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IMPROVED QUANTITATIVE GAS CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF SMALL AMOUNTS OF CHLORINATED HYDROCARBON PESTICIDES IN HUMAN PLASMA

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SUMMARY

A method suitable for multiple analyses of chlorinated pesticides in human plasma in epidemiological studies has been developed. Pretreatment with formic acid, the use of an internal standard and verification of the peaks by means of mass fragmentography are utilized. The method is sensitive and reliable, and detects small differences in a particular person with changes in the extent of exposure to pesticides.

INTRODUCTION

The determination of nanogram amounts of lindane and DDT analogues in human blood still presents analytical problems, particularly with regard to epidemiological studies.

Of the methods described in the literature[1-6] for analyzing pesticides in human blood and plasma, none was suitable for our purposes. Our requirements are that the method should be adaptable to the analysis of small amounts of lindane and DDT in the blood of people occupationally exposed to lindane as compared to the general population. It should also be able to detect small differences due to changes in the extent of exposure of a particular person.

The purpose of the present investigation was to develop a gas chromatographic method fulfilling the above criteria. In addition, a mass fragmentographic technique for the verification and quantitation of DDE in plasma has been developed. This paper describes the experimental procedures; the results of epidemiological and pharmacological studies will be presented elsewhere.

MATERIAL AND METHODS

Blood-sampling

Samples of 10–20 ml of blood were withdrawn into heparinized tubes. Aliquots of 2 ml of plasma were stored immediately at —20°. For the *in vitro* recovery experiments, pooled blood-bank plasma stored at +4° was used.

Extraction procedure

Heptachlorepoxide in hexane was added as an internal standard to the plasma sample (1 ml) in a 10-ml glass tube to give the same concentration in the extraction solvent as in the standard solutions. Anhydrous sodium sulphate (0.5 g) and 1 ml of 97 % formic acid were added and mixed on a Vibri-mixer for about 1 min. Hexane (1 ml) was added and the resulting solution mixed for 5 min with a flask shaker. After centrifugation at 3000 r.p.m. for 5 min, the organic layer was transferred into a 10-ml glass tube and cleaned by shaking with 1 ml of 5 % potassium carbonate solution. A distinct separation of the phases was always obtained, which was not the case when extraction with hexane in the absence of formic acid was performed. After further centrifugation, about 5 μl of the upper layer were injected into the gas chromatograph. In samples with unusually high amounts of pesticides, 0.5 ml of plasma was extracted with 2 or 3 ml of hexane. Alternatively, the hexane phase from the 1:1 extraction was diluted with hexane containing an appropriate amount of internal standard.

Reagents

All chemicals and solvents were of analytical-reagent grade and were used without further purification.

Reference substances (*p,p'*-DDE, *o,p'*-DDT and *p,p'*-DDT) were obtained from Analytical Standards Co., U.S.A., and lindane from Cela, G.F.R. Other reagents were obtained from Merck, G.F.R. Heptachlorepoxide was kindly supplied by Dr. G. WESTÖÖ of The National Food Administration, Sweden.

Cleaning of glassware

All glassware was washed with a detergent (Deconex 11, Borer Chemie, Switzerland), allowed to stand overnight in a mixture of sulphuric acid–nitric acid (4:1) and rinsed consecutively with distilled water, acetone and hexane.

Gas chromatography

The gas chromatograph used was a Varian Aerograph 1400 equipped with a tritium foil electron capture detector. The column was operated isothermally at 205°. The detector and injection port were maintained at 230°.

The analyses were carried out on a 6-ft. (2 mm I.D.) silanized glass column packed with 3 % OV-17 on Gas-Chrom Q (100–120 mesh) and cross-checking was performed on a 6-ft. (2 mm I.D.) glass column filled with 14 % DC 200–QF-1 (2:8) on Gas-Chrom Q (80–100 mesh). The carrier gas (nitrogen) flow-rate was 50 ml/min, which for *p,p'*-DDT gave a retention time of 15–20 min on both columns.

