Determination of 2,4,5-Trichlorophenoxyacetic Acid and Its Propylene Glycol Butyl Ether Esters in Animal Tissue, Blood, and Urine

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A gas chromatographic method for the determination of residues of 2,4,5-trichlorophenoxyacetic acid and its propylene glycol butyl ether esters in tissues and fluids is described. Both compounds were converted to the methyl ester of 2,4,5-T and analyzed by microcoulometric gas chromatography using a column of 15% Dow 710 on Chromport XXX. Average recoveries of 2,4,5-T added to fat, lean tissue, urine, and blood at levels from 0.05 p.p.m. to 20 p.p.m. were 89.3, 89.6, 93.0, and 93.6%, respectively. Corrected recovery of unmetabolized ester added to fat, lean tissue, urine, and blood at levels from 0.5 p.p.m. to 20 p.p.m. averaged 77.9, 70.5, 94.2 and 92.5%, respectively.

The compounds 2,4,5-trichlorophenoxyacetic acid, propylene glycol butyl ether esters (2,4,5-TE) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) are members of a large family of chlorophenoxy compounds which find worldwide use in the control of broadleaf plants. These herbicides are frequently used in close proximity to livestock which are destined for human consumption. For this reason, it is necessary to establish the metabolism, elimination, and residue deposition characteristics of such compounds. In order to investigate the absorption, metabolism, elimination, and residue deposition of 2,4,5-TE and 2,4,5-T, it was necessary to develop analytical techniques which are sensitive, selective, and reproducible.

This report describes a gas chromatographic method for the analysis of 2,4,5-TE and 2,4,5-T in tissue, blood, and urine.

MATERIALS AND METHODS

Herbicides. The herbicides were analytical standards and included 2,4,5-trichlorophenoxyacetic acid, and 2,4,5-trichlorophenoxyacetic acid, propylene glycol butyl ether esters (Dow Chemical Co., Midland, Mich.).

Reagents. Reagent grade diethyl ether, petroleum ether, chloroform, acetonitrile, methanol, and methylene chloride were redistilled in an all-glass system. Ethanol, 95% (U.S.P.), was used as received without further purification.

Boron trifluoride/methanol reagent (Applied Science Laboratories, State College, Pa.), papain, N.F. VIII (Difco Laboratories, Detroit, Mich.), and Florisil, 60/100-mesh (The Floridin Co., Talahassee, Fla.) were used as received without further treatment. The acetate buffer, pH 5.0, contained 11.4 ml. of glacial acetic acid and 7.5 of grams NaOH per liter of water.

Gas Chromatography. A Micro-Tek 2500R gas chromatograph fitted with a Dohrmann Microcoulometric detection system and a Honeywell 1-mv. recorder was used. The column was of stainless steel, 5 feet long and 1/8 inch in diameter, and filled with 15% Dow 710 silicone on acid-washed Chromport XXX (60- to 80-mesh). The column effluent passed through a heated aluminum transfer line into a heated quartz tube, where the organic compounds in the column effluent were combusted in the presence of oxygen. The resulting hydrogen halides were subsequently titrated coulometrically in the Dohrmann T-300 halogen titration cell.

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The operating conditions for the gas chromatographic analysis are as follows:

Column temperatures: 210° C. isothermal; injection port, 225° C.; transfer line, 212° C.; detector inlet, 245° C.; combustion tube, 840° C.

Carrier gas: Prepurified nitrogen (The Matheson Co., LaPorte, Texas), 50 cc. per minute, 42 p.s.i.

Oxygen: 65 cc. per minute, 20 p.s.i.

Sweep gas: Prepurified nitrogen, 20 cc. per minute, 42 p.s.i.

Bias voltage: 240 mv.

Sensitivity range: 450 ohms.

