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Review

Quantitation of monoterpenoid compounds with potential medicinal use in biological fluids

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Abstract

Alicyclic monoterpenes are not only used as flavoring substances, but many have also been used in herbal products and as home remedies. Their formal use as potential drugs has recently been recognized. Consequently, the previous emphasis of the analytical methods on separation and identification of components has now evolved to the development of highly sensitive and specific quantitation methods capable of measuring the parent compounds and metabolites in biological fluids in order to meet the demand in drug development. This review aims to survey and describe major quantitative methods for some of the monoterpenes that possess medicinal applications. This review also comments on the favorable aspects of some of these assay methods as well as future trends. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Monoterpenoids; Terpenoids; Terpenes; Menthol; Camphor; Pulegone; Limonene; Perillyl alcohol

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1. Introduction

The essential oils obtained from flowers, plants, seeds, fruits, and spices have been studied for decades mainly due to their significance in paint, perfumery, flavoring agents, and chemotaxonomy applications. Much of the studies have focused in the study of the composition of these essential oils [1–3], in fruits [4], plants, in vitro cultures [5], and flavoring agents [6]. Its medicinal use, however, has not been formally recognized until relatively recently. A number of monoterpenes have been used either

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directly as pharmaceutical agents or as flavoring agents for pharmaceutical products. For example, menthol, a monocyclic terpene alcohol, has been used in toothpaste, mouthwash, shaving cream, cigarettes, and oral pharmaceutical preparations, and over-the-counter drugs for common cold [7]. Peppermint oil, in which menthol is the major constituent, is a naturally occurring carminative, which relaxes gastrointestinal muscle and is used for the treatment of irritable bowel syndrome (IBS) [8,9]. Mint tea, which contains a high content of menthol among other ingredients, is a herbal remedy for the treatment of digestive disorders [7]. Menthol is a specific stimulant of cold receptors, increases neural discharge by inhibiting the efflux of calcium from the cold receptor, and thus increases the afferent activity of cold sensors. Thus, inhalation of menthol may act to inhibit respiration through stimulation of upper airway cold receptors, and menthol may act to inhibit cough through this mechanism [7]. Camphor and menthol are common ingredients found in topically applied analgesics and rubifacients for the treatment of minor muscle aches and pains [10].

Pulegone and methofuran are monoterpenes found in pennyroyal herb, that have long been used as an abortifacient [11], although its use as such has been associated with significant hepatotoxicity, which has been attributed to its principal monoterpene (*R*)-(+)-pulegone [12]. Menthofuran has been found as a metabolite of pulegone in the rat [13,14]. *R*-Limonene, the principal component of orange peel oil, 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 [15–18] and chemotherapeutic [19,20] properties. Limonene prevents the formation of chemically-induced tumors [21] and exhibits significant antitumor effects [19–22]. Perillyl alcohol (POH) is a metabolite of D-limonene [23–25] as well as a natural product in its own right. It possesses chemopreventive properties against cancer and antitumor activity against mammary, pancreatic, and liver cancers in a rat model [21,26–28]. Biologically, POH and its metabolites inhibited the posttranslational isoprenylation of small G proteins, such as p21 [29,30]. In vivo, POH increases apoptosis in liver cancers in the rat [31] and in pancreatic adenocarcinoma cells in vitro. In most of the medicinal applications, alicyclic monoterpenes were involved. However, few, if any, acyclic monoterpenes have been reported to testify their pharmacologically relevant biological activity. Consequently, the studies on acyclic monoterpenes were limited to their metabolism [32].

Because of the different applications of monoterpenes, various analytical methods have been developed for different purposes. In the cases of studying the composition and identification of the components in essential oils, methods for the separation of the individual components, such as headspace gas chromatography (GC), are found to be important. For pharmacologic studies, however, the development of quantitative assays, for the terpenoid compounds and their metabolites in biological matrices with various isolation and cleanup procedures, is an essential tool. This review focuses mainly on the latter aspects, and various quantitative analytical methods on monoterpenes with potential medicinal use are covered in this review.

