Half lives of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin after EROD-inducing and non-inducing doses

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1 Introduction

The 2,3,7,8-substituted polychlorodibenzo-*p*-dioxins (PCDD) belong to the most persistent substances in mammalians[1]. This persistence is due to a combination of high lipophilicity and correspondingly low water solubility, leading to a sequestration in lipid rich compartments, with resistance to metabolism. The relative contributions of these two properties, however, have not been determined. Furthermore, the influence of various physiological and biochemical changes seen in conjunction with different doses of TCDD is also unknown.
TCDD metabolism is believed to be the rate limiting step for its elimination[2]. It has long been a point of dispute whether TCDD is able to induce its own metabolism[1,3]. Isolated hepatocytes from TCDD pre-treated rats and hamsters are able to metabolise TCDD at increased rates, provided the substrate concentration during incubation exceeds 0,1 µM[4,5]. Overtly toxic doses are needed to attain such high levels in rat liver *in vivo*. The results from studies on biliary excretion in rats are somewhat contradictory. No effect of TCDD pre-treatment was observed within 8 h, a small but significant increase in biliary elimination was seen within 72 h of a second dose of TCDD[6,7].
So far, all investigations on the elimination of TCDD from rats used doses that substantially induced hepatic microsomal enzyme activity. Slight induction (30%) of hepatic cytochrome P450 (CYP450) occurs already at single doses above 3 ng/kg body weight and reaches its maximum (5500%) near 1 µg/kg body weight in female Wistar-rats[8]. To investigate the influence of enzyme induction on the half life of TCDD in male Sprague-Dawley rats we compared the excretion via feces and urine of oral doses of 8 ng and 2 µg TCDD/kg body weight, respectively. To further assess the role of nonbiliary excretion, we investigated the influence of increasing the lipophilicity of the gut contents by addition of 5% hexadecane to the feed. This dietary regimen can increase the transfer of lipophilic substances across the intestinal wall as has been shown for hexachlorobenzene (HCB)[9].

2 Materials and Methods

^{3}H-TCDD (specific activity 26,4 Ci/mmol; radiochemical purity >97% as stated by manufacturer) and TCDD (chemical purity >98% as determined by GC/MS) were purchased from Cambridge Isotope Laboratories (Woburn, MA).
Male Sprague-Dawley rats, age 7-8 weeks, were obtained from Sasco (Omaha, NE) and adapted for 1 week to suspended wire bottom cages (controls) or transparent plastic cages designed to

separately collect feces and urine. They were allowed free access to tap water and finely ground Purina Rodent Chow 5001 (Ralston Purina, St. Louis, MO), or the same food thoroughly mixed by hand with 5% hexadecane (w/w) except for a 24 h fasting period prior to dosing. Animals weighed 240-280 g at the beginning of experiments.

Solutions of ^{3}H-TCDD and TCDD in corn oil were prepared by transferring the appropriate amount dissolved in toluene to a glass vial, evaporating the solvent under a stream of nitrogen, and adding corn oil which was then stirred for 4 days. Solutions were made to contain 2 ng (6,15 fmol; 0,162 µCi) ^{3}H-TCDD/ml corn oil or 0,49 µg (1,52 nmol) TCDD and 10 ng (30,8 fmol; 0,81 µCi) ^{3}H-TCDD/ml corn oil. Groups of 10 animals each received 4 ml/kg body weight of the respective solution or vehicle by oral gavage, amounting to doses of 0 ng, 8 ng and 2 µg TCDD/kg body weight, respectively. In each dose group, 5 animals received regular food, 5 others were kept on hexadecane supplemented chow starting the day after dosing. Urine and feces were collected for 20 days from animals treated with radioactivity. 21 days after dosing animals were sacrificed by CO_2-asphyxiation, their livers excised and weighed, and samples of liver, white adipose tissue, muscle, skin and serum were taken (only liver samples in controls). Liver samples for determination of EROD were frozen in liquid nitrogen immediately after excision.

