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Note

An evaluation of the gas chromatographic estimation of trace quantities of hexachlorophane

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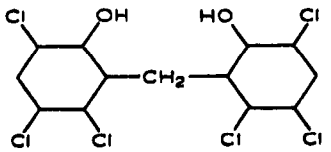
Although the effectiveness of hexachlorophane (2,2'-methylenebis(3,4,6-trichlorophenol); HCP) as an antimicrobial agent is well defined[1], recent events[2,3] have led to speculation regarding its continued general use [1,4–7] and the introduction of new regulations[8]. At the present time, however, HCP continues to be widely used under controlled conditions, notably in hospitals[9]. Following recent toxicity reports[1,10–13] it was considered to be desirable that we should monitor the blood levels arising from the topical application of this drug to patients participating in clinical trials. The preliminary clinical results have been reported elsewhere[14], and the object of this present report is to discuss the problems which became evident during the gas chromatographic estimations.



HCP may be assayed colorimetrically[15] or spectrophotometrically[16], but gas–liquid chromatography (GLC) is undoubtedly the method of choice in the absence of more sophisticated and costly instrumentation. Although it may be chromatographed directly[17,18], the presence of two phenolic groups on this aromatic molecule renders the preparation of some volatile derivative necessary[19] if the problems of long retention times and unsymmetrical peaks are to be avoided. Porcaro[17] has overcome these problems by the use of very short columns (8 to 12 in.) and a high carrier gas flow-rate in the detection of amounts of HCP greater than 5 μg.

The formation of trimethylsilyl (TMS) derivatives[19–21] has been advocated, as have acetylation[22] and methylation[10]. This latter technique was not considered further since the methods applied to HCP have involved the use of diazomethane,

which can be hazardous[23], although it has recently been used by Ferry and McQueen[24] in conjunction with a comparatively short GLC column where it yielded improved peak symmetry over free HCP. The acetylation procedure described by Browning *et al.*[22] was selected as being the most appropriate for this study, and is based upon the partition of whole blood (3.0 ml) with ethyl acetate (10.0 ml), followed by concentration of the extract and its subsequent reaction with a mixture of equal volumes of acetic anhydride and pyridine (0.1 ml).

An instrument fitted with a flame ionisation detector (FID) may be employed in the estimation of HCP in pharmaceutical preparations[19], but for monitoring the trace therapeutic blood levels[2,10] arising from the topical application of this drug, an electron capture detector (ECD) is necessary. Gudzinowicz[25] has evaluated the ^{3}H ECD for the detection of HCP, and this detector was also used by Browning *et al.*[22]; the use of the helium discharge ECD has been reported by Porcaro and Shubiak[20]. The ^{63}Ni ECD used in this present study has been shown to give an erratic response following the direct injection of HCP[20] thus rendering the preparation of some volatile derivative necessary in conjunction with this detector.

EXPERIMENTAL AND RESULTS

A Pye Series 104 gas chromatograph (Pye-Unicam, Cambridge, Great Britain) was fitted with a 1.0 m×4 mm I.D. glass column, packed with 3% Silicone OV-17 on Gas-Chrom Q, 80–100 mesh[26] (Phase Separations Ltd., Queensferry, Great Britain) which was maintained at 265°. The ECD, which comprised 7.5 mC of ^{63}Ni, was heated to 285° and was used in the pulsed mode at 50 μsec pulse space. The carrier gas was high-purity (oxygen-free) nitrogen at a flow-rate of 50 ml/min, and an ECD purge of 10 ml/min. Injections were made into a heated zone above the column at a temperature of *ca.* 300°.

In a typical chromatogram (Fig. 1a) one major peak is observed which correlates with the amount of acetylated HCP (AHCP) injected. Quantitative data were based upon the peak area function:

Peak height × peak width at half its height

The measurements were taken from a tangent drawn across the baseline of this major peak. The calibration graph of this function *versus* concentration of HCP (Fig. 2) gave a comparatively narrow linear concentration range, as would be expected from an ECD of this type. This problem may be overcome by the preparation of a series of calibration graphs at various attenuation settings corresponding to several concentration ranges.

