Trace Analysis of 2,4,5-Trichlorophenoxyacetic Acid, Its Glycineamide, and Their Alkaline Hydrolyzable Conjugates in Mouse Blood, Urine, and Feces

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Abstract □ Chemical methods were developed for the trace analysis of the herbicide 2,4,5-trichlorophenoxyacetic acid, its glycineamide, and their alkaline hydrolyzable conjugates in mouse blood, urine, and feces. The salient elements of the methods are extraction of the free acids with benzene, methylation, cleanup on a silica gel column, and quantification *via* electron-capture GLC. Any unextracted conjugates remaining in the substrates are then subjected to alkaline hydrolysis, and the liberated 2,4,5-trichlorophenoxyacetic acid is assayed. Data are presented concerning recoveries of the compounds from the three spiked substrates. The utility of the procedures is illustrated by a preliminary pharmacokinetic study employing parallel electron-capture GLC and radioassays of the three substrates from mice injected with a single intravenous dose of ^{14}C-2,4,5-trichlorophenoxyacetic acid. GLC characteristics and partition values of the compounds and hydrolysis of the glycineamide under various conditions also are discussed.

Keyphrases □ 2,4,5-Trichlorophenoxyacetic acid—electron-capture GLC and radiochemical analyses, mouse blood, urine, and feces, pharmacokinetics □ GLC, electron capture—analysis, 2,4,5-trichlorophenoxyacetic acid, mouse blood, urine, and feces □ Radiochemistry—analysis, 2,4,5-trichlorophenoxyacetic acid, mouse blood, urine, and feces □ Pharmacokinetics—2,4,5-trichlorophenoxyacetic acid, mice □ Herbicides—2,4,5-trichlorophenoxyacetic acid, electron-capture GLC and radiochemical analyses, mouse blood, urine, and feces

In 1971, the Panel on Herbicides of the President's Science Advisory Committee (1) indicated that additional information was needed to understand the effects of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T, I) in humans and animals; therefore, further research on this compound was initiated. The major objective of studies with I in this laboratory was to ascertain any potential relationships between embryonic and/or fetal exposure to the chemical and the abnormalities. To obtain a pharmacokinetic profile of the chemical in relation to its teratogenic effects, pharmacokinetic data must be available on the unbred female and at various intervals during the gestation period. Therefore, chemical methodology was required for the trace analysis of I, its glycineamide (II, a known conjugate produced under certain conditions in the liver of most mammalian systems), and other possible conjugates in mouse blood, urine, and feces.

Recently, pharmacokinetic studies were conducted using rats, dogs, and humans and single doses of (^{14}C-carboxy)-I (2) and analytical grade I (3). Also, the absorption, elimination, and metabolism of I were studied in rats, pigs, calves, and chickens (4, 5). A method was reported for determining I, 2,4-dichlorophenoxyacetic acid, and related compounds in rat urine based on ethylation, cleanup on silica gel, and analysis of the derivative by electron-capture GLC (6). The liquid–liquid extraction properties of phenoxy acid herbicides from water were investigated (7). However, no methods meeting high sensitivity requirements (low nanogram level) were applicable to the present study.

This paper describes procedures to determine traces of I, II, and their alkaline hydrolyzable conjugates *via* electron-capture GLC under the restrictions imposed by pharmacokinetic studies in mice. The procedures were tested in a preliminary pharmacokinetic study with mice injected with ^{14}C-I by parallel analyses of these substrates *via* liquid scintillation radioassays and electron-capture GLC. Data from these tests, partition values of the compounds, and ancillary information on the effectiveness of acids and bases in hydrolyzing the glycineamide are presented.

EXPERIMENTAL

GLC—A gas chromatograph[1] equipped with a ^{63}Ni-electron-capture detector[2] and a 100-cm glass column (4 mm i.d.) containing 10% OV-101 on Gas Chrom Q (80–100 mesh), conditioned overnight at 275° prior to use, was operated isothermally at 200° in the dc mode. The nitrogen carrier gas flow rate was 160 ml/min. Temperatures of the injection port and detector were 240 and 280°, respectively. Under these conditions, retention times, t_R, of the methyl esters of I and II were 1.40 and 8.00 min, respectively.

Samples of unknown residue content were quantified by relating their peak heights to dilutions of standards of the two esters prepared by methylating 1-mg amounts of the free acids. Samples for injection contained up to 25 and 160 pg of the methyl esters of I and II, respectively, in 5 μl of benzene or hexane–20% benzene.

Where a more rapid and sensitive analysis of only the methyl ester of II was sought, the instrument was operated isothermally at 240°. Under this condition, the t_R was 2.25 min and the response was linear up to about 70 pg/injection.

Radioassays—Radiolabeled samples were counted with a liquid scintillation instrument[3] equipped with a data reduction system. A one-tenth aliquot (0.5–1.0 ml) from each sample assayed by electron-capture GLC was added to a vial containing 12 ml of solubilizer[4] and counted for 20 min. Residual samples (*e.g.*, after hydrolysis and extraction) and the adsorbent from the cleanup column were combusted[5], and any ^{14}C-labeled carbon dioxide was assayed by liquid scintillation.

Materials—Benzene and hexane, pesticide grade, were redistilled in glass and screened for interfering electron-capture GLC peaks by analyzing 5 μl of a 25-fold concentrate. Solvent concentrates having responses less than 1- and 5-pg equivalents of the methyl esters of I and II (per 5-μl injection), respectively, were considered satisfactory.

Diazomethane reagent was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide[6] by using the reagent, kit, and instructions provided by the manufacturer. The ethereal diazomethane was then

[1] Model 5750, Hewlett-Packard Corp., Palo Alto, Calif.
[2] Tracor, Inc., Austin, Tex.
[3] Mark II instrument with model PDS/3 data reduction system, Nuclear-Chicago Corp., Houston, Tex.
[4] PCS solubilizer, Amersham/Searle Corp., Arlington Heights, Ill.
[5] Model JA 101 Oxymat, Teledyne, Inc., Westwood, N.J.
[6] Diazald, Catalog No. D 2800-0, Aldrich Chemical Co., Milwaukee, Wis.