2. Menthol

The first analytical method for quantitation of menthol and related compounds was that of Gleispach and Schandara [33]. This method determined urinary menthol by first using enzyme hydrolysis of menthol glucuronide followed by diethyl ether extraction and GC flame-ionization detection (FID). No internal standard was used for their method. Bell et al. [34] developed a specific gas–liquid chromatography (GLC) method for the quantitation of menthol glucuronide in urine by employing 1,8-cineole as the internal standard. Following hydrolysis, the analytes were extracted with ethyl acetate and the volume of the extract was reduced prior to the analysis by GLC. A 2.7 cm$\times$4 mm glass column packed with polyethylene glycol as the stationary phase was used for the separation and the analytes were detected by FID. Using a similar approach as Bell et al. [34], Kaffenberger and Doyle [35] developed a capillary GC–FID assay method for the analysis of menthol glucuronide in biological matrices. Unlike other previous GC methods, they used phenyl glucuronide as an enzyme-sensitive internal standard. Following the hydrolysis of the urine sample with β-

glucuronidase, menthol and phenol (hydrolyzed internal standard) were extracted with ethyl acetate, and after concentration, the extract was separated by GC with a 30 m×0.25 mm wall-coated methylsilicone column (DB5, J&W) and detected by FID. The reported sensitivity limit was 250 ng/ml and the assay was linear from 25 to 250 μg/ml. The assay was validated and a relative standard deviation (RSD) of 1.2% was obtained. Using this assay, they found that about 40% of the administered dose of 180 mg of menthol by oral route was recovered in the urine as menthol glucuronide in 14 h.

Yamaguchi et al. [36] investigated the metabolism of menthol in Fischer rats, using [^{3}H]-L-menthol. They employed thin-layer chromatography (TLC), GLC, and gas chromatographic–mass spectrometry (GC–MS) techniques in their studies. Following isolation by TLC, excretion of metabolites from urine and feces were assayed on the basis of total radioactivity. The identities of the metabolites were established through derivatization with *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) to form trimethylsilyl derivatives. The conjugates were first hydrolyzed with β-glucuronidase and the aglycones identified by standard spectroscopic techniques. On the basis of the total radioactivity, they have recovered a total of 72% of the administered dose in both urine and feces in 48 h in nearly equal proportions, with most excreted within 24 h. Over 10 mono- and di-hydroxylated and oxidized metabolites such as acids and hydroxylated acids, and their glucuronides were identified based on TLC isolation, derivatization, GC and GC–MS analysis. There were four hydroxylated menthol compounds in rat urine following oral administration. No unchanged menthol could be detected in the urine or bile. The primary metabolite in the liver was menthol glucuronide, which underwent cleavage in the small intestine, and the released menthol was reabsorbed.

Gelal et al. [7] studied the disposition kinetics and biological effects of menthol in humans. They developed a GC–MS selected ion procedure for the analysis of menthol and metabolites in plasma and urine. This method utilized menthol-d_4 as the internal standard, including for the menthol glucuronide conjugate. This labeled internal standard was synthesized via an exhaustive H-^{2}H exchange of L-menthone with deuterium oxide under basic conditions to first form L-menthone-[$^{2}H_3$]. Subsequent reduction of the ketone with lithium aluminum deuteride afforded menthol-[$^{2}H_4$]. For the analysis of the glucuronide conjugate, urine and plasma were first treated with β-glucuronidase to release the conjugate. After enzyme hydrolysis, they used *n*-pentane extraction followed by a rather complex acid–base purification procedure. The analytes were derivatized by pentadecafluorooctanoyl chloride. The resultant derivatives were further cleaned up by treatment with a weak base before they were analyzed by GC–MS. The separation was presumably via a capillary column, although no information on the column was given. The limit of quantitation (LOQ) for menthol was 10 ng/ml for plasma and 100 ng/ml for urine. Electron impact (EI) with a single ion monitoring mode was used for the assay. No assay validation data were provided. Menthol was found to be rapidly metabolized, and only menthol glucuronide was detectable in plasma and urine. The mean plasma half-life of menthol glucuronide ranged from 42.6 to 56.2 min dependent on the formulation given.

We have recently modified the GC–MS assay of Gelal et al. [7] and the modification was in the simplification of plasma extraction and in the mode of GC–MS analysis [37]. Following extraction of the plasma three times with *n*-pentane, the combined organic layers were evaporated using the micro Kuderna–Danish concentrator. A 4-μl aliquot of the final condensate was injected into the GC–MS. A fused-silica capillary column (DB5, 30 m×0.25 mm I.D.) coated with a 0.25-μm thickness of methylsilicone plus 5% phenyl-methylsilicone (DB5, J&W) was used for the GC separation. Ammonia chemical ionization was used for detection. Under these conditions, the most abundant ions were the molecular ions at *m/z* 156 and 160 for menthol and menthol-d_4, respectively, and they were used for the analysis. The assay was validated in human plasma. The LOQ was 10 ng/ml using 0.5 ml of plasma. The extraction recovery was 62.3±14.5% ($n=6$). The assay was linear from 10 to 1000 ng/ml monitored. The within-day RSDs were 14.7% at 20 ng/ml, 6.8% at 200 ng/ml and 8.0% at 1000 ng/ml, all $n=6$. The corresponding accuracy values were 95.3, 97.2, and 104.7%, respectively. The between-day RSD was

6%, as estimated by the slopes of the standard curves ($n = 6$). This assay was applied to a pharmacokinetic study of menthol following mentholated cigarette smoking in women cigarette smokers. No intact menthol was detected.

