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Development of an ion-pair liquid chromatography–high resolution mass spectrometry method for determination of organophosphate pesticide metabolites in large-scale biomonitoring studies

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ABSTRACT

Organophosphate based pesticides are widely used in the agricultural sector, and exposure to these chemicals is common for the general population. Pesticides are toxic due to the inhibition of acetylcholinesterases, and the potential for adverse health effects have been investigated in past and recent studies. Human biomonitoring of organophosphate pesticide exposure is carried out through the determination of the metabolites in urine (dialkylphosphates, DAPs). Hereby we present a new method for determination of the 6 non-specific metabolites dimethyl phosphate (DMP), diethyl phosphate (DEP), dimethyl thiophosphate (DMTP), diethyl thiophosphate (DETP), dimethyl dithiophosphate (DMDTP), and diethyl dithiophosphate (DEDTP) in urine based on off-line solid phase extraction (anion exchange, 96-well plates) followed by ion-pair ultra-performance liquid chromatography time-of-flight mass spectrometry. Recoveries and accuracies in control urine spiked at 5 ng/mL ranged from 48% to109% and from 91% to 115%, respectively. The method limits of detection for the DAPs were 1.2 ng/mL for DMP, 0.38 ng/mL for DEP, 0.20 ng/mL for DMTP, 0.33 ng/mL for DETP, 0.64 ng/mL for DMDTP, and 0.15 ng/mL for DEDTP. The method was applied to samples from a Norwegian mother/child study group (n = 48/56) and the DAPs detection frequencies in urine from mothers and children were about: 40% for DMP, 95% for DEP, 96% for DMTP, 50% for DETP, 15% for DMDTP, and 1% for DEDTP. In both mothers and children, the most abundant DAPs were DMTP (median 2.4/5.2 ng/mL) and DEP (median 2.6/3.4 ng/mL) followed by DMP (median 1.5/2.1 ng/mL). The SG corrected concentrations of DEP and DETP in mothers were statistically higher than in children (p-value < 0.05; Mann-Whitney test) which might suggest a higher exposure to pesticides in these mothers, or significant differences in toxicokinetics between adults and children. The method was proven robust and suitable for large-scale biomonitoring studies.

1. Introduction

Pesticides are primarily used in agriculture in order to control, repel or mitigate weeds, proliferation of insects and diseases. Back in the 40s, a class of chlorinated pesticides (e.g., aldrin, chlordane, DDT, dieldrin, etc.) were extensively used for those purposes [1], but their adverse ecotoxicological effects, and intrinsic persistence and toxicity to invertebrates and vertebrates became a serious threat to human health [2]. Consequently, this class of pesticides was phased out in the late 70s, and most of them banned in the Stockholm Convention in 2001 [3]. The massive use of former chlorinated pesticides has been replaced by organophosphate based pesticides (OPs). As their predecessors, OPs are not selective pesticides and they present serious hazard of acute toxicity to humans [4]. In general, OPs disrupt the human nervous system by acting as inhibitors of enzymes (acetylcholinesterases). Although, the human body eliminates OPs in 48 h via urine and faeces [4], the continuous exposure, and their association to adverse health effects in humans (e.g., neurobehavioral effects [5–7], DNA damage and adverse birth outcomes [6,8]) have raised concerns about their production and use.

Metabolism of OPs typically yields a non-specific hydrolysis product (e.g., $(RO)_2P(O)OH$), as well as an insecticide-specific hydrolysis product from the side chain. The non-specific hydrolysis product is common for 75% of the OPs [9] and can be summarised in 6 dialkylphosphates (DAP): dimethyl phosphate (DMP), diethyl phosphate (DEP), dimethyl thiophosphate (DMTP), diethyl thio-

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phosphate (DETP), dimethyl dithiophosphate (DMDTP), and diethyl dithiophosphate (DEDTP).

Biomonitoring studies usually assess the DAPs in urine because the concentrations of these metabolites are expected to be higher than those from the parent compounds in other matrices, and it gives a better overview of the integrated external exposure to a wide group of OPs. The occurrence of DAPs in humans is well documented and the levels in urine vary from country to country. Recent studies from USA (n = 274), Greece (n = 86), and China (n = 187) reported median or geometric mean urinary concentrations of these DAPs ranging from <LOD-1.9 ng/mL [10], 0.4–7.1 ng/mL [11], and <LOD-17 ng/mL [12], respectively.

There are presently two main analytical methodologies used for the determination of DAPs: a) derivatisation of the DAPs, subsequent clean-up and determination by GC–MS [13,14], and b) determination using LC–MS with or without sample preparation [15,16]. Traditionally the use of GC–MS has provided lower method detection limits (MDL) (0.02-0.15 ng/mL) [11,13,17], although the cost of such low MDL is a tedious and time consuming sample preparation. Thus GC–MS seems impractical for large-scale epidemiological studies and because of this, there is a tendency to switch to LC–MS for polar compounds which ionise well in sources like electrospray (ESI) or atmospheric pressure chemical ionisation (APCI) [18]. Some LC–MS/MS methods for the determination of DAPs in urine are based on anion exchange interactions. Draper et al. [19] coupled a strong anion exchange analytical column to a tandem MS equipped with an APCI source working in negative mode (−ve). The reported MDLs were from low ng/mL to 9.3 ng/mL for DEDTP. Odetokun et al. [9] developed an off-line weak anion exchange (WAX) solid phase extraction (SPE) sample preparation using 0.3 mL of urine, and subsequent determination of DAPs using hydrophilic liquid chromatography (HILIC) coupled to tandem MS equipped with an ESI −ve. This methodology reached MDLs comparable to GC–MS (0.04–0.11 ng/mL), except for DEDTP (1.5 ng/mL).

