VOLUMETRIC ABSORPTIVE MICROSAMPLING FOR THE SCREENING OF OCPs AND PCBs IN µL VOLUMES OF HUMAN BLOOD

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Introduction

Nowadays, human biomonitoring studies for POPs such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs),… are performed on serum specimens[1], with sample sizes of whole blood to be taken from patients ranging from 1 mL to 50 mL. Measurements are carried out by state-of-the-art gas chromatography (GC) isotope dilution (ID) mass spectrometry (MS)[2]. Even if much less invasive than the original surgical abdominal fat removals that were performed in the 1980's, the venipunction of several milliliters of whole blood for analytical purpose is still badly perceived by patients but also often exclude young infants and elderlies from being sampled for obvious health issues, systematically leaving them out of exposure data sets.

In this study, we developed an analytical method for the measurement of selected POPs in ultra-low volume (40 µL) of human blood. The strategy involves the use of volumetric absorptive microsampling (VAMS)[3] to obtain dried-blood sample without homogeneity issues and volume-related sub-punching problems generally encountered when considering dried-blood spot (DBS) samples. This also makes the sample collection independent of the hematocrit content or the volume sampled onto the card. Because of the very low level of invasiveness and ease of use of such microsampling, this has the potential to be used in remote areas for which very little data are currently available but also for screening of large populations in the context of UNEP studies for POP inventories[4]. It would also be extremely valuable for collecting samples from babies, young children, elderlies, and small animals.

A miniaturized SPE procedure has further been developed to isolate 24 OCPs and 6 non dioxin-like (NDL-) PCBs from the 40 µL VAMS samples. That list of analytes has been selected based on their relevance in terms of levels in humans and capacity to be used as markers of exposure[5,6]. Cleaned extracts were analyzed by GC coupled to triple quadrupole (QQQ) MS operating in tandem mode (MS/MS), based on one of our recent validation study for the measurement of PCDD/Fs and PCBs in feed samples[7]. At this stage, PCDD/Fs and HFRs have not yet been included due to their very low background levels in blood (at the sub pg/g lipid), as well as laboratory blank issues, respectively.

Materials and methods

Samples and chemicals. Details on typical dioxin measurement consumables are available in a previous report[8]. A mixture of ^{13}C-labeled pesticides internal standards (ES-5465, Expanded POPs pesticides) containing 25 ^{13}C-labeled OCPs and a mixture of 7 NDL-PCBs containing ^{13}C-labeled congeners (EC-4058) were purchased from Cambridge Isotope Laboratories (CIL, Andover, MS, USA). Quantitation was ensured by isotopic dilution and recovery rates were assessed ^{13}C-labeled PCB-80 and ^{13}C-labeled isodrin (ES-5466) standards (CIL). A 7 points calibration curve for NDL-PCBs was prepared using EC-5179 and EC-4058 standard solutions (CIL) at 0.4-1-4-10-20-40-80 pg/µL and we bought the calibration curve for OCPs (27 compounds) (ES-5464, CIL). We prepared a low-level 8 points calibration curve from the latter, by dilution and addition of ^{13}C-labeled compounds, to obtain the following levels for all pesticides: 0.1-0.2-0.4-0.8-1.2-2-3-10 pg/µL. Blood samples were collected from volunteers. The reference blood sample was collected from a 26 years old volunteer. The associated reference serum was prepared by collecting 40 mL whole blood in dry tubes.

Preparation of dried-blood samples. Blood sample volumes were 40 µL. It consisted in approximately 2 to 3 drops of whole blood to be collected from patients. We used a OneTouch® finger prick device (Johnson & Johnson (J&J), New Brunswick, NJ, USA), mounted with OneTouch UltraSoft® lancettes (J&J) to produce the blood drop. The process was very fast and lasted for less than 1 minute. The blood on the finger was subsequently collected by VAMS technology using a MITRA™ collecting device (Phenomenex, Torrance, CA,

USA) that consisted in a small cube-shaped proprietary wicking adsorbent material attached to a sampling tip (4 tips of 10 µL/sample)[3]. After sample adsorption, tips were lifted and placed in a 96 holes drying box (Phenomenex) for drying and storage.

Extraction. The reference serum sample was extracted in the routine laboratory under ISO 17025 accreditation following a procedure already described[1]. VAMS samples were first left 90 min on a horizontal stirrer plate (400 rpm) in a mixture of water/formic acid/acetonitrile (ACN) 150:100:150 µL allowing solubilization of the blood in the 400 µL total volume. The aqueous media was collected in a 1.5 mL vial and the VAMS sorbent was again solubilized in 40:20:40 µL of the mixture for an additional 30 min on the horizontal stirrer plate. The two fractions (total of 500 µL) were pooled and centrifuged at 5000 rpm for 10 min to remove suspended particles. The sample was then extracted on a 25 mg Discovery DSC-18 SPE cartridge (octadecyl C_{18} stationary phase) (Supelco, Bellefonte, PA, USA) after addition of internal standards directly on the SPE frit. Conditioning of the cartridge was ensured by methanol followed by water. Water was used as washing solvent and, before elution with a mixture of hexane/dichloromethane 70:30 (350 µL), the cartridge was dried for 15 min to remove water. The collected organic fraction was transferred into conical GC vials and the solvent was evaporated on a RapidVap (Labconco, Kansas City, MO, USA) µµ

