In Vivo Studies on the Metabolism of the Monoterpenes *S*-(+)- and *R*-(−)-Carvone in Humans Using the Metabolism of Ingestion-Correlated Amounts (MICA) Approach

Wolfgang Engel[†]

Deutsche Forschungsanstalt für Lebensmittelchemie, Lichtenbergstrasse 4, D-85748 Garching, Germany

The major in vivo metabolites of *S*-(+)- and *R*-(−)-carvone in a metabolism of ingestion correlated amounts (MICA) experiment were newly identified as α,4-dimethyl-5-oxo-3-cyclohexene-1-acetic acid (dihydrocarvonic acid), α-methylene-4-methyl-5-oxo-3-cyclohexene-1-acetic acid (carvonic acid), and 5-(1,2-dihydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one (uroterpenolone) on the basis of mass spectral analysis in combination with syntheses and NMR experiments. Minor metabolites were identified as reduction products of carvone, namely, the alcohols carveol and dihydrocarveol. The previously identified major in vivo metabolite in rabbits, 10-hydroxycarvone, could not be detected, indicating either concentration effects or interspecies differences. Metabolic pathways for carvone in humans including oxidation of the double bond in the side chain and, to a minor extent 1,2- and 1,4 + 1,2-reduction of carvone, are discussed. No differences in metabolism between *S*-(+)- and *R*-(−)-carvone were detected.

Keywords: *Metabolism of ingestion correlated amounts (MICA); urinary metabolites; α,4-dimethyl-5-oxo-3-cyclohexene-1-acetic acid; α-methylene-4-methyl-5-oxo-3-cyclohexene-1-acetic acid; 5-(1,2-dihydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one; carvone; carveol; dihydrocarveol*

INTRODUCTION

Terpenes such as citral, carvone, limonene, terpineol, or cineol play an important role in food. They are responsible for many distinct aromas, in particular those of spices, and their amount is sometimes correlated with the quality of the spice. Their occurrence in foods ranges from some micrograms to even grams per kilogram.

After ingestion, terpenes are well absorbed, together with fat, which acts as the carrier. It is generally accepted that metabolism of terpenes starts with hydroxylation or epoxidation by microsomal monooxidases. Primary metabolites formed are further transformed into more polar compounds, such as diols or carboxylic acids, and excreted via urine as highly water soluble adducts with sulfuric acid or glucuronic acid or as mercapturic acids.

Despite the ubiquitous occurrence and importance of terpenes in food, little is known about the metabolic fate of terpenes in mammals, especially humans. Most of the data available have been obtained from rats (*1*−*4*), rabbits (*5*, *6*), or different antipodean species (*7* and references cited therein) feeding on terpene-rich eucalyptus diets. However, it remains unclear whether the data from these studies can be applied to humans as there may be significant interspecies differences in metabolism. Another shortcoming is that the doses applied to the test animals were much higher (up to 1000 mg/kg of body weight) compared to amounts present in a normal diet. Often the animals have been administered toxic doses of the terpene (*6*) to obtain large quantities of metabolites, for example, for NMR experiments. However, there is some evidence that terpene concentration might have an effect on the structure of the metabolism product itself as previously shown for pine oil metabolites in humans. In one study *cis*- and *trans*-verbenol were found (*8*), and in another case myrtenol, borneol, and bornyl acetate (*9*) appeared to be the major metabolites.

Only a few studies have been undertaken to clarify the metabolism of terpenes in man (*10*). These were either focused on terpenes for use as drugs (*11*) or on a special group of individuals with unusually high exposure to terpenes (*8*, *12*). In addition, Zlatkis and Liebich (*13*) identified terpenes (carvone, limonene, and terpineol) in the urine of unexposed persons, indicating a considerable intake of terpenes via food and cosmetics.

To our knowledge no data are available for terpene metabolism in humans focusing on terpene intake corresponding to a normal diet. The only data available refer to medical treatment, occupational exposure, or accidental poisoning with high amounts of terpenes. The purpose of the present study was, therefore, to obtain initial insight into the in vivo metabolism of low doses of carvone in humans. To obtain data as close as possible to a daily intake situation, the metabolism of ingestion-correlated amounts (MICA) approach was developed. Carvone was chosen for this experiment because it is a constituent of most essential oils and is widely used as a flavor compound, for example, for toothpaste and other cosmetics.

MATERIALS AND METHODS

Chemicals. *S*-(+)-Carvone, *R*-(−)-carvone, carveol, dihydrocarveol, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), sodium chlorite, sodium hypochlorite solution, 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO), and *m*-chloroperbenzoic acid (MCPBA, 70%, technical grade) were from Fluka

[†] Telephone 0049 (0)89 28914375; fax 0049 (0)89 28914183; e-mail wolfy@dfa.leb.chemie.tu-muenchen.de

10.1021/jf010157q CCC: $20.00
Published on Web 07/25/2001

(Buchs, Switzerland). β-Glucuronidase H2 from *Helix pomatia* (100000 units of β-glucuronidase and 4500 units of sulfatase per milliliter) was from Sigma (Steinheim, Germany), and carragheen type 1 was from Serva (Heidelberg, Germany). Glycerol was from Merck (Darmstadt, Germany).

Development of the MICA Experiment. The study was approved by the "Freie Ethikkommission München". Six volunteers [three males (one smoker and two nonsmokers) and three females (two smokers and one nonsmoker)] received a controlled diet starting with lunch 24 h prior to the experiment to avoid uncontrolled intake of terpenes. Any intake of food, sporting activity, and urine volumes were documented by the volunteers. The amounts were adjusted to the eating habits of each participant but were observed strictly throughout the following days of the experiment.

