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Note

Determination of 2-ethylhexyl 2,4-dichlorophenoxyacetate in rat blood and urine by electron-capture gas chromatography and gas chromatography–mass spectrometry

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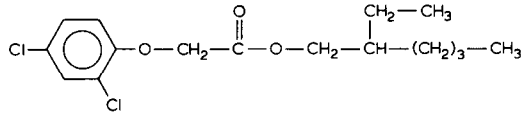
Formulations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-D esters are used as systemic herbicides for the control of broadleaf weeds in cereal crops, pastures and urban areas[1,2]. Plant roots absorb polar (salt) forms of 2,4-D most readily while leves absorb non-polar (ester) forms most readily. The esters of 2,4-D are rapidly converted to the acid by plants[1] and animals[2].

A study was designed to demonstrate that esters of 2,4-D hydrolyze rapidly to 2,4-D acid following oral administration[3]. If similar pharmacokinetic fates are demonstrated then it would suggest that the 2,4-D acid and esters would be toxicologically comparable. The 2-ethylhexyl 2,4-dichlorophenoxyacetate (2,4-D IOE), one of several isoctyl ester isomers, was selected for evaluation. A gas chromatographic–mass spectrometric (GC–MS) method for the determination of the 2,4-D acid in blood and urine had been developed and validated for a previous pharmacokinetic study[4]. Previous analytical methods for 2,4-D esters either could not be reproducibly quantitated or required conversion of the ester to the acid[5,6]. None of these methods allowed the direct comparison of the 2,4-D IOE (parent) and the acid. In this work, methods for the determination of 2,4-D IOE in rat whole blood and urine are described. A gas chromatograph equipped with an electron-capture detector was used for 2,4-D IOE concentrations between 10 and 1000 ng/ml. For higher concentrations a gas chromatograph–mass spectrometer was used in the selected ion monitoring mode.



(2,4-D IOE)

EXPERIMENTAL

Materials

The 2,4-D IOE (Batch No. L072AF1) was supplied by Vertac (Jacksonville, AR, U.S.A.). Distilled-in-glass UV-grade hexane was obtained from Burdick and

Jackson Labs. (Muskegon, MI, U.S.A.). Absolute ethanol (200 proof) was supplied by Union Carbide (Cincinnati, OH, U.S.A.). A Damon/IEC centrifuge, Model HN-SII, was purchased from VWR Scientific (Midland, MI, U.S.A.). Adult Fischer 344 male rats were purchased from the Charles River Breeding Labs. (Wilmington, MA, U.S.A.). The animals were anesthetized with carbon dioxide and fresh blood was withdrawn via cardiac puncture into a syringe containing heparin. Urine was collected from animals housed in a glass Roth-type metabolism cage designed for the separate collection of urine and feces.

Instrumentation

A Varian 3700 gas chromatograph equipped with a ^{63}Ni electron-capture detector and a Hewlett-Packard 3354 data system were used for the quantitative analysis of the samples containing 1 μg/ml and below of 2,4-D IOE. A glass column (2.2 m × 2 mm I.D.) packed with bonded PEG 20M, 80–100 mesh (HNU Systems, Newton, MA, U.S.A.) was used to obtain separation. During the analysis, the column was maintained at 185°C with a nitrogen flow-rate of 25 ml/min. The injection port and detector temperatures were 200°C and 250°C, respectively.

A Finnigan Model 3000 electron-impact gas chromatograph–mass spectrometer equipped with a Model 6000 data system was used for the quantitative analysis of samples above 1 μg/ml. A glass column (1.1 m × 2 mm I.D.) packed with 3% SP-2100 on 80–100 mesh Supelcoport was used to obtain separation. The injection port and column temperatures were 250°C and 220°C, respectively. The helium carrier gas flow-rate was 25 ml/min. The separator and transfer line temperatures were 270°C; the ionization energy was 70 eV, the electron multiplier was operated at $1.1 \cdot 10^3$ V and the preamplifier gain was 10^{-8} A/V. The 2,4-D IOE was determined by selective ion monitoring (SIM) of the fragment ions at *m/z* 220 and 222.

Nomenclature

To reduce any ambiguity in terminology, the following will be defined: control sample, standard, and spiked sample. A control sample is blood or urine to which no 2,4-D IOE has been added. A standard is a solution, containing a known concentration of 2,4-D IOE, to be used as a reference for quantitation. A spiked sample is blood or urine to which 2,4-D IOE has been added.

