Pharmacokinetics of Perillic Acid in Humans after a Single Dose Administration of a Citrus Preparation Rich in d-Limonene Content

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Abstract
d-Limonene, a monoterpene, is widely distributed as a natural nonnutritive constituent of a variety of foods and volatile oils, particularly citrus oils. d-Limonene and its derived metabolites have been shown to possess cancer chemotherapeutic and chemopreventive efficacy in various preclinical model systems. In our previous work, we have found that lemonade prepared with the whole lemon (Mediterranean-style lemonade) contains high levels of d-limonene. The current study is designed to determine the systemic availability of perillic acid, a major and biologically active metabolite of d-limonene, after a single dose administration of Mediterranean-style lemonade. Healthy individuals were recruited to consume 30–40 oz of Mediterranean-style lemonade with a light breakfast. Blood samples were collected for up to 24 h after the completion of lemonade consumption. d-Limonene levels in the juice and perillic acid levels in plasma were determined by reversed-phase high-performance liquid chromatography with UV detection. It was found that 40 oz of lemonade contained 596 mg of d-limonene and 30 oz contained 447 mg of d-limonene. Plasma concentrations of perillic acid reached maximal levels at 1 h after the lemonade consumption and declined rapidly as a function of time with the terminal elimination half-life ranging from 0.82 to 1.84 h. The maximum plasma perillic acid concentration ranged from 2.08 to 13.98 μM, and the levels were undetectable at 24 h after the lemonade consumption. The area under the plasma concentration-time curves of perillic acid ranged from 5.07 to 32.59 (μM·h) after the completion of lemonade consumption. d-Limonene levels in the juice and perillic acid levels in plasma were determined by reversed-phase high-performance liquid chromatography with UV detection. It was found that 40 oz of lemonade contained 596 mg of d-limonene and 30 oz contained 447 mg of d-limonene. Plasma concentrations of perillic acid reached maximal levels at 1 h after the lemonade consumption and declined rapidly as a function of time with the terminal elimination half-life ranging from 0.82 to 1.84 h. The maximum plasma perillic acid concentration ranged from 2.08 to 13.98 μM, and the levels were undetectable at 24 h after the lemonade consumption. The area under the plasma concentration-time curves of perillic acid ranged from 5.07 to 32.59 (μM·h). Our study illustrates that the major metabolite of d-limonene is bioavailable after oral consumption of a citrus preparation rich in d-limonene content.

Introduction
During the last 30 years, research in the field of nutrition and cancer causation has led to exciting, significant progress in providing an understanding of specific risk factors and identifying potential preventive agents in the diet. Nonnutrient compounds in the diet belonging to different categories of chemicals have been found to exert inhibitory effects in experimental carcinogenesis (1–3). Citrus fruits, in addition to providing an ample supply of vitamin C, folic acid, potassium, and pectin, contain a host of active phytochemicals. Of these, d-limonene, which comprises >90% of citrus peel oil, is of significant interest because it has the capacity to inhibit the carcinogenesis processes via a variety of mechanisms (4–6).

The chemopreventive efficacy of limonene during both the initiation and promotion stages of carcinogenesis has been demonstrated in chemically induced rodent skin (7), kidney (8), lung and forestomach (9, 10), and mammary (11–13) tumor model systems. In mammary carcinoma, d-limonene exhibits therapeutic effects against chemically induced mammary tumors in rats, with regression of >80% of carcinomas with little toxicity (11). Limonene also appears to act in a cytostatic fashion. Its removal from the diet results in significant tumor recurrences (11). The postinitiation chemopreventive/tumor suppressive activity may be due, in part, to the inhibition of isoprenylation of cell growth-associated small G proteins such as p21
cd and induction of apoptosis by limonene and its metabolites (11, 12). The initiation-phase chemopreventive effects of d-limonene have been attributed to the modulation of Phase I (14) and Phase II (5) carcinogen-metabolizing enzymes, leading to enhanced detoxification of carcinogens.

