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Identification of the main metabolites of 2-ethylhexanoic acid in rat urine using gas chromatography–mass spectrometry

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ABSTRACT

The metabolites of 2-ethylhexanoic acid, an industrial chemical and the active ingredient in wood preservatives, were investigated in rat urine. Male Wistar rats were given 2-ethylhexanoic acid (2-EHA) in drinking water (600 mg/kg daily) for nine weeks, and then urine specimens were collected and analysed. The compounds were identified by gas chromatography–mass spectrometry in both electron-impact mode and chemical ionization mode. In addition to 2-EHA, ten different 2-EHA-related metabolites were found in the urine of 2-EHA-treated rats. The main metabolite was 2-ethyl-1,6-hexanedioic acid. Urine also contained 2-ethyl-6-hydroxyhexanoic acid and five other hydroxylated metabolites and two lactones, the detailed structures of which have not yet been elucidated. The unsaturated 5,6-dehydro-EHA was also identified; this is the metabolite corresponding to 2-*n*-propyl-4-pentenoic acid, the hepatotoxic metabolite of valproic acid. At least part of the 2-EHA is present in urine as a glucuronide conjugate.

INTRODUCTION

2-Ethylhexanoic acid (2-EHA) is an industrial chemical used *inter alia* in the production of soap. It is also the active ingredient in wood preservatives. The workers in saw-mills are exposed to this agent to the extent that measurable concentrations have been found in their urine after a work shift [1].

2-EHA is a branched carboxylic acid, the structure of which resembles closely that of valproic acid (VPA), a widely used antiepileptic drug. The metabolism of

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VPA in mammals has been studied extensively, and more than twenty metabolites have been identified using gas chromatography–mass spectrometry (GC–MS) [2,3]. One of these metabolites, 2-*n*-propyl-4-pentenoic acid, is known to be toxic to the liver [4] and several patients have even died during the course of VPA treatment [5,6]. Our preliminary results suggest that 2-EHA is also toxic to the liver. Inhibition of the urea cycle, accompanied by hyperammonia, has been observed in rats [7]. The mammalian metabolism of 2-EHA is not as yet known. It is quite possible that these two compounds are metabolized via the same routes.

As an initial step the metabolites of 2-EHA were identified in the urine of rats treated with 2-EHA. GC–MS was applied using both electron-impact (EI) and chemical ionization (CI) techniques. The metabolites of 2-EHA were extracted from rat urine with ethyl acetate and derivatized by methylation or trimethylsilylation. The identification of the metabolites was based on the interpretation of both the EI and CI mass spectra and accurate mass measurement of some specific ions. Ten metabolites were found in urine samples of rats treated with 2-EHA.

EXPERIMENTAL

Chemicals

Bis(trimethylsilyl)trifluoroacetamide (BSTFA, 98.5%), ethyl acetate, 2-ethylhexanoic acid (99.5%), sulphuric acid, sodium hydrogencarbonate and *n*-hexane were obtained from Merck (Darmstadt, Germany) and methanol from Baker (Deventer, Netherlands). The solvents were of analytical grade. For administration in the metabolic study, 2-ethylhexanoic acid was converted into the sodium salt.

In vivo testing

Six-week-old male rats (Han:Wistar, National Laboratory Animal Center, University of Kuopio, Kuopio, Finland), participating the peripostnatal toxicity study (125 ± 25 g), were used for this study. The animals received commercial rat chow (R3-EWOS) *ad libitum*, and they were kept under a light–darkness cycle of 14–10 h in an airconditioned animal room. For nine weeks they were given drinking water that contained 600 mg/kg 2-EHA as the sodium salt, and during this time the daily consumption of water was measured. The dose of 2-EHA was adjusted twice a week according to the most recent body-weight. Control animals received plain water. The animals were placed in metabolic cages at the end of the study, and 24-h urine samples were collected at room temperature and frozen until analysis. Six rats were included in each group.

Sample preparation

A 10-ml aliquot of urine was used for analysis. The urine sample was acidified (pH 2) with 4 *M* hydrochloric acid. This mild acidic hydrolysis was performed for all urine samples. Then the sample was extracted three times with 3 ml of ethyl

acetate. The solvent phases were combined and evaporated to dryness with nitrogen.

Silylation

To a dried sample, 100 μl of BSTFA were added in a glass test-tube. The tube was closed tightly and heated in a water-bath at 70°C for 30 min.