Having established the linear range at each setting, the injection of a standard alongside each sample (corresponding to a concentration within the linear range of the appropriate calibration graph) presents a more practicable technique than the evaluation of a calibration graph together with each series of samples. This latter approach would also introduce problems which may arise from the lack of long term stability and reproducibility of the ECD.

No internal marker is used in this method[22], and it is therefore necessary to establish the reproducibility between successive replicate injections from the syringe. During the preparation of the calibration graph, it was observed that the reproducibility between the peak area functions (defined earlier) arising from duplicate injec-

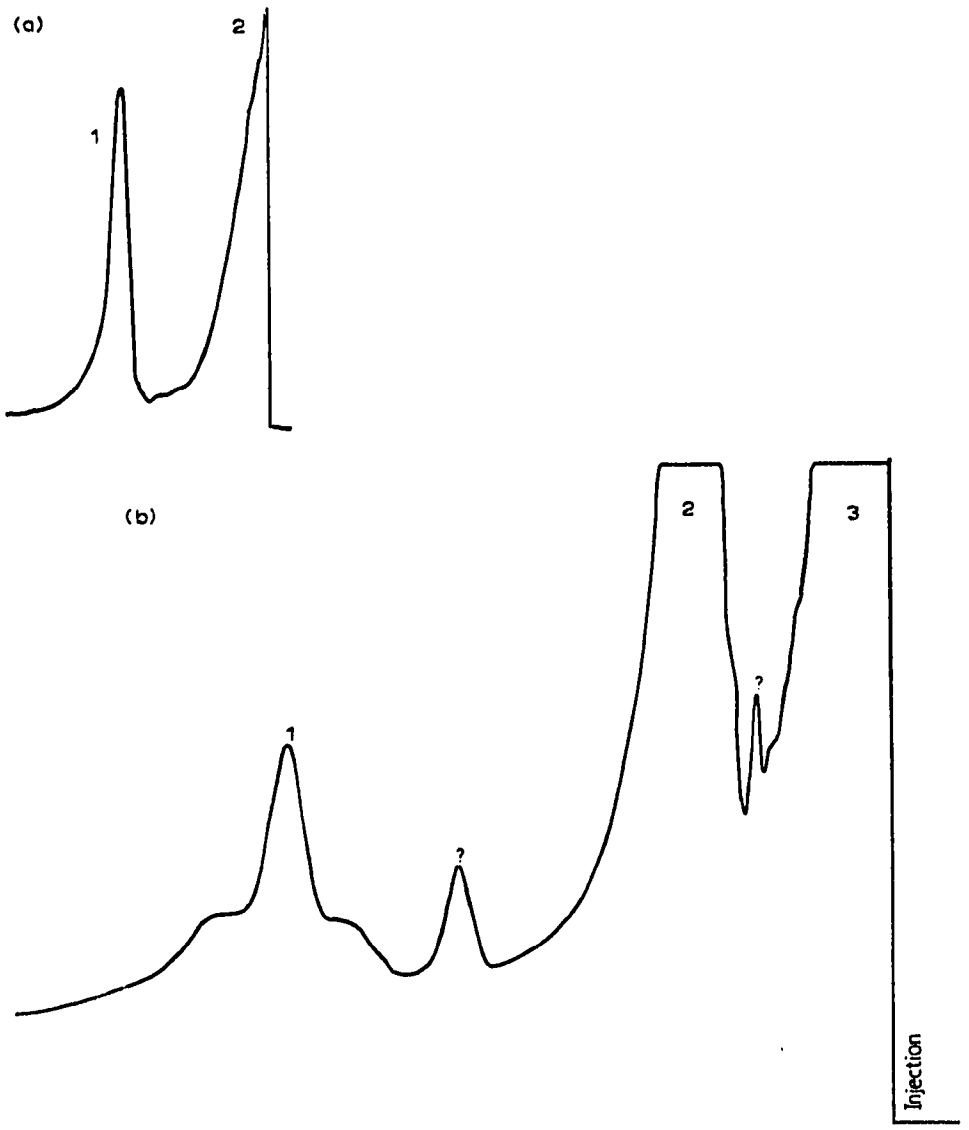


Fig. 1. Chromatogram of 3 μl acetylated hexachlorophane in ethyl acetate. (a) Original concentration, 100 ng/ml. 1= Hexachlorophane; 2= ethyl acetate. (b) Original concentration, 250 ng/ml. 1= Hexachlorophane (secondary peaks); 2= hexachlorophane (major peak); 3= ethyl acetate.

