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REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHIC DETERMINATION OF CHLOROPHACINONE RESIDUES IN ANIMAL TISSUES

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SUMMARY

A sensitive and specific high-performance liquid chromatographic method for the identification and quantitation of chlorophacinone in animal tissues has been developed. Residues were extracted with chloroform–acetone (1:1, v/v). Clean-up of extracts was accomplished with a combined gel permeation–adsorption chromatographic procedure using Bio-Beads SX-3 and incorporating an on-line Sep-Pak silica cartridge. Residues were determined by ion-pair liquid chromatography, with the tetrabutylammonium ion as counter-ion, using a fixed-wavelength UV detector at 280 nm or a diode array detector at 285 nm. Recoveries from spiked liver tissue were around 90% at levels from 0.05 to 1 mg kg^{-1}. A detection limit of 0.001 mg kg^{-1} could be achieved in animal tissues. The diode array detector confirmed identification by matching spectra for residues down to 0.1 mg kg^{-1} and below this level by multi-wavelength monitoring.

INTRODUCTION

Chlorophacinone, 2-[2-(4-chlorophenyl)-2-phenylacetyl]indan-1,3-dione, has been used worldwide as an anticoagulant rodenticide for over twenty years. It inhibits blood coagulation, blocking prothrombin formation and also uncouples oxidative phosphorylation. The acute oral LD_{50} for rats and mice has been recorded[1] as 20.5 mg kg^{-1}. Formulations include concentrates, tracking powders (2%, w/w) and baits of whole grain or crushed cereals (0.005%, w/w).

Poisoning of both wild and domestic animals, including non-target casualties arising from the use of rodenticides, are regularly investigated in this laboratory. Anticoagulant rodenticides are rapidly excreted and residues in the tissues of poisoned animals usually occur at fairly low levels, hence sensitive methods of analysis are required for successful diagnosis of poisoning. Multiresidue methods for the analysis of coumarin-based rodenticides have been described elsewhere[2–4] but a flexible and sensitive method for the analysis of chlorophacinone was required.

Spectrophotometric methods[5,6] have been employed for the analysis of chlorophacinone in bait formulations but suffer from a lack of sensitivity and selectivity.

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Methods based on thin-layer chromatography[7–9] are not suitable for determining low level residues in animal tissue extracts. Bullard *et al.*[10,11] described a sensitive assay for animal tissues which involved oxidation of chlorophacinone with the resulting chlorobenzophenone being analysed by gas chromatography (GC) using electron-capture detection. A second GC method requiring bromination of chlorophacinone prior to analysis has been reported[12]. These methods are lengthy and can suffer from poor recoveries.

High-performance liquid chromatographic (HPLC) methods for the direct analysis of chlorophacinone in formulations have been described in the literature. Grant and Pike[13] used methanol and aq. 0.75% ammonia with a C_{18} pellicular stationary phase but noted that this mobile phase was unsuitable for use with microparticulate octadecylsilane (ODS) packings, which were dissolved. Bennett and Grimes[14] used an ODS-phase with acetic acid–tetrahydrofuran–water (14:2:9, v/v/v) for grain and wax-block baits. Reversed-phase ion-pair chromatography using the tetrabutylammonium ion (TBA^+) as counter-ion has been suggested[15] as a means of assaying the related 1,3-indandione, diphacinone, and this chromatographic mode was employed by Vigh *et al.*[16] for the analysis of chlorophacinone in baits. None of these methods is suitable for use with animal tissue extracts containing residues at a low level. However, the determination of residues in mouse tissue extracts after clean-up by liquid–liquid partition and Florisil column chromatography has been reported by Addison[17] who utilised a LiChrosorb NH_2 packing with an acetonitrile–water (80:20, v/v) mobile phase. Although this method may offer suitable sensitivity the resulting chromatograms appear rather complex and the linear range somewhat limited. This report describes the application of a reversed-phase ion-pair technique to the determination of chlorophacinone residues in animal tissues.

