Journal of Chromatography, 240 (1982) 532–538
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,742

Note

High-performance liquid chromatography of phosfolan, mephosfolan and related compounds

SHERIF A. ABOU-DONIA* and MOHAMED B. ABOU-DONIA*
Department of Pharmacology, Duke University Medical Center, Durham, NC 27710 (U.S.A.)
(First received November 11th, 1981; revised manuscript received January 18th, 1982)

The organophosphorus compounds phosfolan (O,O-diethyl 1,3-dithiolan-2-ylidene phosphoramidate) and mephosfolan (O,O-diethyl 4-methyl 1,3-dithiolan-2-ylidene phosphoramidate) are systemic insecticides that have been used extensively for pest control on cotton, corn, rice, sorghum and sugar cane, especially in the Middle East and Asia[1]. There are several reports concerning the use of phosfolan and mephosfolan against the Egyptian cotton leaf worm (*Spodoptera littoralis*)[2], the pink bollworm (*Pectinophora spp.*) and the spiney bollworm (*Earious insulana*)[3,4].

Numerous methods have been developed for the determination of phosfolan and mephosfolan. These insecticides have been analyzed frequently by spectrophotometry[5,6], gas–liquid chromatography[6] and thin-layer chromatography[6–11]. All these methods have been used mainly for determination of the intact compounds, although in some cases a few metabolites were identified. For example, a sequential thin-layer chromatographic method has been used to analyze phosfolan and mephosfolan and some of their possible metabolites in animals given the ^{14}C-labeled insecticide[12].

The present study describes a high-performance liquid chromatographic (HPLC) method that was developed for quantitative and qualitative analysis of phosfolan, mephosfolan and some of their degradation products. This method offers rapid and accurate analysis of these compounds in metabolic studies and in the analysis of their residues in the environment. In this study, to demonstrate its effectiveness in biological and non-biological systems, the method was used to analyze the residue of these chemicals in human plasma and tomato leaves.

EXPERIMENTAL

Materials

Phosfolan (Cyolane), mephosfolan (Cytrolane), and their related compounds were provided by American Cyanamid (Princeton, NJ, U.S.A.). The following analytical-grade compounds were studied: phosfolan (O,O-diethyl 1,3-dithiolan-2-ylidene phosphoramidate), mephosfolan (O,O-diethyl 4-methyl 1,3-dithiolan-2-ylidene phosphoramidate), hydroxy mephosfolan (O,O-diethyl 4-hydroxymethyl-1,3-dithiolan-2-

* Permanent address: Department of Plant Protection, Al Azhar University, Cairo, Egypt.

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ylidenephosphoramidate), PDIC (propylene dithioimidocarbonate hydrochloride), PDC (propylene dithiocarbonate), EDIC (ethylene dithioimidocarbonate hydrochloride) and EDC (ethylene dithiocarbonate). Stock solutions of these compounds were prepared in methanol (1 mg/ml) and appropriate dilutions were made to produce working standards. Stock and standard solutions were stored at $-20^{\circ}C$ in amber glass vials with no detectable decomposition over a period of 2 months.

High-performance liquid chromatography

Reversed-phase HPLC was performed using a Waters Assoc. liquid chromatograph (Milford, MA, U.S.A.) consisting of two M6000-A pumps, an M660 solvent programmer, a M440 ultraviolet (UV) detector, a U6-K universal injection system and a stainless steel precolumn (10 cm × 2 mm I.D.) containing a reversed-phase μBondapak C_{18} followed by a μBondapak C_{18} column (30 cm × 3.9 mm I.D.). The HPLC-grade solvents were filtered through Millipore membrane filters, type HA or FH, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.) and degassed under vacuum prior to use. Test compounds were injected in 2–25 μl of methanol solutions and separated by gradient elution at room temperature. The initial solvent composition was 5% methanol, 1% glacial acetic acid and 94% water, changing to a final solvent composition of 100% methanol in 60 min. The gradient shape employed was Waters No. 6 gradient shape. The solvent flow-rate was 2.0 ml/min, and the column inlet pressure was 2000 p.s.i. All compounds were detected and quantitated by monitoring the UV absorbance of the column eluates at 254 nm using a 10-mV chart recorder with a chart speed of 1.0 cm/min. Peak areas were measured with a Shimadzu Chromatopak ElA (Shimadzu, Kyoto, Japan) reporting integrator.