Chemotherapeutic activities of pharmaceutical preparations of d-limonene are under evaluation in Phase I/II therapeutic clinical trials (15). d-Limonene, as a drug, is well tolerated in cancer patients at doses that may have clinical activity. One partial response in a breast cancer patient at a dose of 8 g/m²/day was maintained for 11 months, and three additional patients with colorectal carcinoma showed stabilization of disease for longer than 6 months on d-limonene at 0.5 or 1 g/m²/day (15). The favorable toxicity profile and the partial response supports additional clinical evaluation.

The principal sources of d-limonene in the diet are the oils of orange, grapefruit, and lemon (16). It is found naturally in orange juice at an average concentration of 100 mg/liter. d-Limonene (orange oil/essence oil) is also used as a flavoring ingredient for citrus flavor in artificial oils and can be found in nonalcoholic beverages (31 mg/liter), ice cream and ices (68 mg/kg), candy (49 mg/kg), and baked goods (120 mg/kg; Ref. 17).

Dietary intake of d-limonene can vary considerably depending on the types of citrus consumed and the preparation and processing procedures (18). Daily United States per capita consumption of d-limonene, as a result of both its natural occurrence in food and of its presence as a flavor, was estimated to be 0.27 mg/kg body weight/day (16.2 mg/day) for a 60-kg individual (17). However, d-limonene intake may approach 1 mg/kg body weight (60 mg/day) because of high consumption of citrus juice products.
Our previous work has shown that lemonade prepared fresh with whole lemons (Mediterranean-style lemonade) contains d-limonene levels (500ℓ/1027 mg/liter) >10–20 times of those available in commercial citrus juices (18). The objective of this study is to determine whether d-limonene or its derived materials would be available systemically after the oral consumption of Mediterranean-style lemonade. Because perillie acid has been identified to be one of the major metabolites of d-limonene in humans (15, 19) and has been shown to exert potent biological activities (4, 6), we have chosen to monitor the systemic availability of perillie acid after a single dose administration of Mediterranean-style lemonade (see Fig. 1 for the chemical structures of limonene and perillie acid). This study represents a step toward evaluating the protective activity of dietary limonene intervention and may provide information relating to biomarkers of limonene consumption that could be used in epidemiological and nutrition intervention studies (Fig. 1).

Materials and Methods

Study Subjects. Healthy men and women were recruited to participate in the study. To be eligible, the participants were required to be able to give informed consent and were willing to consume 40 oz of lemonade with breakfast. Participants were excluded if they were pregnant, were immunosuppressed by virtue of medication or disease, had serious concurrent illness that interfere with study regimen, or had a history of invasive cancer or systemic chemotherapy within the past 5 years. The study was approved by the University of Arizona Human Subjects Committee. Written informed consent was obtained from all participants. The demographic data of the study participants are summarized in Table 1.

Study Design. All study participants were required to refrain from the ingestion of citrus products 7 days before the study. On the day before the study, study participants were instructed to fast after midnight. Study participants came to the clinic in the morning (7–8 a.m.) and consumed 30–40 oz freshly prepared Mediterranean-style lemonade with bagels and cream cheese. The lemonade and the light breakfast were ingested over 20 min. The Mediterranean-style lemonade was prepared by blending 1 lemon/20 oz of water. Small amounts of sugar were added to minimize the sour taste. The preparation was filtered with commercial household filter and the filtrate was provided to the study participants. Blood samples (10 ml each) were collected before the lemonade ingestion and at time points 1, 2, 4, and 24 h after the completing of the lemonade consumption.

