VARIABLES THAT AFFECT TEQ DETERMINATIONS BY CALUX AND GC-HRMS.

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Introduction

Recently, the CALUX assay (chemical-activated luciferase gene expression) has been proposed as a means to conduct rapid analysis for dioxin and dioxin-like compounds. The genetically modified cell lines used in the CALUX bio-assay respond to dioxin and dioxin-like compounds with the AhR-dependant induction of firefly luciferase. However, the measure obtained by CALUX has to be considered with extreme caution, and everybody using CALUX results must be aware of the assumptions of the method and the importance of sample preparation. The objective of this paper is to focus on parameters that may influence the CALUX measure.

In addition, chemical analysis and CALUX analysis are compared as tools for the evaluation of the toxicity (expressed in TEQ) of a sample.

Substance specific effects

Possible Ah ligands

CALUX analysis will give a response for all compounds able to bind to and activate the Ah receptor. These compounds have been divided into "classical" and "non-classical" Ah ligands by Denison & al.[1]. "Classical" Ah ligands are planar, aromatic and hydrophobic compounds, with a structure close to that of TCDD. Among them, we can cite PCDD/F, PCB, PBB, PBDE, PCT, PCN, PAH, aromatic amines, hexachlorobenzene. Some compounds with physical and structural properties quite different from that of TCDD are also able to bind, in general weakly, to the Ah receptor. These compounds were defined as "Non classical" Ah ligands. Among them, we can cite bilirubin, biliverdin, tryptophan and its metabolites, some compounds from the benzimidazole group, corticosteroid group or imidazolin group. This list is not exhaustive and research to identify AhR ligands continues. When performing CALUX analysis "natural" ligands, e.g. bilirubin present in blood, should not contribute to the response if the objective of the analysis is the determination of the toxicity due to pollutants.

Antagonism and synergy

The TEF principle used in chemical analysis assumes additive contribution of the different AhR ligands. However, important nonadditive interactions between halogenated aromatic hydrocarbons have been observed (see ref 2 for a review[2]). These studies mainly focused on individual polychlorinated biphenyls (PCB 52, 108, 153, 156, 159) or Aroclor 1254 which are able to inhibit the biological response induced by TCDD. The high ratio of antagonist/agonist needed to observe the inhibition is usually observed in various environmental matrices. Note that the antagonism effect depends on the cell lines used[3]. A synergistic interaction was also observed for corticostéroids:

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corticosteroids alone induce a weak response in CALUX, but dramatically enhance the response of TCDD[4].

Procedural effects

When performing a CALUX analysis, the analysts have to choose parameters for the extraction, clean-up and analysis. These choices may have a considerable effect on the results obtained.

Extraction

The extraction determines which AhR ligands are extracted. Hard extraction conditions can lead to complete extraction of some compounds, but also to degradation of some other compounds. On the other hand, soft extraction conditions may lead to only partial recovery of analytes.

Clean-up

The clean-up step determines which compounds are eliminated from the extract and which compounds are delivered to the cell. The effects of clean-up can be divided into two classes:

• A decrease of the response in CALUX due to the elimination of some agonists. One example could be a compound that gives a response in CALUX, but is destroyed during passage over acid silica. Therefore if the extract is treated with acid silica this agonist from the sample does not contribute to the CALUX response.

• A change of the response in CALUX due to the suppression of some antagonistic or synergistic effects. For example, some PCB's do not give any response in the analysis when exposed alone, but their presence in the extract decreases the response of PCDD/F. Consequently, if these PCB's are eliminated from the extract (with a carbon column for example), the response due to the same quantity of PCDD/F in the extract may increase up to a factor of 2 in some cases.

Calculation of the percentage recovery

As no internal standard can be used, the percent recovery may only be estimated. This estimation can be done via the addition of radiolabelled [14]C 2,3,7,8 TCDD to a surrogate recovery sample (same matrix) subjected to the same extraction and clean-up. However, the percent recovery is determined for a different sample than the sample analyzed by CALUX. This implies that the sample preparation should be very reproducible so that the difference between the percent recoveries of the 2 samples is small. Besides this, the percent recovery is estimated only for the 2,3,7,8 TCDD and the percent recovery of other compounds may be quite different. Another possibility is to calculate the recovery from the TEQ level in a quality control sample around the limit of interest.

