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Pharmacokinetics of *d*-limonene in the rat by GC–MS assay

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Abstract

The naturally occurring monoterpene *d*-limonene has been found to inhibit various stages of tumorigenesis in a number of animal models and is now being evaluated as a chemopreventive agent in humans. To date, there are little or no preclinical pharmacokinetics available nor is there a sensitive assay methodology. In this study, *d*-limonene and its dideuterium-labeled internal standard, limonene-d_2, in whole rat blood were extracted with n-pentane which was then concentrated on a Kuderna-Danish concentrator. The residue was analyzed by an ion-trap GC–MS under ammonia chemical ionization. The detection limit of *d*-limonene was 1.0 ng if injected in pure form; however, due to the presence of endogenous *d*-limonene levels (probably from diet), the routine quantitation limit was set at 1.0 μg ml^{-1}. The monitored assay linearity ranged from 1.0 to 30 μg ml^{-1} with within-day CV values of 8.0%, 2.4%, and 2.0% at 1.0, 3.0 and 10.0 μg ml^{-1}, respectively (all at $n=8$), and corresponding accuracy of 100%, 100%, and 101%. The between-day CV values were 12.3, 8.0, and 7.5% at 1, 6, and 20 μg ml^{-1}, respectively (all at $n=8$). Using this assay, pharmacokinetics of *d*-limonene were studied in Sprague-Dawley rats following intravenous and oral administration at 200 mg kg^{-1} each. Blood concentration–time profiles after intravenous administration showed a biphasic decline with a mean initial $t_{1/2}$ of 12.4 min and a terminal $t_{1/2}$ of 280 min. The plasma:red blood cell partition was found to be 0.84. Plasma protein binding of *d*-limonene was found to be 55.3% at 20 μg ml^{-1}. The mean total clearance was 49.6 ml min^{-1} kg^{-1}, the volume of distribution at steady-state 11.7 l kg^{-1}, and median residence time 263 min. The blood concentration–time decline following oral administration also showed a biphasic decline with a mean initial $t_{1/2}$ of 34 min and terminal $t_{1/2}$ of 337 min. The oral bioavailability of *d*-limonene was 43.0%.

Keywords: Chemoprevention; *d*-Limonene; GC–MS assay; Oral bioavailability; Pharmacokinetics

1. Introduction

d-Limonene, *p*-mentha-1,8-diene, is a naturally occurring monoterpene found in various essential oils [1]. This simple compound is widely used as an ingredient in soft drinks, cosmetics, and many other flavoring products. Recent studies have shown that *d*-limonene possesses significant chemopreventive [2–6] and chemotherapeutic [7,8] properties. As a result, considerable research interest has been generated on this compound.

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However, only limited pharmacokinetic information is available [9–11] and what is available was obtained based on a non-specific radioactive analytical method [9] or by an unvalidated gas chromatographic–flame ionization detection method [10,11]. Additionally, no bioavailability data were available. Here we report a sensitive gas chromatography–mass spectrometry (GC–MS) stable isotope dilution method for the quantitative analysis of *d*-limonene in whole blood and the pharmacokinetics and bioavailability of this agent in rats.

2. Materials and methods

2.1. Chemicals and reagents

d-Limonene and n-butyl lithium (1.6 M in n-hexane) were purchased from Aldrich (Milwaukee, WI). Acetonitrile (Optima) and n-pentane (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). [$^2H_3$]Methyl iodide (99.5% D) was purchased from Cambridge Isotope, MA).

