CALUX ANALYSIS IN SMALL AMOUNTS OF HUMAN SERUM

Schroijen C[1], Van Wouwe N[2], Sanctorum H[1], Goeyens L[1,2], Baeyens W[1]

[1]Free University of Brussels, Department of Analytical and Environmental Chemistry, Pleinlaan 2, 1050 Brussels, Belgium; [2]Scientific Institute of Public Health, Juliette Wytsmanstraat 14, 1050 Brussels, Belgium

Introduction

Public health is today a major concern. It is influenced by various interconnected factors like genetic factors, life-style, feeding, social-economic status, medical care and perhaps most of all the environment. The existing interconnections between those factors induce difficulties to identify the diseases' sources in a population.

Polluted environments have already been pointed out for their impact on wellness by various studies in Belgium and worldwide[1,2]. In order to investigate more precisely this factor, The Flemish Ministry of Health in Belgium has settled in 1999 a campaign entitled the Flemish Environment and Health Study (FLEHS). This survey was conducted to assess pollutant concentrations and related health effect biomarkers in humans living in two regions of Flanders (Northern part of Belgium)[3,4]. Results obtained from this study have lead to the conclusion that a larger-scale environmental-health investigation would be useful. This new program was launched in October 2001 and involved the biomonitoring of 4800 participants, residing in 8 different areas of Flanders.

Within this biomonitoring program, dioxin analyses of 1600 adults' samples are performed at the Free University of Brussels (VUB). Dioxin concentrations in serum are determined by the reporter gene expression method CALUX. This method facilitates the assessment of public health risks due to its high throughput rate and low cost compared to chemo-analysis[5]. Although the dioxin analysis of plasma sample by CALUX was already validated[5], the amount of sample available in this study (around 5 mL) has induced small modifications in the sampling preparation. A new validation was therefore performed to allow for reliable results. This paper presents the validation of the CALUX method for the analyses of 5mL human serum and compares these results with the previous validation. Dioxin analyses of human serum carried out within the framework of this extensive Belgian survey will be presented in forthcoming publications.

Materials and Methods

Fat extraction: Five grams of human plasma was mixed with 15 mL of acetone for 2 min in order to denature the proteins. Dioxins and other lipophilic compounds were extracted 3 times with 5 mL of *n*-hexane by shaking for 2 min. The solvent phase was dried upon a pre-conditioned celite column (filled with 0.5 g of celite and 6.5 g of anhydrous sodium sulfate) and collected in a glass tube. The celite column was then washed with 10 mL of *n*-hexane. The total extract was concentrated under a flow of pure air until only the serum fat fraction remained.

Clean up: A 25 mL Pyrex disposable column was filled, from bottom to top, by 1.9 g of anhydrous sodium sulfate (12–60 mesh), 3 g of silica gel impregnated by sulfuric acid (33% w/w) and again 1.9 g of sodium sulfate, and rinsed with 30 mL hexane. A 10 mL Pyrex disposable column was filled from bottom to top by 0.7 g of sodium sulfate, 0.6 g of carbon X-CARB (Xenobiotic Detection Systems Inc., USA) and 0.7 g of sodium sulfate, and rinsed with 5 mL of acetone, 20 mL of toluene and 10 mL of hexane. The acidic silica column was then placed on top of the carbon column. The serum fat containing the dioxin-like compounds was dissolved in 5 mL hexane and loaded on the acid silica gel column and, the recipient rinsed tree times with 5 mL of hexane which were also poured onto the column. The column was then eluted with 15 mL of hexane (the first fraction). When the elution was completed, the acidic silica column was removed and the carbon column eluted with 8 mL of a hexane/acetone (90/10) mixture. This

second fraction was discarded since it is toxic for the cells. The third fraction containing the coplanar PCB was subsequently eluted with 15 mL of hexane/ethyl acetate/toluene (80/10/10), and the fourth fraction containing the PCDD/F was then eluted with 20 mL of toluene. Extracts were concentrated to dryness in a centrifuge under vacuum and resuspended in a known volume of hexane.

