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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PIRIMIPHOS METHYL AND FIVE METABOLITES

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SUMMARY

Pirimiphos methyl is an organophosphorus insecticide which is rapidly metabolised by plants and animals to several modified triesters and free hydroxypyrimidines. A method is described for the determination by reversed-phase high-performance liquid chromatography of pirimiphos methyl and its five major metabolites in plasma and urine. Separations were performed by isocratic and gradient elutions from short columns packed with SAS-Hypersil, a relatively new column packing.

INTRODUCTION

Pirimiphos methyl (N,N-diethylamino-6-methylpyrimidin-4-yl dimethylphosphorothionate, Actellic®, PP 511) is an organophosphorus insecticide used in agriculture and public health, and for the control of pests of stored products. The compound (I in Table I) is extensively metabolised in rats and dogs to other phosphorus-containing triesters, N,N-diethyl-6-methylpyrimidin-4-yl dimethylphosphate (II in Table I), and N-ethylamino-6-methylpyrimidin-4-yl dimethylphosphorothionate (III in Table I). The major excretory products are the parent and modified hydroxypyrimidines: the diethylamino-hydroxypyrimidine (N,N-diethylamino-4-hydroxy-6-methylpyrimidine) (IV in Table I) and the corresponding mono- and di-desethylated hydroxypyrimidines (V and VI, respectively, in Table I)[1].
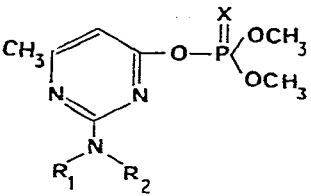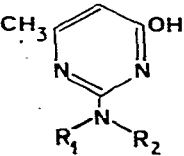
Gas–liquid chromatography (GLC) determinations of pirimiphos methyl and its phosphorus-containing metabolites have been reported[1,2] and a high-performance liquid chromatography (HPLC) method for the estimation of pirimiphos methyl in formulations has been described[3]. However, no method has been reported for the quantitative estimation of the polar hydroxypyrimidines (IV, V and VI). Assay of these compounds in animal tissues and plant materials has been attempted in this laboratory by GLC of derivatives[4]. This method necessitates extraction of the material with methanol, removal of phosphorus-containing compounds from the extract by

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TABLE I

STRUCTURE, ABSORBANCE MAXIMA AND LIMIT OF DETECTION OF PIRIMIPHOS METHYL (I) AND FIVE METABOLITES

The limits of detection were determined for gradient elution of a 25-μl sample of a mixture of compounds I–VI at 235 nm and 0.01 a.u.f.s. (concentrations and conditions as in Fig. 3).

Compound	Structure	$\lambda_{max.}$ (nm)	Limit of detection (ng)
	(pyrimidine ring with CH$_3$, O–P(=X)(OCH$_3$)$_2$, and N–R$_1$R$_2$ substituent)		
I	R$_1$ and R$_2$ = C$_2$H$_5$, X = S	247	10
II	R$_1$ and R$_2$ = C$_2$H$_5$, X = O	242	15
III	R$_1$ = C$_2$H$_5$, R$_2$ = H, X = S	234	20
	(pyrimidine ring with CH$_3$, OH, and N–R$_1$R$_2$ substituent)		
IV	R$_1$ and R$_2$ = C$_2$H$_5$	227	1–2
V	R$_1$ = C$_2$H$_5$, R$_2$ = H	215	1–2
VI	R$_1$ and R$_2$ = H	206	1–2

diethyl ether extraction at low pH followed by extraction of the hydroxypyrimidines at elevated pH and derivatization with either diazomethane, diazoethane or hepta-fluorobutyrylimidazole. A complex misture of derivatives may result from this procedure and recoveries are below 60%.

