Identification of the Major Metabolites of 1,4,7,8-Tetrachlorodibenzo-p-dioxin in Rats

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1. Introduction

The major problems involved with metabolism studies of 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) are its extreme toxicity (LD_{50} = 22-45 μg/kg for rats[1]) and its persistance in animal systems (half life of 31 days in rats[2]). The toxicity and slow rates of excretion of 2,3,7,8-TCDD mean that only μg/kg amounts can be dosed to animals in order to keep them viable during a metabolism study. Isolation and identification of metabolites at these low concentrations are difficult. In previous studies with 2,3,7,8-TCDD[3-5] and several less toxic furan congeners[6-9], the formation of polar metabolites was observed by TLC or HPLC comparison with the parent compound. Digestion of bile with strong acids or enzymes (i.e. β-glucuronidase and arylsulfatase) allowed the determination of some hydrolyzed metabolites by GCMS. In order to identify intact urine, bile, and feces metabolites of dioxins, we have studied the metabolism of a nontoxic congener, 1,4,7,8-TCDD. The low toxicity allowed mg/kg amounts to be dosed and metabolites to be isolated in sufficient quantities to utilize techniques such as NMR and FAB mass spectrometry for identification.

2. Experimental

1,4,7,8-Tetrachloro[7,8-dichlorophenyl-U-^{14}C]dibenzo-p-dioxin (ChemSyn Science Laboratories, Lenexa, KS; radiochemical purity >98%) was diluted with unlabelled 1,4,7,8-TCDD to a specific activity of 10,000 dpm/μg. Each of six male Sprague-Dawley rats (average weight 276 g) was dosed orally with 2 mg ^{14}C-1,4,7,8-TCDD in 0.5 ml peanut oil. A second group of seven male Sprague-Dawley rats (average weight 282 g) were fitted with cannulae in the common bile duct and allowed one day to recover from the surgery. Each of these was dosed orally with 2.4 mg ^{14}C-1,4,7,8-TCDD in 0.5 ml peanut oil. Bile, urine, and feces were collected daily for 72 h. After this time all rats were sacrificed and tissues were collected and frozen.

Aliquots of each urine and bile collection were assayed directly by liquid scintillation counting in Ecolite cocktail (ICN, Costa Mesa, CA). Feces and tissue samples were analyzed for ^{14}C by combustion with a Packard Model 307 oxidizer. Corresponding urine, feces, and bile fractions from the individual rats were pooled for metabolite isolation. Figures 1-3 show the purification steps used for urine, bile, and feces metabolites. Reverse phase HPLC (RP-HPLC) was performed on two 8 x 100 mm C_{18} Delta Pak™ cartridges in series (Waters, Milford MA) eluting with CH_3OH:H_2O or CH_3CN:H_2O gradients. Additional RP-HPLC purification was done on a 4.6 x 250

mm Symmetry™ C_{18} column (Waters, Milford MA) eluting with CH_3OH:H_2O gradients. Feces extracts were fractionated on a silica gel Sep Pak cartridge (Waters, Milford MA) with hexane, CH_2Cl_2:hexane (50:50), CH_2Cl_2, ethyl acetate, and CH_3OH before RP-HPLC. RP-HPLC was monitored with a flow-through radioactivity monitor (RAM).

3. Results

Over 97% of the recovered ^{14}C-1,4,7,8-TCDD was excreted from rats within 72 h of dosing. Poor recovery of ^{14}C from the bile-cannulated rats was due partly to problems in bile collection (one or two rats pulled their cannulas from the collection tubes during each collection period). Table 1 shows the distribution of ^{14}C in the tissues and excreta. Liver, carcass, and the GI tract were the only tissues which contained significant amounts of ^{14}C. The bile-cannulated animals had lower levels of ^{14}C in all tissues. The bile contained over 30% of the dosed ^{14}C indicating significant absorption of the congener. Urine was a minor route of excretion accounting for only 2-3% of the total dose.

Table 1. Recovery of ^{14}C from rats dosed with ^{14}C-1,4,7,8-TCDD.

