2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) poisoning in Victor Yushchenko: identification and measurement of TCDD metabolites

O Sorg, M Zennegg, P Schmid, R Fedosyuk, R Valikhnovskyi, O Gaide, V Kniazevych, J-H Saurat

Summary

Background **2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has a long half-life of 5–10 years in human beings as a result of its high lipophilicity, and little or no metabolism. We monitored TCDD, its form, distribution, and elimination in Victor Yushchenko after he presented with severe poisoning.**

Methods **In late December, 2004, a patient presented with TCDD poisoning; the levels in his blood serum (108000 pg/g lipid weight) were more than 50 000-fold greater than those in the general population. We identified TCDD and its metabolites, and monitored their levels for 3 years using gas chromatography and high-resolution mass spectrometry in samples of blood serum, adipose tissue, faeces, skin, urine, and sweat, after they were extracted and cleaned with different organic solvents.**

Findings **The amount of unmodified TCDD in the samples that were analysed accounted for about 60% of TCDD eliminated from the body during the same period. Two TCDD metabolites—2,3,7-trichloro-8-hydroxydibenzo-p-dioxin and 1,3,7,8-tetrachloro-2-hydroxydibenzo-p-dioxin—were identified in the faeces, blood serum, and urine. The faeces contained the highest concentration of TCDD metabolites, and were the main route of elimination. Altogether, the different routes of elimination of TCDD and its metabolites accounted for 98% of the loss of the toxin from the body. The half-life of TCDD in our patient was 15·4 months.**

Interpretation **This case of poisoning with TCDD suggests that the design of methods for routine assessment of TCDD metabolites in human beings should be a main aim of TCDD research in the metabolomic era.**

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Dermato-Toxicology, Swiss Centre for Applied Human Toxicology, and Department of Dermatology, University Hospital, Geneva, Switzerland (O Sorg PhD, O Gaide MD, Prof J-H Saurat MD)**; EMPA, Swiss Federal Laboratories for Materials Testing and Research, Dübendorf, Switzerland** (M Zennegg PhD, P Schmid PhD)**; and Feofania Hospital, Kiev, Ukraine** (R Fedosyuk MD, R Valikhnovskyi MD, V Kniazevych MD)

Correspondence to: Prof J-H Saurat, Dermato-Toxicology, Swiss Centre for Applied Human Toxicology, and Department of Dermatology, University Hospital, 4 rue Gabrielle Peret-Gentil, 1211 Geneva 4, Switzerland **Jean.Saurat@unige.ch**

Introduction

"If there is no poison, there cannot be poisoning, and there was no trace of it whatsoever".[1] This statement shows the prevailing geopolitical and juridicial context that had impaired the scientific investigation to find out whether Victor Yushchenko, a candidate for the presidential election in Ukraine (figure 1), had been poisoned in 2004. While he was campaigning for the election, he suddenly became severely ill (figure 2) as a result of being poisoned during a dinner in Kiev on Sept 5, 2004.[2,3] However, identification of the poison—pure dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin [TCDD])—was delayed from Sept 5, 2004, until late December, 2004, because the presence of TCDD is not routinely investigated in medical practice in a patient with signs of acute poisoning. Therefore whether forensic investigators would have detected the poison in Victor Yushchenko had he died soon after the intoxication is unknown.

