treated animals received a single dose of 85 mg / kg body weight which is ~ 1 / 3 of the LD_{50} of sumithion (250 mg / kg) while control group received olive oil only. At each interval, three animals from each group were sacrificed without the use of anesthesia and blood was collected. Half of each blood sample was centrifuged to get the serum for enzyme assays. Hearts, kidneys and the second half of the whole blood were subjected to residual analysis. Brain and liver samples were divided into two parts for residual and biochemical analysis.

For residual analysis, organs were homogenized in a CH_2Cl_2-THF (2:1) solvent system then centrifuged and extracted twice with the same solvent. The organic layer extracts of each sample were combined, dried over anhydrous $MgSO_4$ and evaporated. The dry residues were redissolved in acetone and analyzed *via* 12A Shimatzu GC equipped with electron capture detector. The injection and detector temperatures were 230 and 250°C, respectively. The column was packed with chromosorb Q coated with 2 % dexil. Different programming and column temperatures were first applied and found that isothermal column temperature at 180°C gave good resolution of the tested compound peaks.

For biochemical analysis, brain and liver organs were homogenized immediately after sacrificing the animals with 5 mL cold phosphate buffer (pH 7.2, 0.1M) containing 0.5 % triton X-100 and 0.25 M sucrose then centrifuged for 10 minutes at 10,000 rpm in an Eppendorf centrifuge. Alkaline phosphatase (EC 3.1.3.1), ALP, assay was determined in the clear supernatant by the use of *p*-nitrophenylphosphate as a substrate (0.01M) and diethanolamine (1.0 M) containing 0.5 mM $MgCl_2$ as a buffer (pH 9.8). The absorbance change as a result of the reaction of sodium azide with the liberated *p*-nitrophenol for one minute was recorded at 25 °C at 405 nm. ALP specific activity is expressed in U/mg protein where the enzyme unit (U) is the amount of alkaline phosphatase that produces 1.0 μmole *p*-nitrophenol per minute at 25°C. Total protein was determined by the method of Bradford (1976) using Coomassie brilliant blue G-250 dye and bovine serum albumin as a standard. Absorbance was recorded at 595 nm on a Shimatzu 160 A dual-bean UV-VIS spectrophotometer.

A series of single-factor ANOVAs was run by using statistical package SPSS (version 8.0) to test the hypothesis of the pesticide effect on enzyme activity. Fisher's LSD test was used to show significant differences between treatments and controls at

the 0.05 significance level. The hydrophobic parameter, log P (octanol-water partition coefficient), was calculated by using CS Chem 3D (version 5.0), a molecular modeling and analysis program.

RESULTS AND DISCUSSION

To study the toxicity of the recently introduced phosphoramidates **1** - **4** to mammals in comparison with a commercial pesticide, sumithion, all animals received a single dose of one of the tested compounds. Animals of the sumithion group suffered from severe neurological symptoms e.g. muscular cramp, sweating and salivation, leading to death of 40 % of the treated animals in 48 hours. The surviving rats were sacrificed at each interval (0.25, 1 and 2 days).

Animals treated with the phosphoramidates did not show any mortality or neurological symptoms; therefore, the experiment could be extended to 16 days to study the enzyme recovery and the residue persistence. Three animals from each group were sacrificed at 1, 4, 8, 12 and 16 days. Residues of compounds **1** - **4** and sumithion were analyzed by GC analysis where the detection limit, as at 3 times the chromatographic noise (Chamberlain 1985), were 6.8, 4.9, 7.8, 5.9 and 5.5 ppb respectively. The average recovery percents of three determinations for each compound from different tissues spiked with 10 µg/g organ (10 ppm) ranged from 85 to 93% (Table 1). Results, as presented in Fig. 2, indicated that residues of all compounds, including sumithion, decreased with time in all organs. One day after the oral administration, residues of the phosphoramidates **1**, **3** were below 6 ppm while those of the phosphoramidates **2**, **4** did not exceed 2.5 ppm indicating that incorporation of the glutamic acid moiety lowered the phosphoramidate persistence. After four days, compound **2** disappeared in brain and compound **3** was not detected in blood, liver, heart and kidney, while compound **4** vanished in blood and heart. At longer periods, all phosphoramidates were under the detection limit in all studied organs. Sumithion, on the other hand, showed higher residue after one day in all organs (1.92 – 8.01 ppm) while the range decreased to 0.20 – 1.54 ppm in two days.