In addition to the aforementioned analytical methodologies, the retention of OP metabolites on reverse phase columns has been achieved using ion-pair additives (e.g., tetrabutylammonium acetate (TBAA)). The main advantage of using ion-pairing is the enhanced retention factor on C_{18} columns and the possibility to inject urine without, or with very little, sample preparation into hydrophobic stationary phases. However, to this date, the use of ion-pair additives and the lack of sample treatment are detrimental to obtain MDLs comparable to GC–MS methods (0.5–20 ng/mL) [20–23]. In addition, no studies have showed proper robustness of the ion-pair method for large epidemiological studies (e.g., several hundreds or thousands of samples). For these reasons, HILIC has lately been preferred for determination of DAPs using LC–MS [10,24].

The use of high resolution mass spectrometry (HRMS) for the identification and quantification of DAPs has seldom been used [25]. HRMS offers the possibility of screening for a much larger number of compounds in full scan acquisition, high resolving power, mass accuracy and the possibility to perform a retrospective analysis of compounds not considered in the first place. In general, a drawback when using HRMS is the shorter dynamic ranges for quantification compared to triple stage quadrupoles (TSQ) [26,27]. Although, modern HRMS overcome this issue and in terms of sensitivity they are comparable to TSQ [26]. Since TSQ is used for target analyses, most of the methods incorporate a sample preparation to increase the sensitivity. If sample treatment is applied to HRMS methods, it is likely that unknowns are lost, thus compromising the capacity to search for them and carrying out retrospective analyses. Roca et al. [25] developed a reverse phase method coupled to orbitrap without sample preparation to monitor specific and non-specific pesticide moieties in urine, among other pollutants. MDLs for DAPs ranged between 1.6 and 10 ng/mL.

For biomonitoring studies to be conducted on non-exposed subjects, a MDL of 1 ng/mL or less is advantageous [16]. To achieve this goal, it seems like HILIC is the best choice, but since C_{18} columns are extremely robust and widespread in all laboratories, and that there is a lack of methodologies based on ion-pair in the last years, we do believe that there is still room for improvement for methods based on ion-pair chromatography. Typically strong ion-pair additives, such as TBAA, have been employed, but the use of weaker ion-pairs in order to enhance the ionisation of the DAPs has not been properly explored. Therefore, our aim was to develop a sensitive method where: i) sample preparation was carried out using anion exchange sorbent in the format of 96-well plates, ii) a weak ion-pair additive was used to increase retention of DAPs in C_{18} columns, iii) HRMS (time-of-flight) was used to identify and quantify DAPs, and finally iv) the adequateness of the method was demonstrated in a study comprising 356 urine samples from mothers and their children, and the robustness was assessed by analysing in-house control samples during a period of six months.

2. Experimental

2.1. Materials and methods

2.1.1. Chemicals and consumables

Two native (DMTP and DMDTP) and five ^{13}C-labelled standards were obtained as sodium salts, except DETP as potassium salt, and DEDTP and ^{13}C DEDTP as ammonium salts. DMP and DEP were obtained as acids, and all standards were purchased from Chiron (Trondheim, Norway). All standard purities were >98%. Water, acetonitrile (MeCN), methanol (MeOH), and acetone were of LC–MS grade (> 99.9% for organic solvents), and all were purchased from J.T. Baker (Center Valley, PA, USA). Tetrabutylammonium hydroxide (TBAOH; 40% wt in water), triethylamine (TEA, 99%), tripropylamine (98%), acetic acid (AA; 98%), and formic acid (HCOOH, 98%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Strata-X-AW 96-well plates (30 mg) and a manifold were purchased to Phenomenex (Torrance, CA, USA), and an Acquity UPLC® BEH C_{18} column (100 mm × 2.1 mm × 1.7 μm) was purchased from Waters Corp. (Milford, MA, USA).

2.1.2. Urine samples

DAPs have been determined in urine samples from 48 mother-child pairs (48 mothers and 56 children (6–12 years old)) who lived in the Greater Oslo area in 2012 and comprised the cohort of our previous studies [28,29]. The studies were approved by the Regional Committee for Medical Research Ethics, and consents were obtained from all participants.

Children's urine was collected twice a day (morning and evening, total n = 112) while mothers were encouraged to provide more samples in the course of 24 h (2–10 urine samples/participant, total n = 244). All samples were stored in the freezer at −20 °C. Specific gravity (SG) was measured in urine by a pocket refractometer PAL-10 S from Atago (Tokyo, Japan). SG spanned from 1.009 to 1.032 (mean = 1.024) in children and from 1.003 to 1.032 (mean = 1.015) in mothers. The SG corrected concentrations were obtained by applying the formula: $concentration_{SG}$ = concentration x (mean SG-1)/(SG-1) [30].

An in-house control urine sample was prepared by pooling equal volumes of urine from three donors to a final volume of 500 mL. An aliquot of 30 mL was spiked with analytes to obtain a urinary concentration of 5 ng/mL and distributed into 2 mL Eppendorf Flex Tubes®. The aliquots of the in-house control sample were stored in the freezer at −20 °C.