A low volume method for the analysis of dioxins and dioxin-like compounds in serum and whole blood using BDS' DR CALUX® bioassay

Bram Brouwer[1], Edwin Sonneveld[1], Sara Botschuijver[1], Harrie Besselink[1], Bart van der Burg[1]

[1]BioDetection Systems BV (BDS), Amsterdam

Introduction

Biodetection Systems BV's (BDS) DR CALUX® bioassay is a cost-effective and rapid method to measure low levels of dioxins and dioxin-like compounds in various matrices[1,2,3,4]. For the analysis of human serum/plasma samples, existing methods for extraction, clean-up and DR CALUX® bioanalysis require 10 ml of serum/plasma to obtain a limit of quantitation (LOQ) of 20 pg DR CALUX® TEQ/g fat. This low LOQ value is necessary to be able to measure the lower concentration range of the dioxin and dioxin-like compounds found in human serum and plasma. In many epidemiological studies however, the amount of serum/plasma available for dioxin analysis is limited. Therefore, it is desirable to develop an analysis method that requires a lower volume of serum/plasma for dioxins and dioxin-like compounds determination. In some cases only whole blood is available for analysis. Extraction procedures for whole-blood have been described. However these have been reported to result in an overestimation of dioxin content, most likely due to the co-extraction of AhR interacting compounds like porphyrins that contribute to the detected DR CALUX® TEQs. To tackle both problems we developed improved extraction procedures for both matrices.

To ensure continued reliability of the new extract methods in the DR CALUX® bioassay, extraction and clean-up methods for reduced serum volumes and whole-blood were evaluated, selected and validated. In this paper we present method development and validation set-up of this validation study.

Methods and materials

DR CALUX® bioanalysis. The procedure for the DR CALUX® by BDS bioassay is described in details previously[4]. Briefly, the bioassay is performed using a rat hepatoma H4IIE cell line stably transfected with an AhR-controlled luciferase reporter gene construct. Cells were cultured in α-MEM culture medium supplemented with 10% ($^{v}/_{v}$) FCS under standard conditions (37°C, 5% CO_2, 100% humidity). Following fat extraction and clean-up on an acidic silica column, samples are re-dissolved in DMSO. Cells were exposed in triplicate to cleaned extracts for 24 hours in 96-well microtiter plates. After incubation, the cells were lysed. A luciferine containing solution was added and the luciferase activity was measured using a luminometer equipped with 2 dispensers. Each 96-well microtiter plate contained a complete 2,3,7,8-TCDD calibration range (0 – 300 pM 2,3,7,8-

TCDD per well). Total DR-CALUX® TEQ content in the samples analysed was determined by interpolation from the fitted 2,3,7,8-TCDD calibration curve.

Extraction of whole blood samples. *Ten ml of human whole blood samples was extracted by standard shake-extraction procedures with 2 adjustments: more water was added in the extraction to facilitate a better suspension and acidification with HCL was excluded to avoid co-extraction of suspected DR CALUX® interfering compounds. As controls, 10 ml of human serum isolated from the same whole-blood samples was extracted. After extraction, samples were cleaned and analysed using the DR CALUX® bioassay.*

Extraction of low volume (2 ml) serum samples. *Human serum was spiked with a mixture of dioxins, furans and non-ortho-PCBs in such way that processing 10 ml of serum would yield a dioxin and/or dioxin-like content of approximately 70 and 110 pg DR CALUX® TEQ/g serum fat (serum used for spiking contained approximately 30 pg DR CALUX® TEQ/g fat). Two grams of un-spiked and spiked serum samples were extracted using standard shake-extraction procedures, but several adjustments were applied. After sample clean-up the extracts were dissolved either in 1) FCS instead of DMSO, 2) a reduced DMSO volume, 3) combination of 1 and 2. Furthermore,* the effect of a carrier that could serve as an inter-agent between hexane and FCS, DMSO or the combination of FCS and DMSO, was tested (methods 4, 5 and 6) (see table 1). For initial control of the methods, hexane solvent was spiked with various TCDD concentrations (ranging from 0.15 to 1 pg) and extracted by methods 1 to 6. As reference control, 10 ml of human serum was extracted according to standard procedures. Extracts were analysed using the DR CALUX® bioassay.

Table 1 Schematic overview of extraction methods tested for reduced serum volume DR CALUX® bioanalysis

	Final solvent		
	FCS	reduced volume of DMSO	FCS + DMSO
Exclusive inter-agent carrier	Method 1	Method 2	Method 3
Inclusive inter-agent carrier	Method 4	Method 5	Method 6

Sample	Week 1		Week 2		Week 3		Week 4	
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
1	x_1 x_2				y			
2	y				x_1 x_2			
3		x_1 x_2				y		
4		y				x_1 x_2		
5			x_1 x_2				y	
6			y				x_1 x_2	
7				x_1 x_2				y
8				y				x_1 x_2

$$S_{r(rel)} = 100 * \left(\frac{\sqrt{\frac{\sum_{i=1}^{n} (x_{i1} - x_{i2})^2}{2n}}}{\left(\frac{\sum_{i=1}^{n} x_{i1} + \sum_{i=1}^{n} x_{i2}}{2n} \right)} \right) \qquad S_{R(rel)} = 100 * \left(\frac{\sqrt{\frac{\sum_{i=1}^{n} (x_{i1} - y_i)^2}{2n}}}{\left(\frac{\sum_{i=1}^{n} x_{i1} + \sum_{i=1}^{n} y_i}{2n} \right)} \right)$$

S_r(rel) = repeatability (relative)
S_R(rel) = reproducibility (relative)
n = number of samples tested
x_1 = first determination
x_2 = second determination
y = third determination

Figure 1 Validation scheme used to determine the repeatability and reproducibility

Validation. The validation protocol followed was based on Netherlands Standardization Institute (NEN) 7777:2001, incorporating ISO 5725-2/4:1994. A total of 8 samples was selected, extracted, purified and analysed 3 times in triplicate by DR CALUX® bioassay according to the validation scheme presented in figure 1.