TCDD is the most potent member of a group of polyhalogenated aromatic hydrocarbons that includes polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls.[4,5] These lipophilic compounds diffuse freely across cell membranes, and exert their pleiotropic biological effects by binding to the intracellular aromatic hydrocarbon receptor.[6,7] Their toxic effects, particularly those of TCDD, are caused by their high affinity for this receptor, and by their long elimination half-lifes. Because polyhalogenated aromatic hydrocarbons are lipophilic, they accumulate in the lipids in tissues on a physical basis by simple partitioning; this process accounts for their slow elimination in the faeces. Only 17 (including TCDD) of 210 possible PCDDs and PCDFs have chlorine substituents at the lateral positions—ie, carbons 2, 3, 7, and 8, therefore preventing or greatly slowing their bioconversion to polar metabolites during oxidation by the phase I and phase II enzymes. An efficient bioconversion by enzymatic oxidation can take place when two adjacent hydrogen atoms are available, which is not the case when the lateral positions have chlorine substituents.[8–10] Although the metabolism of PCDFs can be induced by TCDD or by themselves,[11,12] TCDD has not been shown to induce its own metabolism.[13] The cytochrome P450 (CYP) monooxygenases CYP1A1, CYP1A2, and CYP1B1 have been shown to be substantially induced in human beings,[14,15] but metabolites of TCDD have not been clearly shown so far. The expected half-life of TCDD ranges from less than 5 years in individuals exposed to high levels—ie, more than 10 000 pg/g lipid weight of TCDD in the blood serum—to more than 10 years in those exposed to less than 50 pg/g lipid weight.[16]

Figure 1: Map of Ukraine

In late December, 2004, we were presented with a patient who was severely affected with probable TCDD poisoning. Without an established specific treatment protocol for such a severe and painful disease, the two possible treatment strategies were to continuously monitor the poison, its form, distribution, and elimination, and to search for medical molecular-based solutions for the organs that were affected by the toxin. We report here the first strategy because the specific wish of the patient was that his case contributed to increasing scientific knowledge about TCDD toxicity.

Methods

We obtained written approval from the patient to release peer-reviewed scientific information about his case. In early January, 2005, we identified TCDD (108 000 pg/g lipid weight) in our 50-year-old patient's blood serum, drawn under controlled conditions at the Geneva University Hospital, Switzerland (table 1), which was more than 50 000 times the average levels of TCDD in the general population.[17] Similar levels were identified by an independent laboratory in a sample taken from the same patient in mid-December, 2004.[18]

The patient's faeces were first lyophilised after homogenisation, whereas solid tissue samples (adipose tissue and skin) were frozen in liquid nitrogen and then homogenised by grinding with a pestle and mortar. Blood, urine, and sweat were frozen. We extracted and cleaned all the samples with different organic solvents, and then analysed the solvent extracts separately for the presence of TCDD metabolites. We mixed the samples with 17 standard ^{13}C12-labelled 2,3,7,8-chlorosubstituted PCDDs and PCDFs before we measured the concentrations of TCDD and its metabolites. Because only the levels of TCDD, and not the other 16 chlorinated congeners, were higher in the patient than the levels in the general population, we only measured concentrations of TCDD in subsequent analyses. We used gas chromatography and high-resolution mass spectrometry to identify and quantify TCDD and its possible metabolites in the samples. The presence of possible TCDD metabolites was investigated on the basis of those predicted by Van den Berg and colleagues,[5] and identified by the analysis of the four most abundant signals of the chlorine isotope patterns within the expected molecular ion clusters in the selected ion monitoring mode during gas chromatography and high-resolution mass spectrometry. Because reference standards for hydroxylated dibenzo-p-dioxins were not commercially available, we used ^{13}C12-labelled 3,3′,4,5′-tetrachloro-4′-hydroxybiphenyl (Cambridge Isotope Laboratories, Andover, MA, USA) for quantification of the possible metabolites. This compound was chosen because it was structurally similar to hydroxylated TCDD metabolites and had a similar fragmentation pattern during electron ionisation mass spectroscopy.[19] Trichloromethoxydibenzo-

Figure 2: Photographs of Victor Yushchenko before poisoning (A), and 3 months (B) and 3·5 years (C) after poisoning with 2,3,7,8-tetrachlorodibenzo-p-dioxin

