BLOOD-SAMPLING METHOD FOR POPS AND OTHER CHEMICALS ANALYSIS IN JAPANESE BIRTH COHORT STUDY, "JAPAN ENVIRONMENT AND CHILDREN'S STUDY"

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Introduction

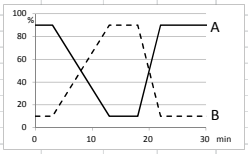
Our modern life is supported by the use of variety of chemicals with beneficial properties. Increase of the indices showing decline of health status in newborn babies/children in recent decades, however, has been raising public concern about potential adverse effects of chemicals or other environmental factors in our daily life. Japanese Ministry of the Environment had been preparing for the start of new birth cohort study, called "Japan Environment and Children's Study". This epidemiological study is intended to clarify possible relationship between chemicals surrounding our daily life and children's health status by detailed chemical analysis of blood/urine /hair samples from mother, father and children[1]. The study started in FY 2010 with 15 local unit centers for the recruit and collection of samples and the core center, National Institute for Environmental Studies (NIES), for the administration and scientific support together with the medical support center, National Center for Child Health and Development[2].

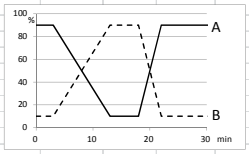
In this cohort study, blood sampling from mothers, fathers and newborn babies (cord bloods) are planned for the analysis of POPs and other chemicals. Part of the samples will be kept in liquid nitrogen vapor phase containers at Time Capsule facility in NIES for future retrospective analysis. The sampling tubes and devices, therefore, have to be selected carefully in order to minimize contamination by not only target chemicals of the study but also any other chemicals of possible concern in future. For the simultaneous analysis of environmental chemicals in human samples, we have developed LC-TOF analytical method and applied the method for the assessment of chemical contamination in various blood tubes/devices. Here we report the results and the selected sampling method for human blood in Japan Environment and Children's Study.

Materials and methods

LCMSMS (AB Sciex 4000QTrap) was employed for the analysis of selected chemicals of concern (fluorosurfactants, alkylphenols and their ethoxylates, phthalate monoesters, etc.) while LC-TOF (Agilent 6224A) was used for the simultaneous analysis of both cationic and anionic chemicals. POPs were analyzed by GC/MS. ICPMS (Agilent 7500 series) was employed for the elemental analysis.

Table 1 LC-TOF conditions (left; positive ion mode, right; negative ion mode)

HPLC		MS		
Instrument	Agilent 1100	Instrument	TOF/LCMS (G6224A)	
Column	**Agilent Zorbax XDB C18 2.1*150mm**	Ion Source	Dual ESI	
Mobile Phase	A: Water(0.1% formic acid)			
	B: MeOH	Source parameter		
Flow rate	200µl/min	Ion Polarity	Positive	
Column heater	40C	Gas temp (C)	325	
Injection Volume	5µl	Gas Flow (l/min)	10	
		Nebluizer (psi)	50	
Gradient		V Cap	3500	
		Fragmenter	125	
		Skimmer1	65	
		OctopoleREPPeak	750	
		Mass range	100-1700	
		Reference Mass	Positive	121.050873
			Positive	922.009798

HPLC		MS		
Instrument	Agilent 1100	Instrument	TOF/LCMS (G6224A)	
Column	**Agilent Zorbax XDB C18 2.1*150mm**	Ion Source	Dual ESI	
Mobile Phase	A: 10mM Ammonium Acetate Water			
	B: AcCN	Source parameter		
Flow rate	200µl/min	Ion Polarity	Negative	
Column heater	40C	Gas temp (C)	325	
Injection Volume	5µl	Gas Flow (l/min)	10	
		Nebluizer (psi)	50	
Gradient		V Cap	3500	
		Fragmenter	125	
		Skimmer1	65	
		OctopoleREPPeak	750	
		Mass range	100-1700	
		Reference Mass	Positive	121.050873
			Positive	922.009798

Vacuum tubes and needles for blood collection were obtained from Becton Dickinson and Co., Nipro Co., Terumo Co., and Top Co. Insepack was a generous gift of Sekisui Chemicals Co. Each tube was rinsed with methanol, and the extract was evaporated, dissolved in 50% acetonitrile/water mixture and filtered, and analyzed by LCMSMS and LC-TOF. Detailed surveys of dioxin, PCB and other POPs were conducted by high resolution GCMS separately. ODS (Zorbax C18, Agilent) was used for the LC-TOF analysis. Several LC conditions were assessed of their potential to detect chemicals in human urine samples. In brief, 0.1 % formic acid – methanol

gradient was found to be suitable for analyzing cationic compounds while ammonium acetate – acetonitrile gradient was selected for anionic compounds. The optimized conditions are summarized in Table 1.

