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Plasma and dermal pharmacokinetics of terpinen-4-ol in rats following intravenous administration

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Terpinen-4-ol, a naturally occurring monoterpene, has been shown to possess antibacterial, antioxidant and anti-inflammatory activities. Furthermore, recent reports have demonstrated that terpinen-4-ol could be developed as new therapies against melanoma either in systemic administration or targeted drug delivery. The purpose of this study was to investigate the pharmacokinetics of terpinen-4-ol in rat plasma and dermal tissue following intravenous (i.v.) bolus injection of terpinen-4-ol at a dose of 2 mg/kg. Unbound concentrations of terpinen-4-ol in dermis were continuously determined by dermal microdialysis. Simultaneously, a conventional blood sampling was performed. The concentrations of terpinen-4-ol in plasma and microdialysates were determined by validated gas chromatography-mass spectrometry. Following i.v. bolus administration, terpinen-4-ol rapidly distributed into the dermis and reached relatively low levels with an average maximum concentration (C_{max}) of 0.10 ± 0.06 μg/ml in comparison with a plasma C_{max} of 6.30 ± 1.90 μg/ml. The free terpinen-4-ol concentrations in dermal tissue were lower than the corresponding total and free plasma concentrations for the entire length of study, indicating that plasma levels do not provide information of actual terpinen-4-ol concentrations in the skin. This study demonstrates that dermal microdialysis is an effective and minimally invasive tool to evaluate the dermal pharmacokinetics of terpinen-4-ol following systemic administration.

1. Introduction

Microdialysis is a sampling technique which allows the continuous monitoring of endogenous or exogenous compounds in the extracellular fluid of tissues. Its principle is based on the passive diffusion of compounds down a concentration gradient across a semi-permeable membrane. This technique was originally developed for sampling of neurotransmitters in rat brain studies (Ungerstedt 1984) and has been applied to dermatological research including dermal pharmacokinetics (Anderson et al. 1991; Benfeldt 1999; Groth 1996). With the use of microdialysis, it is possible to obtain full local pharmacokinetics profile of dermal drug penetration from each sampling site and minimize the sampling burden on a patient in comparison to conventional tissue sampling methods, e.g. skin stripping, skin biopsy and skin blister (Ault et al. 1994; Groth 1996; Kreilgaard 2002).

Terpinen-4-ol, a naturally occurring monoterpene found in many essential oils including *Melaleuca alternifolia* (tea tree) oil and *Zingiber cassumunar* (plai) oil, has been shown to possess antiviral, antibacterial, antifungal, and insecticidal effects as well as antioxidant and anti-inflammatory activities (Astani et al. 2010; Barra et al. 2007; Cha et al. 2007; Wedge et al. 2009; Zuniga et al. 2005). Recent reports have demonstrated that terpinen-4-ol and tea tree oil could be developed as new therapies against melanoma either in systemic administration or targeted drug delivery (Bozzuto et al. 2011; Calcabrini et al. 2004; Giordani et al. 2006; Greay et al. 2010). In the previous studies, skin absorption and elimination kinetics of terpinen-4-ol in its pure form, essential oils and topical formulations were performed using *in vitro* skin stripping and Franz diffusion cell system (Biju et al. 2005b; Cal 2006a, b; Cal and Krzyzaniak 2006; Cal et al. 2006; Cross et al. 2008; Nielsen and Nielsen 2006; Reichling et al. 2006). The results showed that terpinen-4-ol was accumulated in large amounts in hydrophilic skin layer (epidermis with dermis) and dermal tissue served as a natural acceptor for terpinen-4-ol permeating through the skin. Although, the *in vitro* skin penetration of terpinen-4-ol has been extensively studied, the *in vivo* pharmacokinetic data was limited.

Measurement of terpinen-4-ol concentrations in biological matrices is crucial for many studies including pharmacokinetic and bioequivalence studies. Various analytical methods have been reported to estimate terpinen-4-ol concentrations in cosmeceutical formulations (Biju et al. 2005b; Pithayanukul et al. 2007; Reichling et al. 2006), follicular casts (Biju et al. 2005a), plant extracts (Cal 2006b; Cross et al. 2008; Nielsen and Nielsen 2006; Russell and Southwell 2002) and skin tissues

(Cal 2006a; Cal et al. 2006). Here we report the development and method validation of a simple, sensitive and reproducible gas chromatography-mass spectrometry (GC-MS) method for determination of terpinen-4-ol in rat dermal microdialysates and plasma samples. The validated method was subsequently applied to a pharmacokinetic study of terpinen-4-ol in dermis and plasma following intravenous (i.v.) bolus injection at a dose of 2 mg/kg.