Table I—GLC Data for the Quantification of 2,4,5-Trichlorophenoxyacetic Acid (I) and Its Glycineamide (II) as Their Methyl and Ethyl Esters

Compound	Retention Time, min[a] 200°	240°	Upper Limit of Linearity, pg[b] 200°	240°
I methyl ester	1.40	—[c]	25	—[c]
I ethyl ester	1.90	—[c]	44	—[c]
II methyl ester	8.00	2.25	160	70
II ethyl ester	8.80	2.52	250	125

[a] Column oven operated isothermally at stated temperatures. [b] Response was for 5-μl injection at about 5×10^{-10} amp. [c] Solvent interference precluded determination.

dried by percolation through a plug of anhydrous sodium sulfate, redistilled, and stored at −10° until used. A hexane solution of diazoethane also was prepared from *N*-ethyl-*N*,*N*′-dinitroguanidine[7] for ethylating I and II.

Silica gel[8] (oven dried at 110°) was deactivated with 2% buffer[9] (0.05 *M* monobasic potassium phosphate–sodium hydroxide, pH 7). The appropriate amount of buffer was dispersed on the inner surface of a dry, glass-stoppered bottle, and dry silica gel was added. The contents were then mixed by mechanically rolling the bottle for 1 hr. The adsorbent was prepared fresh daily.

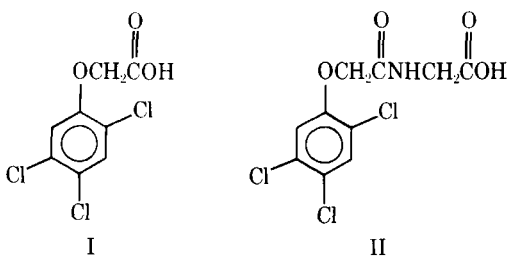
The ^{14}C-2,4,5-trichlorophenoxyacetic acid[10], uniformly ring labeled (1.92 μCi/mg), was assayed as the methyl ester by electron-capture GLC and was essentially identical to the unlabeled standard and free of extraneous GLC peaks.

Animal Chamber—A special sampling chamber for an individual mouse was fabricated from a 2-liter beaker by reshaping the bottom to form a cone (~50 mm deep) and attaching a glass drain tube (~6 mm i.d. × 10 mm long). A disk of stainless steel screen (~2 × 2-mm square openings), approximately the same diameter of the beaker, rested on the bottom of the vessel; a 30-ml culture tube with a screw cap[11] was placed under the drain tip. This arrangement allowed urine to be collected in the tube while feces were retained on the screen. The animal was supported by a heavier disk of larger mesh (8 × 25-mm diamond-shaped openings, measured from opposite corners) equipped with metal legs, which suspended the animal about 25 mm above the bottom screen.

Several animal chambers of this type were used to collect urine and feces from untreated mice for use in developing the analytical methodology *via* electron-capture GLC; they were also used in preliminary pharmacokinetic and other studies with the ^{14}C-I. Composites of samples collected for use in developing the analytical methodology were stored at −10° prior to use; all others were extracted immediately after collection.

Hydrolysis Studies—Samples, 100 ng, of the methyl and ethyl esters of II were subjected to alkaline hydrolysis (1 *N* NaOH) for 2 hr at 85° to determine the completeness of cleaving the compound and of recovering the I reaction product. The hydrolysates were acidified, extracted with benzene, methylated, and assayed by electron-capture GLC. One hundred nanograms of II was also subjected to aqueous acid and alkali treatments at three different temperatures for various periods to determine its stability during extraction and analysis.

Animal Experiments—Unbred female CD-1 mice, 32–33 g, were



I II

[7] Catalog No. E 4160-5, Aldrich Chemical Co., Milwaukee, Wis.
[8] Catalog No. 3405, J. T. Baker Chemical Co., Glen Ellyn, Ill.
[9] Catalog No. S0-B-108, Fisher Scientific Co., St. Louis, Mo.
[10] Mallinckrodt Chemical Works, St. Louis, Mo.
[11] Lined with Teflon (du Pont).

Table II—Extraction Partition Values of the Methyl and Ethyl Esters of 2,4,5-Trichlorophenoxyacetic Acid (I) and Its Glycineamide (II)

Solvent System	I Methyl Ester	I Ethyl Ester	II Methyl Ester	II Ethyl Ester
Chloroform–water	1.00	1.00	1.00	1.00
Chloroform–60% methanol	1.00	1.00	1.00	1.00
Hexane–water	1.00	1.00	0.95	1.00
Hexane–acetonitrile	0.078	0.12	0.011	0.018
Isooctane–80% acetone	0.73	0.81	0.27	0.40
Isooctane–dimethylformamide	0.035	0.048	—[a]	—[a]
Isooctane–85% dimethylformamide	0.12	0.21	0.00	0.01
Heptane–90% ethanol	0.39	0.50	0.050	0.076
Benzene–water[b]	1.00	0.96	1.00	0.98

[a] Detection was by electron-capture GLC. All others were by flame-ionization detection GLC. [b] Solvent interference precluded determination.

individually confined in the special sampling chambers. The mice were allowed free access to food and water throughout the experiment, and control samples of urine and feces were collected for 24 hr preceding the injection of radiolabeled I. The control sample of blood was withdrawn from each mouse just prior to an intravenous injection of 1.86 mg of ^{14}C-I (contained in 100 μl of water–60% ethanol) into the tail vein of the animal; this dosage was equivalent to about 57 mg of I/kg. In the pharmacokinetic studies, samples of blood (5 or 20 μl), urine, and feces were collected at various intervals and assayed.

In experiments prior to the pharmacokinetic studies, mice injected with the ^{14}C-I were confined overnight. Various solvents in different combinations were used to determine the most efficient means of recovering the radioactive urine deposits from the inner surface of the chamber.

