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Gas chromatographic–mass spectrometric analysis of perillyl alcohol and metabolites in plasma

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Abstract

Perillyl alcohol (POH), a metabolite of *d*-limonene and a component of the lavender oil, is currently in Phase I clinical trials both as a chemopreventative and chemotherapeutic agent. In vivo, POH is metabolized to less active perillic acid (PA) and *cis*- and *trans*-dihydroperillic acids [DHPA, 4-(1′-methylethenyl)-cyclohexane-1-carboxylic acid]. Previous pharmacokinetic studies using a GC–MS method detected POH metabolites but not POH itself; thus these studies lacked information on the parent drug. The present report describes a sensitive GC–MS method for the quantitation of POH and metabolites using stable-isotopically labeled internal standards. The residue obtained from CH_2Cl_2 extraction of a plasma sample was silylated. The products were separated on a capillary column and analyzed by an ion-trap GC–MS using NH_3 chemical ionization. POH-d_3 was used as the internal standard for POH while ^{13}C-PA-d_2 was used as the internal standards for the metabolites. The quantitation limits for POH, PA, *cis*- and *trans*-DPA were $<$10 ng/ml using 1–2 ml plasma. The assay was validated in rat and human plasma. The assay was linear from 2 to 2000 ng/ml for POH, 10 to 1000 ng/ml for PA and *trans*-DHPA, and 20 to 1000 ng/ml for *cis*-DHPA monitored. The within-run and between-run coefficients of variation were all $<$8%. Preliminary pharmacokinetic data from a rat following i.v. administration of POH at 23 mg/kg and from a patient receiving POH at 500 mg/m^2 p.o. was also provided. Intact POH, PA, *cis*- and *trans*-DHPA were all detected in plasma in both cases. Two new major metabolites were found in human and one in the rat plasma.

Keywords: Perillyl alcohol; *d*-Limonene

1. Introduction

Perillyl alcohol is a hydroxylated metabolite of *d*-limonene [1–3], which is the major, naturally occurring monoterpene present in orange peel oil. Perillyl alcohol is also found in lavender, mints, and celery seeds. It has been shown that *d*-limonene and metabolites possess chemopreventative and chemotherapeutic activity against human malignancies with low toxicity [4–8]. *d*-Limonene is extensively metabolized (Scheme 1) in animals [1–5] and in humans [9,10] and perillic acid and dihydroperillic acid were found to be the major circulating metabolites [1–3,5,6] and for perillyl alcohol [11]. Metabolites of limonene with intermediate polarity such as perillyl alcohol, perillic acid methyl ester and perillaldehyde were found to be more potent inhibitors of isoprenylation of the p21–26 kDa protein, which is

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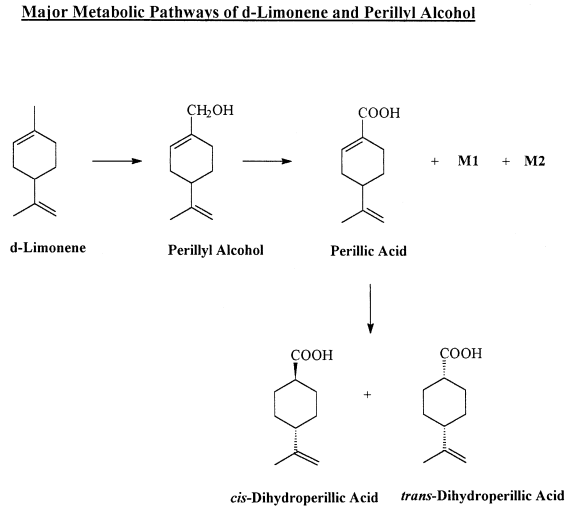

Scheme 1. Major metabolic pathways of perillyl alcohol.

involved in cell growth and proliferation [7], than limonene itself. In vivo, perillyl alcohol was also found to be more active than *d*-limonene [4–7,12–14].

Phillips et al. [11] conducted a pharmacokinetic study of perillyl alcohol in dogs using a GC–MS method with a sensitivity limit of 0.25 μg/ml, but were unable to detect the parent drug in circulation. In order to understand the pharmacokinetic and disposition characteristics of perillyl alcohol, the development of a more sensitive quantitation assay method is necessary. The present report describes a sensitive GC–MS method for the measurement of perillyl alcohol and its metabolites in plasma using the stable isotope-labelled analogs as the internal standards. A derivatization procedure is also used to facilitate the GC separation and mass spectrometric detection.

