A 5-year longitudinal follow up trend in levels of POP plasma concentrations extracted using a 96-well plate method

Jordan Stubleski[1], Petr Kukucka[1,2], Samira Salihovic[3], P. Monica Lind[4], Lars Lind[5], Anna Kärrman[1]

[1] MTM, School of Science and Technology, Örebro University, Örebro, Sweden, 701 82
[2] RECETOX, Masaryk University, Kamenice 5, Brno, Czech Republic, 62500
[3] Department of Medical Sciences, Molecular Epidemiology and Science for Life Laboratory, Uppsala University, Uppsala, Sweden, 75141
[4] Department of Medical Sciences, Occupational and Environmental Medicine, Uppsala University, Uppsala, Sweden, 75185
[5] Department of Medical Sciences, Cardiovascular Epidemiology, Uppsala University, Uppsala, Sweden, 751 85

Introduction

Some persistent organic pollutants (POPs) have been linked to various negative effects such as hormone related cancers and endocrine disruption [1-3], and have been associated with diabetes and risk factors for cardiovascular disease [4, 5]. Biomonitoring studies are an important element in exposure assessment and are frequently used to study temporal trends of POPs including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated biphenyls (PCBs), organochlorine (OC) pesticides, and polybrominated diphenyl ethers (PBDEs) [6, 7]. Longitudinal investigations provide individual-based trends, which may give a more accurate representation of the change in POP levels over time. Nevertheless, biomonitoring studies often include a large sample set with limited sample volume, requiring the use of high-throughput and cost-effective sample preparation methods which use low amounts of sample. The most often used solid phase extraction (SPE) procedures require at least 0.5 mL of plasma and can usually only process between 10 and 30 samples at one time. Simplified methods based on LLE requiring less sample volume (200 µL) and increased sample number (40) have been reported [8, 9]. A new miniaturized SPE method was used to extract 23 Stockholm Convention POPs from 96 plasma or serum samples at once, increasing the efficiency of the sample preparation two to eight-fold compared to the traditional sample preparation procedures, while providing reliable results. The purpose of this project is to process 822 plasma samples using the new miniaturized SPE method and assess the five year (2001-2004 to 2006-2009) longitudinal change in the POPs' plasma concentrations from participants of the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS), a large population-based cohort. Such data are needed to properly assess the fate of POPs in humans and also to assess whether previously set regulations are effective.

Materials and Methods

Plasma samples from 1,016 70 year-old participants (50.2% women) from Uppsala, Sweden were collected between April 2001 and June 2004 for an epidemiological study known as the PIVUS study. The participants were later resampled when they turned 75 years old (n = 822; sampled from 2006 to 2009). All serum samples were collected in the morning after an over-night fast and the samples were stored at -20 °C until analysis. The levels of POPs in plasma samples from the 1,016 70 year-old participants were previously determined [10, 11]. The method developed in this study will be applied to the plasma samples collected when the PIVUS participants turned 75 in order to determine the individual-based change in concentrations of the 23 POPs after a five year follow-up.

All samples and reference materials are stored at -20°C, allowed to thaw at room temperature, and are homogenized by vortex prior to extraction. The miniaturized SPE procedure includes protein precipitation of the 150 µL plasma or serum samples using sulfuric acid and acetonitrile in water followed by sonication. All wells of the SPE 96 well plate (Oasis HLB 60 mg, Waters Corporation, Milford, USA) are preconditioned with methanol followed by water. After the samples pass through the wells, each well is washed with 40% methanol in water. In

order to thoroughly dry the wells the plate is centrifuged for 15 minutes at 4,000 rpm and further dried under vacuum with a nitrogen stream for a minimum of one hour. A 96-well cleanup plate containing 40% sulfuric acid modified silica and sodium sulfate per well is included during the elution of the POPs from the wells using (1:1) dichloromethane:hexane (DCM:hexane). The eluent is quantitatively transferred to a 200µL GC sample vial insert using the DCM:hexane, and the samples are evaporated using a gentle nitrogen stream to the final volume of 20 µL tetradecane. Due to the low levels of POPs present in 150 µL of plasma, highly sensitive detection techniques are required. During the method development and validation period, two different instruments were used for analysis of octachlorinated dibenzo-*p*-dioxin (OCDD), 16 PCBs, 5 OC pesticides, and BDE 47. These included an Agilent 6890 N gas chromatograph (GC) (Agilent Technologies, Atlanta, GA, USA) coupled to a Micromass Autospec Ultima (Waters Corporation, Wilmslow, UK) high resolution mass spectrometer (HRMS) and an Agilent 7890A GC (Agilent Technologies, Atlanta, GA, USA) coupled to atmospheric pressure chemical ionization tandem quadrupole mass spectrometer, Xevo TQ-S (APCI-MS/MS) (Waters Corporation, Wilmslow, UK). Of the 20µL final extract volume, 2µL was injected onto a 30 m × 0.25 i.d. × 0.25 µm DB-5MS capillary column for analysis. The quantification of the POPs was performed by using isotope dilution of ^{13}C-labeled standards.

