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Isotope-dilution gas chromatography-mass spectrometry coupled with injection-port butylation for the determination of 4-*t*-octylphenol, 4-nonylphenols and bisphenol A in human urine

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ABSTRACT

An analytical method that utilizes isotope-dilution gas chromatography-mass spectrometry (ID-GC–MS) coupled with injection-port butylation was developed. The method was validated, and confirmed to be able to determine the presence of three commonly detected endocrine-disrupting chemicals (EDCs: 4-*tert*-octylphenol (4-*t*-OP), 4-nonylphenols (4-NPs) and bisphenol A (BPA)) in human urine with high precision and accuracy. After sample preparation by solid-phase extraction, the extract was introduced into GC–MS via injection-port butylation. The butylated target analytes were identified and quantified by using ion-trap mass spectrometry operating in the selected-ion-storage mode, and employing the measurement of peak area ratios of the butylated target analytes and labeled-analogues in the samples and calibration standards. The labeled-analogues were also used to correct the variations associated with the analysis and matrix effect. The limits of quantitation (LOQs) ranged from 0.1 to 0.3 ng/mL. High precisions for both intra- and inter-day analysis ranged from 1 to 6%, and excellent accuracy (mean recovery) ranged from 92 to 105% on two concentration levels. In human urine, the total concentrations of three selected EDCs varied from 1.28 to 7.14 ng/mL. 4-NPs were detected within all collected samples. The developed method allows accurate analysis of trace-level of EDCs in urine, and these target EDCs could act as useful biomarkers to assess exposure in biomonitoring studies and programs.

1. Introduction

Nowadays, we are exposed to a variety of endocrine disrupting chemicals (EDCs) through industrial pollution, the use of household products, and the ingestion of food. 4-*tert*-octylphenol (4-*t*-OP), 4-nonylphenols (4-NPs), and bisphenol A (BPA) are three well-known EDCs, which have been widely used in both industries and common household application. In particular, these three EDCs are used as surfactants, in the manufacturing of plastics (as plasticizers), in the production of textiles and, in paper and agricultural chemical products [1]. Many studies have observed that they may disrupt the endocrine system and increase the risk of breast cancer in women [2,3]. Moreover, the physical and chemical behaviors of these EDCs are similar to some hydrophobic and semi-volatile organic pollutants, and have been found to accumulate in a variety of aquatic organisms and seafood within the food chain [4–8], as well as have also been found in airborne particles and indoor air [9,10]. These three EDCs have been detected in human serum, blood, breast milk, hair and urine, indicating human exposure to them through inhalation, direct absorption during dermal contact, or ingestion of food [11–16]. The rise in public concern and interest in investigating the adverse effects from long-term exposure to EDCs spurred us on to make an accurate and reliable method for their detection in human urine samples. This type of high matrix containing sample poses specific challenges for quantitation of target analytes [17]. A valuable method that can be used is isotope dilution mass spectrometric technique (IDMS), since it produces high precision, good accuracy, and definable values. As of now, combination of IDMS with GC or HPLC has been used for the determination of organic chemicals in vegetable oils, infant formula, human urine, and plasma [18–23]. The basic principle of IDMS is that a known amount of labeled-analogue for each target analyte is spiked to the sample and the ratio of the target natural analyte and labeled-analogue is measured using a mass spectrometer. The boon of IDMS is that the concentration of the target analyte can be obtained with high accuracy and is relatively less susceptible to interference than other MS techniques. Moreover, the advantage is that the loss of target analyte during the whole analytical procedure does not influence the analytical

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result, thus making IDMS the best form of internal standardization for quantitation of target analytes in complex matrix.

As a continuation of our work to analyze the presence of EDCs in human specimen, and what their presences might mean for human health, these three EDCs were selected in the development of the method based on SPE coupled with isotope-dilution GC–MS plus njection-port butylation. Moreover, the developed method's precision and trueness were examined, and its applicability in the detection of low to trace-levels of target 4-t-OP, 4-NPs, and BPA in the human urine samples was also demonstrated.

2. Experimental

2.1. Chemicals and reagents

Standards: 4-NPs (tech. grade), 4-*t*-OP (purity ≥97%) and BPA (purity ≥ 99%) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Standard stock solutions of each analyte (1000 μg/mL) were prepared in methanol and kept at −4 °C. A mixed standard solution was prepared at concentration of 50 μg/mL by the dilution with methanol for each analyte, which was used for the preparation of working solutions and calibration curves by further diluted with methanol. Isotope-labeled standards: 4-*n*-nonylphenol ring-$^{13}C_6$ (4-*n*-NP- ring-$^{13}C_6$, 100 mg/mL), 4-*t*-octylphenol- ring-$^{13}C_6$ (4-*t*-OP- ring-$^{13}C_6$, 10 mg/mL), and bisphenol A-d_{16} (BPA-d_{16}, 100 mg/mL) were also purchased from Sigma-Aldrich. All other solvents and chemicals were supplied by Merck (Darmstadt, Germany), Sigma-Aldrich, and Mallinckrodt Baker (Phillipsburg, NJ, USA) in high purity grade, and were used without further purification. *E. coli* β-glucuronidase (986400 units/g solid) and *H. pomatia* sulfatase (20230 units/g solid) were purchased from Sigma-Aldrich. Prior to use, the β-glucuronidase was added to 0.1 M ammonium acetate to make a total concentration of 10,000 units/mL. Milli-Q water was produced by Millipore Elix® 10 RO system plus Millipore Synergy® UV system (Millipore SAS, Molsheim, France).