2. Materials and methods

2.1. Chemicals and reagents

(*S*)-(−)-Perillyl alcohol (POH) was purchased from Aldrich (Milwaukee, WI, USA). Perillic acid (PA), *cis*-/*trans*-dihydroperillic acid (*cis*-/*trans*-DHPA, *cis*-/*trans*-4(1′-methylethenyl)cyclohexane-1-carboxylic acid), perillyl alcohol-d_3 (POH-d_3) and perillic acid-d_2-^{13}C (^{13}C-PA-d_2) (Fig. 1) were synthesized according to the literature method [15]. POH-d_3 was used as the internal standard for POH, whereas ^{13}C-PA-d_2 was used as the internal standard for PA and *cis*-/*trans*-DHPA. *Cis*- and *trans*-1-carboxy-4-methylcyclohexane were purchased from Aldrich. The chemical purity of all these synthetic compounds as well as the isotope purity of labeled compounds was 99%. *N,-O*-Bis-(trimethylsilyl)silyltrifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL, USA). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). All other organic solvents and reagents purchased from Fisher Scientific were of reagent grade.

2.2. Sample extraction

To each of a set of 16×125-mm disposable tubes was added appropriate amounts of the internal standards in acetonitrile followed by gentle evaporation of the solvent with a stream of nitrogen. A plasma sample (0.5 ml or less) was then added and the content mixed. The sample volume of less than 0.5 ml was made up to 0.5 ml by addition of blank mouse or human plasma. Acetonitrile (1.0 ml) was added to precipitate the protein. The mixture was centrifuged at 500 *g* for 3 min. The supernatant was transferred to a new set of disposable tubes and was acidified by addition of 10 μl of 3 *M* HCl. The extraction was accomplished with 3×2 ml methylene chloride by vigorously mixing on a vortex-mixer for 10 s, followed by centrifugation at 500 *g* for 3 min. The methylene chloride extracts were combined and evaporated to dryness by a stream of N_2. Forty μl of 60% BSTFA in acetonitrile was then added. The content was allowed to stand at room temperature for 5 min before analysis by GC–MS.

2.3. GC–MS analysis

GC–MS analysis of the analytes was performed on a Finnigan MAT ITS40 (Finnigan MAT, San Jose, CA, USA) coupled to a 3300/3400 Model Varian gas chromatograph (Walnut Creek, CA, USA). The gas chromatograph was equipped with a DB-5 fused-silica capillary column (30 m×0.25 mm)

