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Determination of metrifonate and dichlorvos in whole blood using gas chromatography and gas chromatography–mass spectrometry

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ABSTRACT

Analytical methods for determining metrifonate and dichlorvos in whole blood and a sampling procedure suitable for pharmacokinetic studies in man are described. Metrifonate concentrations were determined after chloroform extraction using gas chromatography–nitrogen–phosphorus detection. The within-assay coefficients of variation were 4 and 9% at 19.4 and 0.8 μmol/l (limits of determination), respectively. Dichlorvos was determined using gas chromatography–mass spectrometry of toluene extracts. The within-assay coefficients of variation were 2 and 5% at 225 and 50 nmol/l (limits of determination), respectively. Since both substances are chemically unstable, the blood was collected by dripping it directly from the vein into 0.74 *M* phosphoric acid.

INTRODUCTION

Metrifonate (2,2,2-trichloro-1-hydroxyethyl dimethyl phosphonate, Fig. 1) is an organophosphorus compound used in the treatment of Schistosoma hae-

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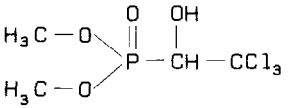
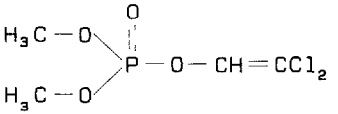


Metrifonate



Dichlorvos

Fig. 1. Structures of metrifonate and dichlorvos.

matobium for more than two decades [1]. Its pharmacological actions are presumed to be mediated by dichlorvos (2,2-dichlorovinyl dimethyl phosphate, Fig. 1) which is formed non-enzymatically from the parent drug [2–5]. Metrifonate is chemically unstable, and in neutral and alkaline aqueous solutions it is transformed to dichlorvos and hydrolysis products [3–5].

As is the case for many drugs used for tropical parasitic infections [6], the standard dose schedule of metrifonate (three doses of 7.5 mg/kg in two-weeks intervals) lacks pharmacokinetic rationale and gives low compliance during mass treatment programmes [7]. Although a simplified schedule (three 5 mg/kg doses in one day) was found to be as effective and safe as the standard dose regimen [8], more knowledge of the pharmacokinetics of metrifonate is mandatory to find the optimal dose schedule.

Specific methods for the determination of metrifonate and dichlorvos in biological materials have previously been reported by Nordgren and co-workers [9,10]. These methods were based on gas chromatography–mass spectrometry and required silylation of metrifonate. Recently Ameno et al. [11] described a gas chromatographic method with nitrogen–phosphorus detection (GC–NPD) for the determination of metrifonate in serum using solid-phase extraction. Our objective was to simplify these methods by using liquid–liquid extractions, avoiding derivatisation, and by using GC–NPD instead of GC–MS. Unfortunately it was found that the sensitivity of NPD was insufficient for the determination of dichlorvos, which is present in much lower concentrations than metrifonate.

Previously, our group [12] described the plasma pharmacokinetics of metrifonate using the assay presented in this paper. According to a recent finding in our laboratory, presented in this paper, both metrifonate and dichlorvos undergo substantial degradation during plasma preparation which usually requires 20 min. In this paper we describe simple and specific methods for the

determination of metrifonate and dichlorvos concentrations in whole blood using appropriate sampling procedures.

EXPERIMENTAL

Chemicals

Metrifonate substance was purchased from The International Dispensary Association (IDA) (Amsterdam, The Netherlands). The internal standard for metrifonate (2,2,2-trichloro-1-hydroxyethyl diethyl phosphonate) was synthesized according to Barthel et al. [13]. Dichlorvos was a gift from Shell Research Limited, Analytical Chemistry Division (Sittingbourne, U.K.). The internal standard for dichlorvos (2,2-dichlorovinyl-di-[$^2H_3$]methyl phosphate) was a gift from Dr. Ingrid Nordgren, Department of Toxicology, Karolinska Institute (Stockholm, Sweden). All other chemicals were of analytical grade.

Equipment

Determination of metrifonate was performed using a Hewlett-Packard 5890 gas chromatograph equipped with a nitrogen–phosphorus detector and connected to an HP 3392A integrator. A 25 m × 0.25 mm I.D. fused-silica capillary column (J & W Scientific, Folsom, CA, U.S.A.) coated with OV-351 was used. The oven temperature was 110°C and the injector block was heated to 250°C. The carrier gas (helium) flow-rate was adjusted to 2 ml/min. Split injection was used with the split flow-rate set at 30 ml/min. All other settings were according to those recommended by the manufacturer of the instrument.