Preparation of Methyl Esters of 2,4,5-T and 2,4,5-TE. Pipet 1 to 5 ml. of the standard or petroleum ether solution of tissue extract into a 10-ml. volumetric flask. Add two drops of a 10% methanolic KOH solution and evaporate to dryness in a water bath. A gentle stream of clean, dry air may be used to expedite evaporation. Add 2 ml. of the boron trifluoride/methanol reagent. Boil in a steam bath for 2 minutes. After the flask has cooled, add 1 to 5 ml. of petroleum ether and swirl to dissolve the sample. Bring up to volume with 2% aqueous sodium sulfate. This causes the petroleum ether solution containing the 2,4,5-T methyl ester to be forced into the neck of the flask thus allowing easy sampling. Inject up to 25 μl. of the 2,4,5-T methyl ester solution into the chromatograph for analysis.

Standard Curves. A series of standards of 2,4,5-T and 2,4,5-TE were prepared by dissolving the technical materials in petroleum ether and making appropriate dilutions. Methyl ester derivatives of both compounds were prepared as described and standard concentration curves established.

Analytical Procedures. PREPARATION, EXTRACTION, AND CLEANUP. TISSUE. Twenty-five-gram portions of lean tissue were sliced into thin layers, frozen, and dried overnight in the freeze-drier. (Fat samples were not freeze-dried.) The dried tissue was removed from the freeze-drier, placed in a Waring Blendor, and homogenized three times with 100-ml. portions of boiling 95% ethanol. Following each homogenization, the ethanol extracts were vacuum filtered through Whatman No. 1 filter paper into a common container and the solid material was returned to the Blendor. Following the third homogenization and filtration, the residue was discarded and the ethanol removed by evaporation over a hot water bath with a dry air stream. (For fat samples, the alcohol extract was chilled in a dry ice–ethanol bath, filtered, and the solidified fat discarded prior to evaporation.) The residue was then taken up in 100 ml. of acetate buffer (pH 5.0) containing 0.5 gram of papain and a few grains of NaCN. The flask was then placed in a 65° C. water bath and digested for

1 hour. Following digestion, the solution was transferred to a 250-ml. separatory funnel and the aqueous solution acidified with 3 ml. of concentrated HCl. The 2,4,5-TE and 2,4,5-T were extracted from the acidic solution with one 50-ml. portion, followed by three 25-ml. portions of methylene chloride. In some cases, centrifugation was necessary to achieve complete layer separation. Water was removed from the methylene chloride by passing the extract through a Na_2SO_4 column. The dried methylene chloride was collected in a 250-ml. Erlenmeyer flask, then evaporated to near dryness. The concentrate was transferred to a 125-ml. separatory funnel with 50 ml. of petroleum ether, and further cleanup accomplished by partitioning three times into 25-ml. portions of acetonitrile. The acetonitrile was then evaporated and the residue transferred to the Florisil elution column with 25 ml. of petroleum ether.

Blood. Whole blood samples of 5 ml. each were placed directly into 125-ml. Erlenmeyer flasks which contained 50 ml. of acetate buffer, 0.5 gm. of papain, and a few grains of NaCN. The flasks were placed in the water bath and the sample was digested for 30 minutes at 65° C. The remainder of the procedure is as described for tissue.

Urine. Twenty-five ml. of 7% HCl in ethanol were added to 125-ml. separatory funnels, containing the 25-ml. urine samples, and mixed by shaking. The 2,4,5-TE and 2,4,5-T were extracted with one 50-ml. portion of chloroform. The chloroform was removed from the extract by evaporation. The chloroform-free residue was transferred with 15 ml. of petroleum ether to the Florisil column for cleanup.

Column Chromatography. The cleanup columns were prepared by adding 7.5 grams of Florisil to a 50-ml. Mohr buret plugged at the bottom with glass wool. The Florisil was then washed with 25 ml. of 2.5% H_3PO_4 in methanol, followed by 15 ml. of methanol. After the methanol had percolated into the adsorbent, 5 grams of anhydrous Na_2SO_4 and a glass wool plug were added above the Florisil. The column was then washed with 25 ml. of diethyl ether followed by 25 ml. of petroleum ether. The concentrated extract was added as soon as the last of the washing solvents had passed below the surface of the top glass wool plug. The unmetabolized esters were eluted with 125 ml. of the 20% diethyl ether in petroleum ether. The metabolite, 2,4,5-T, was then eluted from the column with 150 ml. of 0.5% H_3PO_4 in diethyl ether. The solutions were concentrated to less than 5 ml., transferred to 10-ml. volumetric flasks and diluted to the mark. Aliquots were transferred to 10-ml. volumetric flasks and the herbicides esterified for analysis as described.

