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Pamela L. Crowell · Charles E. Elson · Howard H. Bailey
Abiodun Elegbede · Jill D. Haag · Michael N. Gould

Human metabolism of the experimental cancer therapeutic agent *d*-limonene

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Abstract *d*-Limonene has efficacy in preclinical models of breast cancer, causing >80% of carcinomas to regress with little host toxicity. We performed a pilot study on healthy human volunteers to identify plasma metabolites of limonene and to assess the toxicity of supradietary quantities of *d*-limonene. Seven subjects ingested 100 mg/kg limonene in a custard. Blood was drawn at 0 and 24 h for chemistry-panel analysis and at 0, 4, and 24 h for limonene-metabolite analysis. On-line capillary gas chromatography/mass spectrometry (GC/MS) analysis indicated that at least five compounds were present at 4 h that were not present at time zero. Two major peaks were identified as the rat limonene metabolites dihydroperillic acid and perillic acid, and two minor peaks were found to be the respective methyl esters of these acids. A third major peak was identified as limonene-1,2-diol. Limonene was a minor component. At a dose of 100 mg/kg, limonene caused no gradable toxicity. Limonene is metabolized by humans and rats in a similar manner. These observations and the high therapeutic ratio of limonene in the chemotherapy of rodent cancers suggest that limonene may be an efficacious chemotherapeutic agent for human malignancies.

Key words Limonene · Monoterpene

P. L. Crowell (✉)[1] · H. H. Bailey · A. Elegbede · J. D. Haag
M. N. Gould
Department of Human Oncology, University of Wisconsin Comprehensive Cancer Center, University of Wisconsin-Madison, 600 Highland Avenue, Madison, WI 53792, USA

C. E. Elson
Department of Nutritional Sciences, University of Wisconsin-Madison, 1516 Linden Drive, Madison, WI 53706, USA

[1] *Present address*: Department of Biology, Indiana University-Purdue University at Indianapolis, 723 West Michigan Street, Indianapolis, IN 46202, USA

Introduction

The chemotherapeutic and chemopreventive effects of the monoterpene *d*-limonene (*p*-mentha-1,8-diene, Fig. 1), the predominant constituent of orange-peel oil, have been studied extensively in chemically induced rodent tumor models [1]. Oral treatment of rats harboring chemically induced rat mammary carcinomas with *d*-limonene results in complete regression of >80% of the carcinomas, with host toxicity being limited to reduced weight gain [8, 12]. Interestingly, limonene appears to induce tumor cell redifferentiation via a mechanism that is at present undefined [12]. The expression of mannose-6-phosphate/insulin-like growth factor II receptor and transforming growth factor β1 is increased in limonene-treated, regressing tumors but not in the small number of tumors that are unresponse to limonene [16]. Limonene's mode of action is not likely to involve modulation of endocrine function since limonene-fed rats exhibit normal circulating prolactin concentrations and normal estrus cycles [11].

The chemopreventive efficacy of limonene during both the initiation and promotion stages of carcinogenesis has been demonstrated in chemically induced rodent mammary [7, 11, 19, 25], skin [9], liver [6], lung, and forestomach [26, 27] tumor model systems [1]. The initiation-phase chemopreventive effects of limonene have been attributed to the induction of phase I [20] and phase II [10] carcinogen-metabolizing enzymes, resulting in carcinogen detoxification [20]. The postinitiation chemopreventive/tumor-suppressive activity of limonene may be due in part to the inhibition of isoprenylation of cell-growth-associated small G proteins such as p21ras by limonene and its metabolites [2, 3, 5]. Posttranslational isoprenylation is required for functionality of these proteins, e.g., for transformation by Ras [1, 17].

The metabolism and disposition of limonene has been partially determined. Oral limonene is completely absorbed [14] and has a half-life of approximately 24 h in the rat [3, 14]. Limonene and/or its metabolites distribute throughout the body, showing some preference for fatty tissues [3, 14].

