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Simultaneous analysis of the di(2-ethylhexyl)phthalate metabolites 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid in urine by gas chromatography–mass spectrometry

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Abstract

A gas chromatographic–mass spectrometric method was developed for the quantitative analysis of the three Di(2-ethylhexyl)phthalate (DEHP) metabolites, 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid in urine. After oximation with *O*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride and sample clean-up with Chromosorb P filled glass tubes, all three organic acids were converted to their *tert*.-butyldimethylsilyl derivatives. Quantitation was done with *trans*-cinnamic acid as internal standard and GC–MS analysis in the selected ion monitoring mode (SIM). Calibration curves for all three acids in the range from 20 to 1000 μg/l showed correlation coefficients from 0.9972 to 0.9986. The relative standard deviation (RSD) values determined in the observed concentration range were between 1.3 and 8.9% for all three acids. Here we report for the first time the identification of 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid in human urine next to the known DEHP metabolite 2-ethylhexanoic acid. In 28 urine samples from healthy persons we found all three acids with mean concentrations of 56.1±13.5 μg/l for 2-ethylhexanoic acid, 104.8± 80.6 μg/l for 2-ethyl-3-hydroxyhexanoic acid and 482.2± 389.5 μg/l for 2-ethyl-3-oxohexanoic acid.

Keywords: Di(2-ethylhexyl)phthalate; 2-Ethylhexanoic acid; 2-Ethyl-3-hydroxyhexanoic acid; 2-Ethyl-3-oxohexanoic acid

1. Introduction

Di(2-ethylhexyl)phthalate (DEHP) is the major plasticizer used in Polyvinylchloride (PVC) products to achieve the desired softness, flexibility and stability for the specific applications. It is incorporated into many medical devices, electrical cables, PVC film, clothing and footwear, automotive components, PVC flooring and wall-coverings. In some of the medical products it may even constitute up to 40% by weight of the finished plastic. It has been a priority pollutant for several years because of this widespread use, its large quantities emitted and ubiquitous occurrence in

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the environment [1,2]. It has been shown that DEHP may leach from PVC into the liquids passing through it [3,4], especially for blood and other lipid-containing fluids [5]. At special risk are patients on hemodialysis or receiving blood transfusion [6]. The exposure for these patients was reported to be up to 13 mg DEHP (a single transfusion of platelets) or 74 mg (5 h of hemodialysis) [1]. In 1982 a study conducted by the National Toxicology Program [7] showed that DEHP at high doses was a rodent liver carcinogen. In 1987 DEHP was classified as a ''B2: probable human carcinogen'' by the United States Environmental Protection Agency and its carcinogenity classification of DEHP is currently under review [8]. In contrast to the US EPA, the World Health Organization and the Commission of the European Communities have stated that there is not sufficient evidence to classify DEHP as a possible or probable human carcinogen [9]. Doull and coworkers [1] recently completed a risk assessment of DEHP by the new proposed EPA cancer risk assessment guidelines and recommended that DEHP should be classified as an unlikely human carcinogen. Despite the fact that many studies have been performed to investigate the possible toxicity of DEHP and its metabolites [10–12], questions about health effects of DEHP persist today.

In mammals, including man, DEHP is rapidly hydrolized to mono(2-ethylhexyl)-phthalate (MEHP) and 2-ethylhexanol [13,14]. After hydrolysis of DEHP 2-ethylhexanol is oxidized to 2-ethylhexanoic acid in rats [15,16] and in humans [17,18]. The presence of metabolites due to oxidation of the MEHP side chain were first reported in rats [13,19] and later also in man [20]. Further oxidation products of 2-ethylhexanoic acid found in rat urine were 2-ethyl-5-hydroxyhexanoic acid, 2-ethyl-5-oxohexanoic acid and 2- and 4-heptanone [15,16]. Both ketones were also detected in human urine samples [21–23]. The proposed pathway for ketone formation is β-oxidation of 2-ethylhexanoic acid to two isomeric hydroxy- and oxo-acids with subsequent decarboxylation. For 4-heptanone, the major metabolite found in human urine, this would be 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid [15,16].

