Journal of Chromatography, 338 (1985) 389—395
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2447

DETERMINATION OF METHYL PARAOXON IN DOG PLASMA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

E.P. DE SCHRYVER* and L. DE REU

Heymans Institute of Pharmacology, University of Ghent Medical School, De Pintelaan 185, B-9000 Ghent (Belgium)

and

J.L. WILLEMS

Department of NBC Protection (TDLM-CT), Vilvoorde (Belgium)

(First received July 30th, 1984; revised manuscript received October 16th, 1984)

SUMMARY

A high-performance liquid chromatographic method for the determination of methyl paraoxon in plasma has been developed. Disodium EDTA and aluminon are used to inhibit hydrolysis of methyl paraoxon in plasma. Methyl paraoxon and the internal standard fenitrooxon are extracted from plasma into methylene chloride. Chromatography is performed on a reversed-phase C_{18} column, connected with a fixed-wavelength ultraviolet detector at 280 nm; the compounds are eluted in about 5 min with tetrahydrofuran—acetonitrile—0.01 *M* sodium phosphate buffer, pH 7.4 (12:25:63, v/v/v). Concentrations down to 5 ng/ml methyl paraoxon in plasma can be determined with good precision and accuracy. The method was applied to plasma samples from dogs after intravenous administration.

INTRODUCTION

Methyl paraoxon [O,O-dimethyl (*p*-nitrophenyl) phosphate] is the activated oxygen analogue of methyl parathion, an organophosphate insecticide which exerts its acute toxicity by inhibition of acetylcholinesterase [1]. The main metabolite of methyl paraoxon is *p*-nitrophenol. The structures of the compounds are given in Fig. 1. Knowledge of the plasma concentration of organophosphates could be helpful in the management of intoxicated patients.

Analysis of methyl paraoxon by electron-capture gas chromatography (GC)

0378-4347/85/$03.30

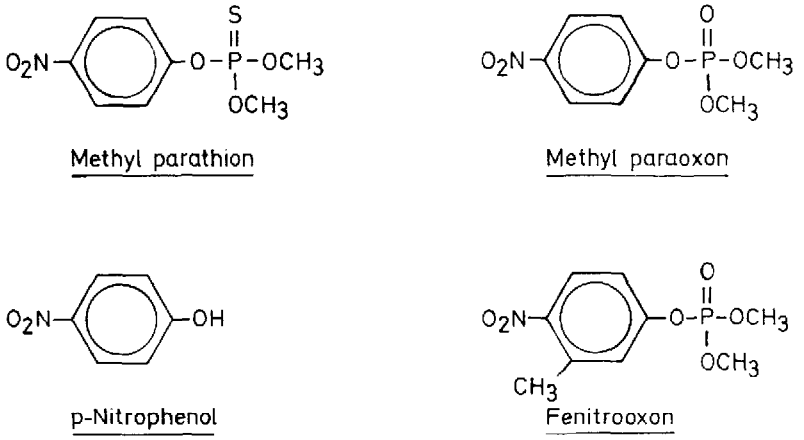

Fig. 1. Structure of methyl parathion, methyl paraoxon, *p*-nitrophenol and fenitrooxon.

[2—4], alkali flame-ionization GC [5] and GC with combination of both detection systems [6] has been described. As methyl paraoxon has a very high UV absorbance, high-performance liquid chromatography (HPLC) with UV detection can be used. An HPLC method, using a column packed with gels and UV monitoring at 260 nm, for the separation of solutions containing methyl parathion and fenitrothion metabolites has been reported [7]. This procedure, however, was not applied to biological samples.

The present paper describes a selective HPLC method for the determination of methyl paraoxon in dog plasma with a simple and rapid extraction procedure, using fenitrooxon [O,O-dimethyl (3-methyl-4-nitrophenyl) phosphate] as the internal standard (Fig. 1). As methyl paraoxon is not stable in plasma, an adequate inhibitor of its hydrolysis was sought. The method has been used to determine plasma concentrations in the dog after intravenous administration of methyl paraoxon.