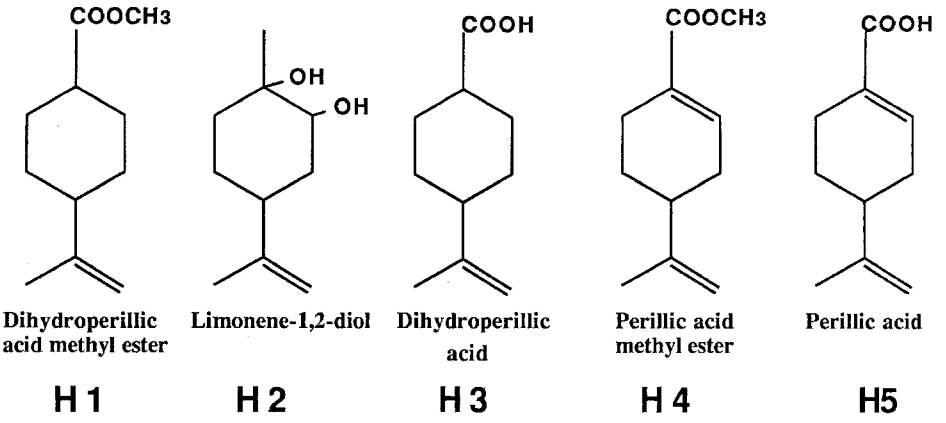

Fig. 1 Chemical structures of human plasma limonene metabolites

Limonene is extensively metabolized by rats, humans, and other species. In both plasma [3] and urine [18], limonene is only a minor component of the total limonene-derived material. The major circulating metabolites of limonene in the rat are perillic acid and dihydroperillic acid [3]. Human and rat urinary metabolites include alcohols, acids, and their glycine and glucuronyl conjugates [18]. Human circulating metabolites of limonene have not yet been identified.

The relative potency of limonene versus its metabolites have been compared in several bioassays. Limonene metabolites have been shown to have greater pharmacological potency than limonene in the inhibition of small-G-protein isoprenylation [1, 5] and tumor cell proliferation [5] and in the chemoprevention of dimethylbenz(a)anthracene-induced rat mammary cancer [4]. These findings coupled with the extensive metabolism of limonene in vivo [3, 18] argue that limonene is a prodrug and its metabolites are the active pharmacological agents in vivo. The high therapeutic index of limonene in the chemotherapy of rodent tumors [12] suggests that it may be efficacious against human malignancies. Thus, it is necessary to assess the metabolism of limonene in humans prior to its evaluation in clinical trials so as to determine whether humans produce the metabolites that have shown greater activity than limonene in other bioassays. We therefore carried out a study in which we identified human plasma metabolites of limonene.

Table 1 Information on the subjects participating in the present study[a]

Subject	Gender	Menopausal status	Limonene dose (mg/kg)	Time points[b] (h postingestion)
2	F	Premenopausal	100	0, 1, 2, 4, 8, 24
3	F	Postmenopausal	100	0, 4, 24
4	F	Premenopausal	100	0, 4, 24
5	F	Premenopausal	100	0, 4, 24
6	M		100	0, 4, 24
7	F	Premenopausal	100	0, 4, 24
8	M		100	0, 4, 24

[a] Subject 1 received 300 mg/kg limonene in orange juice rather than in custard and was not included in the data analysis

[b] Time points at which limonene metabolism was analyzed

Subjects and methods

Limonene

Limonene was given to the subjects in the form of orange-peel oil, which is typically 90%–95% *d*-limonene. The orange oil (Sunkist) used in this study was determined to be 95% limonene by capillary gas chromatography. A comparison of rat metabolism of orange oil (95% *d*-limonene, from Sunkist) versus 99% limonene (Aldrich) indicated that identical metabolites were formed at identical concentrations (data not shown).

Subjects

The subjects were seven paid volunteers, including five women and two men aged a median of 32 years (range, 23–55 years), who had no illness or laboratory abnormality and had given institutionally approved informed consent (Table 1). All subjects had fasted for 12 h and had refrained from ingesting citrus fruits or citrus-flavored soft drinks for 48 h prior to ingestion of the orange oil. Women of child-bearing potential had a negative urine pregnancy test on the day of the study.

