REVERSED PHASE PAPER CHROMATOGRAPHY OF *p*-NITROPHENYL METHYLPHOSPHONATES AND SULFUR ANALOGS

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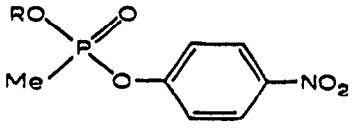
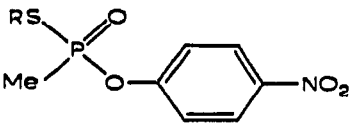
INTRODUCTION

In the course of investigations concerning the structure–activity relationships of organophosphorus compounds in their reaction with hydrolytic enzymes[1,2] a method was required for the analysis of O-alkyl *p*-nitrophenyl methylphosphonates (I), S-alkyl *p*-nitrophenyl methylphosphonothiolates (II) and O-alkyl *p*-nitrophenyl methylphosphonothionates (III).



(I)



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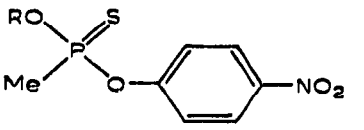


(III)

The separation of parathion and some related phosphates using reversed phase paper chromatography has been reported[3,4]. Because of a comparable lipophilic character of compounds I, II and III a similar technique was used. The three forementioned types of organophosphorus compounds, as well as homologues of I and II (R = Me, Et, *n*-Pr, *n*-Bu and *n*-Pe) separated satisfactorily on filter paper impregnated with silicone oil. A chloroform–ethanol–water system served as the mobile phase. The influence of the concentration of silicone oil on the separation was studied.

For series I and II, a linear relationship between the quantity $R_M = \log (1/R_F - 1)$ and the number of carbon atoms in the alkyl group (R) was found.

It was possible to distinguish the three classes of organophosphorus compounds using two specific chromogenic reagents.

EXPERIMENTAL

Materials

The synthesis of the O-alkyl *p*-nitrophenyl methylphosphonates (I), S-alkyl *p*-nitrophenyl methylphosphonothiolates (II) and paraoxon has been described previously[5,2,6]. The O-alkyl *p*-nitrophenyl methylphosphonothionates (III) were prepared using the method described by KABACHNIK and coworkers[7]. Isoparathion was synthesized according to METCALF AND MARCH[8]. Parathion (Ligtermoet N.V.,

Rotterdam)) was used without further purification. Silicone oil, type DC 550, was obtained from Dow Corning Company.

Horse serum butyrylcholinesterase (acylcholine acylhydrolase E.C. 3.1.1.8) was a freeze-dried product obtained from Organon N.V., The Netherlands. α-Naphthyl acetate used as the substrate and Fast Blue B salt were purchased from the British Drug Houses Ltd. and Edward Gurr Ltd. (London) respectively.

Chromatographic procedure

Whatman No. 1 filter paper was impregnated with 5, 10 or 15 % solutions of silicone oil in hexane by pulling the paper sheets three times through the solution. The wet papers were blotted lightly between filter paper and allowed to dry. The upper layer of a mixture of chloroform–ethanol–water (5:5:3, v/v) served as the mobile phase. The phosphorus compounds were applied on the filter paper from a 0.05 *M* isopropanol solution; 4 μl samples were used for the separation. The ascending technique was used at 25° ± 0.5°. The chromatograms were developed over a period of 16 h after equilibration with solvent vapour for 2 h. They were removed from the tank and dried in a hood for 1 h.

Detection reagents

Sodium hydroxide. The chromatograms were sprayed with a 0.1 *N* aqueous sodium hydroxide solution. In the case of phosphonothionates and phosphates the chromatograms were subsequently heated for 5 min at 110°.

Hanes reagent. The phosphonates were detected with perchloric acid–ammonium molybdate according to HANES AND ISHERWOOD[9] with a heating period of 7 min at 90°. The phosphates needed additional heating for about 2 min.

Gibbs' reagent. The chromatograms were sprayed with a 1 % solution of 2,6-dibromo-*N*-chloro-*p*-quinoneimine in cyclohexane and subsequently heated at 90° for 1 h[10,11].

Dragendorff reagent. The reagent[12], containing a KI–$BiCl_3$ mixture, was applied as a spray. The colour appeared within 15 min.

Butyrylcholinesterase reagent. The paper chromatograms were sprayed first with a butyrylcholinesterase solution [0.4 %, in a 0.1 *M* tris(hydroxymethyl)methylamine buffer, pH 8]. After an incubation time of about 1 min, a second spray was applied with a freshly prepared mixture of α-naphthyl acetate (5 mg in 2 ml of ethanol) and East Blue B salt (10 mg in 8 ml of water).

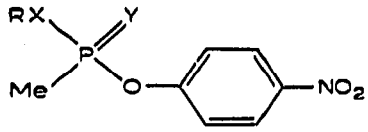
RESULTS AND DISCUSSION

Separation of homologues of series I, II and III

From the R_F values collected in Table I it can be seen that, in general, a satisfactory separation of the homologues of series I and II was obtained on filter paper impregnated with a 5, 10 or 15 % silicone oil solution in hexane. The methyl and ethyl homologues of series I showed no difference in mobility and the methyl and ethyl homologues of series II gave partly overlapping spots on filter paper with a 5 % degree of impregnation. The R_F values of all phosphorus esters decreased when the degree of silicone oil impregnation was increased. In that case an improved separation of the first members of both series I and II was obtained.