Sample Collection and Analysis. Once collected, blood samples were kept in the refrigerator and centrifuged at 4°C within 2 h of collection. After centrifugation, plasma was aliquoted into cryotubes and stored at −80°C for the analysis of perillie acid levels. Plasma perillie acid concentrations were determined using a reversed-phase HPLC3 procedure modified from that reported by Ezennia et al. (20). One ml of authentic plasma samples or spiked plasma standards were mixed with 1 ml of 0.2 m sodium acetate buffer (pH 3.8) and 25-μl internal standard solution (10 μg perillaldehyde/ml sodium acetate buffer). The mixtures were applied to BakerBond Octadecyl SPE cartridges (100 mg; JT Baker, Phillipsburg, NJ) preconditioned with methanol, water, and sodium acetate buffer [0.2 m (pH 3.8)]. After sample application, the cartridges were washed with 1 ml of the sodium acetate buffer. Perillie acid and the internal standard were eluted with 160 μl of methanol twice. The eluent was combined with 100 μl of water, and an aliquot was injected onto the HPLC. Chromatographic separation was achieved using a Supelco LC-ABZ column (150 × 4.6 mm; Supelco, Bellefonte, PA), and a mobile phase consisted of acetonitrile and sodium acetate buffer [25 mM (pH 5.2)] in the ratio of 29:71. The flow rate of the mobile phase was at 1.1 ml/min. The column eluent was monitored with a UV detector at a wavelength of 230 nm. Plasma perillie acid concentrations were quantified using calibration curves prepared with plasma spiked with perillie acid standards. The calibration curve was linear over the concentration range of 0.02 to 2 μg/ml. The inter- and intraday variation for the assay was <10%.

Aliquots of the lemonade preparations were stored at −20°C for the analysis of d-limonene content. The analysis was performed using a reversed-phase HPLC procedure. The lemonade preparation was mixed and diluted with the mobile phase before injecting onto the HPLC. Chromatographic separation was achieved using a Supelco LC-ABZ column (150 × 4.6 mm; Supelco, Bellefonte, PA), and a mobile phase consisted of acetonitrile and sodium acetate buffer [25 mM (pH 5.0)] in the ratio of 70:30. The flow rate of the mobile phase was at 1.1 ml/min. The column eluent was monitored with an UV detector at a wavelength of 230 nm. Plasma perillie acid concentrations were quantified using calibration curves prepared with d-limonene standards diluted with the mobile phase. The calibration curve was linear over the concentration range of 0.5 to 100 μg/ml.

Data Analysis. The following pharmacokinetic parameters of perillie acid were estimated using the WINNONLIN program with the model-independent approach (21): time to reach maximum plasma concentration (Tmax); maximum plasma concentration (Cmax); area under the plasma concentration-time profile (AUC); terminal elimination half-life (t1/2); and terminal elimination rate constant (λz).

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1 The abbreviations used are: HPLC, high-performance liquid chromatography; BMI, body mass index.
Results

Fig. 2 illustrates the HPLC chromatograms from plasma samples collected from one of the subjects at 2 and 24 h after the lemonade consumption. Under the chromatographic conditions used, perillic acid and the internal standard eluted at 21.5 and 26.5 min, respectively. The sample collected at 2 h after the lemonade consumption had a significant peak response corresponding to perillic acid. There was no peak response corresponding to perillic acid in the sample collected at 24 h after the lemonade consumption.

Plasma perillic acid concentration versus time profiles of each individual after consuming freshly prepared lemonade are presented in Fig. 3. The pharmacokinetic parameters of perillic acid are summarized in Table 2. Subject 1 consumed 30 oz of lemonade and all other subjects consumed 40 oz of lemonade. The limonene concentration in the lemonade was determined using a reversed-phase HPLC procedure and was found to be 504 mg/liter. Therefore, study subjects consumed 596 mg of d-limonene from 40 oz of lemonade and 447 mg of d-limonene from 30 oz of lemonade. As illustrated in Fig. 3, plasma perillic acid concentrations increased rapidly and significantly after the lemonade consumption with the peak levels occurred at 1 h after dosing. There was large between-subject variation in the plasma perillic acid levels were not detectable in samples collected at 24 h after dosing.

