ABSTRACT:

Limonene, a monocyclic monoterpene, is present in orange peel and other plants and has been shown to have chemopreventive activities. (+)- and (−)-Limonene enantiomers were incubated with human liver microsomes and the oxidative metabolites thus formed were analyzed using gas chromatography-mass spectrometry. Two kinds of metabolites, (+)- and (−)-trans-carveol (a product by 6-hydroxylation) and (+)- and (−)-perillyl alcohol (a product by 7-hydroxylation), were identified, and the latter metabolites were found to be formed more extensively, the former ones with liver microsomes prepared from different human samples. Sulfinaphenazole, flavoxamine, and antibodies raised against purified liver cytochrome P450 2C5 (P4502C5) 2C9 that inhibit both CYP2C9- and CYP2C19-dependent activities, significantly inhibited microsomal oxidations of (+)- and (−)-limonene enantiomers. The limonene oxidation activities correlated well with contents of CYP2C9 and activities of tolbutamide methyl hydroxylation in liver microsomes of 62 human samples, whereas these activities did not correlate with contents of CYP2C19 and activities of S-mephenytoin 4-hydroxylation. Of 11 recombinant human P450 enzymes (expressed in Trichoplusia ni cells) tested, CYP2C8, 2C9, 2C18, 2C19, and CYP3A4 catalyzed oxidations of (+)- and (−)-limonenes to respective carveols and perillyl alcohol. Interestingly, human CYP2B6 did not catalyze limonene oxidations, whereas rat CYP2B1 had high activities in catalyzing limonene oxidations. These results suggest that both (+)- and (−)-limonene enantiomers are oxidized at 6- and 7-positions by CYP2C9 and CYP2C19 in human liver microsomes. CYP2C9 may be more important than CYP2C19 in catalyzing limonene oxidations in human liver microsomes, since levels of the former protein are more abundant than CYP2C19 in these human samples. Species-related differences exist in the oxidations of limonenes in CYP2B subfamily in rats and humans.

The monocyclic monoterpene (+)- and (−)-limonene enantiomers have been shown to be present in orange peel and other plants and are used as fragrances in household products and components of artificial essential oils (Crowell and Gould, 1994). The (+)-limonene isomeric form is more abundantly present in these plants than the racemic mixture and (−)-isomeric form (Haudenschild et al., 2000). It has previously been shown that (+)-limonene has chemopreventive activities in experimental animal models including rats and mice (Crowell and Gould, 1994; el-Bayoumy, 1994). Some of the hydroxylated metabolites of (+)-limonene, such as sobrerol, carveol, and uroterpenol, have been reported to be more potent than the parent compound in preventing mammary tumors caused by 7,12-dimethylbenz[a]anthracene (Crowell et al., 1992). To better understand the basis of mechanisms of chemopreventive action of limonene, it is of interest to examine the metabolism of limonene in experimental animals and humans.

Metabolism of limonene enantiomers has been studied extensively in experimental animal models in rats, mice, rabbits, guinea pigs, and dogs both in vivo and in vitro (Igimi et al., 1974; Kodama et al., 1974, 1976; Regan and Bjeldanes, 1976; Watabe et al., 1981). Cytochrome P450 P450(P4501) enzymes in liver microsomes of these animal species have been shown to oxidize limonene to several oxidation products such as 1,2- and 8,9-epoxides, carveol (a product by 6-hydroxylation), perillyl alcohol (a product by 7-hydroxylation) (Watabe et al., 1980, 1981; Jager et al., 1999). Recently we reported that limonene enantiomers are oxidized to carveols and perillyl alcohols by CYP2C11 in liver microsomes of untreated rats and by CYP2B1 in those of phenobarbital-treated rats (Miyazawa et al., 2002). Interestingly, a female specific CYP2C12 did not catalyze oxidations of (+)- and (−)-limonene enantiomers by liver microsomes at significant levels. However, it remains unclear which P450 enzymes catalyze limonene oxidations in human liver microsomes.

In this study, we examined oxidations of (+)- and (−)-limonene by P450 enzymes in liver microsomes prepared from different human samples. The metabolites thus formed were analyzed on GC-MS. To determine which P450s are the major enzymes in the oxidations of (+)- and (−)-limonene enantiomers, we used specific P450 inhibitors and antibodies raised against purified human liver P450 enzymes and P450s isolated from Escherichia coli membranes to which human P450 isoform cDNAs have been introduced. Catalytic rates with 11
forms of human P450 enzymes expressed in Trichoplusia ni cells in
the oxidation of limonene enantiomers are also reported.