Hydrolysis of 2,4,5-Trichlorophenoxyacetic Acid, Propylene Glycol Butyl Ether Esters by Raw Muscle. In developing the analytical method for 2,4,5-TE, it was observed that in experiments in which the ester was added to raw tissue, the recovery of the ester was so erratic as to indicate the method was useless. This brought up the question of possible enzymatic hydrolysis of the ester to 2,4,5-T.

To test this hypothesis, six samples of sheep muscle weighing 25 grams each were spiked with 500 μg. of 2,4,5-TE and allowed to stand at room temperature for various time intervals: 1/4, 1/2, 1, 2, and 3 hours, then fast-frozen and freeze-dried. One sample was frozen at the time of spiking and dried immediately.

The samples were then analyzed, as previously described, for both the ester and the metabolite, 2,4,5-T. The results are shown in Figure 1.

Determination of Analytical Efficiency. For 2,4,5-T, known amounts were added to the various tissue samples, allowed to stand for about 30 minutes, then frozen, freeze-dried, and analyzed by the described procedure. Percentage of recoveries was then calculated from the result (Table I).

To determine the efficiency of the procedure for 2,4,5-TE, it was necessary to determine both 2,4,5-TE and the metabolite, 2,4,5-T. Known quantities of 2,4,5-TE were added to the tissues which were then analyzed for both compounds.

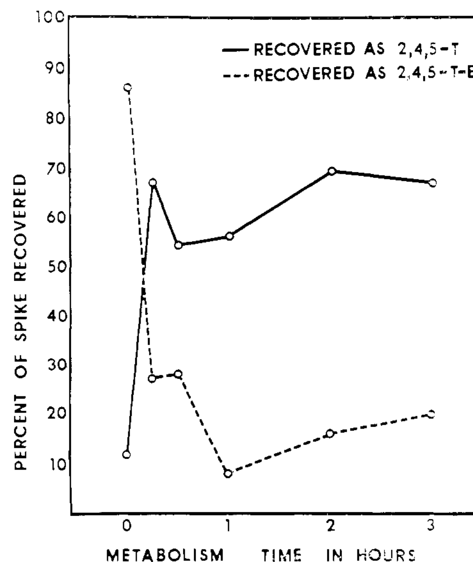

Figure 1. Hydrolysis of 2,4,5-TE to 2,4,5-T by raw muscle

Table I. Percentage Recovery of 2,4,5-T from Tissues Fortified with Known Amounts of 2,4,5-T before Extraction and Cleanup

Tissue	2,4,5-T Added, p.p.m.	Recovery, %[a]
Fat	0.00	0
	0.05	75
	0.10	89
	0.50	87
	1.00	85
	5.00	90
	10.00	94
	20.00	98
	40.00	96
Lean (Muscle, Liver, Kidney)	0.00	0
	0.05	78
	0.10	77
	0.50	96
	1.00	88
	5.00	99
	10.00	97
	20.00	92
Urine	0.00	0
	0.05	86
	0.10	94
	0.50	87
	1.00	93
	5.00	99
	10.00	95
	20.00	97
Blood	0.00	0
	0.05	91
	0.10	88
	0.50	92
	1.00	98
	5.00	95
	10.00	97
	20.00	94

[a] Average of three samples at each level.