Previous studies have shown that MEHP and other DEHP metabolites may even be more active in regard to cellular responses than DEHP itself [24,25], including peroxisome proliferation [26], stimulation of peroxisome proliferator-activated receptor (PPAR) alpha and gamma [27], nephrotoxicity [28] or suppression of cell proliferation [20,29]. It is therefore important to identify all possible DEHP metabolites in humans for further toxicological and molecular genetic studies. Different GC–MS methods for the analysis of 2-ethylhexanol and 2-ethylhexanoic acid have been reported earlier [17,18,30,31], but until now, no method was published for the analysis of 2-ethyl-3-hydroxyhexanoic acid or 2-ethyl-3-oxohexanoic acid. These two postulated DEHP metabolites [16] also have never been identified in human or rat urine. The objective of this study was to develop a GC–MS method for the simultaneous identification and quantitation of the three postulated DEHP metabolites in human urine: 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid.

2. Experimental

2.1. Reagents

N - methyl - *N* - (*tert*. - butyldimethylsilyl)trifluoro - acetamide (MTBSTFA), *O*-(2,3,4,5,6-penta-fluorobenzyl)-hydroxylamine hydrochloride, triethylamine (TEA), *trans*-cinnamic acid and 2-ethylhexanoic acid were purchased from Sigma–Aldrich (Steinheim, Germany). 2-Ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid were synthesized (see synthesis) by the Chemistry Department of the University of Tübingen, Germany. All other chemicals were of analytical grade.

Chromosorb P (acid washed, 80–100 mesh) was obtained from Supelco (Bellefonte PA, USA). A luer-tipped glass tube (1 cm I.D.) packed with Chromosorb P (1.5 g) was washed successively with 0.1 *M* sulfuric acid, methanol, acetone, ethylacetate, dichloromethane and diethyl ether, followed by activation (130°C, 3 h) prior to being used for sample preparation.

2.2. Synthesis

2.2.1. 2-Ethyl-3-oxohexanoic acid

Ethyl 2-ethyl-3-oxohexanoate was prepared according to the literature by self-condensation of ethyl

butyrate in the presence of diisopropylmagnesium bromide [32]. A solution of ethyl 2-ethyl-3-oxohexanoate (2.0 g, 10.7 mmol) was stirred in 5% aqueous potassium hydroxide (10 ml) at room temperature for 12 h. The mixture was extracted with diethyl ether (3×10 ml). The ether phase was discarded and the aqueous phase acidified with 2 *N* HCl under ice-cooling to a pH of about 3. Extraction with diethyl ether (3×10 ml), followed by drying with $MgSO_4$, filtration and evaporation of the solvent gave the acid as a colorless oil; yield 1.02 g (61%). ^{1}H NMR (250 MHz, $CDCl_3$): d=0.78–0.92 (m, 6H, CH_3), 1.46–1.61, 1.74–1.86, 2.40–2.59 (3 m, 2H each, CH_2), 3.35 (dd, J=7.3 Hz, 1H, CH), 11.26 (s, br., 1H, CO_2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): d=11.8, 13.4, 16.8, 21.6, 44.1, 60.1, 175.2, 205.7; MS (EI), m/z (%): 159 (2) [M^++H], 158 (5) [M^+], 141 (5) [M^+-OH], 114 (22) [$C_7H_{14}O^+$], 71 (100) [$H_7C_3CO^+$].

