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ISOLATION AND DETERMINATION OF CHLORINATED ORGANIC PESTICIDES BY THIN-LAYER CHROMATOGRAPHY AND THE APPLICATION TO TOXICOLOGICAL ANALYSIS

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SUMMARY

A sensitive method for the isolation and clean-up of chlorinated organic pesticides from tissues and biological materials is discussed. R_F values of the pesticides in 26 solvent systems, and colours of the spots given with various chromogenic reagents, are reported. The limits of detection, and densitometric measurements, are also discussed. The average recovery has been found to be 94%. The distribution of endrin in different autopsy tissues from five different victims has been studied by the proposed technique.

INTRODUCTION

The ease of availability and widespread use of chlorinated organic pesticides for agriculture has resulted in several accidental, suicidal or homicidal deaths during the last few years. The toxicity of these compounds had been noted[1–4]. Different techniques have been devised for the analysis of chlorinated organic pesticides but they are confined primarily to plant products, soils, food grains and milk products, etc. However, some studies have been made of the isolation, detection and determination of these insecticides from autopsy tissues and other body fluids. Chiba[5] described a method for extraction of these pesticides from stomach contents using benzene as extraction solvent. Partition in acetonitrile–light petroleum was used by Hamence *et al.*[6] for the extraction and purification steps. Dale *et al.*[7] used *n*-hexane for the extraction of pesticides from blood. Some other solvents such as 95% acetonitrile[8], *n*-hexane on an alumina column[9] and hexane–acetone (9:1) (ref. 10) have also been used for the isolation and clean-up of these pesticides from blood and other biological materials.

The identification and detection of chlorinated pesticides was first reported by Mitchell[11] who employed paper chromatography. Paper chromatography using a variety of solvent systems and chromogenic reagents has also been attempted[12–14]. Thin-layer chromatography (TLC) with a series of solvent systems and different chromogenic reagents has been used on plates coated with single or mixed gels[15–21], and combinations of mobile solvent systems such as cyclohexane–acetone[22], cyclo-

hexane–chloroform[23], light petroleum–carbon tetrachloride[24] and hexane–acetone[25] have been used on silica gel G plates. The separation of pesticides on gel plates impregnated with $AgNO_3$ (refs. 26–28) and $CuSO_4$ (ref. 29) has been reported. Attempts have also been made to develop sensitive chromogenic reagents such as 0.5% diphenylamine in ethanol[30], ammoniacal $AgNO_3$ in acetone[31], *o*-toluidine in acetone[32–33], 0.1% diphenylamine in acetone[34] and *o*-dianisidine in acetone[35] for the location of the pesticides. Luckens[36], Noren and Westoo[37] and Coutselinis and Dimopoulos[38] reported methods for the estimation of these pesticides in biological materials. Petrowitg and Wagner[39] used densitometry for quantitative measurements of γ-BHC on TLC plates.

We have previously reported[40–44] the detection and quantitative estimation of endrin and BHC on TLC plates after extraction from autopsy tissues. However, no systematic work has been reported on the isolation, detection and estimation of chlorinated pesticides in autopsy tissues in cases of poisoning. In this paper we report the isolation and clean-up of chlorinated organic pesticides from autopsy tissues and biological fluids and their identification on TLC plates with sensitive chromogenic reagents. Densitometric measurements have been made in order to study the distribution of the poisons in body tissues.

EXPERIMENTAL

Extraction of pesticides from tissues

Isolation. 20 g of tissue were macerated into a fine slurry and transferred to a conical flask fitted with an air condenser. 30 g of anhydrous sodium sulphate and 50 ml of acetone were then added. The flask was placed on boiling water-bath for 30 min, then cooled and the solution was filtered. The procedure was repeated twice, using 25 ml of acetone each time. The filtered acetone layers were combined and transferred to a separating funnel containing 100 ml of distilled water and 20 ml of a saturated solution of Na_2SO_4. The resulting solution was extracted three times with 20, 10 and 10 ml of chloroform, shaking gently for 5 min after each addition.