tions of the same solution of AHCP tended to decrease at higher concentrations with the linearity of the graph.

In view of the limitations of the ECD response, care was taken to ensure that quantitative data were based upon chromatograms in which the peak from the sample and standard alike emerged at a similar height on the solvent tail.

The preliminary results demonstrated the need for a comprehensive evaluation of the acetylation conditions[22]. Aliquots (5.0 ml) of a solution of HCP in ethyl acetate

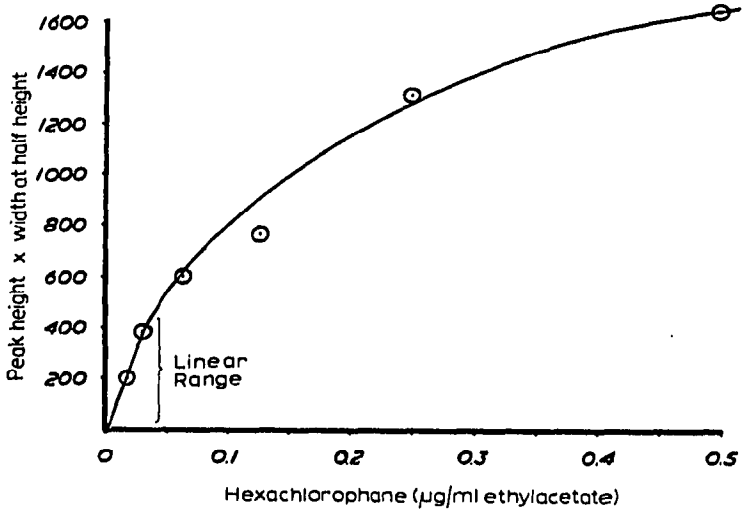


Fig. 2. Calibration graph (0–500 ng/ml).

at a concentration of 0.1 µg/ml were evaporated to *ca.* 0.5 ml and were then acetylated for 0, 5, 10, 15, 20, 25, and 30 min at 60°. Further aliquots (5.0 ml) were evaporated to dryness and volumes of 0.1, 0.2, 0.5 and 1.0 ml, respectively, and each of these was then acetylated for 10 min at 60°.

All the solutions were subsequently evaporated to dryness in a stream of nitrogen, and were reconstituted with ethyl acetate (100 µl) prior to injection into the chromatograph. The results, which are summarised in Tables I and II, respectively, indicate that complete acetylation is achieved very rapidly and is not dependent upon the concentration of HCP at that stage within the working range of the method.

Browning *et al.*[22] evaporated the solution to a volume of 1.1 ml after acetylation, but it was found more convenient to evaporate the solution to dryness in a conical test tube, care being taken to wash the AHCP into the base of the tube with ethyl acetate. The extracts could then be stored in a refrigerator under these conditions for several days without loss of AHCP. Reconstitution was achieved by washing the sides of the tube with 100 µl of ethyl acetate from a microlitre syringe and leaving to stand for *ca.* 5 min at room temperature. The tube was also warmed in the hand to assist the dissolution of the AHCP.

TABLE I

THE EFFECT OF THE ACETYLATION TIME UPON THE RECOVERY OF HEXACHLOROPHANE

	Time (min)				
	0	*5*	*15*	*20*	*30*
Peak function (mm^2)*	728	672	654	692	647

* Defined in the text.