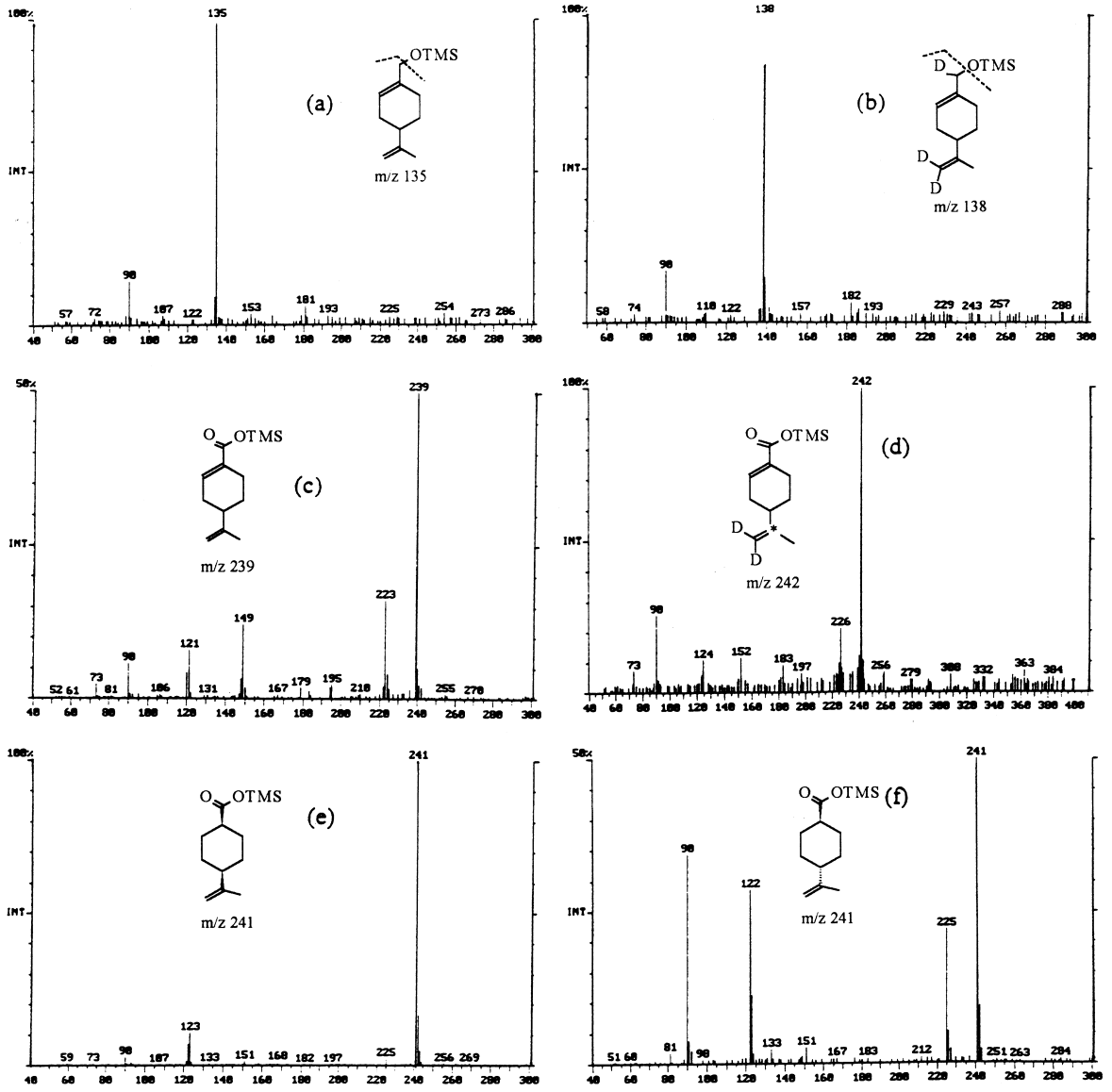

Fig. 1. Mass spectra of the silyl derivative of the analytes: (a) POH, (b) POH-d_3, (c) PA, (d) ^{13}C-PA-d_2, (e) *cis*-DHPA, (f) *trans*-DHPA.

wall-coated with 0.25 mm of cross-linked 95% methyl- and 5% phenyl-polysiloxane (J&W Scientific, Folsom, CA, USA) and an autosampler (Finnigan MAT). Ultra-high purity helium was used as the carrier gas with a head pressure of 15 psi (ca. 10^5 Pa). The injection port and the transfer line were set at 270°C. The oven temperature was initiated at 100°C for 1 min, and was ramped to 250°C at 15°C/min, then increased to 280°C at 30°C/min. The column was held at 250°C for 1 min. Analysis was performed using chemical ionization mode and ammonia was used as the reagent gas. Quantitation was performed in the selected ion monitor mode by measuring the base peak of either MH^+ or the fragment ions characteristic of the trimethylsilylated derivatives of the analytes and their respective internal standards. The peak area ratio method was used in construction of the calibration curves.

2.4. ^{13}C-NMR analysis

^{13}C-NMR spectra were recorded on either a Brucker NR-250 spectrometer at a radio frequency of 62.89 MHz or a Brucker AM-500 spectrometer at a radio frequency of 125.77 MHz with tetramethylsilane as the internal standard and $CDCl_3$ as the solvent.

2.5. Separation of cis- and trans-dihydroperillic acid

A mixture of synthetic *cis*- and *trans*-dihydroperillic acids was separated by thin-layer chromatography. The mixture (10 mg) was dissolved in a small volume of CH_2Cl_2 and the solution was applied onto a precoated silica gel G 60 thin-layer plate (EM Merck, thickness 0.2 mm, 20×20 cm). This plate was developed ascendingly with 50:1 CH_2Cl_2–MeOH on a lined tank for one full-length. Under UV light visualization, two overlapping spots (ca. 50%) (R_f 0.55–0.76) were seen and the upper portion of the faster-running spot (R_f 0.72–0.76) was scrapped off and eluted with CH_2Cl_2. The collected compound (ca. 2 mg) was found to be the pure component corresponding to a retention time of 6.3 min on GC–MS using the condition as described above. The lower portion of the spot was also collected and GC–MS still showed a mixture of *cis*/*trans*-DHPA with retention times of 6.3 and 6.7 min. Attempts to further resolve these components were unsuccessful.

2.6. Assay characterization and validation

Detection limit was measured by reducing the amount of each analyte until the signal-to-noise equaled to four, which is defined as the detection limit. The recovery was estimated by comparing the ratio of the extracted analytes to the unextracted internal standard with that of the unextracted pairs.

Within-run precision for POH, PA, *cis*- and *trans*-DHPA was determined by analyzing six replicates at each concentration on the same day. The concentrations used were: for POH at 2, 4, 20, 200 and 2000 ng/ml, for PA at 40, 200, and 2000 ng/ml, and for *cis*- and *trans*-DHPA each at 20, 100, and 1000 ng/ml. Between-run precision was evaluated by analyzing the variation of the slopes of six standard curves for each analyte obtained on six different days.

