Observation of an Extremely High Dioxin Level in a Human Serum Sample from Ukraine by DR CALUX® which was Confirmed to be 2,3,7,8-Tetrachlorodibenzo-p-dioxin by GC-HRMS.

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Introduction

In the fall of 2004 a Ukrainian politician was hospitalized in the Rudolfinerhaus of Vienna General Hospital, suffering from severe abdominal and back pains, with an unknown etiology. In November of 2004 facial images of the Ukrainian politician were circulating on the internet, which clearly showed acnegenic disfiguration of the face. The facial disfiguration resembled a syndrome called "chloracne", which might have been caused by acnegenic chemicals, such as dioxin. In late November, BioDetection Systems (BDS) contacted the Viennese doctors who were in charge of treating the Ukrainian patient and offered to analyze a blood sample from the patient for the presence of dioxins, or dioxin-like chemicals. A serum sample of patient VY was analysed for dioxin, or dioxin-like activity by BDS' DR CALUX® bioassay and confirmatory analysis was performed in two laboratories by GC-HRMS analysis. Here we report on the results obtained on the serum sample of the patient VY.

Materials and Methods

On Tuesday December 6th of 2004 a DHL package arrived at BDS in Amsterdam, containing two tubes, one filled with about 8 ml of serum and one filled with about 8 ml of blood from the Ukrainian patient, VY. A CD-ROM containing information on the facial and chest disfiguration of the skin was also included. An aliquot of 2 ml was taken from the serum containing tube for solvent extraction and silica-sulphuric-acid clean-up according to BDS' low volume serum protocol[1]. The final residue was taken up in 8 µl of DMSO.

BDS' DR CALUX® assay:

Final serum residue was assessed using the DR CALUX® bioassay, consisting of a rat hepatoma H4IIE cell line stably transfected with an AhR-regulated luciferase gene construct. Conditions for cell culture and a procedure for the DR CALUX® bioassay has been described in detail elsewhere[1]. Data are expressed as pg DR CALUX®-TEQ/g fat, using a serum fat percentage of 0.78% determined by BDS. To evaluate the stability of the DR CALUX®-TEQ activity, measurements were performed at 24 and 48 h of exposure of the cells to the serum residue. Repetition of the extraction, clean-up and analysis of the serum sample was performed, due to the fact that initial results obtained were too high to allow adequate quantification.

GC-HRMS confirmatory analysis:

One ml aliquots were taken from the serum containing tube of patient VY and sent to two GC-HRMS laboratories for confirmation analysis. These laboratories were the RIKILT Laboratory for Food Safety, Wageningen, The Netherlands and the Eurofins/GfA laboratory for dioxin analysis in Münster, Germany.

At RIKILT a mixture of $^{13}C_{12}$-labelled dioxins and dioxin-like PCB standards were added to the one ml aliquot of serum. After incubation, 2 ml of isopropanol was added, followed by two times extraction with 4 ml hexane/diethyl ether (97:3 v/v). The hexane fraction was cleaned up on an alumina column and fractionated on a PGC carbon column. The final residue was concentrated to 10 µl and analysed by GC-HRMS with a mass resolution of 10 000.

Data were presented by RIKILT in pg/g serum and in pgWHO-TEQ/g serum. The data were converted to pgWHO-TEQ/g lipid using the fat percentage of 0.78% of the serum as determined by BDS.

At Eurofins/GfA the serum sample after thawing was digested with 40 ml of deionized water, containing formic acid. The serum fat was extracted using a mixture of ethanol/diethyl ether/hexane. The extracts and solutions were cleaned up by liquid/solid chromatography after addition of sixteen $^{13}C_{12}$-labelled internal tetra- through octa-CDF/D standards. Prior to the GC-HRMS analysis, two further ^{13}C-labelled PCDF/D standards were added to the PCDF/D fraction for the determination of recovery of the internal standards. The quantitative determination of native tetra- through octa-CDF/Ds was achieved via an isotope dilution method. Results are presented as pg/g fresh weight and pg/g lipid weight, using a fat percentage of 0.76% determined by Eurofins/GfA.