Valdez et al. [10] developed a sensitive analytical method for the analysis of camphor, menthol, and methyl salicylate in human plasma and studied the pharmacokinetics and topical bioavailability of these compounds. A Supelco Simplicity-WAX, 30 m× 0.25 mm I.D., 0.25 μm capillary column was used and both GC–FID and GC–MS (mass spectrometry detector), with EI and single ion monitoring (SIM) were used. Plasma was extracted four times with *n*-hexane, and the volume of the extract was carefully reduced before analysis by GC. Anethole was used as the internal standard. Menthol and camphor, but not methyl salicylate, were detected without derivatization. A rather detailed sample processing procedure to circumvent emulsion formation and sample loss due to evaporation was described. An LOQ of 5 ng/ml for camphor and menthol was found and the inter-day RSD was 13.5% for 5 ng/ml, 11.5% for 20 ng/ml, 8% for higher concentrations of camphor. For menthol, the inter-day RSD was 10.2%, 8.9% at 20 ng/ml, and 6.1% and 5.9% for 40 and 100 ng/ml, respectively. The intra-day RSDs for camphor were 8–15.2% for 5 ng/ml, 2.1–5.6% for 20 ng/ml, and 2.1–8.7% for 40 ng/ml. For menthol, they were 6.2–11.2% for 5 ng/ml, 3.7–8% for 20 ng/ml, and 0.8–3.8% for 40 ng/ml. This assay was applied to study the pharmacokinetics of camphor and menthol in human subjects following application of the dermal patches. These authors were able to detect intact menthol and camphor and methyl salicylate with peak levels at 38, 32, and 37 ng/ml for camphor, menthol, and methyl salicylate, respectively. The authors claimed that their successful assay was due to the use of a larger volume of the sample, multiple extraction, and careful concentration before analysis.

Since menthol forms predominantly glucuronide in vivo, Leroy et al. [38] developed a direct measurement of menthol glucuronide in microsomal incubates via a high-performance liquid chromatographic (HPLC) method following a pre-column derivatization procedure. Borneol glucuronide, which was chemically synthesized by a Helferich rection [39], was used as the internal standard. The microsomal incubates containing both the menthol glucuronide and the internal standard were extracted two times with diethyl ether. After drying over anhydrous sodium sulfate, the organic solvent was removed under a stream of nitrogen at a temperature >40°C. The residue was redissolved in acetone and derivatized with 4-bromomethyl-7-methoxycoumarin in 18-crown-6 ether in the presence of potassium carbonate. The resultant reaction mixture was separated on a reversed-phase HPLC column under an isocratic condition with a mobile phase consisting of methanol–water (80:20, v/v). The components were detected with UV at 240 nm. This method was applied to study the enzyme kinetics for the formation of menthol conjugation by microsomal enzyme system. No assay validation data have been provided.

3. Camphor

A couple of HPLC methods for the analysis of camphor or camporated *para*-chlorophenol have been developed for over-the-counter (OTC) preparations and not in biological matrices [40,41]. Gallicano et al. [42] developed a sensitive HPLC procedure with UV detection for the assay of camphor in equine plasma and urine, following 2,4-dinitrophenylhydrazone (DNPZ) derivatization. Camphor was extracted from the biological fluids by diethyl ether, followed by the derivatization for 18 h before analysis. The detection limit achieved for 10 ng/ml in urine (using 40 ml of urine) and 20 ng/ml in plasma (using 5 ml of plasma), with reasonable intra-assay reproducibility, but a large day-to-day variation (23%). No internal standard was employed in their assay method. A parallel GC–FID assay was also developed, but the detection limit was more than 10-fold higher in urine. Using the HPLC assay, low levels of camphor were detected in equine urine and plasma following topical administration of camphor. However, higher plasma and urine camphor levels were found following intratracheal administration. *trans*-Isoketopinic acid was found to be the major urinary metabolite of camphor in the equine. This procedure, although appearing to be sensitive, requires use of a large sample volume and is rather time consuming. The recent GC–FID assay method

developed by Valdez et al. [10], also described above under menthol, provided a better sensitivity for camphor and is less time consuming, capable of handling at least 25 samples per day. This method utilized an internal standard and required 2 ml plasma sample. Robertson and Hussain [43] investigated the metabolism of camphor and related analogs in New Zealand white rabbits cross-bred with albino rabbits. A number of hydroxylated and reduced metabolites, including their glucuronide conjugates, have been identified with standard spectroscopy techniques, such as IR, TLC, GC, and optical rotation. To aid in identification, the formation of dinitrophenyl-hydrazone derivative was used in some cases. Enzyme hydrolysis was also performed in the case of conjugates. The quantitation of these compounds was performed using isothermal GC–FID on a column packed with 10% UC-W 98 (Hewlett-Packard) on chromosorb P and camphane was used as the internal standard. No assay validation information was given.