Administration and evaluation

The subjects ingested 100 mg/kg *d*-limonene in a semisolid mixture of custard-like consistency that was 5% (w/w) orange oil. The custard consisted of dry unflavored custard mix, cold water, cold whole milk, and orange oil. The components were blended at room temperature and then refrigerated for at least 30 min prior to ingestion by the subjects. Subjects ingested the orange oil-containing mixture over a period of 2–10 min. Whole blood was drawn for chemistry panel analysis and for plasma limonene-metabolite analysis prior to and at 4 (metabolism analysis only) and 24 h after ingestion of the orange oil. One subject had additional blood drawn for metabolite analysis at 1, 2, and 8 h postingestion. The chemistry panel consisted of blood urea nitrogen (BUN), creatinine, calcium, magnesium, phosphorus, uric acid, total cholesterol, total protein, albumin, total bilirubin, γ-glutamyl transpeptidase, alkaline phosphatase, aspartate aminotransferase, and lactate dehydrogenase. Subjects were monitored for toxicity for 1 week.

Gas chromatography of plasma extracts

Limonene metabolism was analyzed in organic extracts of plasma by capillary gas chromatography with on-line flame ionization and mass-selective or infrared detection as described previously [3]. The extraction efficiency was >90% for all monoterpenes. Plasma extracts were analyzed on a Hewlett-Packard 5891 gas chromatograph equipped with a 30-m, 0.32-mm inside-diameter, 0.25-μm film-thickness Supelco SPB-5 column with a flame-ionization detector. Where

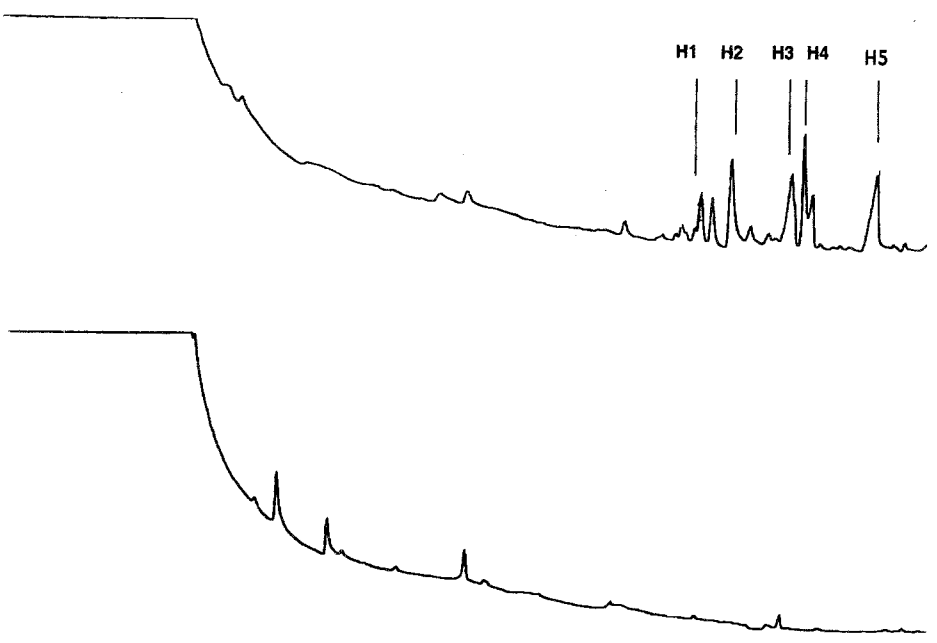

Fig. 2 Capillary gas chromatograms of organic extracts of plasma from subject 4 as obtained by flame-ionization detection at 4 h after (*top*) and just prior to (*bottom*) ingestion of 100 mg/kg *d*-limonene. The two chromatograms, aligned by retention time, were obtained on the same day under the chromatographic conditions described in Subjects and methods. The limonene metabolites, numbered H1–H5, correspond to those shown in Figs. 1, 3, 4, and 5 and in Table 2