Each pesticide showed a specific pattern of affinity towards different tissues, as previously noted for some other organophosphorus pesticides (Garcia- Repetto et al. 1995). Therefore, to test the accumulation in different tissues, the coefficient of distribution in tissue (CDT) was calculated by the formula:

$$CDT = [\text{pesticides}]_{\text{tissue}} / [\text{pesticide}]_{\text{blood}}$$

The CDT provides information on the relative affinity of a compound for different tissues relative to blood (Repetto, 1988); therefore, the CDT was not calculated when residue in blood was zero. The results encountered in Table 2 showed that accumulation (CDT > 1) might occur in the first 24 hours but then decreased as in the case of compound **1** in brain, heart and kidney with CDT equal 5.51, 5.12 and 5.67, respectively. On the other hand, compounds **2** - **4** showed no (CDT < 1) or little accumulation in all organs. In the meantime, no accumulation of sumithion was observed. The relative low persistence and accumulation of the phosphoramidates was comparable to those of a known low persistence pesticide (sumithion) and may be due to their high functionality that facilitates their mobilization and

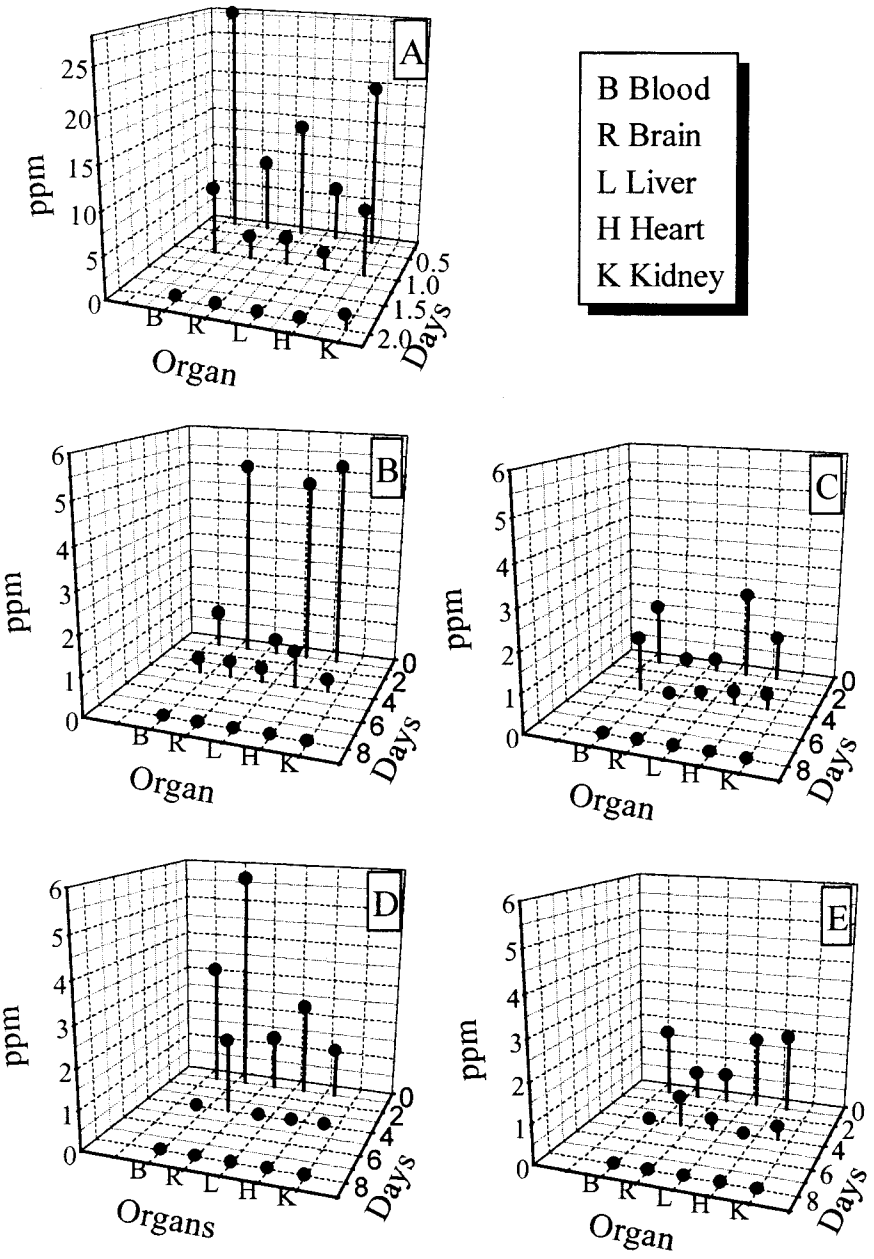


Figure 2. Persistence of sumithion (A) and the phosphoramidates **1** - **4** (B-E, respectively) in different tissues.

Table 1. Average recovering percent of the phosphoramidates **1** - **4** and sumithion.

Compound	Blood	Brain	Liver	Heart	Kidney
1	90.71	87.34	84.66	88.07	85.63
	(±3.53)	(±4.22)	(±5.08)	(±5.84)	(±4.67)
2	88.92	85.94	86.41	90.25	90.56
	(± 3.26)	(± 6.75)	(± 6.29)	(± 4.36)	(± 6.33)
3	92.75	89.27	84.18	90.94	89.37
	(± 1.82)	(± 5.86)	(± 4.16)	(± 3.98)	(± 5.32)
