Research Article

Urinary 2-ethyl-3-oxohexanoic acid as major metabolite of orally administered 2-ethylhexanoic acid in human

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Human metabolism of 2-ethylhexanoic acid (2-EHA), which is a known metabolite of important phthalates, was investigated using 2-EHA-contaminated food. The results of our studies reveal that the major catabolic pathway of 2-EHA in human is β-oxidation. The dominant final urinary metabolite was identified and quantified as 3-oxo-2-ethylhexanoic acid (3-oxo-2-EHA), but only after immediate methylation of the extract from urine and prior to GC-MS analysis. Former studies without the precaution of immediate methylation had found 4-heptanone as the major metabolite, which is obviously an artifact arising from the decarboxylation of 3-oxo-2-EHA.

Keywords: 2-Ethylhexanoic acid / 2-Ethyl-3-oxohexanoic acid / 2-Ethyl-3-hydroxyhexanoic acid / 4-Heptanone / Human metabolism

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1 Introduction

The dialkyl- or alkyl/aryl esters of 1,2-benzenedicarboxylic acid, commonly known as phthalates, are high production-volume synthetic chemicals and ubiquitous environmental contaminants because of their use in plastics and other common consumer products [1]. Di-(2-ethylhexyl) phthalate (DEHP) and di-(2-ethylhexyl) adipate (DEHA) are the most abundant plasticizers in the environment. Their human metabolism is continuously under investigation [2 – 6]. 2-Ethylhexanoic acid (2-EHA) as one of their important metabolites is classified as embryotoxic, owing to results obtained from animal experiments (http://www.bgchemie.de/files/95/ToxBew275-L.pdf).

Food contamination with 2-EHA was observed during our recent studies in samples of baby food and fruit juices for infants [7]. The contamination stemmed from the lids of jars and bottles in which the foods and fruit juices had been packed. Salts of 2-EHA are used as stabilizers during the production of sealing compounds in order to render the seals thermostable [8].

As in biomarker studies, in which the concentration of 2-EHA in urine is monitored, back-calculation of the estimated intake, *e.g.*, of DEHA (from various sources) is done ([9]; Woollen, B. H., Russell, W., Report No: CTL/R/1372, dated 15.5.1998. Survey into the dietary intake of di-(2-ethylhexyl) adipate in Member States of the European Community. Unpublished report submitted to the European Commission, cf. *EFSA J.* 2006, *332*, 1 – 9), it is of general interest to check whether an additional dietary exposure with 2-EHA may influence the analytical data recorded in urine. As metabolites of 2-EHA, 3-oxo-2-ethylhexanoic acid (3-oxo-2-EHA), 3-hydroxy-2-ethylhexanoic acid (3-hydroxy-2-EHA), and 4-heptanone have already been reported [10]. However, from a chemical point of view it is questionable whether the latter is biochemically derived from 3-oxo-2-EHA or formed by decarboxylation in the course of the analysis. To clarify these points, we investigated the urinary excretion of orally administered 2-EHA using a small panel of seven persons. In this paper, the results of this preliminary study are described.

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Abbreviations: 2-EHA, 2-ethylhexanoic acid; **3-Hydroxy-2-EHA,** 3-hydroxy-2-ethylhexanoic acid; **LOQ,** Limits of quantification; **3-Oxo-2-EHA,** 3-oxo-2-ethylhexanoic acid; **DMSO,** dimethyl sulfoxide; **HRCG,** high-resolution gas chromatography

2 Materials and methods

2.1 Chemicals

Chemicals were purchased from Sigma-Aldrich (Steinheim, Germany). (±)-2-Ethylhexanoic acid (2-EHA), 4-hep-

tanone, potassium hydroxide, Diazald™, dimethyl sulphoxide (DMSO), and β-glucuronidase (from *Helix pomatia*) were purchased from Sigma-Aldrich. Heptanoic acid methyl ester (as internal standard) was from Fluka (Deisenhofen, Germany). Analytical grade solvents (purity > 98%) were redistilled and stored on molecular sieve (5 Å) before use. ACN (HPLC grade) was purchased from Fisher Scientific (Loughborough, UK).