indicated, mass spectra were obtained on a Hewlett-Packard 5890 chromatograph equipped with a 5971A mass-selective detector. For monoterpene quantitation, standard curves were generated for limonene and each of the metabolite standards in rat plasma at concentrations ranging from 0.10 to 1.0 m*M*. Perillylaldehyde, which migrated between dihydroperillic acid and perillic acid methyl ester but did not overlap with any of the metabolites, was used as an internal standard. The ratio of the monoterpene peak area/perillaldehyde peak area versus the concentration of monoterpene in the sample was plotted, and the slope of this line was used to determine the concentration of each human limonene metabolite. The slopes of the standard curves generated for all monoterpenes were linear, with correlation coefficients (r) ranging from 0.98 to 1.0. The limit of detection was 5–10 μ*M* for each monoterepene. For monoterpene concentrations below 100 μ*M*, standard addition was used to confirm the identity of the monoterpene. Limonene 1,2-diol (*p*-menth-8-ene-1,2-diol) was synthesized by the hydrolysis of (+)-limonene oxide [5]. Perillic acid, dihydroperillic acid, and their methyl esters were synthesized as described previously [3]. All other monoterpene standards for gas chromatography were purchased from Aldrich at the highest purity available.

Results

Chemistry-panel analysis

No intrasubject change was observed on the chemistry-panel analysis following ingestion of 100 mg/kg *d*-limonene (data not shown). No gradable toxicity was encountered, but the following effects were reported: four of seven subjects reported mild eructation for 1–4 h postingestion, one of seven reported mild satiety for 10 h postingestion, and one of seven noted slight fatigue for 4 h postingestion.

Metabolite identification

The presence of limonene metabolites in plasma was assessed by capillary gas chromatography. The flame-ionization chromatograms from 4-h postingestion time points were first compared with those of the preingestion

Table 2 Retention times and on-line gas chromatography/IR analyses of human limonene metabolites[a]

Compound	Retention time (min)	IR absorbance (cm^{-1})
H1	9.60	ND
Dihydroperillic acid methyl ester	9.60	ND
H2	9.79	ND
Limonene-1,2-diol	9.79	ND
H3	10.46	1770 (s, C = O);3574,1101 (m, OH); 2944 (m)
R3 (dihydroperillic acid)	10.46	1770 (s, C = O);3574,1101 (m, OH); 2944 (m)
H4	10.70	ND
Perillic acid methyl ester	10.70	ND
H5	11.42	1752 (s, C = O);3583,1163 (m, OH); 2948 (w)
R5 (perillic acid)	11.42	1752 (s, C = O);3583,1164 (m, OH); 2943 (w)

[a] The human limonene-metabolite designations H1–H5 correspond to those shown in Figs. 1–5 and in Results. The rat limonene-metabolite designations R3 and R5 correspond to rat metabolites of limonene [3] with the same retention times and mass spectra as those described for the human limonene metabolites

