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Note

Metabolism of 2,4-dichlorophenoxyacetic acid

IX. Gas–liquid chromatography of methyl esters of amino acid conjugates

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Recent investigations have shown that amino acid conjugation plays an important role in the metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D)[1–6]. A number of these conjugates have been isolated and identified from various plant tissues, however, residue analysis usually only involves the quantitation of 2,4-D[7–11]. The amino acid conjugates of 2,4-D are biologically active[5] and analytical methods are needed to analyze for these metabolites or potential metabolites in the natural ecosystem. Recently a method for gas–liquid chromatography (GLC) of the trimethylsilyl derivatives of the amino acid conjugates of 2,4-D has been reported[12,13]. This method is appropriate for characterization and quantification of the conjugates, however, it lacks the sensitivity needed for residue analysis since these derivatives are not suitable for electron capture detection. This manuscript now reports the GLC separation of the methyl esters of 2,4-D, its hydroxylated metabolites and thirteen amino acid conjugates of 2,4-D.

EXPERIMENTAL

Reagents

All solvents used were of highest purity. 2,4-D and Diazald were purchased from Aldrich (Milwaukee, Wisc., U.S.A.). *n*-Octacosane was purchased from Supelco (Bellefonte, Pa., U.S.A.).

4-Hydroxy-2-chlorophenoxyacetic acid (4OH-2Cl) was obtained from Dr. J. Fleeker (North Dakota State University) while 4-hydroxy-2,3-dichlorophenoxyacetic acid (4OH-2,3-D), 4-hydroxy-2,5-dichlorophenoxyacetic acid (4OH-2,5-D) were previously synthesized in this laboratory. All amino acid conjugates of 2,4-D were supplied by Dr. C. S. Feung (Pesticide Research Laboratory, The Pennsylvania State University). The amino acid conjugates of 2,4-D were: 2,4-dichlorophenoxyacetylalanine (2,4-D-Ala), 2,4-dichlorophenoxyacetylarginine (2,4-D-Arg), 2,4-dichlorophenoxyacetylaspartic acid (2,4-D-Asp), 2,4-dichlorophenoxyacetylcysteine (2,4-D-Cys), 2,4-dichlorophenoxyacetylglutamic acid (2,4-D-Glu), 2,4-dichlorophenoxyacetylglycine (2,4-D-Gly), 2,4-dichlorophenoxyacetylhistidine (2,4-D-His), 2,4-dichlorophenoxyacetylisoleucine (2,4-D-Ile), 2,4-dichlorophenoxyacetylleucine (2,4-D-Leu), 2,4-di-

