IDENTIFICATION OF HUMAN METABOLITES OF 2,3,7,8-TCDD

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Abstract
For the first time, two polar metabolites of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were identified in various tissues of a human suffering from severe TCDD intoxication. 2,3,7-trichloro-8-hydroxydibenzo-*p*-dioxin and 1,3,7,8-tetrachloro-2-hydroxy-dibenzo-*p*-dioxin were identified in serum, faeces, and urine by gas chromatography high resolution mass spectrometry. Highest amounts of these metabolites were detected in faeces whereas serum and urine contained only traces. The relative amount of TCDD metabolites excreted via faeces accounts to approximately 50% of the totally eliminated TCDD by this route. As only small amounts of TCDD metabolites were detected in urine, renal excretion of these compounds can be considered as minor elimination pathway. In serum the relative amount of metabolites was 50 times lower than in faeces, probably due to the rapid elimination of the metabolites after formation in the liver by phase I and II enzymes and transferred via the bile to the intestine.

Introduction
Efficient oxidative biotransformation of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F) is possible if two adjacent hydrogens in the molecule enable formation of a hydroxylated tetrachlorodibenzo-*p*-dioxin (TCDD) via an arene oxide intermediate [1-3]. This is not the case for 17 out of the 210 possible PCDD/F congeners, including 2,3,7,8-TCDD, possessing chlorines at all lateral positions (2, 3, 7, and 8). For these congeners biotransformation by phase I and phase II enzymes is highly impeded. Slow biotransformation and high lipophilicity of PCDD/F lead to accumulation and retention in adipose tissues and thus to extremely slow elimination from the body. E.g., the reported elimination half-life of 2,3,7,8-TCDD in humans is in the order of five to ten years [4] . In previous research polar metabolites of 2,3,7,8-TCDD could be detected in the bile of rat and dog, and in human faeces, although chemical structures have not been completely elucidated so far [5-7].
For the first time in a human, metabolites of 2,3,7,8-TCDD were detected and identified by gas chromatography high resolution mass spectrometry (GC/HRMS). Two metabolites, a hydroxytrichlorodibenzo-*p*-dioxin (OH-TriCDD) and a hydroxytetrachlorodibenzo-*p*-dioxin (OH-TCDD), were detected and identified in serum, urine, and faeces of a person severely intoxicated with TCDD [8].

Methods and Materials
Samples: Samples were taken by the involved medical staff and sent to the Swiss Federal Laboratories for Materials testing and Research (Empa). The samples were dispatched frozen on dry ice.
Analytical method: Methods used for the determination of 2,3,7,8-TCDD and its metabolites in different matrices (serum, adipose and dermal tissue, urine and faeces) included the general steps of extraction with organic solvents, gravimetric determination of the lipid content, treatment with concentrated sulfuric acid, derivatization (in case of metabolites), clean-up on activated carbon (AX-21), and quantitative determination by GC/HRMS. To hydrolyze possible urinary glucuronate conjugates urine samples were treated with hydrochloric acid prior to extraction with organic solvents. For quantitative determination of 2,3,7,8-TCDD a mixture of the 17 $^{13}C_{12}$-labelled 2,3,7,8-PCDD/F was used as internal standard. In case of 2,3,7,8-TCDD metabolites no reference compounds were commercially available, neither in the native nor in the labelled form. Therefore, $^{13}C_{12}$-labelled 3,3',4,5'-tetrachloro-4'-hydroxybiphenyl which is structurally very similar to the supposed metabolites was used as an internal standard. Derivatization of hydroxylated metabolites to their corresponding methyl ethers was effected using diazomethane. After chromatographic clean-up on activated carbon (AX-21) and addition of the recovery standard ($^{13}C_{12}$-labelled 1,2,3,4-TCDD) 2,3,7,8-TCDD and its metabolites were quantified by GC/HRMS.