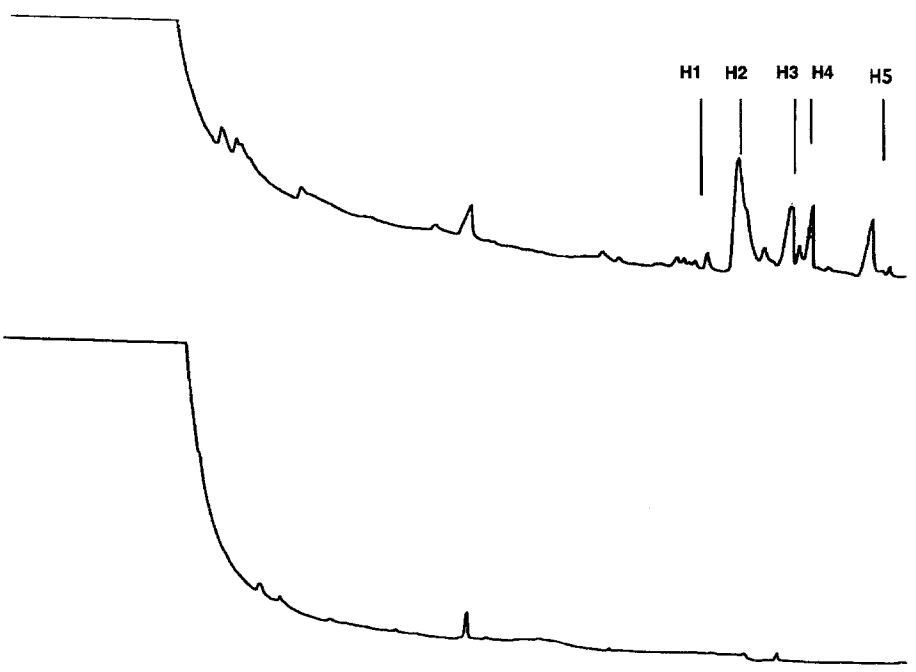

Fig. 3 Capillary gas chromatograms of organic extracts of plasma from subject 8 as obtained by flame-ionization detection at 4 h after (*top*) and just prior to (*bottom*) ingestion of 100 mg/kg *d*-limonene. The two chromatograms, aligned by retention time, were obtained on the same day under the chromatographic conditions described in Subjects and methods. The limonene metabolites, numbered H1–H5, correspond to those shown in Figs. 1, 2, 4, and 5 and in Table 2

time point. As shown in Figs. 2 and 3, several peaks were present in the 4-h chromatograms (Figs. 2, 3; top panels) that were not present in the respective time-zero chromatograms (Figs. 2, 3; bottom panels). These peaks were designated H1–H5 in order of retention time. The retention times (Table 2), vapor-phase infrared (IR) spectra (Table 2), and mass spectra (Figs. 4, 5) were compared with those of the rat plasma limonene metabolites perillic acid, dihydroperillic acid, perillic acid methyl ester, and dihydroperillic acid methyl ester [3]. Four of the five human peaks had the same retention times (Table 2) and mass spectra (Fig. 4) as those of rat limonene metabolites [3], and, where determined, the IR spectra (Table 2) matched as well. On the basis of these criteria, four of

