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A multiclass method for the analysis of endocrine disrupting chemicals in human urine samples. Sample treatment by dispersive liquid–liquid microextraction

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ABSTRACT

The population is continuously exposed to endocrine disrupting chemicals (EDCs). This has influenced an increase in diseases and syndromes that are more frequent nowadays. Therefore, it is necessary to develop new analytical procedures to evaluate the exposure with the ultimate objective of establishing, in an accurate way, relationships between EDCs and harmful health effects. In the present work, a new method based on a sample treatment by dispersive liquid–liquid microextraction (DLLME) for the extraction of six parabens (methyl-, ethyl-, isopropyl-, propyl-, isobutyl and butylparaben), six benzophenones (benzophenone-1, benzophenone-2, benzophenone-3, benzophenone-6, benzophenone-8 and 4-hydroxybenzophenone) and two bisphenols (bisphenol A and bisphenol S) in human urine samples, followed by gas chromatography-tandem mass spectrometry (GC–MS/MS) analysis is proposed. An enzymatic treatment allows determining the total content of the target EDCs. The extraction parameters were accurately optimized using multivariate optimization strategies. Ethylparaben ring-$^{13}C_6$ and bisphenol A-d_{16} were used as surrogates. Found limits of quantification ranging from 0.2 to 0.5 ng mL^{-1} and inter-day variability (evaluated as relative standard deviation) ranging from 2.0% to 14.9%. The method was validated using matrix-matched standard calibration followed by a recovery assay with spiked samples. Recovery rates ranged from 94% to 105%. A good linearity, for concentrations up to 300 ng mL^{-1} for parabens and 40 ng mL^{-1} for benzophenones and bisphenols, respectively, was obtained. The method was satisfactorily applied for the determination of target compounds in human urine samples from 20 randomly selected individuals.

1. Introduction

In the last century, as a consequence of the huge industrial development, wildlife and humans are exposed to synthetic chemicals that can interfere with the normal functioning of the endocrine system. These compounds, commonly called endocrine disrupting chemicals (EDCs), are present in many types of products, such as personal care products (PCPs), pharmaceuticals, sunscreens, foodstuffs, beverage cans, *etc.* Nowadays, a large amount of research groups are working to demonstrate the relationship between human exposure to EDCs and some diseases such as hypospadias, cryptorchidism, testicular cancer, loss in semen quality, breast cancer, many uterine and ovarian diseases and many anomalies in the age of puberty [1–6]. These evidences imply greater efforts to assess human exposure to EDCs.

There are a lot of families of compounds that are able to induce an endocrine disrupting response in the human organism. In the present study, bisphenol A (BPA) and bisphenol S (BPS), parabens (PBs) and benzophenones (BPs), have been selected as the target EDCs, due to their widespread presence in very common products and stuffs that are continuously consumed by population.

BPA is one of the most representative compounds of the EDCs group. It is a highly reactive compound used as the raw material in a large amount of manufactured products, such as polycarbonate plastics, epoxy resins used to line metal cans, and in many plastic consumer products including toys, water pipes, drinking containers, eyeglass lenses, sports safety equipment, dental monomers, medical equipment and tubing and consumer electronics [7]. BPA is one of the highest volume chemicals produced worldwide, with an estimated production of 5.5 million tons [8]. Although humans are frequently exposed to BPA through multiple sources, the diet is considered as the major source of exposure [9].

Another important family of EDCs is parabens. Because of their low toxicity and cost, their inert nature and worldwide regulatory

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acceptance, parabens are widely used in PCPs, pharmaceuticals and food or beverages [10,11]. Individually or in combination, parabens are used in over 13,200 formulations in nearly all types of cosmetics, being the most commonly used methyl and propylparaben [11]. Exposure to parabens may occur through inhalation, dermal contact or ingestion [10,12].

The third group of EDCs considered in this work is benzophenones. There are 12 well-described BPs, namely benzophenone-1 (BP-1) to benzophenone-12 (BP-12), as well as other less usual compounds as 2-hydroxybenzophenone (2-OH-BP) or 4-hydroxybenzophenone (4-OH-BP). In cosmetics and PCPs, BP-1 and BP-3 are usually used in the formulation of nail polishes and enamels. These BPs are also used in the manufacturing of bath, makeup, hair or skin care products and sunscreens [13]. These compounds protect cosmetics and PCPs from damage by absorbing, reflecting, or scattering UV rays. When they are used as sunscreen ingredients, BP-3 and BP-4 protect the skin from UV rays. Human exposures to BPs can be through the skin and ingest, being the most important the dermal route [14].

Disrupting abilities of BPA, PBs and BPs have been demonstrated in many *in vitro* and *in vivo* studies. These compounds are able to induce the proliferation of MCF-7 cancerous cells, demonstrating a clear estrogenic character [15–19]. Animal exposure modeling has proved that these chemicals produce an abnormal sexual development, erratic behaviors and carcinogenesis in adult animals [20–22], and the offspring of exposed individuals can also suffer endocrine disorders during fetal and early post-natal development, therefore permanent adverse effects can be caused [23,24]. In fact, this phenomenon could be present in human population, as it is suggested in several epidemiologic studies where it is shown a negative correlation between prenatal EDCs exposure and measures of cognitive skills in childhood [6,25,26].

