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Synthesis of DEHP metabolites as biomarkers for GC–MS evaluation of phthalates as endocrine disrupters

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Abstract—Phthalates are used primarily as plasticizers to make polyvinyl chloride (PVC) soft and flexible. In recent years the phthalate esters have attracted increasing attention as environmental and biomedical pollutants and, because of their toxicological characteristics. In particular, they are more and more recognized as endocrine disrupters. In this context, we describe herein an efficient synthetic pathway leading to a series of metabolites of di(2-ethylhexyl) phthalate (DEHP). Mono(2-ethylhexenyl) phthalate was used as starting material to obtain these products in good yield, large scale and GC–MS purity. The metabolites of DEHP were synthesized, for the first time, as biomarkers to verify their quantitative determination in human urine and serum by GC–MS analysis for studying the exposure to phthalates and establishing reference values.

1. Introduction

Bis(2-ethylhexyl) 1,2-benzenedicarboxylate [di(2-ethylhexyl) phthalate, DEHP], commonly called phthalate, is used primarily as a plasticizer to make polyvinyl chloride (PVC) soft and flexible. Blood bags, children's toys, vinyl floor, wall covering and car underbody coating are products made with plasticized PVC. Non-polymeric uses of phthalates as fixatives, detergents, lubricating oils and solvents let find them in different products such as cosmetics and wood finishes. Phthalates are also found in foods, which are processed or packaged with plastic materials and in dialysis and intravenous drip fluids as contaminants from the plastic tubing. The amount of the phthalate esters varies between 10% and 60% depending on the product. Since phthalate esters are not chemically bound to the polymer, they are released into the environment and persist for a long time both in water and in air because of their stability. For that reason human population is subjected to absorption of phthalates through inhalation, ingestion, dermal and after medical procedures. Its widespread use leads DEHP to be considered as an ubiquitous ecological contaminant.[1,2] Phthalates are known as endocrine disrupters, an exogenous substance or mixture that alters function(s) of the endocrine system, and consequently causes adverse health effects in an intact organism or its progeny or (sub)populations. Therefore, in recent years phthalate esters have attracted increasing interest as environmental and biomedical pollutants, and because of their toxicological characteristics. It was previously demonstrated that phthalates have peroxisomal proliferative activity. They have teratogenic and feminizing effects, at low levels, in rat and fish.[3–5] They are rapidly metabolized to their respective monoesters and further oxidative products, which are partially glucuronidated and excreted through urine and feces.[6–9] To our knowledge, the extent of exposure of humans to phthalate esters has not yet been sufficiently investigated. Few studies have focused on the exposure to DEHP and to its metabolites[8–12] of workers, of hemodialysis patients,[13–15] or of newborn infants,[16] because of exchange transfusions.[17,18] Latini et al.[19] confirmed a significant and widespread presence of the phthalate DEHP and of its metabolite MEHP, in the blood of newborn infants in an Italian hospital. The DEHP is metabolized to mono(2-ethylhexyl) 1,2-benzenedicarboxylate [mono(2-ethylhexyl) phthalate, MEHP] and this compound can