The diet of each of the following days consisted of the following food. In the morning, each participant received chocolate muesli (50−150 g) with milk (75−200 mL); for lunch, a light meal was prepared consisting of potatoes with a little butter, chicken breast, and a salad with yogurt dressing; every volunteer had to drink 500 mL of milk at lunchtime; in the evening each participant was free to eat any of the following foods: bread, rolls, milk products, and meat products entirely free of spices. For drinks, milk, water, coffee, and tea were allowed. No fruit juices were consumed throughout the experiment. After 24 h of "adjustment", within the next 24 h the diet was continued and the 24 h urine (control) was collected. Then a single dose of carvone (0.5 mmol, ∼1 mg/kg of body weight) was ingested as a solution in full-fat milk (500 mL) at lunchtime, and again a 24 h urine sample (test) was collected. All urine samples were stored immediately in a freezer at −20 °C. Total diet period was 72 h. To avoid intake of terpenes from toothpaste, a terpene-free toothpaste was prepared.

Toothpaste Recipe. Sodium dodecyl sulfate (SDS; 10 g) was dissolved in water (119.6 g; solution 1). Carragheen type 1 (9 g) and ethanol (18 g) were mixed, and glycerol (180 g) was added in small portions under vigorous stirring with a mechanical stirrer (solution 2). The SDS solution 1 (99 g) was added in small portions to solution 2, resulting in solution 3. Finely powdered $CaCO_3$ (90 g) was placed in a mortar, and solution 3 (204 g) was added in small portions. After each addition, the resulting mixture was homogenized thoroughly. The toothpaste contained about 30% $CaCO_3$, 20% water, 40% glycerol, 4% ethanol, 2% SDS, and 2% carragheen type 1.

Isolation of Metabolites from Urine. The frozen urine samples were warmed to room temperature. The pH of the samples was between 6.1 and 6.5 with a total volume ranging from 0.7 to 4.2 L. The differences between test and control urine volume of an individual participant were in the range of ±5%. An aliquot of 100 mL was withdrawn and adjusted to pH 5.0 with concentrated HCl. A solution of glucuronidase and sulfatase from *Helix pomatia* (100 μL) was added to both samples. The samples were hydrolyzed for 72 h at 37 °C and adjusted to pH 2.0 with concentrated HCl, and the metabolites were extracted with peroxide free diethyl ether [2 × 200 mL; total fraction (TF)]. The acids were separated from TF by washing with saturated $NaHCO_3$ solution (3 × 40 mL) and re-extracted with diethyl ether [3 × 50 mL; acidic fraction (AF)] after careful acidification. Phenolic compounds were separated from the organic phase by washing with 0.1 mol/L NaOH (3 × 40 mL) and re-extracted with diethyl ether [3 × 50 mL; phenolic fraction (PF)] after acidification. The remaining ether phase [∼150 mL; neutral fraction (NF)] contained the neutral metabolites. All extracts were concentrated to ∼3 mL by means of a rotary evaporator at 37 °C.

Derivatization Procedures. *Trimethylsilylation.* Trimethylsilylation was performed using *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA). BSTFA (50 μL) was added to the ethereal solution (150 μL) of each fraction and the mixture kept at room temperature for 60 min.

Ethylation. Iodoethane was used as ethylation agent. The ethereal urine sample (150 μL) was reacted with iodoethane (50 μL) in the presence of K_2CO_3 (10 mg) and tributyl hexadecyl phosphonium bromide (10 mg) overnight at room temperature.

Identification of Metabolites. GC-MS runs of each fraction of test and control urine (TF, AF, PF, and NF; derivatized and underivatized) were recorded. Metabolites were localized by mass trace comparison. Each mass trace starting from *m*/*z* 43 to 250 was compared. Substances that were absent in control were considered to be metabolites. Structures were proposed from the obtained mass spectral data and compared to those of authentic samples either commercially available or synthesized as described.

Reference Compounds. Authentic standards of α,4-dimethyl-5-oxo-3-cyclohexene-1-acetic acid (dihydrocarvonic acid), α-methylene-4-methyl-5-oxo-3-cyclohexene-1-acetic acid (carvonic acid), 5-(1,2-dihydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one (uroterpenolone), 5-[1-(hydroxymethyl)ethenyl]-2-methyl-2-cyclohexen-1-one (10-hydroxycarvone), and 2-methyl-5-(1-methyl-1-oxiranyl)-2-cyclohexen-1-one (8,9-epoxycarvone) were prepared according to the procedures described below. All syntheses started from *S*-(+)- or *R*-(−)-carvone, retaining stereochemistry at C-4 of the menthane skeleton.

Synthesis of α,4-Dimethyl-5-oxo-3-cyclohexene-1-acetic Acid (M1). M1 was synthesized in a three-step sequence.

2-Methyl-5-(1-methyl-1-oxiranyl)-2-cyclohexen-1-one (8,9-Epoxycarvone). The synthesis was carried out according to a published procedure (*15*): MS (EI), *m*/*z* (relative intensity) 109 (100), 108 (86), 82 (47), 91 (47), 39 (44), 79 (43), 54 (38), 107 (36), 53 (34), 97 (30), 123 (22).

α,4-Dimethyl-5-oxo-3-cyclohexene-1-acetaldehyde. To a rapidly stirred solution of 5-(1-methyl-1-oxiranyl)-2-methyl-2-cyclohexen-1-one (8,9-epoxycarvone, 3 mmol; 0.5 g) in diethyl ether (50 mL) was added dropwise BF_3 (0.5 mL) in diethyl ether. The dark red reaction mixture was stirred for 30 min at room temperature. The mixture was washed with saturated $NaHCO_3$ solution (10 mL), dried over Na_2SO_4, and evaporated to dryness. Yield was 0.35 g (70%) of crude aldehyde, which was immediately used for the next reaction: MS (EI), *m*/*z* (relative intensity) 108 (100), 109 (90), 82 (42), 79 (35), 80 (34), 54 (27), 39 (24), 81 (23).