2.2 Synthesis of 3-oxo-2-EHA methyl ester and 3-hydroxy-2-EHA methyl ester

The methyl esters of 3-oxo-2-EHA and 3-hydroxy-2-EHA were synthesized *via* their corresponding acids. The acids were achieved by alkaline treatment of the ethyl esters, as previously described [10]. Methylation of the acids using diazomethane was performed to yield the corresponding methyl esters for subsequent qualitative and quantitative analyses.

To obtain 3-oxo-2-EHA, its ethyl ester was synthesized by self-condensation of butyrate and diisopropyl magnesium bromide, as described in [11]. Then the ethyl ester was hydrolyzed by potassium hydroxide as reported in [10]. The spectroscopic data (^{1}H-NMR, high-resolution gas chromatography MS (HRGC-MS)) of the products were in agreement with that of the literature [10]. In addition, the identity of 3-oxo-2-EHA was checked by HPLC-ESI-MS/MS in the negative mode (m/z = 156.9 $[M-H]^-$, product ion m/z 112.7 [–44 U, $-CO_2$] at 15 eV) because of the low stability of the acid (decarboxylation) in the course of HRGC-MS and NMR analyses. 3-Oxo-2-EHA methyl ester was obtained using diazomethane, as described [12, 13]. The identity and purity (98.5% by HRGC-MS) of the methyl ester was proven by HRGC-MS and ^{1}H-NMR. ^{1}H-NMR: DMSO-d_6, 400 MHz: 1.42 (*O*-ethyl, *t*, 7.1 Hz, 3H), 3.78 (CH, *t*, 1H), 2.51 (CH_2, *t*, 7,3 Hz, 2H), 1.49 (CH_2, *m*, 7,1 Hz, 2H), 0.84 (CH_3, *t*, 7.4 Hz, 6H), 1.73 (CH_2, *m*, 2H), 4.11 (*O*-methyl, *q*, 2H). HRGC-MS, m/z (%):172 (5), 87 (27), 71 (100), 59 (26), 43 (60).

3-Hydroxy-2-EHA was synthesized from the corresponding 3-oxo-2-EHA ethyl ester, as described [10]. The formed diastereomers were hydrolyzed using potassium hydroxide. The spectroscopic data (HRGC-MS, ^{1}H-NMR) of 3-hydroxy-2-EHA were in agreement with that of the literature [10]. In addition, the identity of 3-hydroxy-2-EHA was proven by HPLC-ESI-MS/MS (negative mode) (m/z = 158.9 $[M-H]^-$, product ion m/z 86.8). 3-Hydroxy-2-EHA methyl esters were synthesized using diazomethane [12, 13]. The identity and purity (98.7%) of the diastereomers were proven by HRGC-MS. HRGC-MS, m/z (%):131 (47), 102 (67), 99 (44), 87 (100), 55 (31).

2.3 Intervention study design

The test panel of volunteers consisted of seven healthy persons (A – G, two females, five males) aged 23 – 33 (average 26 years). BMI ranged from 18.8 to 32.2 kg/m^2 (average 24.2 kg/m^2). In the first phase of the study, the panel was advised not to eat cheese (may contain 4-heptanone [14]) and to drink only the provided mineral water. Blank urine was collected within 24 h. In the second phase of the study, the test persons were requested to drink a fruit juice sample (1000 mL) containing 2 mg of 2-EHA [7] within 5 min. The drinking and eating limitations mentioned for the first phase were also kept for the second phase of the study. The panel was advised to collect every urine sample in a separate glass bottle during the next 24 h individually. The samples were analyzed immediately or frozen (–18°C) for subsequent HRGC-MS analysis.