TABLE II

THE EFFECT OF CONCENTRATION AT THE ACETYLATION STAGE UPON THE RECOVERY OF HEXACHLOROPHANE

	Volume of ethyl acetate extract at acetylation (ml)			
	0	*0.1*	*0.5*	*1.0*
Peak function (mm^2) *	750	725	726	726

* Defined in the text.

This modification to the technique, with an effective eleven-fold increase in the concentration of AHCP at the GLC stage, permits the detection of a concentration of 100 pg of HCP in 1.0 ml of ethyl acetate, corresponding to a blood level of 330 pg/ml assuming complete extraction from the blood. A chromatogram of AHCP at this level is illustrated in Fig. 3. This limit of detection represents a considerable improvement upon that of 0.05 μg/ml reported in the original paper[22], and is somewhat lower than that obtained by Porcaro and Shubiak [20] and Curley *et al.*[10]. It may be extended by the use of the lower attenuation settings, which do, however, result in excessive tailing of the solvent peak which may mask the AHCP peak.

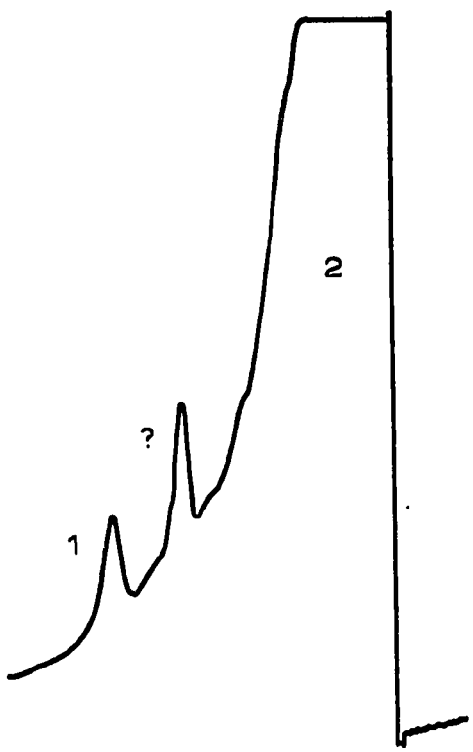


Fig. 3. Chromatogram of 3 μl acetylated hexachlorophane in ethyl acetate. Original concentration, 100 pg/ml. 1 = Hexachlorophane; 2 = ethyl acetate.

In addition to the major AHCP peak, a number of secondary peaks was also observed. They had considerably longer retention times than the major peak, but appeared to be a function of the amount of AHCP injected. These peaks become evident when a more concentrated extract is injected at a comparatively low attenuation setting, as shown in Fig. 1b. The slight leading of the major AHCP peak may be attributed to decomposition in the heated injection zone, rather than the elution of some component with a retention time similar to that of AHCP since the injection of pure solvent did not reveal any 'ghosting' of this peak[27].

Some unidentified peaks appear in all chromatograms, and it is evident that the artefacts on the solvent peak arise from the acetylation mixture. The peak which is eluted just before the major AHCP peak does not originate from the solvent, since it does not give a constant response when several aliquots of pure solvent are injected, and it would not be expected to arise from the column packing material, which was conditioned at 300° before use. Impurities in the carrier gas supply line or plasticisers from the septum[28] cannot be discounted, particularly in conjunction with an ECD, which is far more susceptible to such effects than is the FID. This peak is present in all the chromatograms to a greater or lesser extent, whereas that which is eluted with a retention time slightly longer than that of AHCP is not always observed. One possible origin of these two peaks is, however, the adsorption and retention of a trace of AHCP on the column, and its subsequent elution with each injection of solvent.

This hypothesis is supported by the pattern of anomalous relationships between the peak area function and concentration which were obtained during a preliminary calibration. An explanation may, however, be postulated in terms of the uptake of AHCP by active binding sites on the column system, which may be subsequently eluted with a correspondingly shorter retention time upon the injection of pure solvent. An injection of AHCP would then be partially absorbed onto the regenerated binding sites, resulting in an anomalously low value for that peak area function. The negligible size of the two peaks during the early stages of the work (as in Fig. 1a) and their increase in area as the work progressed (Figs. 1b and 3) lend further support to this explanation.