Clean-up procedure. The separated chloroform layers were combined and transferred to another separating funnel. 20 ml of a 5% aqueous solution of KOH were added and the mixture was shaken for 1 min. The chloroform layer was separated and again shaken for 1 min with 20 ml of 5% KOH. The separated chloroform layer was washed five times with 20-ml portions of distilled water. The washed chloroform layer was dried by passing it through 2 g of anhydrous Na_2SO_4 and then concentrated to 2 ml on a warm water-bath. The remaining solvent was evaporated to dryness in a current of dry air. The residue was dissolved in 1 ml of acetone, and an aliquot portion (10 μl) of the resulting solution was spotted on to a TLC plate.

Extraction of pesticides from stomach washings and urine

20 ml of liquid were filtered into a separating funnel. The residue was washed twice with 10-ml portions of distilled water. The washings were combined with the filtrate and transferred to a separating funnel. 10 ml of a saturated solution of Na_2SO_4 were then added and the funnel was shaken for 5 min with 20 ml of *n*-hexane. The separated *n*-hexane layer was filtered through 2 g of anhydrous Na_2SO_4, and the aqueous layer was extracted twice with 10-ml portions of *n*-hexane. The filtered *n*-

hexane layers were combined, concentrated to 2 ml on a water-bath and dried in a current of dry air. The residue was dissolved in 1 ml of acetone, and an aliquot portion of the resulting solution was spotted on to a TLC plate.

Extraction of pesticides from blood

10 ml of blood were placed in a conical flask containing 20 ml of dilute H_2SO_4. 10 ml of 10% sodium tungstate were added and the flask was maintained at room temperature for 10 min. The solution was filtered and the filtrate was transferred to a separating funnel. The residue was washed twice with 10-ml portions of distilled water, and the washings were combined with the filtrate and then extracted as described for stomach washings.

Apparatus and chemicals

Standard analytical grade reagents and TLC apparatus were used. Standard solutions of pesticides were prepared by dissolving the recrystallized technical grade pesticides in redistilled ethanol.

The following solvent systems were used for the separation of chlorinated organic pesticides:

(I) *n*-Hexane
(II) *n*-Hexane–acetone (19:1)
(III) *n*-Hexane–acetone (9:1)
(IV) *n*-Hexane saturated with acetonitrile (40:1)
(V) *n*-Hexane saturated with dimethylformamide (80:1)
(VI) *n*-Hexane–liquid paraffin (39:1)
(VII) *n*-Hexane–chloroform (49:1)
(VIII) *n*-Hexane–carbontetrachloride (97:3)
(IX) *n*-Hexane–butanone (39:1)
(X) *n*-Hexane–light petroleum (4:1)
(XI) *n*-Hexane–diethyl ether (19:1)
(XII) *n*-Hexane–dioxan (49:1)
(XIII) Cyclohexane
(XIV) Cyclohexane–acetone (99:1)
(XV) Cyclohexane–acetone–ethanol (95:1:4)
(XVI) Cyclohexane–dimethylformamide (19:1)
(XVII) Cyclohexane–liquid paraffin (9:1)
(XVIII) Cyclohexane–liquid paraffin (4:1)
(XIX) Cyclohexane–light petroleum (3:2)
(XX) Cyclohexane–benzene (49:1)
(XXI) Cyclohexane–methylene dichloride (9:1)
(XXII) Cyclohexane–cyclohexanone (49:1)
(XXIII) Light petroleum
(XXIV) Light petroleum–liquid paraffin (9:1)
(XXV) Light petroleum–chloroform (97:3)
(XXVI) Light petroleum–acetic acid (19:1)

TABLE I

R_F 100 VALUES OBTAINED IN DIFFERENT SOLVENT SYSTEMS

M = Main spot, S = secondary spot, T = tailing.