4. Pulegone

Thomassen et al. [13] investigated the contribution of pulegone and menthofuran to the hepatotoxicity of pulegone. A GC–FID method for the analysis of pulegone and menthofuran in plasma was developed. This method used acetophenone as the internal standard. The analytes were extracted from plasma with 2,2,4-trimethylpentane and the analytes separated on a wall-coat 30 m×0.32 mm DB5 capillary column. The extraction recovery was 95% for both analytes and the detection limit for both was about 30 ng/ml. The assay was linear from 0.25 to 25 μg/ml. The between-day RSDs were 4.7 and 8.3% for pulegone and menthofuran, respectively ($n=14$). The pharmacokinetics of pulegone and menthofuran were investigated in the Sprague–Dawley rat, following intraperitoneal administration of pulegone at 150 mg/kg and menthofuran at 100 mg/kg. The mean peak level achieved for pulegone was 7.02 μg/ml and for menthofuran was 1.63 μg/ml with respective terminal half-lives of 2.1 and 6.3 h. In another toxicity study of pulegone and its relationship with glutathione depletion, Thomassen et al. [14] described a similar GC–FID method using a 30 m×0.32 mm wall-coated open tubular (WCOT) fused-silica DB1701 column.

Pulegone was hepatotoxic and pneumotoxic with the *S*-isomer less toxic than the *R*-isomer. Madyastha and Thulasiram [12] studied the biotransformation of *R*- and *S*-pulegone in fungus *Mucor piriformis*. They used solvent extraction, TLC, NMR, IR for metabolite identification and eight metabolites have been identified. No quantitative method was described.

5. Limonene

A GC method was designed with parallel radioactivity and mass spectrometric detection for identification of limonene and metabolites [44], but only limited quantitation data were presented. Crowell et al. [45] studied the metabolism of limonene and identified several metabolites, namely, perillic acid (PA), dihydroperillic acid (DHPA), perillic acid methyl ester, and dihydroperillic acid methyl esters in plasma of rats treated with D-limonene. Two capillary GC methods have been developed for the quantitation of limonene and metabolites in plasma. One was a GC–FID method using a 30 m DB5 column. The second was a GC with an MS and/or an IR detector. Perillyl aldehyde (PCO) was used as the internal standard. The plasma sample was extracted with an equal volume of *n*-butanol–acetonitrile (1:1, v/v). Then, another volume of saturated potassium phosphate solution was added followed by mixing and centrifugation. The separated organic phase was concentrated under a stream of nitrogen and analyzed by GC–MS. Tissue distribution study was also performed using radioactive labeled drug. No assay validation information has been provided.

Poon et al. [46] employed an atmospheric pressure chemical ionization (APCI) liquid chromatographic–mass spectrometry (LC–MS) and LC–electrospray ionization (ESI) MS method with a reversed-phase column for the identification and characterization of limonene metabolites in plasma of patients with advanced cancers. The five major metabolites identified in plasma were: limonene-1,2-diol, limonene-8,9-diol, PA, an isomer of perillic acid (iso-PA), and DHPA. In urine, metabolites identified were: glucuronides of the two isomers of PA, DHPA acid, limonene-8,9-diol, and a monohydroxylated limon-

ene. MS, NMR, and UV were used for metabolite identification. They have also developed an LC–MS method, using the SIM mode, for the quantitation of these metabolites in plasma. In this method, α-terpinene was used as the internal standard. Plasma samples were extracted through elution on a C_{18} BondElut solid-phase cartridge, and after washing with 4×1 ml 10 n*M* ammonium acetate, the analytes were eluted with 300 μl of methanol, of which 100 μl was used for LC–MS analysis. A Finnigan TSQ 700 LC–MS system was used for the analysis and both ESI and APCI were used. The assay was linear from 0 to 5 μg/ml. No assay validation data were provided. Using this assay, peak concentrations of these metabolites in plasma were achieved at: limonene, 1 μg/ml, PA, 0.9–1.5 μg/ml, DHPA, 1.5–1.8 μg/ml, limonene-1,2-diol, 0.9–1.2 μg/ml, all between 4 and 6 h after p.o. administration. Limonene-8,9-diol at 1 μg/ml was achieved 1 h after dosing. Interestingly, no perillyl alcohol (POH) was detected, in spite of the fact that it is a metabolite of limonene.

