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Short communication

Gas chromatographic-electron capture determination of 2,4'-dichlorodiphenylacetic acid from in-vitro adrenal transformations of mitotane and its analogs

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1. Introduction

Mitotane (**1**, Scheme 1), 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD), is used in the treatment of adrenocortical cancer and benign Cushing's syndrome because of its adrenocorticolytic action. Such use, however, is limited by its toxicity and lack of response in many patients. As part of a program to study the mechanism of action of mitotane and to develop new compounds to treat adrenal cancer, it was necessary to develop an analytical method to monitor the in-vitro metabolic transformations of mitotane and related compounds independent of the use of radiolabelled compounds.

Both in-vivo [1-3] and in-vitro assays with adrenal preparations [4-7] have shown 2,4'-dichlorodiphenylacetic acid (**4**) (*o,p'*-DDA) to be the major metabolite of mitotane. There is evidence with mitotane that an acyl chloride (**3**) is formed in the adrenal cortex as an active intermediate [8]. This is a transformation (Scheme 1) that is well established for hepatic activation of compounds containing a dihalogenated methyl moiety [9]. Therefore for mitotane, subsequent to enzymatic hydroxylation of the dichloromethine hydrogen, there would be spontaneous dehydrochlorination to yield an acyl chloride which could acylate adrenocortical bionucleophiles or afford DDA, upon reaction with water.

In an attempt to obtain a more efficacious adrenocorticolytic drug than mitotane, the effect of replacing the aliphatic chlorines in mitotane

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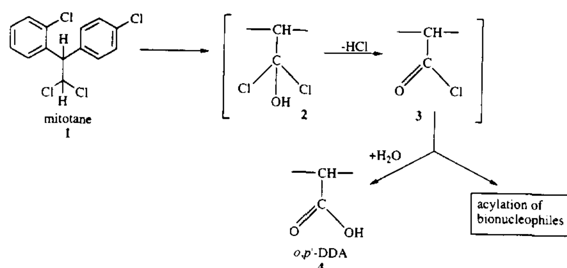
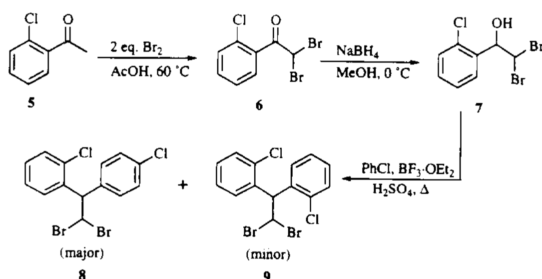


Scheme 1. Metabolic activation pathway for mitotane.

with other halides is being studied. Limited information on the synthesis and activity of such compounds has been reported in the Russian literature [10]. Therefore, one of the compounds noted in that study, the 2,2-dibromomethane analog of mitotane (**8**, Scheme 2), was synthesized. Prior to extensive in-vivo evaluation of this analog and the synthesis of related compounds, in-vitro testing with bovine adrenal cortex mitochondrial homogenates was undertaken. This required a sensitive analysis for the *o,p'*-DDA that would be produced in these homogenates as well as for that recovered after hydrolysis of the acylated adrenal tissue. The assay developed for this purpose was based upon the strong electron-capture property of a trichloroalkyl moiety in gas chromatography–electron capture (GC–EC) assays. This was accomplished by the preparation of trichloroethyl esters of DDA with the use of 2,2,2-trichloroethanol and trifluoroacetic anhydride in the presence of H_2SO_4, in a manner similar to that described for the preparation of phenoxyacetic acid esters [11]. It was of interest to determine whether such acidic metabolites could be measured directly by modification of this GC–EC procedure designed for the assay of phenoxyacetic acid herbicides in water.



Scheme 2. Synthesis of 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dibromoethane.

2. Experimental

2.1. Materials and reagents

o,p'-DDD, *p,p'*-DDD, *p,p'*-DDA, *o*-chloroacetophenone, 2,2,2-trichloroethanol and trifluoroacetic anhydride were purchased from Aldrich Chemical Co. (Milwaukee, WI). *o,p'*-DDA was supplied by the pesticides and industrial chemicals repository of the Environmental Protection Agency (Research Triangle Park, NC). Nicotinamide adenine dinucleotide phosphate in its reduced form (NADPH), sodium dodecyl sulfate (SDS) and *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethane sulfonic acid) (HEPES) were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. 2,2,2-Trichloroethyl ester of *o,p'*-DDA