No.	Compound	I		II		III		IV		V		VI		VII		VIII		IX		X		XI		XII		XIII	
		M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S
1	BHC	9		35		45		14		9		18		11		15		46		18		60		35		27	
		25		48		55		32		18		25		31		30		82		29		72		50		33	
		40		52		60		46		34		34		51		50		92		48		80		66		63	
		48		62		70		64		52		54		85		65		96		65		91		82		66	
		65		78		80		78		75		70		90		80		—		88		—		—		90	
		—		—		—		—		—		—		—		—		—		—		—		—		—	
		—		—		—		—		—		—		—		—		—		—		—		—		—	
2	Lindane	40		52		60		46		18		34		31		30		92		48		80		66		66	
3	Aldrin	82	17	87	47	90	50	85	51	90	13	85	18	92	17	86	15	98	50	85	26	96	70	90	66	86	46
											78				81												
4	Dieldrin	17		47		50		51		18	13	18		19		25		50		26		70		60		46	
5	Endrin	22	2	50	8	58	9	60	18	24	13	25	3	28		29		60	14	33		78		64		62	13
							66																				
6	DDT	78		85		79		81		80		83		60		77		90		78		94		80		84	
		82		—		87		—		—		86		—		88		—		—		—		—		—	
7	Thiodan	5		5		8		30		5		10		15		13		15		8		22		24		9	37
		16		30		65	40	39		17		27		24		28		80		20		55		72		22	51
		30		60		86		80		36		34		36		80		94		46		92		—		87	
8	Heptachlor	83	18	90	80	92	12	89	49	92	25	88	58	84	22	85	43	95	93	86	33	95	89	84	60	82	11
			60		—		78		79		38		—		46		93		—		68		—		—		49
			—		—		84		—		68		—		67		—		—		—		—		—		84
9	Chlordan	7		50T		12		22		25		36		7		43		20		12		18		69T		11	
		18		80		50		36		38		58		22		53		65		22		60		75		18	
		27		90		62		49		54		62		23		67		84T		33		89		84		49	
		35		—		78		79		68		75		46		75		—		68		95		—		82	
		45		—		84		89		92		88		67		85		—		86		—		—		84	
		60		—		92		—		—		—		84		93		—		—		—		—		92	
		75		—		—		—		—		—		—		—		—		—		—		—		—	
		83		—		—		—		—		—		—		—		—		—		—		—		—	
		—		—		—		—		—		—		—		—		—		—		—		—		—	
10	Toxaphen	10		76T		84T		12		40T		65T		18		53T		79T		15		70T		74		5	
		20		—		—		44		—		—		60T		—		—		26		—		—		25T	
		35		—		—		62		—		—		—		—		—		60		—		—		82	
		70		—		—		82		—		—		—		—		—		72		—		—		—	
		—		—		—		—		—		—		—		—		—		—		—		—		—	
11	Kelthane	12		40		43		32	83	15	36	18		13	85	17	80	36		14	77	54		30	87	40	
											80				91		88										
12	Dipterex	0		0		5		0		0		0		0		0		0		0		6		0		0	