Application and extraction of phosfolan, mephosfolan and their related compounds from tomato leaves

Aliquots (5 μl) of methanol solutions (1 mg/ml) of phosfolan, mephosfolan and their related metabolites were spotted on the upper surface of ten tomato leaves. The leaves were placed in a 150-ml Erlenmeyer flask, stoppered tightly and kept for 10 min at room temperature. Then, the leaves were homogenized in 30 ml of hexane–acetone (4:1) for 3 min using a Polytron Ultrasonic homogenizer (Brinkmann, Westbury, NY, U.S.A.). To clean up the solution, 0.1 g of charcoal (Norite A) and 1 g of anhydrous sodium sulfate were added. The flask was swirled gently for 2 min and then stirred by a magnetic stirrer for 5 min. The homogenate was filtered through Whatman No. 1 filter paper which was washed with 5 ml of the solvent mixture, and the filtrate and the wash were combined in the same flask. The solvent was evaporated to dryness using a rotary evaporator at 40°C under reduced pressure and then diluted to 50 μl by methanol. Aliquots of 5 μl were injected into the HPLC columns, eluted and detected as described above.

Application and extraction of phosfolan, mephosfolan and their related compounds from human plasma

Aliquots (5 μl) of methanol solutions (1 mg/ml) of phosfolan, mephosfolan and their related compounds were added to 5 ml of human plasma in 13 × 100 mm screw-capped culture tubes and mixed for 1 min using a vortex mixture. Ethyl acetate (10 ml) was added to the tube, mixed for 1 min, and then centrifuged at 3000 rpm for

5 min. The ethyl acetate layer was transferred to a small flask by Pasteur pipette, and evaporated to dryness using a rotary evaporator at 40°C under reduced pressure. The residue was dissolved to 50 μl of methanol. Aliquots of 5 μl were analyzed by the HPLC procedure described above.

RESULTS AND DISCUSSION

Most organophosphorus insecticides are composed of highly lipophilic molecules; however, biotransformation of these insecticides within the affected organism generally results in the molecule becoming more polar and more water-soluble. This biological modification of organophosphorus insecticides usually results in great changes in the toxicity of the compounds. Therefore, the prime objective of the present study is to develop a rapid and sensitive HPLC method which could be used specifically and quantitatively for the analysis of phosfolan, mephosfolan and some of their metabolites (Fig. 1) in both biological and non-biological systems. To determine the applicability of this method for both systems, it was used to analyze the test compounds in tomato leaves and human plasma.

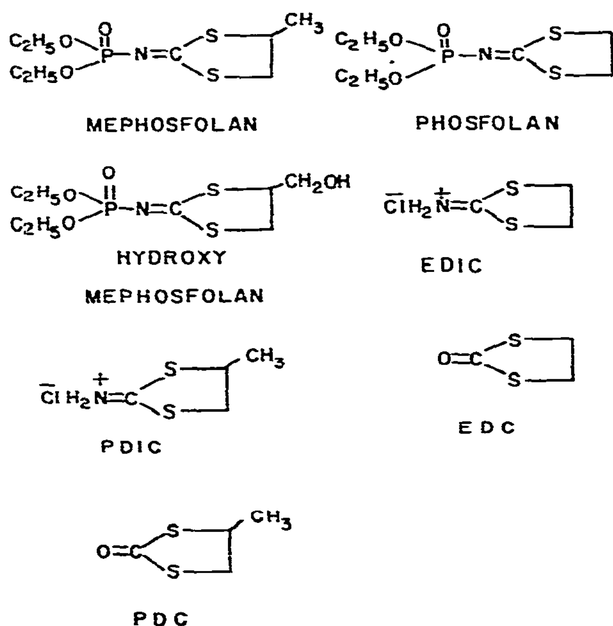

Fig. 1. Chemical structures of phosfolan, mephosfolan and their possible degradation products. Abbreviations are listed under Materials.