Mass fragmentography instrumentation

An LKB 9000 combined gas chromatograph–mass spectrometer was used. This instrument was equipped with the recently described[7] multiple ion detector (MID) and connected to a PDP 12 computer (Digital Equipment Corp.) over an interface developed at the Department of Toxicology. The components were separated on a silanized glass column packed with 5 % SE-52 on Chromosorb W (60–80 mesh). The temperatures of the oven and injection port were kept at 200° and 240°, respectively. The carrier gas (helium) flow-rate was 20 ml/min. The ionizing potential was

50 eV and the trap current 60 μA. The temperature of the ion source was 270°. The separator was maintained at 250°. The multiplier voltage was set at 3.7 kV.

For quantitative mass spectrometry, the MID served as an ion-specific detector for the gas chromatograph. Three selected ions were brought into focus by use of three different accelerating voltages. By switching between these potentials, the intensities of the ions were continuously monitored on three separate channels on a UV recorder.

Two of the isotopic molecular ions, *m/e* 316 and *m/e* 318, were chosen for *p,p'*-DDE and *m/e* 353 for heptachlorepoxide (internal standard). Owing to the differences in the intensities of the focussed ions, the gain of the three channels set to detect *m/e* 316, 318 and 353 was adjusted to give the relative sensitivities 1:1:2, respectively.

The same samples analyzed on the gas chromatograph were analyzed on the mass spectrometer. The hexane phase was evaporated almost to dryness and 20–30 μl of hexane were added. Injections of 2–3 μl were monitored on a UV recorder when eluted from the chromatograph.

Standard curves

Five standard solutions of pesticides in hexane were prepared containing different concentrations of lindane (0.5–4.0 ng/ml), *p,p'*-DDE (2.5–20.0 ng/ml), *o,p'*-DDT and *p,p'*-DDT (5–40 ng/ml) but the same concentration of heptachlorepoxide (10 ng/ml). A 5-μl volume of each standard solution was injected. For the mass fragmentographic studies of *p,p'*-DDE, five standard solutions were prepared containing only *p,p'*-DDE (0.4–2 μg/ml) and heptachlorepoxide (1 μg/ml). The ratio of the peak height of the pesticide to the peak height of the internal standard was calculated and plotted against the respective pesticide concentration. A new standard curve was prepared each day.

RESULTS AND DISCUSSION

Internal standard

The use of an internal standard offers several advantages. If added at the beginning of the procedure, possible losses during extraction are kept under control. This procedure compensates for the variation of the response of the electron capture detector during various days and times of the day. If an internal standard technique is used, it also compensates for the differences in injection volume and evaporation during storage. Heptachlorepoxide has previously been used as an internal standard by NORÉN AND WESTÖÖ[8]. Under our conditions it was found to be suitable as its retention time did not coincide with those of other pesticides and/or impurities. In addition, it is not found in Swedish human plasma or in Swedish foods[9,10]. The use of an internal standard was not taken advantage of in the methods reported earlier[1–6].

Standard curves

Standard curves for lindane, *p,p'*-DDE, *o,p'*-DDT and *p,p'*-DDT are shown in Fig. 1. The slopes of the curves were found to be approximately the same from day to day.

Formic acid pretreatment

DALE *et al.*[5] showed that, in blood from nine persons, pretreatment of the serum with formic acid gave higher yields for DDT than a simple hexane extraction. Feeding rats with ^{14}C-labelled DDT gave a recovery of 98 %.

In our own experience, pretreatment of the sample with formic acid gave higher yields not only of DDT but also of lindane and *p,p'*-DDE (Table I). The reason for this action of formic acid is at present unknown but could possibly be due to the liberation of the chlorinated hydrocarbons from binding sites on the plasma proteins by denaturation of the proteins.

