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Gas chromatographic–mass spectrometric analysis of *d*-limonene in human plasma

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Abstract

d-Limonene shows carminative and cholagogue effects and is used in treatment of gallstone, cholecystitis and angiocholitis. A simple method was developed to determine the concentration of *d*-limonene in human plasma. *d*-Limonene and internal standard (naphthalane, $C_{10}H_{18}$) were extracted with *n*-hexane and then injected to GC–MS. Calibration curves were linear ($r = 0.9990$, $n = 6$) in the range of 2–500 ng/ml for *d*-limonene in human plasma. Limit of detection and quantification were 0.5 and 2 ng/ml, respectively. This rapid and specific method was applied to the clinical pharmacokinetic investigation of *d*-limonene.

Keywords: *d*-Limonene; GC–MS; Analysis; Human plasma

1. Introduction

d-Limonene (*p*-mentha-1,8-diene) (Fig. 1), a monocyclic monoterpene, is a natural component of a variety of foods and beverages. It was found in many fruits (especially citrus fruits), vegetables and spices [1]. As the main odour constituent of citrus, *d*-limonene is used in food manufacturing as a flavoring and added to cleaning products to give a lemon-orange fragrance [2,3]. Large dose of *d*-limonene has been proved to have anticancer activity and minimal toxicity in preclinical and clinical studies [4–12]. Low dose of *d*-limonene shows carminative and cholagogue effects and is used in treatment of gallstone, cholecystitis and angiocholitis [13–17]. As a result, considerable research interest has been focused on this compound. To determine the concentration of *d*-limonene in experimental animals and human, several analytical methods such as gas chromatography-flame ionised detector (GC-FID) [8,11,18,19], high performance liquid chromatography–mass spectrometry (HPLC–MS) [20,21] and GC–MS [22–24] have been reported in the literature. However, these methods cannot easily be applied to determine the concentration of *d*-limonene in human plasma when only a single dose of 300 mg *d*-limonene was given to volunteers for carminative and cholagogue effects, because the sensitivity of these methods is too low or the extraction method is complex. To overcome these problems, a new GC–MS method was developed in this study. This method is sensitive, reproducible and rapid, suitable for the determination of *d*-limonene in human plasma.

2. Experimental

2.1. Chemicals and reagents

d-Limonene reference substance was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). The internal standard, naphthalane ($C_{10}H_{18}$), was bought from the Shanghai Chemical Co., Inc. (Shanghai, China). *d*-Limonene hard capsules were offered by Sanjiu Changzheng Pharmaceutical Co. (Leshan, China). Acetonitrile and *n*-hexane (HPLC grade) were from Fisher Scientific (Pittsburgh, PA). The water was purified

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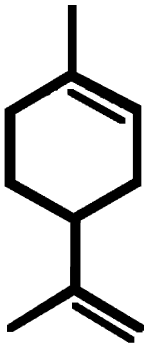

Fig. 1. Chemical structure of *d*-limonene ($C_{10}H_{16}$).

by the Mili-Q system (Bedford, MA, USA). All other chemicals and reagents were of analytical grade.

2.2. Apparatus and GC–MS conditions

An Agilent 6890 gas chromatograph coupled with an Agilent 5973 mass spectrometer, an Agilent Technologies 7683 series autosampler and Agilent ChemStation software (Agilent Technologies, Palo Alto, CA) were used for the analysis. An HP-5 fused-silica capillary column (30 m × 0.25 mm i.d.) coated with a film of methylsilicone and 5% phenyl methylsilicone (thickness 0.25 μm) was used for separation. The injector temperature was 220 °C. The temperature program was started at 60°C, remaining at this temperature for 1 min, and heated at 20 °C/min to 140 °C, remaining there for 2 min. Split injection (2 μl) was conducted with a split ratio of 1:1. High purity helium was used as carrier gas at a flow-rate of 1.5 ml/min. Other instrumental parameters were as follows: the electron energy was set at 70 eV, interface and the ion source temperature were set at 280 °C. The mass spectra of *d*-limonene and naphthalane obtained by the GC–MS systems are shown in Fig. 2. The analytes were monitored by the selected ion monitoring (SIM) mode. The total run time was 7 min.

