NMR Identification of Isomeric 1,3,7,8 TCDD Metabolites in the Rat

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Polychlorinated biphenyls (PCBs), dibenzo-para-dioxins (PCDDs) and dibenzofurans (PCDFs) are ubiquitous environmental trace contaminants arising from various human activities or natural processes. Their toxicity varies greatly according to the specific isomer considered. The PCDD isomer 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) has been called the most toxic anthropogenic chemical known.[1] A great deal of research has been done on the formation, distribution, environmental trace analysis, pharmacokinetics, comparative toxicology and public health implications of these compounds. Comparatively less has been done on their metabolism and mode of action in intact organisms. Since the major route of exposure for humans to trace contaminants of this sort is from the food supply, bioaccumulation of polyhalogenated aromatics in animals raised for food may lead to concerns about food safety issues. A clearer understanding of the metabolism and retention or elimination of this class of compounds in animals is required to address these concerns. Recent advances in analytical chromatography and NMR spectroscopy may allow us to confirm and extend our knowledge in this area.

Metabolic alteration of xenobiotics is generally assumed to lead to the detoxification and elimination of harmful compounds. There are however many exceptions to this generalization, most recent and relevant to PCDDs being the work done on PCB and DDT metabolites. Methylsulfone and hydroxylated metabolites as well as the parent compounds seem to be involved in the mode of action of some of the toxic effects of these compounds.[2)3)4)] Work in this area has made clear the necessity for standard chemical materials[5)] and adequate analytical and identification methods[6)] to help sort out causation in toxicology from correlation.

Of the many theoretically possible metabolites of all the known isomers of PCDDs, very few have been rigorously tested for biological activity. A possible monohydroxy-trichloro metabolite of 2,3,7,8-TCDD, 2-OH-3,7,8-TriCDD, was synthesized and found to be three orders of magnitude less toxic than the parent compound.[7)] Experiments involving dosing guinea pigs with 2,3,7,8-TCDD metabolites in dog bile were complicated by the development of symptoms in control animals (dosed with control bile) and the uncharacterized nature of the dose material.[8)] A possible metabolite isomer 2-OH-1,3,7,8-TCDD was found to bind in vitro to thyroxin-binding protein 4.5-fold more than the physiological ligand.[4)] Clearly, much work remains to be done on the characterization of PCDD metabolites and their possible relation to toxicity in this class of compounds.

A fundamentally important part of any toxicological assay is to know the identity and concentration of the material being tested. This is not always as simple or as straightforward as it sounds when dealing with chemicals that have many possible isomers, or those that may

be biologically bound in vivo or may decompose during routine sample handling. Recent work has shown how useful high resolution NMR spectroscopy can be in distinguishing between various synthesized isomers of PCDDs.[9] The relevance of NMR identifications of isomers to metabolic work was demonstrated by the correct identification of 1,2,7,8-TBDF as the actual dose material that was rapidly metabolized in a study originally thought to be of the 2,3,7,8-TBDF isomer.[10] In the past most PCDDs and their metabolites have been identified by either their chromatographic behavior or GC/MS of their derivitization products.[11][12][13] Advances in chromatography and NMR spectroscopy of trace analytes should permit more direct examination of these compounds in biological matrices. Dosing animals with a series of differently substituted PCDD isomers combined with spectroscopic identification of major and minor metabolites would enable a more detailed picture of their metabolic pathways to be constructed. This in turn may help explain some of the variation in observed TEFs and provide leads and methods for investigating some of the less well understood toxic effects of these compounds.