2.2.2. 2-Ethyl-3-hydroxyhexanoic acid

To a solution of ethyl 2-ethyl-3-oxohexanoate (2.0 g, 10.7 mmol) in ethanol (50 ml) was added sodium borohydride (0.4 g, 10.6 mmol) and the mixture was stirred for 8 h at room temperature. It was acidified to pH 3 by the addition of 1 *N* HCl and extracted with diethyl ether (3×25 ml). The combined organic layers were dried with $MgSO_4$, filtered and concentrated in vacuo to provide 1.71 g (85%) of ethyl 2-ethyl-3-hydroxyhexanoate as a colorless oil (mixture of the *syn*- and *anti*-isomer). ^{1}H NMR (250 MHz, $CDCl_3$): d=0.82–0.89 (m, 6H, CH_3), 1.19 (t, J=7.3 Hz, 3H, CH_3), 1.24–1.66 (m, 6H, CH_2); 2.23–2.31 (m, 1H, CH), 2.48 (s, br., OH), 3.60–3.68, 3.69–3.76 (2 m, 1H, CHOH), 4.06–4.16 (m, 2H, CH_2); ^{13}C NMR (62.9 MHz, $CDCl_3$): d=11.8, 12.1, 13.9, 14.3, 18.9, 19.1, 20.3, 22.7, 36.5, 37.5, 52.6, 52.9, 60.4, 71.7, 171.4, 175.7; MS (EI), m/z (%): 189 (26) [M^++H], 171 (34) [M^+-OH], 145 (42), 116 (100) [$C_7H_{16}O^+$]. A solution of ethyl 2-ethyl-3-hydroxyhexanoate (0.261 g, 1.4 mmol) was stirred in 5% aqueous potassium hydroxide (5 ml) at room temperature for 1 h. The mixture was extracted with diethyl ether (3×10 ml). The ether phase was discarded and the aqueous phase acidified with 2 *N* HCl under ice-cooling to a pH of about 3. Extraction with diethyl ether (3×20 ml), followed by drying with $MgSO_4$, filtration and evaporation of the solvent gave the acid (0.19 g, 88%) as a colorless viscous oil (mixture of *syn*- and *anti*- isomer). ^{13}C NMR (62.9 MHz, $CDCl_3$): d=11.8, 12.2, 13.9, 19.1, 20.0, 22.6, 36.2, 37.4, 52.6, 71.7, 180.3; MS (EI), m/z (%): 145 (90), 116 (100) [$C_7H_{16}O^+$].

2.3. Sample preparation

1.0 ml of urine was mixed with 100 μl of 0.2 μg/ml *trans*-cinnamic acid solution as internal standard and adjusted to pH 2–4 with 0.1 *N* sulfuric acid [33], and then oximated with *O*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride (10 mg) at room temperature for 2 h [34]. The resulting mixture was subjected to an activated Chromosorb P tube, followed by elution with 2 ml diethyl ether and 2 ml ethylacetate. The eluate was collected in triethylamine (TEA, 20 μl) and excess solvent was removed under a gentle stream of nitrogen. Silylation was done with MTBSTFA (20 μl) in isooctane (60 μl) at 60°C for 2 h [33]. All samples were analyzed immediately or stored at 4°C until the next day.

2.4. Gas chromatography–mass spectrometry

Analyses were performed on a gas chromatograph with a mass-selective detector (HP 5890 series II, HP 5971, Hewlett-Packard, Waldbronn, Germany). A DB-1701 fused-silica capillary column (30 m×0.25 mm×0.25 μm, J&W Scientific, Folson, CA, USA) was used with helium as carrier gas, column head-pressure of 60 kPa and splitless injection (18 s). The oven temperature was held at 80°C for 2 min, raised to 230°C at a rate of 4°C/min and then at a rate of 60°C/min to 280°C held for 10 min. The injector and interface temperature were 280 and 300°C, respectively. Mass-selective detection (70 eV, electron impact) was performed in both scan mode (50–650 amu) for identification and in selected ion monitoring mode (SIM) for quantitation.

2.5. Quantitation

For calibration, six standard aqueous solutions with a concentration range from 20 to 1000 μg/l of all three acids were analyzed in the same process as urine samples. The ions m/z 201, m/z 217 and m/z 410 were used for quantitation and the ions m/z 243, m/z 199 and m/z 129 were used for identification of

2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid respectively. For the internal standard *trans*-cinnamic acid the ions m/z 205 and m/z 131 were used for quantitation and identification. Intra-assay imprecision of the method was determined for different urine samples in the observed concentration range by analyzing 10 aliquots from each sample in a row. Recoveries of all three acids were calculated from spiked urine samples. After validation of the GC–MS method 28 urines from healthy controls (adults from local staff with no obvious or known disease and no specific DEHP exposure) were analyzed.

