ELIMINATION OF INTERFERING COMPOUNDS IN PREPARATION FOR ANALYSIS BY AN Ah RECEPTOR BASED BIOASSAY

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Introduction

In general it has been assumed that bioassays are less sensitive to sample clean up than chemical analysis methods. For chemical analysis methods such as gas chromatography/mass spectrometry (HRGC/HRMS) it is necessary to submit samples to extensive and expensive clean up in order to remove compounds that will interfere with the measurement of the compounds of interest. This is especially true for biological samples where compounds such as lipids must be removed to avoid compromising the performance of the instrument. In contrast bioassays tend to be more robust in relation to biological compounds and it is not necessary to remove these compounds in sample clean up. As long as the sample extract does not contain compounds that are acutely toxic to the bioassay, the results should indicate the presence of compounds that bind to and activate the Aryl hydrocarbon receptor (AhR). Concerns about sample clean up for bioassays arise when it is considered that bioassays can respond to a wide range of different compounds. Unlike chemical analysis, which measures the concentrations of specific compounds, bioassays sum the affects of all compounds present in the sample extract that can bind to and activate the AhR. For a crude extract this may include both "classical" and "non-classical" AhR ligands as described by Denison et al.[1] "Classical" Ah ligands are hydrophobic compounds that have a planar, aromatic structure similar to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The "classical" AhR ligands include the polyhalogenated dibenzodioxins/dibenzofurans (PHDD/F), polyhalogenated biphenyls (PHB), polybrominated diphenyl ethers (PBDE), as well as some other polyaromatic hydrocarbons (PAH). Other compounds have been identified that can bind to and activate the AhR, but do not have the same structural characteristics as the "classical" AhR ligands. These "non-classical" AhR ligands include biological molecules such as bilirubin, tryptophan metabolites, and some members of the corticosteroid group.

AhR based bioassays (AhR binding or gene expression assays) do not discriminated between these various active compounds. Therefore, it is necessary to use sample clean up as a way to isolate the compounds that are of interest. Generally, this involves the isolation of PHDD/F and PHB, The two groups of "classical" AhR ligands that are currently widely regulated. Here we report on various sample preparation methods and their efficacy at removing environmental contaminants (pesticides and PAH) as well as selected "non-classical" AhR ligands.

Materials and Methods

Materials

Compounds screened for CALUX activity were purchased from Sigma-Aldrich, Inc. and ChemServices, Inc. TCDD was purchased from Wellington Laboratories.

CALUX Assay

XDS has developed a cell line (mouse hepatoma H1L6.1) that was stably transfected with a vector that contains the gene for firefly luciferase under transactivational control of the aryl hydrocarbon receptor[2]. Serial dilutions of the compounds of interest were prepared in dimethyl sulfoxide. Prior to dosing the cells, the DMSO solutions were suspended in cell culture medium and the medium added to monolayers of the cells grown in 96 well culture plates. In addition to the samples, a standard curve of TCDD was assayed (a two-fold dilution series beginning at 1.55 x 10^{-9} M TCDD). The plates were incubated for 20 hours at 37 ^{o}C in a humidified CO_2 incubator to allow optimal luciferase gene expression. Following incubation, the medium was removed and the cells were examined microscopically for viability. The induction of luciferase activity was quantified using the luciferase assay kit from Promega (Madison, WI).

Identification of interfering compounds

More than two hundred compounds were tested in the CALUX assay for activity. A ten-fold dilution series of each compound starting at 25 milligram/ml DMSO (or highest concentration that was soluble) was tested. It was necessary to test a series of dilutions as some compounds were acutely toxic to the cells and could have resulted in a false negative result if only tested at the highest concentration. Compounds that were active were further submitted to a two-fold dilution series and the CALUX TEQ/ mg compound were determined.

Removal of potential interfering compounds by sample clean up

Active compounds were prepared as a hexane solution and one milligram of the compound was submitted to three different sample clean up methods.

Acid silica only treatment: a hexane solution of the compound was passed over a hexane rinsed 33 % (w/w) sulfuric acid:silica gel column and the eluate was collected in a hexane rinsed glass tube. Following sample addition, the column was rinsed with three column volumes of hexane and the pooled eluate was concentrate under vacuum until nearly dry. The residue was resuspended to four milliliters with hexane and a portion of this was analyzed for activity in the CALUX assay.

Acid silica/XCARB treatment: An acid silica column was prepared as describe above and placed above an XCARB column containing activated carbon. The compound in a hexane solution was added to the acid silica column and the column was eluted as described above. The acid silica column was removed and the XCARB column was rinsed with hexane and then differentially eluted to yield two fractions. The first collected fraction contained PHB and the second fraction contains PHDD/F.