The internal standard, limonene-d_2 [1-methyl-4(1′,1′-dideuterio-isopropylenyl)cyclohexene], was synthesized from the racemic 1-methyl-4-acetylcyclohexene by replacing its carbonyl function with dideuterated methylene moiety using Wittig reaction, similar to the ^{14}C-labeling method of Igimi et al. [9]. The labeled Wittig reagent was first synthesized as follows. To a solution of 1.80 g (6.80 mmol) of triphenylphosphine in 5 ml of dry benzene immersed in a dry-acetone bath was added dropwise a solution of 1.0 g of [$^2H_3$]methyl iodide in 2 ml of dry benzene. The resulting mixture was stirred overnight at room temperature. The mixture was then filtered and the solid washed with dry benzene to yield 2.50 g (89.3%) of [$^2H_3$]methyltriphenylphosphonium iodide. The synthesis was repeated three times to generate an adequate amount of the labeled reagent. Then, to a suspension of 5.37 g (13.2 mmol) of [$^2H_3$]methyltriphenylphosphonium iodide in 10 ml of dry tetrahydrofuran under nitrogen atmosphere in an ice-water bath was added 8.5 ml of 1.6 M n-butyl lithium in hexane. After the solid was dissolved using vigorous stirring, 1.8 g (12.7 mmol) of (±)-1-methyl-4-acetylcyclohexene in 5 ml dry tetrahydrofuran was added dropwise to the solution while maintaining the temperature under 20°C. The resulting mixture was then stirred at 50–60°C for 2 h. After cooling to room temperature, 50 ml of H_2O was added and the resulting mixture was extracted three times each with 50 ml diethyl ether. The combined ether extract was dried over anhydrous magnesium sulfate. Evaporation of the solvent gave a residue which was distilled to yield: 1.52 g (86.9%) colorless liquid, b.p. 165–167°C; ^1H NMR ($CDCl_3$) δ 1.40–2.20 (m, 7H, ring H), 1.66 (s, 3H, HC=C–CH_3), 1.74 (S, 3H, ^2H_2C=CCH_3), 5.40 (br, 1H, C=CH); CI–MS (m/z): 139 (M + 1), 123, 109, 95, 81.

2.2. Gas chromatography–mass spectrometry

A Varian 3400 gas chromatograph (Varian Associates, Palo Alto, CA) with a capillary splitless injector coupled to a Finnigan ITS40 ion trap mass spectrometer (Finnigan-MAT, San Jose, CA) was used for the analysis. A DB-5 fused-silica capillary column (30 m × 0.25 mm i.d.) coated with a 0.25 μm thickness film of methylsilicone and 5% phenyl methylsilicone (J&W Scientific, Folsom, CA). Helium was used as carrier gas and ammonia as the chemical ionization reagent under an emission current of 11 μA. The temperatures of the injection port, transfer line and ion source were set at 270°C, 280°C, and 280°C, respectively. The temperature of the oven was programmed to 60°C for 1 min and then increased to 160°C at a rate of 10°C min^{-1}. Under this condition the retention times of *d*-limonene and limonene-d_2 were both 6.2 min.

2.3. Extraction method

Appropriate volumes of the stock solution of *d*-limonene and limonene-d_2 in acetonitrile were added to 16 × 100 mm disposable test tubes immersed in an ice bath. The solution volume in each tube was adjusted to 1 ml with acetonitrile. Then, to each tube was added 0.5 ml of fresh rat blood. The tubes were immediately mixed by vortex for 30 s and then centrifuged at 1500 × *g* for 8

min at 4°C. The supernatant was transferred to new tubes and 5 ml of n-pentane was added to each tube. The contents of each tube were mixed by vortex for 30 s and then centrifuged at 1500 × *g* for 2 min at 4°C. The n-pentane layer was transferred to a 5 ml Kuderna-Danish concentrator (Ace Glasswares, Vineland, NJ). The concentrator was immersed in a 70°C water bath until most of the n-pentane was evaporated. After cooling, the volume of the concentrated liquid was further reduced to about 0.2 ml under a stream of nitrogen. The entire solution for each tube was transferred to a GC autosampler micro vial. A 5 μl of aliquot was injected into the GC–MS. Since the limonene:internal standard ratio was fixed for each sample, no difference in the results between the order of addition (i.e. compounds to blood followed by acetonitrile versus that above) was expected. This was verified using three different concentrations of 1, 6, and 20 μg ml^{-1}.