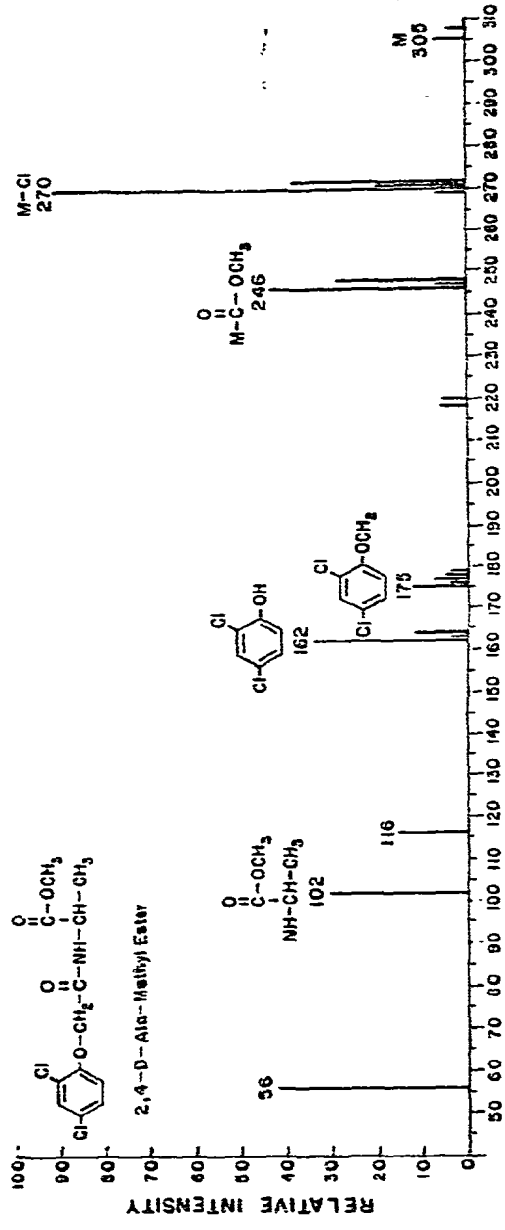

Fig. 1. The mass spectra of the methyl esters of 2,4-D-Ala.

chlorophenoxyacetyllysine (2,4-D-Lys), 2,4-dichlorophenoxyacetylmethionine (2,4-D-Met), 2,4-dichlorophenoxyacetylproline (2,4-D-Pro), 2,4-dichlorophenoxyacetylphenylalanine (2,4-D-Phe), 2,4-dichlorophenoxyacetylserine (2,4-D-Ser), 2,4-dichlorophenoxyacetylthreonine (2,4-D-Thr), 2,4-dichlorophenoxyacetyltryptophan (2,4-D-Trp), 2,4-dichlorophenoxyacetyltyrosine (2,4-D-Tyr), and 2,4-dichlorophenoxyacetylvaline (2,4-D-Val).

Instruments

A MicroTek 220 gas chromatograph equipped with an Infotronics Model II digital integrator was used for this investigation. Silylanized 6 ft. × 4 mm I.D. glass columns were employed with column packings of 2% OV-1 on 100–120 mesh Supelcoport, and 1% OV-17 on 80–100 mesh Supelcoport. Dual flame ionization detectors were used. The instrument conditions were as follows: column temperature varied from 150 to 280°; detector temperature, 280°; inlet temperature, 245°; nitrogen flow-rate, 50–60 ml/min.

Mass spectra were obtained on an LKB-9000 gas–liquid chromatograph interfaced mass spectrometer using a 6 ft. × 3/16 in. O.D. glass column packed with 2% OV-1 on Supelcoport 100–120 mesh, a helium flow-rate of 30 ml/min and a variable column oven temperature.

Preparation of methyl ester derivatives

Diazomethane was prepared from Diazald[14]. Stock solutions of each of the compounds including the internal standard *n*-octacosane were prepared (1 mg/ml in ethanol). One milliliter of each stock solution was added to a 30-ml methylation vial. The alcohol was evaporated under nitrogen at 60°. The residue was dissolved in 10 ml diethyl ether and molar excess of freshly prepared diazomethane was added. After 1 h the ether was evaporated under nitrogen and the methylated products were dissolved in 5 ml benzene (0.2 μg/μl of each compound) prior to analysis.

RESULTS AND DISCUSSION

The amino acid conjugates and hydroxylated derivatives of 2,4-D were completely converted to their methyl esters with diazomethane. The phenolic hydroxyls of 4OH-2Cl, 4OH-2,3-D and 4OH-2,5-D were also methylated under these reaction conditions and their structures were confirmed by mass spectrometry. The basic amino acid conjugates (Lys, Arg, and His) were not soluble in ethyl ether and were not included in this study. All the methyl esters were stable over an 8-day period except for the methyl ester of 2,4-D-Met which was stable in a pure form but unstable in the mixture of methyl esters.