TABLE I

R_F VALUES × 100 WITH THEIR STANDARD DEVIATIONS* OF COMPOUNDS WITH THE GENERAL FORMULA (IV) ON FILTER PAPER IMPREGNATED WITH SILICONE OIL



(IV)

Series	*R*	*X*	*Y*	*Impregnation*		
				5 %	10 %	15 %
I	Me	O	O	84 ± 4	84 ± 5	81 ± 6
	Et			83 ± 2	75 ± 3	73 ± 3
	n-Pr			73 ± 1	64 ± 3	51 ± 9
	n-Bu			64 ± 4	47 ± 4	39 ± 7
	n-Pe			49 ± 5	33 ± 2	26 ± 3
II	Me	S	O	79 ± 3	72 ± 5	68 ± 4
	Et			75 ± 4	60 ± 3	57 ± 4
	n-Pr			63 ± 4	47 ± 4	39 ± 4
	n-Bu			45 ± 4	30 ± 3	22 ± 4
	n-Pe			26 ± 3	15 ± 2	10 ± 2
III	Me	O	S	11 ± 5	5 ± 2	6 ± 1
	Et			8 ± 2	5 ± 1	4 ± 1

* Standard deviations were obtained using the method of DEAN AND DIXON[13].

The methyl and ethyl homologues of series III gave overlapping spots on filter paper with a 5 % degree of impregnation and were inseparable when higher degrees of impregnation were used. Higher homologues of this series were not investigated but it is to be expected that their location will be very close to the starting point.

It turned out to be impractical to use paper sheets with still higher degrees of impregnation as an irregularity in solvent flow occurred.

p-Nitrophenol and the organophosphorus acids, which were occasionally present as impurities due to hydrolysis, were found on the chromatograms with R_F values 0.75–0.80 and 0.90–1.00, respectively.

R_M value

BATE-SMITH AND WESTALL[14] introduced the quantity $R_M = \log ((1/R_F - 1))$ which is proportional to the free energy required to transport a molecule from one of two immiscible solvent phases to the other. The R_M value is directly related to the partition coefficient and made up of additive values of the various groups of which the molecule is composed.

In Fig. 1 the R_M value is plotted *versus* the number of carbon atoms present in the alkyl group (R) of the members of series I and II (R = Me, Et, *n*-Pr, *n*-Bu, *n*-Pe). A satisfactory linear relationship was found in the case of 10 % silicone oil impregnation.

From the graphs represented in Fig. 1 almost identical ΔR_M values per CH_2 group, 0.26 ± 0.01 and 0.29 ± 0.05, were obtained for series I and II respectively.

The positive signs of the ΔR_M values in both series correspond with an increase in lipophilic character which is reflected in an increase in solubility in the stationary phase.

The results found on paper impregnated with a 5 and 15 % silicone oil solution deviated from linearity to a greater extent.

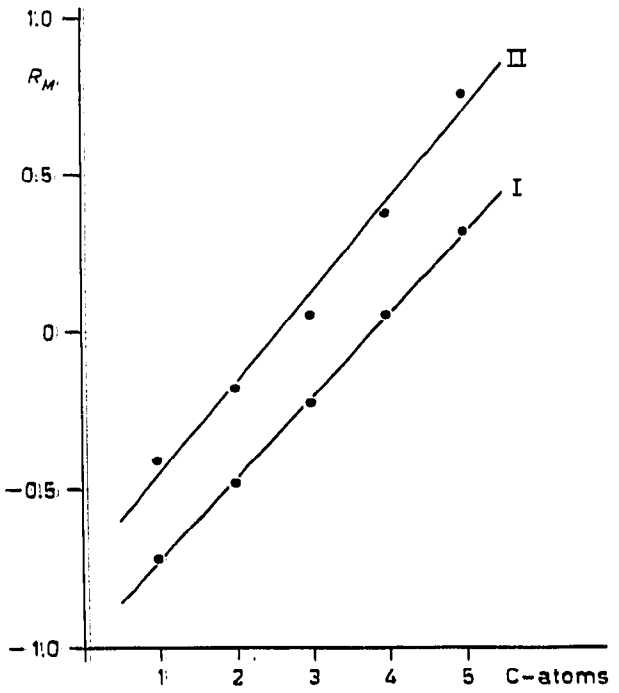


Fig. 1. Plot of R_M *versus* the number of carbon atoms for series I and II on filter paper with 10 % silicone oil impregnation.

Relation between R_F value and structure

As was shown in Table I the R_F values decreased on lengthening the carbon chain of the alkyl group (R) in series I, II and III.