Our laboratory has developed a sensitive and specific GC–MS method for the quantitative analysis of D-limonene in whole blood and investigated the pharmacokinetics of limonene in the rat [47]. This method utilized limonene-d_2, [1-methyl-4(1′,1′-dideuterio-isopropylenyl)cyclohexene], as the internal standard, which was synthesized in the authors' laboratory. Ammonia chemical ionization was used for quantitation of D-limonene. Under these conditions, low fragmentation and high intensity of the molecular ion $[M+1]^+$ was observed. The molecular ion pair at m/z 137 and 139 for limonene and the internal standard, respectively, was used for quantitation. A Finnigan ITS40 ion trap mass spectrometer was used for the analysis. A DB-5 (J&W) 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 was also used. Rat blood or plasma samples were extracted with *n*-pentane. The *n*-pentane layer was transferred to a 5-ml Kuderna–Danish concentrator. 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 microvial in a GC autosampler. A 5-μl aliquot was injected into the GC–MS for analysis. The sensitivity with direct injection of pure D-limonene was as low as 1.0 ng with a signal-to-noise ratio (S/N) of 14. However, due to the presence of an endogenous level of D-limonene in the rat blood (probably from diet origin), the routine sensitivity limit was reduced to 1.0 μg/ml. The linearity of the assay was observed in the range of 1.0 to 30 μg/ml with an average correlation coefficient of 0.99 ($n=6$). The between-day RSD was found to be 3.9% ($n=6$) and the within-day RSD values were 8.0, 2.4, and 2.0% for 1.0, 3.0 and 10.0 μg/ml, respectively ($n=8$). Using this GC–MS method, pharmacokinetics of D-limonene was investigated in the rat following both i.v. and p.o. administrations. Following i.v. administration, the drug was rapidly distributed to the body with an average initial half life ($t_{1/2\alpha}$) of 12.4 min. The elimination was rather slow with an average terminal half life ($t_{1/2\beta}$) of 280 min. Following oral administration, D-limonene reached the highest plasma concentration of 11.3 μg/ml 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 circulation rapidly. The mean area under the curve (AUC) value for i.v. was 5094 μg ml^{-1} min^{-1} and 2190 μg ml^{-1} min^{-1} for p.o. giving an oral bioavailability of 43.0%.

Hardcastle et al. [48] reported the identification, synthesis, characterization, and quantitation of a new human metabolite of limonene, iso-PA. They also reported the synthesis of *R*-perillic acid and evaluation of its biologic activity. Associated with this investigation, the authors developed an LC–APCI–MS method for the quantitation of limonene metabolites in human plasma. Analytes in the human plasma sample were isolated with solid-phase extraction using C_{18} BondElut cartridges. After loading, the cartridge was washed with 4×1 ml 10 m*M* ammonium acetate and the analytes were eluted from the column with methanol. α-Terpinene was used as the internal standard. The separation of components in the plasma extract was achieved on a 5 μm 150 mm×4.6 mm ODS Apex 1 column at a flow-rate of 1 ml/min. The mobile phase consisted of 0.075% aqueous trifluoroacetic acid (solvent A) and methanol (solvent B). A linear gradient elution program was

used: 45% B for 5 min; 45–70% B for 5 min; 70% B for 5 min; 70–80% B for 10 min; and 80% B for 10 min. The eluate was introduced into a Finnigan TSQ 700 operated under an APCI source. Selected ion mode was used and MH^+ of both PA and iso-PA were also used. No characterization of the assay was provided, nor any information on assay validation. Using the LC–APCI–MS assay, peak plasma concentrations of PA and iso-PA in patients receiving doses of 6, 8, 10, 12 g m^{-2} day^{-1} oral limonene were measured. They were: 24.5±15.8 at 12 mg/m^2, 16.4±1.9 at 10 mg/m^2, 9.6±2.2 at 8 mg/m^2, and 7.9±0.2 at 6 mg/m^2 for iso-PA (all in μ*M*). The corresponding levels for PA were: 65.8±4.7, 74.3±43.5, 40.5±22.6, and 30.1±8.7 μ*M*. Inhibition of the isoprenylation enzyme activities by limonene and its metabolites were evaluated. PA and POH were found to be more potent inhibitors of isoprenylation than limonene with IC_{50} values in the low m*M* range. The novel iso-PA has similar IC_{50} as PA.