2.7. Pilot animal study

The femoral and jugular veins of a Sprague-Dawley rat weighing 350 g were cannulated under ether anesthesia. Three hours after recovery, the animal was given neat perillyl alcohol (ca. 22 μl) at 23 mg/kg i.v. via the femoral cannula over 30 s followed by a rinse of 0.5 ml normal saline. At the time schedule of 0, 3, 5, 10, 30, 60, 120, 180, 240, 300, 360, 480, 720, 960, 1200, and 1440 min following dosing, approximately 0.2 ml of heparinized blood each was withdrawn from the jugular vein cannula. Plasma was separated by centrifugation. An aliquot of each plasma sample was appropriately diluted with blank rat plasma to make up to 0.5 ml. Under this condition, the measured concentrations were in the range of the calibration-curve. To each plasma sample was added 200 ng of the internal standard followed by an addition of 1.0 ml acetonitrile to precipitate the protein. The mixture was centrifuged at 2000 *g* for 5 m. The supernatant was removed and frozen at −20°C until analysis.

2.8. Preliminary clinical study

The clinical study is a part of an ongoing Phase I evaluation of POH as a chemopreventative agent at the Cleveland Clinic Foundation. The Phase I protocol was approved by the Institution Review Board (IRB) at the Cleveland Clinic Foundation. Subjects with a personal history of AJCC Stage I–IIIa breast cancer who have undergone definitive resection with curative intent and who have completed all adjuvant radiation therapy, chemotherapy, and reversible hormone therapy are eligible to enroll in this study. All subjects were in good general health with adequate major organ functions and ECOG Performance Status of 0–1.

Patients were treated at the Cleveland Clinic Foundation, Cleveland, Ohio. The current study was limited to patients at the first of the five dose levels. Dose at 0.5 g/m^2 was given as capsules, which were supplied by the National Cancer Institute. Following dosing, blood samples were drawn at 0 (predose), 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0,

and 24 h. Plasma was separated from red blood cells by centrifugation and kept frozen at −20°C until shipment. All frozen specimens were then shipped in a dry-ice container (overnight) to Dr. Chan's Laboratory at the Ohio State University. Plasma samples were analyzed in the same manner as the rat plasma samples.

2.9. Quality control and stability

Two sets of spiked plasma samples consisting of three concentration levels, 20, 50, and 100 ng/ml of POH, PA, *cis*-/*trans*-DHPA were prepared and frozen at −20°C. One frozen set was sent to the Laboratory at the Cleveland Clinic Foundation by overnight courier service in dry-ice. After storage for approximately one week at −20°C, the same samples were returned by courier service to the Ohio State University Laboratory. Another set of samples were maintained frozen at the Ohio State University Laboratory for the same duration until analysis. Drug and metabolite levels were analyzed in these two sets of samples at the same time and the results were compared.

3. Results

3.1. Analysis of perillyl alcohol and its metabolites

The GC–MS mass spectra of POH, PA and *cis*-/*trans*-DHPA and their internal standards, POH-d_3 and ^{13}C-PA-d_2 are shown in Fig. 1. As shown, silylated POH and its internal standard each showed the base peak at m/z 135 and 138 corresponding to their C–O cleavages. MH^+ ions as their TMS derivatives at m/z 239 and m/z 242, respectively, were detected for PA and ^{13}C-PA-d_2 as the base peak, and also for *cis*-/*trans*-DHPA at m/z 241 at 55% relative abundance. These ions were used for subsequent quantitation. On this basis, a typical set of GC mass chromatograms of human and rat plasma extracts is shown in Fig. 2. As shown, all analytes were detected as symmetrical peaks and *cis*- and *trans*-DHPA were well resolved. The retention times of the analytes were: POH/POH-d_3 at 6.2 min, PA/^{13}C-PA-d_2 at 7.3 min, *cis*-DHPA at 6.3 min, and *trans*-DHPA at 6.7 min. The assignments of *cis*- and *trans*-DHPA are described below and coincided with those of Phillips et al. [11]. No interference peaks at the selected ion regions were found in the human or rat plasma blank, except for low levels of signal contributed by the internal standards. These interference contributions were due to incomplete isotope labeling and were all <2%. Thus, the ions monitored at m/z 135, 138, 239, 242 and 241 were considered to be highly specific and sensitive for detecting POH and its metabolites.

3.2. Assignments of cis- and trans-DHPA

Phillips et al. [11] using GC–MS method with electron-impact ionization partially resolved the underivatized *cis*- and *trans*-DHPA. On the basis of the different mass spectral fragmentation patterns of these two isomers augmented by a quantum mechanical calculation to correlate the configuration and mass fragmentation preference, they assigned the smaller component with a shorter retention time as the *cis*-isomer and the larger slower-eluting component as the *trans*-isomer. Since we employed derivatization in our GC–MS assay, direct correlation of our system with those of Philips et al. was difficult. Therefore, we resorted to use an alternative method for the assignment of these isomers. Using thin layer chromatographic separation, we were able to partially resolve the two isomers. The faster running spot was enriched and accumulated. The GC–MS showed that the enriched component had a retention time of 6.3 min. The H-decoupled ^{13}C-NMR spectrum of the synthetic mixture of *cis*- and *trans*-DHPA showed sets of doublets at 180.97 and 181.65 ppm corresponding to the -COOH signals, at 149.76 and 149.96 ppm corresponding to C-1′, at 108.54 and 108.75 ppm corresponding to C-2′, at 44.15 and 44.55 ppm corresponding to C-4, at 39.37 and 42.98 ppm corresponding to C-1, and at above 40 ppm for the remaining carbons and the side chain methyl. The carbon chemical shift assignments were based on the H-coupling, H-decoupling data, and comparison with known assignments of several monoterpenes and cyclohexane systems [16–18]. However, the H-decoupled ^{13}C-NMR spectrum of the above described enriched component failed to show signals with adequate intensity at the downfield region (180 ppm) but signals above 120 ppm were

