Journal of Chromatography B, 1011 (2016) 196–203

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Determination of metabolites of di(2-ethylhexyl) terephthalate (DEHTP) in human urine by HPLC-MS/MS with on-line clean-up

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ARTICLE INFO

Article history:
Received 11 August 2015
Received in revised form 7 December 2015
Accepted 19 December 2015
Available online 24 December 2015

Keywords:
Di(2-ethylhexyl) terephthalate
DEHTP
Plasticizer
Exposure assessment
Urinary metabolite
Human biomonitoring

ABSTRACT

Di(2-ethylhexyl) terephthalate (DEHTP) is used as a substitute for ortho-phthalate based plasticizers like di(2-ethylhexyl) phthalate (DEHP) which are discussed and regulated due to their reproductive toxicity. We developed a fast and rugged method to quantify side chain oxidized monoesters of DEHTP in human urine, namely 5OH-MEHTP, 5oxo-MEHTP, 2cx-MMHTP and 5cx-MEPTP. Sample preparation was kept simple with enzymatic deconjugation and a two column assembly for on-line sample clean up. Metabolites were identified with authentic standards and quantified via isotope dilution LC–MS/MS. The limit of quantification was 0.2 μg/L for 5cx-MEPTP and 5oxo-MEHTP, 0.3 μg/L for 5OH-MEHTP and 0.4 μg/L for 2cx-MMHTP. Accuracy (relative recovery: 95.8–111%) and precision (relative standard deviation: <7%) were highly acceptable. In a pilot biomonitoring study with 34 volunteers (aged 25–61 (median 42), 20 female and 14 male) not known to be occupationally exposed to DEHTP, we could detect 5cx-MEPTP above the limit of quantification in 94% of the samples (median: 0.9 μg/L, maximum: 38.7 μg/L). The other metabolites investigated were detected at a lower rate and at lower concentration levels (5oxo-MEHTP: 21%, maximum: 1.8 μg/L; 5OH-MEHTP: 18%, maximum: 3.4 μg/L; 2cx-MMHTP: 9%, maximum: 0.9 μg/L). All target analytes can be regarded as promising and specific urinary biomarkers for DEHTP exposure. With this method we provide a basis for quantitatively investigating the human metabolism of DEHTP and for performing exposure and risk assessments in the general population and the working environment.

1. Introduction

Di(2-ethylhexyl) terephthalate (DEHTP), CAS Registry No. 6422-86-2, is a substitute for some high molecular weight phthalate plasticizers like di(2-ethylhexyl) phthalate (DEHP), which possess reprotoxic and endocrine disrupting properties. In Europe, the Regulations (EC) No 1907/2006 [1] and (EU) No 10/2011 [2] have restricted the use of DEHP both in toys and childcare articles and in food contact materials. In the U.S., the Consumer Product Safety Improvement Act of 2008 similarly restricted DEHP in toys and childcare products [3,4].

Although DEHTP is structurally similar to DEHP, their toxicological profiles differ considerably. In contrast to DEHP, DEHTP can neither be regarded as a reprotoxic nor an endocrine disrupting chemical [5]. Compared to control groups, rats perinatally treated with DEHTP did not show a significant decrease in testicular testosterone production, which is related to an altered male sexual differentiation as described for DEHP and other phthalates [5,6]. A repeated oral dose study in rats derived a NOEL of 500 mg/kg bw/d based upon increased relative liver weight; peroxisome proliferation in the liver was not noted [7] at the 1% dietary level. However, at higher dose levels, DEHTP has been shown to be an extremely weak hepatic peroxisome proliferator by Topping et al. [8]. In a systemic toxicity study by Wirnitzer et al. [9] in rats, continuous infusion of a lipophilic solution resulting in dose levels of up to 361.6 mg DEHTP/kg bw was used to simulate DEHTP exposure via medical devices such as infusion tubes. Under the given conditions, DEHTP did not show any adverse systemic toxicity. In 2008, the European Food Safety Authority evaluated DEHTP and derived a tolerable daily intake (TDI) of 1 mg/kg bw/day based upon a 2-year combined toxicity/carcinogenicity study [10,11].

Currently, DEHTP is used in a variety of applications such as flooring, cable insulations, toys, medical devices and food contact materials for single or repeated use [10,12]. The specific migration limit (SML) for DEHTP in food contact materials is 60 mg/kg food [2].

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http://dx.doi.org/10.1016/j.jchromb.2015.12.042
1570-0232/

Table 1
Solvent gradient for the chromatographic separation of analytes.

Time (min)	Solvent A (%)	Solvent B (%)
0	90	10
8	90	10
10	50	50
18	45	55
18.1	5	95
23	5	95
23.1	90	10
26	90	10

The increasing importance of DEHTP as a substitute for DEHP and other regulated/restricted plasticizers is reflected in an increase in consumption volumes of DEHTP. In 1990, the U.S. consumed an amount of 11.000 metric tons, rising to 48.000 metric tons in 2012. Data on the consumption of DEHTP in Western Europe are available starting from 2002 with 2.000 metric tons rising to 45.000 metric tons in 2012. Compared to 2012, Western European consumption volumes of DEHTP are predicted to double until 2018 [13].

Similar to other plasticizers, DEHTP can migrate out of the polymer (e.g., PVC) into surrounding media. Consequently, human exposure to DEHTP has to be expected. Therefore, DEHTP has been selected as a substance of interest within the cooperation project between the German Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety (BMUB) and the German Chemical Industry Association (VCI) with the aim to establish new human biomonitoring methods in order to perform exposure and risk assessments for new or emerging chemicals.

