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Determination of Di-(2-ethylhexyl)phthalate (DEHP) metabolites in human hair using liquid chromatography–tandem mass spectrometry

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ABSTRACT

Background: Di-(2-ethylhexyl)phthalate (DEHP) is an endocrine disrupting chemical that is widely used as the major plasticizer for worldwide plastic products. It can cause several toxic effects to human with high dose exposure. In response to the need of human exposure assessment, different biological specimens are taken into account. Compared to blood, urine and other specimens, hair is unique in that it could determine the time period of chemical exposure after several months to years.
Method: The developed method consists of solution incubation, liquid–liquid extraction and stable isotope dilution liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis.
Results: A reliable and sensitive analytical method was developed and validated for the determination of 5 metabolites, mono-(2-ethylhexyl)phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP), mono-(2-ethyl-5-oxy-hexyl)phthalate (MEOHP), mono-(2-ethyl-5-carboxypentyl)phthalate (5cx-MEPP) and mono-[2-(carboxymethyl)hexyl]phthalate (2cx-MMHP) in human hair. Ten authentic hair specimens were successfully determined and quantitated by the developed method.
Conclusion: The developed LC–MS/MS method can successfully determine specific DEHP metabolites in human hair and has a great potential to assess the long term DEHP exposure of human.

1. Introduction

Di-(2-ethylhexyl)phthalate (DEHP) is one of the phthalate plasticizers, and it is widely used for polyvinyl chloride (PVC) product formulation [1]. However, DEHP is a reproductive and developmental toxicant in animals [2] and revealed that it possibly plays a role of endocrine modulator to alter sex steroid hormone levels in humans [3–8]. In May 2011, a great event in Taiwan about the illegal use of the DEHP in clouding agents as additives in beverages and foods for the main purpose of cutting costreceived worldwide attention [9,10]. Therefore, the evaluation of human DEHP exposure is a critical task.

The DEHP metabolites were often used for the monitoring of DEHP to avoid the possible contamination form environmental phthalate. The metabolism of DEHP after exposure in human bodyis rapidly metabolized to the first monoester metabolite, mono-(2-ethylhexyl) phthalate (MEHP) which can be further metabolized through different modification like hydroxylation and oxidation on side-chain to produce mono-(2-ethyl-5-carboxypentyl)phthalate (5cx-MEPP), mono-[2-(carboxymethyl)- hexyl]phthalate (2cx-MMHP) mono-(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP), and mono-(2-ethyl-5-oxy-hexyl) phthalate (MEOHP), then excreted through the urine [11,12]. These 5 compounds are the major metabolites of DEHP and can truly respond the level of DEHP exposure in human [13].

Although blood [14–16] and urine [15,17–22] are the general choice of biological matrixes to assess the level of DEHP exposure in human, both of them only have the short period of detectability after exposure. Hair is an alternative biological specimen that can provide many unique advantages [23,24]. The most significant advantage of hair specimen is longer detection window (usually month to years), enabling retrospective estimation of chronic and past exposure. In addition, it can be used for the segmental hair analysis to investigate the time periods of interesting toxicant exposure. However, as far as we know, there is no report about estimating the DEHP exposure in human hair.

Here, a stable isotope dilution high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method was developed to assess the human DEHP exposure in hair by monitoring the level of MEHP, MEHHP, MEOHP, 5cx-MEPP and 2cx-MMHP. In particular, the authentic hair specimens were determined for proving the practicability of assessing DEHP exposure in human hair.

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2. Experimental

2.1. Chemicals, materials and standards

MEHP, MEHHP, MEOHP, 5cx-MEPP, 2cx-MMHP and their relative isotope labeled internal standards (IS) were purchased from Cambridge Isotope Laboratories, Inc (Andover, MA). All of the reagents such as methanol, acetone, dichloromethane, glacial acid, hydrochloric acid (HCl), and ethyl acetate (EA) were of HPLC or analytical grade and were purchased from J.T.Baker (Phillipsburg, NJ). Phosphate and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO). All of the experimental equipment were washed carefully with acetone and hexane, and then heated at 200 °C for 1 h to reduce possible contamination from phthalates.

2.2. HPLC–MS/MS analysis

The separation of LC was performed with an Agilent 1100 LC system (Santa Clara, CA). The gradient chromatography was carried out using a separate RP-HPLC under the following conditions: analytical column, LUNA C18 column packed with 3 μm particles (50 mm × 2.0 mm) was obtained from Phenomenex (Torrance, CA, USA); column oven, 35 °C; gradient elution was performed with mobile phase A (deionized water with 0.1% formic acid) and mobile phase B (methanol with 0.1% formic acid) at a flow rate of 0.25 ml/min. The gradient program was 0–1.0 min containing 30% B; 1.0–9.0 min, from 30 to 90% B; 9.0–10.0 min containing 90% B; after 10.0 min from 90 to 30% B in 0.5 min and re-equilibrated at 30% B for 3.5 min. The determination of tandem MS using API 3000 triple quadruple mass spectrometer was equipped with a Turbo IonSpray interface operated in negative electrospray ionization (ESI) mode purchased from AB SCIEX (Framingham, MA, USA). All quantitative and qualitative analyses were performed in the multiple reaction monitoring (MRM) scan. Ionization parameter was operated under the following condition: nebulizer gas, curtain gas and collision gas were 10, 8 and 10 arbitrary units, respectively. The heater gas flow and ionspray voltage were set at 7 L/min and −4500 V, temperature was maintained at 400 °C. In order to get optimal ion transmission conditions and ion signals were ramped by a semi-automated ramp procedure to maximize the signal. The conditions varying for particular MRM transition are summarized in Table 1. Data handling and system operations were controlled by the Analyst software 1.4.1.

