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"XENOBIOTIC KINETICS AND TOXICITY AMONG FISH AND MAMMALS"

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ABSTRACT

The purpose of this project is to develop techniques that account for interspecies differences in the pharmacokinetics of xenobiotics. The hypothesis proposed is that toxicity occurs after exposure of the target organ to a characteristic concentration of toxicant for a particular period of time. To test the hypothesis, experiments are proposed to characterize the pharmacokinetics of three representative chemicals (lindane, pentachlorophenol and paraoxon) in small trout via water exposure, and large trout and rats via intravascular injection. Compartmental pharmacokinetic models will be used. The fraction of a dose of each test compound converted to each of its metabolites by the test animals will be determined to account for possible metabolic differences that might contribute to interspecies differences in toxicity. Binding of the test substances in blood to formed elements and plasma proteins will also be characterized. The LC50s and LD50s of the test compounds will be determined and the values will be converted to free concentrations using various pharmacokinetic transformations. The transformation that gives a common concentration for toxicity in the three groups of animals will be an "index of relative exposure" that will provide an estimate of the dose to the target organ rather than the dose to the animal. The area under the free concentration-time curve will be the starting point for development of the exposure index. Successful development of such an index should result in substitution for research purposes of fish for mammalian species, and in a better understanding of interspecies differences in the dosage of chemicals that produce toxicity. The research will also provide useful information about the pharmacokinetic and toxicologic properties of the test compounds.

PROGRESS

Work was focussed on paraoxon, a directly acting inhibitor of acetylcholinesterase (ACHE) and a potent toxicant of the cholinergic nervous system. This compound was chosen for study first because it has a quantifiable toxicity, namely inhibition of ACHE. Simultaneous determination of the concentration-time profile of paraoxon and its inhibition of ACHE was believed to provide a good opportunity to realize the objectives of the project. Maxwell, Vlahacos and Lenz published results of a study of another ACHE inhibitor, soman, that supports this belief (Toxicology Letters 43:175-188,1988). They successfully modeled the soman-induced inhibition of ACHE in the rat after intramuscular injection.

Our first objective was to identify the site of action of paraoxon. While it inhibits ACHE in all tissues, the tissue in which inhibition results in death is not known for certain. The previous progress report (9-15-88 to 6-30-90; AFOSR-88-0345) described results in rat suggesting that the site of action was the CNS, but outside the blood brain barrier.

The HPLC method for determination of paraoxon that we developed proved too insensitive (limit of 1 nM or 275 ng/ml plasma) for the development of the pharmacokinetic model for paraoxon and during the reporting period considerable effort was expended investigating gas chromatographic methods. Recent advances in capillary GC technology have lowered the limits of detection of compounds present in trace quantities. The GC that we used was a Hewlett Packard 5890A, series II, with electron capture, nitrogen-phosphorous and flame ionization detectors, dual capillary injection ports, autoinjector with 100 sample tray, and HP Chemstation and HP model ES/12 PC for instrument control, data acquisition and analysis.

ASSAY DEVELOPMENT PROCEDURES

A series of experiments to develop a method based on the nitrogen-phosphorous detector were carried out. Initially, a 5% phenylmethyl silicone column was used. This column had too high a bleeding rate, which limited the performance of the detector's active element. A methyl type silicone column (25 m by 0.2 mm id and 0.2 μm film thickness) overcame this problem.

To achieve maximum sensitivity, the solvent effect and cold trap methods were utilized in addition to splitless injection. For this purpose the oven temperature programming was optimized as: initial temperature 70 °C, initial time 2 minutes, rate 15 °C per minute, final temperature 190 °C, final time 5 minutes. Injector temperature 210 °C and detector temperature 250 °C. During the procedure, it was noted that the detector active element was very sensitive to the oven temperature change, resulting in an unstable signal and the requirement for longer equilibrium time. To overcome this problem a higher boiling point solvent, isooctane (B.P. 99 °C), was used as the sample solvent, which also enhanced the solvent effect and the cold trap effect.

The sensitivity of the developed method was 6 ng/ml for paraoxon, but the detector was very unstable and rapidly lost sensitivity after replacement of the detector bead; it required changing weekly, which was expensive and made progress difficult. The manufacturer's field representative worked with us to overcome these problems with the N-P detector, but after several months attempting to overcome the instability in the NPD, it was returned to the manufacturer.

We briefly investigated the use of a mass selective detector and capillary GC. The sensitivity achieved was 100 ng/ml, which was not low enough for a pharmacokinetic study. Also, the existing HPLC method was reinvestigated and the sensitivity improved, but only to 200 ng/ml.