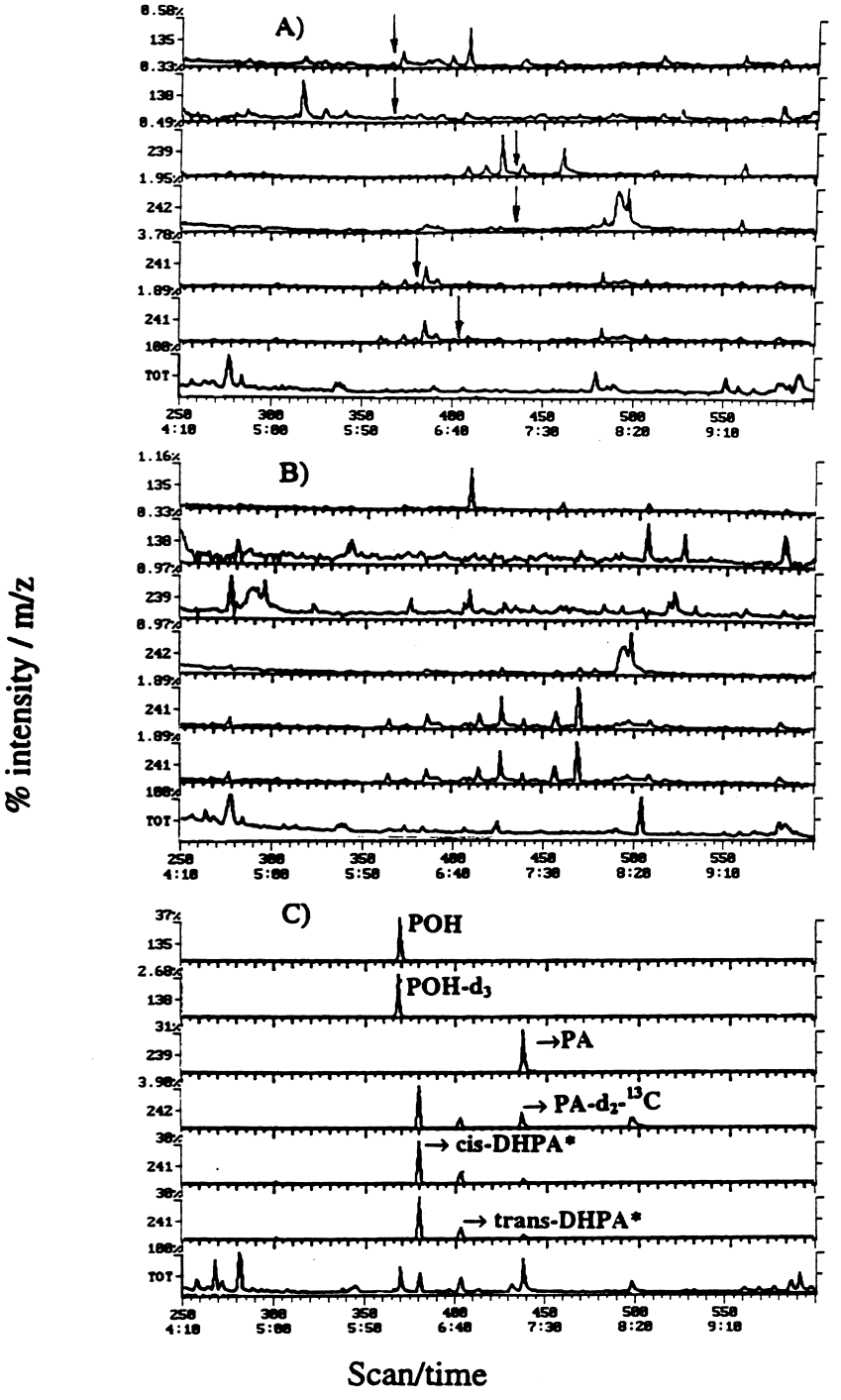

Fig. 2. Representative total and selected ion GC–MS chromatograms of the analytes and their internal standards: (A) Human plasma blank, (B) rat plasma blank, (C) analytes in a rat plasma sample. The ions monitored are labeled on the left vertical axes and the arrows indicated the regions the analytes appeared.