Instrumentation. An Agilent 7000C GC-QQQMS (Palo Alto, CA, USA), operating in tandem mode (MS/MS), equipped with a 7890B GC oven, a programmable temperature vaporization (PTV) inlet, and a 7693A automated liquid sampler (ALS) were used. For maximum sensitivity, the entire amount of sample obtained after extraction in the conical vial (5-10 µL) was injected, allowing. The PTV was operated on solvent vent mode and cooled by liquid CO_2. The GC column was an Rxi-XLB 30 m x 0.25 mm ID x 0.25 µm df (Restek Corp., Bellefonte, PA, USA). The oven temperature program was 50°C for 4.3 min, 50°C/min to 140°C, 10°C/min to 238°C, 2°C/min to 244°C for 5 min, 10°C/min to 310°C for 3 min and a total runtime of 33.5 min. The electron ionization (EI) ion source was heated at 280°C and operated at 60 eV. Quadrupole resolution was set to 'wide' mass, which by default corresponds to peak width of 1.2 Da at half height. The voltage detector was adapted during advanced autotune, in EI high sensitivity mode, to ensure electron multiplication of factor 107 (gain factor of 100), which was reflected by an electron multiplier detector voltage (EMV) of 1990 V. Measurements were carried out in Multiple Reaction Monitoring (MRM) mode after optimization and selection of the base peak as precursor ion and the base peak after collision and fragmentation as product ion. Dwell times were selected and acquisition windows adjusted to optimize acquisition frequency to get 7 to 10 data points per peak[9], which was achieved by setting 40 to 90 ms per transition. We locked retention times to PCB-105 allowing to change and cut the head of the column for other purposes and bring back the system to the original configuration. Two MRM transitions were monitored for each target for quantitation ('Quant transition') and qualification ('Qual transition') purposes. Each Quant/Qual transitions were recorded either from two specific precursor ions or from the same precursor ion (usually 2 Da offset) and two distinct product ions. Quantitation was performed with the Quant transition only and the Qual transition was exclusively used to verify ion ratio between Quant/Qual transitions when sensitivity allowed. Mass Hunter v. B.07.00 was used as acquisition and quantitation software.

Results and discussion:

Determination of limits of quantitation (LOQs). The optimum EI value relied on the compound structure and on whether the precursor ion was produced by breaking a C-C or a C-Cl bond. An intermediate ionization energy of 60 eV was selected, and provided a sensitivity improvement ranging from 14% (endosulfan I) to 86% (delta-hexachlorocyclohexane), compared to the more classical 70 eV value. Because of the absence of noise when using the GC-QQQMS/MS system, instrumental limits of quantitation (iLOQs) were established based on the reproducibility of replicate analysis (n=8) of the lowest calibration point[10]. Method limits of quantitation (mLOQs, Table 1) were further adjusted based on analyte levels (if any) in blanks (mLOQs = average blank level of 10 blanks + 2 SD)[11]. For Aldrin, cis/trans Chlordane, cis/trans Nonachlor, Endosulfan I/II, and Mirex, not present in blanks, mLOQs = iLOQs. Because micro volumes of samples are considered, we often reported sample and blank values very close to each other, with a ratio between 1:1 (thus reported <mLOQ) and 5:1. We therefore worked using a sample-blank paired approach with individual correction. The 40 µL sample size was a method limitation, because of blank levels (iLOQs at the low fg/µL level). Although blank issues an easily be reduced by working in a controlled environment (clean room), we decided not to do so as we wanted the method to be usable in simple lab environment. As it is, the method should therefore rather be considered as a screening approach using ultra-low blood volumes than a 'confirmatory' method.

Table 1: Parallel measurements with the VAMS method and the reference method (real sample).