While paraoxon does not contain a halogen atom, we found by serendipity that the electron capture detector was very sensitive to paraoxon and this detector was much more stable than was the N-P detector. After optimization of all the instrument operating conditions, we achieved a sensitivity of 6 ng/ml for paraoxon and 5 ng/ml for parathion, a widely used organophosphate insecticide that is converted to its active form, paraoxon, in vivo. The method uses a 25 m 5% phenylmethyl silicone column, splitless injection, an automatic injector, and the following temperature program: initial temperature 70 °C, initial time 1 min; ramp at 50 °C per min to 200 °C and hold for 8 min; ramp at 70 °C per min to 250 °C and hold for 3 min. The other operating conditions are:

Injecton port temperature:	250 °C
detector temperature:	300 °C
carrier gas:	nitrogen
total flow rate:	50 ml/min.
septum purge:	0.7ml/min
column head pressure:	12 psi
total run time:	15.3 min.

We used this analytical method to develop an extraction method for simultaneous extraction of paraoxon and parathion from plasma and tissue. The ethyl acetate

extraction method previously developed for HPLC did not work well for this high sensitivity assay, due to many interfering substances that were detected by the ECD. To minimize this problem different protein denaturing agents were tried, such as sodium sulfate, trichloroacetic acid, perchloric acid, and methanol. They did not sufficiently minimize the interferences and the analyte extraction efficiency was low. Solid phase extraction tubes using C18 and C8 packing resulted in about 50 to 60 percent extraction efficiency, but these also produced interfering peaks in the chromatogram. The best method obtained was to extract plasma or tissue homogenate three times with 3 ml isooctane, using methanol and sodium chloride as protein denaturing agents. The extracts were centrifuged for 3 min at 5 °C and 3000 x g. The supernate was collected and evaporated under a stream of nitrogen. The residue was reconstituted in 0.1 to 1.0 ml isooctane, which was subjected to capillary GLC. Malathion was used as the internal standard. The following extraction efficiencies were obtained:

paraoxon	> 93 %
parathion	> 94 %
malathion	> 98 %

Similar high extraction efficiencies have been obtained from homogenates of brain and heart. This high extraction recovery at low concentration requires the use of freshly siliconized glassware. Attached are chromatograms showing paraoxon, parathion and the internal standard malathion. Chromatograms for these substances in injection solvent (Fig. 1), and after extraction from spiked plasma are shown (Fig. 2), along with a plasma blank (Fig. 3), showing that there are no interfering peaks carried over from plasma itself. Assay precision using this extraction procedure: intraday variation (% relative standard deviation) was < 3.9%, and interday variation < 4.3%. A standard curve is also shown (Fig. 4).

The stability of paraoxon and parathion in stored samples of plasma was characterized as follows: rainbow trout plasma was spiked with paraoxon and parathion separately and stored for various times at -20 °C. The plasma samples were assayed for paraoxon and parathion; the results indicated that the paraoxon and parathion were stable in the frozen condition:

Stability of Paraoxon and Parathion in Plasma at -20 °C.

Time (day)	0	1	2	3	4	7	23
Paraoxon (μg/ml)	0.188	0.174	0.187	0.186	0.187	0.176	0.174
Parathion (μg/ml)	0.186	0.182	0.176	0.185	0.185	0.186	0.182

With the above analytical methodology, pharmacokinetic studies were carried out in fish and rats. Rainbow trout were anesthetized using MS222 and fitted with a dorsal aortic cannula in the dorsal buccal cavity, which was exteriorized through the anterior, dorsal region of the head. After a 24 hr recovery period, paraoxon or parathion was administered as a rapid injection into the blood and serial blood samples were removed. The resulting concentration-time profiles of representative experiments are shown in Figs. 5 & 6. The plasma protein binding of the two substances was determined using ultrafiltration. Pharmacokinetic parameter values were determined using both a two compartment model (for parathion) and a "model independent" method (residence time).

The pharmacokinetic parameter values (preliminary estimates) are summarized in the following table:

PHARMACOKINETIC PARAMETERS IN RAINBOW TROUT

		Paraoxon	Parathion
V_{ss}	$ml \cdot kg^{-1}$	1000	1050
CL_B	$ml \cdot hr^{-1} \cdot kg^{-1}$	2885	13.0
$t_{1/2}$	hr	0.535	56.7
f_{free}	%	52.5	0.3

n = 3

The two substances have very similar apparent volumes of distribution, even though the binding to plasma proteins is remarkably different. Parathion has a larger log P value than does paraoxon (3.76 vs. 2.5) and this higher fat solubility would indicate that parathion would tend to concentrate more in fatty tissue and perhaps in other tissues, leading to a larger apparent volume of distribution. However, this tendency was apparently offset by the much higher binding of parathion in the plasma. The free fraction for paraoxon in trout plasma is 175 times that for parathion. This difference in binding appears to account for most of the difference in the total body clearance values, since their ratio is 222. The much longer plasma half life of parathion also is attributable mostly to the much smaller plasma free fraction for this substance. The unbound clearance for both compounds (CL_B/f_{free}) is about 5000 $ml \cdot hr^{-1} \cdot kg^{-1}$, which is about three times the cardiac output in rainbow trout. This would suggest that exchange of these compounds across the gill epithelium is rapid (assuming that much of the clearance is due to branchial elimination) and controlled by cardiac output.