Gas chromatography/high resolution mass spectrometry (GC/HRMS): Quantitative determination of 2,3,7,8-TCDD and its metabolites was achieved by GC/HRMS. GC separation was carried out both on a RTX 5 Sil column and a DB-Dioxin column using helium as carrier gas. The mass spectrometer was operated in electron ionization (EI) mode at 70 eV electron energy and was tuned to a mass resolution of 8'000 to 10'000 (10% valley). For identification of 2,3,7,8-TCDD metabolites, the four most abundant signals of the molecular ion clusters of the corresponding methyl ether derivatives of the expected monohydroxy-trichlorodibenzo-*p*-dioxins (OH-TriCDD) and monohydroxy-tetrachlorodibenzo-*p*-dioxins (OH-TCDD) were recorded in single ion monitoring (SIM) mode. To obtain additional identification criteria for 2,3,7,8-TCDD metabolites the four most abundant signals of the two expected major fragment ions $[M - CH_3]^+$ and $[M - CH_3CO]^+$ were recorded in SIM mode.

Results and Discussion

Identification and quantification of metabolites: The search for possible metabolites of 2,3,7,8-TCDD included biotransformation products formed by oxidation and hydroxylation as reported in the literature [9]. We focused on mono- and dihydroxytetrachlorodibenzo-*p*-dioxins, mono- and dihydroxytrichlorodibenzo-*p*-dioxins, and dihydroxytetrachlorodiphenylethers. The analytical methods were adapted so that the selected target compounds could be identified and quantified in faeces, urine, and serum. Due to the expected low concentrations the search for metabolites using GC/HRMS had to be based on the more sensitive SIM technique instead of full scan low resolution mass spectrometry.

The methyl ethers of two possible 2,3,7,8-TCDD metabolites could be detected in urine: an OH-TCDD and an OH-TriCDD. Unambiguous identification of the chemical structure of the OH-TCDD methyl ether was based on gas chromatographic retention behaviour and mass spectral characteristics using reference compounds. Five out of the six possible OH-TCDD isomers were available as well characterized reference compounds, originally synthesized for the identification of 2,3,7,8-TCDD metabolites in the rat [10, 11] (courtesy donation of Heldur Hakk from the US Department of Agriculture, Fargo USA). The remaining isomer was synthesized in house. To exclude overlapping of chromatographic signals all GC/MS analyses were performed using two different stationary GC phases. Based on the comparison of the mass spectral characteristics and the gas chromatographic retention time one of the metabolites was identified as 1,3,7,8-tetrachloro-2-hydroxy-dibenzo-*p*-dioxin.

For the identification of the unknown OH-TriCDD no reference compounds were available. Therefore, 2,3,7-trichloro-8-methoxydibenzo-*p*-dioxin (CH_3O-TriCDD), which is the methoxy analogue of the most likely OH-TriCDD formed by biotransformation of 2,3,7,8-TCDD, was synthesized and characterized by GC/MS using SIM. The mass chromatograms indicative for the molecular and fragment ions of methylated trichlorohydroxydibenzo-*p*-dioxin (M^+, $[M-CH_3]^+$, and $[M-CH_3CO]^+$) revealed a signal with the same chromatographic retention on two different stationary phases as the signal in the respective mass traces of methylated urine, faeces and serum extracts. Furthermore, the mass spectra of the authentic reference and the derivatized 2,3,7,8-TCDD metabolite were very similar. These data confirm 2,3,7-trichloro-8-hydroxydibenzo-*p*-dioxin as a metabolite being present in urine, faeces and serum.

In summary, the two identified metabolites 2,3,7-trichloro-8-hydroxydibenzo-*p*-dioxin and 1,3,7,8-tetrachloro-2-hydroxy-dibenzo-*p*-dioxin (**Figure 1**) agree with findings reported for biotransformation products of 2,3,7,8-TCDD in the bile of dogs [6] and by *in vitro* studies with rat hepatocyte cultures in which these two metabolites were identified as major transformation products [12]. No dihydroxytetrachlorodibenzo-*p*-dioxins, dihydroxytrichlorodibenzo-*p*-dioxins, and dihydroxytetrachlorodiphenylethers could be detected in urine and faeces. In skin and adipose tissue no OH-TriCDD and OH-TCDD could be detected above the limit of detection.