Results and Discussion

The method used for the sample preparation of the 75 and 80 year age groups is based on a previously developed SPE method which used cartridges containing 150mg Oasis (Waters Corporation) hydrophilic lipophilic balanced (HLB) sorbent [11]. The previously developed method was able to extract 23 Stockholm Convention POPs from 12 samples at one time using 500µL of plasma or serum. In the newly developed method, a 96-well plate format containing 60mg of the Oasis HLB sorbent per well was used to extract the same POPs from 150 µL plasma and serum samples. Method optimization and validation included repeated test extractions using water, newborn bovine serum (NBS), the National Institute for Standards and Technology (NIST) standard reference (serum) material (SRM) 1957, and quality control reference plasma obtained from Örebro University hospital. These materials were used to determine method performance: detection limits for each POP, the method's accuracy, precision, repeatability and reproducibility. The validated method will continue to be applied to plasma samples collected from the PIVUS cohort.

The average recovery of the POPs in the water blanks ranged from 20% to 100%, while recoveries in the NBS blanks, NIST, and reference plasma ranged from 35% to 150%. The method detection limits (MDLs), defined as the average plus three times the standard deviation in concentration of compounds in the blank samples, and reference plasma concentrations obtained from this method are in good agreement with the previously developed method which used 500µL plasma or serum and 150mg Oasis HLB cartridges [10]. Also, the similarity in concentration of the POPs selected in our analysis and those reported by NIST (Figure 1) shows that the high-throughput miniaturized SPE method produces accurate results.

A person's POP body burden can depend on their individual characteristics and exposure, thus longitudinal cohort studies, like the PIVUS study, can provide more characteristic time trends of POP concentrations in humans. A preliminary analysis performed with the first 36 analyzed samples from the 75 year age group showed significant reductions in levels of all analyzed POPs between 20-60% from age 70 to 75, with exception to PCB 206 and PCB 209 (Figure 2). The POPs that showed the largest decrease in plasma concentration were *trans*-nonachlor and the tetra and penta-chlorinated biphenyls, while the higher substituted PCB congeners such as PCBs 194 and 206 showed the least decrease. PCB 209 did not show a decreasing trend. Overall, the results suggest that the regulations placed on the Stockholm Convention POPs included in this study have had a positive effect on lowering the concentrations in humans. These preliminary findings are similar to another longitudinal study (1979-2007) where the concentrations of POPs in Norwegian men were assessed [12], and to the U.S. NHANES biomonitoring results (2003-2008), a large cross-sectional investigation [6].

References

1. Arrebola, J.P., et al., *Associations of persistent organic pollutants in serum and adipose tissue with breast cancer prognostic markers.* Science of The Total Environment, 2016. **566–567**: p. 41-49.
2. Darras, V.M., *Endocrine disrupting polyhalogenated organic pollutants interfere with thyroid hormone signalling in the developing brain.* The Cerebellum, 2008. **7**(1): p. 26-37.
3. He, Y., et al., *Association of breast adipose tissue levels of polychlorinated biphenyls and breast cancer development in women from Chaoshan, China.* Environmental Science and Pollution Research, 2017. **24**(5): p. 4778-4790.
4. Lee, D.-H., et al., *Polychlorinated Biphenyls and Organochlorine Pesticides in Plasma Predict Development of Type 2 Diabetes in the Elderly.* Diabetes Care, 2011. **34**(8): p. 1778.
5. Sjöberg Lind, Y., et al., *Circulating levels of persistent organic pollutants (POPs) are associated with left ventricular systolic and diastolic dysfunction in the elderly.* Environmental Research, 2013. **123**: p. 39-45.
6. CDC. *February 2015 Fourth National Report on Human Exposure to Environmental Chemicals*. 2015 [cited 2015 October 26]; Available from: http://www.cdc.gov/biomonitoring/pdf/FourthReport_UpdatedTables_Feb2015.pdf, http://www.cdc.gov/exposurereport/.
7. van den Berg, M., et al., *WHO/UNEP global surveys of PCDDs, PCDFs, PCBs and DDTs in human milk and benefit–risk evaluation of breastfeeding.* Archives of Toxicology, 2017. **91**(1): p. 83-96.
8. Koponen, J., et al., *Determination of selected perfluorinated alkyl acids and persistent organic pollutants from a small volume human serum sample relevant for epidemiological studies.* Journal of Chromatography A, 2013. **1309**: p. 48-55.
9. Rantakokko, P., et al., *A simple and fast liquid–liquid extraction method for the determination of 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) and 1,1-dichloro-2,2-bis(p-chlorophenyl)-ethylene (p,p'-DDE) from human serum for epidemiological studies on type 2 diabetes.* Journal of Chromatography A, 2009. **1216**(6): p. 897-901.
10. Salihovic, S., et al., *Circulating levels of Persistent Organic Pollutants (POPs) among elderly men and women from Sweden: Results from the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS).* Environment International, 2012. **44**: p. 59-67.
11. Salihovic, S., et al., *A rapid method for screening of the Stockholm Convention POPs in small amounts of human plasma using SPE and HRGC/HRMS.* Chemosphere, 2012. **86**(7): p. 747-753.
12. Nøst, T.H., et al., *Persistent Organic Pollutants in Norwegian Men from 1979 to 2007: Intraindividual Changes, Age–Period–Cohort Effects, and Model Predictions.* Environmental Health Perspectives, 2013. **121**(11-12): p. 1292-1298.