Results and discussion:

(1) Optimization of LC-TOF condition for simultaneous analysis in human urine

In the first stage of the research, optimization of LC-TOF condition for the analysis of urine was conducted by checking the number of detected chemicals. Generally speaking, acidic buffer with weak organic acids are suitable for adding proton to the chemicals to convert them to cations. Among several organic acids frequently used for the LCMS analysis, formic acid showed larger number of peaks compared with other acids and their salts. In specific cases, however, we found oxalic acid, a bi-carboxylate, showed higher sensitivity, possibly because of lower pKa value of the first dissociation, i.e., more acidic character. Similarly we found ammonium acetate buffer – acetonitrile gradient most appropriate for the simultaneous detection of variety of anionic compounds. Typical examples of LC-TOF cationic peak profiles were shown in Figure 1. Several thousands peaks could be easily detected in human urine samples. Clarithromycin (mw=747.48) and its metabolites (A: circled) were detected when a person was treated because of catching cold.

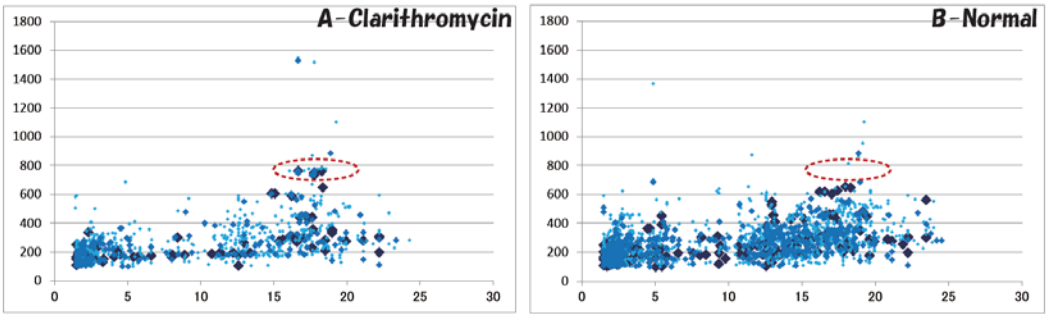


Figure 1 Positive ions detected by LC-TOF from human urine samples
A: urine after ingestion of Clarithromycin, B: normal urine

(2) Selection of blood collection tube for POPs and other chemical analysis

In Japan there are some on-going human biomonitoring programs. Dioxin monitoring, for example, has been analyzing whole blood samples of both men and women[3]. Addition of anti-coagulant, heparin in this case, is necessary for the whole blood collection. As such additives might cause unexpected contamination of chemicals, however, use of serum may be an alternative choice.

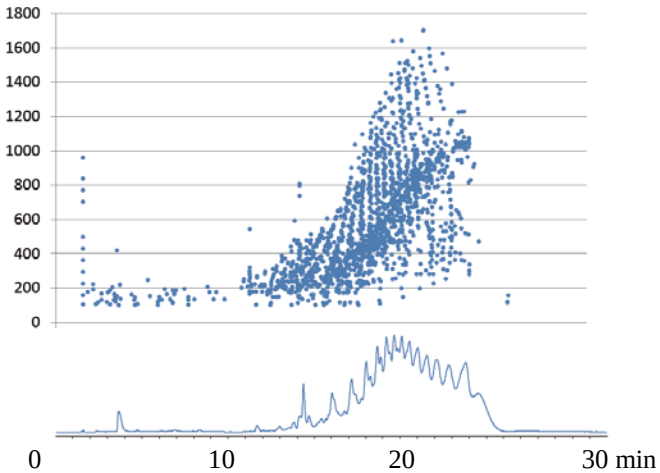


Figure 2 LC-TOF total ion chromatogram of a plain tube extract (lower) and detected peaks (upper)
Upper Figure X axis: retention time (min), Y axis: mass (m/z)

When applying the optimized LC-TOF for survey of blood collection tubes, however, we detected unexpectedly large number of peaks in some of them. Figure 2 showed an example of LC-TOF analysis of rinsed extract from a plain tube for blood collection. As shown in the total ion chromatogram, many peaks could be detected by LC-TOF. Their retention times and masses showed presence of polymers with simple repeated chain structure. Typical mass difference between the two consecutive peaks was 44.026, which corresponds to (-C_2H_4O-) repeat. In other cases the interval apparently corresponded to polyoxypropylene structure. Similar but different peak patterns were found in other vacuum tubes from different companies. Apparently these tubes contain polymers having polyoxyethylene/-propylene tails, such as polyethyleneglycol or polyoxyethylene/-propylene co-polymers.

