A method for analysis of phenolic and neutral organic halogenated substances in plasma

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Introduction

The exposure of the human population to environmental organic pollutants has been of concern for several decades and many documents of potential hazards and risk assessment have been published (1-4). During the last years, the concern for potential endocrine disrupting compounds has been of increased interest (2-4). Several of the compounds that are considered to be endocrine disruptors have a phenolic group, either as such or after metabolic activation. Examples of these are the polychlorinated biphenyl's (PCBs) that have been reported to have both estrogenic (5, 6) and thyroidogenic properties (7). It is therefore essential to have methods for the determination of these compounds in humans, as well as in environmental samples, and blood is a conveniently obtained matrix.

The aim of this project was to develop a non-destructive extraction method for hydroxlyated organic halogenated substances (OHS) in blood plasma, extracting both OHS and lipids efficiently and with a high reproducibility. Further, to combine these features with the ability to extract compounds that are selectively bound to transport proteins in the plasma, such as phenolic compounds.

Materials and Methods

All solvents used were of pesticide grades.
Substances included in the study: 2,6-Dibromophenol, 2,3,4-Trichlorophenol, 2,4,6-Tribromophenol, 2,3,4,5-Tetrachlorophenol, Pentachlorophenol (PCP), 3-Methoxy-2', 5',6 - triCB (3-1), Pentabromophenol (PBP), 2-Methoxy-2',3, 3',4,4'-pentaCB (5-1), 4-Methoxy-2, 2',3, 3',4',5'-hexaCB (6-7), 4-Methoxy-2',3, 3', 4',5, 5'-hexaCB (6-8), 2-Methoxy-2',3, 3',4, 4',5'-hexaCB (6-1), 3-Methoxy-2',3',4, 4', 5,5'-hexaCB (6-4), 4-Methoxy-2,3,3',4',5, 5',6 - heptaCB (7-7), Tetrabromobisphenol A (TBBPA), α-hexachlorocyclohexane (α-HCH), γ-hexachlorocyclohexane (γ-HCH), 2,2',5,6'-Tetrachlorobiphenyl (CB-53), 2,3,6-Tribromodiphenyl ether (BDE-30), p,p'-DDE, Bis (4-chlorophenyl) Sulfone (BCPS), p,p'-DDT, 2,2',4,4'-Tetrabromodiphenyl ether (BDE-47), 2,2',3,3',4,5,5',6-Octachlorobiphenyl (CB-198), 2,3,3',4,4',5,5'-Heptachlorobiphenyl (CB-189), Tris (4-chlorophenyl)methane (TCPM), 2,2',4,4',6-Pentabromodiphenyl ether (BDE-100), 2,2',4,4',5-Pentabromodiphenyl ether (BDE-99), Tris (4-chlorophenyl)methanol (TCPM-OH).

Instruments: Gas chromatography (GC) analysis was performed on a Varian 3400 GC, equipped with an electron capture detector (ECD). The column used was a J&W DB5 column (30m x 0.25 mm I.D.) and 0.25μm film thickness (J&W Scientific, Folsom, CA, USA). Hydrogen was used as carrier gas and nitrogen as makeup gas. The column temperature was held at 80°C for two minutes and then increased by 10°C/min to 300°C, which was kept for ten minutes. The injector temperature was 250°C and the detector temperature was 360°C.
High Resolution - Gel Permeation Chromatography (HR-GPC) was performed on HPLC (Varian 9012) connected to a variable wavelength UV-VIS detector Varian 9050 (Varian, Walnut Creek, CA, USA) operated at 254 nm and a Valco injector (Vici AG, Switzerland) equipped with a 400 μL loop. Separation was performed on two PL gel (5 μ, 50 Å) 7.5 mm (I.D.) columns coupled in series (Polymer Laboratories, Shropshire, UK). The eluent used was n-Hexane / dichloromethane (70/30, v/v) at a flow rate of 1.0 mL/min.
Radioactivity measurements were performed on a Wallac 1409 scintillator (Wallac Oy, Finland). The scintillation cocktail used for lipophilic extracts was scintillator 299 (Packard) and for aqueous samples Ophtiphase II (Wallac Oy) was used.