2.4. Assay validation

Eight concentrations of *d*-limonene in whole blood (0, 1.0, 2.0, 4.0, 6.0, 10.0, 20.0, and 30.0 μg ml^{-1}) were used for each calibration curve and 0.5 ml blood and 5.0 μg of the internal standard were used for each determination. The assay recovery was determined by processing *d*-limonene-spiked whole rat blood (5 and 10 μg ml^{-1}) to obtain the dry residue. Then an appropriate amount of the labeled intemal standard was added to each and the ratios were assayed by GC–MS. These ratios were compared with those from the corresponding control samples without extraction. The between-day variation was evaluated by measurement of spiked rat blood samples at three limonene concentrations of 1, 6, and 20 μg ml^{-1} on eight different days and the coefficients of variation (CV) were calculated. The within-day variation was evaluated at three concentrations of 2.0, 6.0 and 20.0 μg ml^{-1} with eight replicates each. At each concentration the CVs were calculated accordingly. A comparison the averages of the calculated amount with the theoretical amount of *d*-limonene gave the accuracy values.

2.5. Partition ratio between plasma and red blood cells and plasma protein binding

Plasma protein binding of *d*-limonene was determined by the equilibrium dialysis method. A Spectra-Por 1 membrane bag (Fisher Scientific, Pittsburgh) containing 2.0 ml of rat plasma was immersed into a solution of 20 μg ml^{-1} of *d*-limonene in 5 ml of phosphate-buffered saline. After agitating at 4°C for 4 days, 1.0 ml each of the sample was removed from inside and outside the bag. The experiment was performed in duplicate. Limonene concentrations of these samples were determined as before. The percentage protein binding was estimated by the difference in concentration inside and outside the bag and then divided by that inside the bag.

Partition of *d*-limonene between rat plasma and red blood cells was determined by incubation of *d*-limonene in fresh whole rat blood at 5.0 μg ml^{-1} at 37°C for 2 h. After incubation, an aliquot of the blood sample was centrifuged at 1500 × *g* for 10 min and *d*-limonene concentrations in plasma and whole blood were determined. At the same time the hematocrit of the blood sample was determined. The concentration ratio of *d*-limonene in plasma and red blood cells was thus computed.

2.6. Pharmacokinetics of d-limonene in the rat

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 310–370 g were used in the pharmacokinetic study. Before the experiment, the rats were fasted overnight with water given ad libitum. For intravenous administration, both the jugular and the femoral veins of the rats were cannulated with polyethylene tubing under ethyl ether anesthesia. Two hours after cannulation, pure *d*-limonene (200 mg kg^{-1}) was infused slowly through the femoral vein cannula over 5 min and the cannula was washed with 0.1 ml of saline immediately. Blood samples of 0.15 ml each were collected from the jugular vein cannula at 0, 1, 2, 4, 6, 8, 12, 16, 20, 30, 40, 60, 120, 240, 360, 600 min following dosing. The same procedure

was used for oral dosing except that only the jugular vein was cannulated and *d*-limonene (200 mg kg^{-1}) was given by gavage via an intubation tube. The blood samples of 0.15 ml each were collected at 0, 2, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 160, 240, 360, 600 min after dosing. The blood samples were immediately spiked with 0.3 ml of acetonitrile which contained 5.0 μg of limonene-d_2. After mixing by vortex for 30 min, the samples were kept frozen at −20°C until analysis.

2.7. Pharmacokinetic analysis

Plasma concentration–time data were fitted to an appropriate model by the PCNONLIN computer program (Scientific Consulting, Inc., Apex, NC). Pharmacokinetic parameters were thus estimated. The oral bioavailability was calculated by the ratio of the mean area under the curve (AUC) values ($AUC_{p.o.}/AUC_{i.v.} \times 100\%$) in a non-crossover manner.

