Short communication

Serum levels of organochlorine pesticides in the general population of Thessaly, Greece, determined by HS-SPME GC–MS method

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ABSTRACT

In this study, exposure levels of organochlorine pesticides (OCs) were determined in general population residing in Larissa, central Greece. Serum samples from 103 volunteers were analyzed by optimized headspace solid-phase microextraction gas chromatography–mass spectrometry, to detect and quantify OC levels. The most frequently detected analytes were p,p′-DDE (frequency 99%, median: 1.25 ng/ml) and Hexachlorobenzene (HCB) (frequency 69%, median: 0.13 ng/ml). Statistical analysis revealed a significant relationship of p,p′-DDE and HCB levels with age.

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1. Introduction

Organochlorine pesticides (OCs) have been the dominant class of insecticides in agricultural and public health applications for many years. Because of their persistence in the environment and their effects on human health and wild life, OCs have been banned in developed countries from mid 70’s. Although nearly 40 years have passed these substances are still of concern due to their stability and ability to bioaccumulate. Studies across the globe indicate that the presence of p,p′-DDE, a major metabolite of DDT, is detected in more than 95% of the general population (Zubero et al., 2015; Schettgen et al., 2015; Saoudi et al., 2014; Everett and Matheson, 2010).

Adverse health effects resulting from long term DDT exposure have been studied extensively in scientific literature with findings showing a potential relationship with breast cancer, diabetes, decreased semen quality, spontaneous abortion, and impaired neurodevelopment in children (Eskenazi et al., 2009). Moreover, a debate has been started whether even lower background exposures to OCs, and mainly DDT and hexachlorobenzene are linked with impairment of cognitive function in general population leading to dementia and Alzheimer disease (Singh et al., 2013; Medehouenou et al., 2014; Kim et al., 2015; Richardson et al., 2014).

Biomarkers of exposure to OCs have been used widely in exposure and epidemiological studies and their use has many advantages. Because of the long biological lives of OC’s, levels of these substances or their metabolites in human biological samples can represent not only current but also retrospective exposures. Levels of OCs in blood, breast milk, hair and adipose tissue as a measure of retrospective exposure is one of the most reliable laboratory confirmed documentation of past exposures.

Identification and quantification of OCs in serum samples is usually implemented by solid phase extraction or liquid-liquid extraction, followed by GC-ECD or GC–MS analysis. These methods are valid but they can be time consuming and costly since they require many steps during sample pretreatment (conditioning, washing, elution, and solvent evaporation etc). Adopting a solid phase microextraction (SPME) method for biomonitoring of OCs can greatly reduce preparation time, minimize the use of solvents and reduce the cost of the analysis since more than 50 extractions can be performed with a single fiber. To this direction, the aim of this study was to optimize and apply HS-SPME GC–MS analytical method to determine OCs in human serum. Thus we recorded
serum levels of OCs in general population in the city of Larissa, and investigated the link between certain population characteristics with the observed levels of exposure.

2. Materials and methods

2.1. Study population

The population of the present study consisted of 103 volunteers from the city of Larissa, Central Greece. Study subjects were mainly blood donors, who were contacted during voluntary blood donations, employees of University of Thessaly, and volunteers from the nursing home in the city of Larissa. Demographic characteristic were collected with personal interviews during sampling with the use of a questionnaire.

2.2. Sampling and storage

Peripheral venous blood samples were collected from each individual. Blood was extracted by venipuncture and collected on vacutainer blood tubes. The blood was centrifuged and serum was separated from the tube. Samples were stored at −80 °C until analysis.

2.3. Materials

A multicomponent standard solution of OCs (EPA 8081 Pesticide Standard Mix), and standard solutions for HCB, and PCB 101 were obtained from Supelco, USA. Extraction was performed with the use of a Supelco Solid Phase Microextraction Manual Holder supplied with Polydimethylsiloxane (PDMS) 100μM fiber acquired from Supelco. For heating and stirring of the samples a heating magnetic stirrer (VELP SCIENTIFICA, Italy) was used. The stock solutions of OCs, HCB and PCB 101 (used as internal standard) were prepared in methanol at concentrations 1 μg/ml, 0.1 μg/ml and 1 μg/ml respectively. The target analytes for quantification were HCB, Heptachlor, Heptachlor Epoxide, c-chlordane, a-chlordane, p,p′ – DDE, DDD and DDT.