The various derivatives were analyzed by mass spectrometry to confirm their structure and the mass spectra of the methyl esters of 2,4-D-Ala is given in Fig. 1. All the methyl esters analyzed gave strong molecular ions and characteristic fragmentations typical of the amino acid conjugate. These data suggest the mass spectra of the esters may be more useful for identification purposes than the reported mass spectra[3] of the underivatized conjugates. Methyl and ethyl esters were always significant contaminants of the isolation procedure of the amino acid conjugate of 2,4-D. They

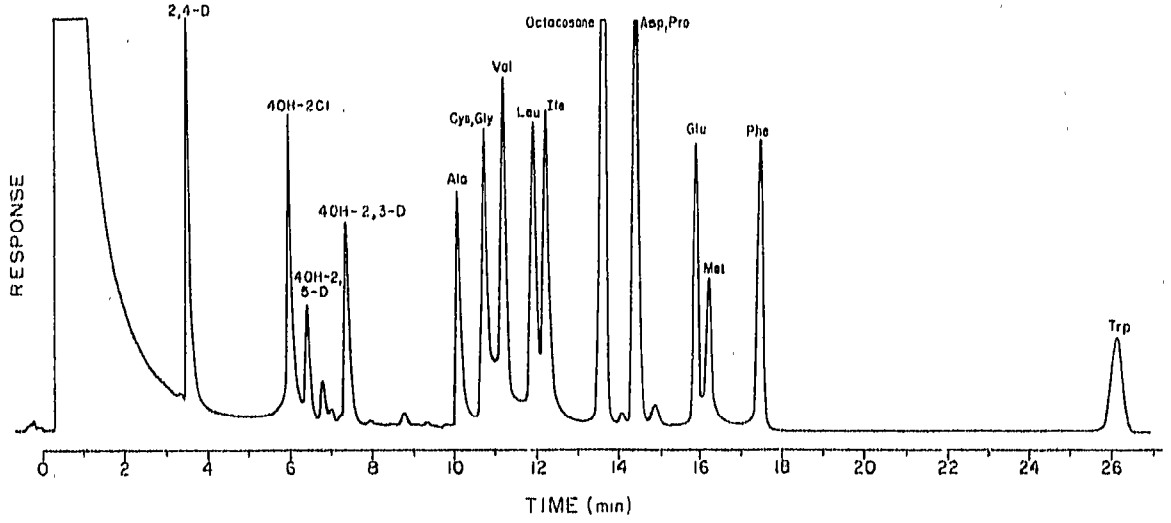

Fig. 2. Gas–liquid chromatogram of methyl esters of 2,4-D-metabolites. Column: 1% OV-17 on 80–100 mesh Supelcoport, 6 ft. × 4 mm I.D. glass. Temperature programmed at a rate of 5°/min up to 280°, initial temperature 150°. Each peak represents *ca.* 2 μg. Flow-rate, 60 ml/min.