2. Investigations, results and discussion

2.1. GC-MS operating conditions

A gas chromatography-mass spectrometry method was developed and optimized to assess the concentrations of terpinen-4-ol in rat dermal microdialysates and plasma samples. In order to develop an extraction method, acetonitrile, dichloromethane, hexane and methanol were tried for dissolving terpinen-4-ol and methyl salicylate which was used as internal standard (IS) and removing endogenous materials, like proteins. The test substances were well soluble in hexane and dichloromethane with good extraction efficiencies. However, hexane was selected as the solvent for extraction because of its low baseline noise and the absence of interfering peaks at the retention times of the analytes when compare to dichloromethane.

The calibration curve for the quantitative determination of terpinen-4-ol was constructed using the peak area of mass fragment *m/z* 93 instead of the 71 which was the most abundant ion. Since the use of *m/z* 93 as a target ion was found to result in better accuracy as compared to the use of ion 71. The optimized GC separation of terpinen-4-ol and IS was achieved within 10 min and the total runtime was 14 min. The retention times of terpinen-4-ol and IS were 8.1 and 9.6 min, respectively.

2.2. Method validation

2.2.1. Specificity

The specificity of the method was evaluated as lack of endogenous interference by analyzing blank microdialysates and plasma samples before terpinen-4-ol administration. No significant interfering peaks from blank samples were observed.

2.2.2. Linearity, sensitivity and carry over

The calibration curves for both matrices showed good linearity within the range of 5–1000 ng/ml. The correlation coefficient was > 0.995 for all validation batches. The limit of quantification for both matrices was established as 5 ng/ml. Accuracy and precision of the lower limit of quantification (LLOQ) in microdialysates were 1.2 and 17.4%, respectively; whereas accuracy and precision in plasma were 2.9 and 11.6%, respectively. Representative chromatograms of blank microdialysates and blank plasma samples spiked with terpinen-4-ol at the limit of quantification are shown in Fig. 1 and 2, respectively. Furthermore, there was no evidence of carry-over effect for both matrices.

2.2.3. Accuracy and precision

Table 1 summarizes the intra-day, as well as the inter-day precision and accuracy of the method. Precision and accuracy were within the acceptable ranges for bioanalytical purposes. Intra-day precision ranged from 2.0 to 6.5% for microdialysates and 4.0 to 8.7% for plasma samples. Inter-day precision ranged from 3.6 to 7.1% and 5.9 to 7.3% for microdialysates and plasma samples, respectively. Intra- and inter-day relative errors (%bias) were less than 1.6 and 3.3% for microdialysis samples,

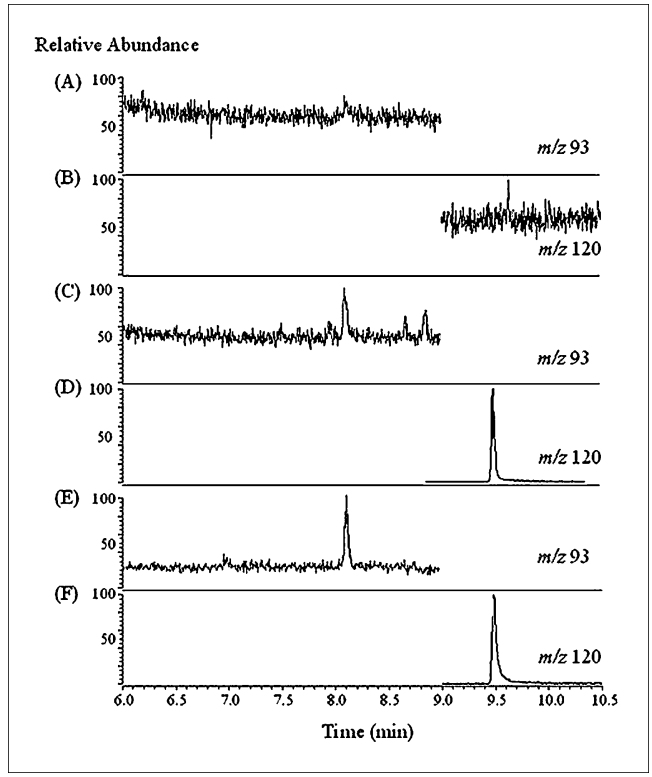

Fig. 1: Representative GC-MS chromatograms of blank microdialysates (A and B); LLOQ of terpinen-4-ol in microdialysate (5 ng/ml) and IS (C and D), real sample at 0–20 min after a single bolus injection of 2 mg/kg terpinen-4-ol (E and F). Peaks of 8.1 and 9.5 min are terpinen-4-ol and IS, respectively

whereas for plasma samples these were less than 4.2 and 1.5%, respectively.