The sample preparation procedure was originated from the previous publication by López et al. (2007) with some substantial modifications. In particular, 10 μl of IS (PCB 101 at 1 μg/ml) were added to 0.5 ml of serum sample. Subsequently 0.5 ml of acetoneitrile were added, the sample was vortexed for 1 min and then centrifuged for 5 min at 15,680 rcf. The 0.5 ml of supernatant was transferred in a 4 ml glass vial containing 0.5 ml of water and 1 ml of K2HPO4 (0.1 M). The analytes of interest were extracted by Solid Phase Microextraction in Head Space mode (HS SPME) with the use of a Polydimethylsiloxane (PDMS), df 100 μM fiber. The fiber was exposed for 30 min to the vapor of heated up to 85 °C liquid phase. After the extraction the fiber was immediately inserted to the GC–MS and desorbed for 5 min at 270 °C and splitless mode of PTV injector.

2.4. Sample treatment and analytes extraction

The analytes characteristic were collected with personal interviews during sampling with the use of a questionnaire.

2.5. GC–MS analysis

A Finnigan Trace GC Ultra/PolarisQ Quadrupole Ion Trap GC/MSn system was used for the quantification of OCs in serum. The gas chromatograph was equipped with a Programmed Temperature Vaporizing Injector (BEST PTV, Thermo Electron Corporation, USA) and a ATTM-5MS 30 m × 0.25 mm column with 0.25 m film thickness of 5% phenyl–95% methylpolysiloxane stationary phase (Alltech Associates, USA). Helium was used as the carrier gas in the constant flow mode at 1 mL/min. The PTV injector temperature was set to 270 °C and and injections was made in a splitless mode. The GC oven program had an initial temperature of 100 °C held for 5 min and then ramped to 160 °C with a heating rate of 15 °C/min, then ramped again to 300 °C at 5 °C/min, held for 2 min and cooled to the initial temperature. GC–MS chromatograms were acquired in SIM (selected ion monitoring) mode of mass analyser.

The quantification of substances was done by internal standard method (PCB 101). Calibration curves were created (concentration range: 0.5–20 ng/ml) for each substance studied. The limits of detection (LOD) and limits of quantitation (LOQ) were calculated according to the ratio signal / noise ratio (S/N) as follows: LOD = 3 S/N, LOQ = 10 S/N. The LOD (in ng/ml) were 0.03 for HCB, 0.04 for Heptachlor, 0.04 for Heptachlor Epoxide, 0.02 for c-chlordane, 0.04 for a – chlordane, 0.01 for p,p′ – DDE, 0.15 for DDD and 0.21 for DDT.

2.6. Statistical analysis

Statistical procedures were carried out by using and the Statistical Package for Social Sciences (SPSS) version 22.0. Continuous variables are presented as median with interquartile range (IQR), and categorical variables are presented as frequencies with the corresponsive percentages.To determine if the variables were normally distributed a test of normality (Kolmogorov-Smirnov) was used. Since OC values were not normally distributed, Mann–Whitney test was used to examine the differences of OCs’ values between population subgroups. A p-value 0.05 or less was considered statistically significant. In the statistical analysis all values below the LOQ were replaced with LOQ/sqrt2 (Succop et al., 2004).

3. Results

The most frequently detected substances were p-p′ DDE and Hexachlorobenzene (detection frequencies 99.03% and 67.96% respectively), while other substances were detected in a small minority of the samples. The respective detection frequencies for a-chlordane, c-chlordane, DDD, DDT, heptachlor epoxide and heptachlor were 16.50%, 4.85%, 4.85%, 1.94%, 0.97% and 2.91% of the population sample. OC values were not normally distributed for none of the pesticides or metabolites measured. The median values were 1.25 (IQR: 0.70–2.58) ng/ml for p-p′ DDE and 0.13 (IQR: < LOD–0.38) ng/ml for HCB.