Methylation

In addition to silylation, the samples were methylated in order to confirm the identity of the 2-EHA metabolites. The dried sample was methylated by adding 300 μl of methanol (in 2% sulphuric acid) and heated in the tube in a water-bath at 70°C for 60 min. Thereafter the sample was allowed to cool to room temperature, and 450 μl of sodium bicarbonate (2%, in water) were added. The sample was extracted three times with 300 μl of *n*-hexane, and the organic phase was used for analysis.

Gas chromatography–mass spectrometry

The samples were analysed in a Hewlett-Packard Model 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) connected to a mass spectrometer. The instrument was equipped with a DB-5 fused-silica capillary column (30 m × 0.24 mm I.D., film thickness 0.25 μm) (J & W Scientific, Folsom, CA, USA). Helium was used as the carrier gas. The injector temperature was 280°C, and the samples (1 μl) were injected using splitless injection (30 s). The oven temperature was held at 80°C for 4 min and programmed at 5°C/min to 220°C. When glucuronides were analysed, the initial temperature was 100°C and the temperature was raised to 280°C at the rate of 20°C/min.

The EI mass spectra were measured with a 70-250SE magnetic sector mass spectrometer (VG Analytical, Manchester, UK). The ion source temperature was 200°C and the electron energy was 20 eV. The resolution of the instrument was adjusted to 10 000.

The CI mass spectra were measured with a VG Trio-2 quadrupole mass spectrometer (VG Masslab, Manchester, UK). The samples were chromatographed as described above. The temperature at the ion source was 200°C and the electron energy was 70 eV. Methane or ammonia was used as the reaction gas. The source pressure was $8 \cdot 10^{-4}$ mbar.

RESULTS

Identification of trimethylsilylated metabolites

The gas chromatogram of the silylated urine extracts from 2-EHA-treated rats shows eleven major 2-EHA related peaks (Fig. 1) that were not found in urine of untreated rats. The main metabolite (XI) was identified as 2-ethyl-1,6-dihexanedioic acid. Other identified peaks include the 5,6-dehydro-EHA (I), two lactones

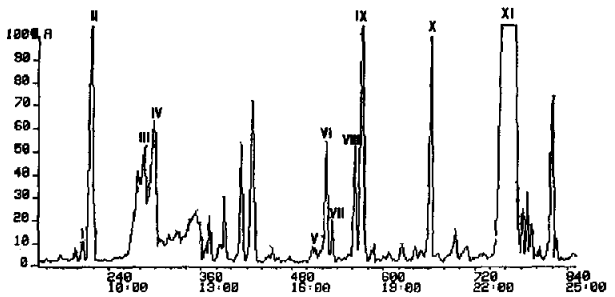

Fig. 1. Gas chromatogram of silylated urine extracts from a 2-EHA-treated rat. Peaks: I = 5,6-dehydro-EHA; II = 2-EHA; III and IV = lactones; V, VI, VII, VIII, IX and X = hydroxylated metabolites of 2-EHA; XI = 2-ethyl-1,6-hexanedioic acid.

(III and IV), six hydroxylated metabolites (V, VI, VII, VIII, IX and X) and 2-EHA (II).

2-EHA (II). The EI mass spectrum (Fig. 2) of the trimethylsilyl (TMS) ester of 2-EHA shows a weak molecular ion peak at *m/z* 216 and an intense $[M - CH_3]^+$ peak at *m/z* 201 (accurate mass 201.1300, calculated 201.1301 for $C_{10}H_{21}O_2Si$), which is typical of TMS derivatives. The peak at *m/z* 73 ($SiMe_3^+$) is typical of the mass spectra of TMS esters and ethers [3]. The peaks at *m/z* 160 ($[M - CH_3CH_2CHCH_2]^{+\cdot}$) (accurate mass 160.0947, calculated as 160.0920 for $C_7H_{16}O_2Si$) and at *m/z* 188 ($M - CH_2{=}CH_2]^{+\cdot}$) (accurate mass 188.1249, calculated 188.1232 for $C_9H_{20}O_2Si$) are formed by McLafferty rearrangement. The ion at *m/z* 57 is presumably due to the butyl moiety. The methane CI spectrum of 2-EHA shows a protonated molecular ion at *m/z* 217 as the base peak, and adduct ions at M + 29 and at M + 41. The major fragment ions are *m/z* 201 ($[MH - CH_4]^+$) and *m/z* 127 ($[MH - HOSiMe_3]^+$) (spectrum not shown).