EXPERIMENTAL

Materials and apparatus

Glass-distilled acetone and chloroform and HPLC-grade methanol and dichloromethane were supplied by Rathburn Chemicals (Walkerburn, Tweeddale, U.K.). Low UV PIC Reagent A, a commercially prepared tetrabutylammonium phosphate ion-pairing reagent buffered at pH 7.5, and Sep-Pak silica cartridges were obtained from Waters Assoc. (Hartford, U.K.). Tetrabutylammonium phosphate was purchased from Sigma (Poole, U.K.) and Bio-Beads SX-3 (200–400 mesh) from Bio-Rad Labs. (Watford, U.K.). All other chemicals were supplied by BDH (Poole, U.K.). An analytical reference standard of chlorophacinone was provided by May and Baker (Romford, U.K.).

An Ultra-Turrax 18 N tissue disperser was used to homogenise tissue samples. A gel permeation chromatographic (GPC) system using Bio-Beads SX-3 in hexane–chloroform–acetone (75:20:5, v/v/v) has been described elsewhere[2]. The HPLC system consisted of a Waters Assoc. ALC 200 liquid chromatograph with a Rheodyne 7120 injection valve (20 μl), and either the incorporated Model 440 fixed wavelength UV-absorbance detector or a Hewlett-Packard 1040A diode array detection system. The column (250 × 4.6 mm I.D.) was slurry packed with ODS-hypersil (5 μm), using a Haskel pneumatic amplifier pump, with propan-2-ol as the slurry medium and methanol as the packing medium.

Extraction

Tissue samples (10 g) were chopped, dried by admixture with anhydrous sodium sulphate and homogenised in chloroform–acetone (1:1, v/v). The extract was filtered, the residual material re-extracted and the second extract added to the filtrate together with washings from the homogeniser. The combined filtrate was carefully evaporated to dryness at 35°C on a rotary evaporator and made up to 10 ml in hexane–chloroform–acetone (75:20:5, v/v/v).

Clean-up

The GPC column was calibrated by applying a reference solution of chlorophacinone in hexane–chloroform–acetone (75:20:5, v/v/v) and then eluting with the same solvent mixture at 5 ml min^{-1}. Fractions (10 ml) were collected and monitored for the rodenticide. Chlorophacinone was eluted from the column between 210 and 380 ml. When animal tissue extracts (5 ml) were applied to the GPC column the first 200 ml of the eluent was discarded and the following 200 ml collected. A further 50 ml of eluent was pumped through the column before a second sample was loaded. The cleaned-up extract was carefully evaporated to dryness at 35°C and re-dissolved in the HPLC mobile phase (1 ml) described below.

Further clean-up was achieved by introducing an on-line silica Sep-Pak cartridge into the GPC system. The PTFE drain tube from the column was coupled directly to the Sep-Pak cartridge using Altex micro plumbing components terminating in a tubing connector. The cartridge was introduced after the first 200 ml of eluent had been passed. When the chlorophacinone fraction had passed through it the cartridge was removed and washed with dichloromethane (7 ml) using a syringe. The dichloromethane was discarded and chlorophacinone eluted from the cartridge with 0.25% formic acid in dichloromethane (40 ml). This cleaned-up extract was carefully evaporated to dryness and re-dissolved in mobile phase (1 ml).

HPLC

Mobile phases were prepared from an aqueous solution of PIC Reagent A and either a solution of PIC Reagent A in methanol or a 0.005 *M* solution of tetrabutylammonium phosphate in methanol. Methanol solutions of PIC Reagent A had to be filtered to remove precipitated buffer salts. All mobile phases were degassed by continuous sparging with helium. A mixture containing 75% of the methanolic component was adopted as the mobile phase for routine use with the ODS-Hypersil column at a flow-rate of 1.1 ml min^{-1}. Elution of chlorophacinone was monitored at 280 nm using the fixed-wavelength detector whilst the diode array detector was used to monitor simultaneously absorbance at 224, 285, 313 and 325 nm. A pilot wavelength of 285 nm was used for peak detection by the HP1040A and this system automatically stored apex and baseline spectra (205–400 nm) of recognised peaks.