2.2.4. Extraction recoveries

Terpinen-4-ol extraction recoveries from microdialysates and rat plasma at three concentration levels are reported in Table 1. The mean recovery of terpinen-4-ol for all microdialysis samples was 98.6% with relative standard deviation (R.S.D.) of < 6.4%. For plasma samples, the mean recovery was 90.3% with R.S.D. of < 7.3%.

2.2.5. Stability

Table 2 summarizes the results of stability of terpinen-4-ol in microdialysates and rat plasma carried out under various conditions. The analyte was found to be stable at ambient temperature for at least 24 h and at −20 °C for one month in both matrices. The post-processed samples were shown to be stable at approximately 25 °C for at least 36 h (autosampler stability). Furthermore, microdialysates and plasma samples spiked with terpinen-4-ol were also subjected to three freeze-thaw cycles. The results showed that the samples could be thawed and refrozen without compromising the integrity of the samples.

2.3. Pharmacokinetic applications

The average protein binding of terpinen-4-ol in rat plasma was 76.7 ± 2.1%, with no dependence on concentrations in the range 0.01–10 μg/ml. For the *in vivo* recovery, the average relative loss was 33.23 ± 6.0%. The results of the non-compartmental and compartmental pharmacokinetic data analysis of terpinen-4-ol in plasma and dermal tissue after i.v. administration are summarized in Table 3. The concentration-time profiles of terpinen-4-ol

Table 1: Precision, accuracy and extraction recoveries of the GC-MS assay for terpinen-4-ol in microdialysates and rat plasma

Sample	Added (ng/ml)	Intra-day ($n=5$)			Inter-day ($n=15$)			Extraction efficiency ($n=5$)	
		Found (ng/ml)	%R.S.D.	%Bias	Found (ng/ml)	%R.S.D.	%Bias	Recovery (%)	%R.S.D.
Microdialysates	10	9.8 ± 0.6	6.5	1.6	10.2 ± 0.7	7.1	−1.5	102.8 ± 6.4	6.2
	500	498.5 ± 14.8	3.0	0.3	511.3 ± 28.5	5.6	−2.3	98.4 ± 6.5	6.7
	750	756.8 ± 15.3	2.0	−0.9	774.9 ± 27.9	3.6	−3.3	94.7 ± 3.7	3.9
Rat plasma	10	10.4 ± 0.9	8.7	−4.2	10.2 ± 0.7	7.3	−1.5	85.2 ± 3.0	3.6
	500	502.0 ± 20.3	4.0	−0.4	503.2 ± 32.1	6.4	−0.6	97.4 ± 5.5	5.7
	750	766.3 ± 39.6	5.2	−2.2	749.5 ± 44.4	5.9	0.1	88.2 ± 3.4	3.9

Table 2: Stability results of terpinen-4-ol in microdialysates and rat plasma

Condition	Added (ng/ml)	Microdialysates			Rat plasma		
		Found (ng/ml)	%R.S.D.	%Bias	Found (ng/ml)	%R.S.D.	%Bias
Room temperature (25 °C, 24 h)	10	9.5 ± 0.1	1.5	5.3	9.7 ± 0.8	8.6	2.5
	500	469.9 ± 3.3	0.7	6.0	532.5 ± 28.1	5.3	−6.5
	750	724.6 ± 13.2	1.8	3.4	753.7 ± 38.8	5.2	−0.5
Frozen-matrix (−20 °C, 1 month)	10	9.6 ± 0.9	9.2	4.4	8.8 ± 0.1	1.8	11.7
	500	486.6 ± 39.0	8.0	2.7	460.7 ± 19.0	4.1	7.9
	750	706.5 ± 22.6	3.2	5.8	683.8 ± 54.9	8.0	8.8
Freeze-thaw (−20 °C, 3 cycles)	10	10.7 ± 0.7	6.4	−6.5	9.5 ± 0.3	3.0	5.2
	500	456.1 ± 5.1	1.1	8.8	506.5 ± 39.4	7.8	−1.3
	750	713.5 ± 20.4	2.9	4.9	738.3 ± 20.3	2.7	1.6
Autosampler (25 °C, 36 h)	10	9.1 ± 0.3	3.4	9.4	11.1 ± 0.4	3.8	−10.6
	500	498.8 ± 19.2	3.9	0.2	525.2 ± 10.0	1.9	−5.0
	750	713.4 ± 15.6	2.2	4.9	803.7 ± 28.7	3.6	−7.2

in plasma and dermal tissue are shown in Fig. 3 with free plasma concentrations based on 76.7% plasma protein binding. The data show that terpinen-4-ol reaches measurable concentrations in both plasma and dermal tissue. However, the concentrations in the dermis were detectable for the first 3.5 h thereafter the dialysate concentrations were below the limit of quantification. As expected, free terpinen-4-ol concentrations in dermal tissue were lower than the corresponding total plasma concentrations for the entire length of study.