6. Perillyl alcohol

Haag and Gould [49] established a GC–FID method for the measurement of the plasma levels of perillyl alcohol (POH) and metabolites. Plasma was extracted with *n*-butanol–acetonitrile (1:1, v/v). The separated organic phase was washed once with potassium diphosphate solution before analysis. Perillyl aldehyde was used as the internal standard. A 30 m×0.32 mm I.D., 0.25 μm thick Supleco SPB-5 capillary column was used for separation. The extraction efficiency of the analytes was >90%. The limit of detection was 10 to 30 μ*M* (1.5–4.5 μg/ml) and the assay was linear, although no dynamic range was given nor any assay validation. The identity of the analytes was verified by GC–MS under electron impact condition. Metabolites identified in the plasma of rats fed with POH and limonene were: PA, DHPA, and perillic acid methyl ester, and dihydroperillic acid ester. No parent drug was detected in plasma of rats treated either with acute or chronic doses of POH, although in the acute treated rat with limonene, the parent drug was detected. Philips et al. [50] reported a GC–MS assay of POH and metabolites (PA and DHPA) in plasma. Perillyl alcohol hexyl ether was used as the internal standard. Plasma was acidified and extracted with tert.-butyl methyl ether. The extract was analyzed by GC–MS under an electron impact condition. The components were separated on a 15 m×0.25 mm wall-coated DB5 capillary column and the fragment ions of the *p*-menthadienoid carboskeleton (m/z 121) and its hydrogen transferred ion (m/z 122) were monitored. The analytes were all baseline resolved except for the *cis*- and *trans*-isomers of DHPA. The assay was validated. The LOQ was 1.5 μ*M* for both PA and DHPA with between-run RSDs of 3.6 and 8.5%, respectively ($n=6$), using 50 μl of plasma. The recovery was in the range of 90%. Pharmacokinetics of POH in the beagle dog was studied following oral administration of POH at 250 mg/kg. No POH was detected by this method and only metabolites PA and DHPA were detected. The terminal $t_{1/2}$ for PA was 3.2 h and for DHPA was 3.4 h. PA levels were higher than those of DHPA.

We have developed a sensitive GC–MS method for the measurement of POH and its metabolites in plasma using the stable isotope-labeled POH and PA as the internal standards [51,52]. A derivatization procedure was also used to facilitate the GC separation and mass spectrometric detection. This method possesses several special features: (1) the use of an ammonia chemical ionization, (2) the use of silylation to form trimethylsilyl (TMS) derivative, and (3) the use of stable-isotope labeled internal standards. A DB5 wall-coated 30 m×0.25 mm capillary column was used for separation. Using this method, POH and its metabolites PA, *cis*- and *trans*-DHPA, and two new metabolites M1 and M2 were also resolved and quantitated. The locations for the *cis*- and *trans*-DHPA on the chromatograph were rigorously assigned. The identities of M1 and M2 were later tentatively assigned as double-bond isomers of PA on the basis of chemical reduction, GC–MS analysis, and in vitro enzyme experiments [53]. Catalytic hydrogenation of M1 and M2 with Pd at atmospheric pressure generated the same *cis*- and *trans*-tetrahydroperillic acids as with PA. On this basis, the metabolic scheme of POH can be depicted as shown in Fig. 1.

The assay was validated in both human and rat plasma. The LOQ for POH was 2 ng/ml and for PA was 10 ng/ml and for *cis*- and *trans*-DHPA was 20

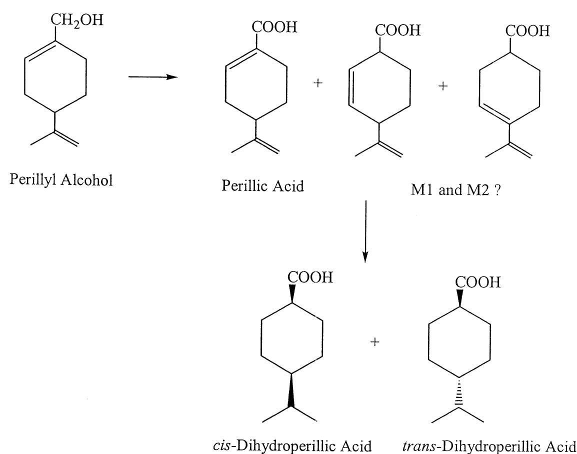

Fig. 1. Major metabolic pathways of perillyl alcohol.