4	90.01	91.39	87.88	86.05	87.16
	(± 2.95)	(± 4.61)	(± 7.03)	(± 4.41)	(± 2.86)
Sumithion	89.19	86.17	85.90	89.56	91.03
	(± 4.66)	(± 3.96)	(± 4.64)	(± 3,78)	(± 6.25)

Results are mean of three determinations (± SD)

Table 2. The CDT values of the phosphoramidates **1** - **4** and sumithion.

Compound	Time (Day)	Brain	Liver	Heart	Kidney
1	1	5.51	0.38	5.12	5.67
1	4	1.12	0.92	2.61	0.87
2	1	0.10	0.17	1.38	0.71
2	4	0.00	0.12	0.23	0.27
3	1	1.80	0.43	0.75	0.39
3	4	-	-	-	-
4	1	0.38	0.42	1.08	1.15
4	4	-	-	-	-
Sumithion	0.25	0.32	0.50	0.23	0.71
Sumithion	1	0.33	0.38	0.24	0.96
Sumithion	2	0.85	0.65	1.12	4.89

excretion. In addition, the presence of different hydrolyzing bonds (P-O-C, P-O-N, CO-O) makes them susceptible to different hydrolase enzymes.

Results of the alkaline phosphatase assays presented in Table 3 and Fig. 3 showed that the enzyme activities were suppressed in serum and liver by all the examined compounds. In most phosphoramidate treatments, the enzyme activity reached the minimum in the first four days then recovered again to its normal level. The enzyme activities were not significantly different from those of the control group beyond 8 days, except for that of compound **1** after 12 days as shown in Table 3. This trend is generally parallel to that of decreasing the residual levels observed during the same period. The enzyme activity in the control animals did not change significantly during the experimental period (16 days). The observed minimum activities were 27.17 – 48.24 % and 58.30 - 80.38 % of the control activities in serum and liver respectively. However, activity was inhibited by sumithion to 9.67 % and 7.42 % in both organs respectively after a period of two days during which most animals died.