Radioactivity in urine and serum was quantified by scintillation counting of duplicate 1 ml aliquots. The radioactivity in urine from rats treated with 8 ng ^{3}H-TCDD/kg body weight was indistinguishable from background levels from the second day after dosing on in all cases and collection of urine was discontinued for those rats. Feces were air dried for 48 h, weighed, ground, and radioactivity was determined after combustion of duplicate samples of 150-300 mg in a Tricarb B306 sample oxidiser (Packard Instrument Co.; Dreieich, FRG). Tissue samples were dissolved in tissue solubiliser according to manufacturers recommendations and radioactivity determined in lysates. Ethoxyresorufin-*o*-decthylase (EROD) activity was determined in microsomal suspensions prepared from liver homogenates as described by Weber et. al.[10].

After completion of these experiments we learned of the instability of ^{3}H-TCDD[4]. HPLC of the ^{3}H-TCDD-standard used in the above experiments revealed it to consist of approximately 70% ^{3}H-TCDD eluting after 72-77 min and 4 main impurities eluting after 30-32 (accounting for 13-14%) and 42-45, 47-49 and 53-55 min (together representing 16-17% of total radioactivity;); Chromatographic conditions: T=25°C; methanol/water 85:15 (v/v); Bakerbond PAH 16-PLUS, 250 x 3.0 (length x i.d.); the main peak at 72-77 min was identified as ^{3}H-TCDD by monitoring the UV-extinction of unlabelled TCDD added prior to chromatography. TCDD and a trichlorodibenzo-*p*-dioxin (TrCDD), but no other chlorinated dioxins were detected in the standard by GC-MS. To assess the influence of these impurities, the standard was purified and the impurities were isolated by preparative HPLC. Dosing solutions were then prepared containing equal amounts of radioactivity of purified ^{3}H-TCDD, isolated impurities and the impure standard, the activity corresponding to 2 ng pure ^{3}H-TCDD (26.4 Ci/mmol) /ml corn oil. Three groups of 5 animals received 4 ml/kg body weight of each solution by oral gavage and feces and urine were collected for 10 days. The animals were sacrificed and radioactivity in feces, liver and serum and liver EROD-activities were determined.

Sigma-minus plots (logarithm of fraction of dose remaining to be excreted vs. day) were established for fecal excretion by individual animals. Elimination constants k_{el} were calculated from these plots omitting the first four days (See: 3. Results). Biological half lives of TCDD elimination were calculated as in ln(2) over k_{el}. Statistical significance of differences between data sets was determined by Student's t-test. Data in text, tables, and figures are given as means ± standard deviation

3 Results

No significant differences ($p < 0.01$) were seen between groups with respect to body weight in either experiment (data not shown). The left panel in figure 1 presents Sigma-minus plots showing the fecal excretion of radioactivity by animals treated with 2 µg or 8 ng TCDD and the different dietary regimens. All animals excreted 30-50% of the initial dose within the first 48 h after dosing, after which excretion followed first order kinetics. Therefore, half lives were calculated omitting the data from the first four days. Table 1 summarises the concentrations found in tissues and serum, percent of dose remaining in liver 21 days after dosing, total urinary excretion and the sum over fecal excretion between day 5 and day 21, and half lives for fecal excretion. A marked shift of radioactivity content from other tissues to liver was observed between low and high dose. For combined urinary and fecal excretion, the half life for the elimination of radioactivity decreased to 21 and 20 days for the groups treated with 2 µg TCDD/kg body weight and hexadecane and regular chow, respectively. EROD activities were determined as 3410 ± 730 and 2540 ± 380 pmol/(mg*min) for the groups treated with 2 µg TCDD/ kg body weight and hexadecane or regular chow, 138 ± 26 and 111 ± 28 pmol/(mg*min) for the groups treated with 8 ng TCDD/ kg body weight and hexadecane or regular chow, and 129 ± 40 and 77 ± 23 pmol/(mg*min) for the control groups given hexadecane or regular chow, respectively. Hence, treatment with 2 µg TCDD/kg body weight elicited approximately 25fold induction of EROD, while treatment with 8 ng TCDD/kg body weight and/or hexadecane showed no effect.