No.	Compound	XIV		XV		XVI		XVII		XVIII		XIX		XX		XXI		XXII		XXIII		XXIV		XXV		XXVI	
		M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S
1	BHC	23		70		40		14		8		29		55		32		23		39		41		29		45	
		50		80		55		28		17		34		82		50		40		46		60		56		58	
		68		88		79		33		25		52		92		66		75		61		66		66		86	
		82		95		89		46		38		62		98		77		85		74		77		86		95	
		90		—		—		58		44		80		—		88		92		86		83		95		—	
		—		—		—		—		52		—		—		—		—		—		—		—		—	
		—		—		—		—		58		—		—		—		—		—		—		—		—	
2	Lindane	68		88		79		33		38		62		82		66		85		61		66	7	66		86	
																							13				
																							24				
3	Aldrin	96	62	97	86	94	57	83	27	68	10	88	60	96	16	91	8	90	52	92	41	80	24	91	8	93	68
															62										43		
4	Dieldrin	62		86		57		27		10		60		62	16	52		52		41		24		43	8	68	
5	Endrin	63	20	89	24	70		32		16	5	61		70	16	66		68	39	51	21	32	5	56	8	79	
6	DDT	88		92		91		67		46		89		93		86		85		85		67		85		95	
		—		—		—		77		54		—		—		—		—		—		76		—		—	
7	Thiodan	20		26		10		7		4	58	22		11	56	10		11	45	17		11	83	16		84	
		42		60		20		15		13	77	50		28		23		26		28		29		37		94	
		66		90		85		80		39		89		95		90		93		73		44		82		—	
8	Heptachlor	92	80	94	—	94	88	83	50	79	5	88	14	95	—	92	81	89	26	82	53	76	12	84	50	89	26
			—		—		—		70		36		74		—		—		57		69		64		92		80
			—		—		—		—		60		—		—		—		77		—		—		—		
9	Chlordan	40		80T		31T		8		5		14		75T		35		15		17		12		20		26	
		80T		—		70		50		20		30		—		74T		26		53		26		50		37	
		92		—		88		65		26		74T		—		81		45		69		38		70T		80T	
		—		—		94		70		32		88		—		92		57		82		54		—		—	
		—		—		—		77		36		—		—		—		77		92		64		—		—	
		—		—		—		83		48		—		—		—		89		—		76		—		—	
		—		—		—				56		—		—		—		—		—		—		—		—	
		—		—		—				60		—		—		—		—		—		—		—		—	
		—		—		—				79		—		—		—		—		—		—		—		—	
10	Toxaphen	32		83T		88T		65T		28		26		83T		85T		58T		72T		28		85T		9	
		85T		—		—		—		39		78T		—		—		80		—		39				72T	
		—		—		—		—		45		90		—		—		—		—		57		—		—	
		—		—		—		—		51		—		—		—		—		—		69		—		—	
		—		—		—		—		60		—		—		—		—		—		—		—		—	
11	Kelthane	48		78		55	90	28		8	52	41		46		42		45		30		20	76	32	88	58	
											62																
											72																
12	Dipterex	0		18		4		0		0		7		5		0		0		0		0		0		0	

The following chromogenic reagents were used for the detection of the pesticides:

(*a*) 1% $AgNO_3$ in ethanol containing 5 ml of concentrated ammonia followed by irradiation with UV light (366 nm) for 10 min

(*b*) 1% Diphenylamine in ethanol followed by irradiation with UV light (366 nm) for 10 min

(*c*) 1% Diphenylamine in ethanol followed by irradiation with UV light (254 nm) for 30 min

(*d*) 5% Diphenylamine in ethyl methyl ketone followed by irradiation with UV light (254 nm) for 15 min

(*e*) 1% *o*-Toluidine in ethanol followed by irradiation with UV light (366 nm) for 10 min

(*f*) 1% *o*-Toluidine in ethanol followed by irradiation with UV light (254 nm) for 30 min

(*g*) 1% *o*-Toluidine in acetone followed by irradiation with UV light (366 nm) for 10 min

(*h*) 1% N-1-Naphthylethylenediamine dihydrochloride in acetone followed by irradiation with UV light (254 nm) for 15 min

(*i*) 0.025% Rhodamine B in ethanol

(*j*) 0.025% Rhodamine B in ethanol followed by a 10% aqueous solution of sodium carbonate

(*k*) 0.025% Rhodamine B in ethanol followed by a 15% aqueous solution of sodium carbonate

(*l*) 0.025% Rhodamine B in ethanol followed by a 10% aqueous solution of sodium carbonate and the wet plate was exposed to UV light (366 nm) for 15 min

(*m*) 1% Light green in acetone

(*n*) 1% Bromophenol blue in acetone

Ethanol used in the above reagents should be free from chloride ions. Glass distilled ethanol was used in order to avoid precipitation of AgCl in reagent *a*. Before irradiation with UV light, the sprayed plates were left to dry at room temperature for 30 min.

Thin-layer chromatography

Glass plates (200 mm × 150 mm) were coated with a 250-μm layer of silica gel G. The plates were dried in air at room temperature and then activated at 110° for 30 min. The plates were used immediately or stored in a desiccator.