Reversed-phase chromatography has been demonstrated as a very powerful method for the separation of complex mixtures of polar compounds in body fluids[5] by hydrophobic interactions with the apolar bonded phase[6]. As an alternative to the octadecylsilane-bonded silica often used in reversed-phase separations the method described here here uses a bonded-phase packing of mixed chain side-groups which allows polar interactions between solutes and the silica microparticles in addition to the hydrophobic interactions characteristic of reversed phases.

A simple method is described for quantitative estimation of the six compounds listed above by reversed-phase HPLC with gradient elution, and for less-complex mixtures with isocratic elution. An example of the measurement of compounds in the blood plasma of several species is described as is the determination of residues in human urine.

EXPERIMENTAL

Apparatus and chromatography

Chromatography was performed on a component system consisting of a Perkin-Elmer Model 601 high-performance liquid chromatograph and a Perkin-Elmer

Model LC-55 variable-wavelength UV detector (Perkin-Elmer, Norwalk, Conn., U.S.A.). The sample was introduced by on-column injection via a septum injector providing flow of solvent coaxial with the injection guide (HETP, Macclesfield, Great Britain), a modification which maximises the "infinite wall effect", of the smooth-bore stainless-steel column, significantly improving resolution[7]. Injections of a 10-μl volume were made for isocratic elutions, but this was increased to 25 μl (combined with reduction in recorder sensitivity from 0.04 to 0.1 a.u.f.s.) for gradient elutions to minimise baseline drift with increasing methanol concentration. 10-μl and 25-μl microsyringes were used for all injections (Hamilton, Bonaduz, Switzerland).

Elutions were performed isocratically with different mixtures of methanol and phosphate buffer (0.1 M, pH 4.5) for elution of simple mixtures and plasma samples. Complex mixtures were eluted with gradients formed from 20% methanol–buffer (v/v) and 55% methanol–buffer (v/v) so that the starting and finishing mixtures would be 20 and 55% methanol, respectively, irrespective of other gradient characteristics. For gradient elution, peak shape was improved by the inclusion of ca. 0.1% perchloric acid in both reservoirs (pH readjusted to 4.5). In all experiments a flow-rate of 1.5 ml/min at a pressure of ca. 800 p.s.i. was used and the column was maintained at room temperature (22°) throughout.

Columns

Short, smooth-bore stainless-steel columns (10 cm × 4.6 mm I.D.) with zero-dead-volume end-fittings (HETP) were slurry-packed with SAS-Hypersil, a mixed short-alkyl-chain bonded reversed-phase microparticulate silica (5 μm) (Shandon Southern Products, Runcorn, Great Britain). A sonicated mixture of 1.7 g SAS-Hypersil in 10 ml methanol–0.1% (w/v) aqueous sodium acetate trihydrate (80%, v/v) was packed into the column by pumping approx 200 ml of methanol–0.1% (w/v) aqueous sodium acetate trihydrate (50%, v/v) through the packing reservoir (HETP) at 5,000 p.s.i. using a pneumatic amplification pump (HS Chromatography, Bourne End, Great Britain). The top of the column was protected by a stainless-steel gauze and plug of quartz wool arranged so that the injected sample was delivered immediately above the centre of the column packing.

Samples

Standard solutions of pirimiphos methyl and related compounds were prepared at a 100 μg/ml (0.01%, w/v) in 50% (v/v) methanol–phosphate buffer (0.1 M, pH 4.5) from analytical standards (ICI Plant Protection Division, Jealott's Hill, Bracknell, Great Britain). Phosphorus-containing compounds were checked for hydrolysis products by thin-layer chromatography [Merck K_{428} precoated plates; diethyl ether–hexane–ethanol (60:30:10) as solvent].

UV absorption spectra of stock solutions of compounds I–VI diluted 1:6 in 50% (v/v) methanol–phosphate buffer (0.1 M, pH 4.5) were measured on a Pye SP1800 scanning spectrophotometer between 190 and 350 nm.