2.3. Sample preparation

To a glass tube containing 0.5 ml plasma, 20 μl *n*-hexane and 20 μl internal standard (IS) solution dissolved in *n*-hexane (1.5 μg/ml) was added at room temperature. Then 1 ml acetonitrile was added. The tubes were vigorously shaken for 3 min (Vortex) and centrifuged at 1000 × *g* for 10 min. The supernatant was transferred to new glass tubes and 0.5 ml *n*-hexane was added to each tube. The mixture was shaken for 5 min and centrifuged at 1000 × *g* for 10 min. The *n*-hexane layer was transferred to the GC autosampler vials, and 2 μl aliquot was injected into a GC–MS system.

2.4. Method validation

The validation of the method was based on the guidelines of the United States FDA [26].

The linearity of the calibration was tested by the analysis of six different calibration series. Calibration curves were constructed by fitting a straight line using the least squares method and $1/y^2$ as weighting factor on the calibration points. For the calibration, the peak area ratio values were plotted in the function of *d*-limonene concentration.

For the determination of inter-day precision and accuracy, six calibration and QC series were analyzed on six different days. The intra-day precision and accuracy were tested with analysis of six parallel samples at the concentration levels of 330, 65 and 4 ng/ml.

Lower limit of detection (LOD) was defined as the lowest concentration, which gives a signal-to-noise ratio of 3 for *d*-limonene. Lower limit of quantitation (LLOQ) was defined as the lowest concentration of the calibration curve that can be determined with not more than ±20% accuracy and precision values.

Extraction recovery of *d*-limonene was tested at the concentration levels of 330, 65 and 4 ng/ml, respectively and it was calculated by comparing the peak area values obtained in the test of intra-day repeatability to the peak area values of reference standard solutions (100%). Recovery of internal standard was determined simultaneously.

The stability of *d*-limonene in plasma was evaluated for 1.5 months at −20 °C, for three freeze and thaw cycles, and for 12 h at room temperature.

2.5. Pharmacokinetic study

2.5.1. Study design and sample collection

This was a single-centre, open-label pharmacokinetic study. The Ethical Committee of West China Medical Center, Sichuan University, (Sichuan, China) approved the study protocol. Each volunteer provided consent before entering the study. Twenty-four healthy Chinese nonsmoking male volunteers, ranging from

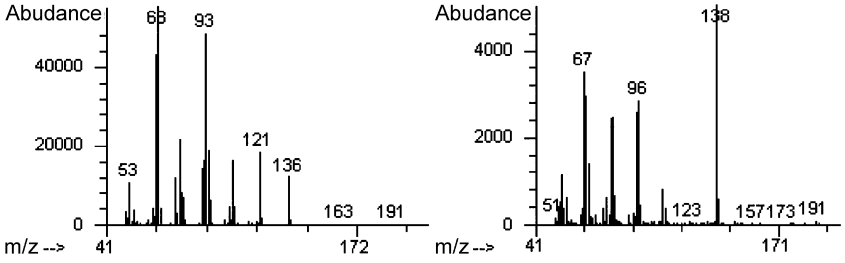

Fig. 2. Mass spectra of *d*-limonene (left) and naphthalane (right).

18 to 27 years old and body weight within 45–70 kg, were enrolled in the study. Medical history, physical examination, ECG and clinical laboratory tests including HIV, hepatitis B and C tests and urine drug screen within two weeks prior to inclusion to the study were evaluated for declaration of the healthy status and a follow-up visit was performed 2–7 days after the study drug intake. The volunteers were not allowed to use any medication within 14 days before and during the study. Meanwhile, in order to minimize exogenous interference of limonene, the volunteers were not allowed to consume alcohol, beverages and food or fruits containing limonene at least one week before and during the study.

