Application of the chemical-activated luciferase expression (CALUX) bioassay for quantification of dioxin-like compounds in small samples of human milk and blood plasma

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1. Introduction

Polyhalogenated aromatic hydrocarbons (PHAHs), such as the polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs), are ubiquitous environmental pollutants that accumulate in the food chain. Humans and vertebrate animals are exposed to these contaminants mainly through dietary intake. In the Netherlands relatively high levels of PCDDs, PCDFs and PCBs have been found in human milk samples[1)] and may therefore pose a threat to the health of human infants. Extensive experimental studies have revealed that most (if not all) toxic actions induced by dioxin-like compounds are mediated via the Ah receptor (AhR) signal transduction pathway[2)]. This common AhR-mediated mechanism of action is the basis for the so-called toxic equivalency factor (TEF) concept[2)], which is nowadays widely used to convert the complex patterns of PCDD/PCDF and PCB congeners generated by GC-MS analysis of e.g. human samples into one value, representing the total dioxin toxic equivalence (TEQ) of that sample, using congener-specific relative toxic potencies[2,3)].

Recently we and others have developed bioassays that can measure the total TEQ value of complex mixtures without the need for extensive clean-up and chemical analysis procedures. The method that we are using at the moment because of its superior sensitivity, ease and fast performance is the chemical-activated luciferase expression (CALUX) system[4,5)]. In this paper, data are presented on the application and validation of the CALUX bioassay to quantify dioxin-like compounds in small aliquots of human milk and plasma, without extensive clean-up procedures.

2. Materials and Methods

Human samples:
Five breast milk samples #1 - #5 were collected in 1995 from random women volunteers from the Rotterdam area, the Netherlands. In addition, aliquots of human milk and plasma (#6 - #11) were derived from larger samples taken in 1990 - 1992 from human women volunteers of the Dutch Mother's Milk Study Cohort[1].

Sample extraction and clean-up procedures:
Two procedures were used to prepare samples for CALUX analysis, the first one representing the procedure as described and used by Tuinstra et al[6], to determine the TEQs in human milk samples from the Dutch Mother's Milk Study Cohort by GC/MS analysis; the second procedure is a much faster and simpler procedure for quick analysis of PHAHs and related AhR agonists in small aliquots of blood samples as described in detail by Murk et al.[7]:
Procedure 1 (neutral pH): essentially involved petroleum ether/diethyl ether (1:1) extraction and subsequent gel permeation, basic alumina and porous graphitised carbon chromatographic purification, resulting in the separate isolation of planar (PCDDs, PCDFs, non-*ortho*-substituted PCBs) and non-planar (mono- and di-*ortho*-substituted PCBs) PHAH compounds. Recovery was assessed at 75% from samples run in parallel, since the biological activity of ^{13}C-labelled PHAH compounds precluded their use as internal standards in the CALUX assay; data were corrected accordingly.
Procedure 2 (acidic): described in detail by Murk et al.[7] and essentially involving n-hexane extraction of human milk and blood serum samples (1 - 1.5 ml) and removal of acid-labile matrix components by passage through a silica column containing 33% (w/w) concentrated H_2SO_4. The efficiency was better than 95% as judged form the recovery of ^{14}C-labelled PCB153[7] (kindly provided by Prof. Åke Bergman, Wallenberg Laboratory, Stockholm University). Therefore no correction for recovery was applied to data obtained using this clean-up procedure.

CALUX assay:
Rat H4IIE hepatoma (H4L1.1c4) cells stably transfected with an AhR-controlled luciferase reporter gene construct (pGudLuc1.1)[5] were grown confluent in 96-well view plates and exposed in triplicate to the PHAH samples and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) concentration standards during 24 hours, using DMSO (0.5% v/v) as a vehicle. The exposed cells were washed with 0.5 × PBS and lysed in 20 µl/well of 10 mM Tris (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid by freezing and thawing the plates. Luciferase activity in the lysates was determined using a Luminoskan 391 luminometer (Labsystems) which automatically injected the luciferin substrate. Confluency of the wells was verified microscopically, which was found to make protein normalization of the luciferase activity values unnecessary; consequently, luciferase activity is reported as relative light units (RLU).