3. Results and discussion

3.1. Mass spectrometry

Ammonia chemical ionization was used for quantitation of *d*-limonene, since under this condition low fragmentation was observed and a high intensity of the molecular ion $[M+1]^+$ was obtained. As shown in Fig. 1, the most abundant ions of *d*-limonene and limonene-d_2 are the molecular ions at m/z 137 and 139, respectively, and this pair of ions was used for quantitation. The internal standard, limonene-d_2, was found to contribute about 0.6% of the ion at m/z 137 due to the less than 100% isotopic labeling. The extent of isotope label was the same as the starting labeled methyl iodide, indicating that no scrambling occurred during the synthesis. The selection of the methylene position for deuterium labeling was based on the relatively simple synthetic method and the stability of these deuterium atoms toward potential exchange.

3.2. Assay validation

The sensitivity with direct injection of pure *d*-limonene was as low as 1.0 ng with a signal-to-noise ratio of 14. However, due to the presence of an endogenous level of *d*-limonene in the rat blood (probably from diet origin) as shown in Fig. 2, the routine quantitation limit was set at 1.0 μg ml^{-1}. We did not determine the endogenous levels of *d*-limonene in the rat, as the levels would be highly variable, depending on diet. However, from a small number of rat blank plasma samples examined, *d*-limonene levels were found to be in the range of 500–600 ng ml^{-1}. The blank value was found to be reduced significantly (nearly ten-fold) if blood was obtained from a rat after overnight fasting.

d-Limonene is a small volatile molecule; to prevent it from evaporating during the concentration process, a micro Kudema-Danish concentrator was used as the evaporation apparatus. The recovery from the entire procedure was found to be 82% at 5 μg ml^{-1} and 78% at 10 μg ml^{-1} concentration. Limonene was found to be stable in plasma at room temperature for at least 72 h and no appreciable loss was found within experimental error during this period. The linearity of the assay was observed in the range of 1.0–30 μg ml^{-1} with an average correlation coefficient of 0.99 ($n=6$). The between-day CV values were found to be 12.3%, 8.0%, and 7.5% at 1, 6, and 20 μg ml^{-1}, respectively (all $n=8$). The within-day CV values were 8.0%, 2.4%, and 2.0% for 1.0, 3.0 and 10.0 μg ml^{-1}, respectively ($n=8$).

3.3. Pharmacokinetics

Two representative blood concentration–time profiles of *d*-limonene in the rat after oral and intravenous administration are shown in Fig. 3. As can be seen, they were best fitted by a two-compartment model according to the Akaiki Information Criterion (AIC). The parameters as calculated by the computer program PCNONLIN are listed in Tables 1 and 2.

Following intravenous administration, the drug was rapidly distributed in the body with an average initial half-life ($t_{1/2\alpha}$) of 12.4 min. The distri-

Table 1
Relevant pharmacokinetic parameters ($n = 6$) of *d*-limonene in rats receiving *d*-limonene intravenously at 200 mg kg^{-1} each

	C_0 (μg ml^{-1})	A (μg ml^{-1})	α (min^{-1})	$t_{1/2\alpha}$	B (μg ml^{-1})	β (min^{-1})	$t_{1/2\beta}$	MRT (min)	AUC (μg ml^{-1} min^{-1})	CL_T (ml min^{-1} kg^{-1})	Vdss (l kg^{-1})
Average	83.17 ± 36.70	68.69	0.1385	12.4	14.44	0.004266	280.2	262.8	5094 ± 3533	49.6 ± 19.1	11.7 ± 6.48
± S.D.		± 36.71	± 0.1471		± 13.45	± 0.004018					
Range				2.0–22.7			58.2–442.0	81.4–503.2			

Table 2
Relevant pharmacokinetic parameters ($n = 6$) of *d*-limonene in rats receiving *d*-limonene orally at 200 mg kg^{-1}