Dichlorvos was analysed using a Hewlett-Packard 5890 gas chromatograph connected to an LKB 2091 mass spectrometer. A 12.5 m × 0.32 mm I.D. fused-silica capillary column (Hewlett-Packard, Palo Alto, CA, U.S.A.) coated with 5% phenyl and 95% dimethyl silicone fluid was used. The carrier gas (helium) flow-rate was adjusted to 5 ml/min. The split flow-rate was set at 35 ml/min. The injector block temperature was 250°C. Splitless injection was used and the split valve was closed at injection and opened 30 s later. The oven temperature was 80°C for 30 s and then raised at 70°C/min to 130°C. The mass spectrometer was set to monitor the ions at *m/z* 185 and 191.

Sampling

Approximately 1.5 ml of blood from a punctured vein of a volunteer were allowed to drip into a 4-ml glass tube containing 1.5 ml of 0.74 *M* (5%, v/v) phosphoric acid. The exact volume was calculated by dividing the weight of the blood by the density factor 1.0595 kg/l [14].

Determination of metrifonate

A 100-μl volume of acidified whole blood was mixed with 10 μl of the internal standard (1.4 μmol/l) and extracted for 10 min with 2 ml of chloroform. After

centrifugation the aqueous phase was aspirated off and the organic phase was evaporated to dryness. The residue was dissolved in 100 μl of chloroform of which 5 μl were injected into the chromatograph. Two sets of six standards each, prepared by spiking drug-free acidified whole blood with metrifonate, were analysed along with the samples and used for quantification. The lower set was used for concentrations of 0.8–4 μmol/l and the higher set for 4–20 μmol/l. The metrifonate/internal standard peak-area ratios were used for quantification. All stock solutions of metrifonate and its internal standard were prepared and stored in 0.74 *M* phosphoric acid.

Determination of dichlorvos

A 500-μl volume of the acidified whole blood was mixed with 25 μl of the internal standard (4.4 μmol/l) and extracted for 10 min with 250 μl of toluene. The tubes were centrifuged and 5 μl of the organic phase were injected. A lower (50–250 nmol/l) and a higher (250–1000 nmol/l) set of standards were analysed along with the samples and were used for quantification. All stock solutions of dichlorvos and its internal standard were prepared in acetonitrile. The exact volume of the substance was measured and the mass calculated using the density factor 1.415 kg/l [15].

Precision, accuracy and extraction recovery

Within-assay precision and accuracy of the methods were estimated by analysing two sets of ten samples each (one set in the high concentration range and one in the low concentration range). Between-assay precision and accuracy of the methods were tested by spiking samples of whole blood with metrifonate (7.77 μmol/l) or dichlorvos (905 nmol/l) daily in duplicates for four consecutive days.

The total analytical recovery of metrifonate was determined at 20 μmol/l by extracting spiked samples without internal standard, evaporating 1 ml of the chloroform layer and reconstituting with 100 μl of chloroform containing internal standard. The peak-area ratios were compared with those of standards prepared in chloroform and injected directly. The extraction recovery of dichlorvos was tested at 1000 nmol/l by extracting spiked samples twice and adding the internal standard to the organic phases after the extractions. The recovery (f) (in %) was calculated from $f = 100(1 - r_2/r_1)$, where r_1 and r_2 are peak-height ratios from the first and the second extraction, respectively. The co-extraction of metrifonate in the dichlorvos assay was estimated by extracting a sample containing 20 μmol/l metrifonate using the dichlorvos procedure. The amount of dichlorvos in this extract was also determined.

Stability

The stability of metrifonate and dichlorvos during storage was tested by keeping acidified plasma and whole blood samples at −18°C for three to five

months. The conversion of metrifonate to dichlorvos during the sampling procedure was tested by spiking whole blood with metrifonate (45 μmol/l), keeping the samples at room temperature for up to 60 min and analyzing for dichlorvos and metrifonate. The presence of dichlorvos in acidified samples spiked with metrifonate was also checked.

RESULTS

Typical chromatograms of metrifonate and dichlorvos are shown in Figs. 2 and 3. No interfering peaks with the same retention times as the analytes were present in blank whole blood samples. The mass spectrum of dichlorvos is shown in Fig. 4. The fragment ions at *m/z* 185 and 191 were used for monitoring dichlorvos and the deuterated internal standard, respectively. We found that the ion at *m/z* 185 gave a higher signal-to-noise ratio than the more abundant ion at *m/z* 109.