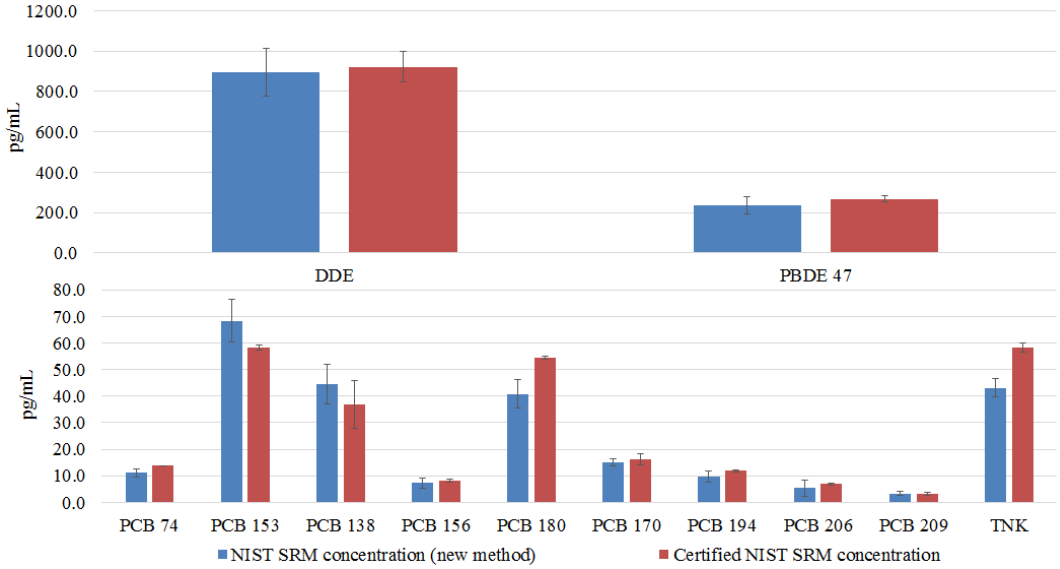

Figure 1: Comparison of the average certified concentrations (pg/mL) of selected POPs in the NIST SRM 1957 to the average concentrations obtained from the new miniaturized solid phase extraction and analysis using the HRGC-HRMS (n=8). The error bars represent the standard deviation in concentration. HCB and OCDD are not included in the figure because their method detection limits were greater than the average concentration determined in the NIST serum. *Trans*-nonachlor is abbreviated as TNK.

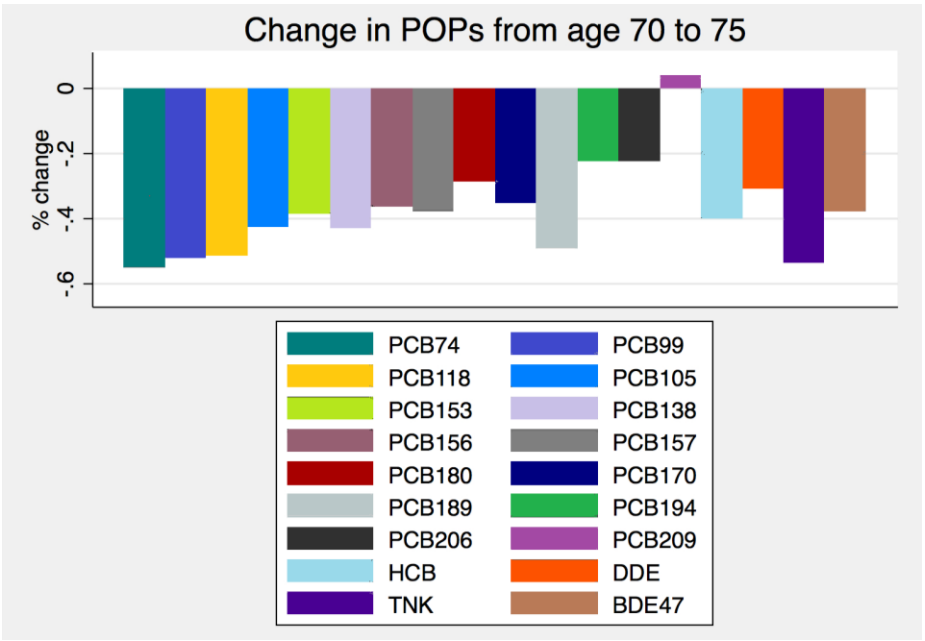

Figure 2: The percent change (shown as the decimal) in 18 of the 23 target analytes detected in the first 36 plasma samples from the Prospective Investigation of the Vasculature in Uppsala Seniors cohort after the five year follow up (2001-2004 to 2006-2009). *Trans*-nonachlor is abbreviated as TNK.