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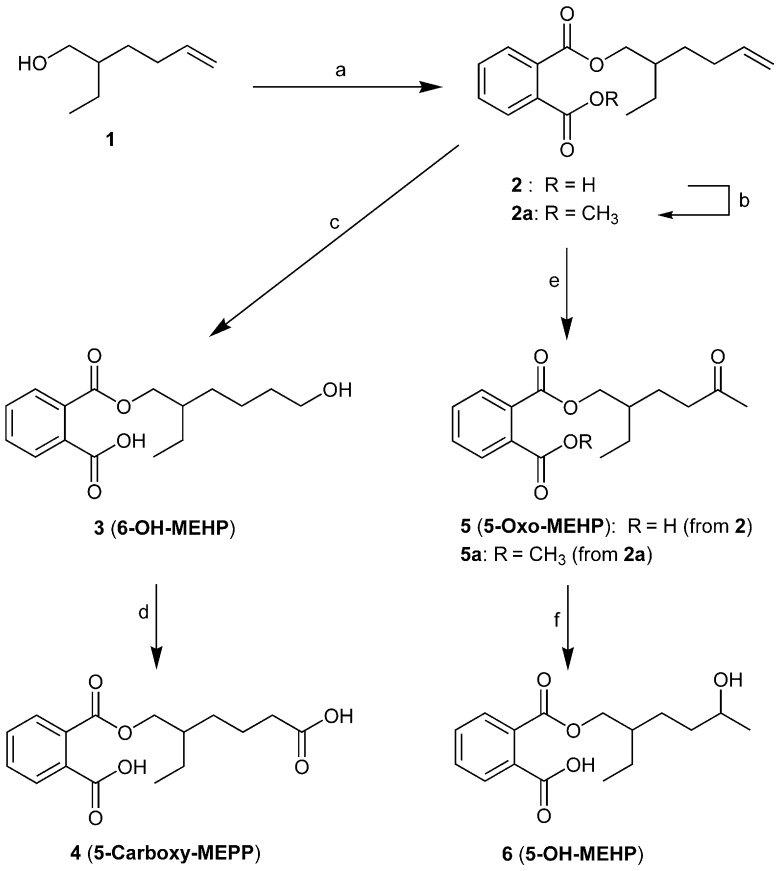
be transformed, by ω-1 oxidation reactions of the monoester alkyl chain, also in mono(2-ethylhexenyl) 1,2-benzenedicarboxylate [mono(2-ethylhexenyl) phthalate, **2**], mono(2-ethyl-5-hydroxyhexyl) 1,2-benzenedicarboxylate [mono(2-ethyl-5-hydroxyhexyl) phthalate, 5-OH-MEHP, **6**],[20] mono(2-ethyl-5-oxohexyl) 1,2-benzenedicarboxylate [mono(2-ethyl-5-oxohexyl) phthalate, 5-oxo-MEHP, **5**],[20] and mono(2-ethyl-5-carboxypentyl) 1,2-benzenedicarboxylate [(mono(2-ethyl-5-carboxypentyl phthalate, 5-carboxy-MEPP, **4**].[21] Biological monitoring of DEHP and their metabolites, not only in urine but also in serum, can be instrumental to investigate the environmental exposure of members of the general population to phthalate esters.[22] For that reason it is evident that the interest in establishing reference tests by using synthetic biomarkers for quantitative determinations of such metabolites in a pool of selected humans.

Therefore, we decided to develop an efficient synthetic pathway leading to a series of secondary metabolites of DEHP to be used in GS–MS analysis for their biological monitoring in human urine and serum.

2. Results and discussion

2.1. Synthesis of DEHP metabolites

Phthalate **2** was obtained starting by a classical esterification reaction of phthalic anhydride with alcohol **1**. The 2-ethylhex-5-en-1-ol (**1**) was synthesized as described in the literature.[23] The crude monoester **2** was used as starting material to achieve four oxidative metabolites of MEHP (Scheme 1). The hydroboration–oxidation process gave the mono(2-ethyl-6-hydroxyhexyl) 1,2-benzenedicarboxylate [mono(2-ethyl-6-hydroxyhexyl) phthalate, 6-OH-MEHP, **3**] by an anti-Markovnikov mechanism. Oxidation of the primary alcohol **3** was undertaken by addition of an acidic aqueous solution containing chromic acid (Jones' reagent)[24–26] to an acetone solution of the alcohol **3**. Oxidation occurred rapidly and lead to compound **4**. The crude acid was purified by several extractions in different solvents. Moreover oxidation of the double bond in compound **2**, undertaken with catalytic amount of $PdCl_2$ in the presence of *p*-benzoquinone following the Wacker reaction,[27] lead to the derivative **5**. The quinone was necessary to reoxidize Pd(0) to Pd(II). The net reaction employs alkene, water and *p*-benzoquinone. Therefore, the catalyst could be recovered at the end of the reaction. The quinone was purified by sublimation before use. Compound **6** was obtained by an efficient reduction of the oxo group of **5** with $NaBH_4$ in an alcoholic solution. The excess of hydride was destroyed with H_2O. All the expected products were obtained in good yield, with chemical purity >95% and were characterized by ^1H, ^{13}C NMR and ESI-MS. Before their use as analytes in GC–MS, all the metabolites were transformed in the corresponding methyl esters by treatment with diazomethane, prepared from



Scheme 1. Reagents and conditions: (a) phthalic anhydride, pyridine (Py), under N_2; (b) CH_2N_2, MeOH/ether (9:1); (c) 1 M B_2H_6 in THF, then H_2O_2, OH^- in THF; (d) CrO_3, H_2SO_4 in acetone; (e) $PdCl_2$, quinone in DMF/H_2O (7:1); (f) $NaBH_4$ in EtOH.