In the morning, after an overnight fast (over 10 h), volunteers were given a single dose of 300 mg *d*-limonene hard capsules with 240 ml water. Subjects were not permitted for water and food intake until 2 h after the treatment. They were also not permitted to lie down or sleep within 4 h after treatment, and standard meals were served 2, 4 and 8 h after the treatment. Venous blood samples from healthy volunteers (approximately 5 ml) were collected into heparinized tubes through an indwelling cannula or venipuncture immediately and at the following time points after the *d*-limonene administration: 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12 h. After immediate chilling, the plasma was separated by centrifugation at 4 °C within 15 min. The samples were then shock frozen and stored at −20 °C until the assay. Frozen plasma samples were thawed in an ice-water bath.

2.5.2. Analysis of pharmacokinetic data

All pharmacokinetic analyses were performed using 3p97 software (the Chinese Society of Mathematical Pharmacology). Pharmacokinetic values for *d*-limonene were estimated by non-compartmental methods. The values of C_{max} and t_{max} were taken directly from the observed data. Individual concentration versus time profiles were plotted, and the terminal disposition rate constant (λ_z) was determined by the log-linear regression of at least three data points judged to be in the terminal phase. The terminal-phase half-life ($t_{1/2}$) was calculated by dividing 0.693 by λ_z. The area under the plasma concentration–time curve from time zero to the time of the last observed concentration (AUC_{0-t}) was calculated by trapezoidal rule. The area under the plasma concentration–time curve from time zero extrapolated to infinity was calculated as $AUC_{0\rightarrow t} + C_t/\lambda_z$, where C_t was the observed plasma concentration at the last measurable sampling time.

3. Results and discussion

3.1. Sample preparation

The mean extraction recovery of *d*-limonene was found to be below 70% when the other organic solvents (e.g. ether, ethyl acetate) were used as extracting solvents in our pilot studies, which did not help to enhance the sensitivity of the analytical method. The sample preparation procedure in this paper was simpler than that reported in the literature [18–24].

3.2. Chromatography and mass spectrometry

Typical chromatograms are shown in Fig. 3 (A–C). The retention times of *d*-limonene and IS were approximately 4.2 and 4.5 min, respectively. Naphthalane was selected as the internal standard for its similarity in the retention and extraction recovery to those of *d*-limonene. Other candidate compound (e.g. limonene-d_2 that is much closer in structure to the analyte) may also be used as IS. But it is unstable and expensive.

The dominant and characteristic ions for *d*-limonene and naphthalane were at *m/z* 93 and *m/z* 138 [25]. In order to enhance the sensitivity of method, SIM mode was selected at *m/z* 93 (from 3 to 4.3 min) and *m/z* 138 (from 4.3 to 5 min). The total run time was only 7 min, which was much shorter than those reported in the literature [18–24].