2.4 Urine sample preparation

After acidification with hydrochloric acid (3.7%) to pH 5, 50 μg of internal standard (heptanoic acid methyl ester) was added to the urine sample (100 mL), prior to extraction. The urine was then extracted three times each with 25 mL diethyl ether. The extract was dried over anhydrous sodium sulphate, filtered, and methylated using diazomethane. Diazomethane was generated from an etheric solution of Diazald™ (5.2 g) in the presence of 2.0 g potassium hydroxide dissolved in 2 mL water, 6 mL ethanol, and 20 mL diethyl ether. The cooled etheric diazomethane solution (–10°C) was poured step by step into the colorless extract of the urinary sample within 30 min until it turned slightly yellow. After careful concentration to exactly 1 mL using a Vigreux column (40°C), the extracts were analyzed by HRGC-MS.

In order to identify the 2-EHA glucuronide, 800 mL urine (after 2-EHA consumption) was extracted three times each with 50 mL ethyl acetate. The organic fraction was dried over anhydrous sodium sulphate, filtered, evaporated to dryness under reduced pressure, and redissolved in 1 mL methanol for subsequent HPLC-MS/MS analysis. The 2-EHA glucuronide could not be found after immediate HPLC-MS/MS (ESI positive) measurement of the samples under study (data not shown).

In addition, samples of 100 mL urine were treated with β-glucuronidase (4 μg) and incubated at 37°C for 1 h as previously described [15]. Finally, 100 mL of urine samples were treated with 10 mL of sulphuric acid (5 M) at 100°C for 1 h as reported [16]. After diethyl ether extraction and subsequent HRGC-MS analysis, in both series of experiments 2-EHA (and therefore the 2-EHA glucuronide) was not detectable (data not shown).

2.5 HPLC-ESI-MS/MS

HPLC-ESI-MS/MS analysis was performed using a Finnigan TSQ 7000 apparatus (Finnigan MAT, Bremen, Germany) with an ESI interface coupled to an Applied Biosystems 140B pump. Data acquisition and evaluation were

conducted on a DEC 5000/33 (Digital Equipment, Unterföhring, Germany) using Finnigan MAT ICIS 8.1 software. HPLC chromatographic separations were carried out on a Eurospher C_{18} column, 100 × 2 mm, with 5 µm particle size (Knauer, Berlin, Germany). The mobile phase consisted of aqueous 0.1% formic acid (A) v/v and ACN (B) as well as, for the identification of potential glucuronides, of aqueous 5 mM ammonium acetate (A) v/v and ACN (B). The gradient applied was 5 – 99% B in 30 min at a flow rate of 0.2 mL/min, and 20 µL injection volume. The analysis was performed in the negative and positive ionization mode. The capillary temperature was 200°C and the spray capillary voltage was set to 3.2 kV. Nitrogen served as both sheath (70 psi) and auxiliary gas (10 U). The mass spectrometer was operated in the full scan mode, *m/z* 150 – 600, with a total scan duration of 1.0 s. MS/MS experiments were performed at a collision energy of 20 – 35 eV, with argon (2.0 mTorr) serving as collision gas. The multiplier voltage was set to 1300 V and for MS/MS experiments to 1800 V, respectively.

2.6 HRGC-MS

An HP Agilent 6890 Series gas chromatograph with split injection (1:20) was directly coupled to an HP Agilent 5973 Network mass spectrometer (Agilent Technologies Inc., CA, USA) using a J&W DB-Wax fused silica capillary column (30 m × 0.25 mm, df = 0.25 µm) and helium (1.0 mL/min) as carrier gas. The temperature program was held at 50°C for 3 min and then raised to 220°C at a rate of 4°C/min. The injector and interface temperatures were 220°C. Mass-selective detection (70 eV, electron impact) was performed in the scan mode (50 – 650 amu). Identifications were performed by comparison of linear retention indices and mass spectral data of sample constituents with that of authentic reference compounds.