Unsaturated 2-EHA (5,6-dehydro-EHA, I). The mass spectrum of a small peak (I, Fig. 1) appearing 15 s earlier than 2-EHA is reminiscent of the spectrum of silylated 2-EHA. However, it shows an abundant ion at *m/z* 199, presumably $[M - CH_3]^+$ (accurate mass 199.1154, calculated 199.1154 for $C_{10}H_{19}O_2Si$) and an M^+ ion at *m/z* 214 (Fig. 2). The methane and ammonia CI spectra of this analyte show abundant ions at *m/z* 215 ($[M + H]^+$) and at *m/z* 232 ($[M + NH_4]^+$), which suggests that the molecular mass of the compound is 214, *i.e.* two units less than that of 2-EHA. The absence of an ion at *m/z* 57 and the presence of an ion at *m/z* 55 (accurate mass 55.0551, calculated 55.0548 for C_4H_7) suggest that the compound is unsaturated 5,6-dehydro-EHA. The McLafferty rearrangement ion at *m/z* 160 indicates that the double bond is at the 5,6-position [8].

Hydroxylated metabolites, lactone form (III, IV). Peaks III and IV are presumably due to lactone formation of hydroxylated 2-EHA. The methane CI spectra of the compound show abundant MH^+ ions as base peaks at *m/z* 143 and adduct ions at M + 29 and M + 41 and a fragment ion at *m/z* 125 ($[MH - H_2O]^+$). The EI spectra show an abundant ion *m/z* 114 (accurate mass for compound III

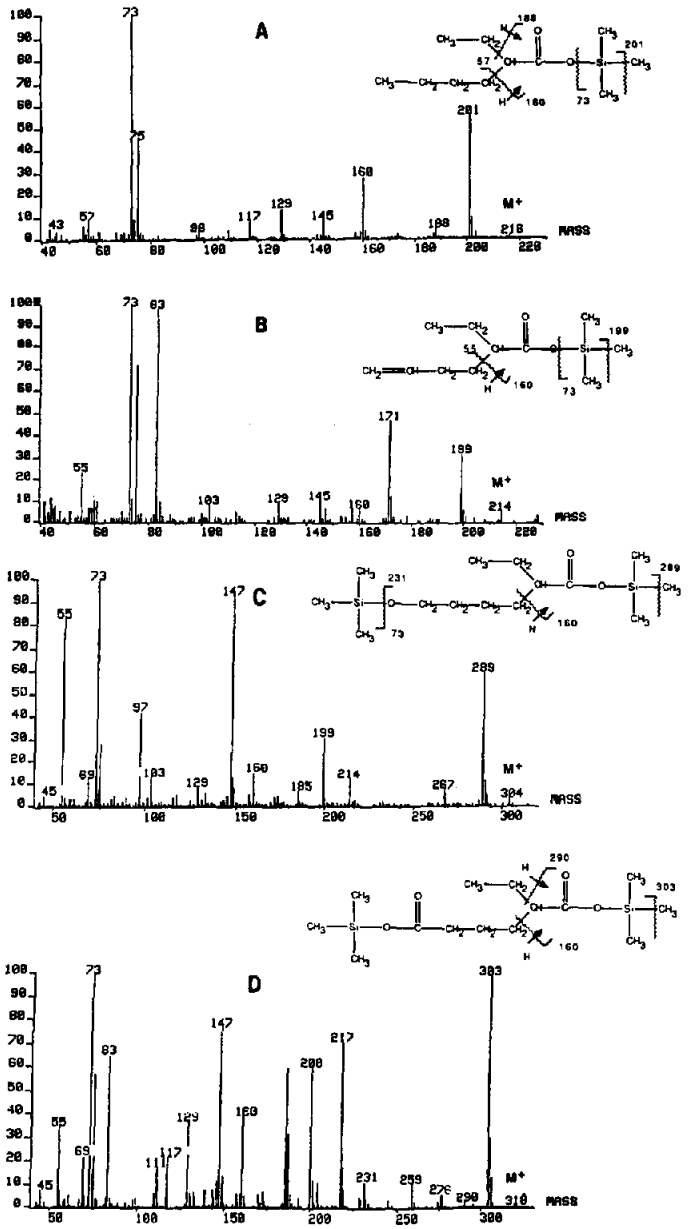

Fig. 2. EI mass spectra of trimethylsilylated derivatives of 2-EHA and its metabolites: (A) 2-EHA; (B) 5,6-dehydro-EHA; (C) 6-OH-EHA; (D) 2-ethyl-1,6-hexanedioic acid.