After oral dosage to rats [14], DEHTP has been shown to be extensively metabolized and rapidly excreted in urine with terephthalic acid (TPA) as the main metabolite in urine (51% of administered dose), followed by oxidized metabolites of 2-ethyl hexanol and mono(2-ethylhexyl) terephthalate (MEHTP). MEHTP and its oxidized metabolites (not further specified) constituted about 9% of the administered oral dose of DEHTP in urine. A recent in vitro study by Silva et al. [15] described, apart from TPA, side chain oxidized monoesters as metabolites of DEHTP. Whereas TPA and 2-ethylhexanol can be formed by various other chemicals, the monoester derived metabolites are specific to DEHTP. Thus, based upon our experiences with the structurally analogous DEHP [16] and in line with Silva et al., we postulated secondary, side chain oxidized monoesters as metabolites of DEHTP as depicted in Fig. 1.

The use of specific DEHTP metabolites as biomarkers in urine is a promising approach to assess human exposure to DEHTP. Similar approaches have been performed for many phthalates and some of their substitutes over the past decade [17–20]. The aim of this study was to develop a fast and reliable method to quantify specific DEHTP metabolites in urine, to use this method in an oral dosage human metabolism study establishing metabolite conversion factors, and thus to provide the basis for future exposure and risk assessment approaches for this substance.

2. Experimental

2.1. Chemicals

1-mono-(2-ethyl-5-hydroxy-hexyl) benzene-1,4-dicarboxylate (5OH-MEHTP), 1-mono-(2-ethyl-5-oxo-hexyl) benzene-1,4-dicarboxylate (5oxo-MEHTP), 1-mono-(2-ethyl-5-carboxyl-pentyl) benzene-1,4-dicarboxylate (5cx-MEPTP), 1-mono-(2-carboxyl-methyl-hexyl) benzene-1,4-dicarboxylate (2cx-MMHTP) and their D4-ring labelled analogues were synthesized by Dr. Belov, Max Planck Institute for Biophysical Chemistry, Germany. All synthesized compounds had a purity >95% determined by 1H-NMR. HPLC-grade water and acetonitrile were purchased from Carl Roth Karlsruhe, Germany. Ammonium acetate (>98%) was purchased from Sigma–Aldrich Steinheim, Germany. β-Glucuronidase from *Escherichia coli* K12 was purchased from Roche Diagnostics Mannheim, Germany. Acetic acid and formic acid were purchased from Merck Darmstadt, Germany.

Table 2
Scheduled MRM-parameters for precursor and product ion pairs. The resolution for Q1 and Q3 was set to unit. MRM detection window was set to 120 s with a target scan time of 1 s. Settling time was 0 s and MR pause was set to 5 s.

	Q1 mass (Da)	Q2 mass (Da)	RT (min)	DP	EP	CE	CXP
5OH-MEHTP							
Quan.	293	77	16.70	−65	−10	−42	−10
Qual.	293	121	16.70	−65	−10	−22	−10
D4-5OH-MEHTP							
Quan.	297	125	16.70	−80	−7	−26	−16
Qual.	297	81	16.70	−80	−7	−48	−10
5oxo-MEHTP							
Quan.	291	121	17.60	−70	−7	−22	−25
Qual.	291	77	17.60	−70	−7	−41	−5
D4-5oxo-MEHTP							
Quan.	295	125	17.60	−80	−7	−23	−9
Qual.	295	81	17.60	−80	−7	−52	−5
5cx-MEPTP							
Quan.	307	165	16.77	−70	−10	−20	−10
Qual.	307	121	16.77	−70	−10	−36	−12
D4-5cx-MEPTP							
Quan.	311	169	16.77	−62	−11	−25	−3
Qual.	311	125	16.77	−62	−11	−37	−9
2cx-MMHTP							
Quan.	307	165	17.53	−40	−10	−15	−9
Qual.	307	121	17.53	−40	−10	−32	−12
D4-2cx-MMHTP							
Quan.	311	169	17.53	−63	−7	−11	−10
Qual.	311	125	17.53	−63	−7	−30	−9

2.2. Standard preparation

Standard stock solutions were prepared by dissolving approximately 10 mg of each analyte (5OH-MEHTP, 5oxo-MEHTP, 2cx-MMHTP, 5cx-MEPTP), weighed exactly, in a 10 mL volumetric flask in acetonitrile. Stock solutions were stored at −20 °C in Teflon® capped glass vials until further use. For analysis, nine calibration standards were prepared ranging in concentrations between 0.2 μg/L and 60 μg/L by diluting the standard stock solutions gradually with high purity water. Internal standard stock solutions were prepared by dissolving approximately 10 mg of each deuterated standard, weighed exactly, in a 10 mL volumetric flask in acetonitrile. The internal standard solution was obtained by mixing 150 μL of a 1:100 dilution of each internal standard stock solution in a volumetric flask and adjusting with water to 10 mL.

2.3. Sample collection and preparation

Urine samples were stored in 250 mL polyethylene containers and kept frozen at −20 °C until further use. Before analysis, samples were thawed at room temperature and vortex mixed to homogenize sample material. An aliquot of 300 μL urine was transferred into a 1.8 mL screw cap vial together with 100 μL ammonium acetate buffer (1 M, pH 6.0–6.4), 20 μL internal standard solution and 6 μL β-glucuronidase from *E. coli* K12 (diluted 1:1 with ammonium acetate buffer). Samples were incubated in a water bath for 2.5 h at 37 °C. After incubation, 10 μL of acetic acid was added to samples to adjust the pH value. Samples were frozen for at least 3 h at −18 °C to precipitate proteins. After precipitation, sam-