Quantification of 2,3,7,8-TCDD metabolites was based on the methylated analogue of $^{13}C_{12}$-3,3',4,5'-tetrachloro-4'-hydroxybiphenyl which, due to its close structural relationship to OH-TriCDD and OH-TCDD, exhibits similar fragmentation behaviour in EI mass spectrometry. Therefore, a response factor of 1 was assumed for quantification based on the total intensity of the two most abundant signals of the molecular ion clusters.

The highest metabolite content was found in faeces; only trace amounts could be detected in urine and serum. The results are summarized in **Table 1**. As shown in **Figure 2** 2,3,7,8-TCDD metabolites contribute significantly to the total amount excreted via the faeces. Approximately 45 to 60% of the total excreted amount of 2,3,7,8-TCDD is in the form of hydroxylated metabolites (faeces 1 to 3). In faeces 4, which was sampled at last, 2,3,7,8-

TCDD is the major compound and the contribution of its metabolites is only approximately 10%. In serum the two metabolites account for less than 2%, meanwhile in the urine samples 2 and 3 they account for more than 99%. In faeces and serum OH-TriCDD is the major 2,3,7,8-TCDD metabolite whereas in urine OH-TCDD is the most abundant metabolite.

Table 1. Concentrations of 2,3,7,8-TCDD and 2,3,7,8-TCDD metabolites in various tissues in pg/g wet weight, and concentration of total metabolites versus 2,3,7,8-TCDD.

Tissue	OH-TriCDD	OH-TCDD	2,3,7,8-TCDD	Metabolite/ 2,3,7,8-TCDD
faeces 1	810	390	1200	1.0
faeces 2	610	160	930	0.83
faeces 3	110	47	110	1.4
faeces 4	33	16	460	0.11
serum 1	12	8.8	980	0.021
serum 2	5.6	5.8	610	0.019
urine 1	5.4	6.8	5.3	2.3
urine 2	1.4	4.4	0.046	126
urine 3	1.5	1.8	0.032	103

Figure 1. Possible biotransformation pathway for 2,3,7,8-TCDD according to the literature [9] with the two identified 2,3,7,8-TCDD metabolites 2,3,7-trichloro-8-hydroxydibenzo-*p*-dioxin and 1,3,7,8-tetrachloro-2-hydroxydibenzo-*p*-dioxin, identified for the first time in human urine, faeces, and serum.

