SPE-HPLC Purification of Endocrine Disrupting Compounds from Human Serum for Assessment of Xenoestrogenic Activity

Philip Sebastian Hjelmborg, Mandana Ghisari and Eva Cecilie Bonefeld-Jørgensen

Unit of Cellular and Molecular Toxicology (CMT), Department of Environmental and Occupational Medicine, Institute of Public Health, University of Aarhus, Denmark

BACKGROUND

Persistent organic pollutants (POPs) is a group of manmade compounds that degrades slowly in the environment (Fig. 1).

The lipophilic nature of POPs causes them to accumulate in fat tissues of animals and humans.

These properties causes POPs to travel over great distances through the food web e.g. East Asian pesticides shows up in polar bears in the arctic and ultimately in humans all over the globe.

POPs has been shown to interfere with male and female sex hormone receptors with the potential to cause adverse health effects such as carcinogenicity and reproductive malfunctions in exposed individuals.

AIM

The aim of the study was to determine the effect of the actual POP mixture on the estrogen receptor (ER) activity using extracts of human serum samples.

A two-step process was applied to achieve this aim:

Firstly, the POPs were extracted from human serum in a way that naturally endogenous hormones would not influence the measurements.

Secondly, the cleaned serum extract were analyzed in a cell culture assay detecting the ER activity (ERE-CALUX).

METHOD

1. SPE-HPLC serum extraction

Serum samples were extracted using solid phase extraction (SPE) columns. These extracts contained all fat soluble compounds including POPs and naturally occurring hormones. A high performance liquid chromatography (HPLC) purification step was added to remove the endogenous hormones since they would give the major response in the ER assay. By this setup we have ascertained that a given response reflects the POPs present in the samples only. A HPLC system with a normal phase column was used for the removal of the endogenous hormones (Fig. 2).

2. ER activity assay (ERE-CALUX)

MVLN cells, stable transfected with an ER luciferase reporter vector, were incubated with the SPE-HPLC extracts in 96-well plates for determination of xenoestrogenic activity. The cell assay responded in a linear fashion to exposure of estrogen-like compounds by releasing a luminescent enzyme (firefly luciferase) that can be detected in a luminescence detector (Fig. 3).

FIGURES

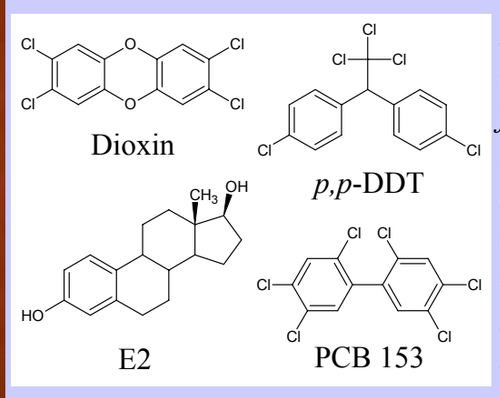


Fig. 1 *Structural comparison of the female sex hormone 17β-estradiol (E2) with examples of compounds with known endocrine disrupting properties.*

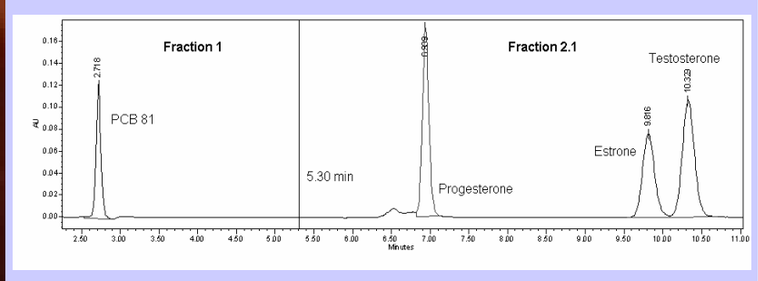


Fig. 2 *HPLC was used to separate POPs from endogenous hormones. The chromatogram shows how a sample PCB is separated from the hormones. The major part of most POPs was found in fraction **F1**.*

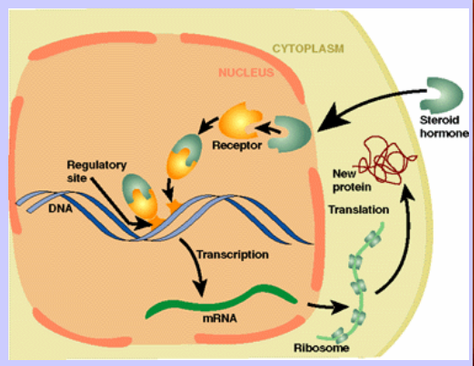
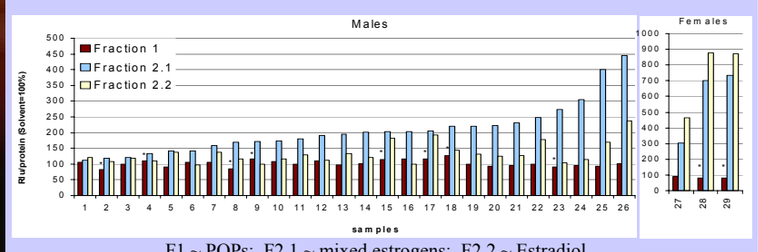