Results and Discussion

Identification of interfering compounds and removal by sample cleanup

After screening over two hundred compounds, 10 were identified that activated the CALUX assay and were potential interfering compounds. Figure 1 shows representative dose response curves for three of the compounds that were tested. Inactive compounds were defined as having maximal activity of less than 10 % of the TCDD maximal response or no clear dose response (o-cresol). Acute toxicity of the compound (acenaphylene) or precipitation of the compound at the highest concentrations (chrysene) often made it difficult to measure a complete dose response curve. Therefore, activity of all tested compounds was measured in the lower half of the dose response curve.

Removal of interfering compounds by sample cleanup

The 10 active compounds were prepared as solutions in hexane and 1 mg of each was submitted to our sample clean up procedure. The resulting fractions were compared to the original solutions to

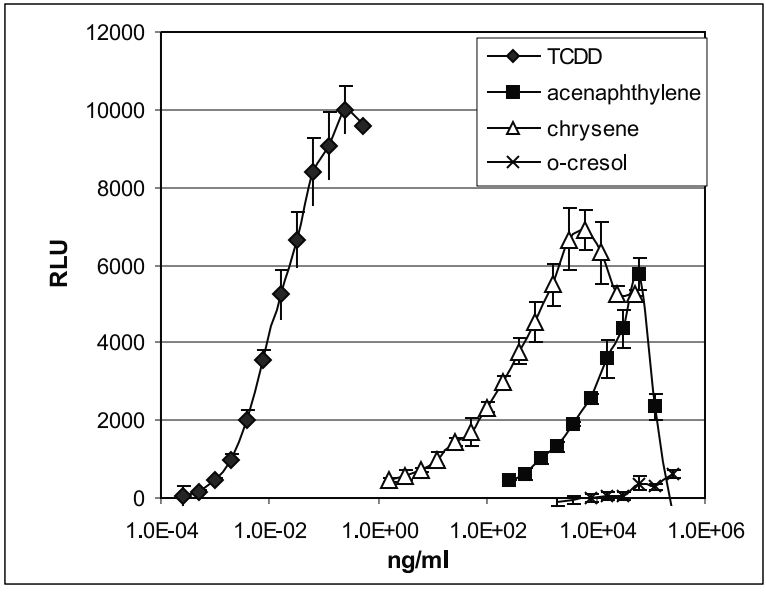

Figure 1. Dose response curves for various polyaromatic hydrocarbons.

determine the percent removal of these potential interfering compounds (Table 1). Removal of activity varied from treatment to treatment with the highest levels of activity present after acid silica only (chrysene – 28 % of activity recovered). The acid silica column in series with the XCARB column provided intermediate results with removal of at least 94 % of activity. This would be sufficient for most samples; however, for samples that are highly contaminated with PAH the sample clean up method can be exhausted and PAH can make significant contributions to activity in the bioassay. When analyzing sediments from near a coke factory (high PAH levels) we found that greater than 90 % of the activity of a sample treated by the dual column method and analyzed in the CALUX assay was associated with PAH. This may in part explain the finding that the CALUX results tend to be higher than the corresponding HRGS/HRMS results which have been reported previously[3].

It is also important that the sample clean up method be streamlined for high sample throughput. Bioassays are valuable as a high-throughput screening method and the sample clean up should allow rapid preparations of samples for analysis. The currently described methods are high throughput sample clean up methods.

Conclusions

Because AhR based bioassays sum the affects of all active compounds that are present, it is necessary to use sample clean up as a means of isolating the compounds that are of interest. The results presented here indicate that it is possible to remove interfering compounds that are AhR active, but are not PHDD/F or PHB. The choice of sample clean up method used can have a significant impact on the amount of activity from these interfering compounds that survives the clean up process and therefore can have an impact on the reported TEQ determination for the sample.

Table 1. Recoveries of pure compounds submitted to sample clean up method. Dioxin fraction and PCB fraction were the respective fractions from an acid silica column and XCARB carbon column in series. Percent recoveries reported as the percent of the original activity applied to the clean up method. ND = no activity detected following clean up.

Compound	Acid silica only % recovery	Dioxin fraction % recovery	PCB fraction % recovery
benzo(a)pyrene	0.002 %	0.02 %	ND
chrysene	28 %	0.2 %	ND
acenaphthylene	ND	0.2 %	ND
benzo(a)anthracene	0.05 %	0.005 %	ND
benzo(b)fluoranthene	26 %	5.7 %	<0.001 %
benzo(k)fluoranthene	<0.001 %	<0.001 %	ND
creosote	ND	ND	ND
p-cresol	ND	ND	ND
dibenzo(a,h)anthracene	<0.001 %	ND	ND
1,2-diphenylhydrazine	ND	ND	ND
fluorene	ND	ND	ND
ideno(1,2,3 cd)pyrene	0.6 %	2.5 %	0.005 %
perylene	ND	ND	ND
2-phenylindole	ND	ND	ND
o-cresol	ND	ND	ND
tryptamine	ND	ND	3 %

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