Table 1
The optimized parameters of LC–MS/MS for 5 metabolites from DEHP.

Analytes	MRM transitions		Optimized parameters				
	Precursor (*m*/*z*)	Product (*m*/*z*)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
MEHP-$^{13}C_4$	280.8	137.0	−40	−165	−10	−23	−7
MEHP	277.0	134.0	−40	−155	−10	−23	−7
		127.2	−40	−165	−10	−25	−6
MEHHP-$^{13}C_4$	297.0	124.2	−40	−165	−10	−28	−6
MEHHP	293.0	121.1	−40	−155	−10	−27	−5
		145.2	−40	−165	−10	−21	−7
MEOHP-$^{13}C_4$	295.0	124.2	−40	−165	−10	−26	−5
MEOHP	291.0	121.2	−40	−155	−10	−26	−5
		143.2	−40	−155	−10	−20	−6
5cx-MEPP-$^{13}C_4$	310.9	159.1	−40	−180	−10	−16	−8
5cx-MEPP	307.0	159.1	−40	−180	−10	−16	−8
		113.2	−40	−180	−10	−40	−5
2cx-MMHP	307.0	159.1	−40	−180	−10	−16	−8
		113.3	−40	−180	−10	−40	−5

Underlined mass delineates MRM quantification transition. MRM—multiple reaction monitoring, DP—declustering potential, FP—focusing potential, EP—entrance potential, CE—collision energy, CXP—collision exit potential.

2.3. Hair

2.3.1. Sample pretreatment

Authentic hair specimens were collected at the posterior vertex of the head from ten individuals. The blank hair was obtained from fifteen laboratory volunteers then mixed together as pooled blank hair. All of hair was decontaminated with 2 ml dichloromethane. After that, hair samples were generally cut into small pieces, and then weighted 25 mg placed into glass tube. The MEHP, MEHHP, MEOHP, 5cx-MEPP and 2cx-MMHP standard-spiked hair samples were prepared for method development and quality control with low (Q_{low}) and high (Q_{high}) concentration at 10 (25 μl of 10 pg/μl) and 80 pg/mg (20 μl of 100 pg/μl), respectively. All of calibrators, pooled blank hair and authentic hair specimens that were all contained 20 pg/mg (50 μl of 10 pg/μl) of $^{13}C_4$-MEHP, $^{13}C_4$-MEHHP, $^{13}C_4$-MEOHP and $^{13}C_4$-5cx-MEPP. No analytical isotope labeled IS was commercially available for 2cx-MMHP, so further quantification of this metabolite was performed by structural isomer of $^{13}C_4$ 5cx-MEPP.

2.3.2. Extraction efficiency in different incubation media

The extraction efficiency of 5 metabolites of DEHP from hair to incubation media was estimated by pooled authentic hair specimens. Eleven different incubation media that include 0.1 mol/l HCl, 0.01 mol/l HCl, 0.1 mol/l phosphate buffer (PB) solution at pH ranges from 5 to 8, methanol and methanol/TFA (8.5:1.5, v/v) mixture were investigated. All the pooled hair samples were incubated with 0.5 ml different incubation media and contained 20 pg/mg of relative IS at 45 °C for overnight. After incubation, the samples were pH adjusted at 3 then through liquid–liquid extraction (LLE) by a 2 ml EA. The organic layer was collected and evaporated to dryness. The dry residues were reconstituted in 50 μl 30% methanol (v/v) and transferred to glass autosampler vials, and then 10 μl of aliquot was injected into HPLC–MS/MS instrument. Finally, the extraction efficiency of different incubation media for 5 metabolites of DEHP in hair was calculated by using calibration curves.

2.3.3. Authentic hair analysis

The authentic hair analysis was carried out by adding 0.5 ml of methanol/TFA (8.5:1.5, v/v) mixture and 50 μL of relative IS at 20 pg/mg, then placed into 45 °C for overnight to extract MEHP, MEHHP, MEOHP, 5cx-MEPP and 2cx-MMHP from hair. The incubated solutions were collected in new glass tubes then evaporated to dryness at 40 °C under a stream of nitrogen. After evaporation, the dry residue was reconstituted and then adjusted the pH value to 3 by glacial acid. The extracts was collected, then evaporated to dryness at 40 °C under a stream of nitrogen. Finally, dry residues were reconstituted in 50 μl 30% methanol and transferred to glass autosampler vials, and then 10 μl of aliquot was injected into a HPLC–MS/MS instrument.

2.4. Method validation

2.4.1. Sensitivity

A series of decreasing concentrations of standard-spiked hair was analyzed to investigate limit of detection (LOD) and limit of quantitation (LOQ) for sensitivity. The LOD was determined as the concentration with a signal-to-noise (S/N) ratio at least 3, and the 2 of independent ion intensity ratios of the 3 ions monitored are within ±20% of the ratio observed in calibration standard. LOQ also was defined as the lowest concentration that met the LOD criteria, S/N ratio of at least 10 and measured concentration within ±20% of the actual value.

2.4.2. Linearity

The analytical method linearity of 5 metabolites of DEHP in hair was performed in the concentration range of 1–100 pg/mg (MEHP, MEHHP and MEOHP) and 5–100 pg/mg (5cx-MEPP and 2cx-MMHP). Calibration curves were acquired with 5 calibrator levels from low to high