These compounds could be classified into two groups according to their separation on HPLC: non-polar compounds (mephosfolan and phosfolan) and polar compounds (PDIC, PDC, EDC and EDIC). Hydroxy mephosfolan exhibited behaviour intermediate between non-polar and polar. Generally, these two groups can be resolved from each other; however, the individual compounds within the polar group may not be readily separated. Because all the compounds have a wide range of

polarity, several HPLC solvents were evaluated to develop a method to distinguish quickly the parent compounds from each other and from their possible degradation products. The best solvent system for the separation of non-polar compounds from each other, as well as from the polar compounds on the reversed-phase μBondapak C_{18} column, was the solvent gradient of 5–10% methanol in water. In this system, non-polar substances moved in good separable distances from each other. Mephosfolan was the slowest followed by phosfolan and then the intermediate hydroxy mephosfolan. By contrast, although the polar compounds eluted faster and separated well from the non-polar substances, they eluted as two peaks with shoulders. EDC appeared as a shoulder on the EDIC peak and PDC appeared as a shoulder on the PDIC peak.

The problem of resolution of EDC from EDIC and PDC from PDIC was solved by adding 1% HPLC-grade glacial acetic acid to the initial eluting water. When this method was used all the pairs of compounds with overlapping peaks were resolved, *i.e.*, PDIC–PDC and EDIC–EDC. Good resolution of the parent phosphates and their degradation products was obtained by reversed-phase HPLC using μBondapak C_{18} columns (Fig. 2). At a flow-rate of 2.0 ml/min, all compounds eluted as sharp symmetrical peaks with baseline resolution. A sharply concave gradient was employed to decrease the retention of the highly non-polar compounds.

The retention times and UV absorbance of the phosfolan and mephosfolan are listed in Table I. These data were highly reproducible with the elution solvents and

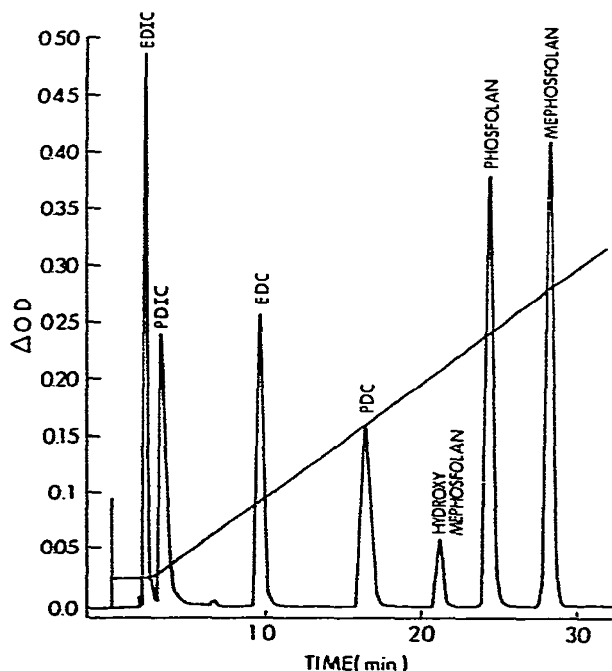

Fig. 2. Separation of phosfolan, mephosfolan and their related compounds by reversed-phase HPLC. Elution from μBondapak C_{18} column using a gradient of 5–100% methanol–water (initial solvent also contained 1% glacial acetic acid) in 60 min. Flow-rate 2 ml/min at 25°C. The shape of gradient used is described by the solid tracing.

TABLE I

RETENTION TIMES AND RELATIVE UV ABSORBANCE OF PHOSFOLAN, MEPHOSFOLAN AND RELATED COMPOUNDS USING HPLC

Experimental conditions are listed under Experimental.

Compound	*Retention time** (min)*	*Relative UV absorbance***
Mephosfolan	29.05	1.00
Hydroxymephosfolan	21.79	0.13
PDIC	3.93	0.59
PDC	17.14	0.52
Phosfolan	25.01	0.96
EDIC	2.56	0.63
EDC	10.36	0.55

* Mean of four successive injections of 5 μg of each compound.

** Values are expressed relative to the UV absorbance at 254 nm of 5 μg of mephosfolan with detector sensitivity at 0.5 a.u.f.s.

column used. The UV detection limit at 254 nm was 10 ng for phosfolan, mephosfolan, EDIC, EDC, PDIC and PDC. Hydroxy mephosfolan had a detection limit of 100 ng.

The relationship between peak area and amount injected remained linear over a 1000-fold range (10 ng–10 μg) (Figure not shown).