3. Results and discussion

A gas chromatographic–mass spectrometric method was developed for the quantitative analysis of 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid in urine. After oximation with *O*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride and sample clean-up with Chromosorb P filled glass tubes all three organic acids were converted to their *tert*.-butyldimethylsilyl derivatives. The tBDMS derivatization of the carboxyl group generates characteristic [M-57] ions in the mass spectra of organic acids, which often is accompanied by a [M-15] ion of low abundance. Fig. 1a shows the mass spectrum of the TBDMS-ester of the internal standard *trans*-cinnamic acid (M_r 262) and Fig. 1b the selected ion chromatogram of a urine sample. The ion m/z 205 [M-57] was used for the different calibration curves and the ion m/z 131 was used as qualifier ion. Fig. 2a shows the mass spectrum of the TBDMS-ester of 2-ethylhexanoic acid (M_r 258) and Fig. 2b the selected ion chromatogram of a urine sample with the ion m/z 201 [M-57] used for quantification and the ion m/z 243 used as qualifier ion for the identification. The calibration curve for 2-ethylhexanoic acid was linear in the range from 20 to 1000 μg/l with a correlation coefficient of $r=0.9987$.

Under the conditions of sample preparation, 2-ethyl-3-hydroxyhexanoic acid only yields the mono-TBDMS derivative (di-TBDMS<0.5% of mono-TBDMS). Incomplete derivatization of hydroxyl groups under these conditions have also been reported for serine and threonine [35]. In none of the urine samples examined di-tBDMS derivatives of 2-ethyl-3-hydroxyhexanoic acid (concentrations from

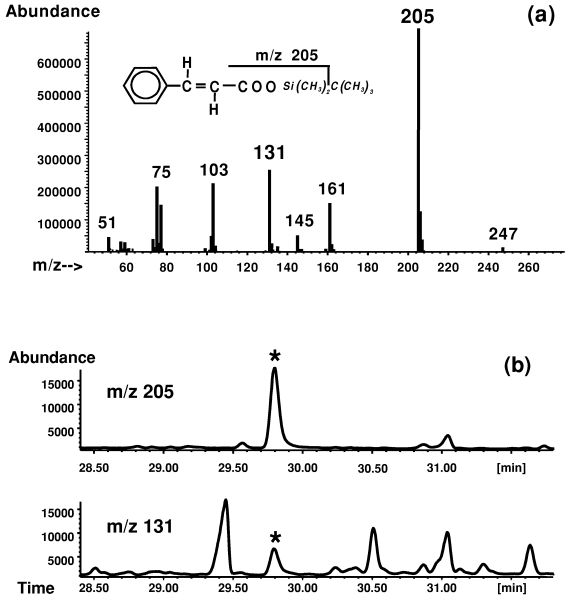

Fig. 1. (a) Mass spectrum of tBDMS-ester of the internal standard *trans*-cinnamic acid (M_r 262); (b) Selected ion chromatogram of a urine sample from a healthy person.

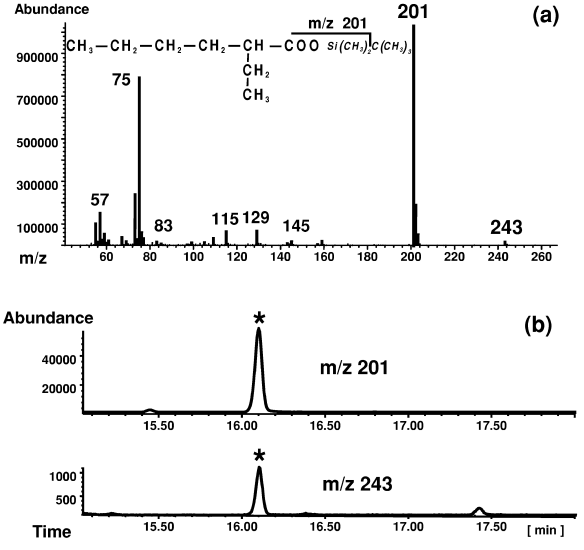

Fig. 2. (a) Mass spectrum of tBDMS-ester of 2-ethylhexanoic acid standard (M_r 258); (b) Selected ion chromatogram of a urine sample from a healthy person (53 μg/l 2-ethylhexanoic acid).