clearly discernible with unequal intensity, consistent with the enrichment. Based on the cyclohexanol system, chemical shift of C-1 appears to be most sensitive to *cis*-/*trans*-isomerism [18]. Thus, ^{13}C-NMR spectra of *cis*- and *trans*-1,4-methyl cyclohexanol show C-1 at 66.2 and 70.0 ppm, respectively, and *cis*- and *trans*-1,4-t-butylcyclohexanol show C-1 at 65.0 and 70.4 ppm, respectively. In both cases C-1s for the *cis*-isomer are several ppm upfield from the *trans*-isomer. The enriched DHPA isomer showed only a singlet at 39.36 ppm of C-1 with the more downfield signal missing. This suggests that the enriched DHPA isomer may be the *cis*-isomer. This possibility was further supported by examination of the ^{13}C-NMR spectra of synthetic *cis*- and *trans*-1-carboxyl-4-methylcyclohexanes which showed C-1 chemical shifts at 31.32 and 34.21 ppm, respectively. Again, the C-1 of *cis*-isomer shows nearly 3 ppm upfield from that of the *trans*-isomer. In order to correlate the GC–MS retention times of the silyl derivatives of these cyclohexane isomers with their stereochemistry, *cis*- and *trans*-1-carboxyl-4-methylcyclohexane were silylated with BSTFA. The GC–MS showed that the *cis*-isomer gave a retention time of 4.12 min while the *trans*-isomer gave a retention time of 4.28 min using the similar temperature programming and column as those of DHPA. On this basis, it was concluded that the DHPA isomer with the shorter retention time was the *cis*-isomer and that with the longer retention time was the isomer with *trans*-configuration. Interestingly, the assignment appeared to be the same as those of Philips et al. [11], based on the relative retention times of the underivatized compounds and the relative amount of the DHPA found as metabolite in vivo.

3.3. Within-run validation

When plotting the peak area ratios between the analyte and the internal standard against the added concentrations of the analytes a linear relationship was obtained within the concentration ranges monitored for all analytes. In both human and rat plasma, for POH linearity was demonstrated from 2 to 2000 ng/ml, for PA and *trans*-DHPA from 10 to 1000 ng/ml, and for *cis*-DHPA 20 to1000 ng/ml. The routine sensitivity limit was found to be 2 ng/ml for POH, 10 ng/ml for PA and *trans*-DHPA, and 20 ng/ml for *cis*-DHPA. The within-run validation data for POH and metabolites in rat and human plasma are shown in Table 1. Also shown in the same table are the accuracy data. As shown, the % C.V. values ranged from 0.5 to 8% with the majority falling below 5% and a mean accuracy value of 98.6%.

3.4. Between-run validation

The between-run variation for POH in human plasma was 5.5% and 6.3% in rat plasma as measured by the slopes of six calibration curves in each medium at six different occasions (Table 2). At each case, the mean intercept is less than 2 ng/ml equivalent. The between-run C.V.s for PA by similar measurements were 5.5% in human plasma and 3.4% in rat plasma, all $n=6$. The between-run C.V.s for *cis*-DHPA in human and rat plasma were 6.9% and 5.1%, respectively ($n=6$). The between-run C.V.s for *trans*-DHPA were 5.0 and 6.3%, respectively ($n=6$) (Table 2). In these cases, the mean intercepts for the calibration curves of the metabolites were less than 5 ng/ml analyte equivalent.

3.5. Recovery and quality control

The recovery values of POH, PA, *cis*- and *trans*-DHPA from the entire procedure were: 98%, 59%, 64% and 72% in human plasma and 90%, 77%, 69% and 76% in rat plasma, respectively. There was less than 3% variation for POH and metabolite levels in plasma samples kept at -20°C over the course of four weeks and there was no appreciable difference in the samples tested through mailing via the courier service.

3.6. Preliminary pharmacokinetics of perillyl alcohol and its metabolites in a rat

Fig. 3 shows a plasma concentration–time profile of POH and metabolites in the rat given POH at 23 mg/kg i.v. As shown, POH was detected in circulation up to 360 min with the initial concentration at 2089 ng/ml. The concentrations declined biexponentially with a terminal half-life of approximately 135 min. PA appeared in plasma in the first sample at 3 min and peaked at 10 at 8893 ng/ml and declined

Table 1
Within-run reproducibility of POH and its metabolites in human and rat plasma[a]