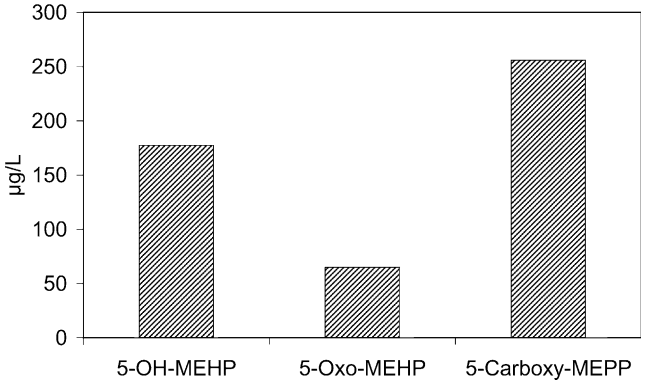


Figure 1. The concentrations of 5-OH-MEHP (**6**), 5-oxo-MEHP (**5**) and 5-carboxy-MEPP (**4**) in urine samples of 50 persons of the general population in µg/L. The 90th percentiles of these analytes, calculated assuming the underlying model to be a log-normal distribution, is shown.

N-methyl-*N*-nitroso-*p*-toluenesulfonamide,[28] and in the corresponding silyl ethers by *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA).

2.2. Biological monitoring of DEHP metabolites in human urine and serum by GC–MS

The secondary metabolites **4**, **5** and **6** were demonstrated to be useful as standards for GC–MS in order to quantify the phthalate metabolites in urine of humans and to establish reference values.

Preliminary data on the quantitative analysis of DEHP and their metabolites were performed on a pool of 50 persons of the general population of south-west Germany, using synthetic metabolites as standard biomarkers for GC–MS analysis. The study showed that in every analyzed person 5-OH-MEHP (**6**), 5-carboxy-MEPP (**4**) and 5-oxo-MEHP (**5**) were present in urine. The 90th percentile of these metabolites, calculated assuming the underlying model to be a log-normal distribution, is reported in Figure 1. In particular, it was shown that beneath phthalic acid, these metabolites had relatively high urinary concentrations, but in all serum samples they were below the detection limit (0.1 µg/L). This means that general population accumulates phthalates from the environment. In fact, phthalate diesters are omnipresent and contaminations of the extracts should be avoided. MEHP was also detected in river water[29] and in solutions that were stored in medical grade PVC bags,[30] while the oxidative metabolites of MEHP were not present in the environment. In the context of a study in environmental medicine, we demonstrated that the oxidative metabolites of MEHP, measured in urine, are suitable biomarkers for monitoring the exposure of the general population to phthalate esters.

3. Conclusions

The described synthetic pathway represents the first efficient synthesis leading to a series of DEHP metabolites. By this methodology, it is possible to obtain mono(2-ethylhexenyl) 1,2-benzenedicarboxylate (**2**), methyl (2-ethyl-5-hexenyl) 1,2-benzenedicarboxylate (**2a**), mono(2-ethyl-6-hydroxyhexyl) 1,2-benzenedicarboxylate (6-OH–MEHP, **3**), mono(2-ethyl-5-carboxypentyl phthalate (5-carboxy-MEPP, **4**), mono(2-ethyl-5-oxohexyl) 1,2-benzenedicarboxylate (5-oxo-MEHP, **5**) methyl (2-ethyl-5-oxohexyl) 1,2-benzenedicarboxylate (**5a**), mono(2-ethyl-5-hydroxyhexyl) 1,2-benzenedicarboxylate (5-OH-MEHP, **6**) in gram scale. Moreover, we demonstrated, for the first time, that the oxidative derivatives of MEHP (DEHP metabolites) can be used as analytes for their determination in GC–MS in urine and serum samples. As an example, these standard biomarkers can be proposed for investigating if exposure to DEHP can have toxicological risks for reproduction in humans.

4. Experimental

4.1. General chemistry methods

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ with a Varian spectrometer at a frequency of 200 MHz and 50 MHz, respectively. Chemical shifts are reported in parts per million relative to TMS and coupling constants (*J*) in hertz (s, d, t, m and br for singlet, doublet, triplet, multiplet and broad, respectively). FT-IR spectra were obtained on a Perkin Elmer Spectrum BX II apparatus. For mass analysis a ThermoFinnigan LCQ Advantage ESI-MS spectrometer was used. Flash column chromatography (FCC) was accomplished on SiO_2 (ICN; 32–63 µm, 60 Å). Thin layer chromatographies were carried out on Merck silica gel 60 F_{254} plastic sheets. Dry solvents were distilled immediately before use.