**Experimental Procedures**

**Chemicals.** (+)- and (−)-Limonene enantiomers, (+)- and (−)-trans–
carveols and (−)- and (−)-perillyl alcohols, were purchased from Wako Pure
Chemical Co. (Osaka, Japan) and were used without further purification; the
purities of these compounds were judged to be >99% on analysis with
GC-MS. NADP⁺, glucose 6-phosphate, and glucose 6-phosphate dehydroge-
nase were purchased from Sigma-Aldrich (St. Louis, MO). Other reagents and
chemicals used were obtained from sources as described previously or of
highest qualities commercially available (Shimada et al., 1999, 2001;
Miyazawa et al., 2002). A GC-MS system equipped with
column (0.25 mm × 30 m; 0.25 μm, coated on 0.25 mm
capillary coupling to Hewlett-Packard 5972 mass spectrometer. The metabolites were
analyzed on GC-MS. In A, concentrations of limonene and protein were
300 μg/ml and 0.5 mg/ml; in B, incubation time was 30 min and substrate concentration was
0.5 mM; and in C, incubation time was 30 min and protein concentration was
0.2 mg/ml. The data sheets provided by the manufacturer.
CYP2C9 was purified to electrophoretic homogeneity from human liver
microsomes as described previously (Shimada et al., 1996). Rabbit antihuman
CYP2C9 antibodies were prepared as described (Kaminsky et al., 1981;
Shimada et al., 1986). Rabbit antiserum raised against purified CYP2C8, 2C9, and
2C19 were the generous gifts from Nihon Nosan Kogyo KK (Yokohama,
Japan).

**Oxidation of (+)- and (−)-Limonene by Human Liver Microsomes and
by Human P450 Enzymes.** Oxidations of (+)- and (−)-limonene enantiomers
by human P450 enzymes were determined as follows. Standard reaction
mixture contained liver microsomes (0.05 mg of protein/ml) or recombinant
P450 (3 pmol/ml) and 200 μM (+)- or (−)-limonene in a final volume of 0.50
ml of 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-
generating system (0.5 mM NADP+, 5 mM glucose 6-phosphate, and 0.5 units
of glucose 6-phosphate dehydrogenase/ml) (Shimada et al., 1986). Incubations
were carried out at 37°C for 30 min and terminated by adding 1.0 ml of
dichloromethane. The extracts (organic layer) were collected by centrifugation
at 3000 rpm for 5 min and were used for analysis with GC-MS for identifi-
cation of the metabolites.

A Hewlett-Packard model 5890A gas chromatograph (Hewlett-Packard,
Atlanta, GA) equipped with a split injector was combined with a direct
coupling to Hewlett-Packard 5972 mass spectrometer. The metabolites were
separated by a TC-WAX FFS (GL Sciences, Tokyo, Japan) column separated by a TC-WAX FFS (GL Sciences, Tokyo, Japan) silica capillary
column (0.25 mm × 30 m; 0.25 μm, coated on 0.25 mm
and 20% glycerol (v/v) (Guengerich, 1994; Shimada et al., 1994). Recombinant
CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2E1, and 3A4
expressed in T. ni infected with a baculovirus containing rat P450 and
NADPH-P450 reductase cDNA inserts were obtained from Gentest Corp.
(Woburn, MA); the P450 contents in these systems were used as described in
the data sheets provided by the manufacturer.

**Results**

**Oxidation of (+)- and (−)-Limonene Enantiomers by Human Liver
Microsomes.** On incubation of (+)-limonene with human liver
microsomes in the presence of an NADPH-generating system, we
detected two metabolites, namely (+)-trans–carveol (a product by
6-hydroxylation) and (+)-perillyl alcohol (a product by 7-hydroxyla-
tion) (Fig. 1). Liver samples from two Japanese and two Caucasians
were used for analysis, and we found that human liver microsomes

It was suggested that (−)-limonene is also metabolized through a similar fashion
by P450 enzymes.

![Fig. 1. Metabolism of (+)-limonene by P450 enzymes.](image)

![Fig. 2. Effects of incubation time (A) and concentrations of protein (B) and
substrate (C) on the oxidation of limonene enantiomers by liver microsomes
of human sample HL-104.](image)

(+)–limonene (C and A) and (−)-limonene (2 and 3) were incubated with
human liver microsomes in the presence of an NADPH-generating system, and the
carveols (open symbols) and perillyl alcohols (closed symbols) formed were ana-
lyzed on GC-MS. In A, concentrations of protein and limonene were 0.2 mg/ml and
0.2 mM, respectively; in B, incubation time and substrate concentration was 30 min
and 0.2 mM, respectively; and in C, incubation time and protein concentration were
30 min and 0.2 mg/ml, respectively.