A nitrogen atmosphere was established over *o,p'*-DDA (200 mg) in a reaction vial (Reacti-Vial 13222 capped with a Mininert valve SC-20; Pierce, Rockford, IL). Trifluoroacetic anhydride (1.5 ml), 2,2,2-trichloroethanol (0.4 ml) and concentrated H_2SO_4 (10 μl) were added through the valve by syringes and the mixture was allowed to react with magnetic stirring for 24 h at room temperature. The reaction mixture was evaporated to half its original volume under nitrogen and removed from the vial with dichloromethane (3×2 ml). The resulting dichloromethane solution was extracted with 0.5 N KOH saturated with dichloromethane (3×5 ml), washed with water saturated with dichloromethane (2×5 ml) and solvent was removed under a stream of nitrogen. Purification of the resulting oil was done on a preparative TLC silica plate (Brinkman 66-44-300-7) with hexane:methylene chloride (2:1) and the separated product band was extracted with methylene chloride to yield an oil. NMR ($CDCl_3$): δ 4.81 (s, 2H), 5.59 (s, 1H) and 7.21–7.43 (m, 8H). Anal. calcd. for $C_{16}H_{11}O_2Cl_5$: C, 46.59; H, 2.69, Found: C, 46.56; H, 2.87.

2.3. 2,2,2-Trichloroethyl ester of p,p'-DDA

The ester was prepared in a similar manner to that of the *o,p'*-ester but with purification by recrystallization from methanol. Melting point: 76–76.5°C. NMR ($CDCl_3$): δ 4.79 (s, 2H), 5.10 (s, 1H) and 7.23–7.34 (m, 8H). Anal. calcd. for $C_{16}H_{11}O_2Cl_5$: C, 46.59; H, 2.69. Found: C, 46.61; H, 2.50.

2.4. 1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dibromoethane

In a one-necked, round-bottomed flask (1 l) were placed 38.9 g (0.252 mol) of *o*-chloroacetophenone (**5**) and 200 ml of glacial acetic acid. To the solution was added 25.8 ml (0.504 mol) of bromine. The reaction mixture was refluxed overnight, cooled to room temperature and solvent was removed under reduced pressure. The residue was distilled (131–134°C, 0.75 mm Hg) to give 66.3 g (84.2%) of the dibromoketone **6**. **(Caution: Severe lachrymator!)** ^1H NMR (360 MHz, $CDCl_3$): δ 6.771 (s, 1H) and 7.26–7.63 (m, 4H). ^{13}C NMR (90 MHz): δ 42.09 (d), 127.13 (s), 130.39 (d), 130.69 (s), 131.03 (d), 132.95 (d), 133.86 (d) and 188.53 (s). IR (neat): 3017.1, 1721.3, 1717.8 and 1434.4 cm^{-1}. MS *m/z* (EI): 139 (100), 111, and 75. High res. MS: calcd. 309.8396; obs. 309.8405.

In a one-necked, round-bottomed flask (1 l) were placed 66.3 g (0.212 mol) of *o*-chlorodibromoacetophenone (**6**) from above and 200 ml methanol. The solution was cooled to 0°C and 16.9 g (0.447 mol) of $NaBH_4$ was added in portions. After the addition was complete, the reaction mixture was stirred at 0°C for 2 h, 200 ml 1 N HCl was added and methanol was removed at reduced pressure. The aqueous layer was extracted with dichloromethane (3 × 75 ml) and the combined organic layers were dried over magnesium sulfate and the solvent removed at reduced pressure. The residue was chromatographed on silica (70–230 mesh) with hexanes:ethyl acetate (9:1) to give 51.7 g (77.5%) of the alcohol **7** as a viscous oil. ^1H NMR (360 MHz, $CDCl_3$): δ 5.462 (d, $J = 2.8$ Hz, 1H), 6.084 (d, $J = 2.8$ Hz, 1H), 7.26–7.38 (m, 3H) and 7.61–7.67 (m, 1H).