EXPERIMENTAL

Reagents

Methyl paraoxon and fenitrooxon were supplied by Ehrenstorfer (Augsburg, F.R.G.) and were purified using preparative column chromatography. Aluminon (aurin tricarboxylic acid ammonium salt) was obtained from Aldrich-Europe (Beerse, Belgium). Disodium EDTA, sodium dihydrogen phosphate, disodium hydrogen phosphate and methylene chloride were supplied by E. Merck (Darmstadt, F.R.G.) and were of analytical grade. Spectrophotometric grade acetonitrile and tetrahydrofuran were used (Carlo Erba, Milan, Italy).

Standard solutions

Stock solutions, corresponding to 1 μg/μl in methanol, were prepared for methyl paraoxon and fenitrooxon. Standard solutions were obtained by diluting the stock solutions to concentrations of 1 or 10 ng/μl in methanol.

Sample preparation

Inhibition. As methyl paraoxon and fenitrooxon are not stable in plasma and

blood, inhibitors mentioned in the literature [8], disodium EDTA (10^{-2} *M*) and a mixture of disodium EDTA (10^{-2} *M*) and aluminon (10^{-3} *M*), were tested. The influence of these inhibitors and of temperature on the stability of the product and the internal standard was evaluated by incubation for 1 h at 0°C, 25°C and 37°C with or without inhibition. Samples were taken at regular time intervals.

Extraction. A 1-ml volume of inhibited plasma (unknown samples, product-free samples or samples containing known amounts of the product) is transferred to a glass-stoppered 5-ml silanized glass tube spiked with either a high (750 ng) or a low (75 ng) amount of the internal standard, dissolved in 75 μl of methanol, depending on the expected concentration range of methyl paraoxon. The sample is extracted with 3 ml of methylene chloride by shaking for 5 min at 50 Hz. After centrifugation at 3000 *g* and 4°C for 10 min, the organic phase is transferred to a 5-ml silanized glass conical tube and evaporated to dryness at room temperature under nitrogen. The residue is stored at −20°C until chromatography.

Chromatography

The chromatography is performed on a microprocessor-controlled Spectra Physics SP-8000 high-performance liquid chromatograph with a fixed-wavelength UV detector operating at 280 nm (Spectra Physics SP-8210), an automatic injector, and a 10-μl sample loop. Separations are achieved using a reversed-phase C_{18} column (250 × 4.6 mm I.D.) packed with 5 μm particle size Spherisorb 5 ODS (Chrompack, Merksem, Belgium) and thermostatted at 40°C. Just prior to chromatography the various extraction residues are redissolved in 30 μl of acetonitrile by vigorous vortexing, and 10 μl are injected onto the HPLC column. With a degassed mixture of tetrahydrofuran—acetonitrile—0.01 *M* sodium phosphate buffer, pH 7.4 (12:25:63, v/v/v), the samples are eluted within 5 min, at a constant flow-rate of 1.5 ml/min (170—210 bars). The use of buffer is necessary to elute *p*-nitrophenol, the main metabolite of methyl paraoxon, with the solvent front. Chromatograms are plotted on a Hewlett-Packard 3390A reporting integrator.

Calibration

For the lower range, inhibited blank plasma samples (1 ml) were spiked with increasing amounts (5—75 ng) of methyl paraoxon and with 75 ng of the internal standard, using standard solutions of 1 ng/μl in methanol. The higher range was calibrated with samples of inhibited blank plasma (1 ml) spiked with increasing amounts (50—1200 ng) of methyl paraoxon and with 750 ng of the internal standard, using standard solutions of 10 ng/μl in methanol. These calibration samples were then taken through the extraction procedure and chromatography described above.

Sample concentrations are calculated by determining peak height ratios of the product, related to the internal standard, and comparing these ratios with the standard curves obtained after analysis of calibration samples.

Absolute recovery

Absolute recovery of methyl paraoxon from plasma is determined by adding

the internal standard after extraction, and comparing peak height ratios with the peak height ratios of a calibration curve. This calibration curve is obtained by injecting acetonitrile solutions containing a fixed amount of the internal standard and varying amounts of methyl paraoxon. Absolute recovery of fenitrooxon from plasma is determined using methyl paraoxon as the internal standard.