	C_{max} (μg ml^{-1})	T_{max} (min)	K_a (min)	$T_{1/2a}$ (min)	α (min^{-1})	$t_{1/2\alpha}$ (min)	β (min^{-1})	$t_{1/2\beta}$ (min)	AUC μg ml^{-1} min^{-1})	F (non-crossover)
Average	11.3 ± 3.6	58	0.02337	37.75	0.02195	33.6	0.002423	337.0	2190 ± 1327	43.0%
± S.D.			± 0.005742		± 0.0050		± 0.001018			
Range		50–60		24.10–50.82		26.5–51.6		191.5–604.3		

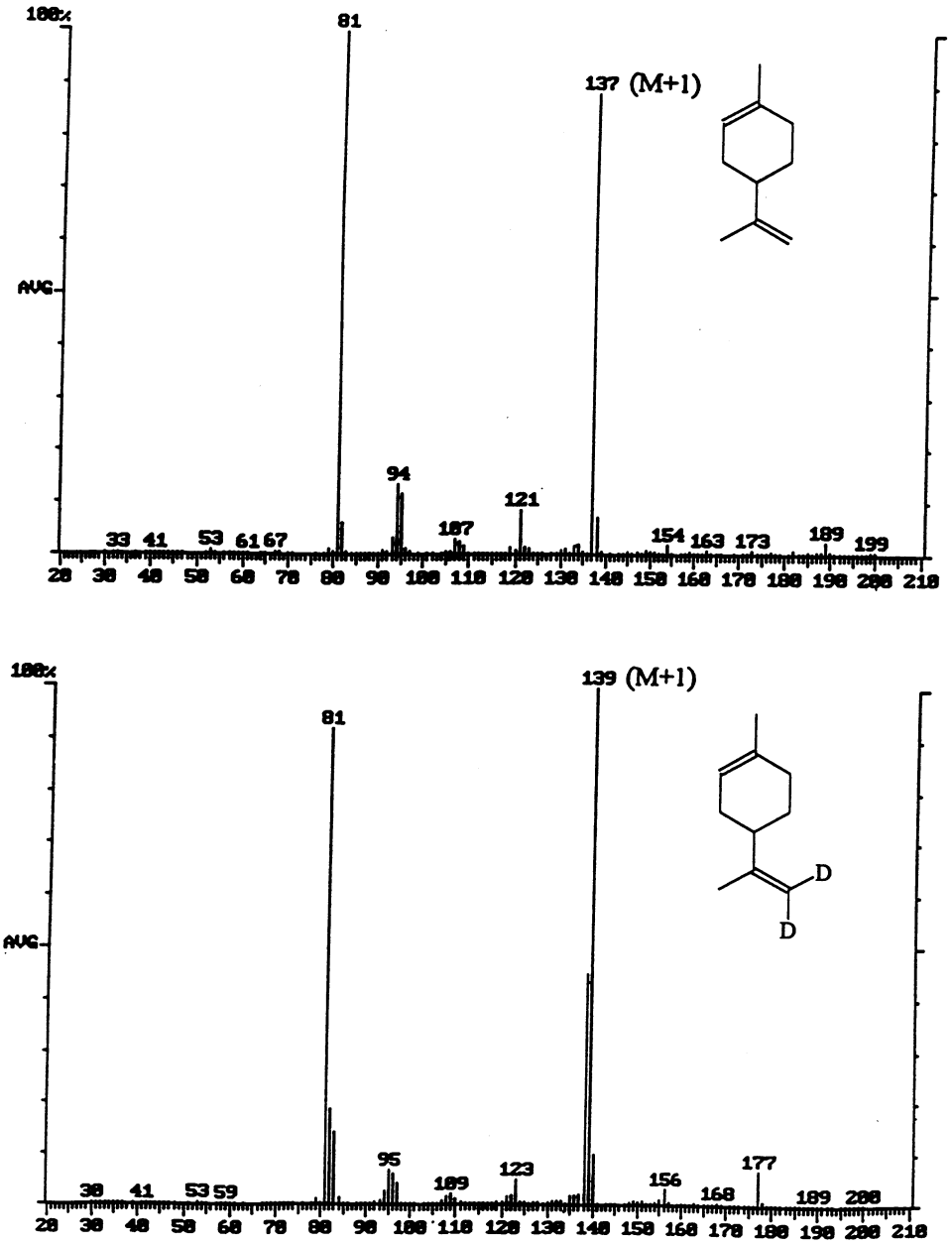

Fig. 1. GC–MS spectra of *d*-limonene (top) and limonene-d_2 (bottom) under ammonia chemical ionization.