In other experiments, trout were cannulated and placed into a solution of parathion. Samples of blood were removed at various times and the concentrations of parathion and paraoxon in plasma were determined, along with their water concentrations. We are working on an integrated pharmacokinetic model for both substances in plasma and exposure water, and results have been obtained in three fish; representative data from a single experiment are shown in Fig. 7. From these data, it appears that parathion is rapidly taken up from water. The plasma/water concentration ratio is on the order of 100 to 200, which reflects the high degree of plasma protein binding of parathion. (It should be noted that the sampling site for plasma is immediately down stream from the gill absorption site.) These data suggest that the permeability of the gill epithelium to parathion is very high and that the rate limiting factor in uptake of parathion is its removal from the absorption site by plasma. The carrying capacity of plasma is high due to the binding. Concentrations of paraoxon, formed metabolically from parathion, were also observed in some samples; its concentration was at the limit of assay sensitivity. The plasma/exposure water ratio for paraoxon is much smaller than for parathion, reflecting the lower degree of plasma protein binding of paraoxon.

To identify the site of action of paraoxon in fish, infusion studies similar to those described in the previous progress report for rat have been initiated. Due to its limited water solubility and lesser potency in fish than rat, a solubilizing agent is needed and we have determined that 5% polysorbate 80 in water can be administered to fish without affecting acetylcholinesterase (AChE) activity. We have infused three fish and measured AChE activity in brain, heart, and jaw muscle after the fish loose their righting reflex. More studies will be needed to identify whether a particular site of action exists for paraoxon.

STUDIES IN RATS

Quantification of paraoxon in plasma. The amounts of paraoxon found in plasma after its administration are very low, lower than observed in fish since rats are more sensitive than are fish and lower doses must be given. Different extraction methods were tried to achieve the maximum extraction efficiency at the very low levels of paraoxon that were expected to be present. Plasma was spiked with known amounts of paraoxon and the internal standard, malathion and subjected to different extraction procedures. Precipitation of the plasma proteins with sodium sulfate and sodium chloride did not give satisfactory results. Precipitation with hexane did not give adequate recovery and ethyl acetate precipitation gave a dirty chromatogram. Solid phase extraction using C18 columns was also attempted. A 70% extraction efficiency was achieved with methanol precipitation and extraction with 1 ml of isooctane thrice. To circumvent the problem of low levels of paraoxon, the extracted compound was finally reconstituted in 50 microliters of isooctane and injected onto the GC. The sensitivity was increased 20 fold by this approach and the lowest amount detected was 0.8 ng.

GC analysis of paraoxon. The analysis was similar to that described for the fish studies. Paraoxon retention time was 9.6 min and that of malathion was 10.3 min. No interfering peaks were observed at the retention times of paraoxon and malathion even though the extracts were concentrated 20 times.

Pharmacokinetic studies. Fisher male rats were anesthetized with 40 mg/kg pentobarbital via the intraperitoneal route and a cannula was inserted into the left jugular vein. After the rats recovered from surgery, they were given atropine sulfate (25 mg/kg) intravenously 15 min prior to being dosed with paraoxon (0.4 mg/kg) in propylene glycol:saline (50:50). At various times (5, 10, 15, 25, 40, 60 min) blood was drawn, centrifuged to separate plasma and prepared for GC analysis. A representative plasma concentration time profile is shown in Fig. 8.

Kinetics of paraoxon in plasma. Preliminary results indicate a monoexponential decay of paraoxon plasma levels with a half life of 0.23 hr^{-1}, K_{el} = 3.01 hr^{-1}, CL = 13.4 liters hr^{-1} kg^{-1} and V_d = 4.4 liters/kg.

FIGURE LEGENDS

Figure 1. Gas Chromatogram showing paraoxon at 9.604 min., malathion at 10.601 min., and parathion at 11.260 min. Organophosphates were dissolved in injection solvent.

Figure 2. Gas chromatogram showing paraoxon, malathion, and parathion after extraction from fish plasma.

Figure 3. Gas chromatogram of blank fish plasma extracted as in Fig. 2.

Figure 4. Standard curves for paraoxon and parathion; malathion was used as the internal standard.