Plasma and dermal concentration-time data were subjected to noncompartmental analysis. All the plasma concentration-time profiles showed a remarkable distribution phase and were fitted with a bi-exponential equation with an estimated elimination half-life ($t_{1/2}$) of 1.80 ± 0.32 h. Unbound concentrations of terpinen-4-ol in dermis reached relatively low levels with an average maximum concentration (C_{max}) of 0.10 ± 0.06 μg/ml in comparison with the plasma C_{max} of 6.30 ± 1.90 μg/ml. The dermal penetration of terpinen-4-ol was assessed by comparing the ratio of the unbound AUC in tissue and the unbound AUC in plasma (fAUC$_{tissue}$/fAUC$_{plasma}$). The mean extrapolated AUC$_{last\text{-}\infty}$ was 4.2% for plasma and 17.0% for microdialysates. Based on the AUC ratios, terpinen-4-ol penetrates into dermal tissue resulting in fAUC$_{dermal}$/fAUC$_{plasma}$ of 0.20 ± 0.06. The terminal half-life was greater in plasma than in dermal tissue (1.80 ± 0.32 h versus 1.17 ± 0.25 h). The mean percentage of free terpinen-4-ol in dermis was 0.03 ± 0.03%.

These finding indicates that plasma levels do not provide information of actual terpinen-4-ol levels in the skin. Using plasma concentrations would overestimate the dermal concentrations and its efficacy. Therefore, direct measurement of free tissue concentrations is necessary. This study also demonstrates that cutaneous microdialysis is an effective and minimally invasive tool to evaluate the dermal pharmacokinetics of terpinen-4-ol following systemic administration.

Table 3: Pharmacokinetic parameters of terpinen-4-ol in plasma and dermal tissue after i.v. bolus administration in rats (2 mg/kg)

Pharmacokinetic parameter	Plasma [a]	Dermal tissue [b]
C_{max} (μg/ml)	6.30 ± 1.90	0.10 ± 0.06
AUC$_{0\text{-last}}$ (μg.hr/ml)	4.07 ± 0.52	0.16 ± 0.05
AUC$_{last\text{-}\infty}$ (μg.hr/ml)	0.18 ± 0.08	0.03 ± 0.01
AUC$_{0\text{-}\infty}$ (μg.hr/ml)	4.25 ± 0.59	0.20 ± 0.04
Clearance (ml/hr/kg)	476.71 ± 58.26	–
$t_{1/2}$ (h)	1.80 ± 0.32	1.17 ± 0.25
V_z (ml/kg)	1245 ± 294	–
MRT (h)	1.16 ± 0.25	1.84 ± 0.65
α(1/h)	16.37 ± 5.36	–
β (1/h)	0.75 ± 0.2	–
fAUC$_{dermal}$/fAUC$_{plasma}$	–	0.20 ± 0.06
%Free terpinen-4-ol in dermis	0.03 ± 0.01	

Data expressed as Mean ± S.D; $n=4$. [a] Based on total concentrations. [b] Based on free concentrations.

3. Experimental

3.1. Chemicals and reagents

(+)-Terpinen-4-ol (≥ 98.5%) and methyl salicylate (≥ 99.5%) were purchased from Sigma-Aldrich (Buchs, Switzerland). GC-MS grade hexane, HPLC grade water and sodium chloride were obtained from Fisher Scientific (Fairlawn, NJ). Drug-free Wistar rat plasma with EDTA was purchased from Innovative Research, Inc., (Novi, MI).

3.2. Instrumentation and chromatographic conditions

GC-MS analysis was performed using a Thermo Finnigan Trace GC 2000 gas chromatograph/quadrupole ion trap mass spectrometer (San Jose, CA) equipped with an AT^{TM} WAX capillary column (0.25 mm ID x 30 m,