were more active in oxidizing at 7-position than at 6-position (Fig. 2).
Formation of these oxidative metabolites increased with incubation
time up to 30 min and protein concentration up to 0.5 mg/ml incuba-
tion mixture (Fig. 2, A and B). Dependence of formation rates on
substrate concentrations of (+)- and (−)-enantiomers of limonene
by human liver microsomes were similar (Fig. 2C). Using five human
liver samples, we found that K_{m} and V_{max} values for the oxidation of
limonenes to respective carveols and perillyl alcohol were very simi-
lar (Table 1). Enzyme efficiencies (V_{max}/K_{m} ratio) were 3.5- and
4.0-fold higher in the formation of perillyl alcohol than that of
carveol.

**Effects of Flavoxamine, Sulfaphenazole, Ketoconazole, and Anti-
P450 Antibodies on Oxidations of Limonenes by Human Liver
Microsomes.** Flavoxamine and sulfaphenazole, known inhibitors of
CYP2C-dependent catalytic activities (Guengerich and Shimada, 1991; Inoue et al., 1997), significantly inhibited 6- and 7-hydroxyla-
tions of limonene enantiomers catalyzed by human liver microsomes
(Fig. 3). However, ketoconazole, a potent inhibitor of CYP3A en-
zymes (Guengerich and Shimada, 1991), weakly inhibited limonene
oxidation activities by human liver microsomes.
TABLE 1

Oxidation of (+)- and (−)-limonene enantiomers to respective carveol and perillyl alcohol derivatives by liver microsomes of five human samples

Data for limonene oxidation activities are means of five human samples and S.D.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Oxidation of (+)- and (−)-Limonene</th>
<th>Formation of Perillyl Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (mM) $V_{max}$ (nmol/min/mg protein) $V_{max}/K_m$</td>
<td>$K_m$ (mM) $V_{max}$ (nmol/min/mg protein) $V_{max}/K_m$</td>
</tr>
<tr>
<td>(+)-Limonene</td>
<td>$0.47 ± 0.19$ $0.17 ± 0.09$ $0.36$</td>
<td>$0.28 ± 0.17$ $0.35 ± 0.30$ $1.25$</td>
</tr>
<tr>
<td>(−)-Limonene</td>
<td>$0.45 ± 0.12$ $0.18 ± 0.10$ $0.40$</td>
<td>$0.24 ± 0.17$ $0.39 ± 0.27$ $1.62$</td>
</tr>
</tbody>
</table>

Correlation between Contents of CYP2C9 and CYP2C19 and Activities of Limonene Oxidations by Liver Microsomes of 62 Human Samples. These above results suggest that CYP2C9 and 2C19 are the important enzymes in the oxidations of limonene enantiomers by human liver microsomes. Correlation between contents of CYP2C9 and CYP2C19 and rates of formation of carveol and perillyl alcohol of (+)-limonene was compared in liver microsomes of 62 human samples (Fig. 5). The mean levels (± S.D.) of CYP2C9 and CYP2C19 in 62 human samples were estimated to be 44 ± 26 pmol/mg of protein (19 ± 12% of total P450) and 3.2 ± 2.6 pmol/mg of protein (11.1 ± 0.7% of total P450), respectively. We found that there were good correlations between CYP2C9 levels and formation of carveol ($r = 0.88$) and (+)-perillyl alcohol ($r = 0.76$) in these human samples (Fig. 5). Limonene oxidation activities were also found to correlate with CYP2C9 marker activities (tolbutamide methyl hydroxylation), but not with CYP2C19 marker activities (S-mephentoyin 4-hydroxylation), in these human samples (data not shown).

Recently, we found that CYP2B1 is a principal enzyme in catalyzing oxidations of limonene by liver microsomes of phenobarbital-treated rats, although CYP2C11 is a major enzyme in liver microsomes of untreated male rats (MIYAZAWA et al., 2002). Since our present study suggested that CYP2B6 did not have any considerable activities for the oxidations of limonene enantiomers, we examined the effects of concentrations of rat CYP2B1 and human CYP2B6 on the oxidation of (+)-limonene in recombinant (T. ni) cells P450 systems. The results showed that CYP2B6 did not catalyze oxidations of (+)-limonene at considerable rates, whereas CYP2B1 catalyzed limonene oxidations at higher rates for the formation of carveol than those of perillyl alcohol (data not shown).