Sample	% of Dose to Conventional Rats	% of Dose to Bile-Cannulated Rats
Urine		
0-24 h	2.7	1.6
24-48 h	0.5	0.2
48-72 h	0.1	0.04
Feces		
0-24 h	57.9	33.1
24-48 h	27.5	9.3
48-72 h	3.4	0.03
Bile		
0-24 h		33.3*
24-48 h		1.1
48-72 h		0.2
Heart	0.001	0.000
Lung	0.012	0.007
Liver	0.634	0.104
Kidney	0.025	0.008
GI tract + contents	1.070	0.038
Carcass	0.779	0.246
Total Recovery	94.7	79.4

*During this collection, two rats pulled their cannulas from the collection tubes resulting in a 5% estimated loss of ^{14}C which was not included in this percentage.

Figure 1. Purification of metabolites in 0-24 h urine (given as % of ^{14}C).

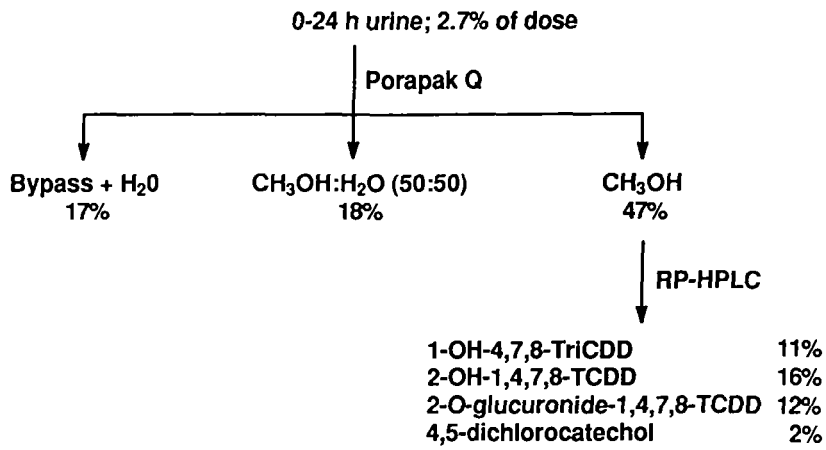


Figure 2. Purification of metabolites in 0-24 h bile (given as % of ^{14}C).

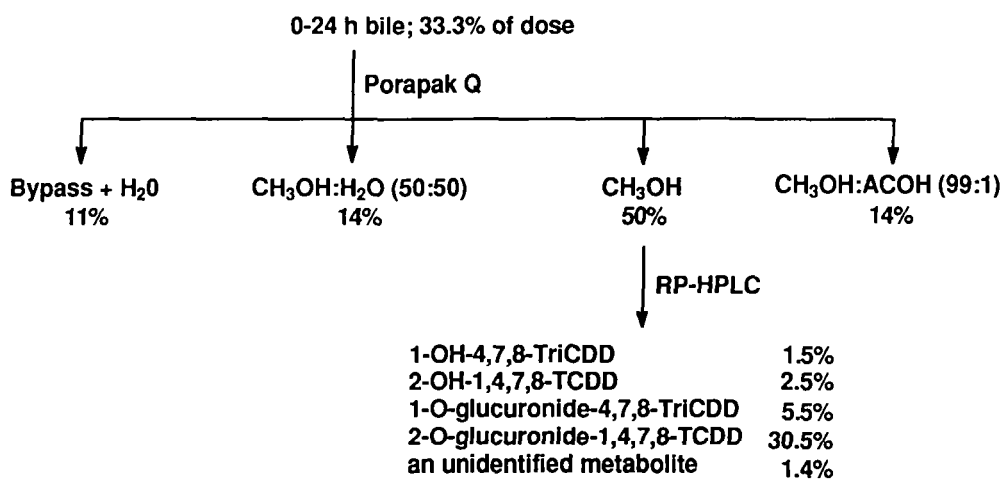
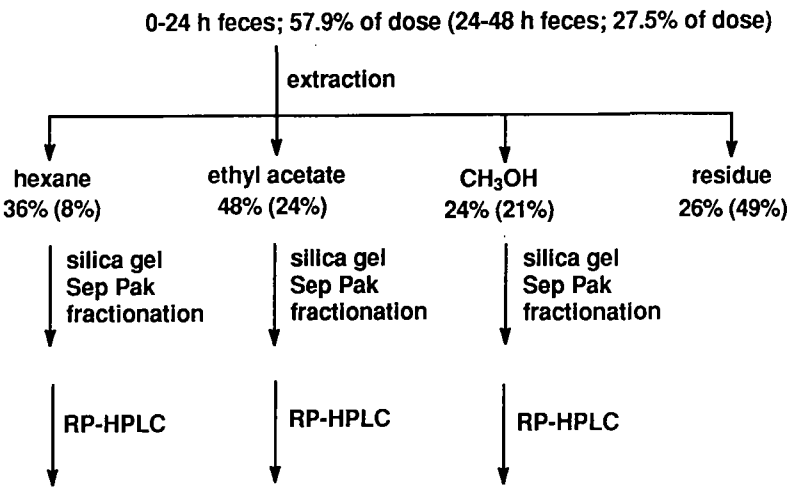