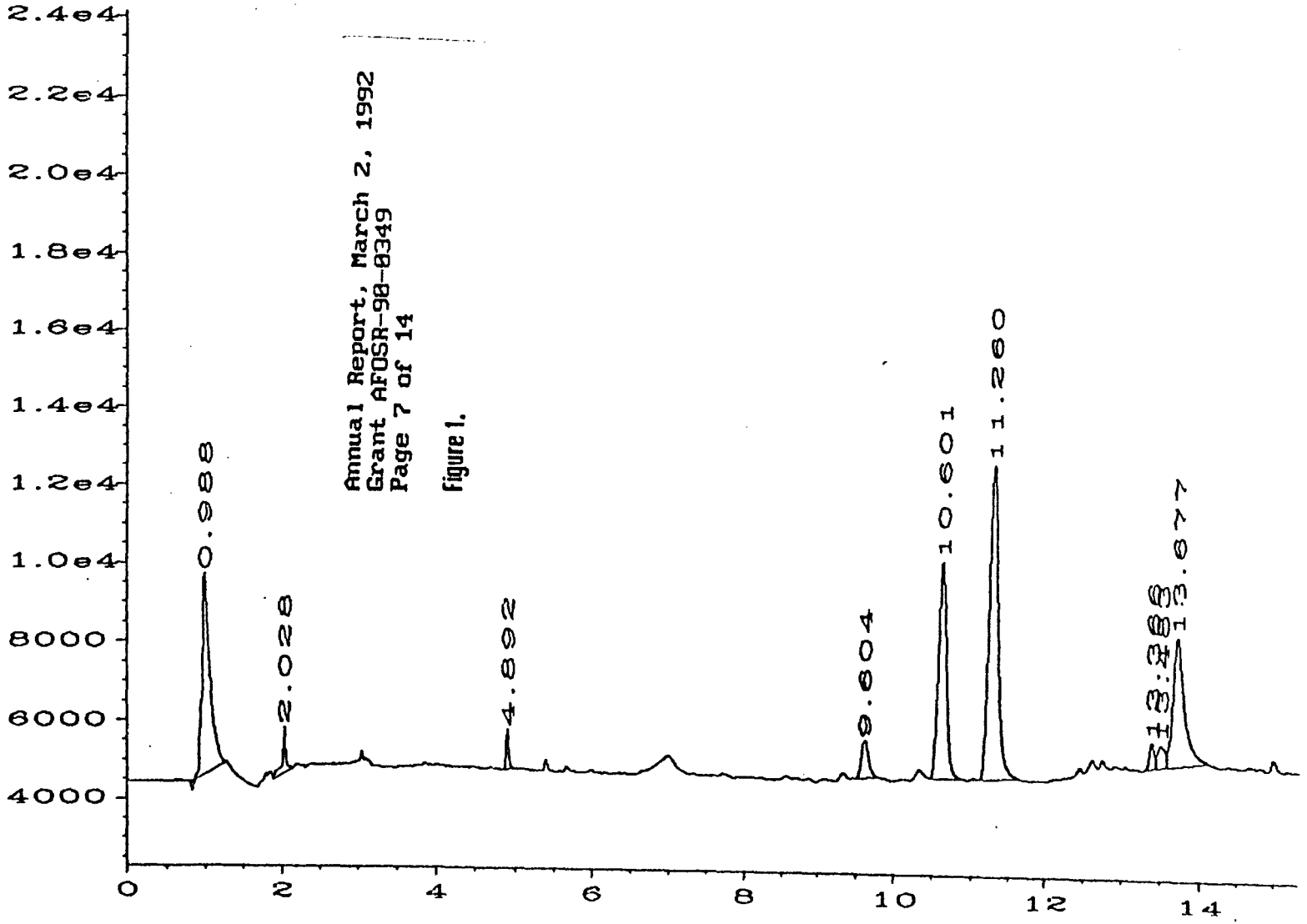
Figure 5. Paraoxon plasma concentration-time profile after intravascular injection of paraoxon into rainbow trout.

Figure 6. Parathion plasma concentration-time profile after intravascular injection of parathion into rainbow trout.

Figure 7. Parathion water and plasma concentrations (solid lines) and paraoxon water and plasma concentrations (dashed lines) after water exposure of a rainbow trout to parathion.

