Metabolism of 1,8-cineole by human cytochrome P450 enzymes: Identification of a new hydroxylated metabolite

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Received 26 April 2004; received in revised form 21 December 2004; accepted 21 December 2004
Available online 17 January 2005

Abstract

Human metabolism of the monoterpene cyclic ether 1,8-cineole was investigated in vitro and in vivo. In vitro, the biotransformation of 1,8-cineole was investigated by human liver microsomes and by recombinant cytochrome P450 enzymes coexpressed with human CYP-reductase in Escherichia coli cells. Besides the already described metabolite 2α-hydroxy-1,8-cineole we found another metabolite produced at high rates. The structure was identified by a comparison of its mass spectrum and retention time with the reference compounds as 3α-hydroxy-1,8-cineole. There was a clear correlation between the concentration of the metabolites, incubation time and enzyme content, respectively. CYP3A4/5 antibody significantly inhibited the 2α- and 3α-hydroxylation catalyzed by pooled human liver microsomes. Further kinetic analysis revealed that the Michaelis–Menten $K_m$ and $V_{max}$ for oxidation of 1,8-cineole in position three were 19 μM and 64.5 nmol/min/nmol P450 for cytochrome P450 3A4, and 141 μM and 10.9 nmol/min/nmol P450 for cytochrome P450 3A5, respectively. To our knowledge, this is the first time that 3α-hydroxy-1,8-cineole is described as a human metabolite of 1,8-cineole. We confirmed these in vitro results by the investigation of human urine after the oral administration of cold medication containing 1,8-cineole. In human urine we found by GC-MS analysis the described metabolites, 2α-hydroxy-1,8-cineole and 3α-hydroxy-1,8-cineole.

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Keywords: Metabolism; 1,8-Cineole; Cytochrome P450; CYP3A; 2α-Hydroxy-1,8-cineole; 3α-Hydroxy-1,8-cineole

1. Introduction

Among numerous groups of naturally occurring compounds examined so far, monoterpenes are known as fragrances and flavouring agents. 1,8-Cineole, a monoterpene cyclic ether which is named eucalyptol, is widely distributed in plants and found in essential oils like Eucalyptus polybractea. It is extensively used for external application in pharmaceutical preparations, e.g., in nasal spray or as disinfectant [1]. Furthermore, it is used in cosmetics, for cough treatment, muscular pain, neurosis, rheumatism, asthma, and urinary stone [2]. Southwell et al. investigated the metabolism of 1,8-cineole in different insect species. Different metabolites such as 2α- and 2β-hydroxy-1,8-cineole, 3α-hydroxy-1,8-cineole and 9-hydroxy-1,8-cineole as well as the diol 2α,9-dihydroxy-1,8-cineole were found [3]. After the administration of 1,8-cineole to rabbits, there were several alcohols and ketones identified in the urine such as 2α/β-hydroxy-1,8-cineole, 3α/β-hydroxy-1,8-cineole, and 3-oxo-1,8-cineole [4]. In addition, it was found that 1,8-cineole is metabolized to several cineolic acids, dihydroxycineoles and hydroxycineolic acids after feeding brushtail possums and male koalas [4–8]. The metabolism of 1,8-cineole was also investigated by liver microsomes of rat and humans as well as by recombinant cytochrome P450 enzymes in insect cells coexpressing human NADPH-P450 reductase. The results suggested that 1,8-cineole, which is oxidized in high rates to 2α-hydroxy-1,8-cineole, is a substrate for CYP3A enzymes in rat and human liver microsomes [2]. So far, 2α-hydroxy-1,8-cineole is the only known metabolite formed by human liver microsomes [9].

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CYP3A4 is the most abundant P450 enzyme in human liver, but is also expressed in extrahepatic tissues such as intestinal mucosa. CYP3A5 is also expressed in nonhepatic tissues and is the dominant CYP3A isoform in the kidney [10]. These two CYP3A enzymes metabolize similar compounds and exhibit high amino acid sequence identity (84%) [11]. Recently, CYP3A4 and CYP3A5 were both detected in human skin with CYP3A4 being more abundant than CYP3A5 [12].