Results and Discussion

DR CALUX® by BDS: The initial DR CALUX® analysis results on the final residue of a 2 ml aliquot of the serum sample from patient VY indicated a very high content of dioxin, or dioxin-like activity (> 20,000 pg DR CALUX®-TEQ/g lipid, data not shown), which was too high to enable a reliable quantification. The final residue was further diluted to obtain results that fitted to the proper range of the calibration curve for quantification[2,3]. Moreover, an additional aliquot of 2 ml serum from patient VY was extracted and cleaned-up for duplicate analysis and for analysis of biostability of the observed high DR CALUX®-TEQ activity. Furthermore, a proportion of the residue from the 2nd aliquot of serum was analyzed for PCB-specific DR CALUX® TEQ, using BDS' C-split method[4]. In Table 1, the DR CALUX® bioassay results for the serum sample of patient VY are shown. Very high DR CALUX®-TEQ activity was observed in the serum of patient VY amounting to an average of 101 pg DR CALUX®-TEQ/g lipid.

Table 1: Results of the DR CALUX® bioassay for the serum sample of patient V.Y.

Sample	**Procedure**	**DR CALUX® TEQ (pg/g fat)**
Serum 1st aliquot	Standard 24 hr	83
Serum 2nd aliquot	Standard 24 hr	118
Serum average result		101 +/- 25
Serum 2nd aliquot	Stability 48 hr	121
Serum 2nd aliquot	C-split (PCB specific)	< LOQ

LOQ: limit of quantification

Stability measurements at 48 hr of exposure of the cells to dilutions of the residue from the 2nd aliquot of serum revealed that the DR CALUX® TEQ activity was bio-stable, i.e., there was no decrease in activity observed between 24 and 48 h of exposure. This is a strong indication that the DR CALUX® TEQ activity is associated with dioxins, or dioxin-like compounds, since other AhR ligands, like most polycyclic aromatic hydrocarbons, plant based products and other labile AhR ligands are metabolized during the 48 hr incubation period by the H4IIe cells. Using the C-split method it is possible to discriminate between PCDF/Ds and dioxin-like PCBs with respect to their contribution to the DR CALUX® TEQ activity observed. The results in Table 1 indicate that no activity could be measured in the flow-through fraction of C-split (dioxin-like PCB-fraction), which further narrowed down the possibilities of culprit AhR-active chemicals to PCDF/Ds.

At this point it was decided to continue the investigations by confirmatory analysis of the observed high DR CALUX® activity, as well as investigating the profile of individual PCDF/D congeners present in the serum sample of patient VY using GC-HRMS. For this purpose two highly qualified and well experienced GC-HRMS laboratories were approached, i.e., the RIKILT laboratory at Wageningen, The Netherlands and the Eurofins/GfA laboratory at Munster, Germany.

Table 2: Comparison of DR CALUX®, and GC-HRMS results for the serum sample from patient V.Y.

Laboratory	Method	Dioxin level (pgTEQ/g fat*)
BDS	DR CALUX®	101
RIKILT	GC-HRMS	109
Eurofins/GfA	GC-HRMS	108

* normalized to 0,78% fat in serum

GC-HRMS-RIKILT

On 14th of December, one week after receipt of the serum sample from the Viennese Hospital by BDS, a one ml aliquot of serum from patient VY was received by the RIKILT laboratory. The GC-HRMS results by RIKILT confirmed the presence of an extremely high level of dioxins in the serum from patient VY, which appeared to be 109 pg WHO-TEQ/g fat, using the serum fat percentage of 0.78% determined by BDS (Table 2). Furthermore, it became clear that the dioxin content in the serum of patient VY consisted almost exclusively of one single PCDD/F congener, 2,3,7,8-tetrachlorodibenzo-p-dioxin (850 pg/g serum,Table 3). Moreover, relatively small amounts of 2,3,4,7,8-PeCDF (0,1 pg/g serum), 1,2,3,7,8-PeCDD (1.5 pg/g serum), OCDD (0.96 pg/g serum) and relatively small amounts of non-ortho PCBs (0.14 pg/g serum) were also found.

Table 3: Profiles of PCDF/Ds, and dioxin-like PCBs in serum of patient V.Y. using GC-HRMS.

