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SEPARATION OF IMPURITIES IN DIAZINON PREPARATIONS AND THEIR EFFECT ON PORPHYRIN BIOSYNTHESIS IN TISSUE CULTURE

ALAN W. NICHOL and SUSAN ELSBURY
School of Applied Science, Riverina College of Advanced Education, Wagga Wagga, New South Wales, Australia 2650,
GEORGE H. ELDER
Department of Medical Biochemistry, Welsh National School of Medicine, Heath Park, Cardiff, U.K.
and
ANTHONY H. JACKSON and K. R. NAGARAJA RAO
Department of Chemistry, University College, Cardiff, U.K.

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Abstract—The impurities present in commercial diazinon preparations have been examined by high performance liquid chromatography with particular reference to the ability of these compounds to cause porphyrin accumulation in cultures of chicken embryo liver cells. Diazinon and its impurities are readily separated on 10 micron Partisil using cyclohexane–dioxan mixtures. The main impurities are tetraethylpyrophosphate, sulphotetraethylpyrophosphate, 2-isopropyl-6-methylpyrimid-4-one, 2-isopropyl-6-methylpyrimidin-4-thione, and 2-isopropyl-4-ethylthio-6-methylpyrimidine. A previously unreported impurity, 2-isopropyl-6-methyl-4-*S*-pyrimidinyl diethylthiophosphate (isodiazinon), was also detected. Both diazinon and isodiazinon cause accumulation of coproporphyrin in cultures of chicken embryo liver cells. Isodiazinon has a greater effect on porphyrin biosynthesis in the cultures than has diazinon. It is suggested that the point of interference with porphyrin biosynthesis is towards the end of the pathway.

Some chlorinated hydrocarbons are known to cause an experimental porphyria in mammals that is similar to the human disease, porphyria cutanea tarda [1]. Recently it has been reported that some cases of porphyria cutanea tarda in Brazil are associated with exposure to an organophosphorous insecticide, diazinon [2]. An independent study of diazinon-associated porphyria cutanea tarda in Australia has shown that some diazinon preparations cause a disturbance in porphyrin metabolism in rats which is biochemically similar to porphyria cutanea tarda [3]. The diazinon preparation used in this work was of 90% purity. A similar trial using diazinon of 97% purity did not cause a disturbance or porphyrin metabolism in rats as reflected in failure of the preparation to cause elevation of urinary and faecal porphyrin levels over a period of sixteen weeks application to the skin [4]. This observation led to an investigation of the impurities in diazinon preparations by high-performance liquid chromatography (HPLC) and of their ability to cause the accumulation of porphyrins in cultures of chicken embryo liver cells.

MATERIALS AND METHODS

HPLC separations were achieved using a Waters Model M-6000A pump equipped with a Cecil CE 272 detector. Analytical separations were carried out using a 250 mm × 4 mm i.d. internally polished stainless steel column packed with 10 micron Partisil using a Micrometric model 705 column packer. Samples were injected in a volume of 20 μl at a concentration of 1 mg/ml in the solvent to be used for the separation. Semipreparative scale separations were carried out on the same absorbent using a column of 500 mm × 10 mm i.d. Prior to testing in the tissue culture system, fractions were evaporated to dryness under nitrogen at a temperature no greater than 50°. Solvents were all of analytical reagent grade unless otherwise stated. Dioxan was dried over sodium wire.

Diazinon of purity designated as 90% was from Ciba–Geigy (Basel, Switzerland). Diazinon of 97% purity and 2-isopropyl-6-methylpyrimid-4-one were also gifts from Ciba–Geigy.

Reference compounds were prepared as follows: tetraethylpyrophosphate (TEPP) and sulphotetraethylpyrophosphate (sulpho-TEPP) by the methods of Toy [5, 6], and 2-isopropyl-4-ethylthio-6-methylpyrimidine by the method of Margot and Gysin [7]. 2-Isopropyl-6-methylpyrimidine-4-thione was prepared by the following method: 2-isopropyl-6-methylpyrimid-4-one (0.5 g) was dissolved in dry pyridine (7 ml). *p*-Toluene sulphonyl chloride (0.75 g) was added and nitrogen bubbled through the solution for 10 min. Nitrogen was now replaced by hydrogen sulphide and bubbling continued for a further 20 min. After flushing the head space of the flask containing the solution with nitrogen the flask was stoppered and the solution allowed to stand overnight. The solution was then poured into water (100 ml) and its pH was adjusted to 4.0 with 10 M

HCl. The product was extracted into chloroform (×3) which was dried over anhydrous sodium sulphate and evaporated to dryness. The residue was crystallized from methanol to yield 2-isopropyl-6-methylpyrimidine-4-thione as colourless needles (0.12 g) m.p. 156–8°, cf. Margot and Gysin [7] 161–2°. Found: C 56.94, H 7.22, N 16.83, S 18.89. Calc. for $C_8H_{12}N_2S$: C 57.10, H 7.19, N 16.65, S 19.05. The NMR spectrum in $CDCl_3$ showed τ values of 1.31 (6H, doublet, CH_3 of isopropyl), 2.30 (3H, singlet, 6-methyl), 3.03 (1H, septet, CH of isopropyl), 7.09 (1H, singlet, H of 5 position of ring).