	Serum		Subcutaneous fat		Faeces		Skin biopsies		Materials extracted from skin		Sweat	Urine
	LW*	WW	LW	WW	LW	WW	LW	WW	LW	WW	WW	WW
4·01	108 000	860	89 000	38 000	..	..	7400	1200	116 000	1400	..	5
5·19	68 500	470	92 000	66 000	..	..	..	..	..	..	..	..
5·85	110 000	720	..	..	..	..	..	..	..	..	..	..
6·64	81 500	700	..	..	..	..	..	..	67 000	2400	..	..
7·40	75 750	680	..	..	..	..	..	..	..	..	..	..
9·40	73 500	550	..	..	..	..	..	..	..	..	..	..
9·80	69 250	600	68 000	50 000	..	..	21 000	1600	..	..	..	..
10·98	58 000	480	..	..	28 000	990	29 000	1150	58 000	3500	..	..
12·66	57 000	400	..	..	..	..	..	..	..	..	..	..
14·37	57 750	390	47 000	24 000	..	..	..	..	14 000	580	..	..
15·52	50 000	390	..	..	..	..	51 000	9200	..	..	..	..
17·36	46 750	400	48 000	39 000	..	..	..	2900	7900	520	..	..
18·67	47 000	320	..	..	..	..	..	..	14 000	240	..	..
20·74	47 000	310	..	..	..	..	..	..	18 000	270	..	..
22·42	..	..	..	..	9900	160	..	..	..	..	..	..
22·98	34 500	260	39 000	29 000	11 000	1100	39 000	930	23 000	1200	..	..
23·01	..	..	..	..	5900	200	..	..	..	..	4	..
23·08	..	..	..	..	10 000	820	..	..	..	..	..	0·05
28·47	31 250	260	30 500	19 000	13 000	135	..	..	..	..	..	0·03
30·77	28 000	230	..	..	..	..	..	..	..	..	..	..
34·12	25 750	180	28 750	23 000	..	..	..	..	..	..	..	..
39·25	20 500	160	23 000	11 000	3800	460	..	160	19 000	330	..	..

*Mean values from gravimetric and blood analyses.

Table 1: **2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) concentrations (pg/g lipid weight [LW] or pg/g wet tissue weight [WW]) as a function of time (months) after the day of poisoning in samples analysed**

p-dioxin and tetrachloromethoxydibenzo-p-dioxin, used as reference compounds for TCDD metabolites, were prepared in situ, whereas five of six possible monohydroxytetrachlorodibenzo-p-dioxins were provided. The lipid content of all samples was measured gravimetrically after evaporation of the solvent.

Equation 1 was used to calculate the decay of TCDD

$$\text{TCDD concentration (t)} = 110\,000\ (\text{pg/g serum lipids}) \cdot e^{-0\cdot045\cdot t}$$

In this equation, t was time expressed in months, TCDD concentration was expressed as pg/g lipid weight, e was 2·71828, and t0 was the date of poisoning. The half-life was calculated from equation 1—ie, ln(2)/0·045=15·4 months. We used a period of 1 year, starting 11 months after the poisoning to try to correlate the TCDD decay curves with the TCDD eliminated or recovered from different routes. The concentrations of TCDD in the lipids at the start and end of this period were calculated with equation 1. The patient's body fat was calculated with a formula reported by Gallagher and colleagues,[20] and corroborated with CT imaging analysis.

The amount of TCDD eliminated in faeces, urine, and sweat during the 12 months of analysis was calculated with equation 2 as follows

$$\Delta m = \int_0^{12} m \cdot e^{-K\cdot t} \cdot dt = \frac{m}{K}(1 - e^{-12K})$$

In this equation, dt was the differential of time, m was the estimated mass of TCDD eliminated in the faeces, urine, or sweat during the first month—ie, the product of TCDD concentration at t0 and the amount eliminated in 1 month, and K was the decay (or rate) constant.