Experimental:

Male Sprague-Dawley rats (300 g) were dosed by gavage with 6.7 mg/kg (2.0mg/0.65 ml corn oil) ^{14}C 1,3,7,8-TCDD (Chemsyn, 63.4 mCi/mmol) that had been adjusted to 0.40 mCi/mmol with cold standard material synthesized in-house. Rats were placed in steel metabolism cages and 24 h feces and urine samples were collected for three days. Animals were then anesthetized and sacrificed by exsanguination (vena cava) and tissue samples were collected for combustion analysis. Urine aliquots were assayed directly by LSC. Feces were frozen and later homogenized in water, extracted once with 10ml/1g water, centrifuged (1500 g) and the solids re-extracted 3X with 10ml/1g ethyl acetate:hexane (1:1). This procedure removed 94% of the total radioactivity in the feces as determined by combustion analysis. All tissues were burned wet, except for the total carcass which was ground and lyophilized before aliquots were taken for burning. HPLC was performed with either an 80%-100% water to methanol gradient for parent and monohydroxy compounds or with 5%-100% water to acetonitrile gradient with 0.1%TFA for more polar compounds. TLC was done on silica gel F-254 plates developed in various solvent systems of hexane/ethyl acetate/tetrahydrofuran. NMRs were obtained from 10 µg of material in sealed capillary tubes in CD_3OD or $CDCl_3$.

Results:

Distribution of radioactivity from a typical experiment (Table 1) shows that most of the dose is excreted in the feces (94% within 72 hrs) and only trace amounts appear in the urine (1.7% within 72 hrs). Replicate experiments to obtain material for metabolite identification (n=7) gave overall recoveries of 78.8 + 8.1% of the dose with much individual variation in percent of dose per day excreted, ranging from 10-75% on the first day and 8-54% on the second day. Analysis of the organic extract of the 0-24 h feces by HPLC showed 25% of the radioactive material to be parent compound and 75% to be a more polar metabolite (identified by GC/MS and NMR). Only the metabolite was present in the 24-48 h and 48-72 h feces, and it was also the main radioactive component (45%) of aqueous feces extracts.

The major metabolite in the feces appeared as one peak in both HPLC gradients, a single spot in several TLC systems, and a single GC peak (GC/MS) with M^+ = 336 and with a typical four chlorine cluster pattern. NMR, however, indicated that the sample was a mixture (Fig 1b) with more peaks in the aromatic region than expected for any of the theoretically possible metabolites. Further experiments with TLC (95:5 hexane/tetrahydrofuran, developed 4X on a 5x20 cm plate) resolved the metabolites into two bands. These isomers had identical mass spectra and HPLC or GC retention times, but by their NMR spectra in CD_3OD were

identifiable as 2-OH-1,3,7,8-TCDD (Fig 1c) and the NIH shift product of the arene oxide intermediate, 1-OH-2,3,7,8-TCDD (Fig 1a). Comparison of spectra of these isomers obtained in $CDCl_3$ with published values for unambiguously synthesized chemical standards[14] support these assignments (allowing for dilution effects), with the 2-OH showing a singlet at 6.75 ppm (lit. value 6.85 ppm) and the 1-OH at 6.57 ppm (lit. value 6.65 ppm).

If only chromatographic behavior and MS spectra had been used to identify these metabolites, it would not have been possible to distinguish between them. That one of these metabolites, 1-OH-2,3,7,8 TCDD, is also a theoretically possible metabolic product of 2,3,7,8-TCDD demonstrates the interrelatedness, complexity and toxicological relevance of isomer identification problems in this series of compounds.

While a great deal has been accomplished in developing reliable methods for very low level detection of PCDDs, it is clear that much remains to be done in the development of methods for analysis and identification of their metabolites. Analytical methods for PCDDs have been deliberately optimized to remove other interfering compounds present. Preliminary experiments indicate that monohydroxy-PCDDs do not come through with PCDDs in the standard CDC cleanup procedure. Many of the theoretically possible metabolites in this group- thiophenols, methylsulfones, dihydroxy- , trichloro-, or ring-opened phenoxyethers- have very similar molecular weights complicated by chlorine isotope patterns making overlapping mass spectra difficult to interpret. Development of optimal extraction and chromatographic methods combined with NMR spectroscopy should enable us to identify metabolite isomers of PCDDs and investigate their biological properties as is being accomplished in the case of PCBs.