Figure 3. Purification of metabolites in each feces fraction (given as % of ^{14}C).



1,4,7,8-TCDD	10.8% (0.3%)	--- (---)	0.7% (---)
1-OH-4,7,8-TriCDD	5.0% (0.8%)	14.4% (2.4%)	2.6% (2.5%)
2-OH-1,4,7,8-TCDD	18% (3.3%)	33.6% (14.4%)	6.0% (4.8%)
4,5-dichlorocatechol	--- (---)	--- (---)	1.0 % (2.3%)

Three metabolites were isolated from the urine in approximately equal amounts: 1-hydroxy-4,7,8-TriCDD, 2-hydroxy-1,4,7,8-TCDD, and 2-O-glucuronide-1,4,7,8-TCDD. The two phenols were derivatized to trimethylsilyl ethers with Regisil® (Regis Tech. Morton Grove, IL) before GC-RAM and GC-MS analysis. Derivatized 1-OH-4,7,8-TriCDD had major ions at m/z 374 ($M^{+\cdot}$, Cl_3), 359 (M-15, Cl_3), and 324 (M-50, Cl_2). Derivatized 2-OH-1,4,7,8-TCDD showed m/z at 408 ($M^{+\cdot}$, Cl_4), 393 (M-15, Cl_4), and 358 (M-50, Cl_3). The ^1H NMR of 1-OH-4,7,8-TriCDD (7.20 s, 7.19 s, 6.87 d, J=9 Hz, and 6.52 d, J=9 Hz; CD_3OD) showed ortho coupling confirming protons in the 2- and 3- positions. The ^1H NMR of 2-OH-1,4,7,8-TCDD (7.24 s, 7.20 s, and 6.61 s; CD_3OD) was compared to synthetic standards[10] for positive identification. The glucuronide conjugate was analyzed by negative ion FABMS and showed m/z at 511 (M-1, Cl_4), 477 (M-35, Cl_3), 335 (M-177, Cl_4), and 301 (M-211, Cl_3). The ^1H NMR spectrum showed three aromatic singlets at 7.26, 7.24, and 7.11 ppm in CD_3OD. Although the NMR does not necessarily confirm the position of the O-glucuronide, we assumed that it was the conjugate product of the major tetrachlorohydroxy metabolite. A small amount of 4,5-dichlorocatechol was also isolated and identified by ^1H NMR (6.84 s; CD_3OD) and GC-MS of the trimethylsilyl ether (m/z 322, Cl_2).