bution volume at steady-state was significantly larger than the volume of the total body water, suggesting extensive tissue binding and distribution. The elimination is rather slow with an average terminal half-life ($t_{1/2\beta}$) of 280 min with significant individual variability. This elimination half-life is in a similar range to that reported by Crowell et al. [10] but differs significantly from the value indicated by the same group more recently [11]. Plasma protein binding of *d*-limonene was found to be 55.3% at 20 μg ml^{-1} and the compound partition between plasma and red

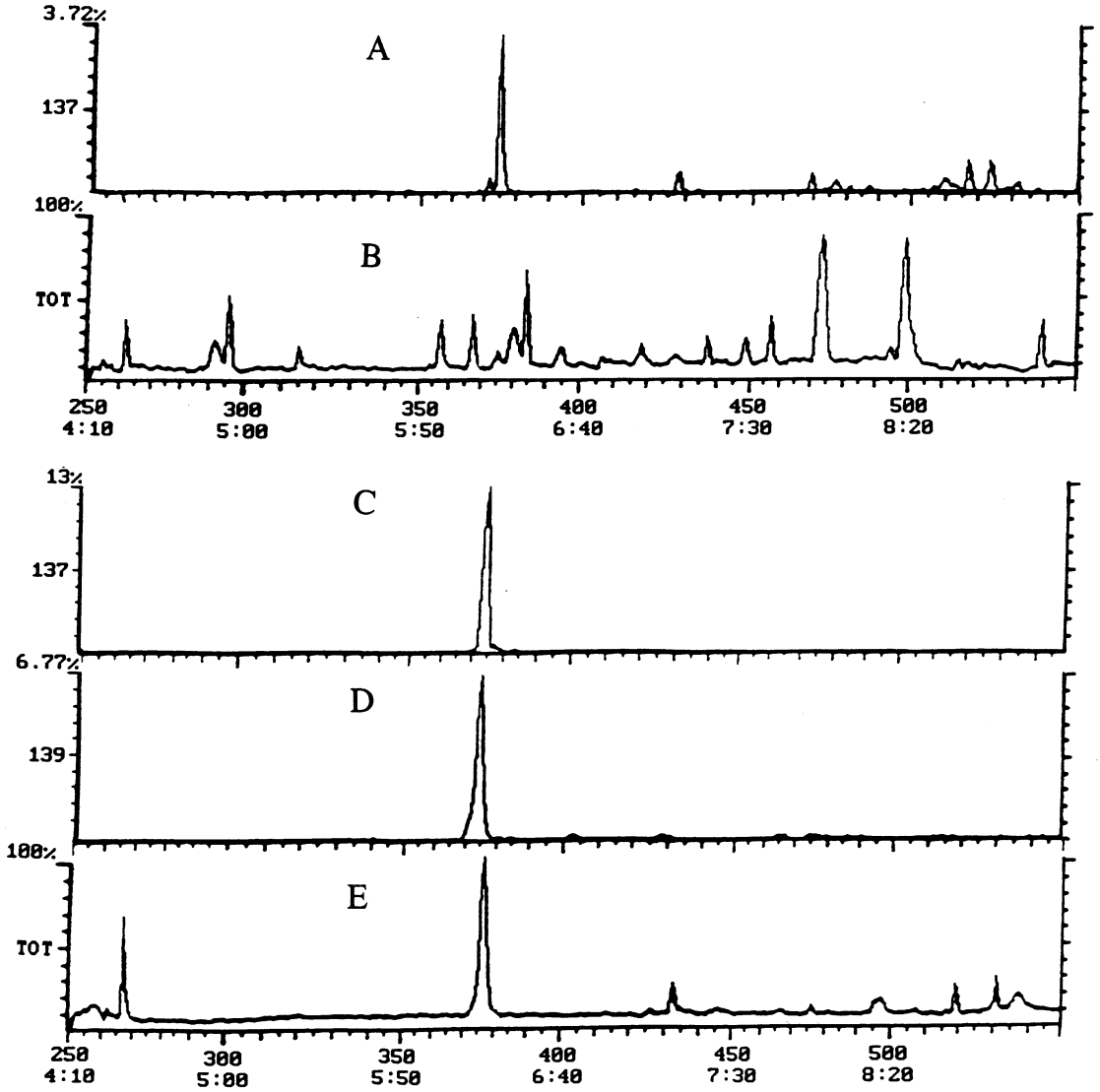

Fig. 2. GC–MS selected and total ion chromatograms of blank and sample rat blood extracts: (A) selected ion chromatogram for *d*-limonene in blank rat blood extract; (B) total ion chromatogram of blank rat blood extract; (C) selected ion chromatogram for *d*-limonene (approx. 10 μg ml^{-1}) in sample rat blood extract; (D) selected ion chromatogram for limonene-d_2 (5 μg ml^{-1}) in sample rat blood extract; (E) total ion chromatogram of sample rat blood. The peak with asterisk shows the presence of low level of *d*-limonene in blank rat blood with its CI mass spectrum confirmed (data not shown). The scale of all peaks on the blank rat blood chromatogram has been expanded relative to those of the sample.

blood cells in a ratio of 1:1.19. Following oral administration, *d*-limonene reached the highest plasma concentration of 11.3 μg ml^{-1} at 58 min, and then declined biexponentially with a mean $t_{1/2\alpha}$ and $t_{1/2\beta}$ of 37.8 min and 337.0 min, respectively. These results indicate that *d*-limonene was orally absorbed and appeared in the circulation rapidly. The mean AUC value for intravenous administration was 5094 μg ml^{-1} min^{-1} and 2190 μg ml^{-1} min^{-1} for oral adminstration, giving an oral bioavailability of 43.0% as estimated by a non-crossover method.