This method was successful in analyzing these compounds in tomato leaves and human plasma (Figs. 3 and 4). All test chemicals were detected in both leaves and

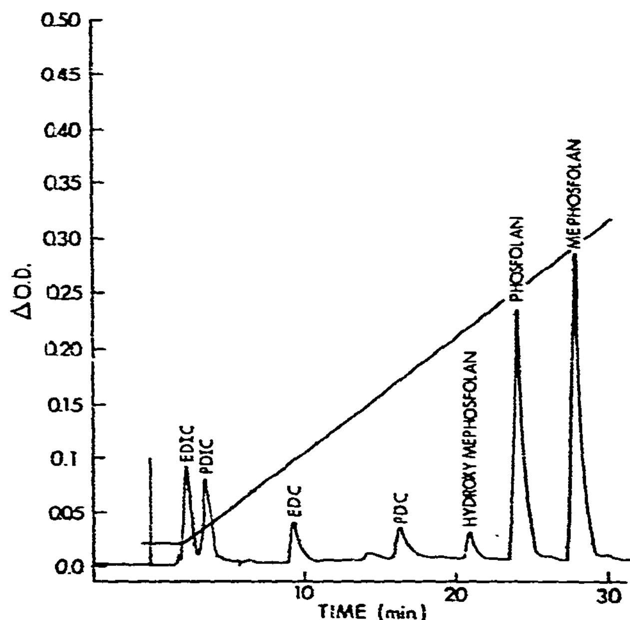

Fig. 3. HPLC profile of phosfolan and mephosfolan and related compounds extracted from tomato leaves. HPLC conditions as in Fig. 2.

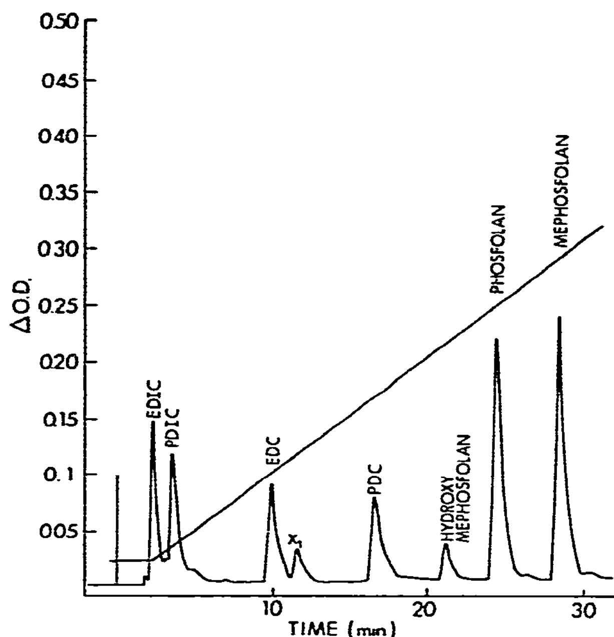

Fig. 4. HPLC profile of phosfolan and mephosfolan and related compounds extracted from human plasma. HPLC conditions as in Fig. 2.

plasma extracts. The percentages of recovery of phosfolan and mephosfolan from tomato leaves were 73 and 83%, respectively. Hydroxy mephosfolan recovery was 62%. All other polar metabolites had much lower recoveries: EDIC 23%, EDC 17%, PDIC 31% and PDC 21%. The low recoveries of the polar compounds may be attributed to the use of charcoal to clean up the tomato extracts from interfering materials. Highly polar compounds also tend to bind to biological tissues. Thus, although the percentages of recovery of the parent compounds from human plasma were similar to those recovered from leaves, the percentages of recovery of the polar compounds from human plasma were higher than from tomato leaves ranging between 33 and 52%. Also the chromatogram obtained from the human plasma extract contained an unidentified peak X_1 (Fig. 4). Since the retention time of this peak was 11.5, it did not interfere with analysis of these compounds.

In conclusion, the method developed is a rapid and sensitive analytical method which resolved phosfolan, mephosfolan and five of their metabolites in a single chromatogram. The present study demonstrates that HPLC fulfills the requirements for quantitative and qualitative analysis of phosfolan and mephosfolan and some of their metabolites.

ACKNOWLEDGEMENT

The supply of phosfolan and mephosfolan by American Cyanamid (Princeton, NJ, U.S.A.) is acknowledged.

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