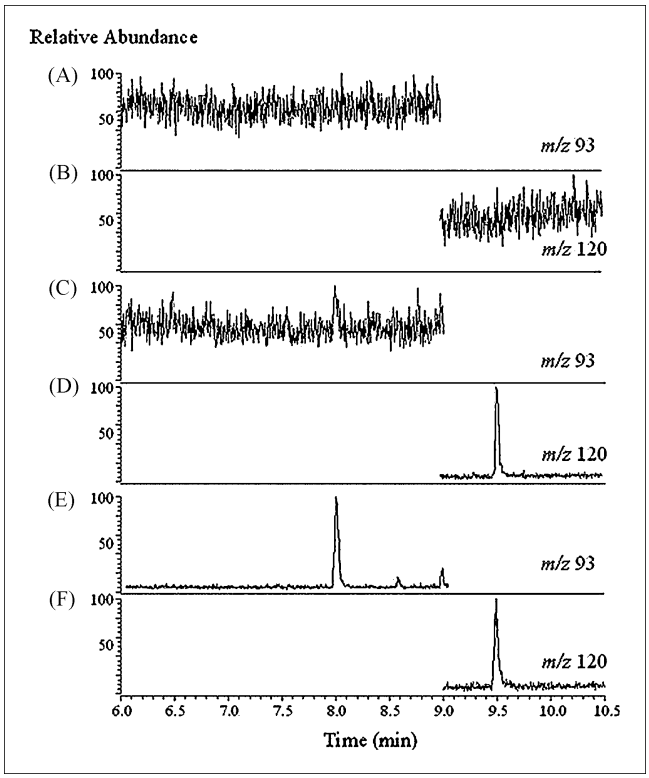

Fig. 2: Representative GC-MS chromatograms of rat plasma (A and B); LLOQ of terpinen-4-ol in rat plasma (5 ng/ml) and IS (C and D), real sample at 10 min after a single bolus injection of 2 mg/kg terpinen-4-ol (E and F). Peaks of 8.1 and 9.5 min are terpinen-4-ol and IS, respectively

0.25 μm film thickness; Alltech, State College, PA). The carrier gas was ultra-high-purity helium (99.999%) at a flow rate of 1 ml/min. Sample injection was performed in the splitless mode. The initial oven temperature was set at 60 °C for 1 min, then increased by 15 °C/min to 200 °C and held for 4 min. The temperature of the injection port, transfer line and ion source were set at 230, 275 and 200 °C, respectively. Electron impact ionization was used with an ionization energy of 70 eV.

The MS was operated in the selected ion monitoring (SIM) mode in order to maximize the sensitivity and robustness of the method. Mass spectra were first obtained in full scan mode (range of acquisition, 50–450 *m/z*), to select appropriate ions for identification and quantification in the SIM mode. The monitored ions were as follows: terpinen-4-o *m/z* 71, 93 and 111, methyl salicylate *m/z* 92, 120 and 152, respectively. The area ratio of 93/120 was used for quantification, whereas the other ions were used as qualifiers.

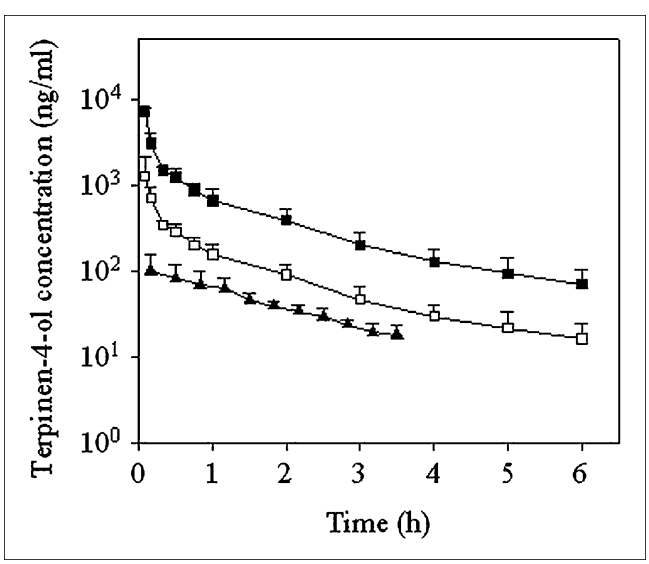

Fig. 3: The concentration-time profile (mean ± S.D.) in plasma (■), free plasma (□) and in the interstitial space fluid (free) of dermal tissue (▲) of terpinen-4-ol following i.v. injection (2 mg/kg)

3.3. Preparation of stock solutions, calibration and quality control samples

Stock solutions of terpinen-4-ol and methyl salicylate which was used as internal standard were prepared in methanol at a concentration of 1 mg/ml. These solutions were diluted with normal saline to obtain appropriate working solutions for preparing calibration and quality control (QC) samples.

Matrix-based calibration standards in rat plasma were prepared by spiking equal volumes (20 μl) of terpinen-4-ol working solutions into blank plasma (100 μl) to yield terpinen-4-ol concentrations of 5, 10, 50, 100, 250, 500, 750 and 1000 ng/ml.

The calibration standard solutions for the microdialysis samples were prepared by diluting the stock solution with normal saline to obtain final terpinen-4-ol concentrations of 5, 10, 20, 50, 125, 250, 500, 750 and 1000 ng/ml. Plasma and microdialysis QC samples were prepared in the same manner as the calibration standards at three concentration levels of terpinen-4-ol (10, 500 and 750 ng/ml).