Fecal elimination of radioactivity by rats given purified ^{3}H-TCDD, the isolated impurities and the impure standard is depicted in the right panel of figure 1. Concentrations of radioactivity in serum, percent of dose remaining in liver 10 days after dosing, cumulative fecal excretion between day 5 and 10, half lives for fecal excretion, and EROD-activities are shown in table 2. While the animals treated with isolated impurities excreted 77% of the total dose during the first four days, elimination

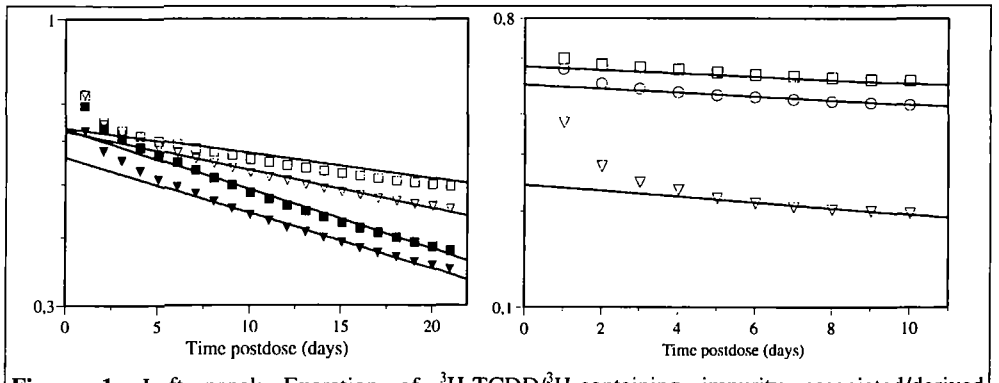

Figure 1: Left panel: Excretion of ^{3}H-TCDD/^{3}H-containing impurity associated/derived radioactivity from male Sprague-Dawley rats given two different nonlethal doses and treated or not treated with hexadecane (sigma-minus plot). Full symbols: Rats treated with 2µg TCDD/kg body weight; open symbols: Rats treated with 8 ng TCDD/kg body weight. Squares: Rats fed diet supplemented with hexadecane; triangles: Controls. Right panel: Excretion of ^{3}H-TCDD/^{3}H-containing impurity associated/derived radioactivity from male Sprague-Dawley rats given the equivalent of 8 ng ^{3}H-TCDD/kg body weight of pure ^{3}H-TCDD, isolated impurities and impure standard. Squares: Purified TCDD; triangles: Isolated impurities; circles: Impure standard. Linear regression was performed omitting data for the first four days. Each point represents the mean from 5 animals. Error bars are omitted for clarity.

Table 1: Tissue concentrations 21 days after dosing, cumulative fecal and urinary excretion between day 5 and day 21 and half lives of fecal excretion calculated from data within the same period for male Sprague-Dawley rats given two different nonlethal doses and treated or not treated with hexadecane

Dose	2 µg TCDD/kg body weight		8 ng/kg TCDD/kg body weight		Unit
Tissue	Hexadecane	Controls	Hexadecane	Controls	
Liver	5 ± 1%*	6 ± 1%*	1.8 ± 0.6%*	2.7 ± 0.8%*	Percent of dose in organ
Serum	6 ± 2%*	8 ± 1%*	3 ± 1%*	4 ± 2%*	Percent of dose per ml (*1000)
Muscle	5 ± 1%	6 ± 1%	7 ± 2%	7 ± 1%	Percent of dose per g tissue (*1000)
Skin	12 ± 4%*	16 ± 1%*	34 ± 11%*	40 ± 10%*	Percent of dose per g tissue (*1000)
White adipose tissue	0.19 ± 0.03%*	0.18 ± 0.01%*	0.7 ± 0.2%*	0.8 ± 0.3%*	Percent of dose per g tissue
Cumulative excretion feces	18 ± 4%	21 ± 3%*	15 ± 1%	10 ± 2%*	Percent of dose excreted between day 5 and day 21
Cumulative excretion urine	4 ± 1%	5 ± 1%	N.D.	N.D.	Percent of dose excreted between day 5 and day 21
Half live of fecal excretion	30 ± 4*	28 ± 3*	44 ± 4*	70 ± 17*	Days
	= significant difference between hexadecane treated animals and controls within the same dose group, $p < 0.01$				
*	= significant difference between animals treated with different doses, $p < 0.01$				

of radioactivity slowed down dramatically thereafter and occurred at rates similar to the other groups. Induction of hepatic EROD did not occur.