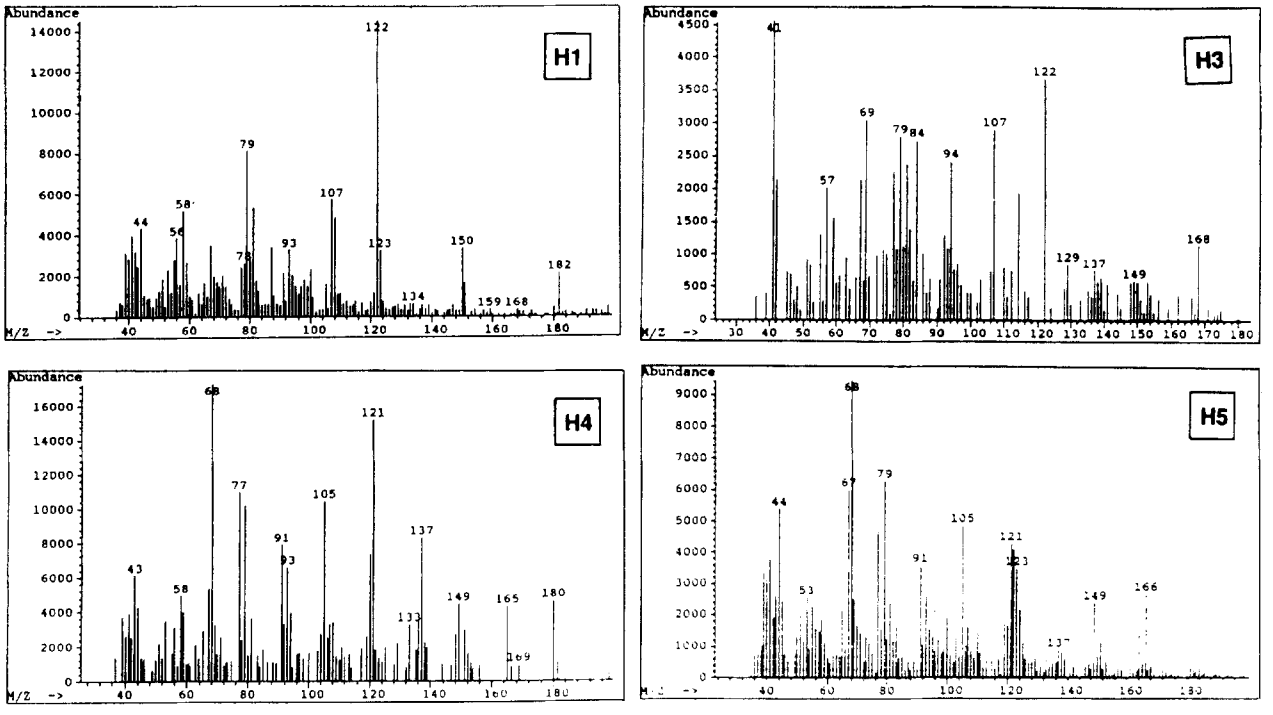

Fig. 4 Mass spectra of limonene metabolites H1, H3, H4, and H5. The mass spectra were obtained by capillary gas chromatography with on-line mass-selective detection as described in Subjects and methods

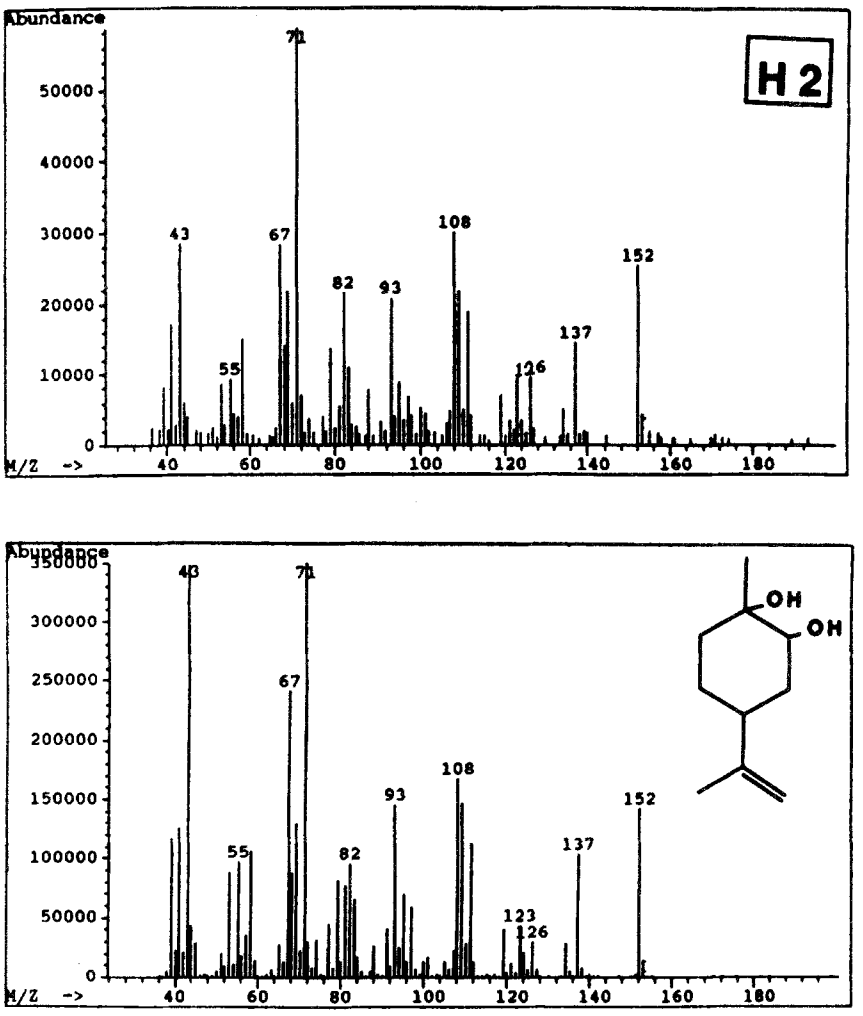

Fig. 5 Mass spectra of limonene metabolite H2 (*top*) and synthetic limonene-1,2-diol (*bottom*). The mass spectra were obtained by capillary gas chromatography with on-line mass-selective detection as described in Subjects and methods

the human peaks were identified as follows: H1, dihydroperillic acid methyl ester; H3, dihydroperillic acid; H4, perillic acid methyl ester; and H5, perillic acid.