3.4. Sample preparation

A 100 μl aliquot of plasma sample was transferred to a 4 ml glass vial followed by the addition of 20 μl of the IS working solution (0.2 μg/ml). Then, 500 μl of hexane was added into the vial. The mixture was vortexed for 30 s and centrifuged at 3000 rpm for 15 min. The upper organic layer (1 μl) was injected into the GC-MS system.

For microdialysates, a 30 μl aliquot of a microdialysis sample was added to a 4 ml glass vial followed by the addition of 20 μl of IS solution (0.5 μg/ml). The sample was extracted by the addition of 200 μl of hexane followed by vortexing for 30 s. The upper organic layer (2 μl) was injected into the GC-MS system.

3.5. Method validation

The method was validated according to the FDA guidance on bioanalytical method validation. The specificity was evaluated by analyzing blank microdialysates and plasma samples from six different rats to test for matrix interfering peaks. The absence of interfering peaks with the same *m/z* ratio at the analyte and IS retention times was verified. Sensitivity was achieved by determining the lower limit of quantification of terpinen-4-ol. The LLOQ was established as the lowest concentration of terpinen-4-ol used in the calibration curve with accuracy and precision of 100 ± 20%. Bias and relative standard deviation (%R.S.D.) were used as measures of accuracy and precision, respectively. Linearity was assessed by plotting terpinen-4-ol to IS peak area ratios versus concentrations of calibration standards. All calibration curves were required to have a correlation value (r^2) of at least 0.995. Precision and accuracy were evaluated by injecting QC samples (10, 500 and 750 ng/ml) in pentuplicate on three different days. Precision was measured by inter- and intra-day R.S.D. (%). The accuracy was evaluated by the deviation or bias (%) of the observed concentration from the expected concentration. The potential for carryover was investigated by injecting blank solvent immediately after analysis of the highest concentration point of the calibration curve.

At the three QC concentration levels, the extraction recoveries were estimated by comparing the peak area ratio of terpinen-4-ol to IS in samples after extraction to the unextracted standard solutions containing the same concentrations in hexane ($n = 3$). The stability of terpinen-4-ol in microdialysates and plasma samples was assessed by analyzing QC samples exposed to different temperature conditions (at room temperature and at −20 °C). To determine the post-preparative stability, three concentrations of QC samples from the first day were kept on the GC-MS autosampler at room temperature for 36 h and then analyzed again. Furthermore, fortified microdialysates and plasma samples were subjected to three freeze-thaw cycles. Samples were frozen for 24 h at −20 °C and then allowed to thaw unassisted at room temperature. After repeating the process two more times, the samples were extracted and analyzed.

3.6. Plasma protein binding

The rat plasma protein binding of terpinen-4-ol was determined using the ultrafiltration method. Aliquots of rat plasma containing various concentrations of terpinen-4-ol (0.01, 0.25, 0.5, 0.75, 1 and 10 μg/ml) were incubated at 37 °C for 30 min. Then, a 500 μl aliquot of plasma was added into the upper part of the centrifugal filter device (Microcon® Ultracel YM-30, Millipore Corporation, Billerica, MA) and centrifuged at 3,000 rpm for 8 min. The ultrafiltration samples were collected and stored at −20 °C until analysis. Triplicates were performed for each concentration. The ultrafiltrate concentration represents the free plasma concentration. The fraction of unbound drug was determined by the following equation: $f_u = C_u/C_t$. Where f_u is the fraction of unbound drug in plasma and C_u and C_t are the unbound and total concentrations of the analyte in plasma, respectively.

3.7. Pharmacokinetic study

The distribution of terpinen-4-ol in rat dermis and plasma after i.v. bolus injection at a dose of 2 mg/kg was investigated using dermal microdialysis with simultaneous conventional blood sampling. The experiment was performed in four male Wistar rats weighing between 300 and 350 g obtained from Harlan Laboratories (Tampa, FL). This study was approved by the Institutional Animal Care and Use Committee of the University of Florida. The protocol adhered to the 'Principles of Laboratory Animal Care' (NIH publication #85-23, revised 1985).