114.0669, and for IV 114.0685, calculated as 114.0689 for $C_6H_{10}O_2$). The respective ion is typical of VPA-lactones [9]. The exact structures cannot be drawn in the absence of model compounds.

Hydroxylated metabolites, pertrimethylsilylated (V, VI, VII, VIII, IX, X).

Peaks V, VI, VII, IX and X in the chromatogram are thought to be silylated hydroxyl metabolites of 2-EHA (Fig. 1). Their molecular masses were determined using methane CI. All these compounds show abundant MH^+ ions at *m/z* 305, and M + 29 and M + 41 adduct ions. All the CI spectra also show an ion at *m/z* 215 ($[M - HOSiCH_3]^+$). The EI spectra of all these metabolites show an ion at *m/z* 289 ($[M - CH_3]^+$) and an intense peak at *m/z* 147, which is typical for compounds containing two TMS groups [10]. Some of the presumed hydroxyl metabolites also show an M^+ ion at *m/z* 304. It is not possible to identify these hydroxy metabolites more fully from these data.

The mass spectrum of the metabolite VIII (Fig. 2C) also shows an ion at *m/z* 214 ($[M - HOSiMe_3]^{+\cdot}$) and at *m/z* 231 ($[M - SiMe_3]^+$). The ion at *m/z* 160 is formed by McLafferty rearrangement (accurate mass 160.0923, calculated 160.0919 for $C_7H_{16}O_2Si$) and suggests that metabolism has occurred in the longer side-chain. The presence of a terminal hydroxyl group was indicated by the ion at *m/z* 103, which is formed by α fission of TMS ether ($[Me_3Si{-}O{-}CH_2]^+$) [11] (accurate mass 103.0535, calculated 103.0579 for $C_4H_{11}OSi$). This ion was not abundant in the mass spectra of other hydroxyl metabolites of 2-EHA. The accurate mass of the $[M - CH_3]^+$ ion was 289.1658, calculated as 289.1655 for $C_{13}H_{29}O_3Si_2$, and the accurate mass of the M^+ ion was 304.1888, calculated as 304.1890 for $C_{14}H_{32}O_3Si_2$. Hence the metabolite is 2-ethyl-6-hydroxyhexanoic acid (6-OH-EHA).

Dicarboxylic acid, pertrimethylsilylated (XI). The main metabolite of 2-EHA (XI) was identified as 2-ethyl-1,6-hexanedioic acid. The CI mass spectrum indicates that the molecular mass of the compound is 318, as major ions are at *m/z* 319 ($[M + H]^+$), at *m/z* 347 ($[M + C_2H_5]^+$) and at *m/z* 229 ($[M - HOSiMe_3]^+$). The EI spectrum of the compound (Fig. 2) has many ions in common with the spectrum of silylated 2-propyl-1,5-pentanedioic acid [10]. However, typical ions for silylated 2-ethyl-1,6-hexanedioic acid are formed by McLafferty rearrangement at *m/z* 160 (accurate mass 160.0924, calculated as 160.0919 for $C_7H_{16}O_2Si$) and *m/z* 290. The supposed molecular ion peak at *m/z* 318 is very weak, but the $[M - CH_3]^+$ ion at *m/z* 303 is abundant (accurate mass 303.1463, calculated as 303.1448 for $C_{13}H_{27}O_4Si_2$).

2-EHA glucuronide. VPA glucuronide has been found in rat urine samples, after silylation, by GC–MS using CI [12]. We identified the corresponding 2-EHA glucuronide using similar methods. The methane CI spectrum of 2-EHA glucuronide (Fig. 3) is very similar to that of VPA glucuronide. The molecular ion $[M + H]^+$ at *m/z* 609 is weak, but the ion at *m/z* 593 ($[MH - CH_4]^+$) is more intense and is accompanied by ions at *m/z* 637 ($[M + C_2H_5]^+$) and at *m/z* 681 ($[M + SiMe_3]^+$). The ions at *m/z* 465 and at *m/z* 375 are typical of pertrimethylsilylated glucuronides [9]. The ion at *m/z* 127 is structurally very informative. It is an aglycone-containing ion that is formed by cleavage adjacent to the carbonyl group [13]. The ammonia CI mass spectrum (not shown) of 2-EHA glucuronide contains an ammonium adduct ion $[M + NH_4]^+$ at *m/z* 626 as the base peak.