In this study, we investigated the human metabolism of 1,8-cineole in vitro by recombinant cytochrome P450 enzymes and human liver microsomes. Furthermore, the human urine of volunteers (n=3) was examined after an oral administration of cold medication containing 1,8-cineole.

2. Materials and methods

2.1. Chemicals

1,8-Cineole was purchased from Fluka (Buchs, CH) and was used without further purification. The purity of this compound was >99% based on analysis with GC-MS. NADP+, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Roche (Basel, CH). Other reagents and chemicals were of the highest qualities commercially available. Authentic components, namely the hydroxy-1,8-cineole metabolites, were donated by Dr. Ray Carman, Department of Chemistry, University of Queensland, and by Dr. Ian Southwell, Wollongbar Agricultural Institute, Wollongbar.

2.2. Enzymes and antibody

Human liver microsomes were purchased from Xenotech (Kansas, USA). Recombinant human CYP3A4 and CYP3A5 and human CYP-reductase coexpressed in Escherichia coli were obtained from CyMep (Dundee, UK); the P450 amounts in the assays were chosen as recommended by the manufacturer. Purified inhibitory monoclonal antibody against human CYP3A4/5 with a protein concentration of 10 mg/ml was purchased from Xenotech (Kansas, USA).

2.3. GC/MS analysis

A Hewlett-Packard model 5860 Series II (Waldbronn, Germany) equipped with a Gerstel programmable temperature vaporizer (PTV)/Gerstel MPS large volume sampler (CIS 3, Gerstel, Mülheim a. d. Ruhr, Germany) was combined with direct coupling to a Hewlett-Packard 5972 mass spectrometer. The metabolites were separated by a RTX-5SIL MS (Restek, Bad Homburg, Germany) silica capillary column (0.28 mm x 30 m, 0.25 μm film thickness) using helium (at 0.7 ml/min) as carrier gas. The column temperature was increased from 37 to 200 °C at the rate of 6 °C/min and from 200 to 330 °C at a rate of 15 °C/min. 40 μl of the sample extracts was injected with an injection speed of 29 μl/min into the liner of the PTV containing silanized glass wool (93 mm x 1 mm I.D., Gerstel) in the solvent vent mode with stop flow (vent flow, 200 ml/min helium). After purging of the organic solvent over 0.5 min at 20 °C and further 0.5 min isotherm, the injector was heated at 600 °C/min to 300 °C and was kept 2 min isotherm in the splitless mode. The GC-MS transfer line was held at 300 °C resulting in an ion source temperature of 180 °C, a quadrupole temperature of 180 °C and an ionization voltage of 70 eV. For detection, the scan mode (30–300 amo) was used.

2.4. Oxidation of 1,8-cineole by human liver microsomes, and human CYP3A4 and CYP3A5 enzymes

The oxidation of 1,8-cineole by human liver microsomes was determined as follows. 10 μl 1,8-cineole was dissolved in 10 ml of 100 mM potassium phosphate buffer (pH 7.4) and stirred vigorously before usage. Standard reaction mixtures containing human liver microsomes (0.4 mg of protein/ml) and 120 μM 1,8-cineole in a final volume of 500 μl 100 mM potassium phosphate buffer (pH 7.4) with a NADPH-generating system (0.1 mM NADP+, 0.8 mM glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase/ml) were used. Incubations were carried out at 37 °C for various time points in an Eppendorf incubator (Hamburg, Germany), and the reaction was terminated by the addition of 1.0 ml ethylacetate. After additional 30 min extraction time and centrifugation for 10 min at 14,000 rpm (Eppendorf Centrifuge, Hamburg, Germany) the recovered organic phase was used for GC-MS analysis. The oxidation of 1,8-cineole with CYP3A4 (134 pmol/ml) and CYP3A5 (28 pmol/ml) was performed exactly as described above.

2.5. Urine collection from volunteers

Urine was collected from two males and one female after an oral intake of cold medication for several days. All volunteers gave informed consent to participate in the study. The urine was hydrolyzed enzymatically and the monoterpene metabolites were extracted by solid phase extraction (SPE) as described previously [13]. Samples were analyzed by GC-MS.