RESULTS AND DISCUSSION

Inhibition of hydrolysis, absolute recovery, precision and accuracy

The in vitro stability of methyl paraoxon in dog blood and plasma was studied at several temperatures. An example of the hydrolysis of methyl paraoxon in dog plasma at 0° C, 25° C and 37° C is shown in Fig. 2. Each point represents the result of a sample worked up in duplicate. At 37° C, the half-life of degradation is about 10 min in dog blood, about 5 min in dog plasma; at 25° C it is about 20 and 10 min for blood and plasma, respectively. Even at 0° C a considerable loss of methyl paraoxon is observed, with a half-life of 90 and 60 min for blood and plasma, respectively. Therefore, for accurate determination of methyl paraoxon in dog plasma the hydrolysis of the product has to be inhibited. Very few data on this problem are found in the literature. Most authors quantitate *p*-nitrophenol, the main metabolite of methyl paraoxon, or apply their method to solutions. Erdös and Boggs [8], however, tried to inhibit the splitting of another organophosphate, paraoxon [O,O-diethyl (*p*-nitrophenyl) phosphate], in human plasma. Disodium EDTA alone was not effective, but degradation was inhibited adequately by adding 10^{-2} *M* disodium EDTA and 10^{-3} *M* aluminon. In our study the same mixture was used to inhibit the degradation of methyl paraoxon and fenitrooxon. After 1 h, 80% of

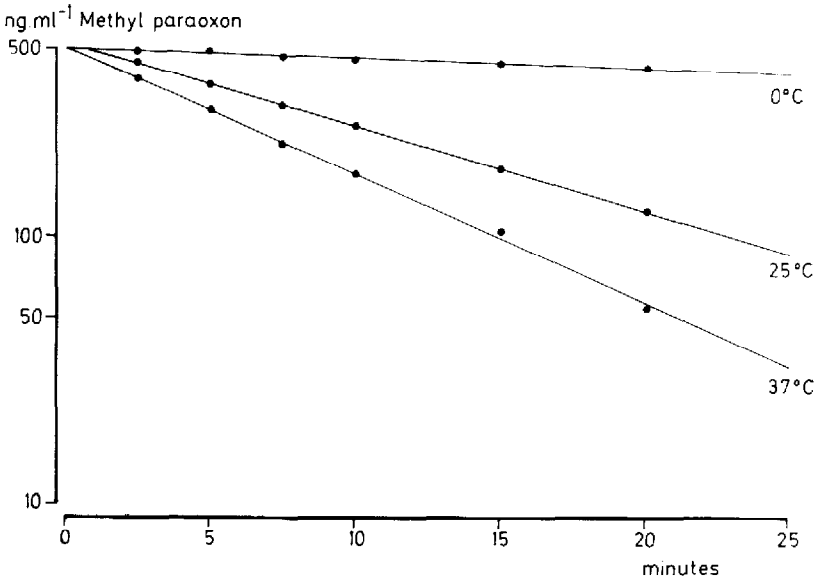

Fig. 2. Hydrolysis of methyl paraoxon in dog plasma at 0° C, 25° C and 37° C as a function of time.

TABLE I

ABSOLUTE RECOVERY OF METHYL PARAOXON AND FENITROOXON FROM PLASMA

$n = 6$.

Amount added* (ng)	Mean recovery (%)	C.V. (%)
Methyl paraoxon		
10	80.4	7.6
30	81.3	4.9
50	85.4	11.9
150	89.4	6.4
400	87.4	6.4
Fenitrooxon		
75	88.7	4.1
500	90.5	5.3

*Quantity added to 1 ml of dog plasma.

TABLE II

WITHIN-RUN ACCURACY AND PRECISION OF THE METHYL PARAOXON ASSAY

Methyl paraoxon* (ng)	n	Relative error (%)	C.V. (%)
15	12	+0.7	5.9
50	12	−4.0	2.7
100	11	+4.4	2.2
400	11	−1.9	3.4

*Quantity added to 1 ml of dog plasma.

methyl paraoxon is recovered at 37°C from blood and plasma, 90% at 25°C. At 0°C no hydrolysis is observed over 1 h. Similar observations were made for fenitrooxon.

The extraction with methylene chloride gives good recoveries (80—91%) of methyl paraoxon and fenitrooxon (Table I).