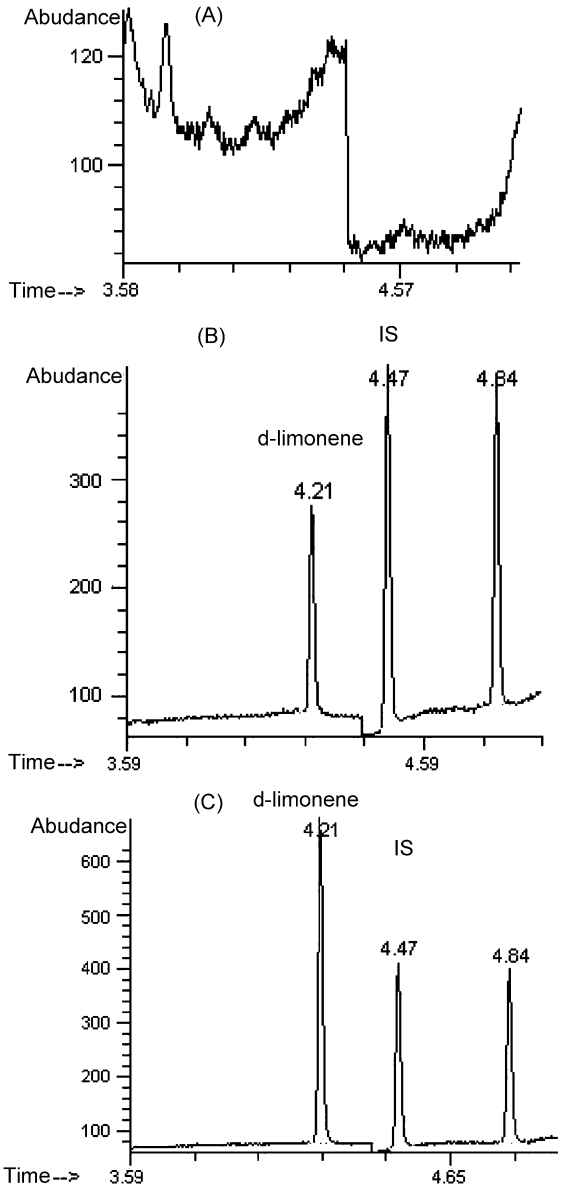

Fig. 3. Chromatograms of: (A), blank plasma obtained from a healthy volunteer; (B), blank plasma spiked with *d*-limonene (9.69 ng/ml); and (C), plasma from a healthy volunteer, 2 h after administration of *d*-limonene (300 mg).

Table 1
Results of regression analysis of the linearity data of *d*-limonene

	Mean ± S.E. ($n = 6$)
Slope	$2.50 \times 10^{-2} \pm 1.15 \times 10^{-2}$
Intercept	$3.33 \times 10^{-2} \pm 2.12 \times 10^{-2}$
Correlation coefficient (r)	$0.9990 \pm 4.26 \times 10^{-5}$

3.3. Method validation

3.3.1. Linearity

Calibration curves of *d*-limonene were linear for plasma over the concentration range of 2–500 ng/ml. Typical equations of calibration curves were $A = 0.025C + 3.33 \times 10^{-2}$ ($r = 0.9990$) (Table 1), where A is peak-area ratio (*d*-limonene/internal standard) and C is *d*-limonene concentration (ng/ml).

3.3.2. Precision and accuracy

The method showed good precision and accuracy. The mean recovery of *d*-limonene was 103.1% (Table 2). The intra-day precision was ≤3.6% and intra-day accuracy ranged from 2.0 to 4.8%. Inter-day precision was ≤4.0% and inter-day accuracy ranged from 3.7 to 6.8% (Table 3).

3.3.3. Extraction recovery

The mean extraction recovery of *d*-limonene from human plasma at low quality control, medium quality control and high quality control (LQC, MQC and HQC) was 99.8 ± 0.72%, which was better than those of the studies reported in the literature [18–24]. The mean relative recovery for IS was 97.2 ± 0.86%

3.3.4. Sensitivity

The LOD defined as the lowest concentration of *d*-limonene, which can be detected (signal-to-noise ratio 3) in human plasma samples was 0.5 ng/ml. LLOQ of *d*-limonene in human plasma samples was 2 ng/ml, which was superior to previous reports [18–24].

Table 2
Absolute recovery of *d*-limonene from plasma samples ($n = 6$)

Concentration added (ng/ml)	Concentration found (mean ± S.D., ng/ml)	Recovery (%)	Relative standard deviation, R.S.D. (%)
4	4.2 ± 0.16	104.5	3.8
65	66.7 ± 1.69	102.7	2.5
330	337.3 ± 8.51	102.2	2.5

3.3.5. Stability

In the short-term stability study, the plasma samples spiked with *d*-limonene were stable at room temperature for 12 h.

In the long-term stability study, the plasma samples spiked with *d*-limonene were stable for 1.5 months at −20 °C and also kept stable after three freeze and thaw cycles.

3.3.6. Specificity

As mentioned above, in order to eliminate the interference originating from diet, especially in the case of limonene-type substances, the volunteers were not allowed to consume alcohol, beverages, food, or fruits containing limonene (such as oranges, peppermint, lemon) for one week before and during the study.

Specificity with respect to plasma components was determined by analyzing six different sources of blank plasma samples collected under controlled conditions. No interference was observed at the retention times of *d*-limonene and IS.