RESULTS AND DISCUSSION

Extraction

The extraction of chlorophacinone residues from animal tissues has been accomplished using acetonitrile on freeze-dried tissues[17] or by homogenisation in acetone[10,11]. However, a mixture of chloroform and acetone (1:1, v/v) was found to be

the most efficient extractant of incurred residues of coumarin-based rodenticides from liver tissue[2]. Preliminary experiments comparing the extraction of incurred residues of chlorophacinone by chloroform–acetone (1:1, v/v) and acetone were made. There was little difference in the amount of residues extracted by these two extractants; acetone yielded marginally higher residues whilst chloroform–acetone tended to give cleaner extracts. Chloroform–acetone was used as the extraction solvent in all subsequent experiments.

Examination of tissues from test animals indicated that liver and to a lesser degree kidney contained the highest residues of chlorophacinone, although residues were invariably low even in these tissues. Liver tissue was chosen for recovery experiments because of its usefulness in the diagnosis of poisoning. Percentage recoveries were determined using goose-liver samples spiked, prior to extraction, with chlorophacinone at levels of 0.05, 0.2 and 1 mg kg^{-1}. After extraction the samples were cleaned-up by the combined GPC–Sep-Pak procedure and the results are shown in Table I. In general recoveries were consistently good giving mean values around 90% at the 0.2 and 1 mg kg^{-1} levels and only slightly lower at 86% for the 0.05 mg kg^{-1} level. Experiments using GPC alone for clean-up at the 1 mg kg^{-1} level gave slightly better recoveries of around 94%.

Clean-up

Clean-up of extracts from animal tissues containing chlorophacinone has been achieved using liquid–liquid partition techniques[10,11] or by adsorption on Florisil[17]. The removal of lipid material from extracts was particularly important in this study as a reversed-phase system was to be used for the determination making it necessary to ensure that the final extracts were soluble in small volumes of the aqueous methanol mobile phase. Gel permeation chromatography using Bio-Beads has been successfully employed to clean-up extracts containing pesticides[18,19] including other rodenticides[2,3,20] and is particularly suited to defatting extracts. The system used by the author for coumarin-based rodenticides[2,3] with hexane–chloroform–acetone (75:20:5, v/v/v) as the eluent for Bio-Beads SX-3 was tested with animal tissue extracts containing chlorophacinone. It proved to be satisfactory in removing fats from the extracts permitting detection of chlorophacinone residues down to fairly low levels.

TABLE I

RECOVERIES OF CHLOROPHACINONE FROM SPIKED LIVER TISSUE

At each level three separate samples of liver tissue were fortified with chlorophacinone prior to extraction. Residues were extracted, cleaned-up by the GPC–Sep-Pak procedure and determined by HPLC as described in the text.

	Percentage recovery (mean ± SD)		
	Fortification level (mg kg^{-1})		
	1	*0.2*	*0.05*
Chlorophacinone	90 ± 3.3*	91 ± 1.3	86 ± 2.6

* Mean of five results.

However, unlike coumarin-based rodenticides, chlorophacinone is not amenable to fluorescence detection and the less specific and less sensitive UV-absorption detection mode was employed. As a result more interferences were observed with the determination of chlorophacinone in extracts cleaned-up by GPC alone. Detection of residues down to levels of 0.2 mg kg^{-1} were possible but to maximise sensitivity further clean-up was desirable. Sep-Pak cartridges have been used to provide additional clean-up in various analytical problems and their use was evaluated for chlorophacinone. Both silica and C_{18} reversed-phase cartridges provided suitable clean-up of spiked liver extracts. Silica cartridges were preferred for animal tissue extracts after GPC but reversed-phase cartridges may prove convenient for use with urine or plasma samples as has been demonstrated for warfarin[21].

Dichloromethane modified with either methanol or acetone was suitable for elution of chlorophacinone from Sep-Pak silica cartridges but such solvents afforded relatively poor clean-up. Dichloromethane acidified with 0.25% formic acid was effective in eluting this rodenticide from the cartridges and yielded a satisfactory clean-up of animal tissue extracts. The recovery of chlorophacinone from tissue extracts was satisfactory using this clean-up off-line and was subsequently found to be just as successful when used in an on-line manner. Chromatograms of an extract cleaned-up by the GPC–Sep-Pak procedure and of a similar extract cleaned-up by GPC only are compared in Fig. 1.