Although there are some differences about EDCs biotransformation depending on exposure via and specific chemical structure characteristics, animal and human organisms are able to transform BPA, PBs and BPS in β-D-glucuronide derivatives easily excreted through the urine because of their high water solubility [14,27–33]. However, free forms of EDCs can accumulate in certain human tissues due to their lipophilic nature producing harmful disrupting effects and passing to the offspring. Several analytical studies have demonstrated that these chemicals are present in human placental tissue [34–36], as well as in human milk [37–39].

Due to the complexity of biological matrices, new methods for sample treatment are needed to ensure good results in exposure analysis. These new procedures have to be simple and fast, and provide enough sensitivity to detect very low quantities of EDCs. The use of the highly-potential microextraction techniques as the dispersive liquid–liquid microextraction (DLLME), developed by Rezaee and co-workers in 2006 [40], have provided good results in complex samples. The fundament of the DLLME has been explained elsewhere, as well as the advantages over the traditional extraction techniques and other microextraction techniques [40]. DLLME has been widely used in the analysis of many types of pollutants and organic compounds in environmental matrices, in food samples and in biological human samples [41,42]. However, DLLME has hardly been used in analysis of EDCs in human samples. Recently, some methods for the determination of BPs and PBs in human urine and serum samples by DLLME–LC–MS/MS or DLLME–LC–DAD have been proposed [43–46].

The aim of the present work is to develop a selective and sensitive DLLME followed by the GC–MS/MS analytical method for the simultaneous determination of six parabens, six benzophenones and two bisphenols in human urine samples. The proposed method was validated and satisfactorily applied to determine the EDCs content (free and total) in samples collected from 20 unknown volunteers.

2. Experimental

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise specified. Water (18.2 MΩ cm) was purified using a Milli-Q system from Millipore (Bedford, MA, USA). Methylparaben (MP), ethylparaben (EP), isopropylparaben (IsPP), propylparaben (PP), isobutylparaben (IsBP) and butylparaben (BP) were supplied by Alfa Aesar (Massachusetts, MA, USA). Bisphenol A (BPA), bisphenol S (BPS), labeled deuterium bisphenol A (BPA-d_{16}), benzophenone-1 (BP-1), benzophenone-2 (BP-2), benzophenone-3 (BP-3), benzophenone-6 (BP-6), benzophenone-8 (BP-8), 4-hydroxybenzophenone (4-OH-BP) and ethylparaben ring $^{13}C_6$ labeled (EP-$^{13}C_6$) were supplied by Sigma-Aldrich (Madrid, Spain). Stock standard solutions (100 mg L^{-1}) for each compound were prepared in methanol and stored at 4 °C in the dark. These solutions were stable for at least four months. Working standards were prepared just before use, diluted with methanol.

The 4-methylumbelliferyl glucuronide, 4-methylumbelliferyl sulfate and β-glucuronidase/sulfatase (*Helix pomatia*, H1) were purchased from Sigma-Aldrich. $^{13}C_4$-4-methylumbelliferone was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). A mixture of $^{13}C_4$-4-methylumbelliferone, 4-methylumbelliferyl sulfate, and 4-methylumbelliferyl glucuronide was prepared in water and stored at 4 °C until use. The enzyme was prepared daily by dissolving 10 mg of β-glucuronidase/sulfatase (3×10^6 U g $solid^{-1}$) in 1.5 mL of 1 M ammonium acetate/acetic acid buffer solution (pH 5.0).

Methanol, ethanol, acetone, ethyl acetate and acetonitrile (HPLC-grade) were purchased from Merck (Darmstadt, Germany). Sodium chloride and ammonium acetate were supplied by Panreac (Barcelona, Spain). Chlorobenzene (ClBz), tricloromethane (TCM), carbon tetrachloride (TCC), potassium chloride, creatinine, sodium sulfate, hippuric acid, ammonium chloride, citric acid, magnesium sulfate, sodium phosphate monobasic monohydrate, calcium chloride dihydrate, oxalic acid, lactic acid, glucose, sodium metasilicate nonahydrate, pepsin and N,O-Bis(trimethylsilyl)trifluoro-acetamide with trimethylchlorosilane (BSTFA/1% TMCS) were purchased from Sigma-Aldrich.

2.2. Instrumentation and software

GC–MS/MS analysis was performed using an Agilent 7890 GC (Agilent Technologies, Palo Alto, *CA*, USA) equipped with a split-splitless inlet and a 7693 ALS autosampler. The detector was an Agilent 7000B triple quadrupole mass spectrometer with inert electron-impact ion source. The mass spectrometer worked in the SRM mode. Electron impact (EI) ionization at −70 eV was used. Agilent MassHunter B.03.02 software package was used for control and data analysis. Helium (99.9999% purity) was used as carrier gas and quench gas (a gas employed in the Agilent 7000 mass spectrometer), and nitrogen (99.999% purity) was used as collision gas; both gases were supplied by Air Liquide España S.L. (Madrid, Spain).

All pH measurements were carried out with a Crison (Crison Instruments S.A., Barcelona, Spain) combined glass-Ag/AgCl (KCl 3 M) electrode using a previously calibrated Crison 2000 digital pH-meter. A MS-100 thermo shaker (Optimum Ivymen System, Cornecta, Spain) was used to make the enzymatic treatment.

Statgraphics Plus version 5.0 software package (Manugistics Inc., Rockville, MD, USA, 2000) was used for statistical and regression analyses (linear mode).

2.3. Sample collection and storage

Human urine samples were collected from 20 volunteers (10 male and 10 female). Samples were anonymized, frozen at −86 °C