Species	Plasma	Theoretical conc. (ng/ml)	Observed Ave. Conc. (ng/ml)	S.D. (n=6)	C.V. (%)	Accuracy (%)
POH	Human	2.0	1.8	0.15	8	90
		4.0	3.7	0.26	7	93
		20.0	21.2	0.7	3.1	106
		200.0	200.4	6.7	3.3	102
		2000.0	1984.6	9.5	0.5	99
	Rat	20.0	22.6	0.8	3.7	113
		200.0	208.9	1.8	0.9	104
		2000.0	2034.9	14.5	0.7	102
PA	Human	40.0	38.9	1.0	2.7	97
		200.0	192.7	2.4	1.2	96
		2000.0	2007.3	10.8	0.5	100
	Rat	40.0	36.0	1.2	3.2	90
		200.0	194.9	3.5	1.8	97
		2000.0	2038.4	29.9	1.5	102
cis-DHPA	Human	20.0	20.9	0.5	2.3	105
		100.0	92.4	3.5	3.8	92
		1000.0	992.5	42.5	4.3	99
	Rat	20.0	19.5	1.1	5.4	98
		100.0	88.4	1.7	1.9	88
		1000.0	999.3	38.6	3.9	99
trans-DHPA	Human	20.0	19.6	0.4	1.9	98
		100.0	98.5	4.3	4.4	99
		1000.0	1082.1	40.2	3.7	108
	Rat	20.0	18.4	0.8	4.4	92
		100.0	98.9	5.5	5.6	99
		1000.0	1073.8	25.4	2.4	107

[a] Half milliliter of plasma was used.

approximately monoexponentially to about 35 ng/ml at 720 min. *Cis*- and *trans*-DHPA appeared in plasma at about 10 min and peaked at about 60 min and also declined monoexponentially. The plasma levels of the *trans*-metabolite were significantly higher than those of the *cis*-isomer. An unknown metabolite, labeled as M2, was also detected in plasma at high levels and this metabolite has the same m/z value as PA but with a retention time of 8.3 min. M2 appeared in plasma at 3 min at 2070 ng/ml PA equivalent and peaked at 10 min at levels over 6000 ng/ml. Although the initial levels of this metabolite were slightly lower than those of PA, the levels after 2 h exceeded those of PA after and were detectable even at 1440 min. The plasma half-life was approximately 3 h.

3.7. Preliminary pharmacokinetics of perillyl alcohol and its metabolites in a patient

The plasma concentration–time profiles of POH and its metabolites were measured in a patient who received a p.o. dose at 500 mg/m^2 and the results are shown in Fig. 4. As shown, POH, PA, *cis*- and *trans*-DHPA were all detectable in plasma. POH was detectable at 3.5 ng/ml at 0.75 h and peaked at 5.0 ng/ml at 1.5 h. The levels then declined mono-exponentially with a half-life of approximately 2.3 h. The AUC value was 22 ng/ml h. The most abundant metabolite in circulation was PA which peaked slowly at 11400 ng/ml at about 1.5 h. PA was formed rapidly following the POH administration, and remained at a steady-state for 1–2 h before

Table 2
Between-run reproducibility of POH and its metabolites in human and rat plasma[a]

Species	Plasma	Theoretic conc. range (ng/ml)	Observed ave. conc. (ng/ml) (S.D.)		C.V. (%)	
			Slope	R^2	Slope	R^2
POH	Human	20.0 to 2000.0	0.0147 (0.000809)	0.9994 (0.00042)	5.5	0.4
	Rat	20.0 to 2000.0	0.0103 (0.000648)	0.9991 (0.00075)	6.3	0.1
PA	Human	10.0 to 2000.0	0.0147 (0.000809)	0.9994 (0.00042)	5.5	0.04
	Rat	10.0 to 2000.0	0.0145 (0.000493)	0.9995 (0.00051)	3.4	0.05
cis-DHPA	Human	20.0 to 1000.0	0.0234 (0.0016)	0.9982 (0.0014)	6.9	0.1
	Rat	20.0 to 1000.0	0.0236 (0.0012)	0.9993 (0.00023)	5.1	0.02
trans-DHPA	Human	10.0 to 1000.0	0.0072 (0.000363)	0.9984 (0.00096)	5.0	0.1
	Rat	10.0 to 1000.0	0.0071 (0.000648)	0.9991 (0.00075)	6.3	0.1

[a] Half milliliter of plasma was used.

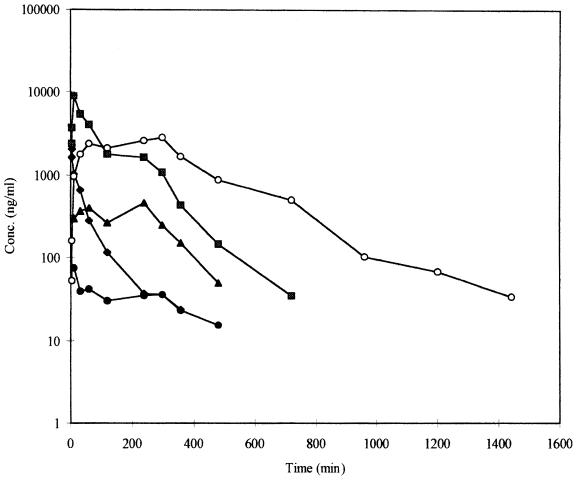

Fig. 3. A set of representative plasma concentration–time profiles of POH and its metabolites in a rat following i.v. administration of POH at 23 mg/kg: (♦) POH, (■) PA, (●) *cis*-DHPA, (▲) *trans*-DHPA, and (○) metabolite M2.