HPLC

The difficulties in chromatographing chlorophacinone in the normal-phase mode using silica and cyano columns reported in the literature[13] have also been

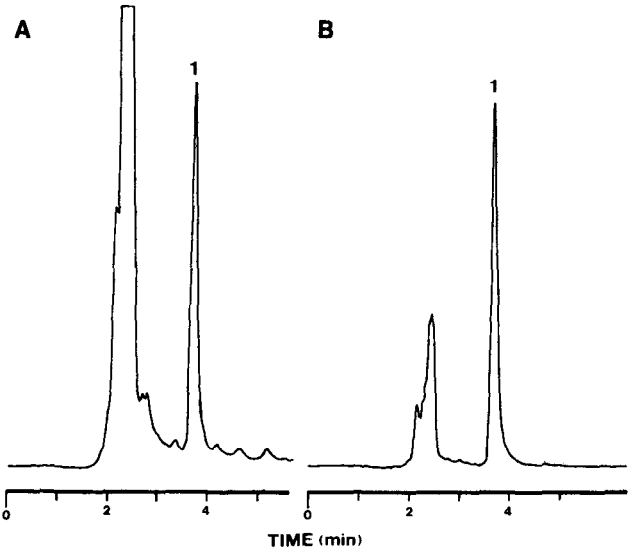

Fig. 1. Ion-pair chromatography of chlorophacinone (1) on ODS-Hypersil. (A) Extract from canine liver containing chlorophacinone (≡ 1.3 mg kg^{-1}), cleaned-up by GPC only. (B) Extract from same tissue cleaned up by combined GPC–Sep-Pak procedure. Mobile phase, 0.005 *M* TBA^+ in methanol–water containing PIC A reagent (75:25, v/v); flow-rate, 1.1 ml min^{-1}; UV detection at 285 nm, 0.07 a.u.f.s.; 20 μl injector loop.

experienced in this laboratory. Similarly reversed-phase HPLC under acidic or neutral conditions has not provided a suitable analytical method although the successful use of a mobile phase consisting largely of acetic acid[14] for this chromatographic mode is notable. Reversed-phase HPLC using methanol and Tris buffer pH8 with μBondapak C_{18} gave better results, permitting the resolution of diphacinone and chlorophacinone but the peaks eluted early and were rather broad making detection limits poor. Addison[17] has used LiChrosorb NH_2 with an acetonitrile–water mobile phase for the determination of chlorophacinone residues in mouse tissue but difficulties were experienced when this method was tried. APS-Hypersil, an amino-phase, was used with acetonitrile–water but even under conditions allowing rapid elution the peak shape was very broad. While evaluating the use of ion-pair liquid chromatography with the tetrabutylammonium ion, for coumarin-based rodenticides[3], it was found that such systems could provide good chromatographic conditions for the analysis of chlorophacinone. Subsequently the use of similar ion-pair systems have been reported for diphacinone[15] and for chlorophacinone[16]. The latter work used tetrahydrofuran as the organic modifier with 0.05 *M* TBA^+ while in the study reported here methanol was preferred as the organic modifier on the grounds of lower toxic hazard and because of its lower cut-off point for UV detection. Initially TBA^+ (0.005 *M*) in the form of PIC Reagent A was used for both methanolic and aqueous solutions but for reasons of convenience and cost tetrabutylammonium phosphate was adopted for methanolic solutions. Experiments were done with various concentrations of TBA^+ in the mobile phase. As the concentration of the counter-ion was increased from 0.005 *M* to 0.02 *M* there was an increase in the retention of chlorophacinone. The elution characteristics of chlorophacinone in the ion-pair system at a 0.005 *M* counter-ion concentration were determined by varying the proportion of methanol in the eluent for isocratic elution. The retention of chlorophacinone by the ODS-Hypersil column was readily controlled by the organic modifier concentration in the mobile phase as had been observed by Vigh *et al.*[16]. The relationship between the capacity factor (k') and methanol concentration is shown in Fig. 2. Chloropha-