Analysis

The first parameter that influences the analysis itself is the cell line. Indeed, the structure of the Ah receptor varies among species and consequently, the affinity of compounds for the Ah receptor varies among species. The permeability of membranes, the AhR level, the AhR nuclear translocator protein levels and/or other transcription factors, the antagonism or synergism effect of some compounds are also species and tissue specific. Therefore the same mixture of AhR ligands could give a different response when exposed in the same conditions to different cell lines [5].

The second parameter is the type of vehicle used to deliver the extract to the cells. According to Sanderson et al. [6], dimethyl sulfoxide is more effective than isooctane in delivering the compounds to the cells, resulting in several fold higher response.

The third parameter is the duration of exposure of the extract to the cells. Some compounds, like PAH or aromatic amines are rapidly metabolized. The response to these compounds can only be

measured after a short incubation (several hours). Following longer exposures the response to these compounds is reduced as they are metabolized.[7]

Data interpretation

When analyzing an unknown sample by the CALUX assay, a calibration curve is established with TCDD standard solutions (response versus log concentration of TCDD) and the best equation fitting the curve is used for calculation of results. The concentration of the sample, expressed as TEQ/sample, is deduced by solving this equation with the response of the sample. However, this approach assumes that the extract analyzed behaves like a diluted or concentrated solution of the standard. This implies that the dose-response curves of the sample and of the standard are parallel and that the maximal achievable response (efficacy) for the standard and sample are identical. In practice, these assumptions are usually not verified, and the concentration measured in the sample is a function of dose. The degree of variation of the measure in function of dose is dependent of the degree of variation between the slopes of the standard and sample dose-response curves [8,9]. In order to limit these possible problems, the acceptable responses in CALUX can be set to less than ½ the maximal response for the TCDD standard curve.

CALUX versus chemical analysis for the evaluation of the toxicity of a sample

The main differences between CALUX and chemical analysis are summarized in the table.

	CALUX	Chemical analysis
Selectivity	Analysis of all AhR ligands in one rapid and inexpensive analysis	Analysis only of the compounds of interest. Time consuming and expensive if all AhR ligands are to be analyzed
Robustness	Sample preparation greatly influences the measure	As long as there is no interferences in GC-HRMS, the sample preparation has no influence on the measure
Antagonism and synergism	Taken into account (in part)	Not taken into account
TEF-REP principle	CALUX-REP lead to a direct measurement based on cellular mechanism of toxicity	WHO-TEF estimated only for some compounds on the basis of several biological tests
Precision	RSD associated with the measure is quite high	RSD associated with the concentration of a congener is quite low. But many compounds have to be determined.
Detection limits	Very sensitive. Contribution of all AhR ligands, even at very low levels	Less sensitive. When some compounds are under detection limits, their contributions to the total TEQ are ignored or

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Additional information	estimated Pattern of contamination, useful for the identification of pollution sources

Chemical analyses are usually taken as the reference method, given the actual measurement of individual congeners of PCDD/F in the sample. CALUX analyses are usually considered as a screening approach, resulting in only an approximation of the toxicity, with a high RSD. However we have to be aware that even if the measure of the concentration of PCDD/F by chemical analysis is more precise, the result expressed as TEQ is also an estimation of the toxicity of the sample since 1) not all compounds with dioxin-like properties are analyzed, 2) the TEF, if determined, is also an estimation 3) non additive interactions are not taken into account 4) not detected compounds do not contribute to the total TEQ or their contribution is estimated.

Conclusions

Sample preparation greatly influences CALUX measurements. Standardization is needed to allow comparison of results obtained in different laboratories.

CALUX and chemical analysis give different information, and are complementary: CALUX is a very powerful method to rapidly evaluate the toxicity of a sample due to dioxin-like compounds and may be employed as a rapid screening for large numbers of samples. This allows for the screening of samples with respect to norms, comparison of sample contamination along a gradient or comparisons of different populations. Chemical analysis gives information on the patterns of contamination, which is valuable for identification of contamination sources and a precise measure of compounds that are subject to regulation. However, it is important to emphasize that both results are only an approximation of the actual toxicity of a sample.

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