Preparation of solutions

Standards containing various concentrations of 2,4-D IOE were prepared by serial dilutions of a stock solution or by the addition of 2.5 μl of an appropriate spiking solution (discussed below) to a known volume of hexane. The stock solution for standard preparation was prepared by accurately weighing approximately 20 mg of 2,4-D IOE into a 10-ml volumetric flask and diluting to volume with hexane. Spiking solutions were prepared by serial dilution with ethanol and were used daily to reduce the possibility of transesterification.

Spiked samples were prepared by adding 2.5 μl of an appropriate spiking solution to an accurately weighed aliquot of blood or urine (nominally 500 mg). Solutions were agitated thoroughly on a Vortex mixer.

Extractions

Blood. The 2,4-D IOE spiked into blood (0.5 ml) was extracted within two minutes after preparation with 1 or 2 ml of dry ice-chilled hexane–ethanol (75–25) mixture. Spiked samples analyzed by GC–electron-capture detection (ECD) were extracted with 2 ml of solvent, in order to fill the autosampler vials (1.5 ml) for automatic injections. Extracted blood samples were kept chilled on dry ice until agitated via a Vortex mixer (1 min) and centrifuged at 1300 *g* (3–5 min). The organic supernatants were transferred into autosampler vials and 1-μl aliquots were injected automatically into the GC–ECD system. Aliquots (2 μl) of supernatants, containing 2,4-D IOE above 1 μg/ml, were manually injected and quantitated by GC–MS.

Urine. The 2,4-D IOE spiked into urine (0.5 ml) was mixed at room temperature with 0.5 ml of ethanol then extracted with 1 or 2 ml of hexane. Spiked urine samples were agitated, centrifuged, and analyzed under the same conditions as the spiked blood samples.

RESULTS AND DISCUSSION

Recovery of 2,4-D IOE at six different concentrations in blood, 0.04–100 μg/ml, ranged from 73.1 to 92.3% and had a mean recovery ± standard deviation (S.D.) of 81.2 ± 9.7% (Table I).

TABLE I

RECOVERY OF 2,4-D IOE FROM RAT BLOOD

*Targeted concentration 2,4-D IOE (μg/ml) in rat blood**	*Recovery of IOE in rat blood (%)***			*Mean recovery (%)*	*S.D.*	*Coefficient of variation*
	1	*2*	*3*			
0.04	85.4	89.6	80.6	85.2	4.5	5.3
0.12	80.3	77.1	73.9	77.1	3.2	4.2
0.4	86.4	68.9	80.3	78.5	8.9	11.3
4.0	82.1	74.6	62.5	73.1	9.9	13.5
40	79.6	80.7	82.4	80.9	1.4	1.7
100	100.0	105.9	71.0	92.3	18.7	20.3
Average				81.2	9.7***	11.3§

* Sample concentrations equal to and below 1 μg/ml were determined by GC–ECD. Sample concentrations above 1 μg/ml were determined by GC–MS.

** Each data point is the mean of two determinations.

*** Average standard deviation (σ) is $\sqrt{(\sigma^2)/n}$.

§ Average coefficient of variation (C.V.) is $\sqrt{(\text{C.V.}^2)/n}$.

Recovery for five 2,4-D IOE concentrations in urine, 0.12–100 μg/ml, ranged from 89.5 to 99.5% with a mean recovery ± S.D. of 95.0 ± 4.1% (Table II).

Representative GC–ECD chromatograms of a control blood extract, spiked blood extract (0.20 μg/ml final concentration), and a reference standard (0.20 μg/ml) are displayed in Fig. 1. Representative GC–ECD chromatograms of a control urine

TABLE II

RECOVERY OF 2,4-D IOE FROM RAT URINE

*Targeted concentration 2,4-D IOE (μg/ml) in rat urine**	*Recovery of IOE in rat urine (%)***			*Mean recovery (%)*	*S.D.*	*Coefficient of variation*
	1	*2*	*3*			
0.12	89.2	98.4	89.7	92.4	5.2	5.6
0.40	93.6	85.2	89.7	89.5	4.2	4.7
4.0	100.4	104.6	93.4	99.5	5.7	5.7
40	98.8	97.7	93.8	96.8	2.6	2.7
100	95.2	96.8	98.0	96.7	1.4	1.4
Average				95.0	4.1***	4.4§

* Sample concentrations equal to and below 1 μg/ml were determined by GC–ECD. Sample concentrations above 1 μg/ml were determined by GC–MS.