Plasma standard curves are linear for the concentrations tested, ranging from 5 to 1200 ng/ml plasma. The average slope for twenty calibration curves assayed over three months is 0.00243 ± 0.00029 (mean ± S.D.), with an average intercept of 0.00495 ± 0.01724 and an average correlation coefficient of 0.9993 ± 0.0014. The accuracy and precision of the procedure was ascertained by adding different amounts of both compounds to blank inhibited plasma and analysing a number of samples with the method described. The results are summarized in Table II.

The lowest acceptable concentration is about 5 ng/ml for a plasma sample of 1 ml, as can be seen in Fig. 3, which shows two chromatograms of extracted plasma (1 ml): (A) blank inhibited dog plasma and (B) blank inhibited dog plasma spiked with 5 ng of methyl paraoxon and 75 ng of the internal standard.

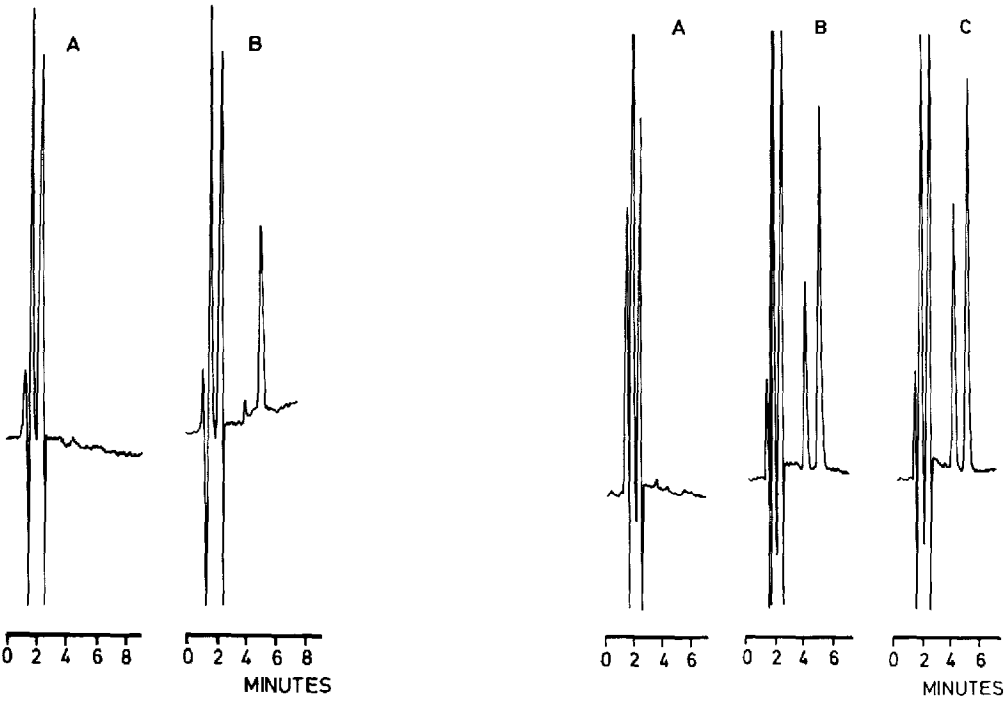

Fig. 3. Chromatograms of extracted plasma: (A) blank inhibited dog plasma; (B) blank inhibited dog plasma spiked with 5 ng of methyl paraoxon (t_R = 3.95 min) and 75 ng of the internal standard, fenitrooxon (t_R = 4.90 min).

Fig. 4. Chromatograms of extracted plasma: (A) blank inhibited dog plasma; (B) blank inhibited dog plasma spiked with 200 ng of methyl paraoxon (t_R = 3.95 min) and 750 ng of the internal standard, fenitrooxon (t_R = 4.90 min); (C) inhibited plasma sample, obtained from a dog 30 min after the injection of 2.5 mg/kg methyl paraoxon; this sample was spiked with 750 ng of the internal standard, fenitrooxon. Note that the sensitivity in this figure is four times lower than in Fig. 3.

Selectivity

As can be seen in Fig. 4A, which shows a representative chromatogram of an extract of blank inhibited dog plasma, there are no interfering peaks. Fig. 4B shows a chromatogram of an extract of blank inhibited dog plasma spiked with 200 ng of methyl paraoxon and 750 ng of the internal standard. The two products are well separated, within a short analysis time. Fig. 4C represents a chromatogram of an extract of inhibited dog plasma, 30 min after the injection of 2.5 mg/kg methyl paraoxon, spiked with the internal standard. There is no interference of hydrolysis products of methyl paraoxon. If a mobile phase containing water instead of buffer pH 7.4 is used, *p*-nitrophenol, the main metabolite of methyl paraoxon, interferes in the HPLC procedure; so the use of buffer is necessary to elute *p*-nitrophenol with the solvent front.