α,4-Dimethyl-5-oxo-3-cyclohexene-1-acetic Acid (Dihydrocarvonic Acid). The synthesis was performed following a general procedure given in ref *16* using crude 2-(4-methyl-5-oxo-3-cyclohexenyl)propanal (∼1 mmol; 166 mg): yield 120 mg (66%); elemental composition (HRMS), $C_{10}H_{14}O_3$; ^{1}H NMR ($CDCl_3$) CH_3−CH (1.23, d, 3H), CH_3−C= (1.79, s, 3H), CH_2,CH,CH_2 (2.2−2.6, m, 6H), −CH=C (6.78, m, 1H), −COOH (11.47, s, 1H); MS (EI), *m*/*z* (relative intensity) 109 (100), 74 (26), 82 (25), 54 (17), 108 (16), 79 (13), 81 (13), 39 (11); ^{13}C NMR ($CDCl_3$, mixture of two diastereoisomers) CH_3−C= (16.71/16.82), CH_3−CH (18.49 signals unresolved), −CH−COOH= (32.08/33.09), −CH−CH_2 (40.66/40.72), −CH_2−CH= (44.06/45.07), −CH_2−C=O (46.61/46.78), CH_3−C= (138.49 signals unresolved), −HC=C− (147.67/147.75), −COOH (183.56/183.76), =C−C=O (202.40/202.48).

Synthesis of α-Methylene-4-methyl-5-oxo-3-cyclohexene-1-acetic Acid (M2). M2 was synthesized in a five-step sequence.

5-[1-(Chloromethyl)ethenyl]-2-methyl-2-cyclohexen-1-one. The synthesis was carried out according to that in ref *17* starting with *S*-(+)-carvone or *R*-(−)-carvone (10 mmol), and the product was further used without purification: MS (EI), *m*/*z* (relative intensity) 149 (100), 82 (78), 142 (69), 39 (57), 107 (48), 105 (44), 93 (41), 91 (40).

Acetic Acid [2-(4-methyl-5-oxo-3-cyclohexenyl)-2-propenyl] Ester. The crude 5-[1-(chloromethyl)ethenyl]-2-methyl-2-cyclohexen-1-one (estimated 8 mmol) was dissolved in *tert*-butyl methyl ether (40 mL). After the addition of tributyl hexadecyl phosphonium bromide (0.1 mmol) and sodium acetate (20 mmol) dissolved in water (10 mL), the mixture was heated under reflux (56 °C) for 10 h. The aqueous phase was removed, and again tributyl hexadecyl phosphonium bromide (0.1 mmol) and sodium acetate (20 mmol) in water (10 mL) were added. Further heating for 10 h led to total conversion of 5-[1-(chloromethyl)ethenyl]-2-methyl-2-cyclohexen-1-one. Purification by column chromatography (*17*) yielded 1.65 g (79% yield

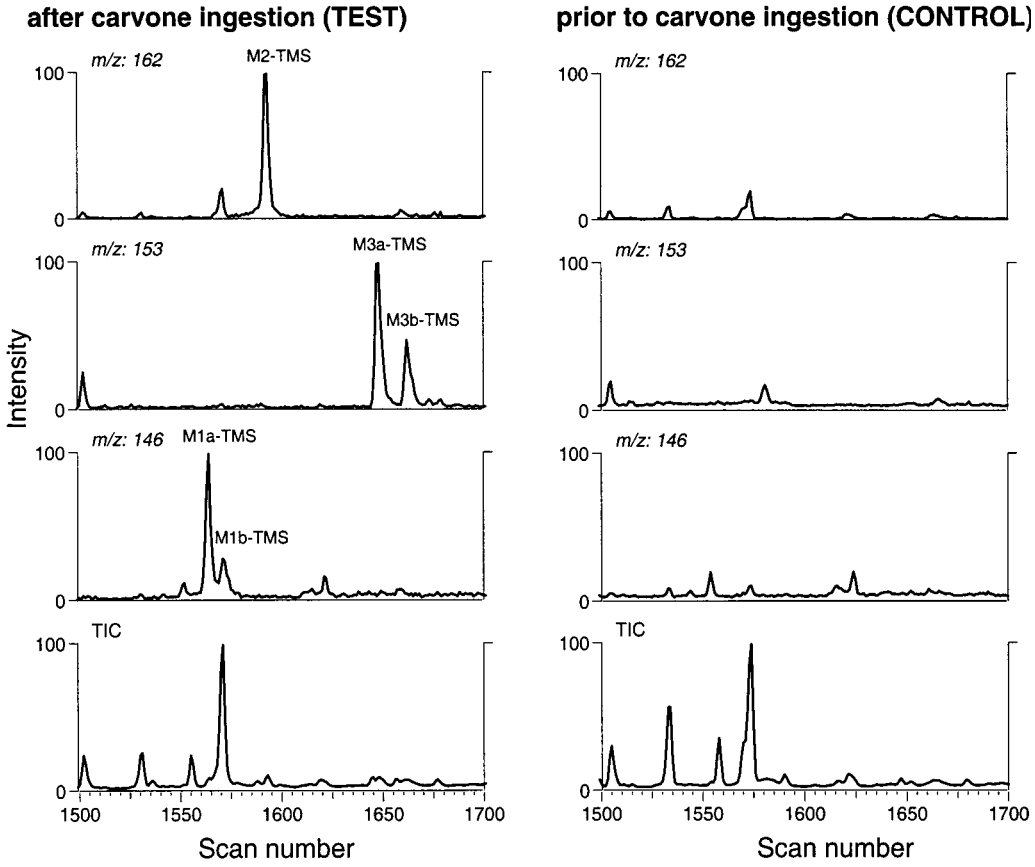

Figure 1. Localization of major metabolites (**M1a**, **M1b**, **M2**, **M3a**, and **M3b**) by mass trace comparison in the acidic fraction (both samples TMS-derivatized).

based on carvone) of the ester: MS (EI), *m/z* (relative intensity) 148 (100), 43 (57), 82 (47), 91 (46), 106 (42), 105 (41), 54 (28), 108 (26).