One human metabolite, H2, was one of the three most abundant metabolites (along with perillic acid and dihydroperillic acid) in all subjects (Figs. 2, 3). The mass spectrum of metabolite H2 is shown in Fig. 5 (top panel). The mass spectrum was somewhat similar to that of two limonene-related diols, *p*-menth-1-ene-8,9-diol (uroterpenol) and *p*-menth-1-ene-6,8-diol (sobrerol; data not shown). Both *p*-menth-1-ene-6,8-diol and metabolite H2 had features typical of terpene alcohols, namely, an undetectable molecular ion peak and prominent peaks of 152 (M^+-H_2O) and 137 (M^+-H_2O and CH_3). On that basis, it

Table 3 Quantitation of human plasma limonene metabolites[a] (ND, Not detected)

Subject	Plasma monoterpene concentration, μ*M*						
	Limonene	Perillic acid	Dihydroperillic acid	Limonene-1,2-diol	Perillic acid methyl ester	Dihydroperillic acid methyl ester	Total monoterpenes
2	ND	32	29	5	ND	ND	66
3	<1	38	33	14	6	<1	91
4	<1	37	35	16	5	<1	93
5	ND	40	42	25	<1	ND	107
6	ND	34	27	12	<1	ND	73
7	ND	29	26	10	ND	ND	65
8	<1	32	38	34	8	ND	112
Average (mean ± SEM)	<1	35±1	33±2	16±4	3±1	<1	87±7

[a] Subjects ingested 100 mg/kg *d*-limonene. At 4 h postingestion, blood samples were taken for metabolite analysis. Detection and quantitation of metabolites was done as described in Subjects and methods

was hypothesized that metabolite H2 might be an isomer of the two diols listed above. Synthetic limonene-1,2-diol (*p*-menth-8-ene-1,2-diol) was analyzed in the same gas chromatography/mass spectrometry (GC/MS) system and was found to have the same retention time (Table 2) and mass spectrum (Fig. 5, lower panel) as metabolite H2, thus confirming the identity of H2 as limonene-1,2-diol.

Metabolite time course and quantitation

The concentration of each human plasma limonene metabolite was determined by an internal standard method, and the results are shown in Table 3. In all subjects, limonene metabolite concentrations were higher at 4 h than at 24 h postingestion (data not shown). For example, subject 2 had blood drawn at 0, 1, 2, 4, 8, and 24 h post ingestion. The highest monoterpene concentrations were detected at 4 h, when perillic acid and dihydroperillic acid were present at 32 and 29 μ*M*, respectively (Table 3). At each of the other time points, the monoterpenes were present at <1-μ*M* concentrations.

In all subjects, the most abundant plasma limonene metabolites detected at 4 h post-ingestion were perillic acid, dihydroperillic acid, and limonene-1,2-diol, whose average concentrations were 35, 33, and 16 μ*M*, respectively (Table 3). At the same time point, limonene represented $<2\%$ of the limonene-derived material in plasma. Perillic acid methyl ester and dihydroperillic acid methyl ester were also present at low concentrations, each representing $<4\%$ of the total limonene-derived material. The relative concentration of limonene-1,2-diol was higher in subject 8 (30% of the limonene-derived material) than in the other subjects (average of 17% of the limonene-derived material); actual concentrations ranged from 5 to 34 μ*M*.

Discussion

We have described the identification of the major circulating metabolites of *d*-limonene in humans. From this work and previously published reports [3, 18], it is quite evident that *d*-limonene is rapidly and extensively metabolized by humans, rats, and other mammalian species. In both humans and rats [3], dihydroperillic acid and perillic acid are major circulating metabolites of limonene, each accounting for $>35\%$ of the total limonene-derived material in plasma. Limonene-1,2-diol is also a major human plasma metabolite (18% of the limonene-derived material) but is present in only small concentrations in plasma of rats consuming limonene (Crowell and Gould, unpublished data). Perillic acid methyl ester and dihydroperillic acid methyl ester are detectable in plasma of both humans and rats after administration of limonene, but they account for $<5\%$ of the total limonene-derived material in each species.