According to patent information, such polymers are used for the coating of tube wall to prevent adhesion of fibrin to the hydrophobic polyethylene telephthalate (PET) wall when coagulation occurred. In fact we found such series of peaks in all the plain vacuum tubes. We did not see such peaks in many of EDTA containing tubes with one exception, while many of heparin tubes were found to contain similar peaks. One company's plain tube was made of glass, which may not need such coating material. However we detected various similar peaks in the rinsed solution, too.

In addition to the peaks, several chemicals of concern were detected in the rinse solution of vacuum tubes as well as butterfly needles. The results were summarized in Table 2. In the table, Polymer means a series of chemicals detected by the LC-TOF, AP means alkylphenols, APEOs means alkylphenol ethoxylates, MAP means monoalkylesters of phthalic acid, and PFCs means perfluorochemicals including PFOS and PFOA. POPs including PFCs, priority chemicals to be analyzed in bloods, were not detected in any of the tubes/needles at all or detected only in negligible levels. Other chemicals, including alkylphenols and their ethoxylates, and phthalate monoesters, on the other hand, were detected in some of the tubes/needles in considerable levels. Although these are not intended to be analyzed in blood, their presence may suggest occurrence of unintentional / uncontrolled pollution during the production of these materials / products. Among them, phthalate esters have been used as plasticizer of PVC for the flexible tubing of the butterfly needle, and also other materials including plastic bags for blood collection. Monoesters are not used for plasticizers but may be present as impurities or decomposed products from diesters during production or sterilization process. Some of the chemicals including alkylphenols might be used as raw materials of some plastics or washing detergent of the mold. Some are also used intentionally as stated above.

Table 2 Results of Analysis of Blood Vacuum Collection Tubes and Butterfly Needles

Company	Type	Tube wall	Stopper	Polymers	AP	APEOs	MAP	PFCs
Blood Collection Vacuum Tubes								
A	Plain	Glass	Elastomer	△	○	◎	◎	
A	EDTA	PET	Elastomer		○	◎	○	
A	EDTA(Trace)	PET	Elastomer	◎	○			
A	Heparin	PET	Elastomer	△		◎	○	
B	Plain	PET	Coated alminum	◎				
B	Plain+Agr.*	PET	Coated alminum	◎	○			
B	EDTA	PET	Coated alminum					
B	Heparin	PET	Coated alminum	◎	◎		○	
C	Plain	PET	Elastomer	△			○	
C	EDTA	PET	Elastomer					
C	Heparin	PET	Elastomer	○			○	
Needles								
A	butterfly	PVC		○	○	○	◎	
B	butterfly	PVC		○				
B	butterfly	PVC		○		○		○
D	butterfly	PVC		○	○		○	
E	butterfly	PVC		○	○		◎	○
	◎	abundant (polymer) or ＞10ng/tube (other chemicals)						
	○	present (polymers) or ＞1ng/tube (other chemicals)						
	△	trace						
	(blank)	not detected						
		no data						

Serum separator is reported to have hydrophobicity and is known to have tendency to absorb hydrophobic chemicals[4]. Also there are some reports showing their interfering effect on the analysis of proteins by mass spectrometry methods[5,6]. Although we did not know direct information on the possible interference of the separator material to the analysis of hydrophobic POPs chemicals, we decided to use tubes without serum separator for the sampling because of its potential interference due to hydrophobicity. As reported in the present study, however, many of the vacuum tubes are found to be coated by some polymers, which prevent adhesion of fibrin, and thus contaminate the blood samples by the material. EDTA-containing plasma collection tubes generally contain smaller number of chemicals detectable by LC-TOF than others, probably because coagulation do not occur and coating of PET wall is not necessary. Curiously one EDTA tube produced for trace element analysis was found to contain huge number of such peaks. We do not know the reason. In addition to these intentional materials, there seem to be uncontrolled pollution of tubes by some of plasticizer-related or molding process-related chemicals. Based on the results of the analysis as reported in the present study, we selected plasma collection vacuum tube containing EDTA (Terumo Co.) for the sampling of blood for chemical analysis. During the research, we also noticed that some washing/coating processes to prevent adhesion of proteins on the PET wall also caused contamination of some of the target chemicals, such as bisphenol A. Good news is that pollution by PFCs, one of the priority chemicals in the study, was not found in any of the tubes examined in the present study, though PFNA were detected in some butterfly needle at ng/needle level. The use of PFCs as surface-active material, including detaching reagent from the mold, occurs frequently in industrial processes. Continuous monitoring of the blood collection/preservation materials is necessary for keeping quality of blood sample handling and preservation process in sufficiently good level for the chemical analysis in future.

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4. See, for example, Bowen RAR, Chan Y, Cohen J, Rehak NN, Hortin GL, Csako G, Remaley AT. (2007) *Clinical Chem.* 51(2): 424–433, and the cited references
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