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IDENTIFICATION OF METABOLITES OF 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN (TCDD) FORMED ON INCUBATION WITH ISOLATED RAT HEPATOCYTES

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SUMMARY - Tritiated TCDD was incubated with isolated rat hepatocytes and the resulting incubation mixture subjected to high performance liquid chromatography (HPLC). The major peak of radioactivity not corresponding to TCDD was incubated with β-glucuronidase. The products of this reaction were derivatized with diazomethane and again subjected to HPLC. Two of the radioactive metabolites isolated by this procedure were found to co-migrate with authentic 1-methoxy-2,3,7,8-tetrachlorodibenzo-p-dioxin and 8-methoxy-2,3,7-trichlorodibenzo-p-dioxin. The identity of these metabolites was also confirmed by gas chromatography-mass spectrometry.

INTRODUCTION

Because of its extreme toxicity and occasional presence in the environment, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a compound about which there is considerable public health concern. Recent studies have provided evidence that TCDD is metabolized both in vivo and in vitro to more polar metabolites, some of which appear to be glucuronides (1-6). Identification of these metabolites may be of value in determining the mechanism of toxicity of TCDD. An important question is whether the various toxic effects of TCDD are the result of the action of the parent compound, a metabolite or metabolites, or both. Identification followed by synthesis of sufficient quantities of the metabolites of TCDD will facilitate answering this question. In this communication, we report the identification of two metabolites of TCDD formed upon incubation with isolated rat hepatocytes.

MATERIALS AND METHODS

Materials - Unlabelled TCDD was a gift from the Dow Chemical Co., Midland, MI. [1,6-^{3}H]TCDD with a specific activity of 50.5 Ci/mmole was

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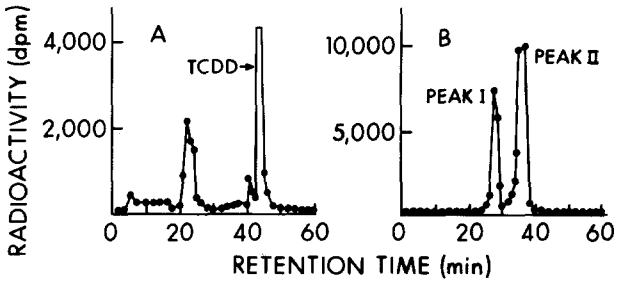

Figure 1-A; High performance liquid chromatography, using a preparative Ultrasphere-ODS column, of methanol extracts of an incubation containing rat hepatocytes and ^{3}H-TCDD. The incubation and chromatographic procedures are described in Materials and Methods. B; Fractions (21-24 min) of Fig. 1-A were combined, reduced in volume and incubated with β-glucuronidase. The resultant incubation was then subject to HPLC as described in Fig. 1-A and Materials and Methods.

obtained from ICN Pharmaceutical, Inc., Cleveland, OH, and purified as previously described (6). The radiochemical purity of the purified [^{3}H]TCDD was greater than 99.6%, as judged by high performance liquid chromatography (HPLC).

4,5-Dichloroguaiacol was prepared as described previously (7). 1-Methoxy-2,3,7,8-tetrachlorodibenzo-p-dioxin (1-MeO-TCDD), 2-methoxy-1,3,7,8-tetrachlorodibenzo-p-dioxin (2-MeO-TCDD), and 8-methoxy-2,3,7-trichlorodibenzo-p-dioxin (8-MeO-triCDD) were synthesized by condensation of 4,5-dichlorocatechol (8) with 1,2,4-trichloro-6-methoxy-5-nitrobenzene (9), with 1,2,4-trichloro-3-methoxy-6-nitrobenzene (9), and with 1,4-dichloro-2-methoxy-5-nitrobenzene (10), respectively, as described by Gray et al. (11).

Incubations - Hepatocytes were isolated from 150-200 g, male, Sprague-Dawley rats (Blue Spruce Farms, Altamont, NY) as described earlier (6). Cell viability was consistently greater than 90% as determined by trypan blue exclusion. A typical incubation mixture contained [^{3}H]TCDD (0.3 μmoles, 40 x 10^{6} dpm) and hepatocytes (2-4 x 10^{6} cells/ml) in 10 ml of Joklik-modified minimum essential medium containing 25 mM Hepes, pH 7.45, and 10% treated horse serum (Grand Island Biological Co., Grand Island, NY). Incubations were carried out under an O_2/CO_2 (95:5, v/v) atmosphere at 37° for 8 hours. The cell-free supernatant obtained upon centrifugation of the incubation mixture at 600 x g for 20 min was lyophilized to dryness and extracted with four 10-ml portions of methanol. The combined methanolic extracts were evaporated under a nitrogen stream, dissolved in a small volume of water, and then subjected to HPLC as described below. Under these chromatographic conditions, unmetabolized TCDD was found to elute from the column with a retention time of 44 min. The fractions corresponding to the radioactive peak eluting with a retention time of 22 min were collected (Fig. 1-A). The combined fractions were evaporated under a nitrogen stream and dissolved in a small amount of water (0.1 ml). The solution of metabolites was then incubated with bovine liver β-glucuronidase (Sigma Chemical Co., St. Louis, MO; 1000 units/ml) in 1 ml of 0.02 M potassium acetate buffer, pH 5.0, at 37° for 20 hr in the presence of ascorbate (1 mM). The resulting incubation mixture was extracted three times with 1-ml portions of ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous sodium sulfate, reacted with diazomethane and subjected to further HPLC as described below.