Blood plasma samples from individuals of several species containing residues of compounds I, II and IV were diluted 5 times in phosphate buffer (0.1 M, pH 7.0) and 0.2-ml samples added to 0.1 ml ice-cold methanol, shaken and centrifuged at 10,000 g for 5 min in a Burkard Koolspin bench centrifuge to remove precipitated protein. Samples of 10 μl of this mixture were injected directly on to the column.

Samples of 100 ml of human urine were shell-frozen in 250-ml round-bottom flasks, freeze-dried and extracted with 80 ml of methanol. The methanol extracts were filtered (Whatman No. 1 filter paper) and the filtrate made up to 100 ml with washings. Samples of 10 μl of these extracts were injected directly on to the column and eluted with a solution of 10 % methanol-0.1 M phosphate buffer (pH 4.5) (v/v) at *ca.* 800 p.s.i.

RESULTS AND DISCUSSION

Previous experience in the authors' laboratories had shown that the quantitative estimation by GLC of the three hydroxypyrimidine degradation products of pirimiphos methyl (IV, V and VI) was an unreliable and time-consuming method since an elaborate clean-up procedure was required before derivatization and even so a complex mixture of derivatives results in methylation or ethylation of the free hydroxypyrimidines. HPLC on reversed phase offered a solution to this problem with the added possibility that by selection of an appropriate reversed phase all the metabolites from the most polar aminohydroxypyrimidine to the least polar fully alkylated parent triester could be resolved in one mixture. UV absorption spectra of the six compounds were measured; the spectra of compound I, II and III are shown in Fig. 1 and those of compounds IV, V and VI in Fig. 2. The wavelength of maximum
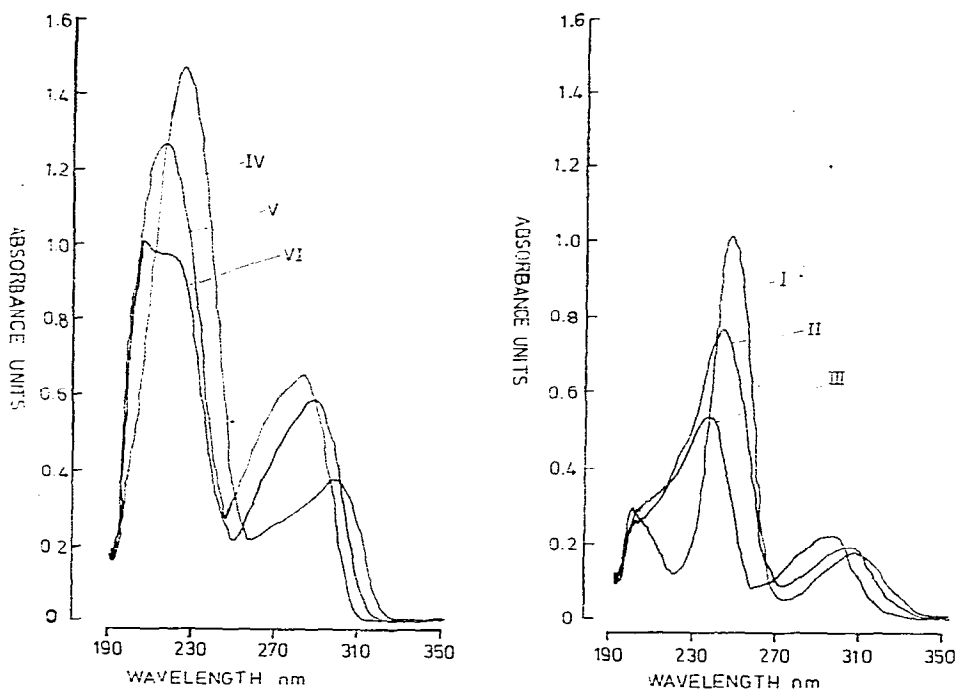

Fig. 1. UV absorption spectra of free hydroxypyrimidines (compounds IV–VI). Concentrations: 14.3 μg/ml in *ca.* 7 % (v/v) methanol–0.1 M phosphate buffer (pH 4.5); temperature, 22°. Pye-Unicam SP1800; 10 sec/cm; 2 nm/sec; 2.0 a.u.f.s.