2 μl of each control sample of BHC, lindane, aldrin, dieldrin, endrin, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), thiodan, heptachlor, chlordan, toxaphen, kelthane and dipterex in acetone (1 μg/μl) was spotted on to a TLC plate 20 mm from one end. The plate was developed in a chamber which had been previously saturated for 2 h. When the solvent front reached 10 cm from the point of application, the plate was removed and dried at room temperature. The plate was sprayed with reagent *a*, dried at room temperature for 30 min and exposed to UV light (366 nm) for 10 min. Gray to black spots against a white background were visible on the TLC plates. The R_F value of each spot was measured. 26 solvent systems were tried for the separation of the chlorinated pesticides and the R_F values are recorded in Table I.

Other chromogenic reagents were also used for the detection of the chlorinated

TABLE II

COLOURS OF THE SPOTS OF CHLORINATED ORGANIC PESTICIDES OBTAINED WITH DIFFERENT CHROMOGENIC REAGENTS

No.	*Reagent*	*Spot colour*
1	*a*	Steel grey against a white background within 10 min
2	*b*	Grey, violet and green against a white background within 10 min
3	*c*	Grey, violet and green against a white background within 30 min
4	*d*	Green to violet against a white background within 15 min
5	*e*	Brown against a white background within 10 min
6	*f*	Light brown against a white background within 30 min
7	*g*	Brown against a white background within 10 min
8	*h*	Brown against a white background within 15 min
9	*i*	Blue against a pink background in daylight and fluorescent orange against a pink background in UV light (254 nm)
10	*j*	Red against a pink background in daylight (same pattern in UV light)
11	*k*	Faint red against a pink background in daylight (same pattern in UV light)
12	*l*	White against a pink background in daylight and blue against a pink background in UV light
13	*m*	Pale yellow against a yellowish green background
14	*n*	Blue against a yellowish green background

organic pesticides (Table II). The colours and the sensitivities of two of the location reagents are in Table III.

Calibration graph. Known amounts (1–20 μg of pesticide per spot) of the control pesticide solution (1 μg per μl) were spotted on to a TLC plate and the spotted plate was developed and sprayed with the chromogenic reagent as described earlier. After irradiation of the plate with UV light (366 nm) for 15 min, the developed spot for each concentration was scanned with a densitometer, and calibration graphs were

TABLE III

DETECTION LIMITS OF THE CHROMOGENIC REAGENTS

sg = Steel grey, v = violet, b = blue, fv = faint violet, vb = violet-blue, gr = green, vbr = violet-brown, br = brown, g = grey, vgr = violet-green, gv = greyish violet.

No.	*Compound*	*Reagent a*		*Reagent b*	
		Spot colour	*Sensitivity (μg)*	*Spot colour*	*Sensitivity (μg)*
1	BHC	sg	1.0	v to vb	1.0
2	Lindane	sg	0.02	fv to vb	0.5
3	Aldrin	sg	0.1	gr	0.2
4	Dieldrin	sg	0.1	gr	0.5
5	Endrin	sg	0.05	gr	0.1
6	DDT	sg	0.1	fv to vbr	0.5
7	Thiodan	sg	0.5	gr to br	1.0
8	Heptachlor	sg	0.1	g to gr	0.05
9	Chlordan	sg	1.0	g to gr	0.2
10	Toxaphen	sg	1.0	v to vgr	1.0
11	Kelthane	sg	0.05	g to vgr	0.5
12	Dipterex	sg	1.0	gv	0.2

constructed which were found to be linear over the range 1–10 μg of each insecticide.

Known amounts of the extracted sample from tissues and other biological materials in acetone were spotted on to a TLC plate together with the control samples. The plate was developed and sprayed with the chromogenic reagent and irradiated with UV light. The pesticides in autopsy tissues and biological materials were identified by comparison of their R_F values with those of the control samples. The densities of the developed spots on the plate were measured with a densitometer, and the concentration of pesticides were obtained from the calibration graphs; thus the amount of pesticide per 100 g of tissue could be calculated. The distributions of endrin in various autopsy tissues from different victims of poisoning were calculated and given in Table IV.