Analysis of Blood—An accurately measured sample of mouse blood was withdrawn from the tip of the animal's tail and immediately delivered into an 8-ml culture tube, containing 0.5 ml of distilled water, by using either a 5- or 20-μl micropipet[12]. Serial samples were taken from the same three animals throughout the experiment. The tube was sealed with a screw cap[11], subjected to vortex mixing for a few seconds until the blood was uniformly dispersed, acidified by adding 0.5 ml of 2 *N* HCl, and again subjected to vortex mixing. Then 5 ml of benzene was added, and the tube was vigorously shaken for 1 min and centrifuged at 2000 rpm for 5 min.

The benzene layer was carefully transferred to a 50-ml glass-stoppered flask with a syringe and cannula, and the aqueous layer in the tube was extracted with an additional 5-ml portion of benzene in an identical manner. [*Note:* Care was taken to withdraw as much of the benzene layer as possible (~4.75 ml) with each extraction; however, none of the aqueous phase should ever be withdrawn into the cannula or syringe. Anhydrous sodium sulfate was shown to absorb nanogram amounts of I from benzene solutions. Therefore, it cannot be used to remove water from the benzene extracts in these procedures.]

The aqueous phase was reserved for subsequent alkaline hydrolysis of possible conjugates. One milliliter of "keeper" solution (100 μg of paraffin oil[13]/ml of benzene) and a boiling bead were added to the flask containing the combined benzene extracts (I and II), and the contents were evaporated just to dryness by using water pump vacuum and a 60° water bath. Residues of the free acids were then converted to their methyl esters by the addition of 2 ml of an ethereal solution of diazomethane. After 15-min, the diazomethane solution was evaporated under water pump vacuum at ambient temperature, and the residue was dissolved in 5 ml of hexane–20% benzene for the subsequent column cleanup or in an appropriate volume of benzene (1 ml or more) for direct injection into the gas chromatograph.

The aqueous phase was subjected to alkaline hydrolysis by adding 0.5 ml of 4 *N* NaOH and heating the sealed tube at 85° for 2 hr with occasional shaking. After the tube had cooled, 1 ml of 4 *N* HCl was added; any I or II freed from the conjugates by the digestion was extracted as I, methylated, and analyzed as previously described. (Any

[12] Drummond Wiretrol, Bolab, Inc., Derry, N.H.
[13] Catalog No. 8501-2850, Hewlett-Packard Corp., Palo Alto, Calif.

Table III—Electron-Capture GLC Analysis of 2,4,5-Trichlorophenoxyacetic Acid (I) and Its Glycineamide (II) from Mouse Blood before and after Silica Gel Cleanup of Free Acid and Hydrolyzed Fractions

Added		Recovered[a]			
		Before Cleanup		After Cleanup	
Compound	ng	ng	%[b]	ng	%[b]
I[c]	0	1.2 ± 0.07	—	1.0 ± 0.44	—
	10	9.2 ± 0.70	80 ± 7	9.2 ± 1.05	82 ± 10
	100	96 ± 2.1	95 ± 2	66 ± 2.1	65 ± 2
	1000	987 ± 11.6	99 ± 1	718 ± 20.2	72 ± 2
I[d]	0	—[e]	—	0.9 ± 0.25	—
	10	—[e]	—	6.5 ± 0.91	54 ± 9
	100	99 ± 3.1	99 ± 3	75 ± 3.1	74 ± 3
	1000	1063 ± 63.5	106 ± 6	863 ± 35.9	86 ± 4
II[f]	0	1.7 ± 0.25	—	1.4 ± 0.00	—
	10	7.6 ± 0.75[g]	59 ± 7[g]	9.9 ± 0.75	85 ± 8
	100	91 ± 3.8	89 ± 4	85 ± 7.9	84 ± 8
II[h] (10 ng = 8.2 ng of I)	0	—[e]	—	0.7 ± 0.16	—
	10	—[e]	—	5.4 ± 3.1	66 ± 4
	100	79 ± 1.2	96 ± 1	68 ± 3.2	83 ± 4

[a] Mean and standard error from triplicate assays. [b] Corrected for background interference in the unspiked sample. [c] Analyzed as the methyl ester of I. [d] Respiked after extraction of I and then hydrolyzed. [e] High background interference precluded assay without column cleanup. [f] Analyzed as the methyl ester of II. [g] High background interference; quantitation is an estimate. [h] Respiked after extraction of II and then hydrolyzed.

conjugate of II freed by the hydrolysis would also be further hydrolyzed to I). GLC analysis of the methylated extracts without column chromatographic cleanup was performed whenever possible, because the recovery was higher and the time per analysis was shorter than analysis with column cleanup. However, total residues of 10 ng or less of the compounds, particularly after alkaline hydrolysis, must be subjected to the cleanup because the high electron-capturing background precludes accurate quantitation at these low levels.

The chromatographic cleanup column[14] consisted of 0.5 g of the silica gel supported by a small plug of glass wool; it was prewashed with 10 ml of hexane. The methylated extract from the free acid fraction or from the alkaline hydrolysis dissolved in 5 ml of hexane–20% benzene was added to the column and allowed to percolate into the adsorbent; then the flask and column were washed with two additional 1.5-ml portions of the solvent. The effluent was discarded, and the original 50-ml boiling flask was placed under the column to receive the subsequent eluate. The column was then eluted with 20 ml of hexane–40% benzene. This eluate, containing the methyl ester of I, was evaporated just to dryness, and the residue was dissolved in an appropriate amount of benzene (1 ml or more) for injection.

When residues of the methyl ester of II were also sought from the column, the adsorbent was deactivated by the addition of 1 ml of absolute methanol and eluted with 10 ml of benzene. The eluate was evaporated and reconstituted in benzene for GLC analysis as described for the methyl ester of I. (*Note:* If a column cleanup of the ethyl ester of I is sought, the extract is added to the column with 15 ml of hexane–20% benzene and the eluate is discarded. The ethyl ester is then eluted from the column with 25 ml of hexane–40% benzene.)