References

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Table 1. Recovery of ^{14}C from a rat given [^{14}C] 1,3,7,8-TCDD by gavage.

Substance	% of Dose
Urine:	
0-24 hr	1.2
24-48 hr	0.31
48-72 hr	0.12
Feces:	
0-24 hr	36.9
24-48 hr	51.7
48-72 hr	5.2
Liver	0.13
Kidneys	0.016
GI Tract	3.5
Heart	0.0004
Lungs	0.0012
Peri-renal Fat	0.044
Carcass	0.52
TOTAL Recovery	99.7

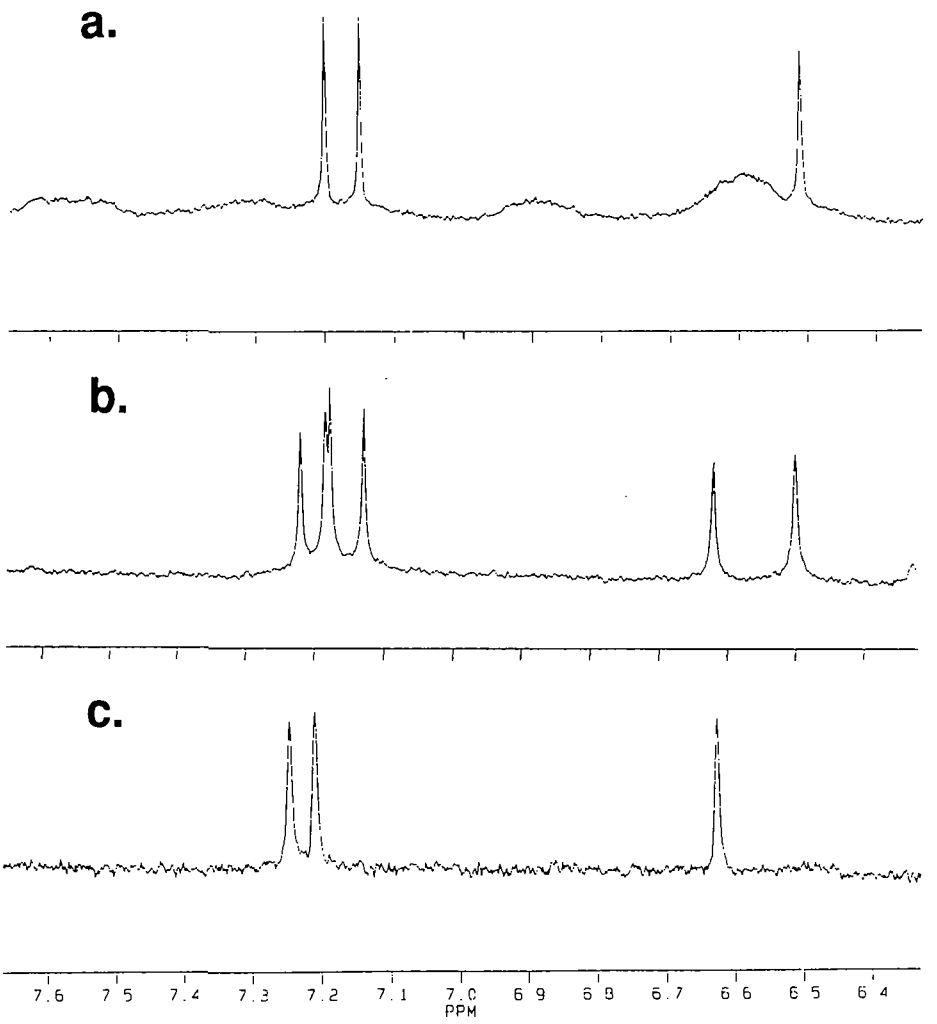

Fig. 1. ^{1}H-NMR of metabolites of 1,3,7,8-TCDD