Talanta 160 (2016) 547–555

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Retrospective analysis of pesticide metabolites in urine using liquid chromatography coupled to high-resolution mass spectrometry

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ARTICLE INFO

Article history:
Received 30 March 2016
Received in revised form
25 July 2016
Accepted 30 July 2016
Available online 31 July 2016

Keywords:
Pesticide metabolites
High resolution mass spectrometry
Urine
Retrospective analysis
Principal component analysis

ABSTRACT

A comprehensive retrospective analysis of pesticide metabolites in urine was developed, using liquid chromatography coupled to Orbitrap high-resolution mass spectrometry (UHPLC-HRMS) that includes both post-run target (suspect screening) and non-target screening. An accurate-mass database comprising 263 pesticide metabolites was built and used for the post-run screening analysis. For non-target analysis, a "fragmentation-degradation" relationship strategy was selected. The proposed methodology was applied to 49 real urine samples from pregnant women. In the post-target analysis 26 pesticide metabolites were tentatively identified, 8 of which (2-diethylamino-6-methyl-pyrimidinol; 3-keto-carbofuran; 4,6-dimethoxy-2-pyrimidinamine; 4-hydroxy-2-isoropyl-6-methylpyrimidine; diethyl malate; diethyl maleate; N-(2-Ethyl-6-methylphenyl)-2-hydroxyacetamide and propachlor oxanilic acid) were confirmed using analytical standards. Likewise, one unknown degradation product, methyl-N-phenylcarbamate was elucidated in the non-target screening. Finally, the real urine samples were grouped according to their origin applying a metabolomic approach.

1. Introduction

The use of pesticides has increased crop yield and reduced insect-borne diseases around the world. After banning or restricting the use of environmental persistent pesticides, non-persistent pesticides, such as organophosphates (OPs), N-methyl carbamates and pyrethroids, have been increasingly used. Nevertheless, the intensive use of these compounds may impact public health, especially of vulnerable populations such as pregnant women and children living in agricultural communities [1]. There is evidence of carcinogenic, neurological, reproductive, immunological and genotoxic effects associated with the exposure to non-persistent pesticides in adults [2]. In addition, several studies have found a greater risk of adverse reproductive effects [3] and delayed or deranged neurobehavioral development in children [4,5]. Biomonitoring is the preferred approach to assess human internal exposure to environmental pollutants. However, the number of studies in pesticide exposure is limited and most of them do not include a wide range of biomarkers. The European Union (EU) is currently promoting HBM (human biomonitoring) across Europe [6]. Their aim is to integrate biomonitoring studies and environmental and health monitoring programmes, to assess human exposure to chemicals in different population groups.

For assessing internal exposure to non-persistent pesticides that have a short-life in the human body, urine is the matrix of choice. Pesticide metabolites in urine are representative of recent exposure because non-persistent pesticides are rapidly metabolized and eliminated in a few days [7].

Conventional targeted analysis of pesticide metabolites in urine using LC-MS/MS is based on establishing a method to determine a list of known analytes, which requires the use of reference standards and a purposeful chromatographic method development [8,9]. As an alternative, LC-HRMS offers the possibility of detecting hundreds of polar contaminants in a quantitative target approach due to its sensitivity and selectivity in full-scan analysis, combined with high-resolving power (> 50,000 FWHM) and accurate mass measurement (1–5 ppm) [10]. Furthermore, it allows the post-run detection of compounds suspected of being present in environmental samples (suspect screening) without reference standards, and the detection of unknown compounds [11,12].

Despite these advantages, to our knowledge only few works

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http://dx.doi.org/10.1016/j.talanta.2016.07.065
0039-9140/

have been published using UHPLC-HRMS for biomonitoring of pesticide metabolites. Cortéjade et al., 2016 [13] developed an analytical method for the targeted screening and multi-residue quantification of 38 environmental contaminants in urine, including 12 pesticides, a single pesticide metabolite (tributyl phosphate) and other compounds of different families. Roca et al., 2014 [14] developed an analytical strategy for biomonitoring of pesticides in urine that included a target analysis of 29 metabolites of pesticides (OPs, pyrethroids, herbicides and carbamates) with LOQs ranging from 0.8 to 3.2 $\mu g\,L^{-1}$, and a reduced post-target screening of different types of metabolites (60 compounds) such as pesticides (12), PAHs (12), phenols (14) and other environmental pollutants. A more in-depth study of the post-target screening for pesticide metabolites is necessary, which should combine target and post-target LC-HRMS strategies. This methodology could be used as a good analytical tool for HBM studies.

In the present study we have developed a comprehensive analytical strategy for pesticide metabolites in urine that combines a previously developed quantitative target analysis, with a massive retrospective screening of pesticide metabolites, using a database containing 263 compounds (Table SI-1) (suspect screening), including metabolites of organophosphates (63), chloroacetamides (36), carbamates (20), triazines (15), triazoles (15), sulfonylureas (11) and other chemical pesticide classes. The method uses a generic extraction based on QuEChERS and UHPLC-HRMS. The analytical methodology was applied to urine samples from pregnant women participating in a study to describe prenatal exposure to pesticides. To our knowledge, there has been no work reported previously on the analysis of pesticide metabolites using a comprehensive database for pesticide metabolites in post-target analysis, and on the use of the non-target analysis.