TABLE IV

DISTRIBUTIONS OF ENDRIN IN VARIOUS AUTOPSY TISSUES

Deceased subject	*Amount (mg per 100 g of tissue)*							
	Stomach	*Large intestine*	*Small intestine*	*Liver*	*Kidney*	*Spleen*	*Heart*	*Lung*
Male	81.00	—	22.00	48.00	45.00	30.00	25.00	—
Female	60.00	—	25.00	22.50	19.50	12.50	32.50	—
Female	61.00	—	22.50	49.00	25.00	20.25	22.50	—
Male	14.50	50.00	66.00	20.00	5.17	12.17	—	—
Child	2.63	—	—	4.00	1.25	1.75	—	1.08

RESULTS AND DISCUSSION

Table I presents the data regarding the TLC separation of 12 common chlorinated organic pesticides obtained using 26 different solvent systems. The large number of solvent systems provided a good separation of the insecticides into sharp and compact spots. From Table I it is clear that the solvent system XVIII can resolve the largest number of breakdown products of most of the chlorinated organic pesticides compared to the separation obtained in other solvent systems, *e.g.*, seven in BHC, nine in chlordan, five in toxaphen and four in kelthane could be distinctly separated. Furthermore, thiodan could be resolved into a maximum of five spots. This would assist in the investigation of the breakdown products and metabolites of these insecticides in autopsy tissues from cases of poisoning. Similarly, solvent system III (Table I) is effective in separating two breakdown products of endrin at $R_F = 0.09$ and 0.66, whereas the parent insecticide has an R_F value of 0.58.

With spray reagent *a* in Table II, steel-grey spots were obtained for all of the pesticides. This spray reagent was more sensitive for lindane, aldrin, dieldrin, endrin, DDT, heptachlor and kelthane. However, spray reagent *b* was superior to the other reagents as it produced differently coloured spots from grey and green to violet which were specific for the chlorinated pesticide. This provides the advantage that the different pesticides can also be identified by the colour of the spots developed on the TLC plate using this single spray reagent *b*. The reagent was most sensitive for endrin which could be detected in amounts as small as 0.1 μg. The colours of the spots ob-

tained for the insecticides and the sensitivity with spray reagents *a* and *b* are recorded in Table III. It is evident that spray reagent *b* is highly sensitive for heptachlor and chlordan which can be detected in amounts as small as 0.05 and 0.2 μg respectively, whereas the sensitivity of spray reagent *a* was 0.1 and 1.0 μg respectively for these two pesticides.

Besides these two chromogenic reagents, some other spray reagents were also tried for the location of the spots of the chlorinated pesticides. The colour of the spots obtained with 14 different chromogenic reagents are recorded in Table II. However, some false spots were obtained for tissue extracts with the spray reagents *i* to *n* in Table II which comprise organic dyes.

With reagents *a* and *b*, the spots of the chlorinated pesticides on the developed plate could also be located by exposing the sprayed plate to bright sunlight for 10 min instead of irradiation with UV light, but in this case less intense spots were observed against a light brown background. Similarly, if instead of irradiating the developed TLC plate with UV light after spraying with one of the spray reagents *a* to *h*, the plate was kept in a hot case at 100° for 1 h, brown spots were observed against a white background.

The extraction procedures described for all of the common chlorinated organic pesticides from biological materials are essentially quantitative. From control experiments, the average recovery by the proposed methods was 94%. In the concentration range 1–10 μg, the calibration graph was linear for all of the chlorinated pesticides studied. At lower concentrations the graph was not linear, although the limit of detection was found to be much lower.

From Table IV, which records the amounts of endrin detected in different autopsy tissues from cases of human poisoning, it is observed that the largest amounts of the poison remained in the stomach and intestine. From the case histories of these fatal poisoning cases, the deaths occurred within 1–2 h of the ingestion of the insecticides. However, endrin was found to accumulate in liver tissues when the death was delayed.

The proposed technique for the isolation of chlorinated organic pesticides from biological materials, and their detection, identification and determination by TLC, has been applied successfully to cases of suspected poisoning by these pesticides. The procedures are reliable and sensitive for routine examination in toxicological analysis.

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