BONDERMAN *et al.*[11] tentatively suggested that *p,p'*-DDE might be bound to lipoproteins. CARLSSON AND KOLMODIN-HEDMAN[12] reported changes in certain

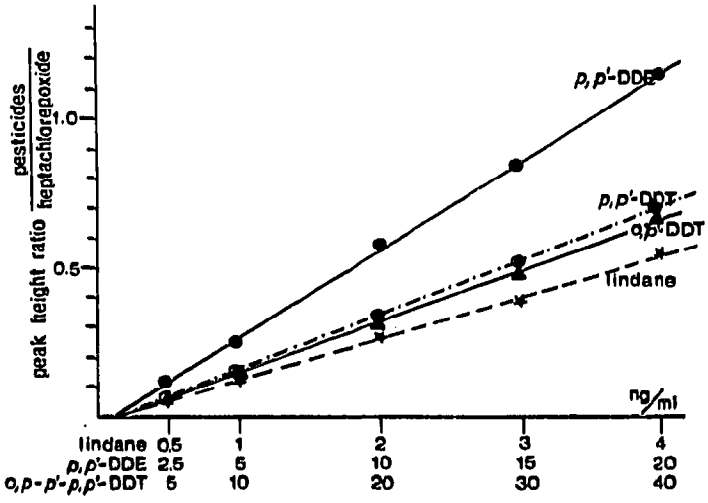

Fig. 1. Standard curves for lindane, *p,p'*-DDE, *o,p'*-DDT and *p,p'*-DDT.

TABLE I

PLASMA LEVELS OF LINDANE, *p,p'*-DDE AND *p,p'*-DDT

Simple hexane extraction compared with hexane extraction with formic acid pretreatment. Pesticide concentrations expressed in ng/ml.

	Hexane + formic acid			*Hexane*		
	Lindane	*DDE*	*DDT*	*Lindane*	*DDE*	*DDT*
	6.2	17	<5	3.8	4.0	0
	7.0	35	<5	1.8	0	0
	9.0	33	<5	5.5	9	0
	9.0	15	<5	6.5	7	0
	3.8	12	<5	5.0	4	0
	6.6	18	<5	9.7	13	0
	12.8	32	<5	7.0	8	0
	9.6	<2.5	0	5.6	3	0
	2.0	14	<5	3.2	10	0
	14.2	14	<5	16.4	14	0
Mean	8.0	19		6.5	7	
S.D.	±3.7	±11		±4.1	±5	
S.E.	±1.2	±3		±1.3	±1.5	

lipoproteins in workers exposed to lindane. Studies are in progress to test the pesticide content of the various ultracentrifuged fractions of plasma.

Concentration step

In our work, a concentration step for the organic phase was unnecessary. This is otherwise a source of loss of pesticide owing to differences in evaporation. BURKE *et al.*[13] reported serious losses when the volumes were reduced to less than 0.5 ml. The evaporation step is especially critical for lindane, which is much more volatile than DDT and its analogues. Should a concentration step be necessary (*i.e.*, low sensitivity of the gas chromatograph as well as low levels of the pesticides), the evaporation method described by STRÖMBERG AND WIDMARK[14] gives the best recovery.

Sensitivity

The minimum detectable absolute amounts of the pesticides in hexane were found to be as follows: lindane 0.0004 ng, *p,p'*-DDE 0.002 ng and *p,p'*-DDT 0.005 ng. For our samples, the lowest practical amounts calculated per millilitre of plasma were as follows: lindane 0.3 ng, *p,p'*-DDE 1 ng and *p,p'*-DDT 3 ng, *i.e.*, 0.3 p.p.b., 1 p.p.b. and 3 p.p.b., respectively.

Reproducibility

Heptachlorepoxide was found to give a reproducible recovery of about 100 %. The reproducibility of a series of analyses of blood-bank plasma is given in Table II. Ten (5 + 5) samples from the same batch were analyzed on two different days.

TABLE II

LEVELS OF LINDANE, *p,p'*-DDE AND *p,p'*-DDT IN POOLED BLOOD-BANK PLASMA

Pesticide concentrations expressed in ng/ml. Ten samples from the same batch, analyzed on two different days.

Number	*Lindane*	*p,p'-DDE*	*p,p'-DDT*
1	0	10	<3
2	0	10	<3
3	0	9	<3
4	0	10	<3
5	0	10	<3
6	0	11	<3
7	0	10	<3
8	0	10	<3
9	0	11	<3
10	0	10	<3

Mean value for DDE = 10 ± 0.6 (S.D.); S.E. = ± 0.2

The mean value of the ten observations was 10 ng/ml, with S.D. ± 0.6 and S.E. ± 0.2. Blood-bank plasma did not contain lindane, and the *p,p'*-DDT level is less than 3 ng/ml (lowest detection limit). Fig. 2 shows a gas chromatogram from a person occupationally exposed to lindane.