Table 1 presents the median concentrations of p-p′ DDE and HCB for subgroups of the population sample, categorized according to demographic characteristics and habits. It is clear that age is a crucial determinant for OCs values with the elders having statistically significant higher exposures. We did not identify any correlation between gender, smoking habit, alcohol consumption and the measured biomarkers of exposure. Individuals who had received only primary or no education had significantly higher HCB levels compared to those who had received higher education. This association was observed probably due to the age difference between the two groups rather than the education level. Individuals who had received primary or no education were approximately 30 years older compared to the other groups, and since age is a strong determinant of HCB levels it is an obvious confounding variable.

4. Discussion

In the present study we applied a HS-SPME GC–MS method for detection and quantification of OCs in serum, in order to reduce the time and cost of the analysis. A previous study regarding hair
Comparison of p-p DDE and HCB concentrations (ppb) with other studies across the globe.

Table 2
Serum concentrations (ng/ml) of OCs according to demographic characteristics.

<table>
<thead>
<tr>
<th>Count</th>
<th>Hexachlorobenzene</th>
<th>p-p DDE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Percentile 25</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>81</td>
</tr>
<tr>
<td>Female</td>
<td>22</td>
<td>0.23</td>
</tr>
<tr>
<td>Age</td>
<td>53-75</td>
<td>25</td>
</tr>
<tr>
<td>47-52</td>
<td>23</td>
<td>0.15</td>
</tr>
<tr>
<td>40-46</td>
<td>27</td>
<td>0.17</td>
</tr>
<tr>
<td>0-39</td>
<td>28</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>Smoking</td>
<td>Regular/daily</td>
<td>49</td>
</tr>
<tr>
<td>Non smoker or less than 1 per day</td>
<td>54</td>
<td>0.14</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>Regular/daily</td>
<td>12</td>
</tr>
<tr>
<td>Less than 1 per day</td>
<td>91</td>
<td>0.13</td>
</tr>
<tr>
<td>Education</td>
<td>Higher</td>
<td>54</td>
</tr>
<tr>
<td>Secondary</td>
<td>39</td>
<td>0.12</td>
</tr>
<tr>
<td>Primary or none</td>
<td>10</td>
<td>0.56</td>
</tr>
</tbody>
</table>

analysis to determine the levels of chlorinated hydrocarbons (DDT and PCBs), has demonstrated that HS-SPME can be substantially simpler and faster compared to conventional methods (Tzatzarakis et al., 2014).

The analytical method was based on a previous publication describing a simple procedure for the determination of PCBs and some OCs in serum (López et al., 2007). Regarding to the sample preparation procedures, we introduced some novel modifications in order to optimize extraction in our own laboratory conditions. Particularly, when we tried to reproduce the previous method, we faced some difficulties with coagulation of the serum proteins during heating and trapping of analytes in clot with substantial reduction of OCs recovery. OCs are hydrophobic compounds that are substantially bound to serum protein and lipids (Gülden et al., 2002). Thus, to increase the efficiency of OCs extraction from serum and prevent their trapping we introduced protein precipitation extraction (PPE) by acetonitrile. We also observed a significant increase in abundance when we tested different centrifugation conditions. When we increased centrifugation rate from 3200 rcf to 15,680 rcf we achieved better separation of phases and observed an approximately 2-fold increase in recoveries. We also tested different durations of SPME and concluded that 30 min is the optimal time for analytes recovery and extraction time. The method requires small amount of sample (0.5 ml of serum), minimum solvent use, and is relatively simple and quick requiring less than 60 min for sample preparation. Moreover we demonstrated that the method is applicable for GC-MS analysis (the original method used GC-ECD) and we showed that it can be used for additional OCs namely heptachlor, c-chlordane, a-chlordane and DDD. The limits of detection are adequate for recording of typical background level in general population.