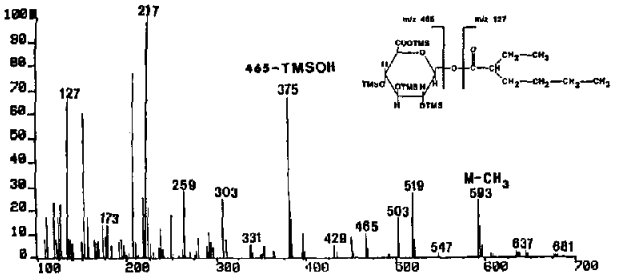

Fig. 3. Methane CI mass spectrum of the trimethylsilylated derivative of 2-EHA glucuronide.

Analysis of 2-EHA and metabolites after methylation

After extraction of the metabolites from urine, the carboxyl groups were methylated and the compounds analysed by GC–MS. This experiment supports the data obtained by analysing the metabolites after trimethylsilylation. The main peak related to 2-EHA in the gas chromatogram (Fig. 4) is methylated 2-ethyl-1,6-hexanedioic acid (peak V). The other main peak (II) originates from unmetabolized 2-EHA. Other peaks indentified were the 5,6-dehydro-EHA (I) and two unknown metabolites (III and IV).

Methylated 2-EHA (II). The EI mass spectrum (Fig. 5) of methylated 2-EHA is very similar to the spectrum of methylated VPA [14]. The M^+ ion at *m/z* 158 is weak (accurate mass 158.0993, calculated as 158.0993 for $C_9H_{18}O_2$). Ions at *m/z* 102 and at *m/z* 130 are due to McLafferty rearrangement (accurate masses 130.0993, calculated as 130.0993 for $C_7H_{14}O_2$, and 102.0616, calculated as 102.0681 for $C_5H_{10}O_2$). The $[M + H]^+$ ion at *m/z* 159 appears as the base peak in the methane CI spectrum.

Unsaturated methylated 2-EHA metabolite (I). The small chromatographic peak in front of methylated 2-EHA presumably originates from the methylated unsaturated 2-EHA metabolite, 5,6-dehydro-EHA (Fig. 5). The ion at *m/z* 55 is abundant. The low abundance of the ion at *m/z* 57 indicates that the longer

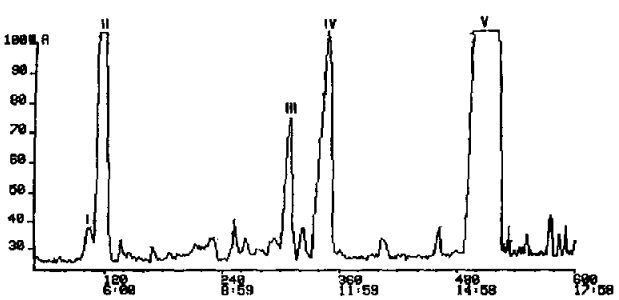

Fig. 4. Gas chromatogram of methylated urine extracts from 2-EHA-treated rats. Peaks: I = 5,6-dehydro-EHA; II = 2-EHA; III and IV = two unknown 2-EHA metabolites; V = 2-ethyl-1,6-hexanedioic acid.

side-chain is unsaturated and there exists an ion at *m/z* 102 (accurate mass 102.0671, calculated as 102.0681 for $C_5H_{10}O_2$), formed by McLafferty rearrangement so that the double bond is at carbon-5 [10]. The accurate mass of the M^+ ion was 156.1121, calculated as 156.1150 for $C_9H_{16}O_2$.

Methylated dicarboxylic acid (V). The main peak in the chromatogram (Fig. 4) is the methylated carboxyl metabolite of 2-EHA, 2-ethyl-1,6-hexanedioic acid. The molecular ion at *m/z* 202 in the EI spectrum (Fig. 5) is very weak, as in the EI spectrum of carboxylated VPA [14]. The ion at *m/z* 174 is presumably formed by loss of $CH_2=CH_2$ from the molecular ion (accurate mass 174.0852, calculated as 174.0892 for $C_8H_{14}O_4$). The ion at *m/z* 171 is $[M - CH_3O]^+$ (accurate mass 171.1009, calculated as 171.1021 for $C_9H_{15}O_3$). The ions at *m/z* 74 and at *m/z* 87 are typical for long-chain carboxylic acid methyl esters [8] (accurate masses 74.0385, calculated as 74.0367, and 87.0420, calculated as 87.0446). The methane CI spectrum shows abundant ions at *m/z* 203 (MH^+) and *m/z* 231 (M + 29).