2. Experimental

2.1. Reagents and chemicals

All solvents used were specific for pesticide residue analysis and of analytical grade. Acetonitrile and methanol were supplied by Scharlab (Barcelona, Spain). Acetic acid (purity 98–100%), β-glucoronidase aryl sulfatase enzyme, and anyhydrous sodium acetate were obtained from Merck (KGaA, Darmstadt, Germany). Deionized water was organically and biologically purified by using a Mili-Q Ultrapure System (Milipore, Darmstadt, Germany). QuEChERS EN extraction kits, containing 4 g $MgSO_4$; 1 g NaCl, 1 g NaCitrate; 0.5 g disodium citrate sesquihydrate, were obtained from Agilent Technologies (Madrid, Spain).

2.2. Standard and stock solution

Certified commercial standards were of high purity (ranging between 75–100%) and purchased from Dr Ehrenstorfer (Augsburg, Germany), Sigma-Aldrich (Barcelona, Spain), Cerilliant-Certificated Reference Materials (Texas, USA), and Cambridge Isotope Laboratories (Massachussetts, USA). Table SI-2 shows the pesticide metabolites and internal standards (IS) used in the target analysis. Stock standard solutions of individual compounds (with concentrations between 20 and 500 $mg\,L^{-1}$) were prepared in acetonitrile by weighing powder solutions and stored at −20 °C. Multi-analyte intermediate standard solutions were prepared by diluting the individual stock solutions with acetonitrile and used for preparing working mixed-standards solutions in acetonitrile: water (10:90 v/v). The concentration of the analytes in working solutions ranged from 1000 to 5000 $ng\,mL^{-1}$ depending on the compound. A working solution of 1000 $ng\,mL^{-1}$ was also prepared containing internal standards.

2.3. Study population

The proposed analytical strategy was applied to 49 real urine samples from pregnant women from two regions of Spain: Sabadell (n=30) and Valencia (n=10), and various regions of Slovakia (n=9). Study subjects were participants in the DENAMIC project, "Developmental Neurotoxicity Assessment of Mixtures in Children" (http://www.denamic-project.eu/). Women participating in the study signed an informed consent form in each phase and the research protocol was approved by the Ethics Committee involved in the study.

2.4. Sample preparation

The sample preparation was performed following a previously developed method [14]. In short, after the homogenization of the whole sample, 5 mL of urine were mixed into a 15 mL tube with 1 mL of 0.2 M acetate buffer (3.1 mL of glacial acetic acid and 9.7 g of sodium acetate diluted in 1 L of deionized water), 20 μL of β-glucuronidase aryl sulfatase enzyme (to hydrolyze possible glucoronide-or sulfatase-conjugated metabolites), and the internal standard solution. The samples were incubated overnight at 37 °C (10–17 h).

After the enzymatic hydrolysis, a simplified QuEChERS procedure was employed to extract metabolites from urine samples. Briefly, 10 mL of acetonitrile, and a pouch of QuEChERS extraction salt packet were added to the hydrolysed sample in a 50 mL polypropylene tube. The mixture was strongly shaken and centrifuged during 10 min at 3500 rpm. The acetonitrile layer obtained was immediately transferred into a 15 mL tube and evaporated to dryness in a water bath at 37 °C under a stream of nitrogen. The dry residue was then dissolved in 200 μL of methanol: water (10/90, v/v) containing 0.1% of acetic acid, placed into a Milipore 0.2 μm Eppendorf and ultra-centrifuged (11,000 rpm, 3 min and 10° C). The final extract was transferred into an injection vial and analysed with the UHPLC-HRMS system.

2.5. UHPLC-HRMS Orbitrap analysis

Chromatographic separation was performed on an Accela liquid chromatography UHPLC system equipped with a Hypersil Gold aQ column (100 mm × 2.1 mm, 1.9 μm) both from ThermoFisher Scientific (Bremen, Germany). The flow rate used was 400 $\mu L\,min^{-1}$ and the injection volume was 10 μL. Separations were performed using a binary gradient. The analysis started with 95% H_2O with 0.1% acetic acid (solvent A). After 1 min, this percentage was linearly decreased down to 45% within 5 min. After that, solvent A was decreased quickly to 0% in 0.5 min and maintained for 1.5 min. The composition was increased to initial conditions in 0.5 min, followed by a re-equilibration time of 12.5 min. The total run time was 20 min

Data acquisition was performed on the Orbitrap mass spectrometer Exactive™ analyzer (Thermo Scientific, Bremen, Germany). The system was equipped with a heat electrospray ionization interface (HESI-II). The ion source parameters were as follow: 3.5 kV (positive mode) and 2.5 kV (negative mode); sheath gas flow-rate: 55; auxiliary gas flow-rate: 10; skimmer voltage: 23 V; heater temperature: 300 °C; capillary temperature: 150 °C; capillary voltage: 45 V and tube lens voltage: 120 V. In this study two independent runs were carried out using electrospray ionization in positive (ESI +) and negative (ESI -) mode with HCD (Higher collision-induced dissociation cell) switching for the fragmentation of ions in a non-selective manner operating with N_2 (> 95%) and employing a collision energy of 20 eV.

The system operating in full-scan mode (50–800 *m/z*) at a resolving power of 25,000 FWHM (scan time=250 ms). No-specific

Please cite this article as: A. López, et al., Talanta (2016), http://dx.doi.org/10.1016/j.talanta.2016.07.065