CALUX Bioassay: The CALUX bioassay was performed at the Scientific Institute of Public Health (PIH) in Belgium. CALUX Cell line was the pGudLuc 6.1. cell line supplied by Xenobiotic Detection Systems Inc. Protocols used for preparing, dosing and reading the plates are described by Van Wouwe et al[5] and Schroijen et al[6]. In brief, 10 calibration points of TCDD in DMSO as well as extracts (transferred in DMSO) are placed on a 96-well culture plate previously seeded with the CALUX cell line. After 20-24 h of incubation, the plate is read using an Orion II luminometer from Bertholds. Results are then transferred to a computer equipped with the Simplicity 4 software. Using the Hill equation on the calibration curve, the relative light units measured on the plate are transformed in TEQ values.

Results and Discussion

Quality controls of the CALUX bioassay

Quality controls of the CALUX bioassay are the same as those previously reported. The homogeneity of the plates was controlled by a verification solution placed in 6 different wells of each plate. Relative standard deviation calculated on these 6 wells could not exceed 15%. In addition, all solvents and material used for the preparation of samples were tested on the CALUX bioassay in order to detect interferences [6].

The only modification performed for this study was on the limit of quantification. In the publication of Van Wouwe et al[5], the part of the calibration curve used for the measurement was set between 0.7 pg TEQ/well and 3 pg TEQ/well. The reason for this limited use of the curve was the less reliable measurements due to higher variability outside these limits. In this study where the amount of sample is limited to 5 mL, such range will induce a non negligible proportion of samples under the limit of quantification (LOQ). The range was then reexamined in order to lower the LOQ. For this, the relative standard deviations observed for 40 standard curves on different plates were calculated for the 10 points of calibration (figure 1). This variation exceeds 30% for the concentration points of 0.098 and 0.195 pg TEQ per well. Above these concentrations the variations range from 2.7 to 22.8%, being more acceptable. However, attention must be paid to measurements in the lowest part of the curve because little variation in RLU induces large variation of TEQ values. Therefore, the limit of quantification for CALUX bioassay was set to 0.3 pg TEQ per well.

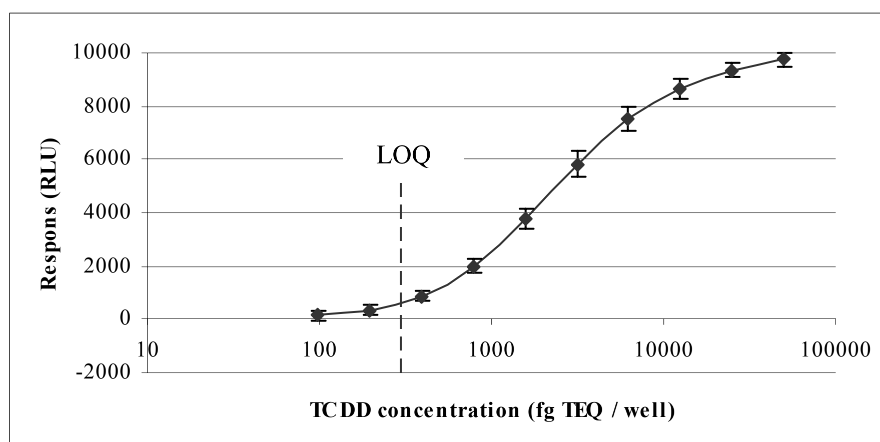

Figure 1: Calibration curve and limit of quantification.

Validation of the CALUX bioassay for human serum samples

Repeatability and reproducibility: The assessment has been performed on the same sample, analyzed 8 times on the first day and twice on 5 other days. This analysis includes every step from the extraction to the exposure and light detection. The repeatability and the within-laboratory reproducibility are calculated according to the ANOVA design as described by Massart et al[7]. The RSD under repeatability and WL reproducibility conditions is 13 and 18%, respectively.

Recovery: A recovery of 82.5 % was obtained by spiking 5 mL plasma sample with a given amount (20 µl) of a ^{14}C-labeled 2,3,7,8-TCDD in *n*-hexane prior to sample preparation. The spiked sample was incubated at room temperature for 45 minutes before extraction, to ensure the distribution of the added dioxins between proteins and lipoproteins[8]. After the extraction and clean-up procedure, the radioactivity in the dioxin fraction collected was compared to the radioactivity in 20 µl of the labeled solution. This recovery is identical to the one observed for the previous validation on 10 mL plasma and was used to correct results for serum samples of the Flemish study.