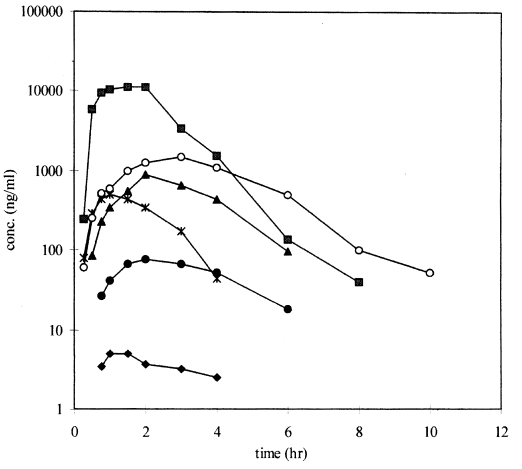

Fig. 4. A set of representative plasma concentration–time profiles of POH and its metabolites in a patient following p.o. administration of POH at 500 mg/m^2: (♦) POH, (■) PA, (●) *cis*-DHPA, (▲) *trans*-DHPA, (*) metabolite M1, and (○) metabolite M2.

declining. The level then declined monoexponentially to about 40 ng/ml at 8 h and became undetectable thereafter. Similar to the case in the rat, *cis*-DHPA levels were lower than those of trans. Two previous-

ly undescribed metabolites were detected in circulation. M1 gave a retention time of 7.7 min and M2 gave a retention of 8.3 min, which appears identical to the one described in the rat. Both of these metabolites gave the MH^+ ion identical to that of PA. Levels of M2 were lower than those of PA but higher than those of M1. M2 peaked at over 1000 ng/ml at 3 h, and M1 peaked at 76 ng/ml at 1 h.

4. Discussion

Previous GC and GC–MS methods failed to detect POH in plasma presumably because of inadequate sensitivity [11]. The reason for the low sensitivity may be due to the use of electron impact mass spectrometry for detection. Excess fragmentation under the EI condition significantly reduced the assay sensitivity. Moreover, POH and its metabolites such as PA are still rather polar. Tailing of their GC peaks may also reduce the assay sensitivity. The present method used a soft ammonia chemical ionization, which could reduce fragmentation and increase the relative abundance of the ion. Additionally, we also found that BSTFA derivatization of POH and its metabolites reduced tailing and further reduced fragmentation and enhanced sensitivity. Another improvement of our analytical method was to use the stable isotopically labeled POH and PA as the internal standard that provided the excellent C.V.s obtained. Thus, the current assay provided more than a hundred-fold improvement in sensitivity over the previous GC–MS method, and made it possible to detect circulating POH following POH administration.

The use of derivatization and a capillary column in this method also permitted the complete separation of *cis*- and *trans*-DHPA (Fig. 3). The earlier eluting peak (6.3 min) was assigned to be the *cis*-isomer, based on the NMR method and this assignment is identical to those of Philip et al. [11] who assigned their earlier eluting component in the partially resolved pair as the *cis*-isomer.

Both the preliminary rat and human pharmacokinetic data show that POH was detectable in circulation for the first time following POH administration. Metabolites of POH were detected in high levels in both rat and human plasma. PA appeared very rapidly in circulation and rose to high concentrations, suggesting that POH underwent rapid metabolism. Both *cis*- and *trans*-DHPA were unequivocally detected in the human and in the rat and their formation as a mixture was previously under debate [11]. Two previously undescribed metabolites were detected in significant levels in human plasma and one also in the rat. These two metabolites as their silylated derivatives gave the parent ion (MH^+) at m/z 239 identical to that of silylated PA but with different retention times. Characterization of these metabolites is currently underway. Thus, the current method is useful to support preclinical and clinical investigations of disposition of perillyl alcohol.

5. Conclusion

A GC–MS stable isotope-dilution method has been developed for the analysis of perillyl alcohol and its metabolites in human and animal plasma and the method has been validated. Unchanged perillyl alcohol in plasma was detected in human and animal circulation for the first time. Isomers of a reduced metabolite DHPA have been total resolved and detected and their previous *cis*- and *trans*-assignments verified by alternate techniques. Two new metabolites were also detected. This GC–MS can be applied to investigate preclinical and clinical pharmacokinetics of perillyl alcohol both as a chemopreventative and chemotherapeutical agent.

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