All calibration curves were linear with coefficients of correlation above 0.99. The linearity of the methods was tested up to 120 μmol/l for metrifonate and to 4500 nmol/l for dichlorvos. The limits of determination were 0.8 μmol/l for metrifonate and 50 nmol/l for dichlorvos which was sufficient to determine the concentrations of the compounds in blood for up to 8 h (approximately

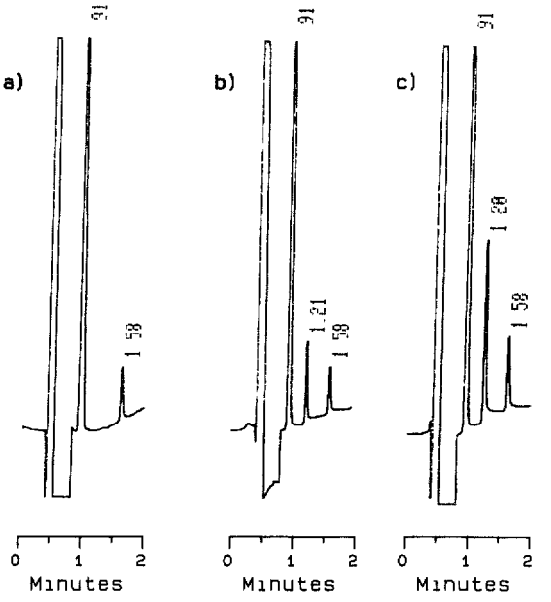


Fig. 2. Typical chromatograms showing (a) a drug-free sample, (b) a sample spiked with 4 μmol/l metrifonate and (c) a whole blood sample from a human subject after a dose of metrifonate. The retention times were 1.2 and 1.6 min for metrifonate and the internal standard, respectively.

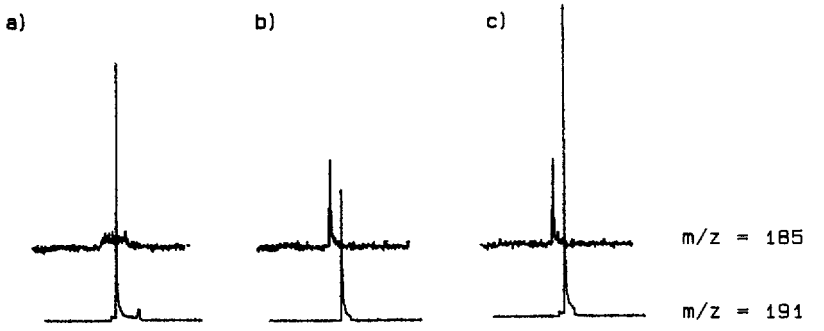


Fig. 3. Typical chromatograms showing (a) a drug-free sample, (b) a sample spiked with 200 nmol/l dichlorvos and (c) a whole blood sample from a human subject after a dose of metrifonate. The retention time was 1.2 min for both dichlorvos and the internal standard.

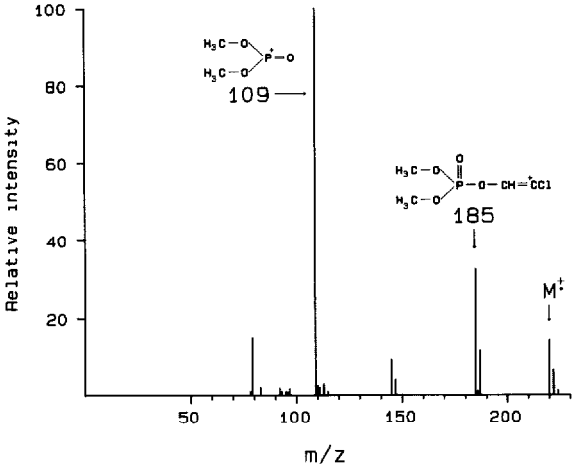


Fig. 4. Mass spectrum of dichlorvos.

four half-lives). The within- and between-assay precision and accuracy of the methods are given in Table I. The extraction recovery is given in Table II. The co-extraction of metrifonate in the dichlorvos assay was found to be approximately 6%. Dichlorvos was also present in that extract at a level corresponding to 0.1% of the added metrifonate concentration.