In a three-necked, round-bottomed flask (500 ml) fitted with a dropping funnel, air condenser, thermometer and an inlet for N_2 was placed 8.00 g (25.4 mmol) of 1-*o*-chlorophenyl-2,2-dibromethanol (**7**) in 200 ml chlorobenzene. The solution was heated to 70°C and a solution of 6 ml BF_3 etherate and 3.5 ml concentrated H_2SO_4 was added dropwise. After the addition was complete, the reaction mixture was stirred for 3 h, cooled to room temperature and the solvent removed at reduced pressure. The residue was taken up in ethyl acetate and treated with sodium bicarbonate until slightly basic. The layers were separated and the organic layer extracted with saturated sodium bicarbonate solution (2 × 50 ml), followed by 50 ml of brine. The organic layer was dried over magnesium sulfate and the solvent removed at reduced pressure. The residue was chromatographed on silica (70–230 mesh) with hexanes:ethyl acetate (19:1). The product was a mixture of *o,p'*- and *o,o'*- isomers **8** and **9**. The mixture was stirred in pentane overnight and the desired *o,p'*- isomer collected by filtration to give 7.21 g (69.2%) with a melting point of 93–94°C. ^1H NMR (360 MHz, $CDCl_3$): δ 5.287 (d, $J = 9.6$ Hz, 1H), 6.238 (d, $J = 9.6$ Hz, 1H), 7.19–7.41 (m, 8H). ^{13}C NMR (90 MHz): δ 45.92 (d), 57.86 (s), 127.15 (d), 127.94 (d), 127.86 (d), 128.81 (d), 129.86 (d), 130.22 (s), 134.02 (s), 137.80 (s) and 138.05 (s). IR ($CHCl_3$): 3067.8, 3050.3, 1594.1 and 1573.4 cm^{-1}. MS *m/z* (EI): 408, 235 (100) and 165. High res. MS: calcd. 405.8526; obs. 405.8534. Anal. calcd. for $C_{14}H_{10}Br_2Cl_2$: C, 41.11; H, 2.46. Found: C, 41.30; H, 2.36.

2.5. Mitochondrial preparations and incubations

Fresh bovine adrenal glands were trimmed of fat and connective tissue and demedullated. The adrenal cortex was homogenized in five volumes of HEPES buffer (50 mM, pH 7.4) and 250 mM sucrose with a Potter–Elvehjem homogenizer. The mixture was centrifuged (1000*g*, 4°C, 20 min) to remove the tissue debris and then the whole homogenate was further centrifuged (10000*g*, 4°C, 20 min). The mitochondrial pellet was washed by resuspending it in HEPES buffer and recentrifuging before the final mitochondrial

pellet was resuspended in HEPES buffer prior to its use. Protein was determined by a modification of the method of Lowry [12].

Buffer (50 mM HEPES, pH 7.4) was added to a 15 ml centrifuge tube containing 1 mg (0.78 mM) of mitotane in ethanol (2%) or in DMSO (0.1%). The incubation mixture contained 2.9 mM $MgCl_2$ and 2.5 mM NADPH in a final volume of 4 ml. The reaction was started by the addition of 10 mg of mitochondrial protein. The mixture was incubated for 2 h at 37°C.

2.6. Extraction and esterification

An aliquot (1 ml) of the incubation mixture was transferred to a 50 ml centrifuge tube followed by addition of a methanol solution containing internal standard (*p,p'*-DDA, 6 μg) and 3 ml of distilled H_2O. The mixture was brought to pH 14 with KOH (0.5 N, 0.4 ml), extracted with ether (3 × 5 ml) and the ether extracts discarded. The remaining solution was acidified to pH 1 with HCl (1 N, 0.5 ml) and extracted with ether (3 × 5 ml). The ether extracts were combined in a 15 ml centrifuge tube and dried under nitrogen. The contents of the tube were extracted with methanol, and the combined extracts transferred to a reaction vial and solvent was removed under nitrogen.

Esterification was performed as described for the reference esters with the following modifications. Less of the reagents were used [trifluoroacetic anhydride (0.6 ml) and trichloroethanol (0.1 ml)] and the reaction vial was heated at 110°C for 35 min. After cooling, the vial was washed and its contents transferred with the aid of isooctane to a 15 ml centrifuge tube. The solvent was removed under nitrogen and isooctane added to reach a volume of 1 ml. The solution was extracted with KOH (0.5 N, 3 and 2 ml) with the aid of vortexing and aliquots (1–2 μl) of the isooctane solution were analyzed by GC–EC (Fig. 1). The amount of *o,p'*-DDA ester was quantitated against the ester produced from the *p,p'*-DDA added as an internal standard before extraction of the acids from the incubation mixtures.