Discussion

Accumulating data show many inhibitory effects of nonnutrient compounds in the diet on carcinogenesis and other chronic diseases (1). A tentative differentiation between dietary patterns showed a 36% advantage in survival for those whose dietary habits corresponded to the Mediterranean diet where consumption of citrus fruit, olive oil, and orange juice was associated with a better prognosis (22). In our ongoing studies on the chemoprevention of cancer, we have a particular interest in the health benefits of the Mediterranean diet, of which citrus is a major component. Observational epidemiological data have consistently suggested that persons such as those living in the Mediterranean area who consume large amounts of fruits and vegetables in general, and citrus fruits in particular, have the lowest incidence rates for cardiovascular diseases and most tumors associated with diet (23, 24). The Mediterranean diet includes frequent exposure to citrus oils, which contain high levels of d-limonene. Food preparation may also play an important role in determining the amount of limonene consumed (18), with parts of the whole lemon fruit often included during cooking to add a desirable sour taste to the food. Dried fruits and fruit peels are consumed as candy or for cake decorations. Despite lemon and other citrus being widespread components of the Mediterranean cuisine, no studies have been conducted to determine the amount of limonene available in these diets or the plasma or tissue levels of limonene or its metabolites in humans consuming these foods.

Mediterranean-style lemonade is prepared from the whole fruit and is widely consumed by the general population in that region. In our previous study, we found that Mediterranean-style lemonade provides a rich source of d-limonene with d-limonene concentrations >20 times of those found in commercial citrus juice or preparations (18). The current study aimed to determine whether the consumption of this citrus preparation would deliver active monoterpenes to the systemic blood. We have selected to determine the systemic exposure of perillic acid, a major metabolite of d-limonene, after the consumption of freshly prepared Mediterranean-style lemonade because p.o. administered d-limonene has been shown to rapidly and extensively convert to its metabolites, and plasma d-limonene levels may not be detectable after low dose administration of d-limonene. In addition, because of the preliminary nature of this investigation, we have developed and validated a simple analytical procedure for determination of plasma concentrations of perillic acid instead of a complex analytical procedure for simultaneous determination of plasma levels of d-limonene and its derived metabolites. Consuming 40 oz of freshly prepared Mediterranean-style lemonade provides a rich source of d-limonene with d-limonene concentration that is 10–20 times higher than intake from regular daily consumption of commercial citrus juice products. Plasma concentrations of the major metabolite of d-limonene, perillic acid, peaked at 1 h after the lemonade consumption, implying that d-limonene consumed was rapidly absorbed and metabolized to perillic acid. Consumption of 40 oz of lemonade (containing 0.596 g d-limonene) resulted in an average peak concentration of 6.2 μM and an average total system exposure...
levels of p21 ras or by inhibiting farnesylation of the protein (4). Plasma at concentrations of perillic acid, dihydroperillic acid, and limonene-1,2-diol were present in H9262.

Consumption of citrus or pharmaceutical preparations of d-limonene are necessary to evaluate the feasibility of conducting intervention trials using such a citrus preparation.

In summary, Mediterranean-style lemonade provides a rich source of d-limonene. Consumption of such a preparation leads to high plasma concentrations of a major and active metabolite of d-limonene. Studies on the safety and tissue exposure of monoterpenes after chronic high consumption of dietary limonene are necessary to evaluate the feasibility of conducting intervention trials using such a citrus preparation.

References

Table 2

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<td>SD</td>
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Notes:
- Subject 1 consumed 30 oz of lemonade and all other subjects consumed 40 oz of lemonade.
- Mean and SD calculated excluding the data from subject 1.

Fig. 3. Plasma perillic acid concentration versus time profiles after the consumption of 40 oz (○) or 30 oz (●) of Mediterranean-style lemonade.

(AUC) of 16.87 µmol/h. The plasma perillic acid levels declined rapidly as a function of time with an average t_{1/2} of 1.38 h.

d-Limonene and its derived monoterpenes have been reported to alter p21 ras expression either by decreasing overall mass composition would affect the plasma levels of perillic acid, we have examined the correlation between the systemic perillic acid levels and BMI from subjects who had consumed 40 oz of lemonade. No significant correlation was observed between the two variables, partly because of the limited sample size. It is of interest that those (subjects 2 and 9) with the lowest BMI (lean individuals) had the highest systemic perillic acid exposure.

In summary, Mediterranean-style lemonade provides a rich source of d-limonene. Consumption of such a preparation leads to high plasma concentrations of a major and active metabolite of d-limonene. Studies on the safety and tissue exposure of monoterpenes after chronic high consumption of dietary limonene are necessary to evaluate the feasibility of conducting intervention trials using such a citrus preparation.


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