The reproducibility of duplicates of human plasma analyzed on different days

is shown in Table III. The plasma samples are from persons occupationally exposed to lindane, as compared to controls. Table III also gives a comparison between duplicate values for DDE and DDT. The difference between the analysis of "pairs" was tested. The differences are not statistically[15] significant from zero for lindane, DDE and DDT, *i.e.*, there is good reproducibility.

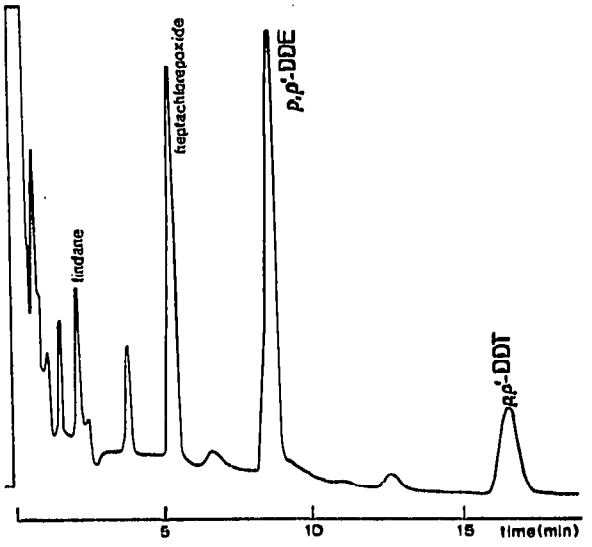

Fig. 2. Gas chromatogram from a person exposed to lindane.

TABLE III

ANALYSIS OF PLASMA FROM WORKERS OCCUPATIONALLY EXPOSED TO LINDANE AS COMPARED TO CONTROLS

Duplicate analyses on two different occasions. Pesticide concentrations expressed in ng/ml.

Initials	*Lindane*		*p,p'-DDE*		*p,p'-DDT*	
	1	*2*	*1*	*2*	*1*	*2*
Exposed						
L.W.	<0.5	<0.5	29	25	9	5
O.B.	22.1	22.0	14	16	[b]	[b]
S.H.	11.3	11.8	11	12	[b]	[b]
E.P.	High	49.6[a]	91	92[a]	3	3[a]
I.H.	0	0	100	95	19	19
K.O.	9.0	6.6	54	60	28	24
B.A.	9.6	9.6	10	15	[b]	[b]
B.R.	1.2	1.1	9	15	4	4
A.S.	High	64.5[a]	11	11[a]	[b]	a,b
A.G.	6.6	4.5	43	45	27	13
P.-O.N.	2.1	4.3	11	19	4	5
Non-exposed						
B.K.H.	0	0	14	14	4	4
N.H.	0.5	0.9	9	10	4	5
C.E.H.	0	0.5	0.8	4	4	3
E.L.	0.8	0.5	11	7	4	3
P.W.	0.5	0	18	11	5	4
D.D.	0.6	0.9	23	22	5	5
C.A.L.	0.5	0.5	36	35	11	9
A.L.	0	0	22	17	9	6

[a] Higher extraction volume.
[b] Not detectable by extraction 1:4 or 1:6.

Effect of using different volumes for the extraction procedure

For samples with a high level of lindane, a 1:1 extraction gave a ratio of pesticide/internal standard that was too high to be used with the normal standard curve. In this event, either a smaller amount of plasma was used or the organic phase from the 1:1 extraction was diluted with hexane containing an appropriate amount of internal standard. The result of the two extraction procedures gave approximately the same values (Table IV) in the ranges reported.

TABLE IV

PLASMA LEVELS OF LINDANE, *p,p'*-DDE AND *p,p'*-DDT

Comparison of various extraction procedures. Pesticide concentrations expressed in ng/ml.