2.7. Hydrolysis of bound material

An aliquot (2 ml) of the incubation mixture was extracted with chloroform:methanol (2:1). The top and bottom layers were discarded and the remaining protein layer was dissolved in 1 ml of 1% SDS and reprecipitated with 5 ml of acetone while undergoing vigorous vortexing. The precipitated proteins were pelleted by centrifugation and the solubilization and precipitation procedure was repeated three more times. The final pellet was dissolved in 1 ml of 6 N HCl and hydrolyzed for 24 h at 110–120°C. At the end of the hydrolysis period, 6 μg of *p,p'*-DDA was added as an internal standard and the 1 ml acidic layer was extracted with ether (3 × 5 ml). The ether extracts were combined, evaporated under nitrogen, esterified and analyzed by GC–EC as described above.

2.8. Instrumentation

Detection and quantitation of the acidic metabolite of mitotane and its *o,p'*-DDD analogs were achieved with a Hewlett-Packard 5830A gas chromatograph equipped with a 15 mCi ^{63}Ni electron-capture detector (model 830) and a glass column (6 ft × 1/4 in × 3 mm i.d.) containing 3% OV-101 on Chromosorb W-HP (100–120 mesh). The following conditions were used: column, 190°C; injector, 250°C; detector, 275°C; carrier gas, 5% methane in argon.

3. Results and discussion

The reference esters obtained from *p,p'*- and *o,p'*-DDA were used to develop the chromatographic system described above. This system resulted in the resolution of the esters in a region of the chromatograph devoid of interferring peaks which might arise from the incubation mixture and reagents used in the analysis. As noted by Mierzwa and Witek [11] in their preparation of phenoxyacetic acid esters, the potential for interference by the strong EC signals of the reagents was controlled by evaporation of the excess trifluoroacetic anhydride and by extraction of the

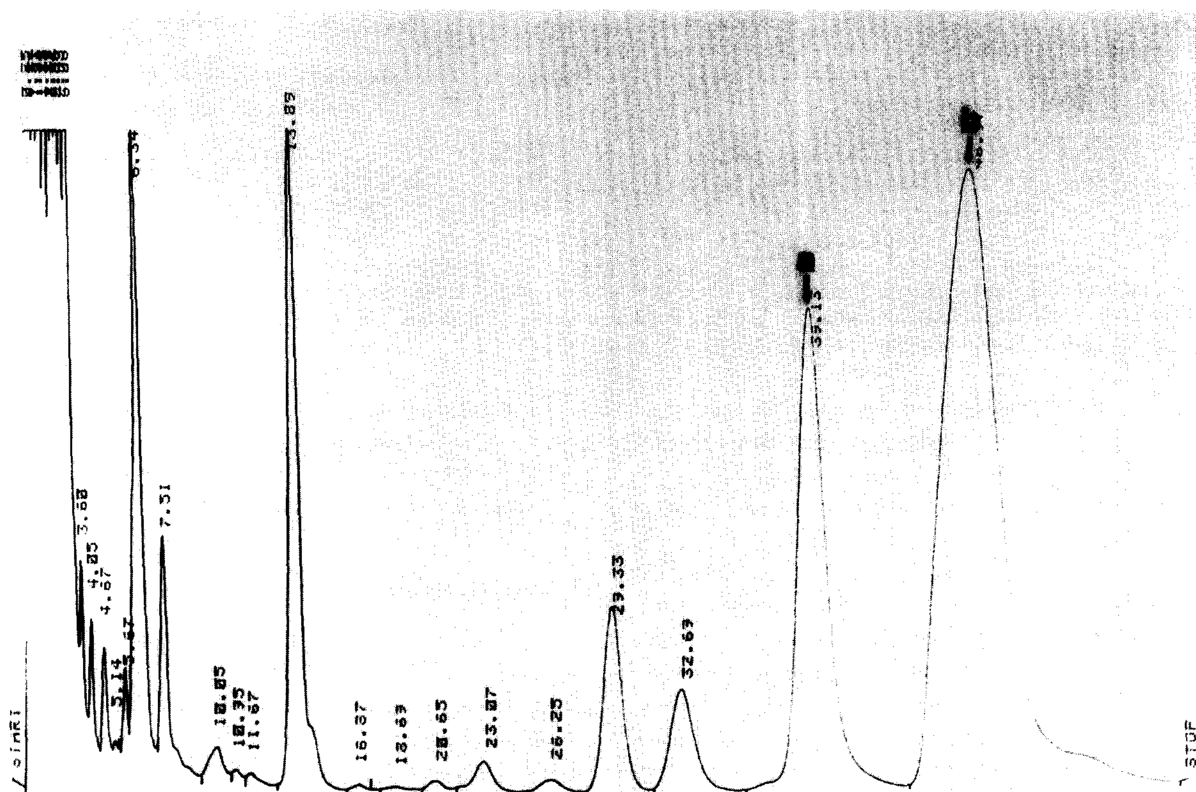


Fig. 1. A typical GC–EC chromatograph for the analysis of *o,p'*-DDA produced from *o,p'*-DDD by a bovine adrenal mitocondrial preparation. Peaks a and b are the 2,2,2-trichloroethyl esters of *o,p'*-DDA (39.13 min) and the internal standard, *p,p'*-DDA (46.97 min) respectively.