Fig. 2. UV spectra of phosphorus triesters (compounds I–III). Concentrations and conditions as in Fig. 1.

absorbance of the parent hydroxypyrimidine is changed by the modifying groups from 207 nm for VI to 248 nm for I: 235 nm was therefore chosen as the best compromise for single-wavelength detection. However the sensitivity towards any particular compound could be significantly increased by measuring the UV absorbance at its own maximum, *e.g.* in the case of compound IV at 227 nm was more sensitive by a factor of 6.3 compared to determination at 254 nm.

Gradient elution

Preliminary chromatography of mixtures of compounds I–VI indicated that isocratic elution was incapable of resolving all six compounds in a reasonable time or of resolving the most polar hydroxypyrimidines and yet preserving good peak shape for pirimiphos methyl. Fig. 3 shows a typical chromatogram for the elution under the best conditions obtained of a 25-μl injection of a mixture of the six compounds. The gradient was run at maximum convexity (1000 arbitrary units) and reached 100% with respect to 55% methanol–phosphate buffer in 16 min. The small, unlabelled peaks observed in gradient-elution chromatograms are considered to be as yet unidentified trace impurities in the standards of compounds II and III. These previously undetected compounds testify to the power of separation of this method.

Isocratic elution

As demonstrated above it was possible to resolve compounds of widely differing polarities as well as closely related pairs of compounds by gradient elution on a reversed-phase system. However in order to enable rapid assay of samples without the necessity of reequilibrating the column to the initial solvent composition between samples the isocratic elution of mixtures was examined. Fig. 4 shows a chromatogram for the elution of compounds IV, V and VI isocratically with a 20% methanol–buffer mixture, and Fig. 5 shows a chromatogram for the elution of the complete mixture with a 55% methanol–buffer mixture. The elution time of pirimiphos methyl was reduced to 16 min at this methanol concentration and the five metabolites were eluted within 7 min, these values being comparable with the time taken for analysis by GLC.

A standard curve of concentration against peak height obtained by isocratic elution at 20% methanol–phosphate buffer of 10-μl samples of dilutions of the parent hydroxypyrimidine stock solution was linear over the range 10–100 ppm. A 10-μl volume of a solution containing 73 ng/μl produced a peak of 50% fullscale deflection (0.1 a.u.f.s.). The limit of detection for this compound was therefore approx. 1 ng (= 0.1 ppm for a 10-μl injection) (see Table I).