The tissue culture system was that of Granick [8] as modified for use with a chemically defined medium by Sassa and Kappas [9]. The medium used was Williams' medium E from Flow Laboratories (Irvine, Scotland, U.K.) supplemented with triiodothyronine (1 μg/ml) and prednisolone phosphate (0.3 μg/ml). When bovine serum insulin was added to the medium a concentration of 1 μg/ml was used. Livers taken from chicken embryos seventeen days after fertilization were disaggregated in calcium and magnesium free Hank's solution using collagenase (Sigma Type I, 0.5 mg/ml) (Sigma Chemical Co., St. Louis, MO). After washing the cells with Williams' medium E the cells were suspended in medium (15 ml per liver) and placed in 3.5 cm diameter Petri dishes (Lux Scientific Catalogue No. 5273, 3 ml per dish). After 24 hr incubation in 5% CO_2 in air, the medium was replaced and the agent to be tested added as an emulsion of volume no greater than 0.1 ml. Emulsions were prepared by dissolving the compound to be tested in ethanol and diluting with Williams' medium E (10:1) immediately before addition to the cultures. Following a further 24-hr incubation period, porphyrin accumulation was measured in the cultures as described by Sinclair and Granick [10] using a Perkin Elmer model 3000 fluorimeter. After the fluorimetric analyses, porphyrins were extracted from the cultures with n-amyl alcohol. Following dilution with two volumes of light petroleum (b.p. 40–60°), the porphyrins were re-extracted into a small volume of 2 M HCl and esterified with methanol:sulphuric acid (20:1). Separation and identification of the porphyrin methyl esters were achieved using the thin layer chromatographic system of Smith [11]. Appropriate reference porphyrins were from Porphyrin Products, Utah.

2-Allyl-2-isopropylacetamide was a gift from Dr F. de Matteis.

LD_{50} determinations were carried out on female rats of 200 g average weight. The method of application was that of Gaines [12]. Only deaths occurring within 48 hr of dosing the rats were recorded. Statistical data of Diechman and Le Blanc [13] were used.

Field desorption mass spectra were determined with a Varian CH-5D double focussing mass spectrometer using a wire current of 10–20 mA and a source temperature in the range 100–150°; spectra were recorded with a SS100 data system and Statos recorder. NMR spectra were determined with a Perkin Elmer R34 90 MHz instrument using $CDCl_3$ solutions with tetramethylsilane as internal standard.

RESULTS

Separation of diazinon impurities by HPLC

The separation of 90% diazinon into its components is shown in Fig. 1. Separation of the less polar

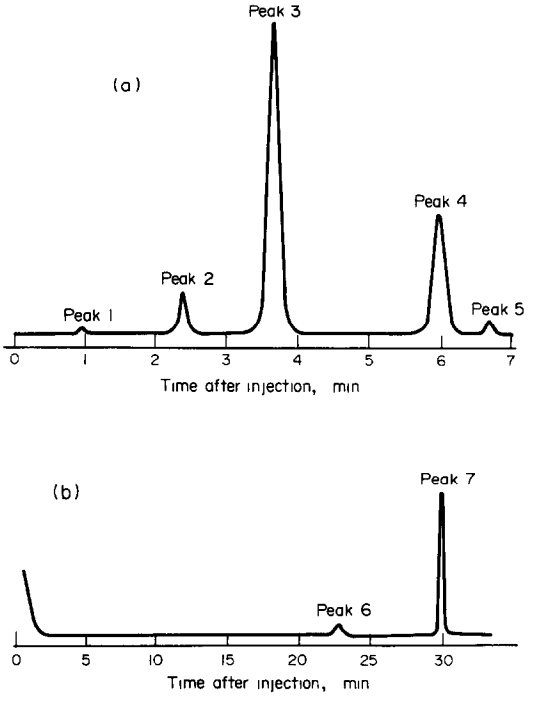


Fig. 1. HPLC of 90% diazinon. (a) Solvent, 3% dioxan in cyclohexane. Detector set at 260 nm. (b) Solvent, 15% dioxan in cyclohexane. Detector set at 220 nm.