Quality control for serum samples: The fat extraction and clean-up was done in a series of 33 serum samples, 1 control serum and 1 procedural blank. The control serum is an aliquot of human serum, which was analyzed 20 times to determine the average dioxin concentration and the corresponding control chart limits. For the procedural blank, 5 mL milli Q water was used instead of serum. Before detection, 4 µl verification solution were added to this extract. Each set of sample results was accepted when the response of the procedural blank was within the range set by the six verification solution values placed on the plate and when the response of the control serum is within the control chart limits.

Correlation between CALUX and GC-HRMS: Following the validation, 11 human plasma samples were analyzed to compare TEQ values given by the chemical assay GC-HRMS and the bio-assay CALUX. GC-HRMS results were obtained from the IPH and were calculated on basis of the 17 PCDD/Fs congeners and the WHO-toxic equivalence factors for humans[5]. The CALUX analyses of the dioxin fraction were performed at the VUB. A significant correlation was established between the results (R = 0,74; figure 2). Although, higher CALUX results were expected due to the method's ability to detect all compounds with affinity for the AhR. These results could be explained by the fact that measurements took place in the lowest part of the calibration curve.

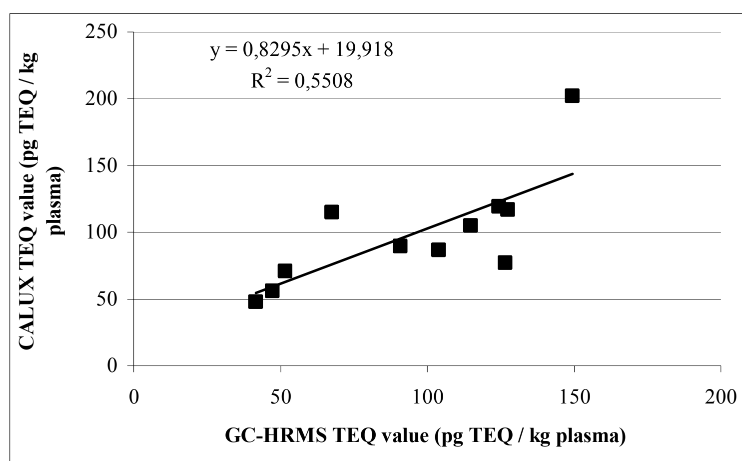

Figure 2: Comparison between CALUX results and GC-HRMS results.

Acknowledgements

The author is grateful to the other members of the Scientific Institute of Public Health.

References

1. Fierens S, Mairesse H, Hermans C, Bernard A, Eppe G, Focant JF, De Pauw E. *Journal of Toxicology and Environmental Health, Part A* 2003;66:1287.
2. Meneses M, Schuhmacher M, Domingo JL. *Environment International* 2004;30:481.
3. Koppen G, Covaci A, Van Cleuvenberg R, Schepens P, Winneke G, Nelen V, van Larebeke N, Vlietinck R, Schoeters G. *Chemosphere* 2002;48:811
4. Covaci A, Koppen G, Van Cleuvenberg R, Schepens P, Winneke G, van Larebeke N, Nelen V, Vlietinck R, Schoeters G. *Chemosphere* 2002;48:827.
5. Van Wouwe N, Windal I, Vanderperren H, Eppe G, Xhrouet C, Massart A-C, Debacker N, Sasse A, Baeyens W, De Pauw E, Sartor F, Van Oyen H, Goeyens L. *Talanta* 2004;63:1157.
6. Schroijen C, Windal I, Goeyens L, Baeyens W. *Talanta* 2004;63:1261.
7. Massart DL, Vandeginste BGM, Buydens LMC, De Jong S, Lewi PJ, Smeyers-Verbeke J. In: *Handbook of Chemometrics and Qualimetrics Part A*, Elsevier,2003:388.
8. Patterson DG, Fürst P, Henderson LO, Isaacs SG, Alexander LR, Turner WE, Needham LL, Hannon H. *Chemosphere* 1989;19:135.