The frequent surgical interventions during which many skin biopsies were taken and cutaneous lesions were removed also represented routes of TCDD elimination. Equation 3 was used for the calculation of

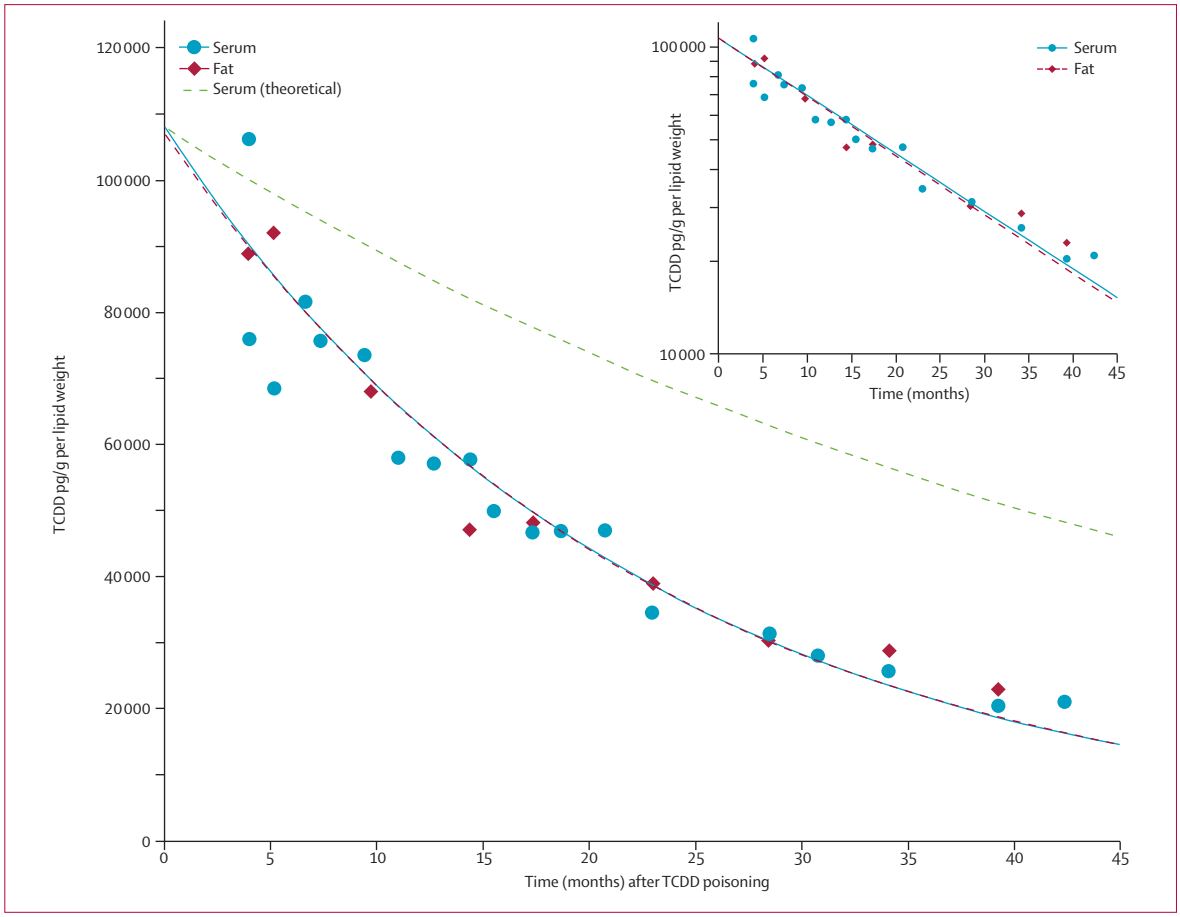

Figure 3: **Elimination decay curves of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in serum lipids and subcutaneous fat**
A theoretical decay curve is shown with values reported in individuals exposed to high levels of TCDD (y-axis is log scale in inset graph).[16] Green dotted line represents the expected decay curve for individuals not at risk of TCDD exposure. The fitted equation of TCDD decay in blood serum was (108 000±6000) (ppt) $e^{(-0.0449 \pm 0.005)t}$, and in fat was (107 000±5000) $e^{(-0.0443 \pm 0.0035)t}$.

