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Quantification of PCDD/Fs and dioxin-like PCBs in small amounts of human serum using the sensitive H1L7.5c1 mouse hepatoma cell line: Optimization and analysis of human serum samples from adolescents of the Flemish human biomonitoring program FLEHS II

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ABSTRACT

Since the CALUX (Chemically Activated LUciferase gene eXpression) bioassay is a fast and inexpensive tool for the throughput analysis of dioxin-like compounds in a large number of samples and requires only small sample volumes, the use of this technique in human biomonitoring programs provides a good alternative to GC–HRMS. In this study, a method for the separate analysis of PCDD/Fs and dioxin-like PCBs (dl-PCBs) in human serum with the new sensitive H1L7.5c1 mouse hepatoma cell line was optimized.

Sample dilution factors of 5 and 2.4 were selected for routine analysis of respectively the PCDD/Fs and dl-PCBs. The validation studies showed that repeatability and within-lab reproducibility for the quality control (QC) standard were within the in-house criteria. A long-term within-lab reproducibility of 25% for the PCDD/F fraction and 41% for the dl-PCB fraction for the analysis of pooled serum samples, expressed as pg BEQ/g fat, was determined. CALUX recoveries of the spiked procedural blanks were within the acceptable in-house limits of 80–120% for both fractions and the LOQ was 30.3 pg BEQ/g fat for the PCDD/Fs and 14.5 pg BEQ/g fat for the dl-PCBs. The GC–HRMS recovery of a C13-spiked pooled serum sample was between 60 and 90% for all PCDD/F congeners and between 67 and 82% for the non-ortho PCBs. An adequate separation between both fractions was found. The CALUX/GC–HRMS ratio for a pooled serum sample was respectively 2.0 and 1.4 for the PCDD/Fs and the dl-PCBs, indicating the presence of additional AhR active compounds. As expected, a correlation was found between human serum samples analyzed with both the new H1L7.5c1 cell line and the more established H1L6.1c3 cell line. The geometric mean CALUX-BEQ values, reported for the adolescents of the second Flemish Environment and Health Study (FLEHS II) recruited in 2009–2010, were 108 (95% CI: 101–114) pg CALUX-BEQ/g fat for the PCDD/Fs and 32.1 (30.1–34.2) pg CALUX-BEQ/g fat for the dioxin-like PCBs.

1. Introduction

Although emissions of PCDD/Fs and PCBs have decreased during recent years, these compounds are still environmental pollutants of concern: (1) since PCDD/Fs and dioxin-like PCBs are persistent in the environment, accumulate in fat tissue and in the food chain, have hormone disrupting properties and are carcinogenic [1,2] and (2) because emissions in certain locations in Flanders are still high

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[3]. Therefore, it is important to include the analysis of these compounds as exposure biomarkers in human biomonitoring programs.

In 2007, a second cycle of the Flemish Environment and Health Study (FLEHS II) started and more than 40 biomarkers of exposure (i.e., metals, persistent organic pollutants, perfluorinated compounds, . . .) and 10 effect markers (i.e., hormones) were measured in 650 samples, recruited from 14 to 15 year-old adolescents (n = 200), adults between 20 to 40 years (n = 200) and mother-child pairs (n = 250) [4]. Since only a small amount of serum (5 mL) was available for the PCDD/F and dioxin-like PCB determination, screening of these samples by GC–HRMS analysis was not possible, since the individual congeners would be below the quantification limit when using such low sample volumes. The CALUX (Chemically Activated LUciferase gene eXpression) bioassay provided a good alternative, since it requires only a small amount of serum to analyze the total amount of dioxin-like compounds in the extract.

This publication presents an optimized method for the separate analysis of PCDD/Fs and dioxin-like PCBs in human serum with the newly developed and more sensitive third generation CALUX (H1L7.5c1) mouse hepatoma cell line [5,6]. The H1L7.5c1 cell line was specially designed to analyze low concentrations of PCDD/Fs and PCBs in small sample volumes. With the less sensitive H1L6.1 cell line, which was commonly used in previous biomonitoring studies [7–9] and food/feed analysis [10,11], only a single-point analysis of the whole extract was often used and it was not possible to measure the dioxin-like PCB fraction, since most samples were below the quantification limit (LOQ) [9]. In this study, for the first time, dioxin-like PCBs could be measured in serum samples with the improved H1L7.5c1 cell line with a high percentage of the samples above the LOQ. Dose-response analysis using different dilutions of serum sample extracts allowed determination of an optimal dilution factor to facilitate screening analysis and to minimize sample volumes needed for analysis. The use of this new H1L7.5c1 cell line will also allow optimization of CALUX protocols for the analysis of both PCDD/Fs and dioxin-like PCBs in various matrices, especially those with low concentrations and/or small sample volumes like food and feed or human samples (i.e., blood and milk).