Analytical Methods - HPLC was performed on a Waters Associates instrument (Milford, MA) fitted with a solvent gradient system controller. For isolation of the glucuronide metabolites of TCDD (Fig. 1-A and 1-B), a preparative Altex Ultrasphere-ODS column (Beckman, Berkeley, CA; 10.0 mm x 25 cm) was used. The column was eluted with a 30-min linear gradient from water to methanol. The flow rate was 3 ml/min and 3-ml fractions were collected. For isolation and analysis of the aglycones and methylated aglycones (Fig. 2-A and 2-B), an analytical Altex Ultrasphere-ODS column (4.6 mm x 25

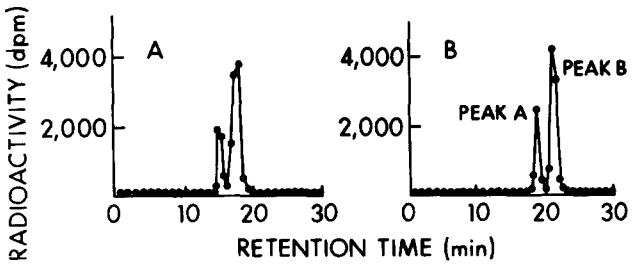

Figure 2. High performance liquid chromatography of the combined fractions corresponding to Peak II in Fig. I-B using an analytical Ultrasphere-ODS column. Fractions corresponding to Peak II in Fig. I-B were combined, evaporated under a nitrogen stream to dryness and the residue was dissolved in methanol (0.2 ml). An aliquot of the methanolic solution was examined by HPLC (A). The remaining methanolic solution was treated with a large excess of diazomethane at room temperature for 20 hr in a sealed vial and subjected to HPLC (B). The chromatographic conditions were described in Materials and Methods.

cm) was used. The column was eluted with a 20-min linear gradient from 80% methanol in water to 100% methanol. The flow rate was 1.5 ml/min and 0.75-ml fractions were collected. The methanol and water used for HPLC were acidified by adding 0.2 ml of 88% formic acid to 1000 ml of each solvent prior to use. Aliquots of the fractions from HPLC were dissolved in ACS scintillation cocktail (Amersham Corp., Arlington Heights, IL) and counted on a Packard Model 460 automatic scintillation counter equipped with an automatic external standard.

Electron-impact mass spectra were obtained using a Finnegan Model 4000 GC-mass spectrometer fitted with a 30-m fused silica capillary column (J & W Scientific, Inc., Rancho Cordova, CA) coated with SE-30. Helium gas was used as the carrier at a flow rate of 5 ml/min. The initial column temperature of 50° was maintained for 30 sec after injection. Thereafter the column temperature was increased to 255° at a rate of 20°/min. The elution of compounds was detected by monitoring the total ion current. The mass spectrometer was operated at a filament current of 50 μA and at 70 eV.

RESULTS AND DISCUSSION

A typical HPLC elution profile of the radioactivity in the cell-free supernatant from the incubation of [^{3}H]TCDD with rat hepatocytes is shown in Fig. 1-A. Fractions corresponding to the metabolite(s) of TCDD (21-24 min) from several incubations were combined and subjected to enzymatic hydrolysis using β-glucuronidase. Approximately 60% of the total radioactivity in these combined fractions could be extracted with ethyl acetate after β-glucuronidase treatment. HPLC of the ethyl acetate extracts showed that β-glucuronidase treatment yielded at least two radioactive products (Fig. 1-B).

The compounds 4,5-dichlorocatechol and 4,5-dichloroguaiacol are potential metabolites of TCDD. Under the chromatographic conditions described in Fig. 1, 4,5-dichlorocatechol and 4,5-dichloroguaiacol eluted from the column at retention times of 26.4 and 27.8 min, respectively. However, treatment of

the combined fractions corresponding to Peak I, Fig. 1-B (25-30 min), with excess diazomethane afforded products which were less polar (longer retention times) than 1,2-dichloro-4,5-dimethoxybenzene which is the product of diazomethane treatment of the catechol as well as the guaiacol. Because of limited amounts of material, the identity of the metabolites present in Peak I, Fig. 1-B, was not pursued further.