Initials	*Lindane*		*p,p'-DDE*		*p,p'-DDT*	
	1	*2*	*1*	*2*	*1*	*2*
R.E.	3.9[a]	4[b]	21[a]	23[b]	3[a]	3[b]
A.G.	0.5[a]	0.5[b]	31[a]	26[b]	5[a]	—
O.B.	High[c]	22.0[b]	12[c]	16[b]	6[c]	3[b]
E.P.	High[b]	49.6[g]	91[b]	92[g]	3[b]	3[g]
A.N.	6.1[c]	6.0[d]	29[c]	29[d]	37[c]	39[d]
J.A.	2.6[c]	2.7[d]	High[c]	30[d]	15[c]	15[d]
S.P.	0	0	High[f]	143[e]	42[f]	42[e]
E.N.	0	0	High[f]	120[e]	34[f]	30[e]
K.J.	0	0	High[f]	139[e]	34[f]	30[e]
R.B.	0	0	High[f]	114[e]	21[f]	30[e]

[a] 1:1 extraction, dilution of hexane phase 1:4.
[b] 1:4 extraction.
[c] 1:1 extraction.
[d] 1:1 extraction, dilution of hexane phase 1:2.
[e] 1:2 extraction, dilution of hexane phase 1:2.
[f] 1:2 extraction.
[g] 1:8 extraction.

Polychlorinated biphenyls (PCB)

PCB are often known to interfere with the analyses of DDT in fat samples[16]. A method for determining PCB compounds, DDT and its analogues in food has been described by WESTÖÖ AND NORÉN[17]. The presence of PCB in Swedish food, fish and water has been reported[9,10,18,19].

In the present investigation, the samples were cross-checked on two columns, OV-17 and DC 200–QF-1. To check for a possible PCB peak under the DDT area, the hexane phase was shaken with potassium hydroxide (5 % solution in ethanol). DDD and DDT were then converted to DDE, when a possible PCB peak would be unmasked. No peaks interfering with the calculation of DDT were obtained. The retention time of lindane is too short to be disturbed by PCB. For the identification of *p,p'*-DDE, see the following section on mass fragmentography.

Mass fragmentography

The technique of mass fragmentography has been described in several publications[7,20,21] and recently it has also been applied to quantitative problems[22–24].

The quantitative measurement of *p,p'*-DDE by mass fragmentography was

performed in order to confirm the results obtained with the electron capture detector.

The mass fragmentograms of the reference substances and of an extract added with heptachlorepoxide are shown in Fig. 3. Fig. 3 shows that the plasma sample contains *p,p'*-DDE; this is confirmed by both the retention time and the relative intensity of the two ions of *m/e* 316 and 318. The mass fragmentographic method was used on 30 plasma samples in which the concentration of *p,p'*-DDE was found to be in the range 6–60 ng/ml. Comparisons with the results obtained by electron capture detection gave very good agreement between the two methods, $r = 0.99$ (Fig. 4). The specificity of this technique eliminated the uncertainty of compounds being hidden under high *p,p'*-DDE peaks.

