Determination of Chlorophacinone and Diphenadione Residues in Biological Materials

Roger W. Bullard,* Gilbert Holguin, and James E. Peterson

The anticoagulants chlorophacinone (2-[(p-chlorophenyl)phenylacetyl]-1,3-indandione) and diphenadione (2-(diphenylacetyl)-1,3-indandione) have been determined in animal blood plasma, animal tissues, milk, foliage, and grain samples. Sample preparation is material dependent, but the remainder of the procedure applies for all analyses. Chlorophacinone and diphenadione are oxidized, and the respective p-chlorobenzophenone and benzophenone products are analyzed by gas-liquid chromatography with electron-capture detection. The lower limit of detectability ranges from 10 ppb for plasma samples to 0.5 ppb for milk samples.

Chlorophacinone (2-[(*p*-chlorophenyl)phenylacetyl]-1,3-indandione) and diphenadione (2-(diphenylacetyl)-1,3-indandione) are two of the better known rodenticides of the 1,3-indandione anticoagulants (Saunders *et al.*, 1955; Rowe and Redfern, 1968; Lund, 1971). Diphenadione has also been used therapeutically as a prothrombopenic agent (O'Reilly and Aggeler, 1970) and as a systemic injected into cattle for control of vampire bats (Thompson *et al.*, 1972).

Investigation of these compounds as livestock systemics at our laboratory required the analysis of many blood, milk, and tissue samples in which concentrations ranged from trace levels to 50 ppm. Spectrophotometric procedures had been reported for diphenadione (Caswell, 1959; Ozolins *et al.*, 1963; Danek and Kwiek, 1964; Hollifield and Winefordner, 1967) and chlorophacinone (Chempar Chemical Co., 1971), but none of the procedures were sensitive, precise, and flexible enough for our analyses. We therefore developed a method in which chlorophacinone and diphenadione, after oxidation, can be determined by gas-liquid chromatography (glc) down to 10 ppb or less in a variety of biological materials. The primary use of the method has been in the analysis of residues in mammalian body fluids and tissues, but, because of our laboratory's interest in anticoagulant rodenticide baiting, it has been expanded to include foliage and grain samples.

EXPERIMENTAL SECTION

Materials. Solvents were Mallinckrodt Nanograde; granular anhydrous sodium sulfate and reagent chemicals were Mallinckrodt Analytical Reagent grade. Diphenadione (diphacinone) was supplied by Velsicol Corp. and chlorophacinone by Chempar Chemical Co. Both were recrystallized twice from acetone-water solutions before use in standard solutions.

Preparation. *Plasma Samples.* Venous blood was collected in a vessel coated with sodium oxalate (approximately 4 mg/ml of blood) and centrifuged at 2000 rpm; the plasma fraction was collected and held frozen until analysis. Ten milligrams of ascorbic acid and 0.5 ml of 3 *N* HCl were added to each plasma sample (1-3 g), and the volume was adjusted to 3.5 ml with water. Plasma proteins were then precipitated by adding 15 ml of acetone and removed by centrifugation. The precipitate was rinsed once with 15 ml of acetone, and the supernatant and rinse were combined in a 25 × 150 mm screw-capped (Teflon-lined) culture tube. (Although recovery could have been improved somewhat by a second rinse, the solvent volume would become unwieldly in the subsequent evaporation step.)

Other Samples. Animal tissue, milk, and plant samples were processed similarly. Tissue (25 g) was cut into small pieces, mixed with 5 times its weight of powdered anhydrous sodium sulfate, and refrigerated for 1 hr, and the mixture blended. Fresh milk (30–50 g) was placed in a tared dish and weighed; after 5 hr evaporation to near dryness in a fume hood, the material was reweighed, mixed with 10 times its weight of powdered anhydrous sodium sulfate, cooled in a freezer at least 1 hr, and ground to a dry, free-flowing powder with a mortar and pestle. Foliage was cut into small pieces, dried in a mechanical convection oven overnight at room temperature, blended, and ground to 40-mesh size with a Wiley mill. Grain samples were ground directly.

After dehydration and grinding, aliquots of the sample preparations (30 g for the tissue-sodium sulfate mixture, all of the milk-sodium sulfate mixture, and 1–5 g for foliage and grain) were each placed in a glass-stoppered 125-ml erlenmeyer flask and extracted by shaking for 10 min with 60 ml of acetone that contained 0.2 ml of concentrated HCl and 10 mg of ascorbic acid. Then 30 ml of the extract was transferred to a 25 × 150 mm culture tube for cleanup.