2.2. Sample collection and SPE procedure

The collection of human urine used as samples came from 21 healthy female volunteers within our department, their ages ranging from 20 to 29 years old. Prior to their utilization, all samples were stored at −20 °C.

The sample preparation process was as follows: one mL of an aliquot of urine was pipetted into a 2.0 mL conical tube, then mixed with 100 μL of 1.0 M ammonium acetate (pH 6.8) and 10 μL of enzyme solution (containing 10000 units/mL of â-glucuronidase and 2500 units/mL of sulfatase). The mixture was gently mixed and incubated for 2 h at 37 °C. After enzymatic de-conjugation, the SPE procedure was performed with Bond Elut pH cartridge (50 mg, 1.0 mL, Agilent, Santa Clara, CA, USA) and operated on a VacMaster vacuum extraction device (IT Sorbent Technology, Cambridge, UK). Before extraction, the cartridge was conditioned using 2 mL of methanol and rinsed by 2 mL of deionized water. Then, 1.0 mL of de-conjugation urine sample spiked with isotope-labeled standard mixture (final concentration 10 ng/mL for each labeled-analogue) was loaded through the cartridge at a rate of about 0.5 mL/min. The cartridge was washed with 2.0 mL of 10% methanolic solution, and subsequently air-dried under vacuum for at least 5 min. All target analytes were then eluted by 1.0 mL of dichloromethane/isopropanol (3:7, v/v) solution. The extract was completely evaporated to dryness under a stream of nitrogen. The residue was then re-dissolved in a solution containing 25 μL of methanol plus 25 μL of 20 mM tetrabutylammonium hydroxide (TBA-OH, as a butylating agent), and subjected to injection-port butylation GC–MS analysis.

2.3. Injection-port butylation GC–MS analysis

The injection-port butylation GC–MS analysis was performed using a Varian 450 GC connected to a Varian 220 ion-trap mass spectrometer (Walnut Creek, CA, USA) operating in the selected ion storage (SIS) mode under electron ionization for quantitation. A ChromatoProbe (Varian) and a temperature-programmed injector were used to introduce 10 μL of sample, as described elsewhere [24]. Temperature of the injection-port was maintained at 100 °C for 1.0 min for the reaction of butylation and solvent vaporization. After the vaporization, the temperature was quickly elevated to 250 °C (the temperature ramping rate at 200 °C/min) to allow the derivatives to be introduced into the GC analytical column. Since the ChromatoProbe device with a disposable micro-vial was used in this study, neither retention effect of the reagent nor thermally degraded components from the sample matrix within the injection-port was detected [24]. Separation was carried out in a DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Agilent). GC temperature program was set as follows: 100 °C holding for 5 min; a temperature ramp of 10 °C/min up to 200 °C; and another temperature ramp of 15 °C/min up to 300 °C (held for 3.3 min), totaling to 25 min. The temperature of the transfer line was set at 280 °C. Full-scan EI spectra were acquired under the following conditions: scan time 1 s, mass range 100–500 *m/z,* ion trap temperature 160 °C, solvent delay 14 min, an automatic gain control target set at 20,000, and emission current 20 μA (at 70 eV electron energy).

2.4. Method evaluation

The above mentioned method, after development, was evaluated using spiked deionized water and urine sample #7. The standard solution was spiked with both deionized water and urine samples (final concentration 10 ng/mL for each target analyte). The limits of detection (LODs) and limits of quantitation (LOQs), defined as signal/noise (S/N) ratios of 3 and 10, respectively, were determined through SPE coupled with injection-port butylation GC–MS procedures of the spiked real samples, as described in previous reports [25]. Precision and trueness were evaluated by intra- and inter-day analyses of the spiked urine samples. The value of intra-day precision was found by analyzing five urine samples spiked with the standard mixture on the same day ($n = 5$), while the value of inter-day precision was found on five consecutive days ($n = 5$). Trueness was calculated as the mean recovery of these spiked samples.

3. Results and discussion

3.1. Mass spectra of butylated target analytes

Firstly, suitable GC separation and stable mass spectra of butylated 4-*t*-OP, 4-*t*-OP- ring-$^{13}C_6$, 4-NPs, 4-*n*-NP- ring-$^{13}C_6$, as well as dibutylated BPA and BPA-d_{16} were obtained to provide the optimal detection sensitivity and specificity. Fig. 1 displays the SIS chromatogram of butylated target analytes with their corresponding labeled-analogues, and the mass spectra of butylated (a) 4-t-OP, (b) 4-NPs, and (c) dibutylated BPA detected in a spiked urine sample #7. No molecular ions were observed in these butylated target analytes under electron-impact ionization. The base peak was observed at $[M–71]^+$ for butylated alkylphenols (including 4-*t*-OP- ring-$^{13}C_6$), such as *m/z* 191 for 4-*t*-OP, *m/z* 197 for 4-*t*-OP- ring-$^{13}C_6$, and *m/z* 205 for 4-NPs, which can be attributed to the losses of C_5H_{11} in the alkyl moiety from the molecular ion. Since 4-NPs containing a mixture of branched nonyl groups, intensive characteristic ions at $[M–71–(CH_2)_n]^+$ were also observed, assigned to the loss of