2.7 Quantitation

For the determination of 3-oxo-2-EHA, 3-hydroxy-2-EHA, and 4-heptanone, calibration curves were performed using heptanoic acid methyl ester as internal standard (100 mg/L) and blank urine. Aliquots from a stock solution of 3-oxo-2-EHA methyl ester (3.3 mg/L), 3-hydroxy-2-EHA methyl ester (3.9 mg/L), 4-heptanone (1.0 mg/L), and heptanoic acid methyl ester as internal standard (100 mg/L) were added to blank urine (100 mL). The compounds were quantified by means of calibration curves (HRGC-MS peak area divided by internal standard area *versus* quotient of acid and internal standard concentration). Linearity for 3-oxo-2-EHA, 3-hydroxy-2-EHA, and 4-heptanone ranged from 0.1 to 1.5 mg/L, 0.2 to 1.0 mg/L, and 0.3 to 1.5 mg/L urine, respectively. Limits of quantification (LOQ) ranged from 0.1 to 0.3 mg/L, whereas limit of detection was 1 µg/L. All the experiments were performed in triplicate.

2.8 NMR

^{1}H-NMR spectra were recorded on a Bruker AVANCE 400 NMR spectrometer (Rheinstetten, Germany). DMSO-d_6 (stored on molecular sieve, 5 Å) was used as solvent. Data evaluation was performed using Mestre-C 4.4.1.0 software.

3 Results and discussion

In our first series of experiments, 4-heptanone, previously reported as an important metabolite of 2-EHA [15, 17, 18] was found in high amounts. In fact, HRGC-MS analysis revealed 4-heptanone as the main urinary metabolite of 2-EHA; however, shown by a number of additional experiments (data not shown), as decarboxylation artifact caused by thermal treatment in the course of sample preparation. Using fast methylation of all urinary acids by diazomethane, prior to concentration of the extract, in the urine samples under study, 4-heptanone was observed only as a minor metabolite whose amount ranged below the calibration curve (less than 0.1 mg/L), whereas 3-oxo-2-EHA was by far the major metabolite detected in the urine samples. In agreement with previous findings reported by Wahl *et al.* [10], we could also detect the reduced form of 3-oxo-2-EHA, *i.e.*, 3-hydroxy-2-EHA, but, as with 4-heptanone, this metabolite occurred in too low concentrations to be quantified in every urine sample.

In Fig. 1, a representative HRGC-MS profile of an analyzed urine is given. Table 1 summarizes the amounts of the urinary metabolites of 2-EHA. The 2-EHA glucuronide could not be found either by direct analysis or after enzymatic and acidic sample treatment (data not shown), and also the free 2-EHA was not detectable. Quantitative determination of 3-oxo-2-EHA revealed amounts ranging from 1.3 to 2.4 mg in urine collected within 24 h after the intake of the 2-EHA dose (test person E not considered). In addition, the quantity of 2-EHA equivalent based on the amount of 3-oxo-2-EHA was calculated and set in relation to the intake of 2 mg of 2-EHA. On an average, 98% or – neglecting the extreme value from test person E – 84% of the administered 2-EHA was recovered as 3-oxo-2-EHA in the urine.

Figure 2 shows the time course of the urinary 3-oxo-2-EHA. Within the range from 2 to 7 h after the intake of 2-EHA, the highest amount of 3-oxo-2-EHA was measured for the test persons A to F, respectively. After 12 h, the majority of test persons had excreted the metabolites of 2-EHA, *i.e.*, the values recorded for 3-oxo-2-EHA ranged in the area of their individual blank values. Considering the unusually high recovery value of the test person E (Table 1), it has to be assumed that this subject has ingested additional 2-EHA with the nutrition in the course of the study.

There is valuable information about the metabolism of 2-EHA in rodents and humans [15, 19, 20]. In some cases, it was compared with the body distribution of the structu-