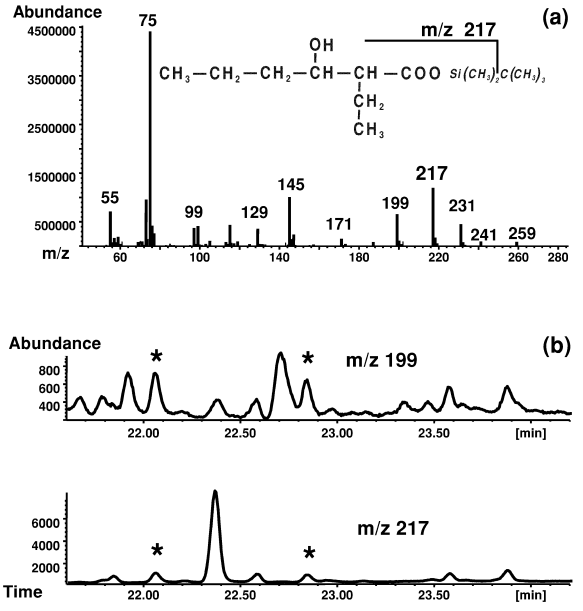

Fig. 3. (a) Mass spectrum of tBDMS-ester of 2-ethyl-3-hydroxyhexanoic acid standard (M_r 274); (b) Selected ion chromatogram of a urine sample from a healthy person (50 μg/l 2-ethyl-3-hydroxyhexanoic acid).

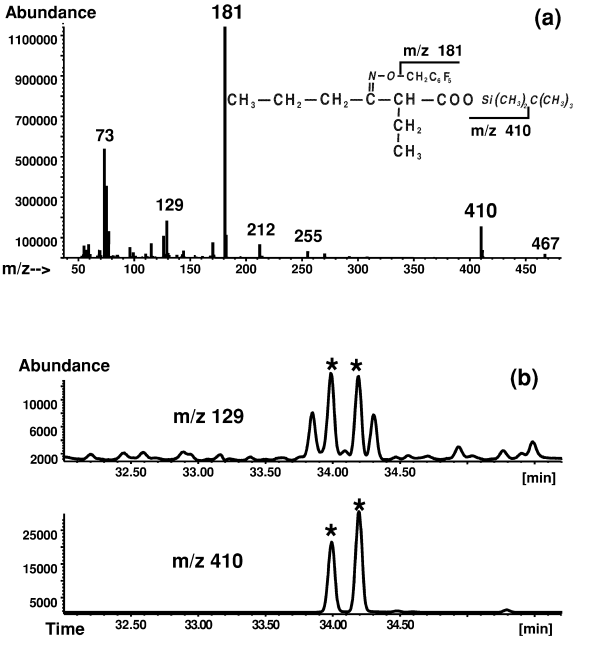

Fig. 4. (a) Mass spectrum of the *O*-(2,3,4,5,6-pentafluorobenzyl)oxime-tBDMS-ester of 2-ethyl-3-oxohexanoic acid standard (M_r 467); (b) Selected ion chromatogram of a urine sample from a healthy person (308 μg/l 2-ethyl-3-oxohexanoic acid).

25 to 224 μg/l) have been detected. Fig. 3a shows the mass spectrum of the TBDMS-ester of 2-ethyl-3-hydroxyhexanoic acid (M_r 274) and Fig. 3b the selected ion chromatogram of a urine sample. The ion m/z 217 [M-57] was used for quantitation and the ion m/z 199 for identification. As the synthesis of 2-ethyl-3-hydroxyhexanoic acid was done by $NaBH_4$-reduction of the racemic 2-ethyl-3-oxohexanoic acid ethyl ester the product yielded two diastereomeric forms which were separated on the GC column used in this method. There was an equal distribution of the two diastereomers in the standard solutions (peak area). In all the urine samples analyzed always both diastereomers were present and the sum of both was used for the quantitation (Fig. 3b, peaks at 22.05 and 22.85 min). The calibration curve was linear in the range from 20 to 1000 μg/l with a correlation coefficient of $r=$ 0.9915.

Fig. 4a shows the mass spectrum of the *O*-(2,3,4,5,6-pentafluorobenzyl)oxime-TBDMS-ester of 2-ethyl-3-oxohexanoic acid (M_r 467) and Fig. 4b the selected ion chromatogram of a urine sample. The formation of double peaks is in accordance with the *syn*- and *anti*-isomers formed by oximation of the reacting oxo group. Although the most abundant ion is m/z 181, which formally corresponds to the pentafluorotropylium ion $[C_6F_5CH_2]^+$,best quantitation results were obtained when using the area sum of the baseline separated peaks of the ion m/z 410 [M-57]. The ion m/z 129 was used for identification. The calibration curve was linear in the range from 20 to 1000 μg/l with a correlation coefficient of $r=$ 0.9986.