5-[1-(Hydroxymethyl)ethenyl]-2-methyl-2-cyclohexen-1-one (10-Hydroxycarvone). The synthesis was carried out according to that in ref *17*. A yield of 1.0 g (86%) of 5-[1-(hydroxymethyl)-ethenyl]-2-methyl-2-cyclohexen-1-one was obtained: MS (EI), *m/z* (relative intensity) 148 (100), 39 (70), 106 (70), 41 (59), 53 (56), 54 (56), 91 (50), 82 (49), 105 (48), 133 (18), 135 (16), 166 (10).

α-*Methylene-4-methyl-5-oxo-3-cyclohexene-1-acetaldehyde.* The synthesis was performed according to that in ref *16*. 5-[1-(Hydroxymethyl)ethenyl]-2-methyl-2-cyclohexen-1-one (1 mmol; 166 mg) was used. The resulting crude aldehyde was immediately used for further synthesis without any purification. No byproducts were present as checked by HRGC-MS: MS (EI), *m/z* (relative intensity) 108 (100), 135 (61), 164 (53), 82 (40), 54 (38), 39 (35), 121 (25), 91 (25).

α-*Methylene-4-methyl-5-oxo-3-cyclohexene-1-acetic Acid (Carvonic Acid).* The oxidation of α-methylene-4-methyl-5-oxo-3-cyclohexene-1-acetaldehyde to the corresponding acid was performed according to the procedures described in refs *18* and *19*. The crude aldehyde was used without further purification, and 2-methylbutene was used as chlorine scavenger. The target compound α-methylene-4-methyl-5-oxo-3-cyclohexene-1-acetic acid (105 mg; yield based on the alcohol 58%) was obtained as a colorless liquid: elemental composition (HRMS), $C_{10}H_{12}O_3$; ^{1}H NMR ($CDCl_3$) CH_3−C= (1.80, s, 3H), CH_2,CH_2−ring (2.4−2.7, m, 4H), CH−ring (3.25, m, 1H), =CH′H″ (5.71, m, 1H), =CH′H″ (6.44, m, 1H), −CH_2−CH= (6.77, s, 1H), −COOH (9.34, s, 1H); MS (EI), *m/z* (relative intensity) 162 (100), 54 (78), 134 (70), 82 (69), 39 (65), 135 (55), 109 (52), 53 (45), 91 (40), 180 (43), 41 (34), 108 (26). Possibly due to rapid polymerization, only uninterpretable ^{13}C NMR data were obtained.

Synthesis of 5-(1,2-Dihydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one (M3). To a magnetically stirred solution of *2-methyl-5-(1-methyl-1-oxiranyl)-2-cyclohexen-1-one* (5 mmol; 0.83 g) in THF (10 mL; freshly distilled) was added a mixture of H_2SO_4 (200 mg) in water (10 mL). After 2 h at room temperature, no epoxide was detectable by HRGC-MS. The reaction mixture was diluted with water (20 mL) and adjusted first to pH 6 by adding $NaHCO_3$ and finally acidified to pH 3 by dropwise addition of 85% H_3PO_4. The aqueous mixture was washed with diethyl ether (2 × 20 mL) to remove acidic and neutral byproducts. The combined organic phases were washed with water (5 mL; adjusted to pH 3 with 85% H_3PO_4), and the combined aqueous phases were evaporated under reduced pressure to yield the desired diol together with Na_2SO_4 and NaH_2PO_4. The diol was separated from the salts by washing the residue with a mixture of ethanol and diethyl ether (20 mL total volume; 50:50, v/v). Column chromatography on a diol phase (Bakerbond Diol, 40 μm, pentane/diethyl ether gradient) gave pure 5-(1,2-dihydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one (uroterpenolone) as a viscous colorless liquid: yield 0.75 g (81%); ^{1}H NMR (DMSO-d_6) CH_3−C= (1.01, s, 3H), CH_3−C−OH (1.66, s, 3H), CH_2,CH_2,CH−ring (2.1−2.5, m, 5H), −CH_2−OH (3.16−3.35, m, 2H), −OH (4.21, s, 1H), −OH (4.59, s, 1H), −CH=C− (6.83, m, 1H); ^{13}C NMR (DMSO-d_6) CH_3−C= [15.21/15.23 (DEPT +)], CH_3−C−OH [21.63/21.82 (DEPT +)], −CH_2−CH= [26.15/26.58 (DEPT −)], −CH_2−C=O [38.41/38.93 (DEPT −)], −CH−C−OH [40.78/41.02 (DEPT +)], −CH_2−OH [67.12/67.18 (DEPT −)], HO−C−CH_2−OH [72.01/72.05 (DEPT 0)], CH_3−C= [133.61/133.69 (DEPT 0)], −HC=C− [145.87/146.15 (DEPT +)], =C−C=O [199.53/199.78 (DEPT 0)]; MS (EI), *m/z* (relative intensity) 109 (100), 110 (47), 153 (42), 43 (27), 75 (21), 135 (20), 95 (15), 103 (15).

NMR Spectroscopy. NMR spectra were recorded on a Bruker AM 360 (Bruker, Karlsruhe, Germany) in $CDCl_3$ (unless otherwise stated) with TMS as internal standard (δ = 0 ppm).