GC/MS analysis:
GC/MS analysis of human breast milk samples was performed as described elsewhere[6]

TEQ calculations:
TEQs from GC-MS data were calculated using recently published consensus TEF values[3,8]. CALUX-based TEQs were calculated by comparison of the luciferase activity induced by the sample against a dose-response curve generated from TCDD concentration standards simultaneously analyzed (Fig. 1). Mean and standard error (S.E.) values were calculated from the individual pg TEQ/g fat values thus obtained from each of three replicates.

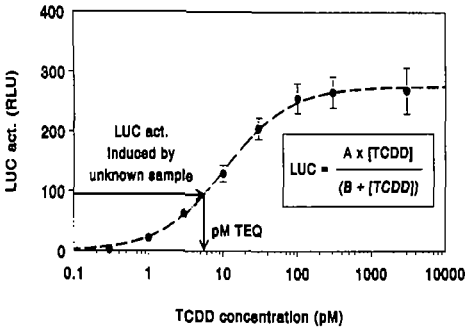

Fig. 1. Calculation of TEQs. An appropriate dilution of the sample was tested in the CALUX assay to generate less than half of the maximal luciferase induction level. Simultaneously, a range of TCDD concentration standards were run and a dose-response curve generated by curve-fitting using the equation for 1-site receptor-ligand binding[9], as indicated (A = maximal luciferase induction level; B = EC_{50} value of TCDD). The estimates for the parameters A and B was subsequently used to calculate the amount of TEQs present in the sample from its luciferase induction level.

3. Results

CALUX versus GC/MS analysis of TEQs

To validate the use of the CALUX assay for complex environmental samples, the planar PHAH fraction (PCDDs, PCDFs and non-*ortho*-substituted PCBs) was isolated from five human breast milk samples (#1 - #5), using clean-up procedure 1, and the TEQ content of this fraction was determined by either CALUX or GC/MS analysis (Table 1). The TEQ values found by CALUX analysis were in the same range as the TEQs calculated on the basis of PHAH congener levels determined by GC/MS analysis; less than a factor 2 discrepancy between CALUX- and GC/MS-based TEQs was observed except for one sample (#5).

Table 1. TEQ levels determined by CALUX and by GC/MS analysis in the planar PHAH fraction of human milk samples as obtained by clean-up procedure 1.

sample nr.	CALUX pg TEQ/g fat (± S.E.)	GC/MS pg TEQ/g fat*)
1	20.4 ± 0.8	12.0
2	38.3 ± 4.7	23.0
3	42.3 ± 2.0	23.7
4	47.7 ± 1.7	33.2
5	57.6 ± 3.7	16.1

*) Singular determinations; no standard errors available.

A simple clean-up procedure, suitable for CALUX analysis.

Clean-up procedure 1 is too laborious, expensive and time-consuming to allow routine analysis of large numbers of (milk) samples. Therefore, clean-up procedure 2, which was developed and successfully applied as a fast, simple and cheap procedure for the preparation of blood plasma samples for CALUX analysis[7], was tested as a possible alternative. To this end, six breast milk and corresponding blood plasma samples (#6 - #11) from the same individuals from the Dutch Mother's Milk Study Cohort were subjected to CALUX analysis using clean-up procedure 2. The TEQ levels of the breast milk samples were found to be comparable to, and differed less than a factor 2 from the TEQ levels measured in blood plasma samples from the same individuals (Fig. 2).