the two-phase kinetics (subscripts refer to the phases) shown by these skin biopsies and removed lesions

$$\Delta m = \int_{11}^{23} [(m_1 \cdot e^{-K_1 \cdot t}) + (m_2 \cdot e^{-K_2 \cdot t})] \cdot dt = \frac{m_1}{K_1}(e^{-11K_1} - e^{-23K_1}) + \frac{m_2}{K_2}(e^{-11K_2} - e^{-23K_2})$$

The two-phase kinetics can be explained by an accumulation phase from the blood and fat in the first stage, which lasted several months, followed by an exponential decay. About 200 samples of materials extracted from the skin were removed for analysis each month during 12 months. These materials contained skin (epidermis and dermis), blood, fat, and dermal cysts (webappendix).

See Online for webappendix

Role of the funding source

The sponsor of the study had no role in study design, data gathering, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

We measured the concentrations of TCDD in serum lipids and subcutaneous fat samples from Victor Yushchenko over 3 years. The decay curves of TCDD in serum lipids and subcutaneous fat samples, calculated with first-order kinetics (figure 3), were similar, providing confirmation that TCDD was in equilibrium between serum lipids and subcutaneous fat.

The concentrations of TCDD in the lipids at the start and at end of the period of analysis were 66 000 pg/g and 38 000 pg/g, respectively. TCDD burden at the start and at end of the period of analysis was 990 µg and 740 µg, respectively. The amount of TCDD eliminated from the body during this time was 250 µg.

Table 2 shows the fitted values (ie, to the analytic curves) for the estimated TCDD eliminated per month (m_1) and the decay constant (K_1). The amount of TCDD eliminated, using equations 2 and 3, in the faeces, urine, and sweat, and during the surgical procedures that were done during 1 year was about 150 µg, representing 60% of total (250 µg) eliminated by the

	Equation	A (pg/g wet weight; SD)	m_1 (µg per month; SD)	m_2 (µg per month; SD)	K_1 (per month; SD)	K_2 (per month; SD)	R^2
Blood serum	$c(t)=Ae^{-K_1 t}$	940 (50)	..	..	0·053 (0·005)	..	0·883
Subcutaneous fat	$c(t)=Ae^{-K_1 t}$	61 000 (10 000)	..	..	0·036 (0·01)	..	0·674
Faeces	$c(t)=m_1e^{-K_1 t}$	..	10·9 (5)	..	0·042 (0·027)	..	0·563
Urine	$c(t)=m_1e^{-K_1 t}$	..	0·18 (2)	..	0·043 (2)	..	*
Sweat	$c(t)=m_1e^{-K_1 t}$	..	0·21 (2)	..	0·043 (2)	..	*
MES	$c(t)=m_1e^{-K_1 t}-m_2e^{-K_2}$	..	14·56 (4·18)	14 (3·88)	0·065 (0·031)	0·144 (0·68)	0·790

A=TCDD concentration in blood serum and subcutaneous fat on date of poisoning. Subscripts refer to the phase of the two-phase reaction. m=estimated amount of TCDD eliminated per month in faeces, urine, sweat, and materials extracted from skin (MES). K_1=mean of decay constants for serum, fat, and faeces. R^2=Coefficient of determination. *Not enough timepoints to fit the curve, hence m_1 was calculated from the first available data and the estimated production of urine or sweat per month.

Table 2: **Equations for the calculation of concentrations of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) eliminated from the body and in tissue samples as a function of time**

body during the same time (table 1). A substantial amount of TCDD eliminated had thus to be accounted for by its metabolism.