The bile contained two major metabolites and three minor ones. No evidence for parent compound was seen in the bile. Over 30% of the ^{14}C in the bile was the same glucuronide conjugate found in the urine, 2-O-glucuronide-1,4,7,8-TCDD, as confirmed by -FABMS and ^1H NMR comparison. The other major metabolite was identified as 1-O-glucuronide-4,7,8-TriCDD by -FABMS which showed M-1 at 477 (Cl_3) and M-177 at 303 (Cl_3). The ^1H NMR showed ortho coupling in the aromatic region again indicating protons in the 2- and 3- positions (7.43 s, 7.38 s, 7.11 d, J=9 Hz,

6.93 d, J=9 Hz; DMSO-d_6). On our 400 MHz NMR in CD_3OD, the 2- and 3-protons had coincident chemical shifts and so coupling could not be observed.

Two of the minor components of the bile were identified by GC-MS and ^1H NMR as 1-OH-4,7,8-TriCDD and 2-OH-1,4,7,8-TCDD. These free hydroxy compounds may be artifacts due to cleavage of sulfate esters or the glucuronides during processing. The other minor metabolite was the most polar by RP-HPLC but could not be identified. It showed two single aromatic protons by NMR (7.25 and 7.24 ppm; CD_3OD) suggesting a conjugated dichlorocatechol or additional substitution on the dioxin ring. Derivatization methods did not render a product amenable to GC analysis nor did hydrolysis with 3.8 N HCl followed by derivatization.

The feces from conventional rats contained over 85% of the dose within 48 hours. Similar metabolites were found in the 0-24 h and 24-48 h collections; however, the amounts of each varied with time. 1,4,7,8-TCDD accounted for 11% of the ^{14}C in the 0-24 h feces and was identified by GC-MS (M^+ 320, Cl_4) and ^1H NMR (7.13 s and 6.95 s; $CDCl_3$) comparison to the original material. In the 24-48 h feces < 0.5% was unchanged parent. 2-OH-1,4,7,8-TCDD and 1-OH-4,7,8-TriCDD were the two major metabolites found in both the 0-24 and 24-48 h feces and were identified by GC-MS and ^1H NMR as before. 4,5-Dichlorocatechol was also found as a minor metabolite in the feces. A larger portion of the 24-48 h fraction (49%) remained as insoluble residue compared to the 0-24 h fraction (26%).

4. Conclusion

^{14}C-1,4,7,8-TCDD was extensively metabolized to hydroxylated products in the rat; however, no metabolism was observed on the 7,8-substituted ring. The major hydroxylated metabolite (2-OH-1,4,7,8-TCDD) was most likely formed via an arene oxide in the 2,3-position, since no NIH shifted products were observed. Studies of 2,3,7,8-TCDD in dogs[5] and 1,3,7,8-TCDD in rats[10] have shown only NIH shifted hydroxylation products formed via arene oxides in the 2,3- and 3,4-positions. Hydrolytic dechlorination resulted in the other major metabolite of 1,4,7,8-TCDD, namely 1-OH-4,7,8-TriCDD. Similar chlorine replacements have been shown for 2,3,7,8-TCDD[5], 2,3,7,8-TCDF[9], and pentaCDFs[7]. Unchanged parent was found in the first 24 hour feces accounting for 6.8% of the total dose. In rat bile, the hydroxylated metabolites were present almost exclusively as glucuronide conjugates. This is in contrast to a study of 2,3,7,8-TCDD in dogs which appeared not to produce biliary glucuronide conjugates[5] and a study of 2,3,4,7,8-pentaCDF in rats which suggested no biliary conjugates were formed[6]. To our knowledge, this is the first time intact glucuronide conjugates of a dioxin or furan have been identified by mass spectrometric and ^1H NMR methods. No sulfate conjugates of the hydroxylated metabolites were found in the bile.

Unlike previous studies of 2,3,7,8-TCDD and related compounds[5,7,9], dihydroxy metabolites and metabolites formed by cleavage of a single ether bond of the dioxin ring were not observed. Cleavage of both ether bridges to give the 4,5-dichlorocatechol occurred to a minimal extent. Metabolites arising from the glutathione pathway were not found in bile, urine, or feces. Although some reports have been made of sulfur-containing metabolites of dioxins and furans[7,11,12] after extremely harsh treatment (H_2SO_4, pH=1, 80°C), the glutathione pathway does not appear to be a major detoxication route for dioxins.

5. References

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