Kinetic analysis were performed in recombinant CYP2C9, 2C19, and 3A4 in the oxidations of (+)- and (−)-limonene enantiomers (Fig. 6). $K_m$ values for the formation of carveols and perillyl alcohols by these P450 enzymes were around 0.3 mM, except that $K_m$ value for the formation of carveol by CYP2C9 was somewhat higher (0.57 mM) (Table 3). $V_{max}$ values were higher in CYP2C19 than those in CYP2C9 and 3A4 in the oxidations of (+)- and (−)-limonene enantiomers. As a result, enzyme efficiencies ($V_{max}/K_m$ ratio) were always higher in CYP2C19 than those of CYP2C9 and 3A4 (Table 3).

Discussion

In this study, we found that (+)- and (−)-limonene enantiomers were oxidized to respective carveol (6-hydroxylation) and perillyl alcohol (7-hydroxylation) metabolites by CYP2C9 and CYP2C19 in human liver microsomes. Although enzyme efficiencies ($V_{max}/K_m$ ratio) were much higher in CYP2C19 than those of CYP2C9 in the formation of carveols and perillyl alcohols from limonene enantiomers by recombinant P450 systems, CYP2C9 was found to be more active than CYP2C19 in catalyzing limonene enantiomers by human liver microsomes with the following lines of evidence. First,
phenazole, a specific CYP2C9 inhibitor, significantly suppressed the activities of oxidations of limonene enantiomers catalyzed by human liver microsomes. Second, anti-CYP2C9 IgG, which was obtained from rabbits immunized with purified human liver CYP2C9 (Shimada et al., 1986), completely inhibited limonene oxidations by liver microsomes. Third, antisera raised against CYP2C9 (purified from membranes of E. coli) inhibited very significantly the oxidations of (+)- and (−)-limonene enantiomers by human liver microsomes. It should, however, be mentioned that antibodies raised against CYP2C19 (purified from membranes of E. coli) inhibited more effectively than anti-CYP2C9 for the oxidation of (+)-limonene by liver microsomes (Fig. 4). The data sheets provided by the manufacturer (Nippon Nosan Kogyo Co.) suggested that anti-CYP2C19 (at 2 μl sera) inhibited S-mephenytoin 4-hydroxylation by more than 80%, whereas anti-

![Image 1](https://example.com/image1.png)

**FIG. 4.** Effects of preimmune sera (○), anti-2C9 sera (△), anti-CYP2C8 sera (●), anti-CYP2C9 sera (□), and anti-CYP2C19 sera (■) on the formation of (+)- and (−)-carveols (A and C) and (+)- and (−)-perillyl alcohols (B and D) from (+)- and (−)-limonene, respectively, by human liver microsomes of HL-104.

Activities are represented as percent of controls (in the absence of antibody).

![Image 2](https://example.com/image2.png)

**FIG. 5.** Correlation between contents of CYP2C9 (A and B) and CYP2C19 (C and D) and catalytic activities of liver microsomes of 62 human samples toward oxidation of (+)-limonene.

In A and B, contents of CYP2C9 were compared with formation of (+)-carveol and (+)-perillyl alcohol, respectively, in these human samples; in C and D, contents of CYP2C19 were compared with formation of (+)-carveol and (+)-perillyl alcohol, respectively, in these human samples.

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Oxidative metabolism of (+)- and (−)-limonene enantiomers to respective carveol and perillyl alcohol derivatives by recombinant human P450 enzymes together with NADPH-P450 reductase expressed in T. ni cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidations of (+)- and (−)-limonene enantiomers by recombinant human P450 enzymes were determined by the methods as described under Experimental Procedures. Substrate concentrations used were 200 μM. Data are means of duplicate determinations and range.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P450</th>
<th>Oxidation of (+)-Limonene</th>
<th>Oxidation of (−)-Limonene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+)-Carveol</td>
<td>(+)-Perillyl alcohol</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>0.07 ± 0.02</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>0.11 ± 0.02</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.08 ± 0.02</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>CYP2C18</td>
<td>0.47 ± 0.11</td>
<td>1.52 ± 0.08</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.09 ± 0.04</td>
<td>0.25 ± 0.03</td>
</tr>
</tbody>
</table>
CYP2C9 sera (at 2 μl) inhibited by only 40% diclofenac 4’t-hydroxyla-
tion catalyzed by human liver microsomes. Finally, limonene oxid-
action activities were found to correlate with contents of CYP2C9, but
not CYP2C19, in liver microsomes of 62 human samples. The reasons
why CYP2C9 is more active than CYP2C19 in the oxidations of
limonene by liver microsomes may be related to higher contents of
this P450 species in human livers. Our estimate suggested that the
mean levels of CYP2C9 in liver microsomes of 62 human samples
were about 14-fold higher than that of CYP2C19, probably resulting
in more important roles of CYP2C9 than CYP2C19 in catalyzing
limonene oxidations by human liver microsomes.