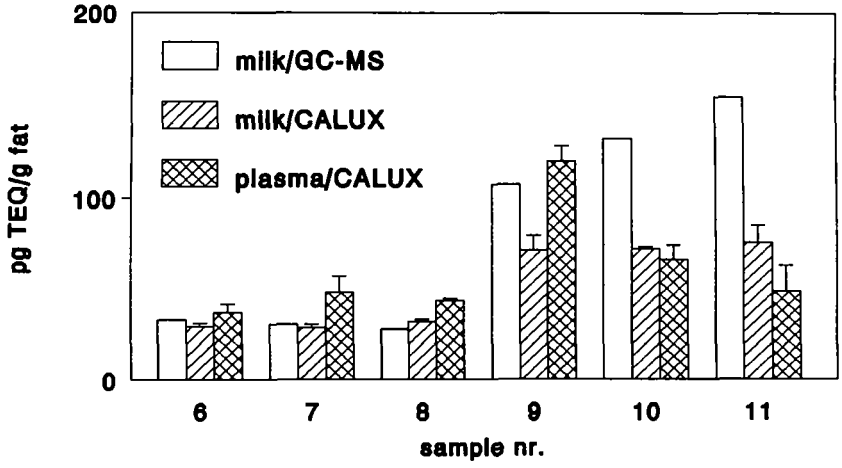

Fig. 2. Application of a simple acidic clean-up (procedure 2) to prepare human milk and blood plasma samples for CALUX analysis. Comparison of CALUX-based and GC/MS-derived TEQ levels.

Moreover, although it involved passage of the samples through an acidic silica column, application of procedure 2 to these breast milk and blood plasma samples yielded TEQ levels in the same range (Fig. 2) as were found by GC/MS analysis of the milk samples upon clean-up by the pH neutral procedure 1. Additional evidence for the validity of procedure 2 was obtained by parallel clean-up of a portion of four other breast milk samples (#1 - #4) using either procedure 1 or 2, and subsequent CALUX analysis, allowing a direct comparison of both procedures. Although procedure 2 would destroy possible acid-labile compounds, similar or slightly higher TEQ levels were found with procedure 2 as with the pH neutral procedure 1 (data not shown).

Correlation between TEQs determined by CALUX and GC/MS analysis.

Together, the data presented in Table 1 and Fig. 2, generally show less than a factor 2 discrepancy between CALUX- and GC/MS-based TEQs (sample #5 was the only exception observed) and reveal a statistically significant positive correlation ($r = 0.71$) between the TEQ levels obtained by both methods (Fig. 3).

4. Discussion

Together with the simple, fast and cheap clean-up procedure using sulphuric acid-silica column chromatography, and given the small sample volume needed (1 - 2 ml), the CALUX assay represents a very sensitive assay suitable for the rapid (pre)screening of large numbers of human milk and blood plasma as well as other environmental samples[7,10] for AhR agonistic activity.

The validity of the CALUX assay for the quantification of AhR agonist activity in

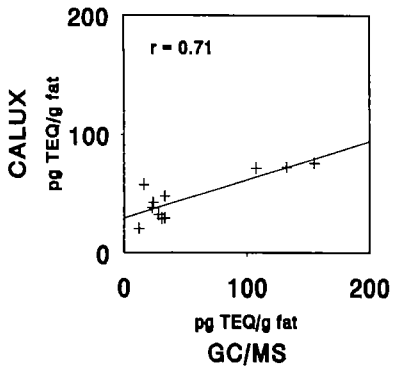

Fig. 3. Correlation between TEQ levels as determined by CALUX and GC/MS analysis.

complex environmental samples was confirmed by the observation that the TEQ levels in human milk samples determined by CALUX analysis correlated positively and showed less than a factor 2 discrepancy with the TEQs calculated from GC/MS data. These observations are consistent with a similar positive correlation and absolute comparability of CALUX- and GC/MS-based TEQs in fish and otter liver reported by Murk et al.[11]. In addition, the TEQ levels in breast milk and blood plasma of the same individuals were found to be in the same range, as to be expected when expressed on a per g fat basis, since blood plasma and milk fat levels tend to be in equilibrium[12].

The planar PHAH (PCDDs/-PCDFs/non-*ortho* PCBs) TEQ levels as determined by GC/MS (mean = 21.6 pg TEQ/g fat) in human milk samples #1 - #5, which have recently been collected, seem to be slightly lower than the levels (46.3 pg TEQ/g fat) previously found[6] in the Dutch Mother's Milk Study Cohort samples collected in 1990 - 1992. This observation is consistent with the decrease of TEQ levels in mother's milk observed by other research groups[13,14].

5. Acknowledgements

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