** Each data point is the mean of two determinations.

*** Average standard deviation (σ) is $\sqrt{(\sigma^2)/n}$.

§ Average coefficient of variation (C.V.) is $\sqrt{(C.V.^2)/n}$.

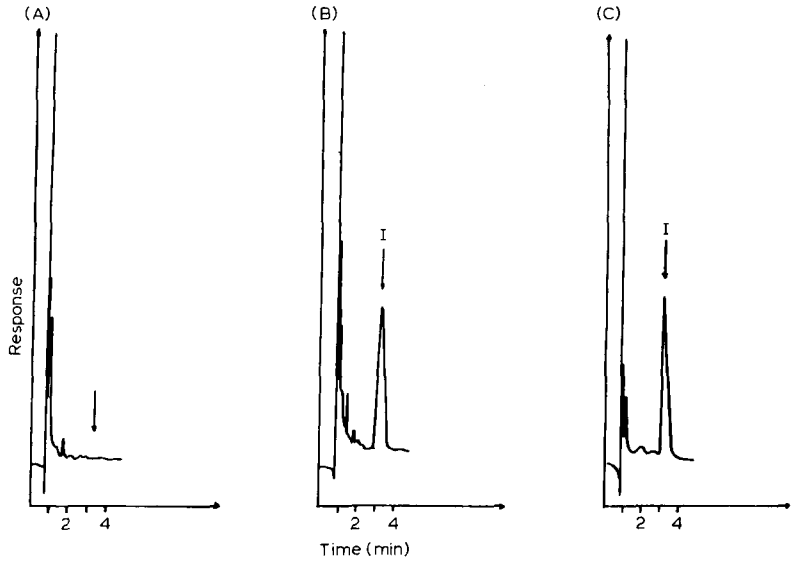


Fig. 1. Representative chromatograms for the determination of 2,4-D IOE in rat blood by GC–ECD. (A) Control blood extract. (B) Spiked blood (0.20 μg/ml final concentration). (C) Reference standard (0.20 μg/ml). Peak I = 2,4-D IOE.

extract, spiked urine (0.20 μg/ml final concentration) and a reference standard (0.20 μg/ml) are displayed in Fig. 2. No interference peaks were observed in either the control blood or urine extracts. The GC–ECD response was linear from 12–288 ng/ml of 2,4-D IOE in hexane, as determined by the linear regression correlation coefficient of 0.9989. The GC–ECD retention time for 2,4-D IOE was 3.7 min.

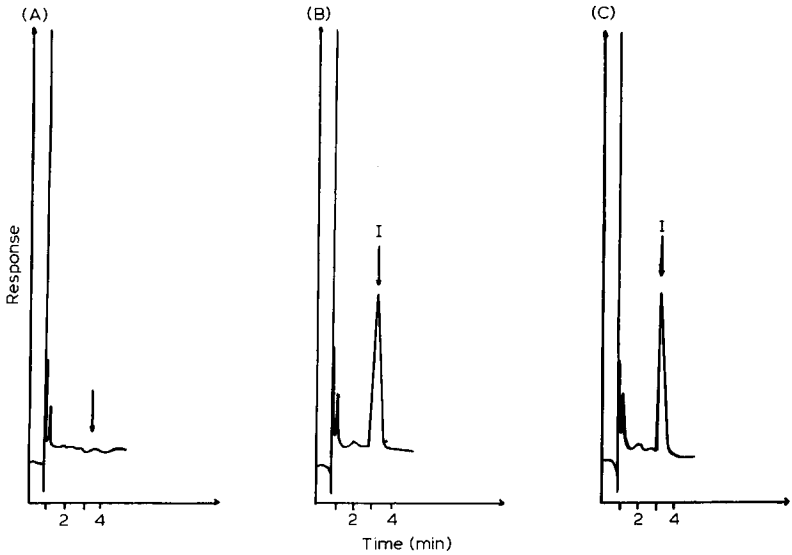


Fig. 2. Representative chromatograms for the determination of 2,4-D IOE in rat urine by GC–ECD. (A) Control urine extract. (B) Spiked urine (0.20 μg/ml final concentration). (C) Reference standard (0.20 μg/ml). Peak I = 2,4-D IOE.