Two metabolites—2,3,7-trichloro-8-hydroxydibenzo-p-dioxin (OH-TriCDD) and 1,3,7,8-tetrachloro-2-hydroxydibenzo-p-dioxin (OH-TCDD)—were detected in faeces, serum, and urine, whereas none were detected in fat and skin (table 3; figure 4). The decay constants for TCDD, determined from the concentrations of OH-TriCDD and OH-TCDD in faeces and urine on different dates, were 0·0736 per month for faeces and 0·0684 per month for urine. The amounts of the two metabolites eliminated in the faeces and urine were 90 µg and 5 µg, respectively, giving a total of 95 µg, when we used equation 2 with m equal to 11·3 µg per month for faeces and 560 ng per month for urine, and the respective decay constants. The average molecular mass of the two metabolites (321 g/mol) was almost the same as that of TCDD (322 g/mol), as were the average concentrations of the metabolites in faeces and urine, which meant that the total amount of the two metabolites was equivalent to 95 µg of TCDD, or 38% of 250 µg eliminated from the body.

	Sample	OH-TriCDD (pg/g wet weight)	OH-TCDD (pg/g wet weight)
11·00	Faeces	810·0	390·0
23·00	Faeces	610·0	160·0
28·50	Faeces	110·0	47·0
39·25	Faeces	33·0	16·0
9·75	Subcutaneous fat	≤15·0	≤10·0
17·50	Subcutaneous fat	≤9·3	≤9·3
15·52	Skin	≤5·9	≤14·0
34·12	Skin	≤3·2	≤8·0
39·25	Skin	≤36·0	≤91·0
4·00	Blood serum	12·0	8·8
6·50	Blood serum	5·6	5·8
15·50	Blood serum	≤2·1	≤2·1
23·00	Blood serum	≤1·7	≤2·1
23·75	Blood serum	≤2·9	≤3·3
28·50	Blood serum	≤0·81	≤1·2
4·00	Urine	5·4	6·8
23·00	Urine	1·4	4·4
28·50	Urine	1·5	1·8

OH-TriCDD=2,3,7-trichloro-8-hydroxydibenzo-p-dioxin. OH-TCDD=1,3,7,8-tetrachloro-2-hydroxydibenzo-p-dioxin.

Table 3: **Concentrations of metabolites of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in samples as a function of time (months) after TCDD poisoning**

Discussion

Of 17 PCDDs and PCDFs analysed in Victor Yushchenko's blood, only TCDD levels were higher than those in the general population, indicating an acute intoxication with pure TCDD. Victor Yushchenko is one of two people reported to be exposed to high levels of TCDD. The other person, a young woman with acute centrofacial inflammatory dermatosis, which had begun in autumn 1997 after she developed non-specific mild gastrointestinal symptoms, came to the University of Vienna Medical School, Austria, in March, 1998; this woman had a blood serum TCDD concentration of 144 000 pg/g lipid weight.[21] This patient was exposed to pure TCDD in her food, and developed an acute gastrointestinal syndrome during a known period, whereas victims of Agent Orange during the Vietnam war,[22] industrial accidents, such as the Icmesa factory in Seveso, Italy,[23,24] or environmental disasters, such as in Yusho, Japan,[25] were exposed to a mixture of chemicals. The biological half-life of TCDD seemed to depend mainly on its tissue concentration. Even if 30% of 38% of TCDD eliminated by the body might have been related to repeated surgical procedures, we calculated a half-life that was much shorter than the generally predicted 36–120 months in moderately exposed individuals. The shorter half-lifes in other individuals exposed to high TCDD levels in Vienna, Austria,[26] and Italy,[23,24] also support the inverse association of the half-life with tissue concentration. The analysis of the effects of treatments given specifically to increase the excretion of TCDD in the faeces by manipulation of the lipid excretion (with olestra and orlistat) on the shorter half-life is difficult, and studies

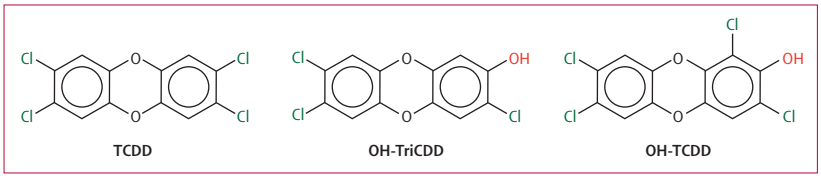

Figure 4: **Structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and its metabolites 2,3,7-trichloro-8-hydroxydibenzo-p-dioxin (OH-TriCDD) and 1,3,7,8-tetrachloro-2-hydroxydibenzo-p-dioxin (OH-TCDD)**

that are specifically designed to assess these effects are needed.