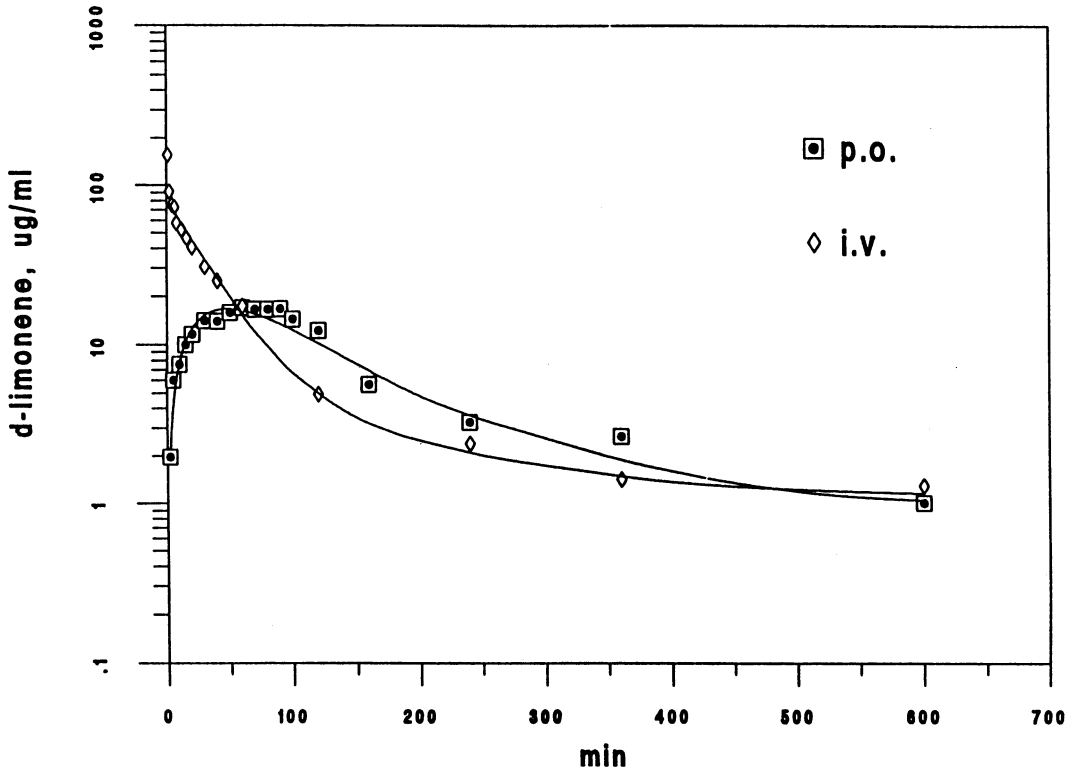

Fig. 3. The representative concentration–time curves of *d*-limonene in Sprague-Dawley rats receiving 200 mg ml^{-1} of *d*-limonene i.v. (◇) or p.o. (□); symbols represent measured concentrations and lines were from curve-fitting.

Although *d*-limonene has been widely used in the cosmetic and flavoring industry for a long time, its human consumption is likely to increase because of its potential health benefit. However, sparse pharmacokinetic information, especially pertaining to its oral bioavailability, is available. The present study provides some pharmacokinetic data and an estimate of its oral availability in an animal model, recognizing that there probably is significant species variability in pharmacokinetic properties of *d*-limonene.

4. Conclusion

In summary, a sensitive and specific GC–MS method has been developed for the analysis of *d*-limonene in the rat whole blood, using limonene-d_2 as the internal standard. Using this analytical method, the pharmacokinetics of *d*-limonene after intravenous and oral administration have been studied in the rat. Pharmacokinetics of *d*-limonene in the Sprague-Dawley rat following intravenous administration at 200 mg kg^{-1} showed a biphasic profile with a mean terminal $t_{1/2}$ of 4.7 h. Oral bioavailability of *d*-limonene was estimated to be 43.0% by a non-crossover method.

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References

[1] R. Croteau, Chem. Rev. 87 (1987) 929–954.
[2] C.E. Elson, T.H. Maltzman, J.L. Boston, M.A. Tanner, M.N. Gould, Carcinogenesis 9 (1988) 331–332.
[3] F. Homburger, A. Treger, E. Boger, Oncology 25 (1971) 1–10.
[4] T.H. Maltzman, L.M. Hurt, C.E. Elson, M.A. Tanner, M.N. Gould, Carcinogenesis 10 (1989) 781–783.
[5] C.J. Moore, W.S. Kennan, B.C. Wang, M.N. Gould, Proc. Am. Assoc. Cancer Res. 32 (1991) 131.
[6] L.W. Wattenberg, J.B. Coccia, Carcinogenesis 12 (1991) 115–117.
[7] J.A. Elegbed, C.E. Elson, M.A. Tanner, A. Quereshi, M.N. Gould, J. Natl. Canceer Inst. 76 (1986) 323–325.
[8] J.D. Haag, M.J. Lindstrom, M.N. Gould, Cancer Res. 52 (1992) 4021–4026.
[9] H. Igimi, M. Nishimura, Xenobiotica 4 (1974) 77–84.
[10] P.L. Crowell, S. Lin, E. Vedejs, M.N. Gould, Cancer Chemother. Pharmacol. 31 (1992) 205–212.
[11] P.L. Crowell, C.E. Elson, H.H. Bailey, A. Elegebede, J.D. Haag, M.N. Gould, Cancer Chemother. Pharmacol. 35 (1992) 31–37.