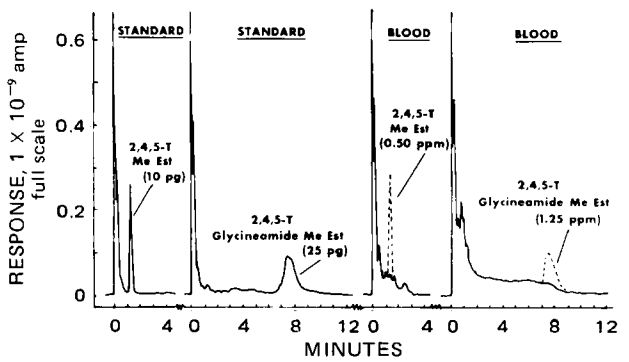
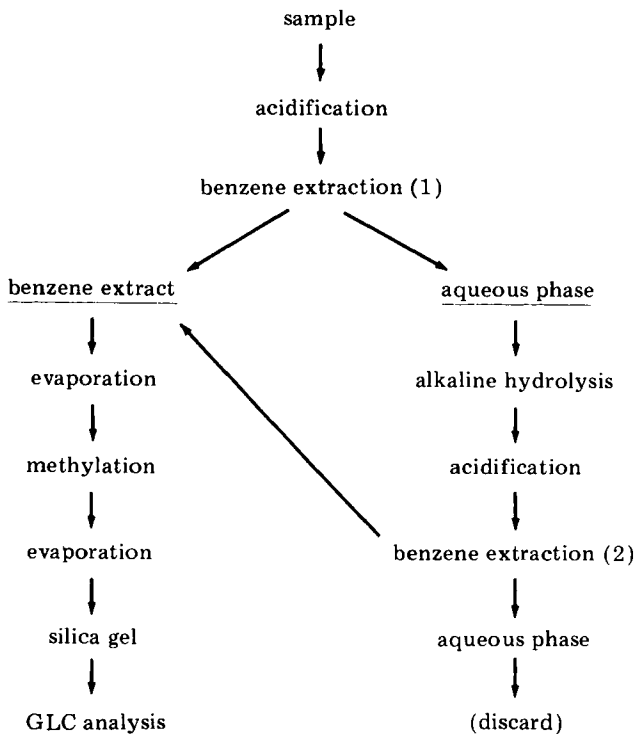


Figure 1—*Gas chromatograms of methyl ester (MeEst) standards of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), its glycineamide, and cleaned-up blood extracts in benzene spiked with the standards (injection = 5 μl).*

[14] Catalog No. 420,000, Kontes Glass Co., Vineland, N.J.

Analysis of Urine—The urine samples were collected in the 30-ml tube of the animal chamber. The animal and its metal support were removed, and any feces collected on the screen were reserved for separate analysis. The screen and walls of the chamber were then washed three times with 5-ml portions of 1 *N* HCl and twice with 5-ml portions of benzene. The 30-ml culture tube containing the urine and washings was removed, sealed, vigorously shaken for 1 min, and centrifuged at 2000 rpm for 5 min. The benzene layer was transferred to a 50-ml glass-stoppered flask, and the aqueous layer was extracted with two additional 10-ml portions of benzene; the combined extracts were evaporated, methylated, and analyzed as described for the blood.

The aqueous phase was subjected to alkaline hydrolysis by adding



Scheme I

Table IV—Electron-Capture GLC Analysis of 2,4,5-Trichlorophenoxyacetic Acid (I) and Its Glycineamide (II) from Mouse Urine before and after Silica Gel Cleanup of Free Acid and Hydrolyzed Fractions

Added		Recovered[a]			
		Before Cleanup		After Cleanup	
Compound	ng	ng	%[b]	ng	%[b]
I[c]	0	35.3 ± 1.16	—	5.1 ± 0.00	—
	10	43.6 ± 0.00	83 ± 0	15.3 ± 1.01	102 ± 10
	100	147 ± 0.0	112 ± 0	113 ± 3.8	108 ± 4
	1000	1200 ± 10	116 ± 1	987 ± 130	98 ± 13
I[d]	0	15.0 ± 2.25	—	0.68 ± 0.13	—
	10	18.5 ± 0.17	35 ± 2	5.6 ± 0.14	49 ± 1
	100	97 ± 2.2	82 ± 2	110 ± 1.7	109 ± 2
	1000	1053 ± 13	104 ± 1	1043 ± 46	104 ± 5
II[e]	0	12.3 ± 2.24	—	5.8 ± 0.70	—
	25	27.0 ± 1.07	59 ± 4	29.4 ± 1.34	94 ± 5
	250	246 ± 7.5	94 ± 3	259 ± 8.3	101 ± 3
II[c,f] (25 ng = 20.4 ng of I)	0	15.0 ± 2.25	—	0.7 ± 0.13	—
	25	26.3 ± 1.52	55 ± 7	20.3 ± 0.91	96 ± 4
	250	218 ± 7	100 ± 3	205 ± 18	100 ± 9

[a] Mean and standard error from triplicate assays. [b] Corrected for background interference in the unspiked sample. [c] Analyzed as the methyl ester of I. [d] Respiked after extraction of I and then hydrolyzed. [e] Analyzed as the methyl ester of II. [f] Respiked after extraction of II and then hydrolyzed.

2.5 ml of 10 *N* NaOH and heating the sealed tube at 85° for 2 hr with occasional shaking. After the tube had cooled, 2.5 ml of 12 *N* HCl was added, and the aqueous phase was extracted with three 10-ml portions of benzene. The combined extracts were then prepared and analyzed as described for blood. Column chromatographic cleanup of the samples was performed as described for blood. The cleanup was usually required for all samples containing residues of 10 ng or less.

Analysis of Feces—The excrement obtained from the screen of the animal chamber was transferred to a 12-ml culture tube, and three glass beads (5 mm diameter) and 3 ml of 1 *N* HCl were added. The sealed tube was subjected to vigorous vortex mixing for 1 min. After the addition of 5 ml of benzene, the acidified sample was gently shaken for 1 min and centrifuged at 2000 rpm for 5 min; the benzene layer was transferred to a 50-ml glass-stoppered flask as described for blood. The extraction was repeated with two additional 5-ml portions of benzene, and the combined extracts were prepared and analyzed as described for blood.