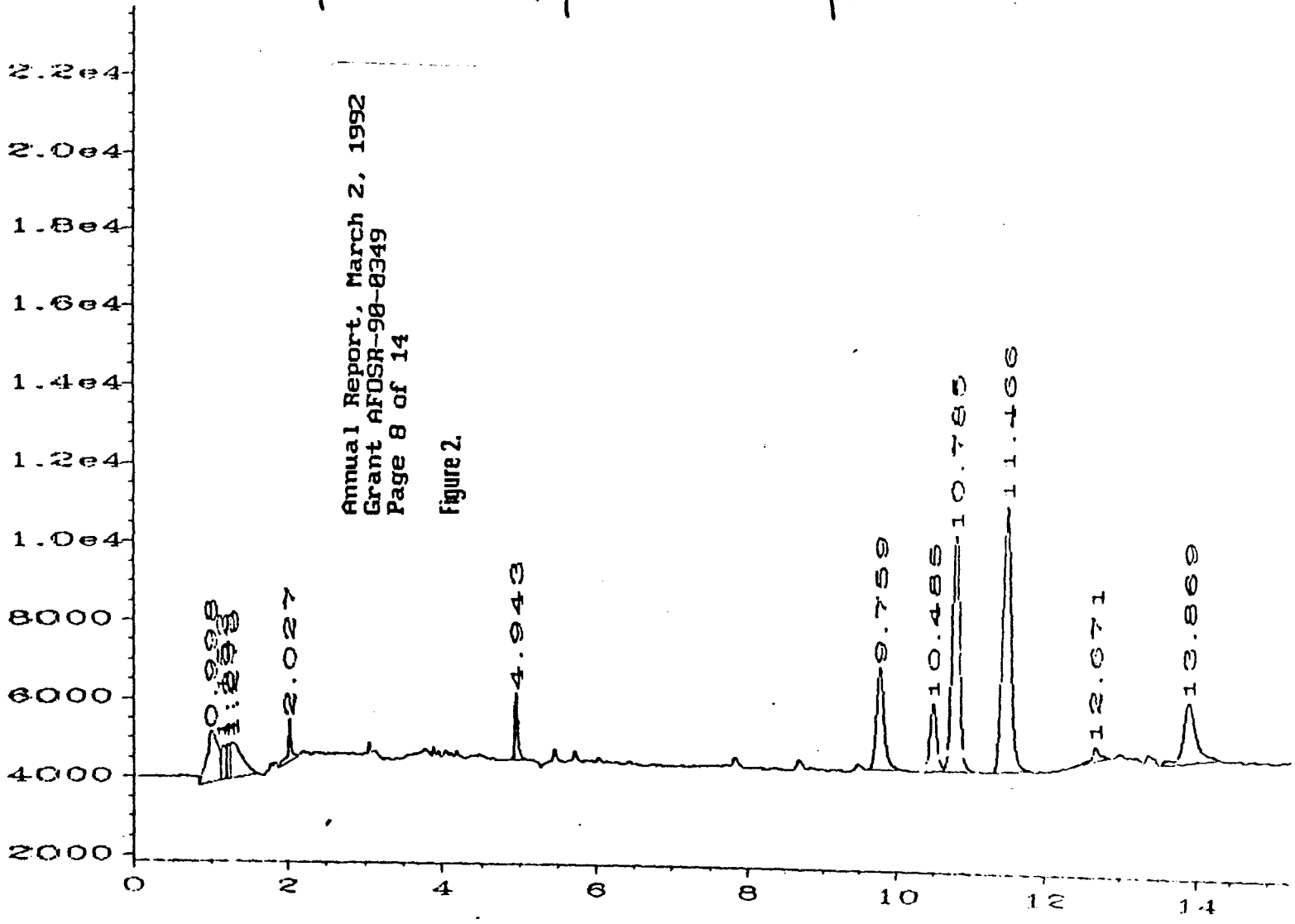
Figure 8. Paraoxon plasma concentration-time profile after intravenous injection of paraoxon to rat.

figure 1.