Table II. Percentage Recovery of 2,4,5-T and Its Propylene Glycol Butyl Ether Esters (2,4,5-TE) from Tissues Fortified with Known Amounts of 2,4,5-TE before Extraction and Cleanup

Tissue	2,4,5-TE Added, p.p.m.	Recovery, % As 2,4,5-TE	As 2,4,5-T (Uncorrected)	Analytical Efficiency for 2,4,5-TE
Fat	0.0	0	0	...
	0.5	45	28	66.4
	1.0	52	18	66.0
	5.0	62	20	79.7
	10.0	58	25	79.0
	20.0	65	20	81.7
	40.0	75	15	88.9
Lean (Muscle, Liver, Kidney)	0.0	0	0	...
	0.5	10	72	40.0
	1.0	30	47	64.4
	5.0	13	68	41.5
	10.0	9	78	46.0
	20.0	4	85	52.6
Urine	0.0	0	0	...
	0.5	90	0	90.0
	1.0	88	0	88.0
	5.0	97	0	97.0
	10.0	95	0	95.0
	20.0	98	0	98.0
Blood	0.0	0	0	...
	0.5	78	10	87.5
	1.0	80	15	94.5
	5.0	74	20	93.5
	10.0	67	28	94.2
	12.5	77	15	91.4
	20.0	80	12	91.7

To calculate the efficiency of the procedure for 2,4,5-TE, the quantity of 2,4,5-T recovered was corrected to 100% efficiency based on the data in Table I. The corrected 2,4,5-T recovery was then subtracted from 100% to give the part of the total spike which was not converted to 2,4,5-T. This value was then divided into the percentage of the original spike, which was recovered as 2,4,5-TE, and the result reported as analytical efficiency for 2,4,5-TE (Table II).

RESULTS AND DISCUSSION

Retention time for the methyl ester of 2,4,5-T was 1.5 minutes with no interfering peaks (Figure 2). Control samples produced base line readings in the critical region. As little as 1.0 ng. equivalent of 2,4,5-T or 1.5 ng. equivalent 2,4,5-TE could be detected. The equations for the linear regression of the standard curves for 2,4,5-T and 2,4,5-TE were $\hat{X} = 0.21(Y) + 2.03$ and $\hat{X} = 0.29(Y) - 0.19$, respectively, where $\hat{X}$ is the expected peak height (per cent of full scale) and Y is nanograms of herbicide equivalent injected.

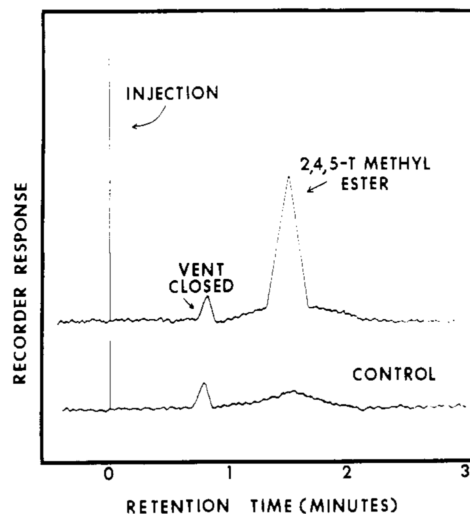

Figure 2. Chromatograms of extracts of control fat, and of omental fat to which 2,4,5-T was added

Like 2,4-D, the trichlorophenoxy compounds were efficiently extracted from tissue with hot ethanol (Clark *et al.*, 1967). Filtration of the alcohol extract was enhanced considerably by freeze-drying the tissue prior to extraction. Also, troublesome emulsions were eliminated, which sped up the filtration step. In addition, the solid residue was much easier to transfer back to the Blendor following filtration.

As with 2,4-D, digestion of the extracted residue with papain was necessary in order to quantitatively recover the 2,4,5-T compounds (Clark *et al.*, 1967).

Average recovery of 2,4,5-T added to fat, lean tissue, urine, and blood at various levels were 89.3, 89.6, 93.0, and 93.6%, respectively (Table I). In recovery experiments in which the ester was added to raw tissue, it was necessary to analyze for both the ester and the 2,4,5-T acid. As shown in the rate of hydrolysis experiment (Figure 1), the degree of conversion from ester to acid depends largely upon the tissues involved and the time the herbicide is in contact with the raw tissue before freezing. Recovery of the unmetabolized ester from fat, lean tissue, urine, and blood averaged 77.9, 70.5, 94.2, and 92.5%, respectively.

LITERATURE CITED

Clark, Donald E., Wright, Fred C., Hunt, LaWanda M., J. AGR. FOOD CHEM. **15**, 171 (1967).

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