The aqueous phase was subjected to alkaline hydrolysis as described for blood, except that 1 ml of 6 *N* NaOH was used. After hydrolysis, 1 ml of 8 *N* HCl was added; then the sample was extracted, prepared, and analyzed as described for blood. The column cleanup of fecal extracts on silica gel also was performed as described for blood; this cleanup was required for extracts containing residues of 100 ng or less.

Recovery Experiments—Forty-four 24-hr collections of urine and feces from untreated mice in the sampling chambers yielded a mean volume of 0.48 ± 0.26 ml of urine and 0.19 ± 0.05 g of feces/animal/day. The urine was composited and diluted with distilled water to produce 0.48 ml of undiluted mouse urine/3 ml of solution. The feces were composited and mixed by grinding with a mortar and pestle to ensure uniformity. Portions of these samples and fresh mouse blood (20 μl) were used as substrates in the development of analytical methodology based on electron-capture GLC analysis.

Amounts, 0.5 ml, of distilled water, each containing the equivalent of 20 μl of whole mouse blood, were added to triplicate 8-ml culture tubes containing 0-, 10-, 100-, or 1000-ng amounts of I or 0-, 10-, or 100-ng amounts of II as dry residues. After vortex mixing, the samples were analyzed for free acids as described. After extraction, the aqueous samples were again spiked at the same levels by adding 50 μl of acetone containing the appropriate amount of acid or glycineamide. These samples then were subjected to alkaline hydrolysis and analyzed as described.

Triplicate 0.19-g portions of the composited feces in 12-ml culture tubes or 3-ml portions of the diluted urine composite in 30-ml culture tubes were spiked with 0, 10, 100, or 1000 ng of I or 0, 25, or 250 ng of II in 50 μl of acetone. After vortex mixing, the samples were analyzed as described. The aqueous phases were then spiked again in an identical manner, hydrolyzed, and analyzed as described.

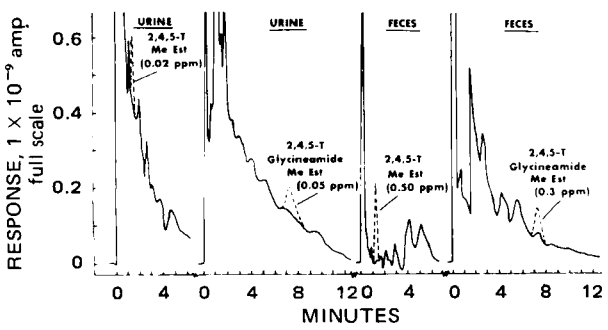


Figure 2—*Gas chromatograms of cleaned-up urine and feces extracts spiked with the methyl esters (MeEst) of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and its glycineamide. (Extracts containing 10 and 25 pg of the methyl esters of 2,4,5-trichlorophenoxyacetic acid and its glycineamide, respectively, were injected in 5 μl of benzene.)*

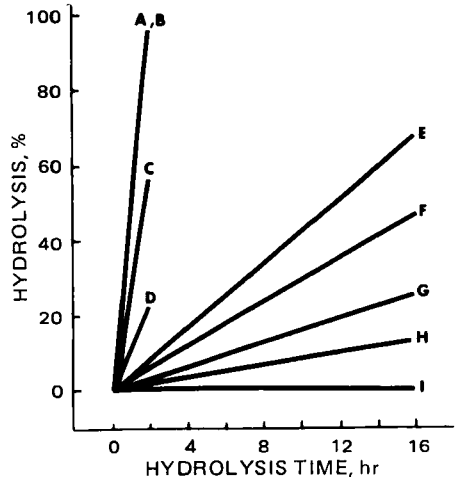


Figure 3—*Percent hydrolysis of the glycineamide of 2,4,5-trichlorophenoxyacetic acid under various conditions. Key: A, 1 N NaOH at 85°; B, 5 N HCl at 85°; C, 1 N HCl at 85°; D, 0.5 N NaOH at 25°; E, 5 N NaOH at 5°; F, 1 N NaOH at 5°; G, 0.5 N NaOH at 5°; H, 0.1 N NaOH at 25°; and I, 0.1 N NaOH at 5° or 0.1, 1, or 5 N HCl at 5 and 25°.*

Table V—Electron-Capture GLC Analysis of 2,4,5-Trichlorophenoxyacetic Acid (I) and Its Glycineamide (II) from Mouse Feces before and after Silica Gel Cleanup of Free Acid and Hydrolyzed Fractions

Added		Recovered[a]			
		Before Cleanup		After Cleanup	
Compound	ng	ng	%[b]	ng	%[b]
I[c]	0	18[d]	—	1.0 ± 0.67	—
	10	26[d]	80	8.4 ± 0.79	74 ± 8
	100	128[d]	110	91 ± 3.8	90 ± 4
	1000	983 ± 10	96 ± 1	950 ± 23	95 ± 2
I[c,e]	0	34.3 ± 13.9	—	0.7 ± 0.29	—
	10	45.1 ± 1.00	108 ± 10	7.8 ± 0.10	71 ± 1
	100	116 ± 13	82 ± 13	104 ± 6	103 ± 6
	1000	1006 ± 1	97 ± 0	1013 ± 6	101 ± 1
II[f]	0	71.4 ± 0.0	—	23.8 ± 4.0	—
	25	138 ± 6	266 ± 24	75.3 ± 2.9	105 ± 12
	250	297 ± 6	90 ± 2	274 ± 2	100 ± 1
II[c,g] (25 ng = 20.4 ng of I)	0	—[h]	—	0.7 ± 0.29	—
	25	—[h]	—	18.5 ± 3.21	87 ± 16
	250	194 ± 26	95 ± 13	208 ± 3	102 ± 2

[a] Mean and standard error from triplicate assays. [b] Corrected for background interference in the unspiked sample. [c] Analyzed as the methyl ester of I. [d] High background interference; quantitation is an estimate. [e] Respiked after extraction of I and then hydrolyzed. [f] Analyzed as the methyl ester of II. [g] Respiked after extraction of II and then hydrolyzed. [h] High background interference precluded assay without column cleanup.