It should be stressed that no unidentified peaks coincide with the elution of AHCP, since injections of pure solvent yield only the two peaks which have already been discussed. The effect of these peaks upon the quantitative data will be minimal, provided standard reference solutions are injected alongside each sample.

The 'on-column' retention of free HCP has been observed by Browning *et al.*[22], but no such problems were reported from AHCP. The choice of Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.), which is one of the most inert supports currently available[29] would be expected to minimise any absorption problems. The precision of the method as a whole was subjected to a comprehensive evaluation by the original authors[22] and has not been investigated further.

It is essential that attention should be directed towards the potential problems associated with this assay, and their resolution, if valid results are to be obtained.

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REFERENCES

1 *Brit. Med. J.*, 1 (1972) 705.
2 *F.D.A. Drug Bulletin*, U.S. Department of Health, Education and Welfare, Rockville, Md., December, 1971; also February, 1972.
3 R. D. Kimbrough and T. B. Gaines, *Arch. Environ. Health*, 23 (1971) 114.
4 *Brit. Med. J.*, 1 (1972) 579.
5 *Pharm. J.*, 209 (1972) 481.
6 *Pharm. J.*, 210 (1973) 286.
7 *Pharm. J.*, 211 (1973) 23, 27.
8 *The Medicines (Hexachlorophane Prohibition) Order, 1973; Statutary Instrument No. 1120.* Her Majesty's Stationary Office, London, 1973.
9 N. M. Kanof, *J. Amer. Med. Ass.*, 220 (1972) 409.
10 A. Curley, R. E. Hawk, R. D. Kimbrough, G. Nathenson and L. Finberg, *Lancet*, 2 (1971) 296.
11 R. D. Kimbrough, *Arch. Environ. Health*, 23 (1971) 119.
12 L. M. Abbott, P. M. Buckfield, D. G. Ferry, D. S. Malcolm and E. G. McQueen, *Aust. Paediatr. J.*, 8 (1972) 246.
13 V. D. Plueckhahn, *Drugs*, 5 (1973) 97.
14 J. C. Edwards, N. D. Greenwood and B. Williamson, *Symp. Acne, Vick International, Slough, Bucks., November, 1972.*
15 V. D. Johnston and P. J. Porcaro, *Anal. Chem.*, 36 (1964) 124.
16 R. W. Daisley and C. J. Olliff, *J. Pharm. Pharmacol.*, 22 (1970) 202.
17 P. J. Porcaro, *Anal. Chem.*, 36 (1964) 1664.
18 R. C. Bachmann and M. R. Shetlar, *Biochem. Med.*, 2 (1969) 313.
19 J. V. Wisniewski, *Facts Methods*, 8 (1967) 10.
20 P. J. Porcaro and P. Shubiak, *Anal. Chem.*, 40 (1968) 1232.
21 P. J. Porcaro, P. Shubiak and M. Manowitz, *J. Pharm. Sci.*, 58 (1969) 251.
22 R. S. Browning, J. Grego and H. P. Warrington, *J. Pharm. Sci.*, 57 (1968) 2165.
23 G. D. Muir (Editor), *Hazards in the Chemical Laboratory*, Royal Institute of Chemistry, London, 1971, Ch. 5, p. 112.
24 D. G. Ferry and E. G. McQueen, *J. Chromatogr.*, 76 (1973) 233.
25 B. J. Gudzinowicz, *The Analysis of Pesticides, Herbicides and Related Compounds using the Electron Affinity Detector*, Jarrell-Ash Co., Waltham, Mass., 1965.
26 L. L. Alber, *J. Ass. Offic. Anal. Chem.*, 52 (1969) 1295.
27 R. A. Simonaitis and G. C. Guvernator, *J. Gas Chromatogr.*, 5 (1967) 49.
28 B. T. Croll, *Chem. Ind. (London)*, (1971) 789.
29 J. F. Palframan and E. A. Walker, *Analyst (London)*, 92 (1967) 71.