Intra-assay imprecision of the method was determined with different urine samples in the concentration range from 33.4 to 573.6 μg/l for all three acids by analyzing 10 aliquots of each sample in a row. The relative standard deviation (RSD) values determined were between 1.3 and 8.9% for all three acids with slightly but not significant better values for 2-ethylhexanoic acid.

Recoveries of all three acids were calculated from spiked urine samples. The recoveries of 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid and 2-

ethyl-3-oxohexanoic acid (with original concentrations of 23.8, 36.1 and 123.8 μg/l) were from 102.0 to 107.5%, 102.8 to 105.3% and 106.9 to 108.6% respectively based on spiked experiments with final concentrations up to 74.8, 138.9 and 558.2 μg/l respectively. The signal-to-noise ratio of the ions used for quantitation (ions *m/z* 201, 217 and 410) was higher than 10 for all three acids at a concentration of 2 μg/l, which is below any concentration found so far in urine samples.

In all 28 urine samples from healthy persons we found all three acids in different concentrations. Here we report for the first time the identification of 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid in human urine next to the known DEHP metabolite 2-ethylhexanoic acid. Table 1 shows the mean and median concentrations of all three acids expressed as μg/l and μg acid/g creatinine, which often is used in clinical studies to account for the different concentrations of urine specimens. In samples of urine from sawmill workers being exposed to 2-ethylhexanoic acid as a wood preservative the observed concentration range was reported to be 12.7 to 6881.4 μg 2-ethylhexanoic acid/g creatinine with a median of 127.4 μg/g [18]. After a single exchange transfusion in newborn infants the serum DEHP level was in the range from 6.1 to 21.6 mg/l with a median 2-ethylhexanoic acid of 174 μg/l in urine [17]. This seems reasonable with the here reported median concentration of 52 μg/l in urine of not specifically DEHP exposed adults.

The existence of both 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid in human urine, shown here, together with the occurrence of 2-and 4-heptanone in human urine reported earlier [21–23] provide further evidence for the postulated metabolism of DEHP to 2- and 4-heptanone as final products in humans [15,16] next to this known metabolic pathway in rats [12,16]. Especially the fact, that both diastereomers of 2-ethyl-3-hydroxyhexanoic acid are present in human urine make it very unlikely to be of endogenous origin (e.g. endogenous production of the D(−)-3-Hydroxybutyrate enantiomer). The contradictory results of 4-heptanone being elevated in diabetes [21] or not [36] could arise from the fact that the hospitalized diabetic patients [21] received intravenous infusions or were even on hemodialysis, thereby being exposed to pronounced amounts of DEHP, whereas the healthy control group did not receive any intravenous infusions. It has recently been shown, that if both groups are not hospitalized there is no difference in 4-heptanone concentrations in urine [23,37].

4. Conclusions

With the method described in this paper it is now possible to analyze 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid in urine with good reproducibility and sensitivity. This method should also be applicable to the analysis of any other aliphatic, hydroxy- or oxocarboxylic acid in urine.

The public interest in possible adverse effects of DEHP still persists today. The European Commission's decision of 7 December 1999 to prohibit soft PVC toys and childcare articles for children under three years of age shows the importance of validated methods for the analysis of DEHP and all its metabolites in humans and the need for further toxicological and molecular genetic studies. Here we report for the first time the identification of the postulated DEHP metabolites 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid in human urine next to the known DEHP metabolite 2-ethylhexanoic acid. Now further studies can be

Table 1
Concentrations of the three organic acids in human urine ($n=28$)

DEHP metabolite	Mean±SD [μg/l]	Mean±SD [μg/g creatinine]	Median [μg/l]	Median [μg/g creatinine]
2-Ethylhexanoic acid	56±14	68±38	52	57
2-Ethyl-3-hydroxyhexanoic acid	105±81	106±66	77	86
2-Ethyl-3-oxohexanoic acid	482±390	525±347	336	347

performed in groups being exposed to high amounts of DEHP with risk assessment studies including not only DEHP and MEHP concentrations, but rather all the known metabolites.

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