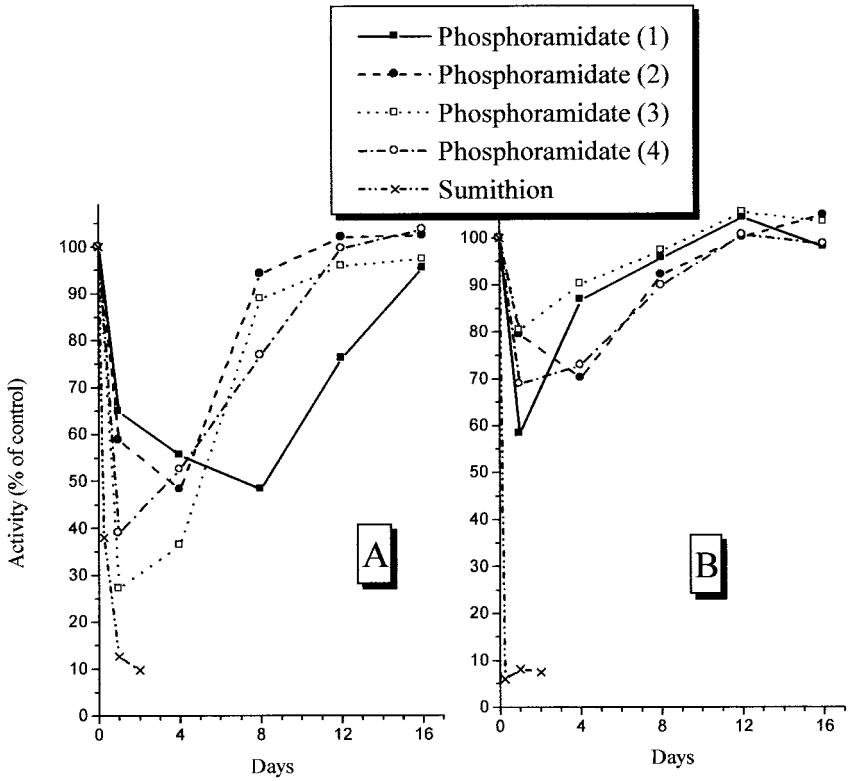


Figure 3. Inhibition and recovery of alkaline phosphatase upon treatment with the phosphoramidates and sumithion in serum (A) and liver (B).

The results indicated that although both the phosphoramidates and sumithion showed a comparable low persistence and accumulation, they differed remarkably in their neurotoxic and biochemical effects represented by their neurological symptoms and inhibitory effects on alkaline phosphatase (discussed above) and acetylcholinesterase (*Ali et al. unpublished*). This variation could be explained by the higher hydrophobicity of sumithion and its metabolites, 3-methyl-4-nitrophenol. It was established that hydrophobicity represented by log P is an important factor and strongly correlated to the toxicity of phenols and many other toxins (Hansch and Leo 1995). Log P showed that 3-methyl-4-nitrophenol is more hydrophobic than the metabolites of the examined phosphoramidates, phenol and catechol (1.85, 1.48 and 1.09, respectively). Incorporating amino acids in the phosphoramidates even enhances their hydrophilicity. Therefore, it could be concluded that the highly functionalized phosphoramidates have some advantages of having low persistence and biochemical effects in different tissues in addition to giving relatively non-toxic metabolites, which minimize their hazardous effects on the environment and contacted mammals.

Table 3. The activity percentages of alkaline phosphatase relative to control.

Time (Day)	Serum	Liver	Serum	Liver
Control	100.00 (± 6.81)	100.00 (± 3.91)		
	Phosphoramidate (1)		Phosphoramidate (2)	
1	64.85 (± 3.09)*	58.30 (± 3.04)*	58.78 (± 2.44)*	79.43 (± 4.34)*
4	55.50 (± 3.77)*	86.79 (± 2.38)*	48.24 (± 3.49)*	70.19 (± 2.83)*
8	48.24 (± 1.92)*	95.66 (± 4.62)	94.15 (± 4.38)	92.08 (± 2.32)*
12	76.11 (± 4.38)*	104.34 (± 5.57)	101.87 (± 4.75)	100.19 (± 3.62)
16	95.32 (± 4.54)	98.11 (± 4.38)	102.23 (± 4.81)	104.83 (± 5.93)
	Phosphoramidate (3)		Phosphoramidate (4)	
1	27.17 (± 1.76)*	80.38 (± 4.19)*	39.04 (± 1.40)*	68.87 (± 2.47)*
4	36.30 (± 2.15)*	90.19 (± 4.17)*	52.46 (± 3.16)*	72.83 (± 4.26)*
8	88.76 (± 3.16)*	97.17 (± 4.19)	76.81 (± 3.51)*	89.81 (± 3.08)*
12	95.78 (± 4.52)	105.47 (± 4.08)	99.53 (± 1.55)	100.57 (± 4.25)
16	97.19 (± 3.16)	103.40 (± 4.32)	103.51 (± 5.67)	98.68 (± 3.91)
	Sumithion			
0.25	37.94 (± 2.39)*	5.96 (± .43)*		
1	12.65 (± 1.54)*	8.04 (± .74)*		
2	9.67 (± 1.41)*	7.42 (± .43)*		

Results are mean of three experiments (± SD)
* significantly different from control at $p = 0.05$

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