Cleanup and Derivitization. After the extract in each tube was evaporated to dryness on a Buchler Rotary Evapo-Mix, 20 ml of 0.6 *N* sodium hydroxide and 20 ml of methylene chloride were added, and the tube was shaken for 5 min with a Burrell Wrist-Action Shaker. After separation, the upper phase was removed by syringe and discarded and the lower (methylene chloride) phase was evaporated to dryness. The same procedures were then followed for a partition between acetonitrile and *n*-hexane (20 ml each). The lower (acetonitrile) phase was again evaporated to dryness.

Chlorophacinone and diphenadione oxidize to form *p*-chlorobenzophenone and benzophenone, respectively, which are readily analyzed by glc. Chromium trioxide solution, prepared by dissolving 1.5 g of CrO_3 in 1 ml of water and adding 59 ml of acetic acid, was used to form these products. The solution (3 ml for plasma and 8 ml for all other samples) was added to each 25 × 150 mm culture tube containing the dry cleanup residue. The mixture was refluxed 30 min at 93°. If the mixture begain to turn green, indicating incomplete oxidation, more CrO_3 solution was added.

After refluxing, the oxidation mixture and water rinses (totaling 20 ml) were transferred to a 60-ml separatory funnel and partitioned with 20 ml of *n*-hexane. The aqueous layer was discarded and two additional water partitions (20 ml each) removed the remaining traces of the acetic acid. The hexane extract and two 3-ml rinses of the separatory funnel were then dried by passing them through a 300 × 10 mm i.d. chromatography tube packed with 10 g of granular anhydrous sodium sulfate. The extract and rinses were collected in a 250-ml Kuderna-Danish evaporative concentrator, concentrated to near dryness, and brought with hexane to an appropriate volume for glc.

U.S. Fish and Wildlife Service, Wildlife Research Center, Federal Center, Denver, Colorado 80225.

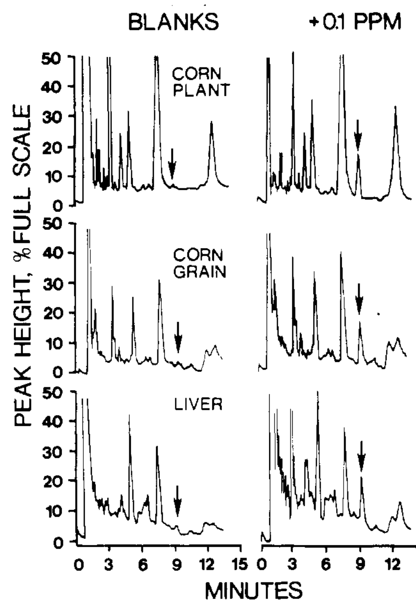

Figure 1. Gas-liquid chromatograms of diphenadione-fortified plant and animal tissue samples. The arrow represents the retention time of the oxidation product, benzophenone.

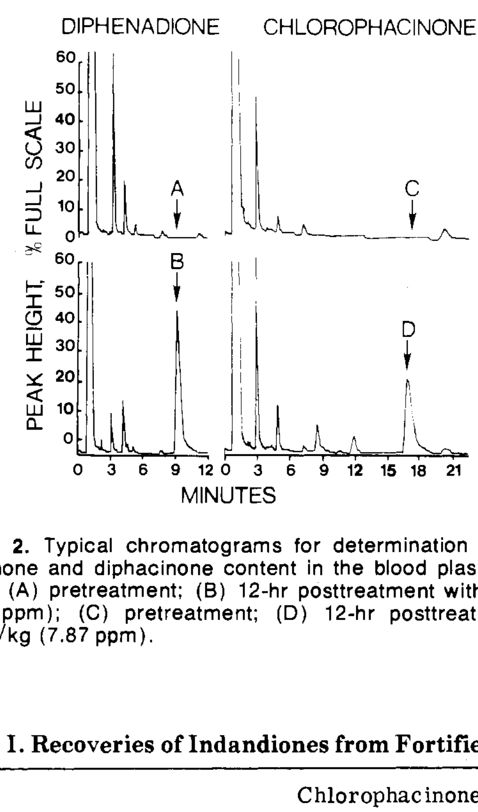

Figure 2. Typical chromatograms for determination of chlorophacinone and diphacinone content in the blood plasma of test cattle: (A) pretreatment; (B) 12-hr posttreatment with 3 mg/kg (6.34 ppm); (C) pretreatment; (D) 12-hr posttreatment with 10 mg/kg (7.87 ppm).