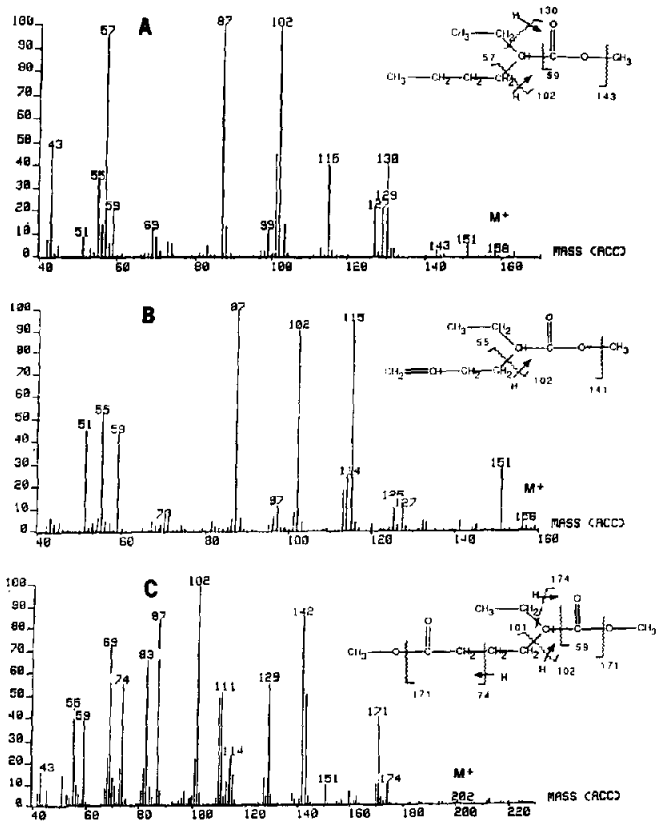

Fig. 5. Mass spectra of methylated 2-EHA and its metabolites: (A) 2-EHA; (B) 5,6-dehydro-EHA; (C) 2-ethyl-1,6-hexanedioic acid.

DISCUSSION

These results suggest that 2-EHA is metabolized in the rat to several metabolites. The main metabolite appears to be 2-ethyl-1,6-hexanedioic acid, which corresponds to the dicarboxylic metabolite of VPA that has been found in large amounts in urine specimens from VPA-treated rats [10].

One of the hydroxylated 2-EHA metabolites (VIII, Fig. 1) was identified as 2-ethyl-6-hydroxyhexanoic acid. The occurrence of this compound could be predicted as 2-EHA must first be oxidized to a 6-hydroxy compound before further oxidation to the 6-oxo derivative and 6-carboxylic acid [15]. The 6-hydroxy compound and 6-carboxylic acid were both identified after silylation and methylation of the urine samples, but the 6-oxo derivative was not identified.

There were five other hydroxylated metabolites and two lactones, the detailed structures of which cannot be determined on the basis of these data. Synthesis of these metabolites is necessary for their further identification. In addition to the metabolites, unmetabolized 2-EHA was present in rat urine.

The stage of conjugation of these metabolites remained uncertain because only mild acidic hydrolysis was performed on the urine samples, and standards for quantitative determination were not available. A glucuronide conjugate was identified for 2-EHA with CI, suggesting that at least part of the 2-EHA is excreted in urine as this conjugate. In the rat, most of the valproic acid is excreted in the urine as a glucuronide [12].

One metabolite of valproic acid is 2-*n*-propyl-4-pentenoic acid, which is hepatotoxic [4]. This compound has recently been shown to be produced via ω-1-hydroxylation and subsequent dehydration, catalysed by an enzyme system including cytochrome P-450 [3]. 2-EHA seems to have the same kind of metabolite, 5,6-dehydro-EHA. 2-*n*-Propyl-4-pentenoic acid acts as an active inhibitor of both cytochrome P-450 and β-oxidation enzymes in rat liver [16,17]. It remains to be studied whether it is 2-EHA or one of its metabolites that causes the liver effects observed [7].

2-EHA is an intermediate in the metabolism of the plasticizer di(2-ethylhexyl) phthalate [18,19]. These results indicate that 2-EHA is yet further metabolized and 2-EHA is not the final step in the metabolism of this plasticizer.

These preliminary results indicate that 2-EHA undergoes intensive metabolism in mammals. The exact pathways of the metabolism, as well as their significance for toxicity, remain to be further elucidated.

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