Table VI—Electron-Capture GLC and Radiochemical Analysis of 2,4,5-Trichlorophenoxyacetic Acid (I), Its Glycineamide (II), and Alkaline Hydrolyzable Conjugates in Blood from Mice Injected with ^{14}C-2,4,5-Trichlorophenoxyacetic Acid (Nanograms per Microliter of Blood)[a]

Sampling Time	Assay	I	II	Alkaline Hydrolyzable Conjugates	Total as I
Pretreatment	Electron-capture GLC	0.31 ± 0.06	0.20 ± 0.03	0.26 ± 0.40	0.73
	Radiochemical	3.73 ± 1.61[b]	—	12.0 ± 1.6	15.7
5 min	Electron-capture GLC	950 ± 431	0.36 ± 0.12	0.41 ± 0.64	951
	Radiochemical	904 ± 410[b]	—	23.0 ± 15.1	927
30 min	Electron-capture GLC	672 ± 101	0.41 ± 0.17	0.19 ± 0.32	673
	Radiochemical	690 ± 109[b]	—	3.53 ± 1.88	694
1 hr	Electron-capture GLC	450 ± 126	0.36 ± 0.08	<0.26 ± 0.40[c]	450
	Radiochemical	423 ± 125[b]	—	8.72 ± 2.60	432
2 hr	Electron-capture GLC	381 ± 111	0.73 ± 0.31	5.37 ± 3.12	387
	Radiochemical	355 ± 104[b]	—	6.18 ± 5.41	361
4 hr	Electron-capture GLC	489 ± 157	1.01 ± 0.14	11.8 ± 3.9	502
	Radiochemical	472 ± 157[b]	—	6.03 ± 4.41	478
8 hr	Electron-capture GLC	279 ± 65	1.05 ± 0.15	5.80 ± 3.63	286
	Radiochemical	266 ± 71[b]	—	26.6 ± 8.62	293
24 hr[d]	Electron-capture GLC	68.9	0.05	2.24	71.2
	Radiochemical	57.8[b]	—	<12.0[c]	69.8
48 hr[d]	Electron-capture GLC	60.1	0.13	1.46	61.7
	Radiochemical	50.4[b]	—	0.23	50.6
72 hr	Electron-capture GLC	47.5 ± 34.0	0.43 ± 0.55	2.95 ± 3.19	50.8
	Radiochemical	41.3 ± 30.8[b]	—	11.5 ± 1.5	52.8

[a] Mean and standard error from three mice. Results are corrected for pretreatment sample background. [b] The ^{14}C-assay did not resolve the individual free acids; therefore, this result represents both. [c] None detected above background. [d] Data from a single and different mouse treated in the same manner. Physical condition of the other three animals precluded sampling at this interval.

RESULTS AND DISCUSSION

The analytical methods previously described were developed specifically for the mouse substrates to be assayed in pharmacokinetic studies with I (Scheme I). The binding of the free acids to proteins, especially in the whole blood, is considered to be relatively weak, since acidification with hydrochloric acid and extraction with benzene yielded good recoveries from samples spiked with both I and II. Nevertheless, the acidification and extraction of free acids should be expedited and the residues should be methylated and stored as the esters to prevent losses due to the tendency of nanogram amounts of the free acids to become associated with various surfaces. The aqueous phase remaining after extraction of the free acids may be stored in a refrigerator until alkaline hydrolysis.

Data concerning the electron-capture GLC characteristics of the methyl and ethyl esters of I and II are presented in Table I. Although ethylation of the compounds was not required in this study, information pertaining to the ethyl esters may be useful for assaying other substrates having background interferences at the t_R's of the methyl esters.

Typical chromatograms of standards of the methyl esters of I and II and extracts of untreated mouse blood before and after being spiked with the esters of the two compounds are presented in Fig. 1. Similar chromatograms of mouse urine and feces are presented in Fig. 2.

Partition values (the fraction of solute partitioning into the nonpolar phase of an equivolume immiscible binary solvent system) for the methyl and ethyl esters of the two compounds were determined as described by Beroza and Bowman (8, 9). The partition values of the methyl and ethyl esters of I and II, which are extremely useful in developing extraction and cleanup methods and confirming identity at trace levels, are presented in Table II. The partition values for I and II in chloroform–water or benzene–water (pH 0–2) were in line with those reported (7) for I (*i.e.*, 0.96–1.0). The identity of trace levels of I and II extracted from the blood and excreta of animals dosed with I in the present studies was confirmed by using partition values.

In tests pertaining to the efficiency of hydrolyzing the methyl and ethyl esters of II with aqueous sodium hydroxide at 85° for 2 hr, recoveries of I accounted for 98 and 97% of the compounds, respectively. Since no trace of intact II could be detected, these hydrolysis conditions were adopted for the analytical procedure.

Table VII—Electron-Capture GLC and Radiochemical Analysis of 2,4,5-Trichlorophenoxyacetic Acid (I), Its Glycineamide (II), and Alkaline Hydrolyzable Conjugates in Urine from Mice Injected with ^{14}C-2,4,5-Trichlorophenoxyacetic Acid (Micrograms per Sampling Interval)[a]