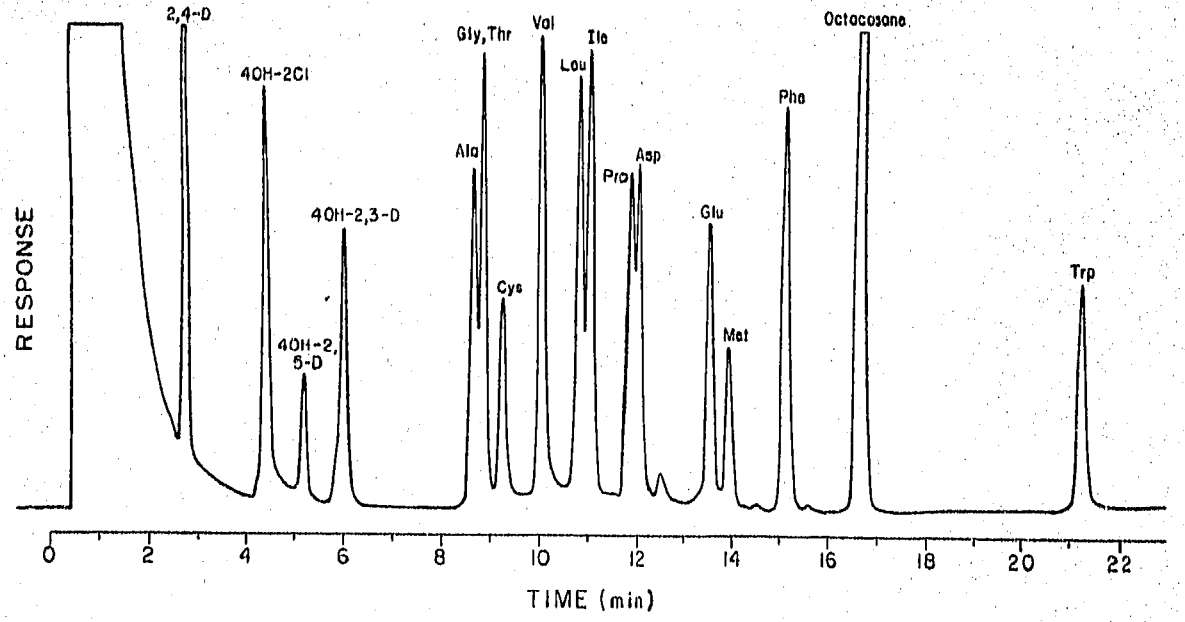

Fig. 3. Gas–liquid chromatogram of methyl esters of 2,4-D-metabolites. Column: 2% OV-1 on 100–120 mesh Supelcoport, 6 ft. × 4 mm I.D. glass. Temperature programmed at a rate of 5°/min up to 280°, initial temperature 150°. Each peak represents *ca.* 2 μg. Flow-rate, 60 ml/min.

were inadvertently produced in the thin-layer chromatographic (TLC) isolation procedures.

The separation achieved on GLC of the methyl esters of the amino acid conjugates of 2,4-D and other hydroxylated metabolites of 2,4-D on 1% OV-17 (80–100 mesh Supelcoport) is illustrated in Fig. 2. Twelve of the 16 methyl esters (2,4-D, 4OH-2Cl, 4OH-2,5-D, 4OH-2,3-D and 8 amino acid conjugates, Ala, Val, Leu, Ile, Glu, Met, Phe and Trp) were completely separated in 26 min. The compounds which overlapped were (1) 2,4-D-Cys and 2,4-D-Gly, and (2) 2,4-D-Asp and 2,4-D-Pro. The separation achieved on 1% OV-17 would be sufficient to analyze for all of the identified 2,4-D metabolites (2,4-D, 4OH-2Cl, 4OH-2,5-D, 4OH-2,3-D, 2,4-D-Asp, 2,4-D-Glu, 2,4-D-Ala, 2,4-D-Val, 2,4-D-Leu, 2,4-D-Phe and 2,4-D-Trp).

The GLC separation achieved on 2% OV-1 (100–120 mesh Supelcoport) is shown in Fig. 3. Ten methyl ester derivatives were separated completely (2,4-D, 4OH-2Cl, 4OH-2,5-D, 4OH-2,3-D, 2,4-D-Cys, 2,4-D-Val, 2,4-D-Gly, 2,4-D-Glu, 2,4-D-Met, 2,4-D-Phe, and 2,4-D-Trp). Five derivatives partially separated, and only two compounds did not separate (2,4-D-Gly and 2,4-D-Thr). The relative retention times of each compound to internal standard (octacosane) for both separations are given in Table I. When both columns are used all of the 16 compounds examined can be separated.