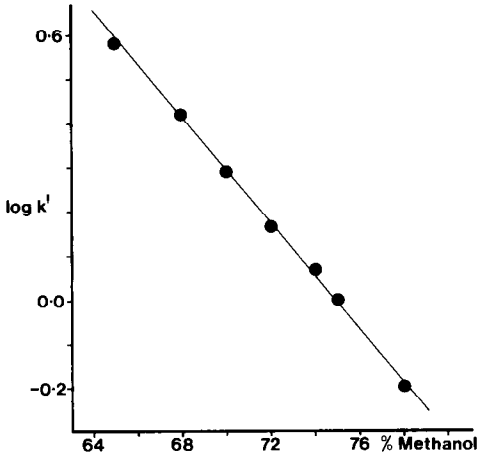

Fig. 2. Influence of organic modifier (methanol) concentration on the capacity factor (k') of chlorophacinone. Mobile phases prepared from 0.005 *M* TBA^+ in methanol and aqueous PIC Reagent A. Other chromatographic conditions as described for Fig. 1.

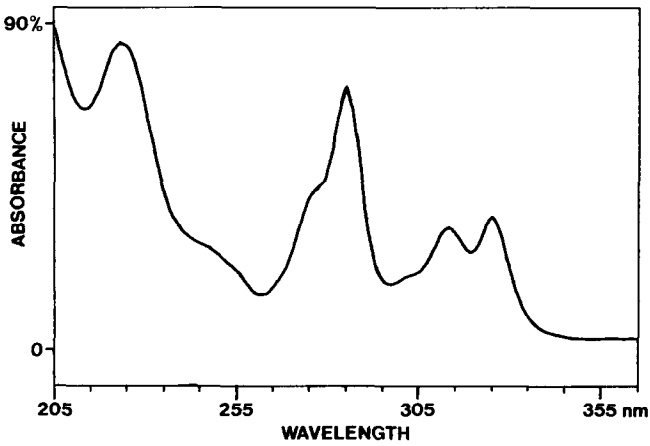

Fig. 3. Normalised UV absorption spectrum of chlorophacinone (highest absorbance set at 90%) obtained from HPLC of a reference standard (160 ng) under the chromatographic conditions described for Fig. 1. Absorbance at 205 nm was 0.078 a.u.

cinone eluted later than diphacinone with all mobile phases used and was fully resolved from diphacinone under the conditions employed for routine analysis.

An UV spectrum of chlorophacinone taken on-line by the HP1040A detection system (Fig. 3) indicated the most suitable wavelengths for detection. The UV responses obtained by the fixed-wavelength detector and diode array detection system are shown in Table II. The response of the HP1040A at 285 nm almost matched that of the fixed-wavelength detector operated at 280 nm. Considering these results it is somewhat surprising that Addison[17] found detection at 254 nm offered sensitivity an order of magnitude better than at 280 nm.

Use of the diode array detection system had the advantage that on-line confirmation of peak identity was possible by overlaying and matching a normalised spectrum with the corresponding spectrum taken from the peak obtained with a chlorophacinone standard. This procedure is particularly useful with chlorophacinone which has a very distinct and characteristic UV spectrum. Such confirmation was possible for residue levels down to around the 0.1 mg kg^{-1} level; approximately

TABLE II

COMPARISON OF UV RESPONSES OF THE FIXED WAVELENGTH AND DIODE ARRAY DETECTORS OBTAINED WITH CHLOROPHACINONE

Responses determined as peak heights derived from HPLC of chlorophacinone (432 ng) on ODS-Hypersil using 0.005 M TBA^+ in methanol–aqueous PIC Reagent A (68:22, v/v) at 1.5 ml min^{-1}.