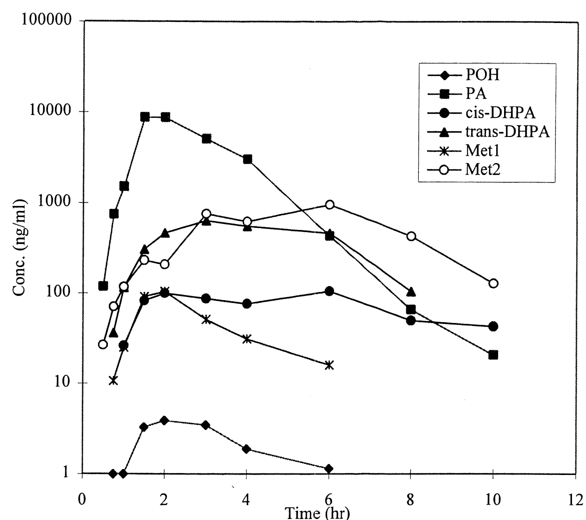

Fig. 2. Plasma concentration–time profiles of perillyl alcohol (♦), perillic acid (■), *cis*- (●) and *trans*- (▲) dihydroperillic acids, and two new metabolites M1 (*) and M2 (○) (tentatively, isomers of perillic acid) in a patient receiving an oral dose of perillyl alcohol at 500 mg/m^2.

ng/ml. The assay was linear from 2 to 2000 ng/ml for POH, for PA and *trans*-DHPA from 10 to 1000 ng/ml, and for *cis*-DHPA 20 to 1000 ng/ml. The within-run RSD values ranged from 0.5 to 8% with the majority falling below 5% and a mean accuracy value of 98.6%. The between-run variation for POH in human plasma was 5.5% and 6.3% in rat plasma. The between-run RSDs for PA were 5.5% in human plasma and 3.4% in rat plasma, all $n=6$. The between-run RSDs for *cis*-DHPA in human and rat plasma were 6.9 and 5.1%, respectively ($n=6$). The between-run RSDs for *trans*-DHPA were 5.0 and 6.3%, respectively ($n=6$). 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. Using this GC–MS assay, plasma POH was detectable in all patients receiving 500 mg/m^2 p.o. with a mean peak level of 5.7 ng/ml, occurring at 1.4 h. The mean terminal $t_{1/2}$ for POH was 1.9 h and that for PA was 4.1 h. The descending order of the magnitude of the area-under-the plasma concentration–time curve (AUC) values is: PA>M2>*trans*-DHPA>*cis*-DHPA=M1>POH (Fig. 2) [54].

Boon et al. [55] investigated cytotoxicity and biotransformation of POH in PC12 cells and in the rat. POH and its metabolites were analyzed by HPLC, using a 100 mm×3.0 mm I.D. Inertsil 5 ODS-2 column equipped with a Chrompack ODS-2 guard column (10×2 mm) at a flow-rate of 0.45 ml/min. The mobile phase used consisted of 0.1 *M* $NaNO_3$, 0.01 *M* KBr, 0.01 *M* citric acid, 0.1 m*M* EDTA-Na_2, adjusted to pH 3 and MeOH (67:33, v/v). The analytes were detected by electrochemical detection. Quantitation was performed on POH, PCO, and PA with calibration curves, and conjugates were measured after glucuronidase hydrolysis. The detection limit for POH, PCOOH, and PCO was 10 pmol or 1.5 ng. No assay validation data were provided, other than a description of linearity in the calibration curve.

An isocratic HPLC–UV procedure was developed for the quantitation of PA in plasma [56]. The internal standard used was Biochanin A. Plasma was treated with two volumes of acetonitrile. The resultant supernatant was diluted by $NaHCO_3$ before being analyzed by HPLC. The components were separated on a Waters Nova-Pak C_{18} 15 cm×0.39 cm I.D. 4 μm column preceded by an RP18 precolumn with a flow-rate of 1.1 ml/min. The mobile phase was acetonitrile–0.05 *M* ammonium acetate buffer pH 5.0 (36:64, v/v). The detection wavelength was programmed at 217 nm for PA and 261 nm for the internal standard. Plasma pharmacokinetics were studied in CD2F1 mice given i.v. POH at 46.8 mg/kg. The assay was validated to give

a within-run RSD of 0.13 to 5.96% and a recovery of 106–118%. The limit of detection was 15.6 ng/ml and LOQ of 125 ng/ml. The plasma concentration–time profile was monoexponential with a mean $t_{1/2}$ of 16.9 min.

