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RESEARCH PAPER

Determination of Seven Urinary Metabolites of Organophosphate Esters using Liquid Chromatography-Tandem Mass Spectrometry

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Abstract: A simple method was developed for simultaneous determination of seven urinary metabolites of organophosphate esters (OPs) using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Based on different physical and chemical properties of these OP metabolites, their enrichment and clean-up were performed through solid phase extraction to obtain high-efficient solid phase extraction cartridges, and the washing and elution conditions were optimized. At the same time, the kinetic parameters and mass spectrometry parameters were investigated for getting the qualitative and quantitative characteristic ion pairs for analysis of each metabolites. The results showed that the Oasis WAX solid phase extraction cartridge was suitable for sample enrichment and clean-up, and the optimal elution solvents were 2 mL of 5% ammonia in methanol and 2 mL of methanol. The recoveries of six analytes ranged from 60.5 to 104.0%, whereas the recovery of diethyl phosphate ranged from 17.8% to 36.2%. The complete baseline separations of seven analytes were achieved under optimized chromatographic conditions. The limits of detection and limits of quantification of the seven analytes ranged from 0.005 to 0.2 μg L^{-1} and 0.02 to 0.5 μg L^{-1}, respectively. The intra-day and inter-day precision results (RSD ≤ 15.4%) showed that this method had good stability and reproducibility. This method was subsequently used to determine OP metabolites in 10 urine samples from the general population in Guangzhou city. The concentrations of the seven OP metabolites in urine samples ranged from 0.5 to 6.7 μg L^{-1}.

Key Words: Liquid chromatography-tandem mass spectrometry; Organophosphate esters; Urine; Metabolites

1 Introduction

Due to the gradual prohibition of brominated flame retardants, organophosphate esters (OPs) have been widely used as substitutes, and the production and consumption of OPs have rapidly increased[1]. OPs are used as additives inflame retardants, plasticizers and anti-foaming agents in a variety of industries, including construction, textile, electronics, chemical and petroleum industry[2]. It has been reported that OPs have become a global environmental pollutant and are distributed in various environmental matrices, such as air, water, dust, soil, sediment and biota samples[3]. Toxicological studies have verified that OPs could induce neurotoxicity, carcinogenicity, teratogenicity and endocrine disrupting effects. Given the wide distribution of OPs and their potential human health effects[4,5], it is necessary to monitor human exposure levels to OPs and provide basic data for human health risk assessment.

According to the results of *in vivo* and *in vitro* studies, OPs are absorbed and rapidly metabolized into their dialkyl and diaryl phosphate analogs, and OP diesters are the major detected metabolites[6,7], which are mainly excreted through

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urine after metabolism in human body. Therefore, determination of OP diesters in human urine is an important method for assessing human exposure levels to OPs. In general, most studies have focused on the establishment and improvement of analytical methods for urinary OP metabolites, and few studies have investigated human exposure to OPs. To date, two major instrumental analytical methods have been used to determine OP diesters. One is gas chromatography coupled to tandem mass spectrometry (GC-MS/MS). Due to the polarity of OP diesters, these target compounds require derivatization. For example, Schindler *et al*[8,9] used solid phase extraction (SPE) for the initial enrichment and clean-up of urine sample, and then the target OP diesters were derivatized with pentafluorobenzylbromide, and further clean-up through SPE. The extracts were subsequently analyzed by GC-MS/MS. The pretreatment procedure of this method was complicated, and thus was unsuitable for large-scale epidemiological investigations. Another analytical method is liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Su *et al*[10] developed a highly sensitive method based on ultra-high pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) to determine OP diesters in human urine. Four non-chlorinated OP diesters were directly determined using UHPLC-electrospray (−)-MS/MS, while UHPLC-ESI (+)-MS/MS was used to determine 3 chlorinated OP diesters following methylation using diazomethane. The sensitivity for determination of OPs metabolites was significantly increased. However, due to the complicated matrix effects of urine, the derivatization reaction requires a higher level of technology to ensure its repeatability. Cequier *et al*[11] reported a high-throughput method based on ultra-performance LC coupled with time-of-flight mass spectrometry. The urine samples did not require pretreatment and were directly injected into the instrument. The entire analysis took less than 3 min; however, the method suffered from severe matrix effects and their limits of detection were high. In addition, van den Eede *et al*[12] and Cooper *et al*[13] also reported a rapid method based on SPE and LC-MS/MS for simultaneous determination of six urinary OP diesters. Urine samples were enriched by SPE using a weak mixed anion exchange sorbent and directly analyzed by LC-MS/MS. To date, these aforementioned methods satisfied the basic requirements for OP diester analysis, but had some disadvantages, such as severe matrix effects, higher limit of detection and few OP diesters were detected. Therefore, in the present study, based on the environmental investigation of OPs in China[14,15], seven OP metabolites in human urine were selected as target compounds. A simple and rapid method was established by optimization of SPE cartridges, sample clean-up and instrumental parameters.

2 Experimental

2.1 Instruments and reagents

An Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to API4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) and a Heraeus™ Labofuge™ 200 centrifuge (Thermo Fisher Scientific, Dreieich, Germany) were used in this study. A solid phase extraction device with 12 holes was obtained from Supeclo (Bethlehem, PA, USA). A Milli-Q Unique-R10 water purification system was obtained from Research Scientific Instruments Co. (Millipore, USA). Oasis WAX Extraction Cartridges (60 mg, 3 mL) were obtained from Waters company (Milford, MA, USA).

For standards used in this study, diethyl phosphate (DEP) and dibutyl phosphate (DBP) were purchased from Chem Service Inc. (West Chester, PA, USA). Diphenyl phosphate (DPhP), bis(2-chloroethyl) phosphate (BCEP), bis(1-chloro-2-propyl) phosphate (BCPP), D_{10}-DPhP, D_8-BCEP and D_{12}-BCPP were purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Bis(1,3-dichloro-2-propyl) phosphate(BDCPP) was purchased from Wellington Laboratories (Guelph, Canada). Dibenzyl phosphate (DTP) was obtained from Tokyo Chemical Industry, Japan.

Methanol (LC grade) was obtained from Merck (Darmstadt, Germany). Acetic acid (LC grade) was obtained from Tedia Company (Fairfield, OH, USA). Ammonia (28%–30%) and anhydrous sodium acetate (analytical grade) were obtained from Anpel (Shanghai, China).

2.2 Urine sample collection

All urine samples were collected between October 2014 and July 2015 from the Center for Reproductive Medicine of Nanfang Hospital, Southern Medical University, China. All participants were informed about this study and provided a signed informed consent. In this study, ten urine samples were analyzed for method validation. Urine was collected in clean polyethylene plastic bottles and stored at -80°C until analysis. In addition, fifty volunteers from our laboratory were recruited and approximately 10 mL urine was collected from each volunteer. These urine samples from volunteers were blended to form a mixed urine matrix for method optimization.

2.3 Urine sample pretreatment

Urine samples were thawed and centrifuged at 3000 r/min for 10 min. Two milliliters of supernatant were placed in a glass tube and 10 ng of surrogates (D_{10}-DPhP, D_8-BCEP and D_{12}-BCPP) were added. Urine pH was adjusted to 5.0 with 200 μL 0.1 M sodium acetate buffer. The urine was homogenized and placed in the dark for 6–8 h. Before sample extraction, the SPE column was conditioned with 2 mL of methanol, 2 mL of 5% ammonia in methanol and 3 mL of sodium acetate buffer. After urine sample was loaded, the SPE column was washed with 2 mL of 30% methanol in water (pH

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