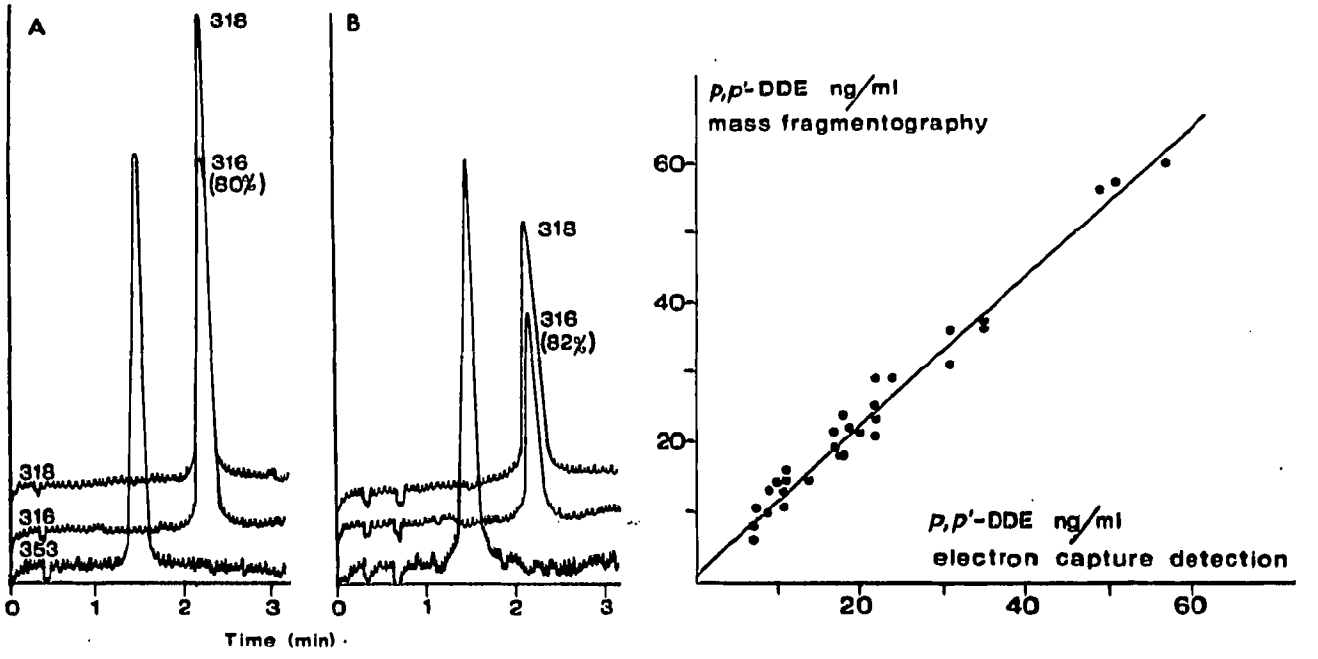

Fig. 3. Mass fragmentographic recording of (A) reference substances, heptachlorepoxide (*m/e* 353) and *p,p'*-DDE (*m/e* 316, 318); (B) extract from plasma with added heptachlorepoxide. The extract contained 20 ng of *p,p'*-DDE per millilitre.

Fig. 4. Relationship between the concentration of *p,p'*-DDE in plasma samples analyzed with electron capture and mass fragmentographic detection techniques.

Mass fragmentographic quantitation of *p,p'*-DDT and lindane has presented some problems, owing to the partial decomposition of DDT and losses of lindane at the necessary evaporation step. Further experiments are in progress.

Interfering peaks

Peaks with the same retention times as that of aldrin and the double retention time of DDT were sometimes recorded. Some batches of formic acid, even from the same company and with the same declared purity, interfered in the calculation of lindane. The batch of formic acid should therefore always be pre-tested.

Plasma levels as an index of exposure

NACHMAN *et al.*[3] used a modified DALE *et al.*[1] method and compared the levels of *p,p'*-DDT and *p,p'*-DDE in whole blood and plasma in 50 samples. In 88 % of the

samples the plasma value was double that of whole blood (within 3 p.p.b.). If the levels are expected to be low, plasma is therefore preferable. Moreover, the chromatograms show less impurities.

The levels of lindane in plasma (Table II) seem to correspond to the rated degree of exposure to lindane (to be published). MILBY *et al.*[25] reported a correlation between blood levels of lindane and acute exposure. For *p,p'*-DDE levels a correlation with DDT is observed[1,2], and for *p,p'*-DDT it depends on a recent exposure[2,26].

CONCLUSIONS

(1) Lindane levels in human plasma seemed to reflect the extent of exposure.

(2) Pretreatment of plasma with formic acid before extraction gave higher yields of lindane, *p,p'*-DDE and *p,p'*-DDT.

(3) Heptachlorepoxide proved to be a suitable internal standard.

(4) Recovery experiments and duplicate analyses showed good reliability in the method described.

(5) The quantitation of *p,p'*-DDE in plasma by means of the electron capture detector has been confirmed by mass fragmentography.

(6) Peaks in plasma at the retention times of *p,p'*-DDE and *p,p'*-DDT due to PCB were negligible in the samples analyzed.

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