TABLE I

RETENTION TIME OF METHYL ESTERS RELATIVE TO AN INTERNAL STANDARD (*n*-OCTACOSANE) ON 2% OV-1 AND 1% OV-17

Compound	*Relative retention time*	
	OV-1	*OV-17*
2,4-D	0.160	0.220
4OH-2Cl	0.251	0.435
4OH-2,5-D	0.296	0.478
4OH-2,3-D	0.353	0.545
2,4-D-Ala	0.499	0.748
2,4-D-Gly	0.512	0.797
2,4-D-Thr	0.512	—
2,4-D-Cys	0.538	0.792
2,4-D-Val	0.591	0.829
2,4-D-Leu	0.646	0.881
2,4-D-Ile	0.662	0.901
2,4-D-Pro	0.718	1.058
2,4-D-Asp	0.732	1.058
2,4-D-Glu	0.813	1.167
2,4-D-Met	0.846	1.190
2-4-D-Phe	0.911	1.289
2,4-D-Trp	1.273	1.913
n-Octacosane	1.000	1.000

All of the derivatives analyzed gave a linear response with the flame ionization detector over a range of 2–10 μg (Fig. 4) and are suitable for quantification using an internal standard[15]. Additional studies are needed to adapt these data to the sensitivity of electron capture detection and to develop a practical residue analysis procedure.

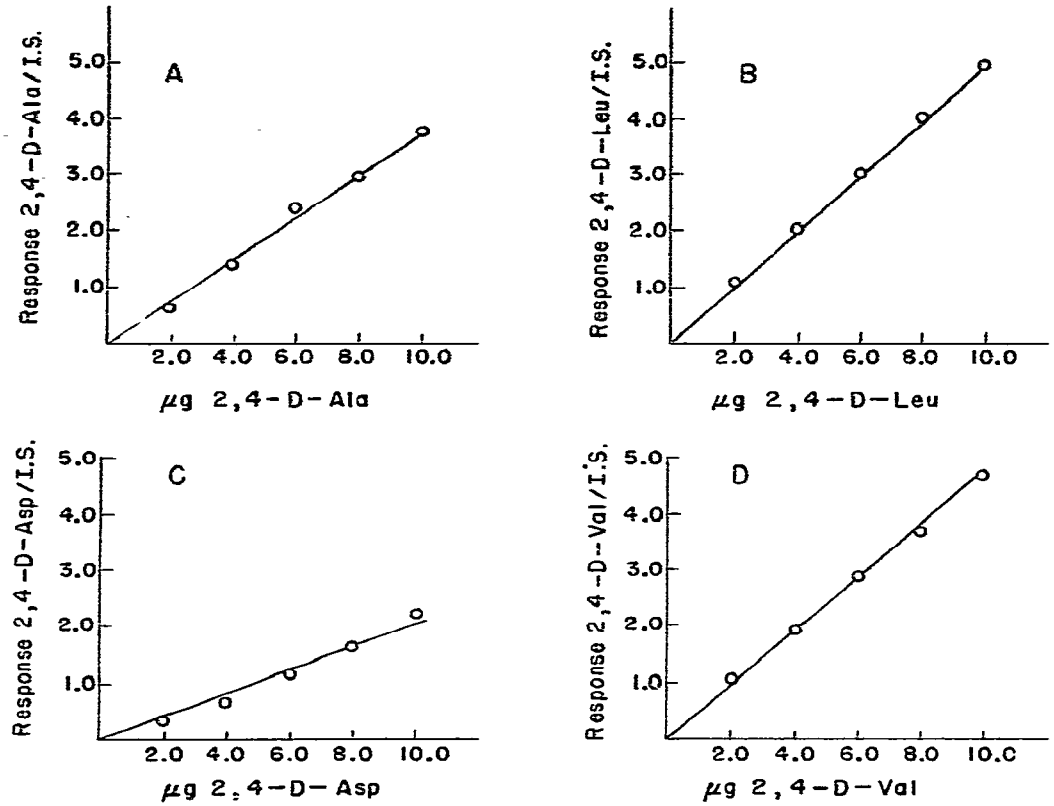

Fig. 4. Relative response of methyl ester derivatives *vs.* μg derivatives injected. (A), 2,4-D-Ala; (B), 2,4-D-Leu; (C), 2,4-D-Asp; and (D), 2,4-D-Val.

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