2. Materials and methods

2.1. Chemicals and standards

Hexane (for dioxins and PCBs, minimum 96%), acetone (Pesti-S grade, minimum 99.9%) and toluene (for dioxins and PCBs, minimum 99.8%) were purchased from Biosolve (The Netherlands). Ethyl acetate pestanal and silica gel 60 for column chromatography were purchased from Sigma–Aldrich (Germany). Sulphuric acid (95–97%, ACS reagent), Celite 545 (0.02–0.1 mm) and DMSO were obtained from Merck (Germany). Anhydrous sodium sulphate was purchased from Boom (The Netherlands) and the X-CARB from XDS (USA). The standard solution of 2,3,7,8-TCDD (50 ng/mL) was purchased from Campro Scientific (The Netherlands).

2.2. Analytical procedure

2.2.1. Extraction and clean up

The extraction and clean up procedure for the analysis of PCDD/Fs and dioxin-like PCBs was based on the protocol used by Schroijen et al. and Van Wouwe et al. [12,13] with some small adaptations. Briefly, 5 mL of human serum was weighted and mixed with 15 mL of acetone for 2 min in order to denature the proteins. PCDD/Fs, PCBs and other lipophilic compounds were extracted 3 times with each time 5 mL of hexane using shaking for 2 min, followed by centrifugation for 2 min. This hexane solvent phase was filtered upon a pre-conditioned celite column, filled with 0.5 g (1.3 cm^3) of celite and 6.5 g (4.3 cm^3) of anhydrous sodium sulphate and conditioned with 30 mL of hexane. The hexane fractions containing serum lipids were collected in a glass tube and the celite column was then washed with 10 mL of hexane, which was also collected in the tube, to elute all lipids from the column. After extraction, the samples were gently evaporated at 40 °C under a flow of pure air until only the serum lipids remained. The amount of fat was weighted and the extract was redissolved in 5 mL hexane and cleaned up on a pre-conditioned multi-layer silica column coupled in series with a carbon column.

The silica gel column (25 mL) was filled from bottom to top with glass wool, 1.9 g (1.3 cm^3) sodium sulphate, 3.0 g (4.3 cm^3) of 33% (w/w) sulphuric acid silica gel and 1.9 g (1.3 cm^3) sodium sulphate. The carbon column (10 mL) was filled with glass wool, 0.7 g (0.5 cm^3) sodium sulphate, 0.34 g (1 cm^3) X-CARB and 0.7 g (0.5 cm^3) sodium sulphate. Before loading the sample extract, the silica column was rinsed with 30 mL hexane, while the carbon column was conditioned with respectively 5 mL acetone, 20 mL toluene, 10 mL hexane. After sample addition, the glass tube containing the serum lipids was rinsed with 2 times 5 mL hexane and finally the column was eluted with 15 mL hexane. After full elution, the upper acidic silica column was removed and the carbon column was eluted with 8 mL of a hexane/acetone (90/10) mixture. Because this fraction was toxic to the cells, it was discarded [14]. The coplanar PCBs were eluted from the carbon column with 15 mL of hexane/ethyl acetate/toluene (80/10/10), followed by elution of the PCDD/Fs with 20 mL toluene. The PCDD/F and PCB fractions were evaporated until dry (40 °C) and redissolved in a defined volume hexane. The samples were stored at room temperature until CALUX analysis.

2.2.2. CALUX bioassay

The CALUX (Chemical Activated LUciferase gene eXpression) assay is a reporter gene mammalian cell bioassay. The recombinant cells used in the bioassay contain a stably transfected AhR-responsive firefly luciferase reporter gene, which responds by the induction of luciferase. The cell lines used in the bioassay were the recombinant mouse hepatoma cell lines H1L6.1c3, stably transfected with pGudLuc 6.1 containing 1 dioxin responsive domain (1 DRD), and the more sensitive H1L7.5c1 mouse hepatoma cell line, stably transfected with pGudLuc 7.5 and containing 5 dioxin responsive domains (5 DRDs) [5,6]. Cell treatment and measurement were based on the protocols described by Windal et al. [14] and the XDS method 4435 from 2008. Briefly, the cells were grown in cell culture plates containing 15 mL RPMI 1640 supplemented with 8% FCS and 1% penicillin/streptomycin (Gibco, UK). After trypsinization, the cells were counted and diluted to a concentration of 55–65 E4 cells/mL and each well of the 96-well plate was seeded with a 200 μL cell suspension in RPMI. After 24 h of incubation (37 °C, 5% CO_2), the 96-well plate was seeded with the standard solutions and sample extracts. Therefore, the target compounds in the sample extracts were transferred to 4 μL DMSO by evaporation of hexane at 40 °C using a vacuum centrifuge. Then, 400 μL RPMI medium was added and the mixture was dosed to the cell lines in duplicate (2 wells, 188 μL/well). When 188 μL of a standard solution, blank or sample extract in RPMI with 1% DMSO was added to every well, the plate was again incubated for 20–24 h. Afterwards, the medium was removed, the wells were rinsed with 75 μL PBS buffer pH 7.4 (Gibco, UK) and the cells in each well were visually inspected under the microscope in order to evaluate if the extract was toxic to the cells. If not, 50 μL lysis reagent (Promega, USA) was added to each well and the plate was shaken for 5 min. After a 10 min incubation period in the luminometer (Glomax, Promega, USA), 50 μL luciferin reagent (Promega, USA) was injected and the light output was given in relative light units (RLUs) (integration