Table 1. Identity and HPLC retention times of diazinon impurities on 10 micron Partisil

Compound	Retention time (min) Cyclohexane 3% dioxan	Cyclohexane 15% dioxan
Sulpho-TEPP (Peak 1)	1.0	
2-Isopropyl-4-ethylthio-6-methylpyrimidine (Peak 2)	2.4	
Diazinon (Peak 3)	3.6	
Isodiazinon (Peak 4)	6.0	
2-Isopropyl-6-methyl pyrimidine-4-thione (Peak 5)	6.8	
2-Isopropyl-6-methyl pyrimid-4-one (Peak 6)		22.4
TEPP (Peak 7)		29.6

components was best achieved using 3% 1,4-dioxan in cyclohexane, and of the more polar components using 15% 1,4-dioxan in cyclohexane. An alternative solvent system that was found useful for separating the less polar compounds was cyclohexane–chloroform (60:40, v/v). Greatest detection sensitivity for the pyrimidine components was achieved at 260 nm. The identity and retention times of the peaks are given in Table 1. Identification of the components of the mixture was achieved for 2-isopropyl-6-methylpyrimid-4-one, 2-isopropyl-6-methyl-pyrimidine-4-thione, and 2-isopropyl-4-ethyl-thio-6-methylpyrimidine by isolation and crystallization of the compounds following multiple semipreparative separations. The compounds showed melting and mixed melting points, i.r. spectrum and retention times identical with those of the corresponding compound prepared by the literature method. TEPP and sulpho-TEPP showed identical i.r. spectra to peaks 6 and 1 respectively.

Table 2 gives characteristics of the u.v. spectra of the components of the diazinon mixture which may be used for their quantitative determination following determination of the peak areas.

Characterization of peak 4

Initial attempts, aimed at the purification by vacuum distillation of the compound giving rise to the fourth peak (Fig. 1) seen in the '90% diazinon' HPLC pattern, resulted in the decomposition of this compound. A crystalline product was obtained which was characterized on the basis of melting point, i.r. and NMR spectrum as 2-isopropyl-4-ethylthio-6-methylpyrimidine. HPLC of the products also revealed a peak with an identical retention time to that of sulpho-TEPP. The instability of the compound was also apparent from the fact that gas chromatography on OV-17 at a temperature of 180° showed only two peaks corresponding to sulpho-TEPP and 2-isopropyl-4-ethylthio-6-methyl-pyrimidine.

Further attempts at characterization of the compound were made using material resulting from multiple semipreparative separations on Partisil followed by rechromatography until only a single peak was seen. Solvent was removed by evaporation *in vacuo* at a temperature not greater than 40°C.

The compound was a pale yellow oil. The i.r. spectrum showed ν_{max} (liquid film) 2960, 2910, 2830 (N—H stretch) and 1290 cm^{-1} (P=O stretch). The latter assignment is based on the absence of this peak in the spectrum of diazinon but its presence in that of '*S*-ethyl diazinon' [14]. The NMR spectrum in $CDCl_3$ showed τ values of 1.30 (6H, doublet), 1.35 (6H, triplet), 2.47 (3H, singlet), 3.14 (1H, septet). 4.26 (4H, multiplet), and 7.42 (1H, singlet); cf. diazinon 1.30 (6H, doublet, CH_3 of 2-isopropyl), 1.35 (6H, triplet, CH_3 of ethoxy), 2.45 (3H, singlet, 6-methyl), 3.10 (1H, septet, CH of isopropyl), 4.33 (4H, multiplet, CH_2 of ethoxy), 6.63 (1H, singlet, H of 5 position of pyrimidine ring).

The field desorption mass spectrum of the compound showed a molecular ion of *m/e* 304, as did diazinon. For this reason the compound is designated as 'isodiazinon'.

Details of the ultra violet absorption spectrum of isodiazinon are given in Table 2.