Sig. 2 in D:\HPCHEM\1\DATA\062R0203.D

spiked fish plasma sample



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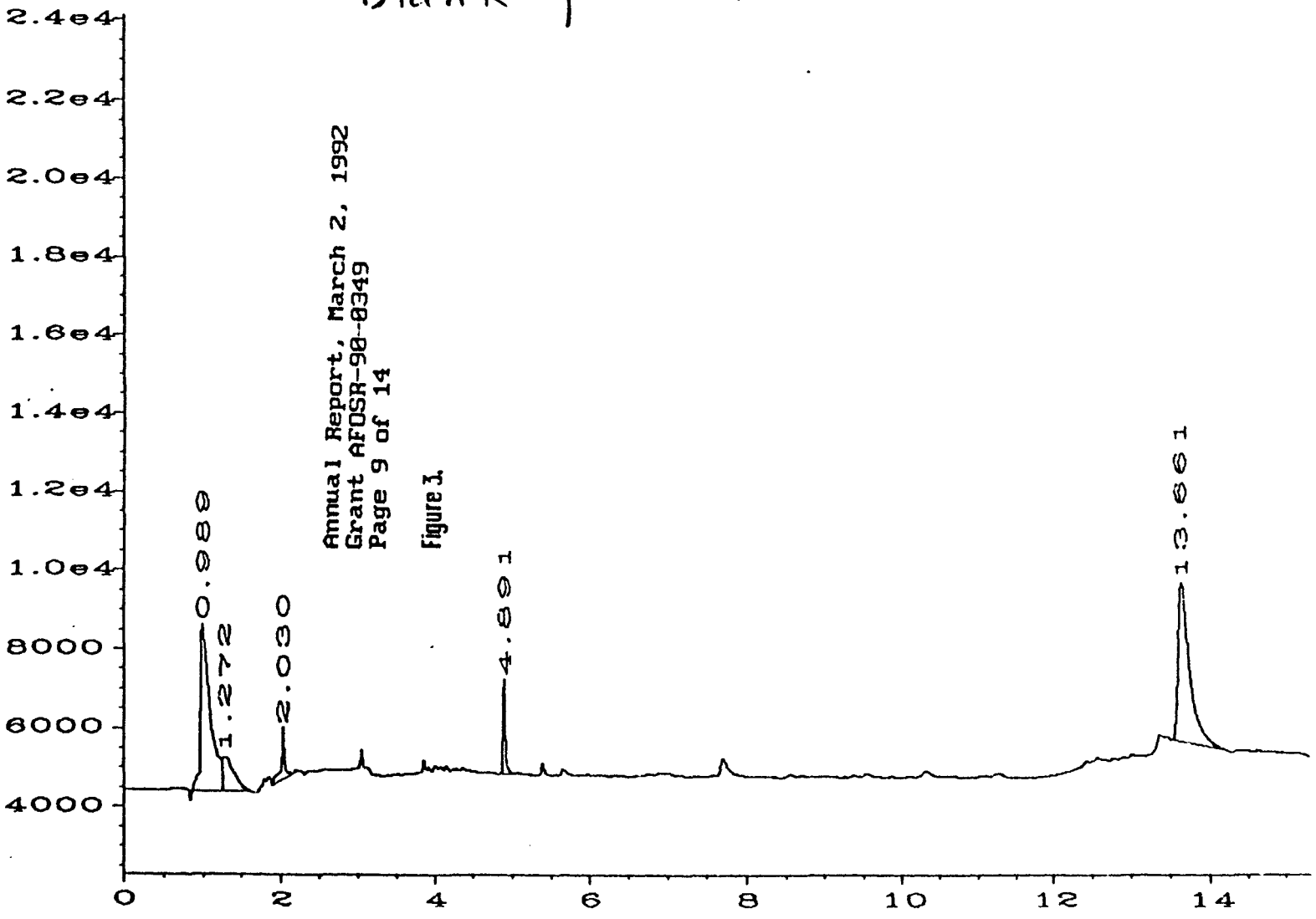
Blank plasma



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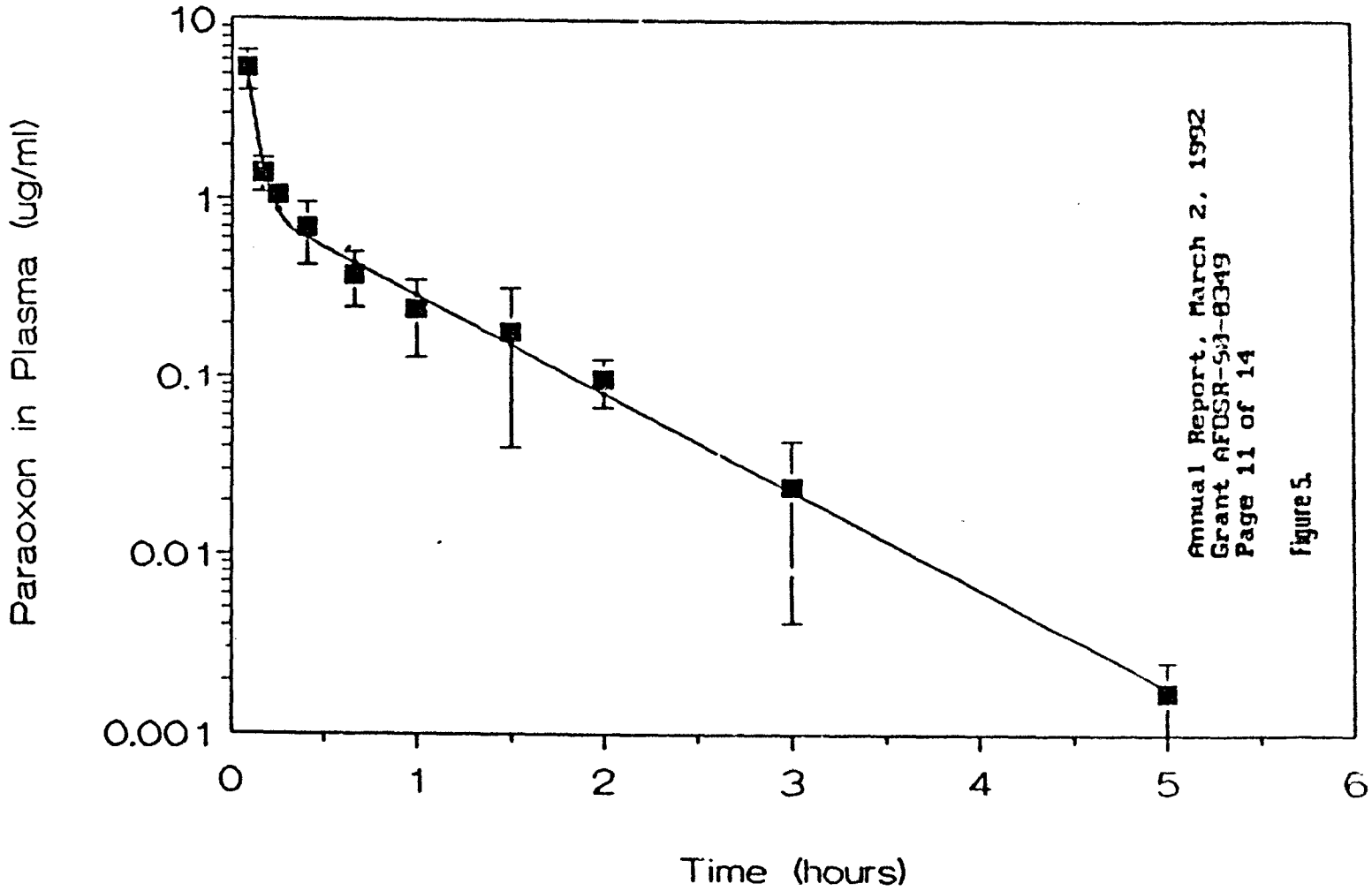
paraoxon plasma standard curve
R-sq=0.9998
plasma1
plasma2
plasma3
area ratio
amount ratio
2.00
1.60
1.20
0.80
0.40
0.00
0.00
0.26
0.52
0.78
1.04
1.30