The Wallenberg plasma extraction (WPE) method developed can be summarised as: Plasma (5g) was denaturated by HCl and iso-propanol after which the organohalogenated substances (OHS) were extracted with hexane:metyl *tert*-butyl ether. The extract was separated into phenol and neutral compounds by partitioning between KOH and hexane. Phenolic compounds were derivatized with diazomethane prior to further clean-up. As lipid removal steps for both neutral and methylated phenolic compounds, three alternative methods were used: a non-destructive method, using High Resolution-Gel Permeation Chromatography (HR-GPC) and two destructive methods using sulphuric acid treatment or sulphuric acid impregnated silica gel chromatography. The samples were analysed by gas chromatography.
Extraction efficiency of OH-PCB: To determine the denaturation efficiency of proteins, rats were dosed i.v. with ^{14}C-labelled 4-hydroxy- 3,3',4',5-tetrachlorobiphenyl.This substance is known to have a high affinity to a thyroxin transport protein in plasma (8). After 4 hours, the rats were sacrificed and the blood sampled. The plasma was extracted and the distribution of the ^{14}C-activity was determined.
Lipid extraction efficiency evaluation: Plasma was also extracted according to Bligh and Dyer (B&D) (9). This method, generally considered to be an efficient method for lipid extraction, uses chloroform and methanol as extraction solvents (10).
Recovery experiment: For each of the three clean-up methods plasma, 10 replicate samples (5 g) were spiked with standard solution. 5 replicate samples were analysed to determine the background levels of the investigated compounds.

Results and Discussion
The denaturation and extraction of the radiolabelled 4-hydroxy- 3,3',4', 5-tetrachlorobiphenyl was greater than 98%, determined as ^{14}C in the organic phase.

The reproducibility of the lipid extraction methods was high, a variance of 1.64 % from the mean value 0.41% for 31 replicate samples (lipid weight). In comparison, the 5 replicate samples being extracted according to B&D, resulted in a mean value of 0.32% (lipid weight) with a variance of

7.93%. The lipid yield was thus highly reproducible by the WPE method and, compared to the B&D method, higher yields were obtained.

The final recoveries of the different neutral compounds included in the evaluation ranged from 56 and 103 % after treatment including the sulphuric acid (Figure 1). BCPS is completely partitioned in to the sulphuric acid but can be recovered by addition of water, prior to extraction (ref till detta). TCP-OH acts as a neutral compound but is not stable in sulphuric acid. The low recovery of BDE-30 is due to losses in the evaporation steps, whereas the HCH's are lost in partitioning procedure of the phenolic compounds as they are labile in alkali. Using the HRGPC as a lipid removal method resulted in mean recoveries ranging from 65 to 98%. This method is non-destructive and is the only method that allowed analyses of TCP-OH.

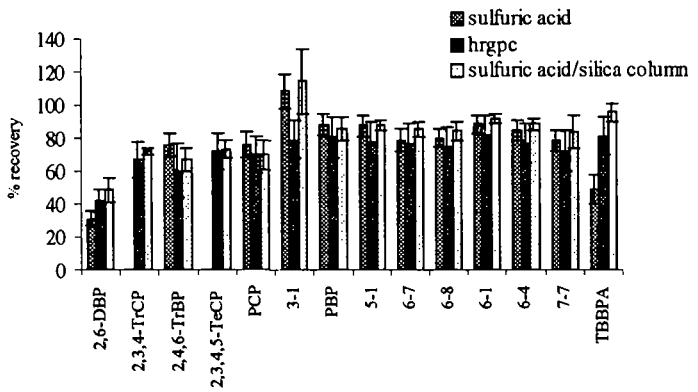

Figure 1. Comparison of recoveries of neutral compounds using the three different clean-up methods.

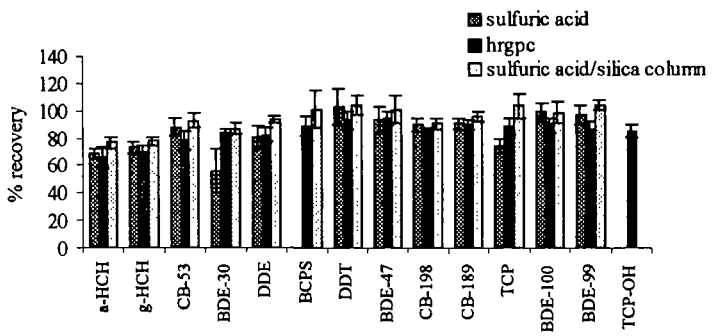

Figure 2. Comparison of recoveries of the phenolic compounds using the three clean-up methods.

Mean recoveries between 42-81% were obtained for the phenolic compounds using HRGPC as the clean-up step. The low recovery for 2,6-DPB in all the clean-up methods is due to losses in the evaporation steps. The sulphuric acid treatment resulted in recoveries between 32-109%. For the TBBPA, the low recovery may in part be explained by partitioning in to the sulphuric acid. Recoveries obtained using the sulphuric acid column were between 49-115 %.

The WPE-method is a convenient method for extraction of both lipids and neutral adn phenolic compound, resulting in a high and reproducible recovery. For lipid removal, different methods may be chosen depending on the analytes to be included in the analyses. The HRGPC method is recommended when labile compounds are to be analysed since it is a non-destructive method. However, unless an automated system can be set up, the method is labour- and time-consuming. The classic treatment with sulphuric acid for lipid removal allows parallel sample treatment and gives high yields. The sulphuric acid/silica gel column also allows parallel sample treatment, and may be automated, in addition to give good recovery for all compounds except for compounds acting as Lewis bases and compounds labile to the acid.

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