The TCDD concentrations in serum lipids and in subcutaneous fat recorded in this patient were similar during the 3 years of decay. Our set of nine analyses of TCDD and its metabolites in nine samples of adipose tissue and concurrent levels in blood, for the same person at various time points, confirm the findings of previous studies—ie, the existence of an equilibrium between these two compartments in man.[27] Our results in addition to the findings of previous studies should have an important implication for the design of strategies to monitor the exposure of individuals to toxins with new metabolomic approaches.

The measurement of TCDD concentrations in blood, adipose tissue, faeces, urine, and skin with time indicates that the samples extracted from TCDD-induced skin lesions contained large amounts of TCDD (table 2). These findings suggest that the toxin-induced skin lesions might represent a compartment that was not previously reported. Nonetheless, we have established that the main route of TCDD elimination in our patient was in the faeces, as previously reported in rodents.[28–30]

To understand the discrepancy between the amounts of TCDD eliminated in the faeces with time and its faster than predicted elimination half-life, we used a technical approach to search for TCDD metabolites in the samples from our patient. We identified two of five possible hydroxylated metabolites that were predicted by Van den Berg and colleagues.[5] These metabolites accounted for less than 40% of total TCDD eliminated in a man exposed to high levels of TCDD. The highest levels of metabolites were detected in faeces, whereas only traces were found in the blood serum. The metabolite to TCDD ratio was 50-fold lower in the blood serum than in faeces. These findings indicate that these metabolites were unlikely to have been ingested with TCDD, and that TCDD is slowly metabolised, probably by the liver and skin. In the skin, the genes encoding the CYP1A1 and CYP1A2 hydroxylases were highly induced, as assessed with quantitative PCR, whereas the enzymatic activity of CYP1A2 was substantially induced in the blood serum with the Cooperstown (5+1) cocktail method[31] (data not shown). High concentrations of TCDD might be needed to activate these phase I enzymes,[8,15,32] and therefore might explain why the TCDD half-life depends on the degree of exposure to TCDD. Although their chemical structure had not been elucidated, the possible occurrence of TCDD metabolites has previously been shown in rats,[33] dogs,[19] and human beings[34] with radiolabelled TCDD. Hydroxylated metabolites of the brominated analogue of TCDD—ie, 2,3,7,8-tetrabromodibenzo-p-dioxin—have been identified in rat bile.[35]

Although not done previously, levels of TCDD and its metabolites in tissue, faeces, and body fluids should be monitored in a patient with severe dioxin poisoning because they are indicators of what the follow-up period and treatment strategy should be. The poisoning of Victor Yushchenko with TCDD has changed from a story reported in the news to a medical model. This model of TCDD poisoning indicates that methods need to be designed for the routine analysis of TCDD metabolites in human beings, and the main aims of research into TCDD poisoning in the metabolomic era should be the analysis of factors that are involved in the metabolism of this toxin.[36]

Contributors

OS contributed to the study design, literature search, data analysis and interpretation, writing the report, and drawing figures. JHS contributed to the study design, literature search, patient care, data analysis and interpretation, and writing the report. MZ and PS contributed to literature search, data analysis and interpretation, and writing the report. OG, RF, RV, and VK participated in gathering samples and patient care. All authors have seen the final version of this report.

Conflicts of interest

We declare that we have no conflicts of interest.

Acknowledgments

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