parathion plasma standard curve
R-sq=0.9999
plasma1
plasma2
plasma3
area ratio
amount ratio
3.50
2.80
2.10
1.40
0.70
0.00
0.00
0.30
0.60
0.90
1.20
1.50

Figure 4.

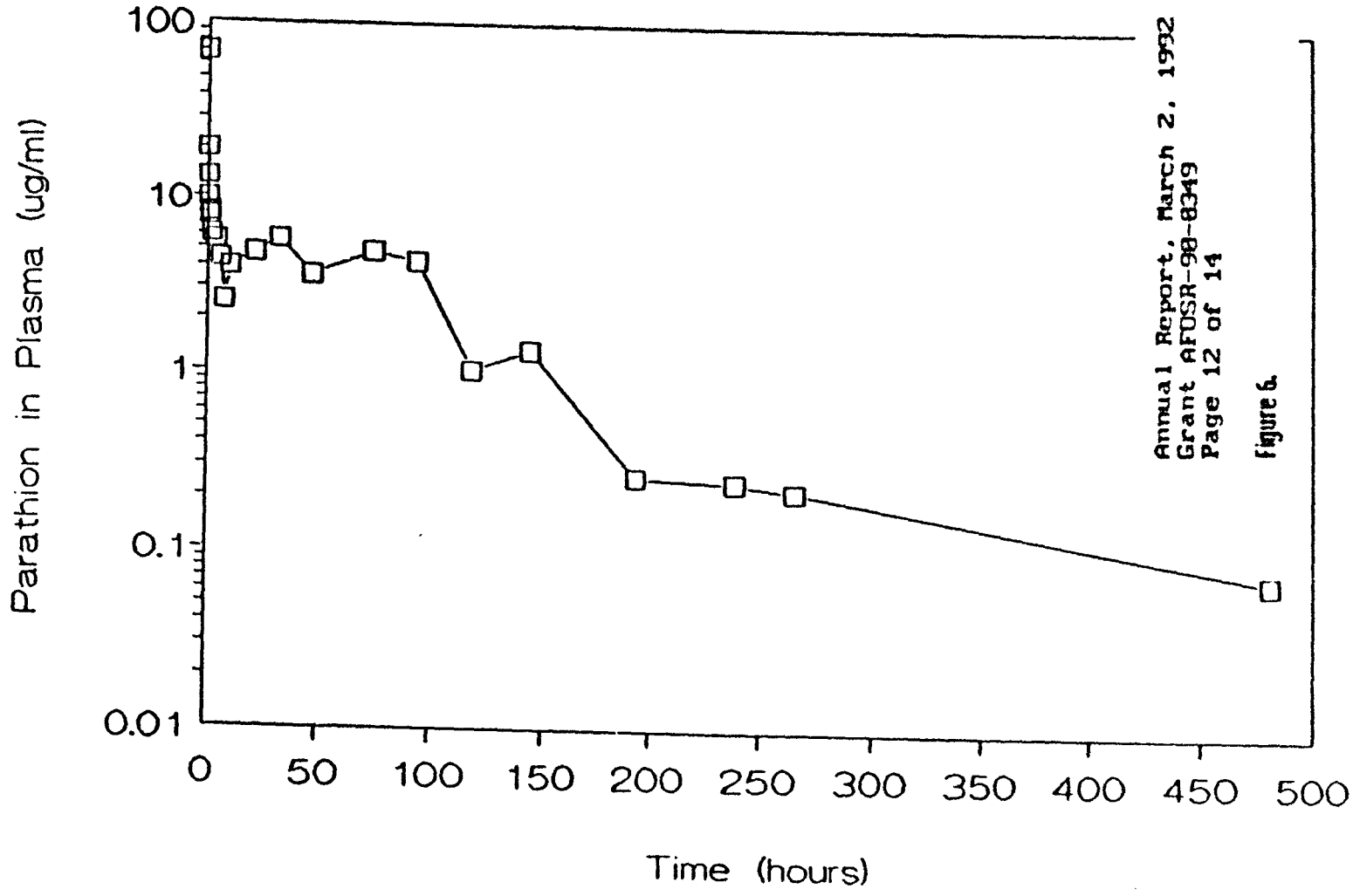