excess trichloroethanol in KOH solutions. The reference esters also served to confirm the retention times of the esters found in the analyses of *o,p'*-DDA produced by adrenal incubations.

The method was tested with known amounts of *o,p'*-DDA and with *p,p'*-DDA as an internal standard to correct for differences in extraction and synthesis yields. The usual procedure with *p,p'*-DDA as the internal standard, but in the absence of *o,p'*-DDD or its dibromo analog, was used. The recovery of *o,p'*-DDA and the reproducibility of the method as shown in Table 1 proved to be satisfactory.

The sensitivity of the procedure was also examined with the addition of only 0.1 μg of *o,p'*-DDA and with the reduction of the internal standard to 0.3 μg of *p,p'*-DDA in the usual incubation mixture. The incubation mixture was processed in the same way as in the standard analytical procedure with the exception that the final isooctane extract was concentrated to dryness, taken up in 100 μl of isooctane, and 2 μl of the isooctane solution injected for GC–EC detection. This modification represented a 5–10 fold concentration of the isooctane extraction after esterification. Although this approach was not designed for quantitative measurements, it consistently afforded the ability to detect the ester produced from 2.0 ng of *o,p'*-DDA. Under these conditions, an extraneous peak between the ester peaks is evident which, with further concentration, would prevent baseline resolution of the peaks. Nevertheless, the ability to detect the ester produced from 0.1 μg of *o,p'*-DDA could equal, with our usual use of 1 mg of DDD substrate per incubation, the detection of a 0.01% metabolic yield of DDA. This sensitivity,

Table 1
Determination and recovery of *o,p'*-DDA added to incubation mixtures[a]

Added *o,p'*-DDA (μg)	Found[b] (μg)			Recovery (%)	RSD (%)
15.0	14.2	15.1	14.7	97.8	3.08
6.00	5.92	6.16	5.92	100.0	2.31
2.00	2.07	2.05	1.94	101.0	3.47

[a] *o,p'*-DDA and 6 μg of the internal standard, *p,p'*-DDA, were added to 4 ml of the incubation mixture containing $MgCl_2$ (2.9 mM), NADPH (2.5 mM) and mitochondrial protein (10 mg) in HEPES buffer (50 mM, pH 7.4).
[b] Each of the three determinations is the mean of three injections.

and the reproducibility of the assay in the range 2–15 μg (Table 1), indicated that the GC–EC analysis of trichloroethyl exters should have general application for the detection and measurement of metabolically-produced acids.

The yields of *o,p'*-DDA, as it relates to the metabolism and protein binding of mitotane, compared with its dibromo analog are summarized in Table 2. The direct metabolic yields of *o,p'*-DDA and for that recovered after the hydrolysis of the protein fractions are greater for mitotane than for its dibromo analog. The detection of *o,p'*-DDA as a metabolite and its recovery after binding to the protein fraction for these compounds are consistent with those presented for mitotane in Scheme 1. Changes in halogen substitution at the β carbon could affect the rate of β hydroxylation and subsequent dehydrohalogenation as well as the relative stability and activity of the resulting acyl halides. It is planned to synthesize such halide analogs and to compare their extent of metabolism and binding by the GC–EC procedure described in this paper.

Table 2
Comparison of the metabolism of mitotane and 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dibromoethane

Substrate	% *o,p'*-DDA from:	
	Incubation mixture[a]	Hydrolysis of the protein fraction[b]
Mitotane	1.25 ± 0.28	1.82 ± 0.33
DiBr analog	0.41 ± 0.10	0.47 ± 0.14

[a] Values are the average of four injections for the analysis of *o,p'*-DDA from three independent incubations where [substrate] = 0.78 mM and mitochondrial protein = 2.5 mg ml^{-1} for an incubation time of 2 h.
[b] Values are the average of three injections of the analysis of the *o,p'*-DDA recovered from the hydrolysis of the protein fraction from each of the three incubations.

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