3. Results

3.1. Identification of metabolites from 1,8-cineole formed by human liver microsomes and recombinant human CYP3A4/3A5

Initially, the metabolism of 1,8-cineole was examined by human liver microsomes in the presence of a NADPH-generating system. The metabolites formed were analyzed
by GC-MS equipped with El-MS. We found two main metabolites which were identified by their MS-spectra as 2α-hydroxy-1,8-cineole and 3α-hydroxy-1,8-cineole (Fig. 1). Component identification was made on the basis of mass spectral fragmentation, retention time comparison with authentic constituents, spiking with authentic constituents and mass spectral matching with commercial libraries (NIST, Wiley). Besides the chromatographic behaviour, in panel C (Fig. 1) the spectral data indicates the formation of a 3α-hydroxylated metabolite. The molecular mass of the metabolite was increased by the introduction of an oxygen atom from 154 to 170 and the fragments 155 (M+–CH₃), 137 (M+–CH₃O=H₂O+CH₃) and 127 (M+–C₃H₇) are described in the literature [3]. The chirality of the metabolites was not determined because quantities were too small for isolation and direct measurement and chiral GC have only been partially successful [3,14].

3.2. Influence of incubation time, P450 enzyme concentration and 1,8-cineole concentration on 3α-hydroxylation of 1,8-cineole by human CYP3A4/CYP3A5

The formation of 3α-hydroxy-1,8-cineole in the presence of human CYP3A4 and CYP3A5 with an NADPH-
generating system varied depending on incubation time, P450 concentration and substrate concentration (Figs. 2 and 3, respectively).

Using different incubation times, we found a linearity of the 1,8-cineole 3α-hydroxylation activities catalyzed by human CYP3A4 and 3A5 up to 30 min. Increasing of the

CYP3A4 1,8-Cineole 3α-hydroxylation

Fig. 2. Dependence on incubation time (A), enzyme contents (B) and 1,8-cineole concentrations (C) of 1,8-cineole 3α-hydroxylation activities catalyzed by human CYP3A4. (A) Concentrations of 1,8-cineole and CYP3A4 were 120 µM and 134 pmol/ml, respectively. (B) The incubation time and concentration of 1,8-cineole were 30 min and 120 µM, respectively. (C) The incubation time and CYP3A4 concentration were 30 min and 134 pmol/ml, respectively.

CYP3A5 1,8-Cineole 3α-hydroxylation

Fig. 3. Dependence on incubation time (A), enzyme contents (B) and 1,8-cineole concentrations (C) of 1,8-cineole 3α-hydroxylation activities catalyzed by human CYP3A5. (A) The concentrations of 1,8-cineole and CYP3A5 were 1.2 mM and 28 pmol/ml, respectively. (B) The incubation time and concentration of 1,8-cineole were 30 min and 1.2 mM, respectively. (C) The incubation time and CYP3A5 concentration were 30 min and 28 pmol/ml, respectively.
P450 enzyme concentration or the 1,8-cineole concentration lead to increasing 3α-hydroxy-1,8-cineole formation. In the absence of human CYP3A4 and CYP3A5 we detected no 1,8-cineole hydroxylation at all.

### 3.3. Kinetic analysis of 1,8-cineole 3α-hydroxylation by human CYP3A4/3A5

Kinetic analysis of the 1,8-cineole 3α-hydroxylation catalyzed by human CYP3A4 and CYP3A5 were performed. The Michaelis–Menten $K_m$ values for this reaction were 19 μM for human CYP3A4 and 141 μM for human CYP3A5, the $V_{\text{max}}$ values were determined to be 64.5 nmol/min/nmol P450 for human CYP3A4 and 10.9 nmol/min/nmol P450 for human CYP3A5. Finally, the efficiencies of catalysis ($V_{\text{max}}/K_m$ value) were 3.4 nM⁻¹ min⁻¹ for CYP3A4 and 0.08 nM⁻¹ min⁻¹ P450 for CYP3A5. For comparison the results for 1,8-cineole 2α- and 3α-hydroxylation are shown in Table 1.