Hydrolysis of isodiazinon

Isodiazinon (0.05 g) was dissolved in 5% methanolic potassium hydroxide (5 ml) and refluxed for 1 hour. The solution was diluted with water (10 ml) adjusted to pH 4.0 with acetic acid and extracted with chloroform (×3). After drying over anhydrous sodium sulphate, the chloroform extract was evaporated to dryness and the residue crystallized from

Table 2. Millimolar extinction coefficients ($10^{-3}\varepsilon$) for diazinon and its impurities at three wavelengths

Compound	$10^{-3}\varepsilon$* 220 nm	260 nm	λ_{max}†
Diazinon	3.65	0.91	4.16(247) 6.69(249)[a] 3.77(246)[b]
Isodiazinon	9.14	7.50	8.59(271) 9.92(293)[a] 9.73(286)[b]
2-Isopropyl-4-ethylthio-6-methylpyrimidine	4.77	4.58	4.67(256), 6.71(278) 6.33(229), 15.59(300)[a] 5.42(256), 7.49(276)[b]
2-Isopropyl-6-methyl pyrimidine-4-thione	5.00	2.29	10.41(291), 7.63(335) 10.38(295)[a] 13.49(299)[b]
2-Isopropyl-6-methyl pyrimid-4-one	5.32	3.80	4.21(270) 7.89(228)[a] 3.49(266)[b]
Sulpho-TEPP	0.105	—	—
TEPP	0.092	—	—

* The solvent was cyclohexane–15% dioxan except for determinations at λ_{max} where, in addition, ε was determined in 0.01 M HCl in ethanol (designated 'a') and 0.01 M NaOH (designated 'b').

† The position of the absorbance maximum in nanometers is given in parentheses.

methanol. The product (0.02 g, m.p. 161–2°) showed identical NMR and i.r. spectra to the spectra of 2-isopropyl-6-methylpyrimidine-4-thione.

The effect of ageing on impure diazinon

The increasing toxicity of some diazinon preparations has been observed by Gaines [12]. The progressive change in composition of 90% diazinon over a period of one year is apparent from Table 3. The table shows the composition of the preparation at the beginning and end of this period as calculated from the data given in Table 2. Using HPLC in both solvent systems, 97% diazinon showed a single peak. This chromatographic behaviour did not change over the period in question. Values of LD_{50} for all of the diazinon preparations and for 'isodiazinon' are given in the table.

The effect on porphyrin accumulation in tissue culture of diazinon and its impurities

The effect of diazinon and its impurities on porphyrin accumulation by chicken embryo liver cells is shown in Table 4. Most compounds were tested both in the presence and absence of insulin. The induction of cytochrome P-450 synthesis in chicken embryo liver cells is minimal in the absence of insulin

Table 3. Effect of ageing on the LD_{50} and composition of '90% diazinon'

Compound	Percentage composition		LD_{50} (mg/kg)
'90% diazinon' (at receival)	Diazinon	94.0	170
	Isodiazinon	0.8	
	2-Isopropyl-4-ethylthio-6-methylpyrimidine	0.06	
	2-Isopropyl-6-methyl pyrimidine-4-thione	0.7	
	2-Isopropyl-6-methyl pyrimid-4-one	2.5	
	Sulpho-TEPP	0.6	
	TEPP	0.4	
'90% diazinon' (stored for 1 year at room temperature under nitrogen)	Diazinon	74.0	30
	Isodiazinon	15.0	
	2-Isopropyl-4-ethylthio-6-methylpyrimidine	3.2	
	2-Isopropyl-6-methyl pyrimidine-4-thione	1.0	
	2-Isopropyl-6-methyl pyrimid-4-one	1.2	
	Sulpho-TEPP	2.5	
	TEPP	2.8	
Diazinon	Pure by HPLC		470
Isodiazinon	Pure by HPLC		65

Table 4. Effect of diazinon and its impurities on porphyrin accumulation in cultures of chicken embryo liver cells

Agent*	Amount of porphyrin† accumulating (pmoles/mg/24 hr) Insulin present	Insulin absent	Major porphyrin accumulating
No agent other than solvent	18	15	Proto
Diazinon	900	730	Copro
Isodiazinon	1700	1600	Copro
Sulpho-TEPP	540	220	Copro and proto
2-Isopropyl-4-ethylthio-6-methylpyrimidine	190	75	Copro and proto
2-Isopropyl-6-methyl pyrimidine-4-thione	180		Copro and proto
2-Isopropyl-6-methyl pyrimid-4-one	55		Proto
TEPP	25	20	Proto
2-Allyl-2-isopropyl acetamide (AIA)	800	250	Proto

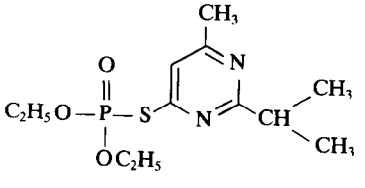
* The final concentration of all agents was 20 μg/ml in the cultures except for AIA which was 100 μg/ml.

† Values are means for 6 culture dishes. The spread of values was in all cases less than 10% of the mean.

[15]. Diazinon, isodiazinon and sulpho-TEPP caused a marked increase in porphyrin accumulation. The absence of insulin from the medium only marginally decreased the level of porphyrin accumulation caused by diazinon and isodiazinon. In contrast, the other impurities, including the very toxic tetraethylpyrophosphate, has little effect on porphyrin biosynthesis.