Determination of metabolites in plasma samples

Diluted plasma samples (5 times in phosphate buffer) from several species including rat, rabbit, sheep, hen, quail and man were obtained containing residues of pirimiphos methyl and pirimiphos methyl oxon (II) (with which they had been spiked) and also varying levels of compound IV resulting from hydrolysis of II in the plasma. It was possible to determine quantitatively the levels of these compounds in the plasma by injection of a 10-μl sample of the plasma diluted 2:1 in methanol. A typical chromatogram for the determination of compounds II and IV in rat plasma is shown in Fig. 6.
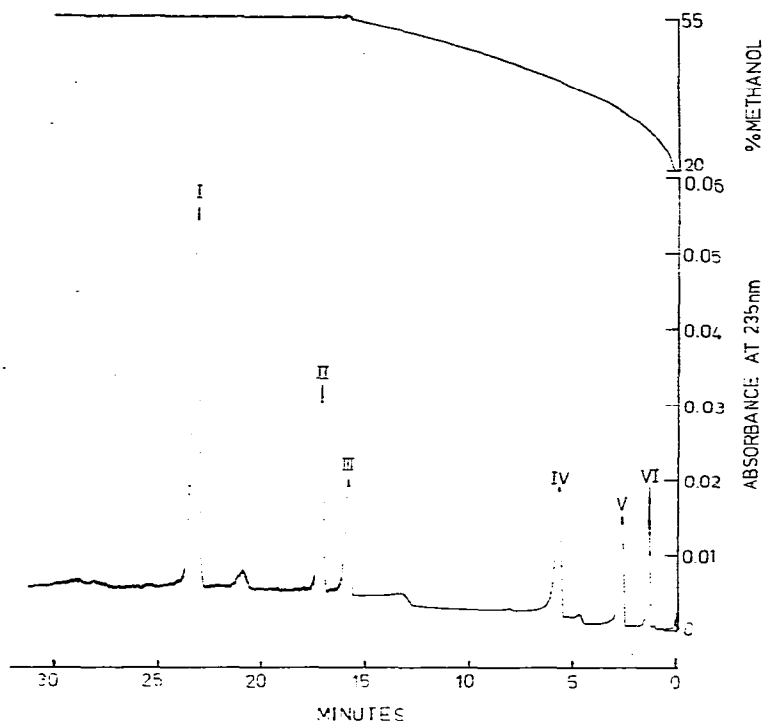
Fig. 3. Chromatography of a mixture of compounds I–VI. Column: 5 μm SAS-Hypersil, 10 cm $\times$ 4.6 mm I.D.; flow-rate 1.5 ml/min; temperature, 22°. Gradient elution from 0 to 100%, 55% (v/v) methanol–0.1 