A pharmacokinetic study has recently been conducted under the Phase I clinical trial of POH as a chemotherapeutic agent [57]. The drug was administered as a soft gelatin capsule containing POH and soybean oil. The dose was escalated from 800, 1200, 1600 mg m^{-2} $dose^{-1}$, given four times per day. Pharmacokinetics were performed on course 1 on days 1 and 2 and the sampling schedule was to 6 h after the ingestion of the first dose then to 8, 23, and 25 h for the remaining doses. The pharmacokinetics was also performed to 6 h after the ingestion of the first dose on days 15 or later courses. The GC–MS procedure of Philips et al. [50] was adopted for the pharmacokinetic studies. No POH was detected in plasma in this study, but it was detected in urine. Two major metabolites PA and DHPA peaked at 1–2.5 h and 2–3.5 h post ingestion, respectively. The $t_{1/2}$ for the metabolites was 1–2 h for PA and 1.5–2.5 h for DHPA. Plasma metabolite levels (C_{max} and AUC) were higher for PA than for DHPA. The metabolite levels, in general, increased with an increase in dose and there was no evidence of drug accumulation. In fact, there was a trend towards decreasing metabolite levels on day 29, although it was not statistically significant. Some patients also showed lower metabolite levels with prolonged use. About 8–9% of the total dose was recovered in the urine in the first 24 h following dosing, of which less than 2% of the recovered dose was POH and the majority was PA (nearly 90%) and DHPA (10–15%). No difference in excretion was found in patients taking the drug with and without food.

7. Other monoterpenes

Many terpenoids such as β-ionone, limonene, borneol, linalool, α-terpineol [58,59], α-terpineol and isobornyl acetate [60], 1,8-cineol [61,62], camphor, β-pinene, and camphene [63], α-pinene [64], and geraniol [64] have been found to induce drug metabolic enzymes [65]. Madyastha and Srivatsan [65], in addition to the identification of hydroxylated menthol metabolites, investigated the binding of L-menthol to uninduced and phenobarbital-induced rat liver microsomes. A number of isolation and purification techniques including TLC, column isolation, capillary GC–FID, NMR, IR, and MS was used for identification of menthol-hydroxylated metabolites, but no quantitative analytical method was described. Southwell and Flynn [66] investigated the metabolism of several monoterpenoid compounds including β-pinene, *p*-cymene, and 1,8-cineole in brushtail possum. Urine and feces metabolites were examined. α-Pinene was found to be metabolized to myrtenic acid and *trans*-verbenol, β-pinene to myrtenic acid, *p*-cymene to *p*-cresol and cumic acid, and 1,8-cineole to *p*-cresol, 9-hydroxycineole and cineol-9-oic acid. NMR, optical rotation, UV, MS, and IR were used for identification of these metabolites. Column chromatography and GLC were also used for the isolation, purification, and characterization GLC. No quantitation procedure was described.

8. Discussion

Monoterpenes are highly volatile and lipophilic compounds. Most of the analytical methods focus on using gas chromatography-related techniques. These compounds generally lack a strong chromophore and the detection methods used are mostly FID and MS, and in a limited case, infrared detector. The high volatility presents a potential problem of sample loss during work up. This problem has been circumvented by using a highly volatile organic solvent, such as pentane, for extraction. Additionally, in many cases, the sample loss can be minimized by using a micro Kuderna–Danish concentrator to remove the bulk volatile organic solvent. Although the use of mass spectrometry as a detection technique is usually considered to be highly sensitive, the use of electron impact ionization for detection of monoterpenes will result in a loss of sensitivity. Monoterpenes are highly branched hydrocarbons that undergo extensive fragmentation under electron impact ionization, resulting in a decrease of ion intensity especially for the molecular ion. The use of chemical ionization has largely minimized this problem, and in many cases, a substantially intense molecular ion can be achieved. Additionally, the use

of derivatization such as silylation can help to reduce fragmentation and increase the intensity of higher mass ions. Finally, for all of these manipulations, the use of an isotopically labeled internal standard will provide better reproducibility. These approaches have been used for the assay of several monoterpenoid drugs such as limonene, perillyl alcohol, and menthol in our laboratory as well as in others. For the first two drugs, we have detected significant levels of the intact drugs in circulation in vivo following drug administration, a finding different from many previous investigations. The detection of intact drug facilitates interpretation and characterization of the pharmacokinetic behaviors of these agents, as characterization of the pharmacokinetic behaviors of these compounds based on metabolite levels will usually be complicated by the presence of a precursor–successor relationship (metabolite pharmacokinetics).

A number of monoterpenoid compounds undergo extensive glucuronidation in vivo. Conventionally, the conjugates are quantitated following enzymic hydrolysis and the aglycones assayed by GC or GC–MS. With the advent of the LC–MS methods, the monoterpenes and their glucuronides can now be quantitated directly by LC–MS or LC–MS–MS. Thus, there is a distinct advantage for the use of the LC–MS method for quantitation of monoterpenes and metabolites. A limited number of analytical methods have been published, but the use of LC–MS analytical method is still not widespread. For the LC–MS method, a careful sample processing technique is still required because of the high volatility of the analytes.

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