Rat metabolism of limonene closely parallels that of humans in terms of both the specific metabolites formed and the time course in which they are formed [3, 18] (Table 3). In the present study, the concentrations of limonene metabolites were found to be highest at 4 h postingestion. By 24 h in all subjects and by 8 h in one more frequently monitored subject, the concentrations of limonene metabolites were lower than those detected at the 4-h time point. Similar time courses have been described in rats, where the highest concentrations of limonene-derived material are present between 2 and 12 h postingestion and are declining by 24 h postingestion [3, 18]. Thus, all available data suggest that the rat is a suitable model for human limonene metabolism and disposition.

The proportion of limonene-1,2-diol relative to the remainder of the circulating limonene-derived material was higher in male subject 8 than in male subject 6 or any of the female subjects. Thus, the differences in limonene-1,2-diol concentration cannot be attributable to gender or to pre- or postmenopausal status. The differences in limonene-1,2-diol concentrations observed among humans may be due to heterogeneity in limonene-metabolizing enzyme levels. Although the enzymes responsible for the metabolism of limonene to limonene-1,2-diol have not yet been identified, heterogeneity among humans has been reported for enzymes that metabolize polycyclic aromatic hydrocarbons such as cytochrome P-4501A1 and μ-class glutathione-S-transferase [13, 22, 23]. Limonene may be metabolized in part by one or more of these enzymes. Administration of limonene to rats increases liver carcinogen-metabolizing enzyme activities, including cytochrome P-450 [20] and glutathione-S-transferase [10, 24].

Limonene caused no gradable toxicity to humans at a single dose of 100 mg/kg. Rats ingesting limonene at a daily dose of 8 g/kg in their diet exhibit complete regression of $>80\%$ of chemically induced mammary tumors [12]. Thus, the doses tested in the human study described herein are well below the monoterpene doses associated with tumor regression in rats [12]. However, the lack of gradable toxicity observed in humans ingesting 100 mg/kg limonene suggests that higher doses of limonene may also be well tolerated. In Japan, limonene has been used locally in the gallbladder of humans for the purpose of dissolving gallstones at daily doses of up to 20 g/patient, or approximately 285 mg/kg, with no overt toxicity [15]. In the present study, mild effects were reported following ingestion of the limonene in custard (e.g., satiety, fatigue). However, it is possible that these effects were due to the rich custard rather than to the limonene.

Metabolites of limonene have been shown to be more potent pharmacological agents than limonene itself in several experimental systems. First, the relative effects of limonene and its metabolites on protein isoprenylation and tumor cell proliferation have been compared in cultured cells. Perillic acid [2], dihydroperillic acid [2], and all other known limonene plasma metabolites [5] were found to be more effective inhibitors of protein isoprenylation and tumor cell proliferation [5] than is limonene. Second, the relative chemopreventive effects of limonene and two of its urinary metabolites [18], carveol and uroterpenol, were compared [4]. When given orally during the initiation phase of dimethylbenz(*a*)anthracene-induced rat mammary

carcinogenesis, both hydroxylated urinary metabolites of limonene exhibited greater chemopreventive activity than did limonene [4]. Thus, on the basis of these bioassays, limonene metabolites appear to be the pharmacological agents responsible for tumor regression [12] and cancer chemoprevention [6, 7, 9, 11, 19, 25–27] in vivo. Since humans produce all of the known rat plasma metabolites [3] in approximately the same proportions [Table 3], the most pharmacologically active monoterpenes are present in human plasma as well. Thus, it will be possible to give the prodrug limonene to humans in upcoming phase I clinical trials [21] rather than directly giving a more potent limonene metabolite.

The pharmacological dose and chromatographic methods described herein should prove useful in the evaluation of limonene pharmacokinetics during future clinical trials. The chemotherapeutic and chemopreventive efficacy of limonene and related monoterpenes in experimental cancer-therapy models [1] and the known biological activities of human limonene metabolites [2, 5] suggest that limonene and related monoterpenes may be effective chemotherapeutic agents for human malignancies.

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