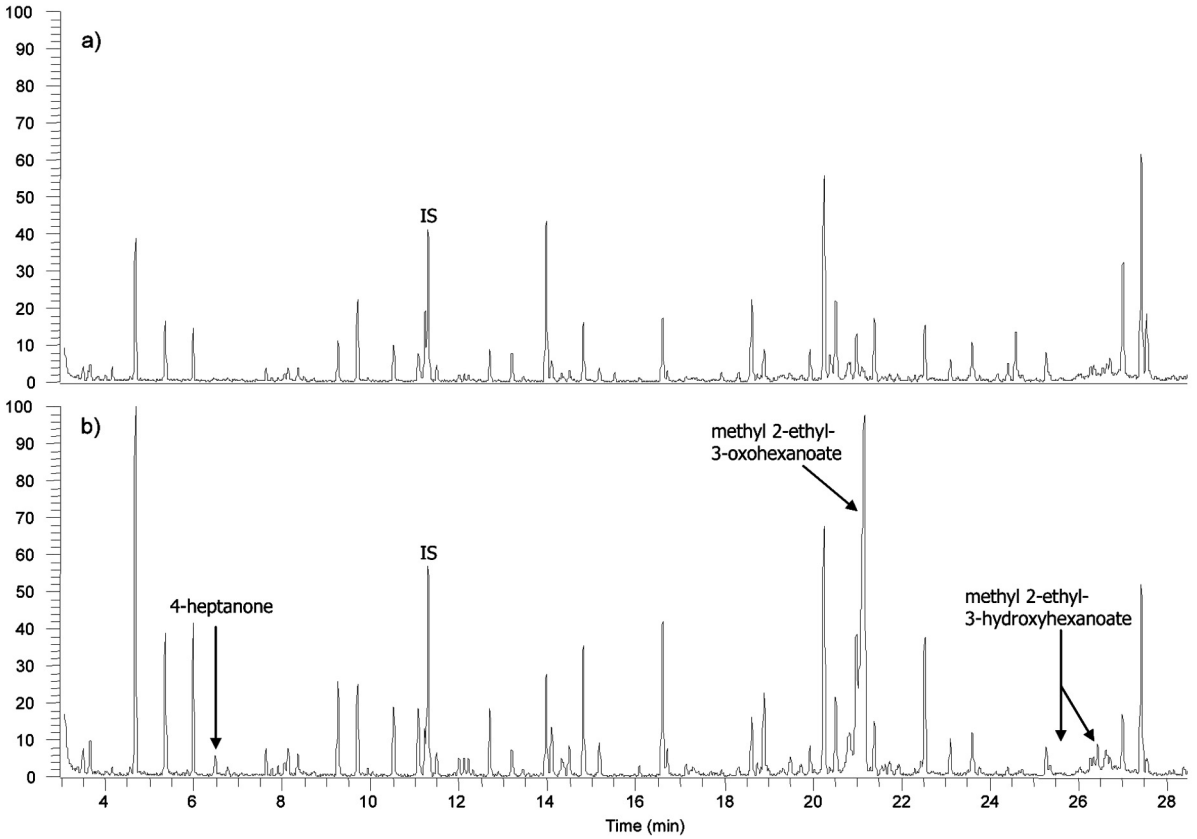

Figure 1. HRGC-MS profiles (TIC) of the analyzed urine from test person C (a, blank; b, after administration of 2-EHA). IS, internal standard (experimental conditions, *cf.* Section 2).

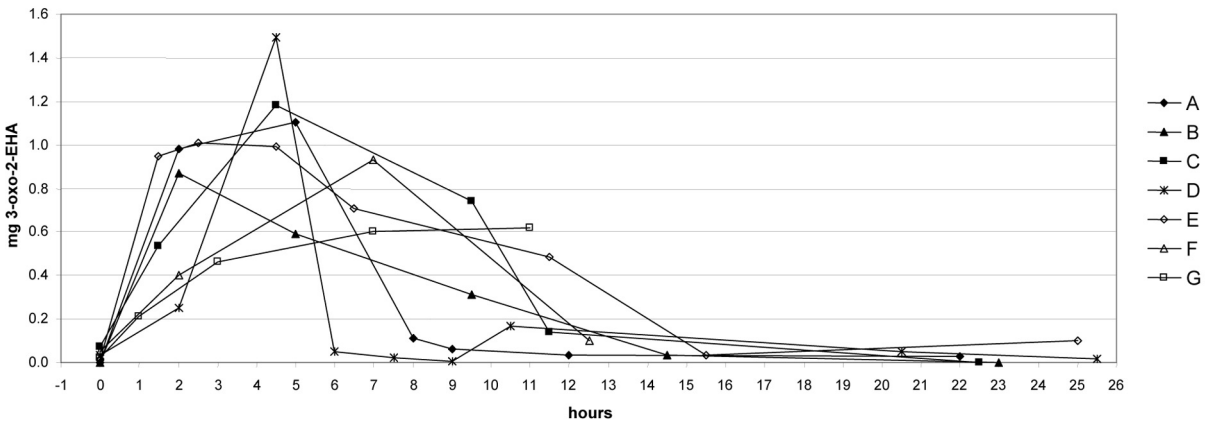

Figure 2. Time course of the urinary excretion of 3-oxo-2-EHA after administration of 2 mg each of 2-EHA to the panel members A–G.