4.1.1. 1-Hydroxy-2-ethylhex-5-ene (1). This compound was synthesized as described in the literature.[23]

4.1.2. Mono(2-ethylhexenyl) 1,2-benzenedicarboxylate (2). 2-Ethyl-5-hexen-1-ol (**1**) (2.2 g, 17.2 mmol), phthalate anhydride (2.54 g, 17.2 mmol) and pyridine (1.6 mL) were refluxed under nitrogen for 3 h. The mixture was quenched with cold water and extracted with Et_2O. The organic phase was washed twice with a solution of 10% HCl to remove pyridine. Finally, the mixture was extracted with 0.4 M K_2CO_3. The aqueous basic layer was acidified to pH 1 with 1 M HCl and then extracted with Et_2O. After drying with Na_2SO_4 and evaporation of the solvent under vacuum, compound **2** was obtained as a colourless oil (4.48 g, 94% yield) and used without further purification in the next step. R_f (Et_2O/hexane 1:1, UV) = 0.15. ESI-MS: calcd for $[M+H]^+$ 277.3. Found 277.9. ^{1}H NMR δ 0.90 (3H, t, J = 8 Hz, CH_3), 1.24–2.14 [7H, m, CH_2= $CH(CH_2)_2CHCH_2CH_3$], 4.25 (2H, d, J = 6 Hz, $COOCH_2$), 4.96 (2H, dd, J = 10, J = 1.8 Hz, CH_2=), 5.02 (1H, br, OH), 5.76 (1H, m, CH=), 7.61 and 7.81 (4H, 2 × m, aromatic CH). ^{13}C NMR δ 10.9 (CH_3), 23.6 (CH_3CH_2), 29.9 ($CH_2CH{=}CH_2$), 30.9 ($CH_2CH_2CH{=}$ CH_2), 38.1 (CH_3CH_2CH), 68.0 ($COOCH_2$), 114.5 ($CH{=}CH_2$), 128.6–133.4 (aromatic C), 138.5 ($CH{=}CH_2$), 168.2 ($COOCH_2$), 172.3 (COOH).

4.1.3. Methyl (2-ethyl-5-hexenyl) 1,2-benzenedicarboxylate (2a). A fresh diethyl ether solution of CH_2N_2 was slowly added, at 0 °C, to compound **2** (0.57 g, 2.06 mmol) in MeOH/ether (30 mL, 9:1) until the colour of the solution remained yellow. The excess of CH_2N_2 was flushed out with a nitrogen stream and the solvent removed under vacuum obtaining product **2a** as a pale yellow oil (0.59 g, 98% yield). R_f (AcOEt/petroleum ether 2:1, UV) = 0.74. ESI-MS: calcd for $[M+H]^+$ 291.3. Found 291.9. ^{1}H NMR δ 0.91 (3H, t, J = 7.4 Hz, CH_3), 1.4–2.15 [7H, m, CH_2=CH(CH_2)$_2$CHCH_2CH$_3$], 3.88 (3H, s, $COOCH_3$), 4.24 (2H, d, J = 6 Hz, $COOCH_2$), 4.96 (2H, dd, J = 8.2, J = 1.8 Hz, CH_2=), 5.76 (H, m, CH=), 7.61 and 7.81 (4H, 2 × m, aromatic CH). ^{13}C NMR δ 10.4 (CH_3), 23.1 (CH_2CH_3), 29.7 (CH_2CH=CH_2), 30.7 (CH_2CH_2CH=CH_2), 38.1 (CH_3CH_2CH), 38.2 (COOCH_3), 68.0 (COOCH_2), 114.4 (CH=CH_2), 128.3–133.4 (aromatic C), 138.0 (CH=CH_2), 167.4 (COOMe), 168.1 ($COOCH_2$).