High-Resolution Gas Chromatography (HRGC)-Mass Spectrometry (MS). HRGC was performed using a DB-5 capillary column (30 m × 0.32 mm; J&W Scientific, Folsom,

CA) in a gas chromatograph type 8000 (ThermoQuest, Egelsbach, Germany). Sample injection was performed using the split technique at a temperature of 230 °C and a split ratio of 1:25. The volumes injected ranged from 1 to 20 μL. The temperature during HRGC was held at 35 °C for 1 min and raised to a final temperature of 230 °C at a rate of 6 °C/min. Mass spectra were recorded on an MD 800 quadrupole mass spectrometer (ThermoQuest) in the electron ionization (EI) mode at 70 eV.

HRGC-High-Resolution Mass Spectrometry (HRMS). HRGC was performed on a DB-5 capillary column (30 m × 0.25 mm; J&W Scientific) in a gas chromatograph type 5890 (Hewlett-Packard, Heilbronn, Germany). Sample injection was performed using the cold on-column technique. The volumes injected ranged from 0.2 to 0.5 μL. The temperature during HRGC was held at 35 °C for 1 min and raised to 230 °C at a rate of 6 °C/min. Mass spectra were recorded with a Finnigan MAT 95S (Finnigan, Bremen, Germany) mass spectrometer in the EI mode at 70 eV, in the chemical ionization (CI) mode at 115 eV with isobutane as reagent gas, and in high-resolution (HR) mode using perfluorokerosene as internal standard.

RESULTS AND DISCUSSION

Urine samples of six volunteers (three males, three females) were collected for 24 h before (control) and after (test) ingestion of 1 mg of carvone/kg of body weight as described under Materials and Methods. Because it can be assumed that the metabolites of carvone have been condensed with either sulfuric acid or glucuronic acid during phase II reactions, the samples were treated with glucuronidase and sulfatase to liberate the apolar metabolites presumably formed in phase I reactions. After extraction with diethyl ether and separation into acidic, phenolic, and neutral compounds, each fraction was analyzed by HRGC-MS either before or after trimethylsilylation or ethylation.

By comparison of single mass traces in all fractions of underivatized control and test urine samples, two metabolites were localized in the NF, none in the PH, and three in the AF. The same results were obtained for the trimethylsilyl (TMS) derivatized samples, indicating complete localization of all extractable major metabolites. Only a small amount of unmetabolized carvone was found in all test samples.

Detection of Metabolites in the AF. In the TMS derivatizated AF the presence of metabolites was indicated best by *m*/*z* 162, 153, and 146 (Figure 1). Due to the very low amount of carvone ingested in this MICA experiment, none of the metabolites in AF would have been detectable by comparison of the TIC alone (lowest pair of chromatograms in Figure 1).

Metabolite M1. Due to coelution with other compounds present in the AF, no clear mass spectrum of M1 could be obtained from the underivatized test sample. The mass spectrum obtained for **M1-TMS** (TMS derivatized metabolite 1) showed characteristic signals at *m*/*z* 131 and 146 (Figure 2). From HRMS the structure of the ion *m*/*z* 146 was in agreement with propionic acid-TMS, probably resulting from a McLafferty rearrangement of the molecular ion. The presence of *m*/*z* 109 indicated the unchanged ring structure of carvone. On the basis of these suggestions, the structure of **M1** was proposed as α,4-dimethyl-5-oxo-3-cyclohexene-1-acetic acid.

Ethylation of **M1** (**M1-ET**) resulted in the mass spectrum shown in Figure 2. The McLafferty rearrangement product *m*/*z* 102 was the base peak suggested to stem from propanoic acid ethyl ester. To confirm the structure, **M1** was synthesized and directly analyzed by