Preliminary toxicokinetic investigations

The method described has been used to measure plasma levels of methyl paraoxon in dogs. Atropine (1 mg/kg) was given intramuscularly 30 min before

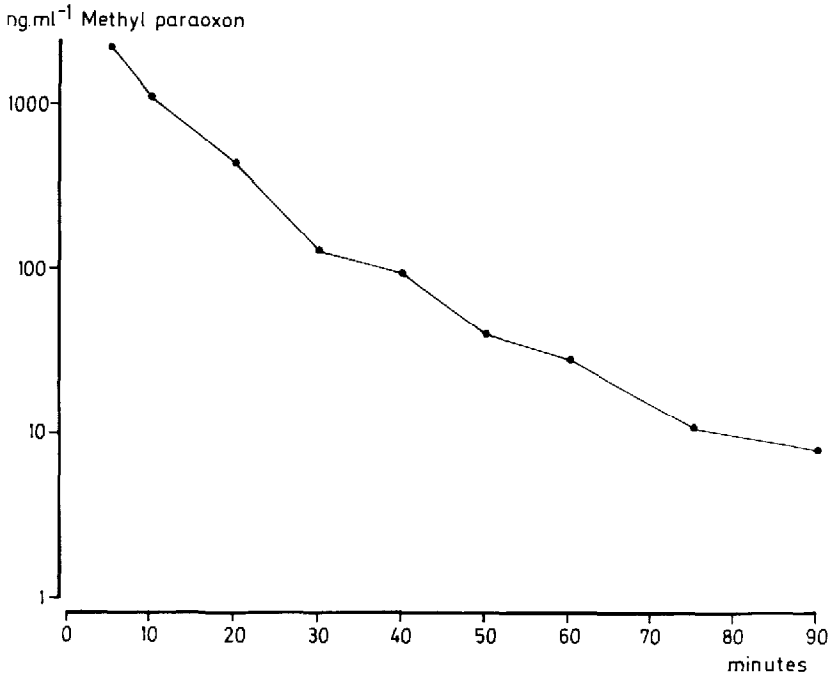

Fig. 5. Semilogarithmic plot of methyl paraoxon plasma concentrations versus time after intravenous injection of 2.5 mg/kg of the product.

intravenous injection of methyl paraoxon at a dose of 2.5 mg/kg. The plasma concentration—time curve in a dog is depicted in Fig. 5. The concentration of methyl paraoxon can be determined accurately, since dog blood samples can be collected with a syringe and added to tubes containing disodium EDTA and aluminon placed in an ice bath, without loss of methyl paraoxon.

The proposed method allows toxicokinetic studies of methyl paraoxon, since inhibition of the hydrolysis, extraction from plasma, sensitivity, analytical recovery and selectivity are satisfactory.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministry of Defence, Belgium. The authors thank Prof. Dr. M. Bogaert, Dr. F. Belpaire and Dr. M.T. Rosseel for critical discussion of the experiments.

REFERENCES

1 J. Miyamoto, Agric. Biol. Chem., 28 (1964) 411.
2 T. Nakatsugawa, N.M. Tolman and P.A. Dahm, Biochem. Pharmacol., 17 (1964) 1517.
3 J.F. Thompson, J.B. Mann, A.O. Apodaca and E.J. Kantor, J. Assoc. Off. Anal. Chem., 58 (1975) 1037.
4 K.D. Rainsford, Pestic. Biochem. Physiol., 8 (1978) 302.
5 P.A. Cammer and R.M. Hollingworth, Biochem. Pharmacol., 25 (1976) 1799.
6 W.W. Sans, J. Assoc. Off. Anal. Chem., 61 (1978) 837.
7 T. Abe, Y. Fujimoto, T. Tatsuno and J. Fukami, Bull. Environ. Contam. Toxicol., 22 (1979) 791.
8 E.G. Erdős and L.E. Boggs, Nature, 190 (1961) 716.