In blood without acid neither metrifonate nor dichlorvos was stable. The metrifonate concentration decreased by 50% in 1 h while that of dichlorvos increased to 8% of the original metrifonate concentration. When incubating dichlorvos in whole blood its concentration decreased by 50% in 10 min. After acidification and storage of samples at −18 °C metrifonate was stable for three months, and after twenty months the concentration had decreased by ca. 40%. In the same samples the concentration of dichlorvos was stable for one month and had decreased by ca. 10% in twenty months.

TABLE I

WITHIN- AND BETWEEN-ASSAY PRECISION AND ACCURACY OF METRIFONATE AND DICHLORVOS IN WHOLE BLOOD

Compound	Added (μmol/l)	Found (μmol/l)	Coefficient of variation (%)
Within-assay variation (n=10)			
Metrifonate	0.777	0.765	9
	19.4	18.2	4
Dichlorvos	0.050	0.047	5
	0.226	0.217	2
Between assay variation (n=4)			
Metrifonate	7.77	8.12	9
Dichlorvos	0.905	0.896	5

TABLE II

EXTRACTION RECOVERY OF METRIFONATE AND DICHLORVOS IN WHOLE BLOOD

Compound	Concentration (μmol/l)	Mean recovery (n=4) (%)
Metrifonate	20	87
Dichlorvos	1	99

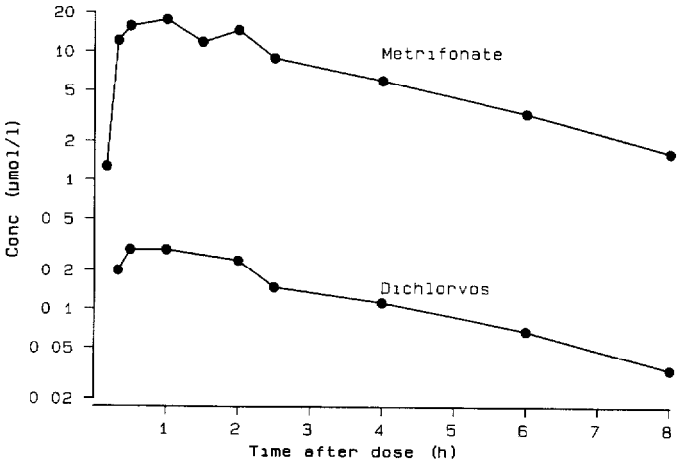


Fig. 5. Concentration versus time curves for metrifonate and dichlorvos in a human subject having received 7.5 mg/kg metrifonate.

The utility of the assays was demonstrated by giving a single dose of 7.5 mg/kg metrifonate to one subject and analyzing the blood collected as described above. The levels of the two substances followed each other well, those of metrifonate being 56 (S.D. = 5) times higher than those of dichlorvos in all samples (Fig. 5). The elimination half-life of metrifonate was 2.3 h.

DISCUSSION

The degradation of metrifonate and dichlorvos, which takes place at significant rates in blood after sampling [3], causes serious problems in the determination of the two compounds. Nordgren and co-workers [9,10] added [$^2H_6$]metrifonate to the samples before the assay and monitored [$^2H_6$]dichlorvos in order to compensate for the formation of dichlorvos during analysis. This procedure does not compensate for the degradation of metrifonate to other products, nor for the degradation of dichlorvos, which both occur to a considerable extent. In spite of that, the concentration ranges were similar to those in the subject studied by us (Fig. 5). In a previous study on the pharmacokinetics of metrifonate [12] we used plasma samples that had been acidified immediately after centrifugation. According to more recent findings in our laboratory both metrifonate and dichlorvos undergo rapid and substantial degradation during plasma preparation, i.e. centrifugation, which makes plasma less suitable for analysis. In order to obtain reliable results of the analysis it therefore became necessary to use a sampling procedure that minimizes these reactions. By adding blood directly to phosphoric acid the pH is lowered to ca. 1, thus immediately inhibiting enzymic reactions as well as the base-catalyzed conversion of metrifonate to dichlorvos. With this procedure the total collection of 1.5 ml of blood took less than 10 s but the time for each drop from leaving the vein until acidification is much less.

In conclusion, this study presents simple methods for the determination of metrifonate and dichlorvos for pharmacokinetic purposes and a reliable procedure to stop the degradation of the drugs during sampling and storage.

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