4 Discussion

The rapid excretion of 70-80% of the dose of isolated impurities and the following slow elimination of the remainder points towards two pharmacokinetically very different impurities or groups of impurities in the impure standard, one rapidly metabolised, the other poorly. The half life for the elimination of 2,3,7-TrCDD was established as approximately 1.5 days, matching the fast elimination of the former[11]. From the excretion of isolated impurities it can be estimated that approximately 7% of the initial dose of radioactivity remaining to be excreted on day 4 in the low dose groups of the experiment investigating the elimination of TCDD with hexadecane represented the slow component of impurities. While the difference between half lives in the two control groups at high and low dose could be due to a more rapid metabolism of the slow component of impurities

Table 2: Liver content and serum concentrations of radioactivity 10 days after dosing, cumulative fecal excretion of radioactivity between day 5 and day 10, half lives of fecal excretion calculated from data within the same period and EROD-activities in liver homogenates for male Sprague-Dawley rats given the equivalent of 8 ng ^{3}H-TCDD/kg body weight of purified ^{3}H-TCDD, impurities isolated from an impure ^{3}H-TCDD-standard or the impure standard

Tissue	Purified ^{3}H-TCDD	Isolated impurities	Impure standard	Unit
Liver	23 ± 10%*	0.51 ± 0.05%	15 ± 9%*	Percent of dose in organ
Serum	6 ± 2%*	2.2 ± 0,4%	6 ± 1%*	Percent of dose per ml (*1000)
Cumulative excretion feces	4.5 ± 0.9%	3.7 ± 0.5%	4.4 ± 0.5%	Percent of dose excreted between day 5 and day 10
Half live of fecal excretion	54 ± 6*	30 ± 9	46 ± 6*	Days
EROD-activities	81 ± 35	78 ± 37	103 ± 22	Pmol resorufin/(mg protein*min)
*= significantly different from group treated with isolated impurities, $p < 0.01$				

at the higher dose, the difference in cumulative excretion of 11% is larger than the estimated amount of impurity remaining. It cannot be ruled out, however, that the difference caused by the administration of hexadecane in the dose groups receiving 8 ng TCDD/kg body weight is due to a more rapid excretion of impurities.

The notion of an autoinduction of TCDD-metabolism and hence increased excretion in the dose range of 2 µg TCDD/kg body weight challenges the view commonly expressed in literature, but is supported by the results of Poiger and Buser[6]. These authors found a slight but significant increase in biliary excretion (9.7 ± 1.9% vs. 7.0 ± 0.9%) within 72 h after administration of ^{3}H-TCDD caused by pre-treatment with 10 µg TCDD/kg body weight i.p. 8 days prior to challenging. Kedderis et. al. found no increased biliary excretion within 8 h of administration of ^{14}C-TCDD by pre-treatment with 100 nmol TCDD 3 days earlier[7]. It seems, however, likely that a difference as small as experienced by Poiger and Buser within 72 h would be extremely difficult to detect in a period as short as 8 h.

The difference between the half lives for fecal excretion as a result of treatment by hexadecane at a dose of 8 ng TCDD/kg body weight and the lack of such difference at 2 µg TCDD/kg could be explained by different rate limiting steps at different doses. If indeed, as postulated by Neal et. al.[2], autoinduced metabolism is the rate limiting step at the higher dose, the increase of lipophilicity of the contents of the gut by hexadecane will not lead to enhanced excretion of TCDD. When the dose is too low to effect autoinduction, luminal transfer across the intestinal wall is likely to become the rate limiting step, and increased elimination from the animals body by hexadecane will occur.

The results presented here lead us to the conclusion that while autoinduced metabolism might be the rate limiting step in the fecal elimination of TCDD in Sprague-Dawley rats at doses that markedly induce hepatic EROD, direct excretion into the intestinal lumen is probably the rate limiting step at doses that do not induce hepatic CYP450.

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6 References

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