Sampling Interval, hr	Assay	I	II	Alkaline Hydrolyzable Conjugates	Total as I
Pretreatment	Electron-capture GLC	0.01 ± 0.00	0.06 ± 0.05	<0.01 ± 0.00[b]	0.07
	Radiochemical	0.02 ± 0.01[c]	—	0.03 ± 0.02	0.05
0–6	Electron-capture GLC	1.86 ± 1.45	<0.06 ± 0.05[b]	<0.01 ± 0.00[b]	1.92
	Radiochemical	1.83 ± 1.41[c]	—	0.05 ± 0.02	1.88
6–24	Electron-capture GLC	13.7 ± 5.0	11.7 ± 6.25	<0.01 ± 0.00[b]	23.3
	Radiochemical	29.8 ± 11.5[c]	—	2.16 ± 0.98	32.0
24–30	Electron-capture GLC	7.10 ± 4.86	3.61 ± 4.18	<0.01 ± 0.00	10.1
	Radiochemical	9.16 ± 5.70[c]	—	1.03 ± 1.05	10.2
30–48	Electron-capture GLC	61.7 ± 76.6	28.3 ± 25.7	38.1 ± 39.9	123
	Radiochemical	83.8 ± 92.2[c]	—	39.8 ± 41.5	124
48–52	Electron-capture GLC	35.8 ± 54.6	7.28 ± 8.94	13.6 ± 2.9	55.4
	Radiochemical	39.5 ± 56.7[c]	—	14.2 ± 2.4	53.7
52–72	Electron-capture GLC	40.0 ± 32.4	22.0 ± 17.2	36.5 ± 12.8	94.5
	Radiochemical	59.4 ± 46.7[c]	—	37.1 ± 13.9	96.5

[a] Mean and standard error from three mice. Results are corrected for pretreatment sample background. [b] None detected above background. [c] The ^{14}C-assay did not resolve the individual free acids; therefore, this result represents both.

Table VIII—Electron-Capture GLC and Radiochemical Analysis of 2,4,5-Trichlorophenoxyacetic Acid (I), Its Glycineamide (II), and Alkaline Hydrolyzable Conjugates in Feces from Mice Injected with ^{14}C-2,4,5-Trichlorophenoxyacetic Acid (Micrograms per Sampling Interval)[a]

Sampling Interval, hr	Mean Sample Weight, mg + *SE*	Assay	I	II	Alkaline Hydrolyzable Conjugates	Total as I
Pretreatment	538 ± 108	Electron-capture GLC	0.01 ± 0.00	0.04 ± 0.03	0.01 ± 0.00	0.05 ± 0.02
		Radiochemical	0.02 ± 0.01[b]	—	0.02 ± 0.01	0.04 ± 0.01
0–6	43.6 ± 42.1	Electron-capture GLC	1.44 ± 1.07	<0.06 ± 0.01[c]	0.10 ± 0.07	1.54 ± 1.14
		Radiochemical	1.52 ± 1.05[b]	—	0.10 ± 0.06	1.62 ± 1.11
6–24	89.0 ± 7.4	Electron-capture GLC	4.62 ± 0.78	<0.04 ± 0.02[c]	0.89 ± 0.61	5.51 ± 0.71
		Radiochemical	5.56 ± 0.82[b]	—	1.01 ± 0.60	6.57 ± 0.48
24–30	57.7 ± 29.1	Electron-capture GLC	2.07 ± 1.14	<0.04 ± 0.03[c]	0.73 ± 0.42	2.80 ± 1.56
		Radiochemical	2.75 ± 1.55[b]	—	0.85 ± 0.53	3.60 ± 2.08
30–48	262 ± 203	Electron-capture GLC	16.9 ± 11.8	1.47 ± 1.96	7.10 ± 9.23	25.2 ± 21.7
		Radiochemical	19.4 ± 13.8[b]	—	7.30 ± 9.32	26.7 ± 22.4
48–52	25.8 ± 14.8	Electron-capture GLC	0.99 ± 0.44	<0.05 ± 0.01[c]	0.11 ± 0.06	1.12 ± 0.45
		Radiochemical	1.09 ± 0.52[b]	—	0.10 ± 0.06	1.19 ± 0.54
52–72	363 ± 265	Electron-capture GLC	7.30 ± 6.70	0.12 ± 0.10	2.56 ± 2.40	9.95 ± 9.07
		Radiochemical	7.96 ± 7.86[b]	—	2.50 ± 2.39	10.5 ± 10.2

[a] Mean and standard error from three mice. Results are corrected for pretreatment sample background. [b] The ^{14}C-assay did not resolve the individual free acids; therefore, this result represents both. [c] None detected above background.

The extent of hydrolysis of II under various conditions is illustrated in Fig. 3. Aqueous sulfuric acid (0.01, 0.05, 0.10, 0.50, and 1.0 *N*) failed to yield any detectable hydrolysis at 25° for 2 hr. Also, aqueous hydrochloric acid (0.1, 1.0, and 5.0 *N*) failed to hydrolyze the glycineamide at 5 or 25° over 16 hr. However, significant hydrolysis occurred with aqueous sodium hydroxide (0.1–0.5 *N*) at 25°. These data indicated that residues of II should not be mixed with sodium hydroxide unless hydrolysis is intended. Therefore, samples containing II or conjugates were immediately acidified and extracted to ensure the integrity of the analytical results.

Results from blood, urine, and feces of untreated mice, spiked and analyzed *via* electron-capture GLC, are presented in Tables III, IV, and V, respectively. Excellent recoveries were obtained from all three substrates spiked with 100- and 1000-ng amounts of I analyzed both before and after the silica gel cleanup. Results from the aqueous sample residues respiked prior to hydrolysis indicated that the previous extractions had efficiently removed I and that the additional I was recovered from the hydrolysis procedure. Samples of blood or urine containing 100 ng or more of I should be analyzed without utilizing the silica gel cleanup to obtain higher recoveries and to speed the analysis. The background interference in urine was reduced from about 35.3- to 5.1-ng equivalents of I by utilizing a silica gel column; therefore, the cleanup is required where low levels of residues are sought. Feces should be cleaned up at all residue levels below 1000 ng.

Good recoveries were obtained for II from the three substrates spiked at all levels and especially after the extracts were cleaned up on silica gel. Results from the analysis of the residual aqueous substrates respiked with II prior to hydrolysis and assayed as I demonstrated that the previous extraction of II was complete and that hydrolysis of II and recovery of the I moiety were accomplished.

Rinses of the animal chambers with various amounts, combinations, and sequences of aqueous sodium hydroxide (1 *N*), hydrochloric acid (1 *N*), benzene, methanol, acetone, ether, and aqueous (ethylenedinitrilo)tetraacetate (1 mg/ml) were performed to determine the most efficient means of conveniently recovering the radioactivity deposited by the urine from mice injected with ^{14}C-I. The use of aqueous sodium hydroxide followed by benzene yielded a slightly better recovery (99.4%) of the total radioactivity than did aqueous hydrochloric acid followed by benzene (97.3%). Nevertheless, the use of hydrochloric acid was adopted to prevent any possible hydrolysis of II or conjugates by the alkali during the rinsing process.