rally similar valproic acid, an antiepileptic drug, as well [21]. Besides glucuronidation, the peroxisomal β-oxidation pathway of 2-EHA is favored for humans (Fig. 3) [15]. In rats ω_1- and ω-oxidation products such as 2-ethyl-1,6-hexanedioic acid, 2-ethyl-6-hydroxyhexanoic acid, 2-ethyl-5-hydroxyhexanoic acid, 2-ethyl-5-hexanoic acid, and 2-ethyl-5-oxohexanoic acid have been found after administration of large doses of 2-EHA [17]. As shown in Fig. 3, in humans an oxidative step-by-step degradation of 2-EHA to form 3-hydroxy-2-EHA and 3-oxo-2-EHA including decarboxylation to 4-heptanone has been described [15]. The authors have stated that the metabolization products of 2-ethylhexanol from DEHP-containing plasticizers are 3-hydroxy-2-EHA acid and 3-oxo-2-EHA besides the

Table 1. Urinary metabolites determined by HRGC-MS after oral administration of 2-EHA to the panel members A – G. Values for 3-oxo-2-EHA summarized over 24 h and recoveries (calculated as 2-EHA related to administered 2-EHA (2 mg)) are given (av, average value)

Subject	4-Hepta-none	3-Hydroxy-2-EHA	3-Oxo-2-EHA (mg in 24 h)	Recovery (%)
A	+	+	2.1	97.0
B	+	+	1.7	75.6
C	+	+	2.4	108.6
D	+	+	1.9	86.0
E	+	+	3.9	179.0
F[a)]	+	+	1.3	60.2
G[a)]	+	+	1.7	79.2
av	+	+	2.1	97.9

+ Substance identified but amount below LOQ.
a) Only 12 h values available.

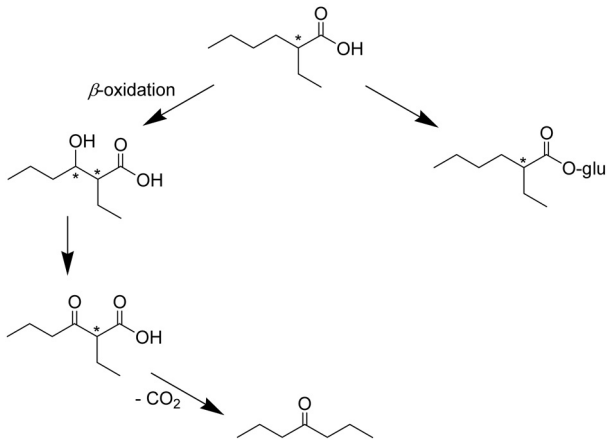

Figure 3. Proposed metabolic pathway of 2-EHA in human *via* β-oxidation [15] (simplified).

observed 4-heptanone [15]. Consequently, for 2-EHA the same metabolization pathway *via* β-oxidation was observed in our study. A possible reason why no 2-EHA-glucuronide was detected in our study in contrast to others, might be given in the different amounts of 2-EHA administered.

4 Concluding remarks

Our findings confirm the results of previous studies that the major catabolic pathway of 2-EHA in human is β-oxidation. The dominant final urinary metabolite was identified and quantified as 3-oxo-2-EHA, but only after immediate methylation of the extract from urine and prior to GC-MS analysis. Former studies without the precaution of immediate methylation had found 4-heptanone as the major metabolite, which is obviously an artifact arising from the decarboxylation of 3-oxo-2-EHA.

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