Results and Discussion

Human whole-blood extracts correlated well with their representative serum extracts (data not shown). In contrast, the addition of acid during extraction increases the final DR CALUX® TEQ in the whole blood sample. Applying the adjusted serum extraction protocol (addition of extra water and exclusion of HCl) for whole blood samples is promising and is under validation.

Methods 1 to 4 for the extraction of reduced serum volumes were not suitable for the analysis of dioxins and dioxin-like compounds in two grams of serum whereby the desired LOQ is maintained at 20 pg DR CALUX® TEQ/ gram fat (data not shown). For all these extraction procedures, the final DR CALUX® analysis results did not comply with the performance criteria set for the bioassay[5]. In contrast, methods 5 and 6 resulted in final extracts which both could be used for proper DR CALUX® bioanalysis. Method 5 produced better recoveries as determined by extraction of dioxins, furans and non/mono-ortho PCBs spiked hexane samples (see figure 2). Serum samples were extracted using both method 5 and 6 and compared to extraction of 10 ml of the same serum sample using the traditional method for extraction. The coefficient of determination for method 5 versus the traditional method is substantially better than for method 6 versus the traditional method (see figure 3).

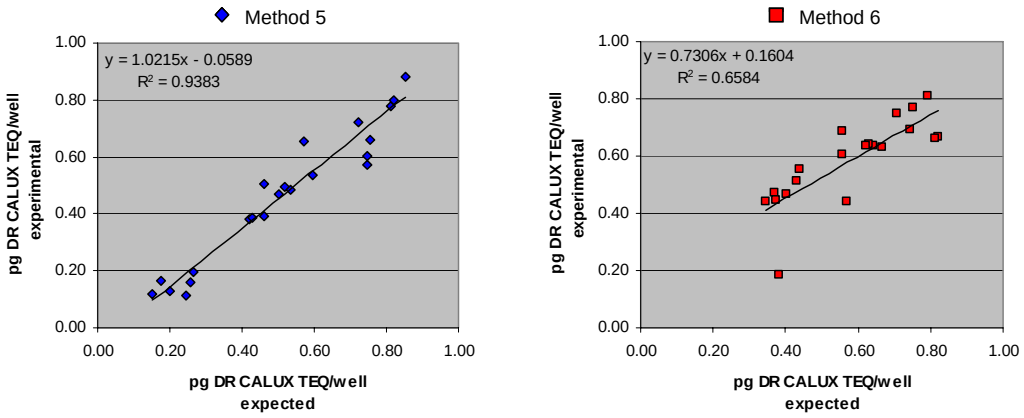

Figure 2. Correlation between expected DR CALUX® TEQs (spiked hexane) and experimental DR CALUX® TEQs obtained by extraction methods 5 and 6. Various dioxins, non-ortho PCBs and mono-ortho PCBs spiked hexane samples were extracted according to method 5 and 6 followed by DR CALUX® bioanalysis.

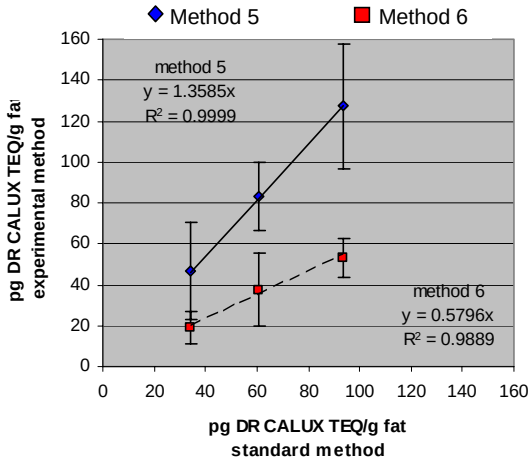

Figure 3 Correlations between extractions of 10 ml of serum according to the standard procedure and extractions of 2 ml of serum according to either method 5 or method 6.

Overall conclusions

- Using the extraction methods developed, comparable results were obtained between serum and whole blood analysis by DR CALUX®
- As compared to the extraction and clean-up procedure for serum samples, extraction of whole blood samples include addition of extra water and exclude the addition of HCL.
- The new procedure for extraction, clean-up and DR CALUX® bioanalysis for serum samples allowed the use of reduced volume sizes (2 ml serum) while maintaining a limit of quantitation of 20 pg DR CALUX® TEQ/g extracted fat
- The low volume analysis methods for dioxin and dioxin-like chemicals by DR CALUX® bioanalysis developed in this study allows for large-scale epidemiological surveys

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