One day before the experiment, the abdominal fur of the rat was carefully removed with an electric animal hair clipper. On the day of experiment, the animal was anesthetized with 4% isoflurane for 5 min in an induction chamber and maintained in anesthesia throughout the entire study with 2% isoflurane by nose cone. The rat was placed on a temperature-controlled heating pad in the dorsal position to maintain its body temperature at 37–38 °C. A microdialysis probe (CMA 30, 10-mm membrane length, and 6 kDa MWCO) was implanted in the dermis of the abdominal region of the rat. The probe was connected to a microinjection pump (Harvard Apparatus 22 injection pump, model 55–4150) and perfused with normal saline at a flow rate of 2 μl/min. After equilibration period of 30 min, the probe was calibrated using retrodialysis technique (Stahle et al. 1991) by perfusing with terpinen-4-ol (10 ng/ml) for 30 min. Then the probe was flushed with normal saline for at least 30 min to allow for the calibration solution to clear from the probe and tissue prior to dosing. Following washout, 2 mg/kg of terpinen-4-ol was administered via i.v. bolus into the lateral tail vein. Microdialysates were collected at 20-min intervals for 6 h after dosing. Twelve blood samples (200 μl) obtained from the tail vein were collected in EDTA-containing tubes at 0 (pre-dose), 5, 10, 20, 30, 45 min, and 1, 2, 3, 4, 5 and 6 h after dosing. Blood samples were centrifuged at 10,000 rpm for 15 min. The plasma was immediately separated and stored at −20 °C until analysis.

3.8. Pharmacokinetic data analysis

Noncompartmental and compartmental analyses were performed using WinNonlin version 5.3 (Pharsight Corporation, Mountain View, CA).

3.8.1. Noncompartmental analysis

The following parameters were calculated for each rat, and the mean and standard deviation (S.D.) of each parameter were determined.

Plasma. The terminal elimination rate constant (λ_z) was estimated from the slope of the terminal exponential phase of the logarithmic plasma concentration-time profile using at least four data points. Terminal elimination half-life ($t_{1/2}$) was calculated as $\ln(2)/\lambda_z$. The area under the concentration-time curve from 0 to the last measured value ($AUC_{0\text{-last}}$) was calculated using the log-linear trapezoidal rule, and $AUC_{0-\infty}$ was calculated as $AUC_{0\text{-last}} + C_{last}/\lambda_z$, where C_{last} is the last concentration measured. Clearance (CL) was calculated as the dose divided by the $AUC_{0-\infty}$. The volume of distribution (V_z) was calculated as the CL divided by λ_z. The mean residence time ($MRT_{0-\infty}$) was calculated as the area under the first moment curve ($AUMC_{0-\infty}$)/$AUC_{0-\infty}$.

Dermal. The midpoint of the sampling interval was used as time-point for calculations. Unbound concentrations in the dermal tissue from each rat were calculated from the measured microdialysate concentrations and the individual probe recovery. The pharmacokinetic parameters were calculated using the same formula as for plasma samples.

The penetration of terpinen-4-ol into the dermal tissue was assessed by comparing the ratio of the unbound AUC in tissue and the unbound AUC in plasma ($fAUC_{tissue}/fAUC_{plasma}$). The percentage of free AUC of terpinen-4-ol per amount of administered in dermis was calculated as $100 \times fAUC_{tissue}$/absolute amount of terpinen-4-ol administered.

3.8.2. Compartmental analysis

The plasma concentration-time data following i.v. bolus injection were subject to compartmental model analysis. The model was selected based on goodness of fit using the Akaike's Information Criterion (AIC) and Schwarz Criterion (SC) residual analysis, and overall correlation coefficient. Plasma concentration-time data for each rat were fitted using a two-compartment open body model described by the following equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

where C_p is the total plasma concentration at time t, α and β are the hybrid constants for the distribution and elimination phases, respectively, A is the y axis intercept for the distribution phase and B is the y axis intercept for the linear elimination phase.

The values of pharmacokinetic parameters obtained from the noncompartmental approach were compared by the ANOVA with HSD-Turkey post hoc comparisons. A P-value of < 0.05 was considered statistically significant.

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References

Anderson C, Andersson T, Molander M (1991) Ethanol absorption across human skin measured by *in vivo* microdialysis technique. Acta Derm Venereol 71: 389–393.

Astani A, Reichling J, Schnitzler P (2010) Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. Phytother Res 24: 673–679.

Ault JM, Riley CM, Meltzer NM, Lunte CE (1994) Dermal microdialysis sampling *in vivo*. Pharm Res 11: 1631–1639.

Barra A, Coroneo V, Dessi S, Cabras P, Angioni A (2007) Characterization of the volatile constituents in the essential oil of *Pistacia lentiscus* L. from different origins and its antifungal and antioxidant activity. J Agric Food Chem 55: 7093–7098.

Benfeldt E (1999) *In vivo* microdialysis for the investigation of drug levels in the dermis and the effect of barrier perturbation on cutaneous drug penetration. Studies in hairless rats and human subjects. Acta Derm Venereol Suppl (Stockh) 206: 1–59.