Gas-Liquid Chromatography. Determinations were performed on an Aerograph 1520B gas chromatograph equipped with a 0.0625-in. i.d. injection port liner and a tritium foil electron-capture detector. The column was a 100 ft long × 0.03 in. i.d. stainless steel capillary coated with OV-101 containing 5% Igepal CO-880. Operating parameters were: injection port, 225°; column, 175°; and nitrogen flow, 12 ml/min. Nitrogen makeup gas (23 ml/min) was added between the column and detector.

Under these conditions, benzophenone and *p*-chlorobenzophenone had retention times of 9.2 and 17 min, respectively. Figures 1 and 2 represent typical chromatograms. The samples were quantitated by comparison of the peak height with that of an appropriate standard. A regression equation based on the glc analysis of fortified samples was established for each material and used subsequently to predict the concentration of unknown samples.

RESULTS AND DISCUSSION

Protein binding to plasma albumin is characteristic of coumarin anticoagulants (O'Reilly and Aggeler, 1970), which behave much like the indandiones. Because most biological materials contain protein, all samples were subjected to mild acid hydrolysis. In addition, ascorbic acid is required in plasma samples to prevent complexing of the drug with the oxidized heme that is present as a result of hemolysis (Schulert and Weiner, 1954) and was added to other samples as a precaution against similar complexing.

Both chlorophacinone and diphenadione proved unsuitable for conventional glc analysis, but oxidation provides derivatives with short retention times and excellent electron-capture characteristics. The results of our oxidation process (Table I) were consistent with a recent report of chromic acid oxidation of *gem*-diphenyl substituted compounds to benzophenone (Vessman *et al.*, 1970). The efficiency of the conversion decreases as the anticoagulant concentration increases; this is assumed to be a property of the reaction mechanism. In initial studies we found yields to be constant but different for each concentration. Yields were unchanged after 30-min reaction time at temperatures from 60 to 140°; we chose 93° simply because it is easiest to maintain on multiple heating racks.

Our early methodological development and most of our analyses have been with plasma samples. Table I shows the recovery from fortified plasma samples. Regression equations are derived for each sample material from such data. The equations therefore incorporate all corrections for recovery, molecular weight change, oxidation yield, etc.

For chromatography of plasma and most tissue samples, packed columns containing XE-60, OV-225, OV-17, or

Table I. Recoveries of Indandiones from Fortified Bovine Plasma

	Chlorophacinone			Diphenadione		
Added ppm	% oxidation yield[a]	ppm found ± SD[b,c]	Mean % recov.[c]	% oxidation yield[a]	ppm found ± SD[b,c]	Mean % recov.[c]
0.000		0.000 ± 0.000		0.00	0.000 ± 0.000	0.00
0.050	111.03	0.026 ± 0.004	52.00	97.76	0.044 ± 0.002	88.00
0.100	88.75	0.066 ± 0.006	66.00	99.81	0.082 ± 0.004	82.30
0.250	76.41	0.192 ± 0.004	76.80	95.59	0.195 ± 0.008	78.12
0.500	70.38	0.349 ± 0.024	69.80	86.62	0.366 ± 0.028	73.10
1.000	69.21	0.692 ± 0.008	69.97	63.87	0.752 ± 0.062	75.20
Mean			66.91			79.34

[a] Yield determined in a separate experiment where blanks, in triplicate, were fortified just before oxidation. [b] Mean and standard deviation of four samples. [c] Values have been corrected for the oxidation yield and molecular weight conversion of the derivative measurement.

OV-101 coated on Gas-Chrom Q can be used, but we found that none of these provided adequate resolution with milk samples. Rather than add an additional cleanup step for milk or change columns for different samples, we use the 100 ft × 0.03 in. capillary column for all analyses.

Although whole blood can be analyzed by the glc procedure used for plasma, the chromatograms are more complex and variable, and an abundance of ascorbic acid must be added to prevent heme complexing. In a preliminary test using blood from a lamb dosed with 1 mg/kg of diphenadione, results were poor when whole blood was processed as described for plasma, but when the ascorbic acid was increased from 10 to 100 mg, recovery from whole blood was improved 38% and the residue value determined for whole blood was within 3% of that determined for plasma (corrected for 37.5 hematocrit value). In another test, no diphenadione could be detected in the blood cell fraction removed by centrifugation when it was washed with physiological saline to remove all remaining traces of plasma. Therefore, since the plasma fraction appears to contain essentially all the anticoagulant and residue levels in plasma can be converted by the appropriate hematocrit value to levels in whole blood, we use the simpler plasma analysis.