4.1.4. Mono(2-ethyl-6-hydroxyhexyl) 1,2-benzenedicarboxylate (3). B_2H_6 (1 M) in THF (5.5 mL) was added, drop by drop at 0 °C under nitrogen, to a solution of compound **2** (1.52 g, 5.5 mmol) in anhydrous THF (50 mL). After stirring at room temperature for 1 h, excess of hydride was destroyed with H_2O (0.1 mL). After 5 min, 3 N NaOH (0.5 mL) and 30% H_2O_2 (0.5 mL) were added and the reaction mixture was stirred for 1 h at 50 °C. The mixture was extracted with Et_2O, washed with brine and dried over Na_2SO_4 and $FeSO_4$. The crude alcohol, isolated after evaporation of the solvent under vacuum, was purified by FCC. Product **3** was obtained as an oil (1.44 g, 89% yield). R_f (AcOEt/petroleum ether 1:1, UV, $KMnO_4$) = 0.3. ESI-MS: calcd for $[M+H]^+$ 295.3. Found 295.4; calcd for $[M+Na]^+$ 317.3. Found 317.3. ^{1}H NMR δ 0.90 (3H, t, CH_3), 1.44 [9H, m, $CH_3CH_2CH(CH_2)_3CH_2OH$], 2.92 (1H, br, OH), 3.84 (2H, m, CH_2OH), 4.23 (2H, m, $COOCH_2$), 7.56 and 7.74 (4H, 2 × m, aromatic CH). ^{13}C NMR δ 11.1 (CH_3), 22.0 (CH_3CH_2), 24.0 [$CH_2(CH_2)_2OH$], 29.9 [$CH_2(CH_2)_3OH$], 31.9 (CH_2CH_2OH), 38.9 ($CHCH_2CH_3$), 61.9 (CH_2OH), 67.4 (COOCH_2), 128.5–132.9 (aromatic C), 168.6 ($COOCH_2$), 170.1 (COOH).

4.1.5. Mono(5-carboxy-2-ethylpentyl) 1,2-benzenedicarboxylate (4). Jones' reagent (1 M, 2 mL), prepared from CrO_3 (6.7 g) in 96% H_2SO_4 (6 mL) and H_2O (50 mL), was added, drop by drop at 15–20 °C, to compound **3** (553 mg, 1.88 mmol) in anhydrous acetone (6 mL). The precipitated reduced chromium salt was centrifuged off. The solution was quenched by addition of H_2O and extracted with Et_2O that was dried over Na_2SO_4. Methanol or $NaHSO_3$ was added to the brown solution to eliminate residue Cr(VI) salts. The crude acid **4** was extracted with 0.4 M K_2CO_3. The aqueous layer, acidified to pH 1 with 1 M HCl, was extracted with $CHCl_3$ and dried over Na_2SO_4. Compound **4** (546 mg, 94% yield) was obtained as a colourless oil by evaporation of the solvent under vacuum. R_f (AcOEt/hexane 1:1, UV) = 0.5. ESI-MS: calcd for $[M-H]^+$ 309.3. Found 309.0. ^{1}H NMR δ 0.90 (3H, t, CH_3), 1.42 (4H, m, (CH_2)$_2$$CH_2$COOH), 1.65 (3H, m, C$HCH_2CH_3$), 2.38 (2H, m, C$H_2$COOH), 4.22 (2H, m, OCH_2), 6.25 (2 × COOH), 7.8–7.5 (4H, m, aromatic CH). ^{13}C NMR δ 10.9 (CH_3), 23.6 (CH_2CH_2COOH), 30.0 ($CH_2CH_2CH_2$COOH), 34.2 ($CHCH_2CH_3$), 38.5 (CH_2COOH), 67.7 (OCH_2), 132.9–128.7 (aromatic C), 168.1 ($COOCH_2$), 172.1 (CH_2COOH), 179.8 (aromatic C).

4.1.6. Mono(2-ethyl-5-oxohexyl) 1,2-benzenedicarboxylate (5) and methyl (2-ethyl-5-oxohexyl) 1,2-benzenedicarboxylate (5a). Compound **2** (2.46 g, 8.9 mmol) or compound **2a** (2.58 g, 8.9 mmol) was added, drop by drop in 5 min, to a solution of $PdCl_2$ (16 mg, 0.089 mmol) and *p*-benzoquinone (1.06 g, 9.8 mmol) in DMF/H_2O (7:1, 24 mL). After stirring overnight at room temperature, the reaction mixture was quenched with 3 N HCl (60 mL) and extracted with ether. The organic phase was washed with 10% NaOH and brine. The aqueous basic layer was acidified to pH 1 with 3 N HCl and then re-extracted with Et_2O. The crude product was purified by FCC.