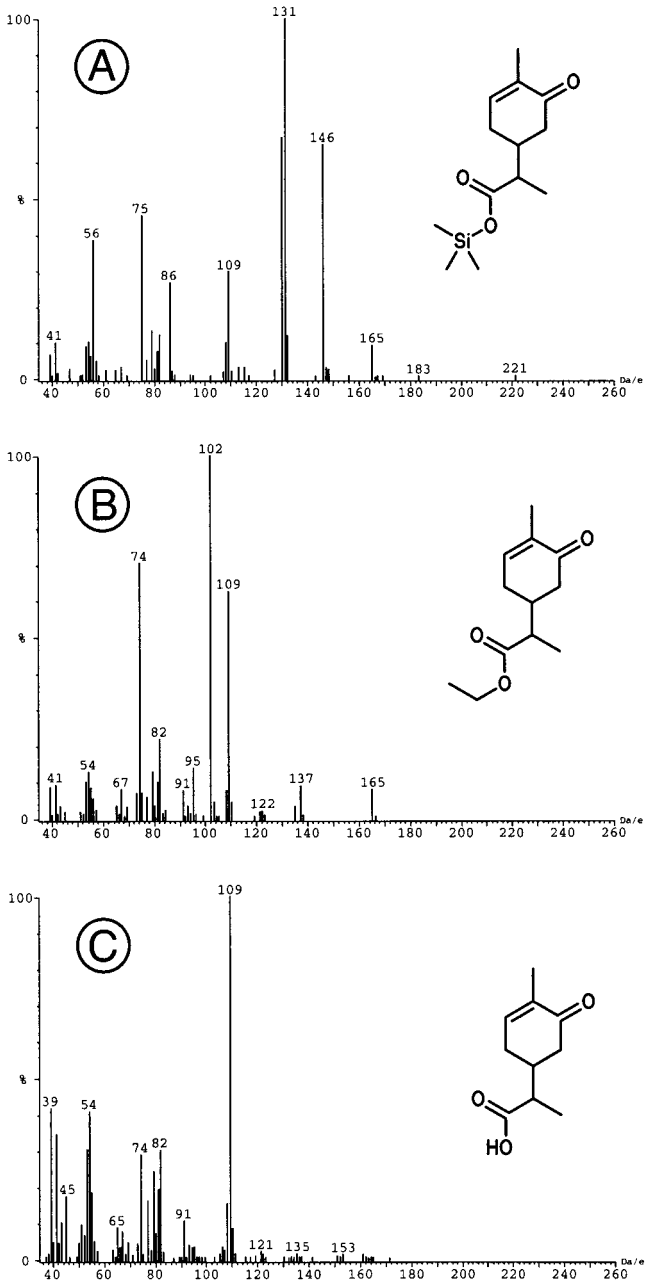

Figure 2. Mass spectra of (A) α,4-dimethyl-5-oxo-3-cyclohexene-1-acetic acid trimethylsilyl ester (**M1-TMS**), (B) α,4-dimethyl-5-oxo-3-cyclohexene-1-acetic acid ethyl ester (**M1-ET**), and (C) α,4-dimethyl-5-oxo-3-cyclohexene-1-acetic acid (**M1**).

HRGC-MS (Figure 2). The base peak *m*/*z* 109 corresponds to the 2-methyl-2-cyclohexenone part of the molecule. Ethylation or trimethylsilylation of synthesized **M1** gave spectra identical with the ones of the urine metabolite (data not shown).

Compared to the synthetic route published (*14*) a new, more direct synthetic approach for the preparation of α,4-dimethyl-5-oxo-3-cyclohexene-1-acetic acid was developed (Figure 3). By analogy to the known limonene metabolite, dihydroperillic acid, **M1** was assigned as dihydrocarvonic acid.

Both diastereoisomers of **M1** were obtained by synthesis due to the new asymmetric center introduced at C-8 of carvone (Figure 3). These could be separated by HRGC; however, no preparative separation was performed because both diastereoisomers were present in

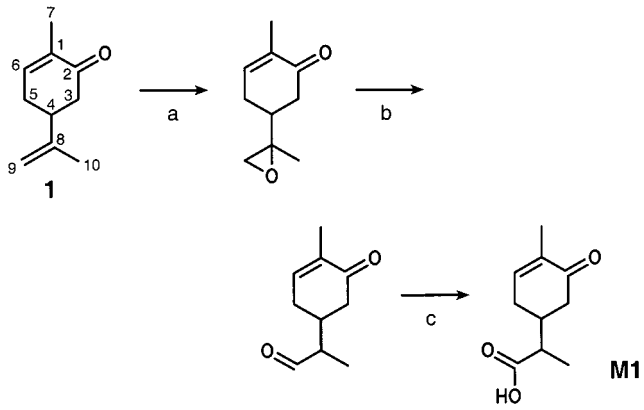

Figure 3. Synthesis of α,4-dimethyl-5-oxo-3-cyclohexene-1-acetic acid (**M1**): (a) MCPBA/CH_2Cl_2/room temperature, yield 80%; (b) $BF_3 \times Et_2O$/room temperature, yield 70%; (c) NaOCl, TEMPO, tributyl hexadecyl phosphonium bromide/CH_2Cl_2, H_2O/0 °C, yield 66%.

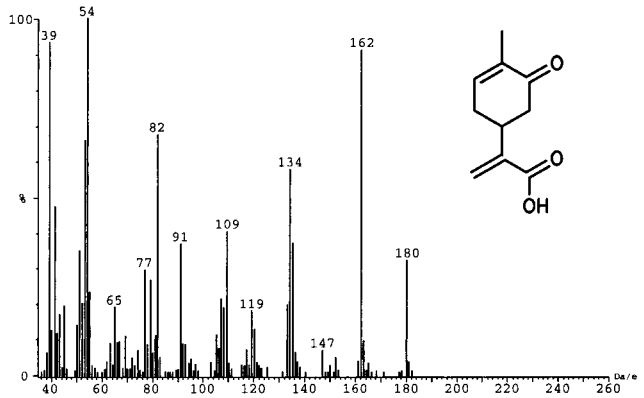

Figure 4. Mass spectrum of α-methylene-4-methyl-5-oxo-3-cyclohexene-1-acetic acid.

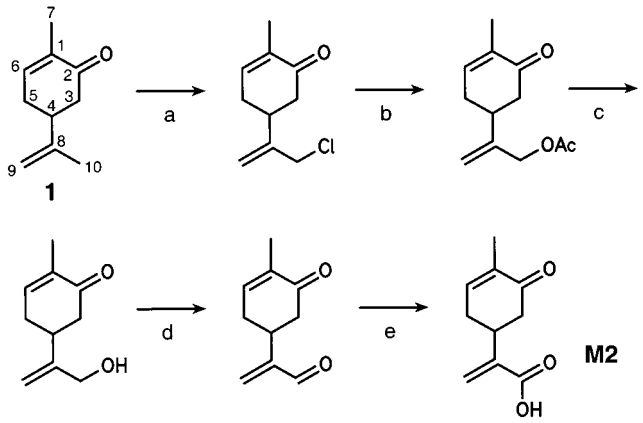

Figure 5. Synthesis of α-methylene-4-methyl-5-oxo-3-cyclohexene-1-acetic acid (**M2**): (a) NaOCl, CO_2 (solid)/H_2O, CH_2Cl_2/room temperature, yield 80%; (b) NaOAc, tributyl hexadecyl phosphonium bromide/TBME, H_2O/reflux, yield quantitative; (c) K_2CO_3/H_2O/room temperature, yield 86%; (d) NaOCl, TEMPO, NaBr/CH_2Cl_2, H_2O/0 °C; (e) $NaClO_2$/tBuOH, H_2O, methylbutene/room temperature, yield (d + e) 58%.