Recombinant CYP3A4 was found to be one of the enzymes in
metabolism of limonene enantiomers. Although the enzyme
efficiencies (V_{max}/K_m ratio) for the oxidation of limonene enantiomers
by recombinant (T. ni cells) CYP3A4 were found to be similar to
those by recombinant CYP2C9, the contribution of this P450 isoform
seemed to be minor in human liver microsomes (Table 3). These were
supported by the inability of ketoconazole, a potent inhibitor of
CYP3A4 (Guengerich and Shimada, 1991), to inhibit limonene oxida-
tions catalyzed by human liver microsomes.

We have recently shown that there are sex-related differences in
the metabolism of limonene by rat liver microsomes and that the male-
specific CYP2C11 has higher catalytic rates for the oxidation of
limonene than female-specific CYP2C12 (Miyazawa et al., 2002).
Such differences may, in part, be related to the occurrence of male-
specific nephrotoxicity caused by limonenes in rats (Lehman-Mckee-
man et al., 1989; Borghoff et al., 1990; Hard, 1998). In fact, some of
the limonene metabolites are shown to be more potent to interact with
α2-μ-globulin than the parent compound (Lehman-Mckeeman et al.,
1989; Borghoff et al., 1990). In humans, however, it has been reported
that there are few sex-related differences in the contents and catalytic
properties of individual P450 enzymes (Shimada et al., 1994).

(+)‐Limonene has been shown to have chemopreventive activities
in experimental animal models (Crowell et al., 1992; Crowell and
Gould, 1994; el-Bayoumy, 1994). One of the mechanisms
underlying chemoprevention of limonenes may be related to inacti-
nation of the ultimate carcinogens by inducing phase II enzymes. It is
not known at present whether the metabolites of limonenes, such as
sobrerol, carveol, and uroterpenol, are more potent than the parent
compound in preventing mammary tumors caused by 7,12-dimethyl-
diben[α]anthracene. It has also been shown that (+)limonene itself
does not induce P450 enzymes but induces phase II enzymes such as
glutathione S-transferase and UDP-glucuronosyltransferase enzymes
(Ariyoshi et al., 1975; el-Bayoumy, 1994). One of the mechanisms
underlying chemoprevention of limonenes may be related to inacti-
nation of the ultimate carcinogens by inducing phase II enzymes. It is
not known at present whether the metabolites of limonenes, such as
carveol and perillyl alcohol, induce phase II enzymes in mammalian
tissues.

In this study, we found that CYP2B6 did not catalyze oxidations
of limonenes at significant rates. This is in contrast with the results of rat
studies in which CYP2B1, a homologous enzyme to CYP2B6, cata-
lizes limonene oxidations at high rates and anti-CYP2B1 significantly
inhibits limonene oxidations by liver microsomes of phenobarbital-
treated rats (Miyazawa et al., 2002). Such species-related differences
in the metabolism of xenobiotic chemicals by the same family of P450

---

**TABLE 3**

<table>
<thead>
<tr>
<th>Formation of Carveol</th>
<th>Formation of Perillyl Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_m$ (mM)</td>
<td>$V_{max}$ (nmol/min/nmol P450)</td>
</tr>
<tr>
<td>(+)-Limonene</td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.57</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>0.27</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.27</td>
</tr>
<tr>
<td>(-)-Limonene</td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.30</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>0.25</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Kinetic parameters of oxidation of (+) and (-) limonene enantiomers by recombinant human P450 were determined by the methods as described under Experimental Procedures.

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**Fig. 6.** Dependence on concentrations of oxidations of (+)-limonene (A and B) and (-)-limonene (C and D) to respective (+)- and (-)-carveol and perillyl alcohol by recombinant human CYP2C9 (●), CYP2C19 (□), and CYP3A4 (○).
enzymes are of interest when the biological and pharmacological data are to be extrapolated from experimental animals to humans (Wrighton et al., 1995; Iwatsubo et al., 1997).

In summary, present results showed that both (+)- and (−)-limonene enantiomers are oxidized to respective carvole and perillyl alcohol derivatives by CYP2C9 and 2C19 in human liver microsomes. Our results also showed CYP2C9 plays more important roles than CYP2C19 in catalyzing limonene oxidations. Although recombinant CYP3A4 catalyzed limonene oxidations at considerable rates, contribution of this P450 isoform may be minor, since ketoconazole, a potent CYP3A4 inhibitor, inhibited weakly limonene oxidations catalyzed by liver microsomes. The exact roles of CYP2C enzymes in the biological significance of limonene enantiomers when humans are exposed to these chemicals are unknown at present.

References