PCDF/D congener	Results RIKILT (pg/g serum)	Results Eurofins/GfA (pg/g serum)
2,3,7,8-TCDF	<0.05	<0.24
1,2,3,7,8-PECDF	<0.10	<0.11
2,3,4,7,8-PECDF	0.11	<0.14
1,2,3,4,7,8-HXCDF	<0.10	<0.16
1,2,3,6,7,8-HXCDF	<0.10	<0.16
2,3,4,6,7,8-HXCDF	<0.10	<0.16
1,2,3,7,8,9-HXCDF	<0.10	<0.16
1,2,3,4,6,7,8-HPCDF	<0.25	<1.06
1,2,3,4,7,8,9-HPCDF	<0.25	<1.06
OCDF	<0.50	<2.66
2,3,7,8-TCDD	850	842
1,2,3,7,8-PECDD	1.5	1.09
1,2,3,4,7,8-HXCDD	<0.10	<0.16
1,2,3,6,7,8-HXCDD	<0.10	<0.16
1,2,3,7,8,9-HXCDD	<0.10	<0.16
1,2,3,4,6,7,8-HPCDD	<0.25	<1.06
OCDD	0.96	<2.66
Total amount dioxin (LB)	850	843
Total amount dioxin (UB)	850	843
3,4,5-4' CB (CB 81)	0.11	
3,4-3',4' CB (CB 77)	0.29	
3,4,5-3',4' (CB 126)	1.3	
3,4,5-3',4',5' CB (CB 169)	0.96	
Total amount Non-ortho PCBs (LB)	0.14	
Total amount Non-ortho PCBs (UB)	0.14	
3,4,5-2',4' CB (CB 123)	<10	

2,4,5-3',4' CB (CB 118)	<10
2,3,4,5-4' CB (CB 114)	<10
2,3,4,-3',4' CB (CB 105)	<10
3,4,5-2',4',5' CB (CB 167)	<10
2,3,4,5-3',4' CB (CB 156)	<10
3,4,5-2',3',4' CB (CB 157)	<10
2,3,4,5-3',4',5' CB (CB 189)	<10
Total amount Mono-ortho PCBs (LB)	0.000002
Total amount Mono ortho PCBs (UB)	0.02
2,4-4' CB (CB 28)	<100
2,5-2',5' CB (CB 52)	<100
2,4,5-2',5' CB (101)	<100
2,4,5-3',4' CB (CB 118)	<100
2,4,5-2',4',5' CB (CB 153)	<100
2,3,4-2',4',5' CB (CB 138)	<100
2,3,4,5-2',4',5' CB (CB 180)	<100

GC-HRMS-Eurofins/GfA

On 14th of December, a one ml aliquot of serum from patient VY was also send to the Eurofins/GfA laboratory for GC-HRMS analysis. Again, the GC-HRMS results from Eurofins/GfA confirmed the presence of an extremely high level of dioxins in the serum of patient VY which amounted to 108 pg WHO-TEQ/g fat, using a fat percentage of 0.78% as determined by BDS (Table 2). Furthermore, the PCDF/D pattern (Table 3) was almost identical to the pattern observed by RIKILT, i.e., almost exclusively 2,3,7,8-tetrachlorodibenzo-p-dioxin and a small quantifiable concentration of 1,2,3,7,8-PeCDD.

In conclusion, the serum sample obtained by BDS from the Viennese doctors involved in treating the Ukrainian patient VY contains an extremely high level of 2,3,7,8-tetrachlorodibenzo-p-dioxin of about 100 000 pg TEQ/g fat. The DR CALUX® bioassay results were fully confirmed by the GC-HRMS analyses, within 10% of variation. As far as we know this is the second highest level of contamination with dioxin ever reported. These study results provide a plausible diagnosis for patient VY who most likely suffers from dioxin poisoning. Treatments aimed at enhanced elimination of the 2,3,7,8-TCDD from the body as well as follow up investigations are strongly recommended.

References

1 Brouwer, B., Sonneveld, E., Botschuiver, S., Besselink, H.and van der Burg, B. (2004) A low volume method for the analysis of dioxins and dioxin-like compounds in serum and whole blood using BDS' DR CALUX® bioassay. *Organohalogen Compounds*, 66:687-690.

2 Behnisch, P.A., Allen, R., Anderson, J., Brouwer, A., Brown, D.J., Campbell, T.C., Goeyens, L., Harrison, R.O., Hoogenboom, R., Van Overmeire, I., Traag, W. and Malisch, R. (2001) *Organohalogen Compounds*. 50:59-63. .

3 Jonas, A., Besselink, H and Brouwer, B. (2005) Introduction of a linear 2,3,7,8-TCDD curve-fit as a means to improve performance characteristics of the DR CALUX® bioassay. submitted to Dioxin 2005.

4 Besselink, H., Jonas, A., Pijnappels, M., Swinkels, A., Fjellanger, K. And Brouwer, B. (2003). Comparison of DR CALUX® and HRGCMS-derived TEQs: Introduction of conversion factors. *Organohalogen Compounds*, 60:203-206.