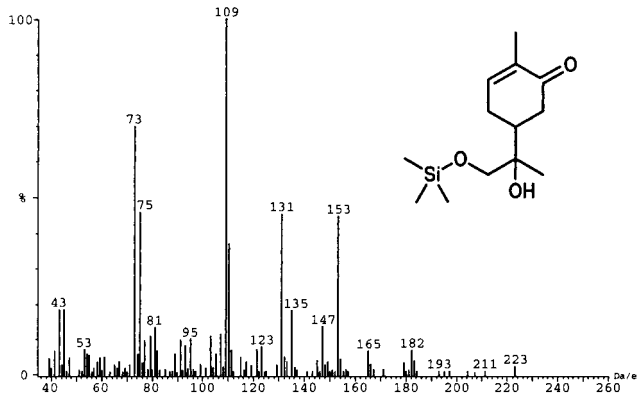

Figure 6. Mass spectrum of trimethylsilylated 5-(1,2-dihydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one (**M3-TMS**).

human urine (Figure 1). Metabolite **M1** was mentioned once in the literature (*14*); however, no NMR or MS data were given.

Metabolite M2. Because metabolite **M2** was present in higher concentrations, a mass spectrum of underivatized **M2** could be obtained directly from test urine (Figure 4). By MS-CI a molecular weight of 180 was confirmed. By HRMS the elemental composition was determined to be $C_{10}H_{12}O_3$. The loss of water (M − 18) forming the base peak *m/z* 162, however, is quite unusual for a carboxylic acid, which normally loses an OH radical, that is, forming M − 17. Because only one diastereoisomer of **M2** (Figure 1) was detected, the double bond of the side chain must still be present, leaving two possible structures for **M2**: one as suggested in Figure 4 and another with the carboxyl group at C-7. The latter structure, however, can be ruled out due to the intense fragment *m/z* 82 indicating the unchanged α-methyl-α,*β*-unsaturated keto moiety of carvone.

To confirm these assumptions, the proposed structure was synthesized (Figure 5) yielding identical GC retention times, MS-EI, and MC-CI spectra as **M2** isolated from test urine. **M2** was, therefore, identified as α-methylene-4-methyl-5-oxo-3-cyclohexene-1-acetic acid and is named carvonic acid. This is the first report of this compound, which has not been described previously.

Metabolite **M2** seems to be somewhat unstable, because during NMR analysis the concentrated solution of the acid appeared to polymerize because both methylene signals decreased considerably within 24 h, leading to new signals in the range of 1−3 ppm.

Metabolite M3. Although present in the AF after standard workup, **M3** surprisingly turned out to be nonacidic. The fragment *m/z* 109 in **M3-TMS** (Figure 6) indicated the presence of the unchanged ring structure of carvone. The fragment *m/z* 153 suggested a sterically hindered underivatized 1-hydroxyethyl group attached to the ring. The intense fragment *m/z* 73 indicated the presence of at least one TMS group and, consequently, of a second less hindered hydroxy group. Combining all structural features, the structure of **M3** was proposed to be 5-(1,2-dihydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one (Figure 6). Its occurrence in the AF could be explained by its high water solubility, thus being extracted from the ether phase by the aqueous solution of $NaHCO_3$ used for removing the acids. Its nonacidic character was confirmed by washing TF with 1 mol/L H_3PO_4 instead of the $NaHCO_3$ solution, leading to nearly complete removal of **M3** compared to only traces of the acidic metabolites **M1** and **M2**.

To avoid the formation of chlorinated products during the synthesis (Figure 7) of 5-(1,2-dihydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one, hydration of 8,9-epoxycarvone was performed with diluted H_2SO_4 in a mixture of THF and water. The use of ethanol as suggested in ref *20* has to be avoided because substantial amounts of ethoxy derivatives are produced. A

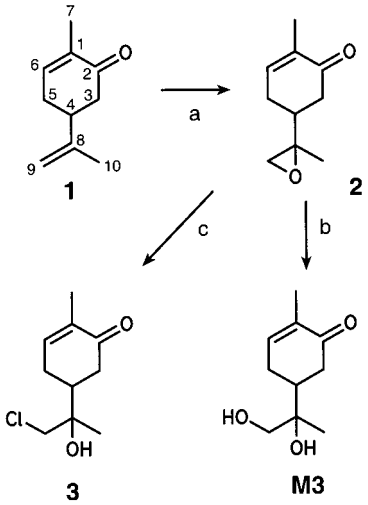

Figure 7. Synthesis of 5-(1,2-dihydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one: (a) MCPBA/CH_2Cl_2/room temperature, yield 80%; (b) H_2SO_4/THF, H_2O/room temperature, yield 81%; (c) HCl/acetone, H_2O.

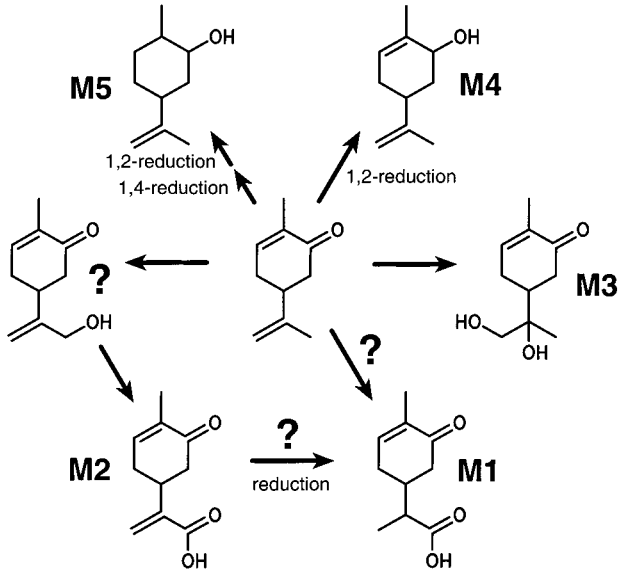

Figure 8. Proposed metabolism scheme leading to unconjugated primary metabolites of carvone in humans.

sample of 5-(1,2-dihydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one (**M3**, Figure 7) prepared according to this method and derivatized with BSTFA showed identical GC retention times and MS-EI and MC-CI spectra as **M3-TMS** isolated from test urine. **M3** was, therefore, identified as 5-(1,2-dihydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one. In analogy to the limonene metabolite uroterpenol, uroterpenolone is suggested as a trivial name.