Results of the preliminary pharmacokinetic studies with mice injected with ^{14}C-I, as determined by parallel electron-capture GLC and radiochemical assays, are presented for blood, urine, and feces in Tables VI, VII, and VIII, respectively. No results are reported for II *via* radioassay; since the radioassay did not resolve I and II, the value reported for I includes both compounds. The concentration of I and its products found in the blood was highest (>900 ng/μl) at the first sampling period (5 min after injection) and declined to about 50 ng/μl during the 72-hr test period, as expected after intravenous injection. Residues of II and the alkaline hydrolyzable conjugates accounted for only a small percentage of radioactivity of the material present, which existed primarily as I.

Results from the urine assays indicated that only trace amounts of I and its products were excreted during the 6-hr period immediately after injection. However, during the 6–24-hr interval and thereafter, appreciable amounts of I, II, and conjugates were excreted. Low levels

of I and lesser amounts of conjugates were found in the mouse feces at all intervals of sampling. No significant amount of II was obtained from the feces.

Radioassay results from the blood, urine, and feces generally correlated well with those from the electron-capture GLC procedure. Radioassays of the residual substrates (after hydrolysis and extraction), column adsorbents, and discarded solvents indicated that essentially all radioactivity had been extracted from the samples and that losses during the analytical procedure were negligible.

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ACKNOWLEDGMENTS AND ADDRESSES

Received December 8, 1975, from the *Department of Health, Education, and Welfare, Food and Drug Administration, National Center for Toxicological Research, Jefferson, AR 72079*

Accepted for publication February 17, 1976.

The authors acknowledge the assistance of Judith E. McAninch, Percy M. Draper, Kenneth L. Rowland, Daniel M. Nestorick, James B. LaBorde, and Johnny J. Bazare, Jr., in conducting ancillary studies related to the development of analytical methods and animal sampling techniques.

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Mechanistic Studies on Transcorneal Permeation of Pilocarpine

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Abstract □ The mechanism of corneal pilocarpine penetration was studied in the albino rabbit using radiochemical techniques. The apparent rate and extent of pilocarpine accumulation in the aqueous humor and the various cell layers of the cornea were determined for both intact and abraded eyes. For the first time, drug levels were monitored in the epithelium and stroma-endothelium of the intact cornea using a tissue-scraping technique. In addition, a new postinstillation rinsing method was devised to evaluate the rate of corneal uptake. The results demonstrate a dual role for the corneal epithelium, both as a barrier to drug penetration and as a reservoir for drug in the intact cornea. The transcorneal pilocarpine flux is slower than the data appear to indicate, and previous overestimates of the apparent absorption rate constant are due to parallel elimination processes occurring at the absorption site. Pharmacokinetic parameters were determined for each tissue to generate an overall mechanism for corneal permeation.

Keyphrases □ Pilocarpine—mechanism of corneal penetration, rate and extent of accumulation in aqueous humor and various cell layers, rabbits □ Corneal penetration mechanism—pilocarpine, rate and extent of accumulation in aqueous humor and various cell layers, rabbits □ Ophthalmic cholinergic agents—pilocarpine, mechanism of corneal penetration, rabbits

The cornea is an easily accessible tissue of the body. However, because of its small size and great degree of specialization, studies on corneal drug transport have been limited, with the net result that the understanding of transcorneal permeation is sketchy. Studies of inorganic ion movement have been fairly extensive (1) due to their great physiological importance to the cornea, and these ions are transported primarily through specialized transport systems associated with the epithelial potential. A relatively large number of studies also have been performed using organic molecules, both drug and nondrug, as recently described (2). However, these studies have been generally restricted to simple quantitation of drug penetration to a specific target tissue, often the aqueous humor, without elucidating the mechanism of corneal permeation. The experimental design in most of these studies does not permit anything but qualitative speculation regarding corneal penetration. The present study examines the corneal kinetics of pilocarpine in an effort to ascertain its transcorneal mechanism.

BACKGROUND

The prevailing theories on corneal penetration of pilocarpine often include, all or in part, a number of principal speculations: (*a*) the existence of a barrier to penetration in the lipophilic corneal epithelium, (*b*) rapid uptake and transport of pilocarpine by the cornea, (*c*) controlled release of pilocarpine to the anterior chamber by the endothelium, and (*d*) the presence of a depot, or reservoir, of pilocarpine somewhere in the cornea.

For most drugs, the existence of a barrier in the corneal epithelium is well documented (2, 3). This barrier is often considered to vary in magnitude according to the solubility character of the drug. A very water-soluble drug is unable to penetrate this barrier, and a very lipid-soluble drug penetrates it easily but is unable to leave; therefore, some degree of solubility in both aqueous and lipid media is deemed desirable for optimum transcorneal permeation (4, 5). Recently, the location of this barrier function was specifically attributed to the outermost cell layer of the epithelium (6, 7), and the sensitivity of this barrier to surfactants has been carefully studied (8–10).

The premise that pilocarpine has a rapid corneal penetration rate has been professed by a number of investigators whose conclusions were derived from both *in vitro* (11, 12) and *in vivo* (13) data. The basis for this assumption is usually the observation that pilocarpine exhibits early peak times in the target tissues. In addition, the flux of pilocarpine from the cornea to the anterior chamber has been attributed to some property, as yet undisclosed, of the corneal endothelium (12).

The speculation that pilocarpine accumulates somewhere in the corneal tissues arises from analogy to early studies with fluorescein (14, 15) and accurate determinations of aqueous humor drug dynamics after dosing with pilocarpine (16). In the latter case, the elimination rate constant for pilocarpine from the anterior chamber was significantly smaller than the normal aqueous humor turnover rate, and the presence of a depot in the cornea was offered as an explanation.