### 3.4. Inhibition of 1,8-cineole 2α- and 3α-hydroxylation by human liver microsomes

Subsequently, we studied the 2α- and 3α-hydroxylation rates by human liver microsomes in the presence of a CYP3A inhibitor. CYP3A4/5 antibody inhibited the 2α- and 3α-hydroxylation (Fig. 4). The 3α-hydroxylation was almost completely inhibited in the presence of 0.25 mg/ml CYP3A antiserum.

### 3.5. 1,8-Cineole 2α- and 3α-hydroxylation in vivo

To validate the in vitro findings we analyzed the urine of three volunteers (two males, one female) who had taken a cold medication containing 1,8-cineole for 5 days. Prior to the analysis by GC-MS the urine was prepared by solid phase extraction. Comparison of the resulting mass spectra from the peaks with the MS spectra library confirmed clearly the formation of the 2α- and 3α-hydroxylated 1,8-cineole metabolites (Fig. 5). No product formation was found in controls and before the intake of the medication (not shown). This was in concordance with our results using pooled human liver and CYP3A4 and CYP3A5, but in all urine samples the 2α-hydroxy-1,8-cineol content was significantly higher than the 3α-hydroxy-1,8-cineole content.

### 4. Discussion

Various monoterpenoids are metabolized by liver microsomes of different mammalian species. Limonene is attacked at the methyl group in the 7-position to form perillyl alcohol or at the 6-position on the ring system to form carveol [15,16]. The first human metabolite described for limonene was uroterpenol [17,18] which was formed also by the larvae of the common cutworm [19]. Furthermore, it has been shown that verbeneone was catalyzed by human liver microsomes to 10-hydroxyverbeneone [20].

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Table 1: Kinetic analysis of the 2α-hydroxylation and 3α-hydroxylation of 1,8-cineole by human CYP3A4 and CYP3A5

<table>
<thead>
<tr>
<th>Recombinant</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (nmol/min/nmol P450⁻¹)</th>
<th>$V_{\text{max}}/K_m$ (nM⁻¹ min⁻¹)</th>
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<tbody>
<tr>
<td></td>
<td>2α-OH</td>
<td>3α-OH</td>
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<td>P450</td>
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<td>19</td>
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<td></td>
<td>64.5</td>
<td>14.9</td>
<td>4.8</td>
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<tr>
<td></td>
<td>3α-OH</td>
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<td></td>
<td>10.9</td>
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Fig. 4. Influence of Anti-CYP3A antibody on the 2α-hydroxylation (■) and 3α-hydroxylation (●) activities of 1,8-cineole in the presence of human liver microsomes.
These results indicate that natural environmental compounds are differentially oxidized in several species. The structures of these chemicals determine which molecules are attacked at a definite position by P450 enzymes [21]. The metabolism of 1,8-cineole was studied previously in rats and rabbits. Two alcohols, namely 2-hydroxy-1,8-cineole and 3-hydroxy-1,8-cineole, were excreted in the urine [1,4]. More recently, Miyazawa et al. [2,9] reported that 1,8-cineole is metabolized by rat and human liver microsomes as well as by several recombinant cytochrome oxidases to only 2-hydroxy-1,8-cineole. In this study we demonstrated in vitro and in vivo that 1,8-cineole is converted to the expected 2α-hydroxy-1,8-cineole but also to 3α-hydroxy-1,8-cineole.

The following lines of evidence suggest that 1,8-cineole is also oxidized at the 3-position forming 3α-hydroxy-1,8-cineole (Scheme 1). First, the incubation of 1,8-cineole with human liver microsomes, human CYP3A4 and CYP3A5 revealed two metabolites which were identified by GC-MS as 2α-hydroxy-1,8-cineole and 3α-hydroxy-1,8-cineole. Second, anti-CYP3A4/5 antibody significantly inhibited the 1,8-cineole 3α-hydroxylation and 2α-hydroxylation activities catalyzed by human liver microsomes. Finally, both hydroxylated metabolites were detected in the urine of three persons after oral intake of medications containing human liver microsomes, human CYP3A4 and CYP3A5.