Substitution of a P–O bond by a P–S bond increases the lipophilic character of the molecule. Accordingly the phosphonothiolates (II) gave lower R_F values when compared with those of the corresponding phosphonates (I) as is shown in Table I. A much greater effect was found when the P = O bond was replaced by a P = S bond. This is demonstrated in Table II for the compounds I, II and III (R = Et). R_F values of paraoxon (diethyl *p*-nitrophenyl phosphate), isoparathion (O,S-diethyl *p*-nitrophenyl phosphorothiolate) and parathion (diethyl *p*-nitrophenyl phosphorothionate) are added for comparison. Partition coefficients obtained for paraoxon (4.2) and parathion (2.13) in the system hexane–water[15] are in agreement with the results recorded in Table II; a high partition coefficient corresponds with a low R_F value.

Replacement of the methyl group directly attached to the phosphorus atom by an ethyl group results in a more pronounced lipophilic character of the molecule and as a consequence a drop in R_F value is found. The reverse is observed when an ethyl group is substituted by an ethoxy group (Table III).

TABLE II

R_F VALUES × 100 OBTAINED ON PAPER IMPREGNATED WITH A 5% SILICONE OIL SOLUTION

Compound	R_F × 100
I, R = Et	83
II, R = Et	75
III, R = Et	8
Paraoxon	70 (74)*
Isoparathion	48 (47)
Parathion	2 (4)

* Figures in parentheses are from METCALF AND MARCH[3].

Detection

Besides the identification by R_F values, it was possible to distinguish the three classes of organophosphorus compounds I, II and III using two specific chromogenic reagents: Gibbs' and Dragendorff's. Phosphorothiolates and phosphorothionates give yellow and red coloured spots respectively, using Gibbs' reagent. No colour is observed with organophosphorus compounds which contain no sulfur[10,11]. Similar results were obtained with the compounds I, II and III. Dragendorff reagent is generally used to detect ammonium or sulfonium compounds. In the investigation described here it was applied as a specific reagent in order to distinguish the series I, II and III on account of their differences in sensitivity. It gave orange spots on a faint yellow background. The lower detection limits were ~ 30 μg for the thiolates and ~ 10 μg for the phosphonates and phosphates. Thionates did not react up to a quantity of 150 μg.

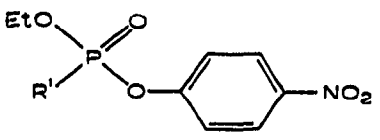
The detection results with phosphonates (I), phosphonothiolates (II) and phosphonothionates (III) were in agreement with those obtained for comparable compounds such as paraoxon, isoparathion and parathion.

Using a combination of Gibbs' and Dragendorff reagents the identification of *p*-nitrophenyl methylphosphonates could be performed as is shown in Table IV.

The sodium hydroxide solution was used to check the presence of a *p*-nitrophenyloxy group or *p*-nitrophenol. Hanes reagent detected the presence of a phosphorus atom. Most of the investigated organophosphorus compounds possess cholin-

TABLE III

R_F VALUES × 100 OF COMPOUNDS WITH THE GENERAL FORMULA (V) ON PAPER IMPREGNATED WITH A 5% SILICONE OIL SOLUTION

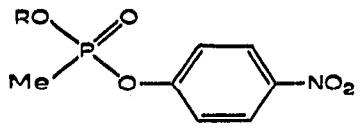
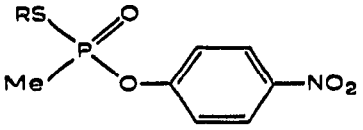
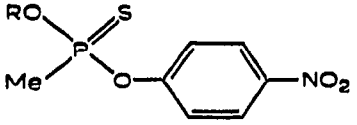


(V)

R'	R_F × 100
Me	83
Et	55
EtO	70

TABLE IV

DETECTION OF *p*-NITROPHENYL METHYLPHOSPHONATES AND THEIR SULFUR ANALOGS

Structure	*Reagent**	
	Gibbs'	*Dragendorff*
	—	orange ++
	yellow ++	orange +
	red ++	—

* ++ = Normal sensitivity, + = low sensitivity, — = no reaction.

esterase inhibiting properties. The butyrylcholinesterase reagent showed the presence of inhibitors, giving white spots on a mauve background.

The detection reagents described permitted a satisfactory estimation of the purity of some *p*-nitrophenyl methylphosphonates and their sulfur analogs.

ACKNOWLEDGEMENT

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SUMMARY

On account of differences in lipophilic character it is possible to separate *p*-nitrophenyl methylphosphonates and their sulfur analogs by reversed phase paper chromatography. Whatman No. 1 filter paper was impregnated with silicone oil 550; the upper layer of a chloroform–ethanol–water (5:5:3, v/v) system served as the mobile phase.

The relation $R_M = \log (1/R_F - 1)$ holds for homologous series of O-alkyl *p*-nitrophenyl methylphosphonates (I) and S-alkyl *p*-nitrophenyl methylphosphonothiolates (II) up to the *n*-pentyl homologues.

Using a combination of Gibbs' and Dragendorff reagents it was possible to distinguish the phosphonates, phosphonothiolates and phosphonothionates.

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