In this study we confirmed that DDT metabolite p-p’ DDE is detectable in serum in almost everyone residing in the city of Larissa, Central Greece, in contrast with its parent compound DDT which was detected at a very low frequency (1.94%). OCs have also been recorded in hair samples of Greek populations in previous studies (Tsatsakis et al., 2008a, 2008b). Their serum levels recorded in our study, either reflect past exposures to DDT or most likely have resulted from dietary and environmental exposure to DDE. Compared with other studies from Greek populations, DDE levels in Larissa were lower than those found in Attica region (Kalantzi et al., 2011) and those found in a large group of pregnant women in a cohort study conducted in the Island of Crete (Vafeiadi et al., 2014). When compared with data from large National Bio-monitoring programmes the DDE levels were higher than those found in Germany and in France and comparable with the levels of exposure of the US population. Also compared with general population from Thailand (Teeyapant et al., 2014), where DDT was used for malaria control until 1999, p-p’ DDE levels were approximately 10 times lower. Hexachlorobenzene levels were slightly higher compared with other European and US populations.

An overview of these comparisons is given in Table 2. Our analysis also revealed a strong relationship of p-p’ DDE and HCB concentrations with age. There is a wide consensus of the existing scientific evidence concerning the increase of OC values with age (Schettgen et al., 2015; Porta et al., 2008; Jakszyn et al., 2009; CDC, 2009). This association is explained by the long biological half lives of some OCs combined with the fact that significantly greater exposures occurred in the past decades when DDT and other OCs

Table 2
Comparison of p-p DDE and HCB concentrations (ppb) with other studies across the globe.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sampling years</th>
<th>Country</th>
<th>N</th>
<th>Matrice Age</th>
<th>p-p’ DDE</th>
<th>Hexachlorobenzene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kalantzi et al., 2011; Vafeiadi et al., 2014</td>
<td>2007</td>
<td>Greece</td>
<td>61</td>
<td>Serum Mean 40 (SD: 11.2)</td>
<td>2.46 (SD: 2.09)</td>
<td>0.23 (SD: 0.26)</td>
</tr>
<tr>
<td>CDC, 2009</td>
<td>2003–2004</td>
<td>USA</td>
<td>1368</td>
<td>Serum 20+</td>
<td>3.08 (SD: 3.60)</td>
<td>0.11 (SD: 0.10)</td>
</tr>
<tr>
<td>Saoudi et al., 2014</td>
<td>2006–2007</td>
<td>France</td>
<td>386</td>
<td>Serum Mean 45.4 (SE: 1.2)</td>
<td>0.58 (SD: 0.15)</td>
<td>0.09 (SD: 0.09)</td>
</tr>
<tr>
<td>Teeyapant et al., 2014</td>
<td>2011</td>
<td>Thailand</td>
<td>484</td>
<td>Serum Mean 48 (SD: 10)</td>
<td>10.00 (95% CI: 8.07–11.9)</td>
<td>0.36 (SD: 0.63)</td>
</tr>
</tbody>
</table>

* Original values were expressed as lipid adjusted and converted to ng/ml assuming a concentration of lipids in serum 6.5 g/l.
where still in use. The ban of DDT and other pesticides characterized as Persistent Organic Pollutants (POPs) in the developed countries led to a essential decline in the body burden of these toxicants in general population which is reflected in the lower concentration on younger individuals. However, circulating concentrations of OCs, even at low background levels, similar to those found in our study, are still of concern. Recent investigations, suggest that exposure levels typically observed in general population might be related to various health effects, with many studies focusing on metabolic effects and diseases such as type 2 diabetes (Salihovic et al., 2016). Also, effects of background prenatal exposure to OCs to the offspring are still being investigated. Prenatal serum levels of DDE and HCB were associated with excess adiposity and higher blood pressure levels in early childhood, in a recently published mother–child study in Crete, Greece (Vafeiadi et al., 2015). The most plausible mechanisms of OC’s toxicity among others involve endocrine disruption, oxidative stress and epigenetic modifications (Mrema et al., 2013).

Concluding, our results reveal that general population of Thessaly region has been exposed to OCs and mainly p,p’ DDE and hexachlorobenzene, and continues to be exposed to these substances. Levels of exposure are within the range reported by other studies and it could be stated that they are typical background levels of a developed country.

References