	UV response (milli-absorbance units)							
	Wavelength (nm)							
	220	*224*	*254*	*275*	*280*	*285*	*313*	*325*
Fixed wavelength	–	–	47	–	74	–	–	–
Diode array	70	76	22	39	–	63	30	32

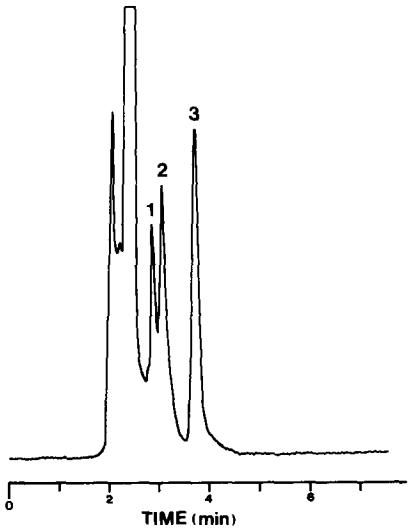

Fig. 4. Ion-pair chromatography of a kidney extract, from a test animal, containing chlorophacinone, peak 3, ($\equiv$ 0.11 mg kg^{-1}). Peaks 1 and 2 were possible metabolites of chlorophacinone. Chromatographic conditions as for Fig. 1 but detector sensitivity was 0.004 a.u.f.s.

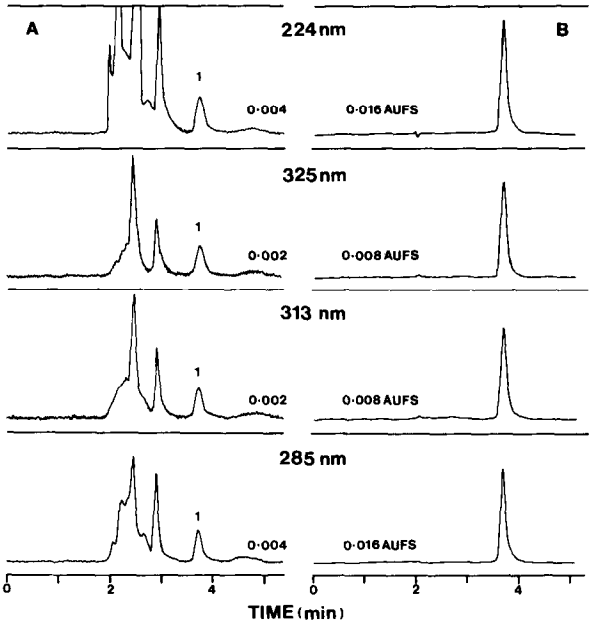

Fig. 5. Multiwavelength monitoring of chlorophacinone. (A) Muscle tissue extract containing chlorophacinone, peak 1, (3.5 ng $\equiv$ 0.014 mg kg^{-1}). 50 μl injector loop used. (B) Chlorophacinone standard (32 ng). Chromatographic conditions as for Fig. 1.

10 ng on column. A chromatogram of a kidney extract from a test animal, containing chlorophacinone at this level is shown in Fig. 4. Two peaks eluting ahead of chlorophacinone in this chromatogram had almost identical UV spectra to that of this rodenticide and are thought to correspond to metabolites of chlorophacinone. Similar peaks have been observed in other liver and kidney extracts from test animals but not in tissue extracts arising from chlorophacinone poisoning incidents. Beyond the limit of application of this confirmation technique some measure of confirmation was still possible using absorbance ratioing of the wavelengths simultaneously monitored by the diode array detector (Fig. 5).

The minimum detectable amount of chlorophacinone was approximately 200 pg and the response was linear up to 1 μg on column. A practical detection limit of 0.001 mg kg^{-1} could be achieved in most animal tissues when a 50-μl injection loop was used. Incurred chlorophacinone residues have been successfully monitored in the low ppb range in liver, kidney and muscle tissue extracts. Residues ranged from 0.004–1.3 mg kg^{-1} for liver tissue, 0.004–0.19 mg kg^{-1} for kidney tissue and 0.011–0.014 mg kg^{-1} for muscle tissue.

The method described here has provided a sensitive, specific and flexible analytical technique for chlorophacinone in animal tissues. No long term problems with stability of the ODS column have been observed provided that the buffer salts and counter-ion are flushed out after use. Under the elution conditions employed the method was essentially free of interferences from the related compound diphacinone, which has an almost identical UV spectrum to that of chlorophacinone, and from any remaining co-extractives.

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