![Fig. 5. Full scan GC-MS chromatograms of urine extracts from one female (C) and two males (A, B) after the oral administration of 1,8-cineole. The chromatographic peaks of 2α-hydroxy-1,8-cineole (a) and 3α-hydroxy-1,8-cineole (b) formed via the in vivo metabolism of 1,8-cineole are shown.](image)

Scheme 1. Proposed metabolism of 1,8-cineole by human P450, human CYP3A4/CYP3A5 and in vivo.
1,8-cineole. To our knowledge, this is the first time that these hydroxylated 1,8-cineole metabolites were detected in human urine.

Miyazawa et al. [2] used ten different P450 enzymes expressed in insect cells to determine which P450 enzymes are the major catalysts for the 1,8-cineole hydroxylation. CYP3A4 had the highest activity for the 1,8-cineole 2-hydroxylation followed by CYP3A5 while the other enzymes exhibited only very low activities. Therefore, we investigated the 2- and 3-hydroxylation of 1,8-cineole by human CYP3A4 and CYP3A5. Interestingly, we found for CYP3A4 a higher 3α-hydroxylation activity than 2α-hydroxylation activity although Miyazawa et al. did not find hydroxylation in 3-position. However, we detected higher 2α-hydroxylation activities with human liver microsomes and in vivo we found higher 2α-hydroxy-1,8-cineole concentrations than 3α-hydroxy-1,8-cineole concentrations. This suggests that other enzymes in the human body may be also catalysts of the 2-hydroxylation. The potent inhibition of the 2α- and 3α-hydroxylation of human microsomes by CYP3A antibody shows that both enzymes are major 1,8-cineole oxidizing enzymes in the human liver.

The V\text{max} value determined by Miyazawa et al. for 1,8-cineole 2-hydroxylation by recombinant human CYP3A4 was 48 nmol/min/nmol P450 [2]. This value is similar to the V\text{max} (58.1 nmol/min/nmol P450) determined in this study for CYP3A4 1,8-cineole 2α-hydroxylation. For CYP3A5 1,8-cineole 2α-hydroxylation we found a four-fold lower V\text{max} value (14.9 nmol/min/nmol P450) than for CYP3A4. Kinetic analysis of the 1,8-cineole 3α-hydroxylation activities showed different K\text{m} and V\text{max} values for human CYP3A4 and CYP3A5 presented in Table 1. It is likely that 1,8-cineole is more specific for CYP3A4 activity. The different K\text{m} values for CYP3A5 catalyzed 2- and 3-hydroxylation are significant different. This is surprising because it is caused only by another position of the hydroxyl group at the ring system. The V\text{max}/K\text{m} determined for CYP3A4 was 3.4 mmol·min\(^{-1}\)·mol\(^{-1}\) P450 (CYP3A5: 0.08 mmol·min\(^{-1}\)·mol\(^{-1}\) P450). In comparison to the V\text{max} values of other monoterpens metabolized by P450 enzymes the values described here for CYP3A4 are high. For example for the metabolism of limonene to carveol or perillyl alcohol the V\text{max} values were between 0.16 and 1.1 nmol/min/nmol P450 for recombinant human CYP3A4 [11,12].

In conclusion, the present study shows that 1,8-cineole is catalyzed by human CYP3A4 and CYP3A5 enzymes to 2α-hydroxy-1,8-cineole and 3α-hydroxy-1,8-cineole. Our findings show that in humans the ring carbon oxidation by cytochrome P450 enzymes is favored. The in vivo relevance is supported by the detection of both metabolites in the urine samples after an oral intake of 1,8-cineole. The metabolites can be used as urinary marker for intake of 1,8-cineole in the same way as verbenol for α-pinene exposure [13,22–24].

Acknowledgment

This work was supported by a grant from the Deutsche Forschungsgemeinschaft. We thank N. Fricke and M. Möller for technical assistance as well as W. Dott for helpful discussion. The authors are indebted to Dr. Ray Carman and Dr. Ian Southwell for providing the hydroxycineole samples and unpublished data.

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