IA Paraoxon Avg. FW= 371 g

Dose= 5.7 mg/Kg



IA Parathion FW= 483 g

Dose= 8 mg/Kg



Water Exposure to Parathion

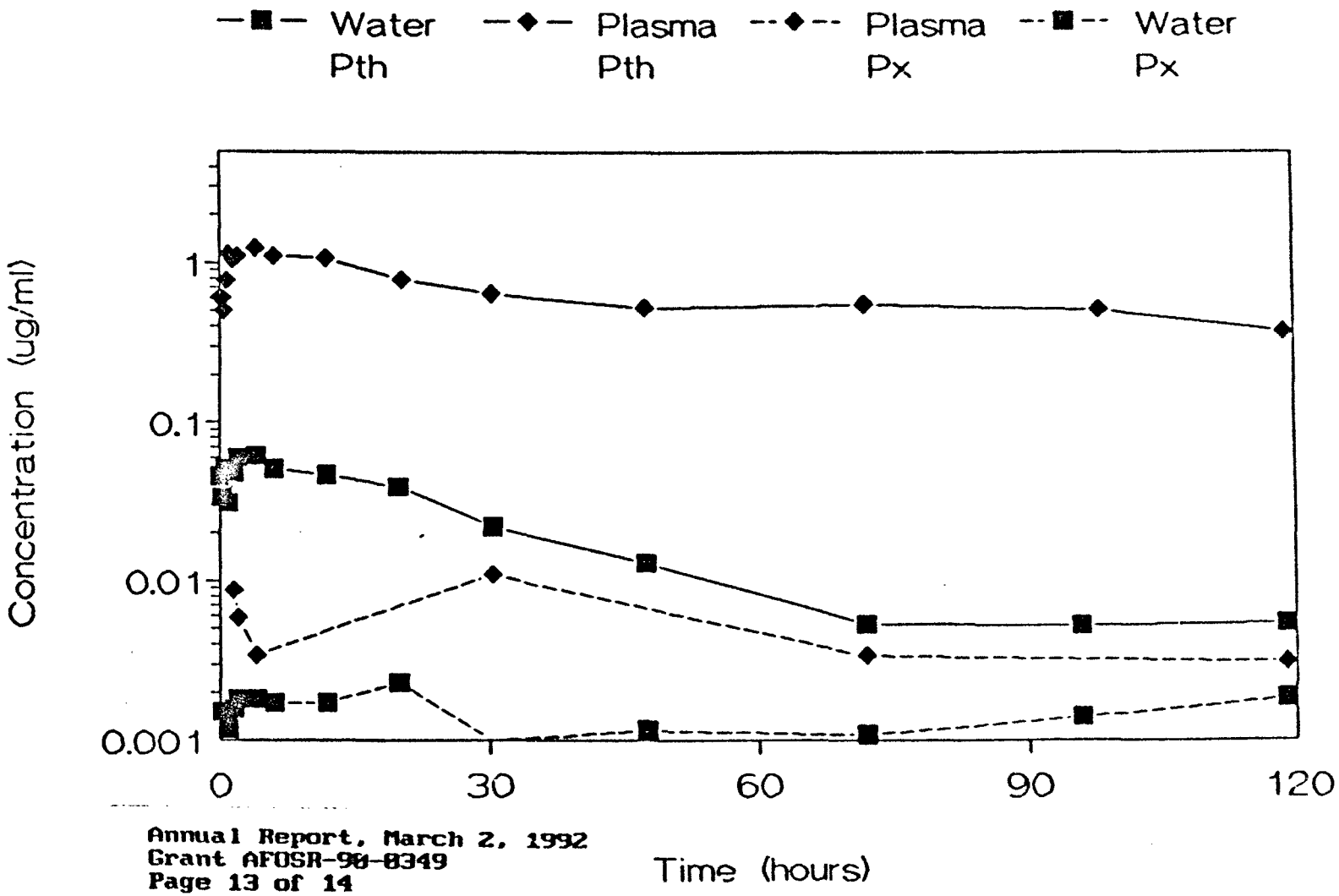


Figure 7.

Paraoxon IV in rats
Dose (0.400mg/kg)