The synthetic procedure published for **M3** (*15*) starts with hydrolysis of epoxidized carvone (**2** in Figure 7) using hydrochloric acid in aqueous acetone. However, when we repeated this step, 5-(2-chloro-1-hydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one (**3**, Figure 7), resulting from the addition of hydrochloric acid to the epoxide, was formed as the main product (data not shown). Additional evidence for the proposed structure was obtained by reaction of **3** with base, leading to recovery of the epoxide (**2**). This behavior is typical for 1-hydroxy-2-chloro compounds, whereas diols cannot be converted into epoxides by simple treatment with a base.

Furthermore, standard NMR techniques have to be selected carefully to distinguish unequivocally between 5-(2-chloro-1-hydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one (**3**) and 5-(1,2-dihydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one (**M3**). In the case of H−D exchange with a deuterated protic solvent such as D_2O or CD_3OD, the remaining ^1H and the observed ^{13}C NMR signals can be assigned to both compounds because the chemical shifts differ only slightly and the OH signals are absent. In the case of an aprotic deuterated solvent such as $CDCl_3$, the signal for OH protons is often broad, preventing proper integration and thus clear discrimination between **3** and **M3**.

To prove the presence of two different hydroxy groups, the NMR data were obtained from a sample dissolved in DMSO-d_6, yielding both OH signals as two sharp singlets. It has to be mentioned that **M3** is entirely insoluble in $CDCl_3$ and, therefore, the previously published NMR data (*15*) obtained from a sample dissolved in this solvent are questionable.

Metabolites in the NF. In the NF, two compounds were identified, namely, carveol (**M4**) and dihydrocarveol (**M5**). The stereochemistry of both metabolites was not further investigated; however, other diastereoisomers of **M4** and **M5** were detectable to a minor extent, indicating that metabolic reduction of carvone is not stereospecific.

From the structures of the metabolites of carvone identified in this study it is obvious that α-hydroxylation or epoxidation of a double bond does not seem to be possible if there is a carbonyl group in conjugation. Therefore, in carvone, obviously only the double bond in the side chain is oxidized. This behavior is consistent with the chemical reactivity of both double bonds in carvone toward epoxidation by peracids forming exclusively the 8,9-epoxide. It cannot be excluded that the epoxide is generated in vivo as a primary product, followed by further oxidative or hydrolytic transformations. However, no epoxide was found in the urines.

In rabbits only 10-hydroxycarvone (*6*) was identified as the key metabolite of (±)-carvone. Furthermore, only the (+)-isomer of 10-hydroxycarvone was detected after the application of racemic carvone, suggesting different metabolisms of (+)- and (−)-carvone or isomerization of (−)-carvone to the (+)-isomer prior to metabolism. In our experiment using much lower doses of carvone all metabolites were identical after the application of either *S*-(+)-carvone or *R*-(−)-carvone. Additionally, no qualitative differences were observed among the six persons. Because the stereochemistry of the metabolites still has to be determined, differences between *R*-(−)- and *S*-(+)-carvone metabolism cannot be ruled out. However, it seems more likely that stereochemistry at C4 of carvone (**1**, Figure 3) is not affected by metabolism.

The alcohol, 10-hydroxycarvone, identified in rabbits (*6*) might be an intermediate in humans. It may be formed in the first stage of oxidative metabolism (Figure 8), being further transformed to carvonic acid (**M2**). However, further oxidation seems to be very efficient in humans, because 10-hydroxycarvone was not detected. The observed differences between rabbits and humans may also be explained by a simple concentration effect; the doses applied were 1 mg/kg of body mass in this MICA experiment and 700 mg/kg in the case of rabbits (*6*).

It remains to be determined whether the unsaturated carboxylic acid **M2** is finally reduced to **M1** or if there

is an independent pathway leading directly from carvone to **M1** (Figure 8) as mimicked during synthesis (Figure 3). In the first step during in vivo metabolism, carvone might also be converted to the epoxide and after rearrangement to the saturated aldehyde might be finally oxidized to **M1**, making this route an alternative to the reduction pathway starting with **M2**.

The neutral metabolites carveol (**M4**) and dihydrocarveol (**M5**) might be formed by a competing 1,2- and 1,4-reduction of carvone (Figure 8). The enolized product of the 1,4-reduction, dihydrocarvone, might be further reduced to dihydrocarveol. However, even though no exact quantification was performed, the amounts of **M4** and **M5** were apparently much lower than those of **M1**, **M2**, and **M3**, suggesting that reductive metabolism is only a side pathway.

The results suggest that low-dose metabolic experiments in humans, as it is implied in MICA, are the most realistic and thus the best model available for evaluation of in vivo metabolism in humans. Further studies with carvone site-specifically labeled with stable isotopes are in progress to gain more insight into the oxidation mechanism.

ACKNOWLEDGMENT

I thank Bettina Fickert, Ines Otte, Valerie Rota, Michael Czerny, and Magnus Jezussek for being patient volunteers and Peter Schieberle for helpful comments during revision of the manuscript. I gratefully acknowledge the support of Edith Kaldyk (NMR), Oliver Frank (NMR), Ines Otte (MS), and Julius Schönauer (MS).

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Received for review February 7, 2001. Revised manuscript received April 9, 2001. Accepted June 12, 2001.

JF010157Q