Figures 1 and 2 illustrate typical experimental uses of the procedure. It has been used routinely in analyzing plasma, liver, kidney, muscle, fat, and milk samples, and satisfactory results have also been obtained with foliage and grain samples from wheat, oats, and corn. The lower limit of detectability for these materials ranges from about 10 ppb for plasma samples down to 0.5 ppb for milk samples.

An additional option is available for plasma samples in which the anticoagulant is known and the samples after preparation and cleanup contain 5 μg or more. We have routinely analyzed such samples by redissolving the dry sample residue from cleanup in 3 ml of acetonitrile, transferring to a quartz cuvette, and analyzing by uv spectrophotometry with a Beckman Model DK-2A recording spectrophotometer at 325 nm. (If the anticoagulant is not detectable by this means, 2 ml can be recovered from the cuvette and analyzed by glc.) Concentrations in unknown samples are determined by substituting the absorbance value into a linear regression equation derived from analysis of fortified plasma samples. Although this method will not distinguish between chlorophacinone and diphenadione or determine concentrations below about 5 ppm, it considerably simplifies the routine analysis of sample series where it is appropriate.

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LITERATURE CITED

Caswell, R. L., *J. Ass. Offic. Anal. Chem.* **42,** 104 (1959).
Chempar Chemical Co., unpublished data, 1971.
Danek, A., Kwiek, J., *Diss. Pharm.* **16,** 359 (1964).
Hollifield, H. C., Winefordner, J. D., *Talanta* **14,** 103 (1967).
Lund, M., *J. Hyg.* **69,** 69 (1971).
O'Reilly, R. A., Aggeler, P. M., *Pharm. Rev.* **22,** 35 (1970).
Ozolins, N., Egerts, V., Krauja, A., *Latv. PSR Zinat. Akad. Vestis, Kim. Ser.,* 675 (1963).
Rowe, F. P., Redfern, R., *Ann. Appl. Biol.* **61,** 322 (1968).
Saunders, J. P., Heisey, S. R., Goldstone, A. D., Bay, E. C., *J. Agr. Food Chem.* **3,** 762 (1955).
Schulert, A. R., Weiner, M., *J. Pharmacol. Exp. Ther.* **110,** 451 (1954).
Thompson, R. D., Mitchell, G. C., Burns, R. J., *Science* **177,** 806 (1972).
Vessman, J., Hartvig, P., Strömberg, S., *Acta Pharm. Suecica* **7,** 373 (1970).

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Gas–Liquid Chromatographic Determination of Sencor (Metribuzin) and Its Major Metabolites and Photoproduct

G. R. Barrie Webster,* Sagietta R. Macdonald,[1] and Leonard P. Sarna

A new method has been developed which enables simultaneous glc analysis of the *as*-triazinone herbicide Sencor (metribuzin) (21087-64-9) and its three major metabolites and degradation products. Best results were obtained using 3% Silar 5CP on Chromosorb W (acid washed, DMCS treated). Tritium electron capture detection was more sensitive (0.01 ng of Sencor minimum), but was not selective with respect to soil coextractives. Coulson conductivity detection was less sensitive (1 ng of Sencor minimum), but provided selective quantitation of nitrogen containing compounds; no interfering peaks were observed in soil extracts. An improved method of glc analysis for Sencor alone using the Melpar flame photometric detector is described. Linear response over the range 3 to >150 ng of Sencor was observed. No interfering peaks were observed in soil extracts; the quantifiable minimum was 1 ng of Sencor.

The new herbicide Sencor (BAY 94337, metribuzin) has been registered for several years in Canada for use against broad-leafed and grassy weeds in potatoes, and is now registered in the United States for similar use on soybeans. Use on other crops is being investigated (Saidak, 1974; Duke and Hunt, 1972; Osgood, 1972). The efficacy of Sencor is complemented by its low mammalian toxicity (acute oral LD_{50}^{rat} 1960 mg/kg; Chemagro Corporation, 1971). Sencor has shown no carryover in United States trials, but has shown a tendency, following use in some areas of western Canada, to persist in the soil causing injury to subsequent crops which are not tolerant to Sencor (Stobbe, 1972; Bowden, 1973).

Contribution No. 1 from the Pesticide Research Laboratory, Department of Soil Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2.
[1] Present address: Freshwater Institute, Winnipeg, Manitoba, Canada R3T 2N6.