Fig. 3 *The firefly luciferase protein (enzyme) is released when the ER is activated by estrogens, or by estrogen mimicking compounds.*



F1 ~ POPs; F2.1 ~ mixed estrogens; F2.2 ~ Estradiol

Fig. 4 *Estrogenic activity of SPE-HPLC fraction 1 (F1) and 2 (F2.1 and F2.2) of human serum samples analyzed in parallel ERE-CALUX assays.*

The ERE-CALUX activity of SPE-HPLC serum extracts from men (1-26) and women (27-29) increased highly in fraction 2.1 and 2.2 (compared to F1) because of the presence of female sex hormones.

** statistically significant ($p<0.05$) different from solvent control (100%).*

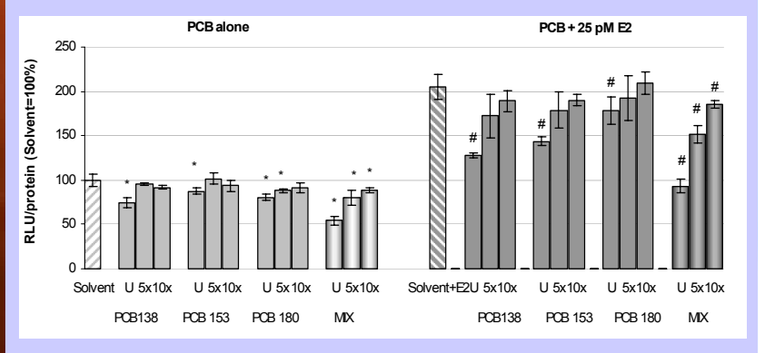


Fig. 5 *Effects of PCB congeners on ER-mediated transactivation. MVLN cells were exposed to PCBs at the given concentrations (PCB138: 7.6 μM, PCB153: 11.1 μM, PCB180: 6.86 μM and MIX: 7.6+11.1+6.86 μM) directly (U) and after 5 and 10 times dilution (5x, 10x), ± 25 nM E2. The protein normalized luciferase activities of the solvent control (0.1% Ethanol) were set to 100%. Data are expressed as the mean ± S.D.*

** and # statistically significant ($p<0.05$) different from solvent control or 25nM E2 reference control, respectively.*

RESULTS

1. The recovery of PCB congeners in spiked serum samples was up to 86% making the extraction method suitable for the study.

2. ER-luciferase activities of SPE-HPLC extracts verified that the endogenous estrogens were separated from the F1 POP fraction (Fig. 4). The ER response measured in actual serum samples were in the linear range of the dose-response curve (not shown).

3. Direct exposure of cells *in vitro* (Fig. 5), and upon SPE-HPLC *ex vivo* analysis of serum spiked with PCB 138, PCB 153 and PCB 180 showed similar effects on ER-transactivation (not shown).

4. E2-competition titrations showed that the xenoestrogenic effects were mediated via the ER (not shown).

Summary. Thus the present method is a validated tool to assess the combined effect of lipophilic POPs where additive/synergistic and agonistic/antagonistic effects are integrated giving an overall estimate of exposure and body burden.

SUMMARY & CONCLUSION

To our knowledge the combination of SPE-HPLC purification with the ERE-CALUX assay to assess the combined xenoestrogenic effect of lipophilic POPs in human serum has not previously been reported.

We have thoroughly evaluated this system, in which the ER additive/synergistic and agonistic/antagonistic effects of POPs are integrated giving the net xenoestrogenic bioactivity.

The complex nature of POP exposure patterns and accumulation levels of known and unknown compounds necessitates assessment tools other than the pure chemical analysis in which single compounds are detected individually.

The additive/synergistic and/or antagonistic effects of the large number of compounds present in human serum cannot be predicted on the basis of individual compounds considering their mutual interactions.

The present study demonstrates how to assess the combined net effect of lipophilic POPs in human serum giving an overall image of their potential biological xenoestrogenic activity.

ACKNOWLEDGEMENTS

We thank our colleagues from *Unit of Cellular and Molecular Toxicology*: Manhai Long and Tanja Krüger for scientific support, and Anne Keblovszki and Birgitte Sloth Andersen for excellent technical assistance. The study was supported by grants from the European Commission: INUENDO, www.inuendo.dk, QLK4-CT-2001-00202 and the Board of Danish Environmental Protection Agency.