		DB method			Reference method		
	mLOQ ng/L blood	Level ng/L blood	Recovery rate %	Within-lab reproducibility (n=5)	Level ng/L blood	Recovery rate %	Bias
Pentachlorobenzene	128	48.7	28	11	<mLOQ	67	
alpha BHC	1140	416.2	34	18	<mLOQ	3	
Hexachlorobenzene	196	59.0	34	14	29.8	8	98
gamma BHC (Lindane)	5223	2218.0	38	18	<mLOQ	3	
beta BHC	243	109.7	27	18	<mLOQ	0	
PCB-28(ind)	1438	<mLOQ	27	-	11.4	69	
delta BHC	73	<mLOQ	27	-	<mLOQ	3	
Heptachlor	36	<mLOQ	78	-	<mLOQ	0	
PCB-52(ind)	4704	<mLOQ	17	-	<mLOQ	42	
Aldrin	40	<mLOQ	39	-	1.5	8	
DDE 2,4'	44	<mLOQ	30	-	0.6	126	
PCB-101(ind)	21342	<mLOQ	41	-	<mLOQ	103	
trans-Chlordane	23	<mLOQ	39	-	<mLOQ	100	
cis-Chlordane	29	<mLOQ	39	-	<mLOQ	100	
Endosulfan I	34	<mLOQ	33	-	<mLOQ	90	
trans- Nonachlor	11	<mLOQ	40	-	6.7	96	
DDE 4,4'	71	106.5	32	21	254.2	140	-58
Dieldrin	75	<mLOQ	34	-	<mLOQ	0	
DDD 2,4'	85	37.6	35	30	<mLOQ	22	
Endrin	14	<mLOQ	43	-	<mLOQ	0	
DDT 2,4'	81	33.2	36	22	<mLOQ	2	
cis- Nonachlor	38	<mLOQ	39	-	<mLOQ	86	
DDD 4,4'	78	42.1	37	31	<mLOQ	1	
PCB-153(ind)	3952	60.4	57	17	78.1	136	-23
Kepone (Chlordecone)	1742	<mLOQ	19	-	567.8	96	
Endosulfan II	1000	<mLOQ	0	-	<mLOQ	0	
DDT 4,4'	83	32.2	39	20	<mLOQ	0	
PCB-138(ind)	1875	64.4	42	14	36.8	101	75
PCB-180(ind)	1573	81.8	66	7	91.2	75	-10
Mirex	21	<mLOQ	35	-	1.6	54	

Sampling and extraction. Micro volume sampling can be carried out using regular DBS paper and sub-punching but without control on the exact sampled blood volume. This is especially true when considering adult population blood containing various hematocrit levels (HCT). Using a commercially available popular DBS paper, we measured blood volumes from replicated 6 mm circle punches and found an average volume of 8.7±0.9 µL (n=10, 10.3% RSD) for a single blood sample. The use of volumetric absorptive microsampling (VAMS) system allowed to have better control on blood volumes (4.0% RSD), independently of HCT level [3,12]. After drying of the tips, the blood was eluted from the VAMS using formic acid to hydrolyze lipids[13] and enhance the extractability of compounds of interest. Further clean-up of the 500 µL centrifuged volume was performed using C_{18} SPE cartridge (25mg/2 mL) in 96-well pate format for parallel extraction of several samples. Recovery rates ranged at 10-70%. In total, a volume of 2 mL of solvents per sample was required, including conditioning and washing steps.

Performances. A same young individual was sampled for both serum (40 mL) and blood (40 µL) and was used as a reference sample (non-fortified) (Table1). Excepted for 4,4'-DDE, the analyte profile is respected. Larger uncertainty arose for PCB-153 and PCB-138 due to high levels in the blank. In this context where we typically measured samples/blank ratio from 1 to 5 at very low levels, the method cannot provide the commonly targeted performances in terms of trueness (±20%) and within-lab reproducibility (15% RSD).

The Table 1 shows that for the 5 reference targets, the bias is between -58 and +98% but with very good within-lab reproducibility for all targets from 7% to 31%. We also report good recovery rates for all targets. The method was suitable for all PCBs and OCPs. The low amount of blood used has the advantage that low amount of interferences were also present, therefore facilitating the extraction and not requiring a further clean-up after the SPE. Nearly all relative standard deviations (RSDs) were above 15%, the commonly accepted value. Looking at these expected high RSD as well as the small gap between blanks and samples, the DB method is to be considered as a first easy-to-use screening method in case of suspected issues, such as hot spots or environmental issues, before further deeper investigation with more classical methods.

Screening of real samples. We applied the sampling technique to screen 11 Belgian non-exposed individuals from 7 to 65 years old. As expected, many congeners were below mLOQs. From the 30 analytes, detection rate ranged between 3 and 47%. We also see a higher sum of all contaminants for older people, which is consistent with the fact that those molecules are poorly metabolized and that their levels are supposed to increase over time.

The advantage of the method is also addressed in terms of cost. The consumables necessary were 2 mL solvents per sample, the VAMS tips (200 EUR/96 tips), the C-18 cartridges (200 EUR/96-well), and the instrumental/human cost. The extraction was therefore a very fast (3 hours for multiple samples in parallel) and an extremely cheap solution.

Conclusions

A minimally-invasive, very fast, and cost effective method to screen people for 30 organochlorine pesticides and non dioxin-like PCBs is reported. Because of the very low levels measured with regards to the background, the method is subject to a higher variability in comparison with the standard method. Yet, the method, supported by adequate analytical parameters such as the limits of quantitation, delivers an alternative to the confirmatory analysis in order to easily gather data from people in remote area, from young infants, or for purposes requiring fast response in case of suspected issues.

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