Biju SS, Ahuja A, Khar RK (2005a) Tea tree oil concentration in follicular casts after topical delivery: determination by high-performance thin layer chromatography using a perfused bovine udder model. J Pharm Sci 94: 240–245.

Biju SS, Ahuja A, Rafiullah MR, Khar RK (2005b) A validated HPTLC method for determination of tea tree oil from cosmeceutical formulations. J Pharm Biomed Anal 38: 41–44.

Bozzuto G, Colone M, Toccacieli L, Stringaro A, Molinari A (2011) Tea tree oil might combat melanoma. Planta Med 77: 54–56.

Cal K (2006a) How does the type of vehicle influence the *in vitro* skin absorption and elimination kinetics of terpenes? Arch Dermatol Res 297: 311–315.

Cal K (2006b) Skin penetration of terpenes from essential oils and topical vehicles. Planta Med 72: 311–316.

Cal K, Krzyzaniak M (2006) Stratum corneum absorption and retention of linalool and terpinen-4-ol applied as gel or oily solution in humans. J Dermatol Sci 42: 265–267.

Cal K, Kupiec K, Sznitowska M (2006) Effect of physicochemical properties of cyclic terpenes on their ex vivo skin absorption and elimination kinetics. J Dermatol Sci 41: 137–142.

Calcabrini A, Stringaro A, Toccacieli L, Meschini S, Marra M, Colone M, Salvatore G, Mondello F, Arancia G, Molinari A (2004) Terpinen-4-ol, the main component of *Melaleuca alternifolia* (tea tree) oil inhibits the *in vitro* growth of human melanoma cells. J Invest Dermatol 122: 349–360.

Cha JD, Jeong MR, Jeong SI, Moon SE, Kil BS, Yun SI, Lee KY, Song YH (2007) Chemical composition and antimicrobial activity of the essential oil of *Cryptomeria japonica*. Phytother Res 21: 295–299.

Cross SE, Russell M, Southwell I, Roberts MS (2008) Human skin penetration of the major components of Australian tea tree oil applied in its pure form and as a 20% solution *in vitro*. Eur J Pharm Biopharm 69: 214–222.

Giordani C, Molinari A, Toccacieli L, Calcabrini A, Stringaro A, Chistolini P, Arancia G, Diociaiuti M (2006) Interaction of tea tree oil with model and cellular membranes. J Med Chem 49: 4581–4588.

Greay SJ, Ireland DJ, Kissick HT, Levy A, Beilharz MW, Riley TV, Carson CF (2010) Induction of necrosis and cell cycle arrest in murine cancer cell lines by *Melaleuca alternifolia* (tea tree) oil and terpinen-4-ol. Cancer Chemother Pharmacol 65: 877–888.

Groth L (1996) Cutaneous microdialysis. Methodology and validation. Acta Derm Venereol Suppl (Stockh) 197: 1–61.

Kreilgaard M (2002) Assessment of cutaneous drug delivery using microdialysis. Adv Drug Deliv Rev 54 Suppl 1: S99–121.

Nielsen JB, Nielsen F (2006) Topical use of tea tree oil reduces the dermal absorption of benzoic acid and methiocarb. Arch Dermatol Res 297: 395–402.

Pithayanukul P, Tubprasert J, Wuthi-Udomlert M (2007) *In vitro* antimicrobial activity of *Zingiber cassumunar* (Plai) oil and a 5% Plai oil gel. Phytother Res 21: 164–169.

Reichling J, Landvatter U, Wagner H, Kostka KH, Schaefer UF (2006) *In vitro* studies on release and human skin permeation of Australian tea tree oil (TTO) from topical formulations. Eur J Pharm Biopharm 64: 222–228.

Russell M, Southwell I (2002) Monoterpenoid accumulation in *Melaleuca alternifolia* seedlings. Phytochemistry 59: 709–716.

Stahle L, Segersvard S, Ungerstedt U (1991) A comparison between three methods for estimation of extracellular concentrations of exogenous and endogenous compounds by microdialysis. J Pharmacol Methods 25: 41–52.

Ungerstedt U (1984) Measurement of neurotransmitter release by intracranial dialysis. In: Marsden CA (ed.) Measurement of Neurotransmitter Release *in vivo*, Chichester, p. 81–105.

Wedge DE, Tabanca N, Sampson BJ, Werle C, Demirci B, Baser KH, Nan P, Duan J, Liu Z (2009) Antifungal and insecticidal activity of two *Juniperus* essential oils. Nat Prod Commun 4: 123–127.

Zuniga B, Guevara-Fefer P, Herrera J, Contreras JL, Velasco L, Perez FJ, Esquivel B (2005) Chemical composition and anti-inflammatory activity of the volatile fractions from the bark of eight Mexican Bursera species. Planta Med 71: 825–828.