The fractions corresponding to Peak II, Fig. 1-B (34-37 min), were found to contain at least two metabolites when examined by HPLC using an analytical Ultrasphere-ODS column (Fig. 2-A). Diazomethane treatment of the fractions corresponding to Peak II, Fig. 1-B, resulted in the elution profile of radioactivity shown in Fig. 2-B. The fractions corresponding to Peak A and to Peak B, Fig. 2-B, were combined separately and evaporated to dryness. The residues were dissolved in small amounts of acetone and subjected to gas-chromatography-mass spectrometry (GC-MS).

The gas chromatogram of the fractions corresponding to Peak A revealed a single peak with a retention time of 11.5 min. The mass spectrum indicated the compound contained three chlorine atoms and one methoxy group (Fig. 3-A). The gas chromatographic retention time and mass spectra was consistent with authentic 8-MeO-triCDD (Fig. 3-B). Furthermore, the radioactive Peak A, Fig. 2-B, was found to co-migrate with synthetic 8-MeO-triCDD when examined by analytical HPLC.

Using the same gas chromatographic conditions, the fractions corresponding to Peak B, Fig. 2-B, afforded a single peak with a retention time of 14.7 min. The mass spectrum indicated the methylated metabolite contained four chlorine atoms and one methoxy group (Fig. 3-C). The two possible isomeric methoxy derivatives of TCDD, 1-MeO-TCDD and 2-MeO-TCDD, were synthesized and examined using GC-MS. Using the gas chromatographic conditions described in Fig. 3-C, these two methoxy derivatives of TCDD were not separated. However, both had a retention time identical to that of the metabolite described in Fig. 3-C. The mass spectra of these two methoxy derivatives of TCDD are quite similar, the only major difference being in the

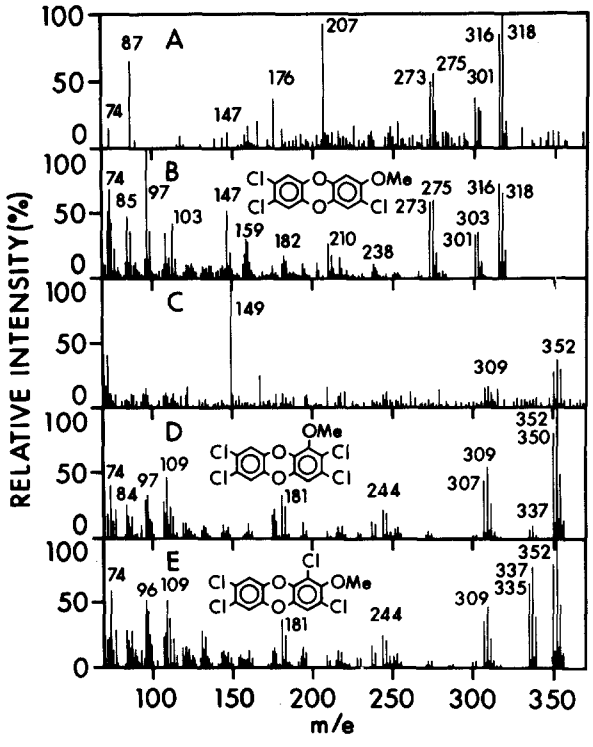

Figure 3. Mass spectra of methylated metabolites and authentic methoxy derivatives of TCDD. A; Peak A, Fig. 2-B, B; authentic 8-methoxy-2,3,7-trichlorodibenzo-p-dioxin, C; Peak B, Fig. 2-B, D; authentic 1-methoxy-2,3,7,8-tetrachlorodibenzo-p-dioxin, E; authentic 2-methoxy-1,3,7,8-tetrachlorodibenzo-p-dioxin. See Material and Methods for analytical methods.

intensity of the fragment ion clusters (m/e 335; $M^+ - CH_3$) relative to the molecular ion clusters (Fig. 3-D and 3-E). The mass spectrum of the methylated metabolite (Fig. 3-C) was consistent with that of 1-MeO-TCDD. 1-MeO-TCDD and 2-MeO-TCDD can be separated using an analytical HPLC column. The retention times of 1-MeO-TCDD and 2-MeO-TCDD are 21.5 and 22.8 min, respectively, when the column is eluted as described in Fig. 2. The radioactive Peak B, Fig. 2-B, was found to co-migrate with 1-MeO-TCDD.

Thus, 1-hydroxy-2,3,7,8-tetrachlorodibenzo-p-dioxin and 8-hydroxy-2,3,7-trichlorodibenzo-p-dioxin have been identified as metabolites of TCDD resulting from incubation with isolated rat hepatocytes.

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