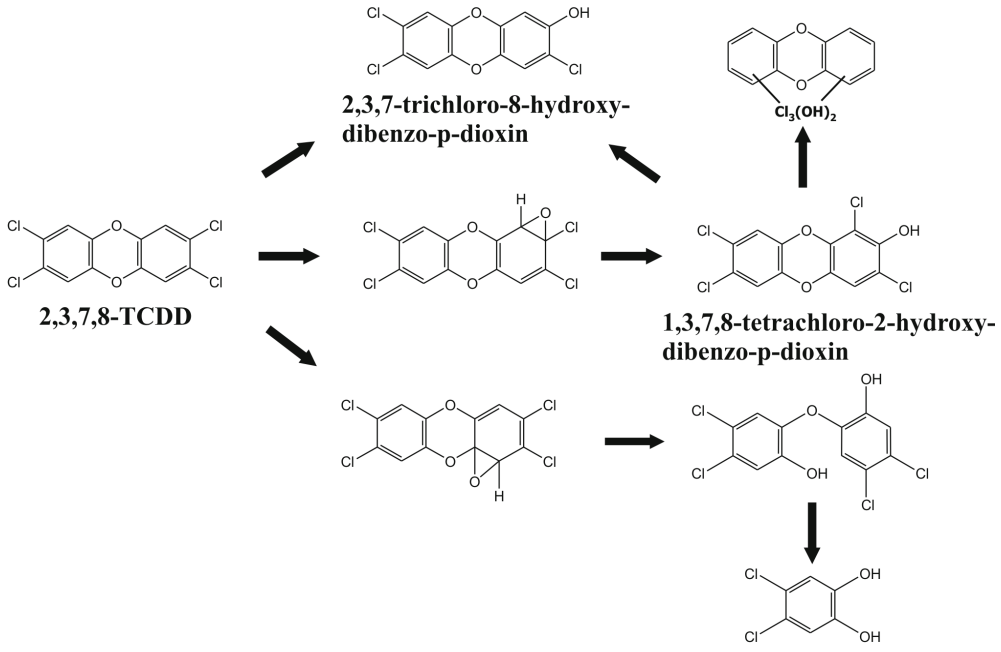
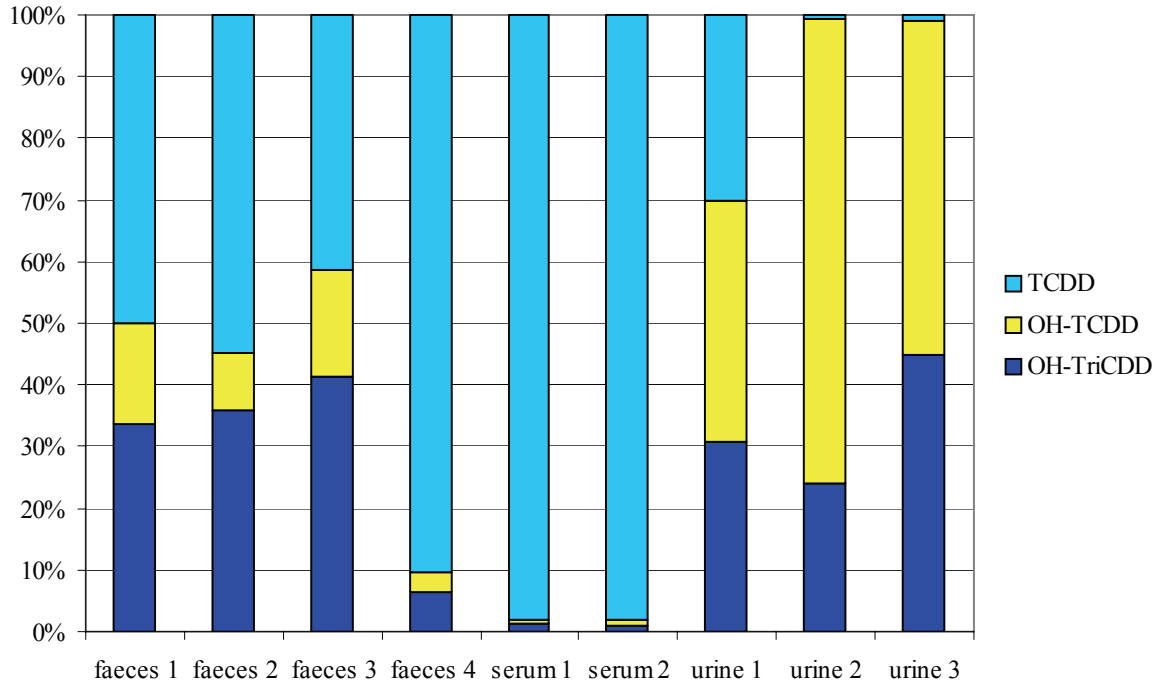


Figure 2. Relative contribution of 2,3,7,8-TCDD**,** 2,3,7-trichloro-8-hydroxydibenzo-*p*-dioxin (OH-TriCDD) and 1,3,7,8-tetrachloro-2-hydroxydibenzo-*p*-dioxin (OH-TCDD) to the analyzed samples. The samples were taken at different times after the intoxication.



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