The determination of 2,4-D IOE concentrations above 1 μg/ml was achieved by GC–MS using SIM. Ions at *m/z* 220 and 222 were the largest fragment ions which yielded suitable response and had an identifiable chlorine isotope ratio.

Representative GC–MS chromatograms of control blood extract, control urine extracts, spiked blood (40 μg/ml), spiked urine (40 μg/ml), and a hexane standard (40 μg/ml) are displayed in Fig. 3. No interfering peaks were observed in the blank blood or urine extracts. The retention time of 2,4-D IOE by GC–MS was approximately 90 sec.

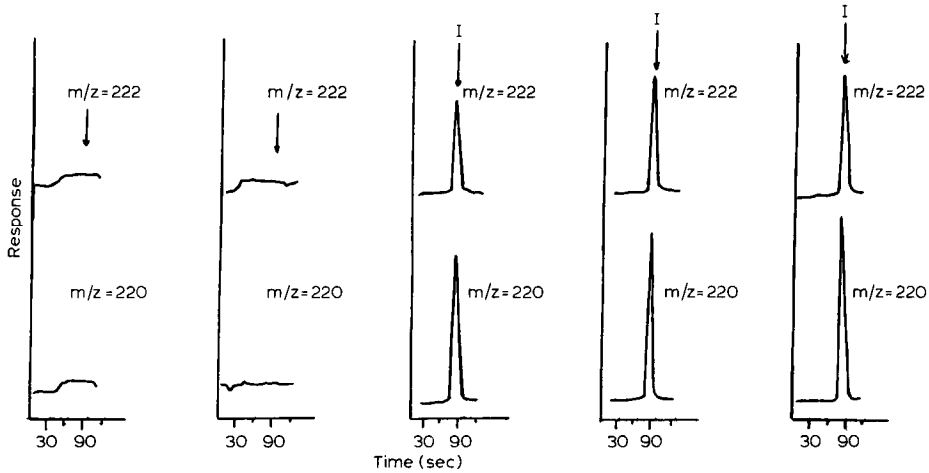


Fig. 3. Representative chromatograms for the determination of 2,4-D IOE in rat blood and urine by GC–MS with SIM. (A) Control blood extract. (B) Control urine extract. (C) Spiked blood (40 μg/ml). (D) Spiked urine (40 μg/ml). (E) Reference standard (40 μg/ml). Peak I = 2,4-D IOE.

GC–MS analysis gave speed and flexibility over a wide range of concentrations. The limit of detection by GC–MS was 250 ng/ml. The GC–ECD analysis supplied a 25 fold increase in sensitivity (limit of detection 10 ng/ml) but had a limited linear range (< 1 μg/ml). All 2,4-D IOE concentrations were quantitated by external standard calculations using peak height or peak area response.

Recovery of 2,4-D IOE from blood was maximized by extraction of the compound within 2 min after sample preparation. Five aliquots of 2,4-D IOE in blood (40 μg/ml) were extracted at five time intervals (0, 2, 5, 15 and 30 min). The recovery dropped dramatically after 2 min. Recovery of 2,4-D IOE in blood at 0 and 2 min was 85% while recovery at 5–30 min ranged between 4 to 11%. The cause of this drop in recovery was not elucidated but could be due to enzymatic hydrolysis of the ester and/or binding of 2,4-D IOE to components of the blood.

CONCLUSIONS

The method described in this paper is rapid, sensitive and specific for determining 2,4-D IOE in rat blood and urine for the concentration ranges of 0.04–100 μg/ml and 0.12–100 μg/ml, respectively. The quantitation of 2,4-D IOE at concentrations of 1 μg/ml and below was achieved by GC–ECD and had a limit of quantitation of approximately 10 ng/ml. The 2,4-D IOE concentrations above 1 μg/ml were analyzed by electron impact GC–MS utilizing SIM. The limit of quantitation for GC–MS was approximately 250 ng/ml. The mean recovery of 2,4-D IOE from blood and urine was 81.2 ± 9.7% and 95.0 ± 4.1%, respectively.

ACKNOWLEDGEMENT

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