M phosphate buffer in 16 min at max. convexity. Standard mixture (25 μl) containing: 1.05 μg I; 0.65 μg II; 0.575 μg III; 0.175 μg each of IV, V and VI. Wavelength, 235 nm, sensitivity, 0.1 a.u.f.s.
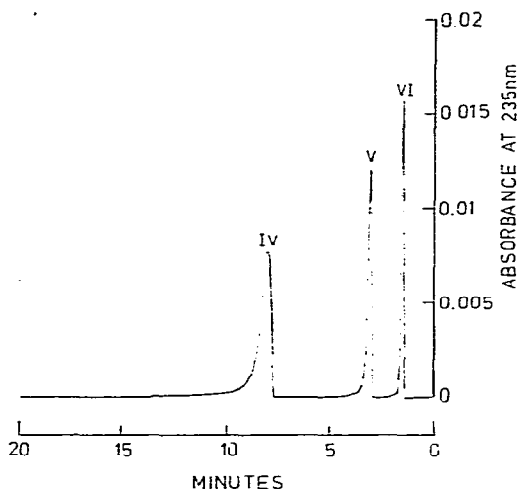
Fig. 4. Chromatography of free hydroxypyrimidines. Column, temperature and flow-rate as in Fig. 3. Isocratic elution at 20% (v/v) methanol–0.1 M phosphate buffer (pH 4.5). A 10-μl mixture containing: 0.33 μg each of IV, V and VI. Wavelength, 235 nm; sensitivity, 0.04 a.u.f.s.
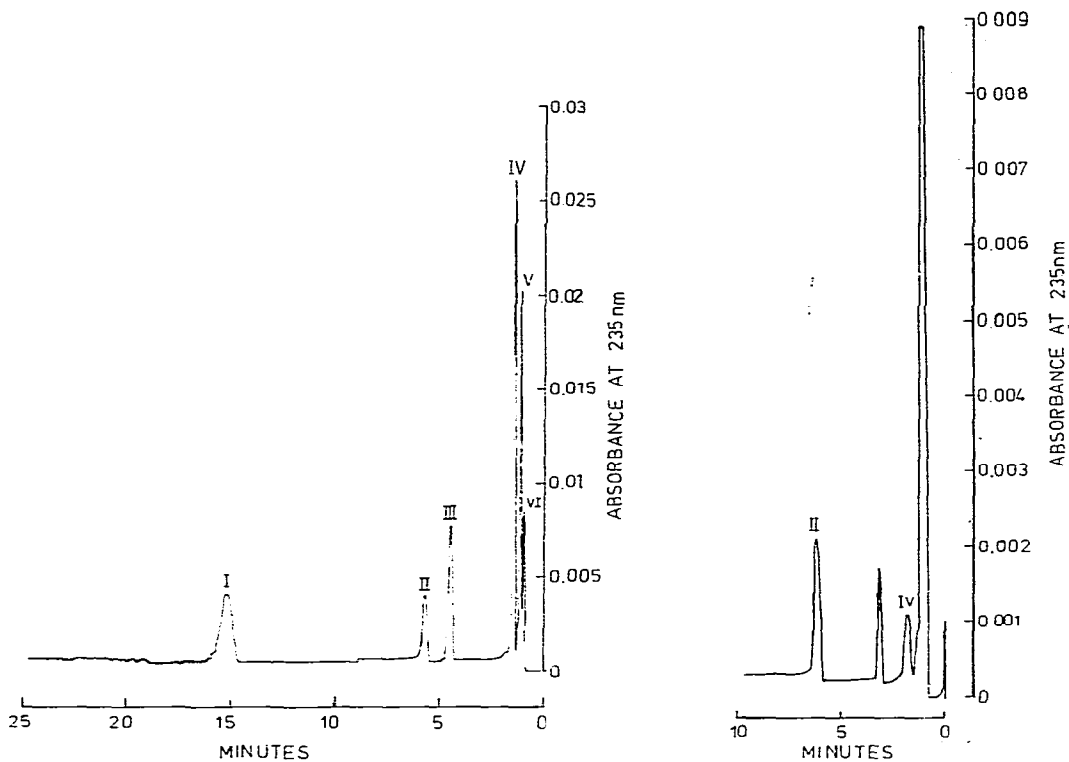
Fig. 5. Chromatography of compounds I–III by isocratic elution. Column, temperature and flow-rate as in Fig. 3. Isocratic elution at 55% (v/v) methanol–0.1 M phosphate buffer (pH 4.5). A 10-μl mixture containing: 0.42 μg I; 0.26 μg II; 0.34 μg III; 0.07 μg eazch of IV, V and VI. Wavelength, 235 nm; sensitivity, 0.04 a.u.f.s.