Compound **5**: oil (1.06 g, 41% yield). R_f (AcOEt/petroleum ether 2:1, UV) = 0.2. ESI-MS: calcd for $[M-H]^+$ 291.3. Found 291.1. IR (KBr) cm^{-1}: 3500–2500 (OH), 2964.5 (CH), 1714.2 (C=O). ^{1}H NMR δ 0.92 (3H, t, CH_3), 1.3 (2H, m, CH_2CH$_3$), 1.69 (3H, m, CHCH_2CH$_2$CO), 2.19 (3H, s, $COCH_3$), 2.52 (2H, m, CH_2CO), 4.2 (2H, m, OCH_2), 7.64 and 7.8 (4H, m, aromatic CH). ^{13}C NMR δ 11.1 (CH_3CH_2), 23.7 (CH_3CH_2), 24.7 (CH_2CH_2CO), 30.0 (aliphatic CH), 38.2 (CH_3CO), 40.6 (CH_2CO), 63.5 (OCH_2), 128.7–131.5 (aromatic C), 168.1 ($COOCH_2$), 170.6 (COOH), 210.6 (CH_3CO).

Compound **5a**: oil (1.52 g, 56% yield). R_f (AcOEt/petroleum ether 2:1, UV) = 0.2. ESI-MS: calcd for $[M+H]^+$ 306.3. Found 306.9. ^{1}H NMR δ 0.92 (3H, t, CH_3), 1.4 (2H, m, CH_2CH$_3$), 1.63 (3H, m, CHCH_2CH$_2$CO), 2.11 (3H, s, $COCH_3$), 2.45 (2H, m, CH_2CO), 3.88 (2H, m, $COOCH_3$), 4.2 (2H, m, OCH_2), 7.54 and 7.7 (4H, m, aromatic CH). ^{13}C NMR δ 10.9 (CH_3CH_2), 23.7 (CH_3CH_2), 24.7 (CH_2CH_2CO), 29.8 (aliphatic CH), 38.2 (COCH_3), 40.7 (CH_2CO), 52.5 (COOCH_3), 63.5 (OCH_2), 128.7–131.2 (aromatic C), 167.6 ($COOCH_2$), 167.8 ($COOCH_3$), 208.5 (CH_3CO).

4.1.7. Mono(2-ethyl-5-hydroxyhexyl) 1,2-benzenedicarboxylate (6). A solution of $NaBH_4$ (117 mg, 3.16 mmol) in ethanol (2 mL) was slowly added to compound **5** (231 mg, 0.79 mmol). After stirring at room temperature for 2 h, the mixture was quenched with H_2O (1 mL). The aqueous basic layer was acidified to pH 1 with concd HCl and then extracted with *n*-hexane. The crude alcohol, isolated after concentration under vacuum, was purified by FCC affording **6** as an oil (110 mg, 48% yield). R_f (EtOAc/petroleum ether 2:1, UV, $KMnO_4$) = 0.3. ESI-MS: calcd for $[M+H]^+$ 294.3. Found 294.8. ^{1}H NMR δ 0.90 (3H, t, CH_3), 1.25 (3H, d, J = 6.2, CH_3CHOH), 1.45 (6H, m, CH_2CH_2CHOH and CH_3CH_2), 1.75 (1H, m, CHCH_2CH$_3$), 4.07 (2H, m, OCH_2), 4.36 (1H, m, CHOH), 6.25 (1H, br, OH), 7.41 and 7.74 (4H, m, aromatic CH). ^{13}C NMR δ 11.1

(CH_3CH_2), 23.9 (CH_3CHOH), 24.7 (CH_3CH_2), 27.2 (CH_2CH_2CHOH), 35.7 ($CHCH_2CH_3$), 39.3 (CH_2CHOH), 66.7 (*C*HOH), 69.5 (OCH_2), 128.8–131.8 (aromatic C), 168.4 ($COOCH_2$), 170.6 (COOH).

4.2. GC–MS analysis of DEHP metabolites in human urine and serum

For enzymatic hydrolysis of the glucuronidated phthalate metabolites, the urine samples were incubated with β-glucuronidase for 20 h. Thereafter, serum and urine samples were acidified to pH 2–3, extracted twice with ethyl acetate and derivatized with diazomethane and BTSFA [*N*,*O*-bis(trimethylsilyl) trifluoroacetamide]. For separation of the analytes a 20 m capillary column (DB1, J & W Scientific, 0.18 mm i.d. and 0.18 μm film thickness) was used. The column oven temperature was initially kept isothermal at 40 °C for 1 min; followed by an increase to 80 °C at a rate of 20 °C/min, to 200 °C at a rate of 10 °C/min, to 280 °C at a rate of 5 °C/min and kept at 280 °C for 5 min. Quantitative analysis of DEHP and its metabolites was performed by selected ion monitoring gas chromatography/mass spectrometry,[14] operating in negative ion chemical ionization mode to monitor the negative ion at *m*/*z* 148. The limit of detection was approximately 0.1 μg/L for the DEHP metabolites.

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