As it is known, substances from diet may interfere with the analysis very easily, especially in the case of limonene-type substances. Preliminary studies were conducted prior to test to find out whether the interference from diet could be eliminated under dietary restriction conditions. The results indicated that there was an interfering compound peak at the retention time of *d*-limonene in the chromatograms obtained from blank human plasma when there was no dietary restriction. After dietary restriction, the peak became smaller as time went on and disappeared after three to five days. Therefore, it is dietary restriction that ensures the specificity of this study and in order to minimize the interference from blank plasma, seven days of dietary restriction were required.

Furthermore, since most cleaning products contain limonene, it was necessary to avoid limonene in cleaning products.

3.4. Pharmacokinetic study

Method suitability was demonstrated during method application on a pharmacokinetic study of *d*-limonene after a single oral dose of *d*-limonene (300 mg) to 24 healthy adult male Chinese subjects. Fig. 4 shows mean plasma concentration profile for *d*-limonene. The evaluated pharmacokinetic parameters are summarized in Table 4.

The maximum concentration of *d*-limonene in measured plasma ranged from 40.1 to 327.4 ng/ml. The average value of C_{max} was much lower than that observed in most of the other studies [18–24]. The t_{max} values (ranging from 0.75 to 3.0 h) was relatively smaller than that (ranging from 1.0 to 6.0 h), reported in the paper [18]. $AUC_{0-\infty}$ values were somewhat less variable

Table 3
Precision and accuracy of *d*-limonene in plasma for intra-day and inter-day ($n = 6$)

Concentration added (ng/mL)	Intra-day			Inter-day		
	Concentration found (mean ± S.D., ng/ml)	Precision R.S.D. (%)	Accuracy R.M.E. (%)	Concentration found (mean ± S.D., ng/ml)	Precision R.S.D. (%)	Accuracy R.M.E. (%)
4	4.2 ± 0.15	3.6	4.8	4.3 ± 0.17	4.0	6.8
65	66.9 ± 1.85	2.8	3.0	67.8 ± 1.96	2.9	4.2
330	336.6 ± 8.32	2.5	2.0	342.2 ± 9.24	2.7	3.7

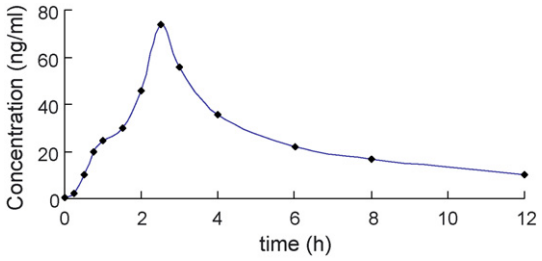

Fig. 4. Plasma concentration–time profile of *d*-limonene in 24 healthy volunteers following a single oral dose of 300 mg.

Table 4
Pharmacokinetic parameters after administration of 300 mg *d*-limonene hard capsules to 24 healthy volunteers

	rrC_{max} (ng/ml)	rrt_{max} (h)	rr$t_{1/2}$ (h)	rrAUC$_{0\text{-}t}$ (ng h/ml)	rrAUC$_{0\text{-}\infty}$ (ng h/ml)
Mean	rr93.6	rr2.50	rr3.42	rr275.8	rr311.9
S.D.	rr64.8	rr2.20	rr1.60	rr110.1	rr108.8

ranging from 136.1 to 573.0 ng h/ml. The AUC value was much lower than that observed in most of the other studies [18,20]. The difference may be due to the lower *d*-limonene dose in this study. The fact that the mean apparent volume of distribution (V_d) was 359.4 l indicated that the concentration of *d*-limonene in extravascular tissue was higher than that in the vascular compartment. The oral clearance (Cl_{oral}) was found to be ranging from 53.7 to 312.1 l/h.

4. Conclusions

In this paper, a novel and simple GC–MS method was developed for the determination of *d*-limonene in human plasma. It involves the simple and cost effective liquid–liquid extraction and meets the requirements of pharmacokinetic applications. This method has also shown some advantages such as the easy sample preparation procedure and high sensitivity. The method was successfully applied to the clinical pharmacokinetic research of *d*-limonene.

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