DISCUSSION

The water catalyzed conversion of diazinon to 2-isopropyl-4-ethylthio-6-methylpyrimidine, tetraethylpyrophosphate and sulphotetraethylpyrophosphate, as well as other products, has been reported by Margot and Gysin [7]. The role of the potent acetylcholinesterase inhibitors, TEPP and sulpho-TEPP, in the phenomenon of increasing acute toxicity of some diazinon preparations has been commented on by Meier *et al.* [16]. This report indicates that the previously unreported impurity, isodiazinon, may also participate in this phenomenon. Isodiazinon is unstable, especially when heated, and is no doubt an intermediate in the formation of 2-isopropyl-4-ethylthio-6-methylpyrimidine which is also a major impurity in the diazinon preparations studied in the present work. The i.r. spectrum of isodiazinon differs from that of diazinon in that the absorbance band at 1290 cm^{-1} is absent in diazinon. This band is also seen in *S*-ethyldiazinon [14], suggesting the presence of the P=O grouping in isodiazinon. The NMR spectrum of isodiazinon differs substantially from that of diazinon only in the position of the proton attached to the 5-position of the pyrimidine ring which is shifted down field by 0.8 ppm. This suggests the presence of a substituent on the ring system with different magnetic properties to those of diazinon itself. The hydrolysis of isodiazinon to 2-isopropyl-6-methylpyrimidine-4-thione confirms that the different substituent has sulphur directly attached to the pyrimidine ring. This information, together with the NMR and mass spectra, indicates that isodiazinon is 2-isopropyl-6-methyl-4-*S*-pyrimidinyl diethylthiophosphate (I) cf. diazinon (II).



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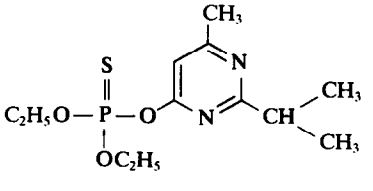

II

The importance of the thiol–thiono ester rearrangement in the production of highly toxic thiol esters has been recognised in a number of organophosphorus compounds. The presence of thiol ester isomers in malathion preparations was a cause of widespread poisoning by this insecticide in Pakistan [17]. The thiol esters are more powerful alkylating and phosphorylating agents than the thiono esters and hence are more potent inhibitors of acetylcholinesterase than their thiono isomers. The LD_{50} of isodiazinon (Table 3) indicates that its presence in impure diazinon preparations may be one reason for the higher toxicity of these preparations than of pure diazinon.

The presence of isodiazinon in the '90% diazinon' preparation provides the most satisfactory explanation for the earlier observation that dermal application of this diazinon preparation gave rise to a disturbance of porphyrin metabolism in rats. In tissue culture this compound is almost twice as effective in causing porphyrin accumulation as is diazinon. Organophosphate thiono esters are known to have a destructive effect on cytochrome P-450. de Matteis [18] has shown that parathion donates its sulphur atom to the enzyme during oxidative desulphurization. Although this observation is in agreement with the fact that all of the compounds found to cause marked accumulation of porphyrins in tissue culture contain labile sulphur, it does not provide a complete explanation for porphyrin accumulation. Two observations point to the involvement of other factors. Firstly, the absence of insulin does not appreciably affect porphyrin accumulation due to either diazinon or isodiazinon in culture. Porphyrin accumulation due to sulpho-TEPP is, however, significantly lower in the absence of insulin. As insulin appears to be required for the induction of cytochrome P-450 synthesis [15], this suggests that the two pyrimidine organophosphorous compounds may act at an earlier point in the porphyrin biosynthesis pathway than the synthesis of the cytochrome P-450 holoenzyme. Secondly, the major porphyrin which accumulates in cultures treated with diazinon and isodiazinon is coproporphyrin. Compounds which interfere with cytochrome P-450 synthesis by inhibiting ferrochelatase or by rendering iron unavailable to this enzyme cause the accumulation of protoporphyrin IX in the cultures. An example is 2-allyl-2-isopropylacetamide [19]. The accumulation of coproporphyrin in the cultures again suggests that diazinon and isodiazinon may interfere with porphyrin biosynthesis at an earlier point in the pathway. It is, however, unlikely that the point of interference is the enzyme uroporphyrinogen decarboxylase. If this were the case, uroporphyrin rather than coproporphyrin would be expected to accumulate. The greater activity of isodiazinon suggests that interference with porphyrin biosynthesis may be linked to the more active phosphorylating and alkylating properties of the organophosphate thiol ester isomers.

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