Fig. 6. Chromatography of rat plasma. Column, temperature and flow-rate as in Fig. 3. Isocratic elution at 50% (v/v) methanol–0.1 M phosphate buffer (pH 4.5). Sample: 10 μl diluted rat plasma (see text) containing compounds II and IV. Wavelength, 235 nm; sensitivity, 0.01 a.u.f.s. The unlabelled peaks correspond to compounds present in control plasma.

Repeated injections of samples containing large amounts of protein eventually caused deterioration of column performance evidenced by poor peak shape, especially at larger retention times. Excess protein may be removed from samples in methanol by cooling to −4° and centrifugation of the precipitated protein but even without this precaution the column retains good performance for injection of over 200 10-μl samples. Where determination of hydroxypyrimidines only was required this was less of a problem since the column retained good performance for the resolution of these compounds well in excess of 200 samples. Columns which have deteriorated may be partially restored by repacking the initial 1 or 2 cm of the column with fresh packing.

Determination of metabolites in human urine

Determination by HPLC on SAS-Hypersil of metabolite V in human urine was attempted in order to assess the usefulness of this technique in measuring the rate of excretion of pirimiphos methyl metabolites in cases of potential accidental poisoning

with the technical insecticide. Samples of urine spiked at different concentrations with pirimiphos methyl were extracted and chromatographed as described above: chromatograms for urine samples obtained from two different individuals are shown in Fig. 7. At a concentration of 10% methanol–buffer (v/v) in the eluting solvent the peak corresponding to metabolite V is well resolved from peaks detected in the control urine sample. Furthermore the method appears to offer a limit of detection of *ca.* 2 ppm in the extract (here equivalent to the same concentration in the urine) as seen in the chromatograms where the peak corresponding to metabolite V represents 36.6 and 2.3 ppm in the extracts of urine from individuals X and Y, respectively. It is noteworthy that interfering substances in the urine varied considerably between individuals and time of collection, more dilute urine presenting less interfering peaks. The analysis of more concentrated urine samples would benefit from an extended clean-up procedure.

HPLC on reversed phase thus appears to offer a rapid and reliable method for the estimation of pirimiphos methyl and its metabolites. The method is especially useful for the determination of hydroxypyrimidine residues in solution in plasma and
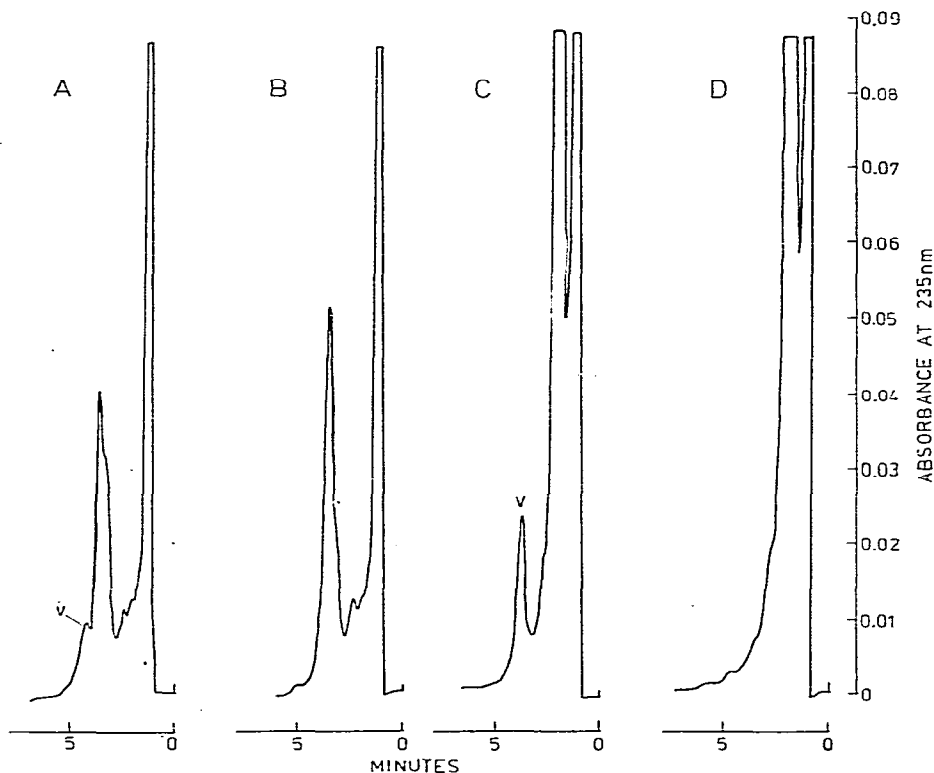

Fig. 7. Chromatography of human urine spiked with compound V. Column, temperature and flow-rate as in Fig. 3. Isocratic elution at 10% (v/v) methanol–0.1 M phosphate buffer (pH 4.5). Wavelength, 235 nm; sensitivity, 0.1 a.u.f.s. (A) A 10-μl urine extract from individual X containing compound V at 2.3 ppm. (C) A 10-μl urine extract from individual Y containing compound V at 36.6 ppm. (B) and (D) 10-μl control urine samples from individuals X and Y, respectively.

urine but similar residues in solid plant and animal tissues are easily extractable in methanol[1] and should prove straightforward to assay by direct injection of methanol extracts.

ACKNOWLEDGEMENTS

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