DERMAL AND PLASMA PHARMACOKINETICS OF TERPINEN-4-OL, THE MAIN ACTIVE INGREDIENT OF *ZINGIBER CASSUMUNAR* (PLAI) OIL

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ABSTRACT

The purpose of this study was to investigate the pharmacokinetics of terpinen-4-ol in rats following intravenous (i.v.) bolus injection of terpinen-4-ol and topical administration of plai oil, derived from the rhizomes of *Zingiber cassumunar* Roxb. Total plasma and unbound dermal concentrations were measured by microdialysis and conventional blood sampling, respectively. After i.v. bolus administration (2 mg/kg), terpinen-4-ol rapidly distributed into the dermis and reached relatively low levels in comparison with those of plasma. The plasma concentration-time profile can be described by a two-compartment model. The dermal pharmacokinetic study of terpinen-4-ol following topical application of plai oil was performed under non-occlusive conditions. The oil was topically applied at a dose of 2, 4 and 8 mg/cm^2 plai oil corresponded to the amount of 1.2, 2.4 and 4.8 mg/cm^2 terpinen-4-ol, respectively. Terpinen-4-ol demonstrated linear pharmacokinetics in dermal tissue across the investigated doses range. This study demonstrates that microdialysis is an effective and minimally invasive tool to evaluate the dermal pharmacokinetics of terpinen-4-ol following i.v. or topical administration.

KEY WORDS : TERPINEN-4-OL / PHARMACOKINETICS / MICRODIALYSIS / *ZINGIBER CASSUMUNAR*

175 pages

การศึกษาเภสัชจลนศาสตร์ในผิวหนังและพลาสมาของเทอร์พิเนนโฟออลซึ่งเป็นสารสำคัญหลักในน้ำมันไพล

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บทคัดย่อ

วัตถุประสงค์ของการการศึกษานี้เพื่อศึกษาเภสัชจลนศาสตร์ของเทอร์พิเนนโฟออลในหนูทดลอง เมื่อให้โดยการฉีดเข้าเส้นเลือดดำที่หาง และให้โดยการทาน้ำมันไพลบนผิวหนัง ซึ่งน้ำมันไพลนี้ได้มาจากการกลั่นด้วยไอน้ำของเหง้าไพล (*Zingiber cassumunar* Roxb.) หลังจากที่ให้เทอร์พิเนนโฟออลในขนาด 2 มิลลิกรัม/กิโลกรัมโดยการฉีดเข้าเส้นเลือดดำที่หาง พบว่าเทอร์พิเนนโฟออลมีการกระจายตัวไปที่ผิวหนังอย่างรวดเร็ว และมีระดับความเข้มข้นในผิวหนังต่ำกว่าพลาสมาตลอดการศึกษา กราฟความสัมพันธ์ระหว่างระดับความเข้มข้นของเทอร์พิเนนโฟออลในพลาสมากับเวลาสามารถอธิบายได้ด้วยแบบจำลองเภสัชจลนศาสตร์แบบสองห้อง การศึกษาเภสัชจลนศาสตร์ของเทอร์พิเนนโฟออลในผิวหนัง หลังจากทาน้ำมันไพลบนผิวหนังบริเวณหน้าท้องของหนูทดลองในปริมาณ 2 4 และ 8 มิลลิกรัม/ตารางเซนติเมตร พบว่าเทอร์พิเนนโฟออลมีการกระจายตัวไปที่ผิวหนังอย่างรวดเร็ว พื้นที่ใต้กราฟระหว่างความเข้มข้นของเทอร์พิเนนโฟออลกับเวลาตั้งแต่เวลาศูนย์ถึงอินฟินิตี้ต่อขนาดน้ำมันไพลที่ให้ทั้งสามขนาด ไม่มีความแตกต่างอย่างมีนัยสำคัญแสดงให้เห็นว่าเทอร์พิเนนโฟออลมีเภสัชจลนศาสตร์ในผิวหนังแบบเชิงเส้นในช่วงขนาดของน้ำมันที่ทำการศึกษานอกจากนี้การศึกษานี้แสดงให้เห็นว่าวิธีไมโครไดอะไลซิส เป็นวิธีที่มีประสิทธิภาพในการนำมาศึกษาเภสัชจลนศาสตร์ของเทอร์พิเนนโฟออลในผิวหนังของหนูทดลอง เมื่อฉีดเข้าเส้นเลือดดำหรือทาน้ำมันไพลบนผิวหนัง

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LIST OF ABBREVIATIONS

%	percent
α	hybrid constants for the distribution phase
β	the hybrid constants for the elimination phases
λ_z	terminal elimination rate constant
A	surface area
A	the y axis intercept for the distribution phase and
AAALAC	Assessment and Accreditation of Laboratory Animal Care
AIC	Akaike's Information Criterion
ANOVA	analysis of variance
$AUC_{0\text{-last}}$	The area under the concentration-time curve from 0 to the last measured value
$AUC_{0-\infty}$	The area under the concentration-time curve from 0 to infinity
$AUMC_{0-\infty}$	area under the first moment curve
B	the y axis intercept for the linear elimination phase
C	analyte concentration
°C	degree Celsius
$C_{dialysate}$	concentration of analyte in the dialysate
C_{last}	last concentration measured
C_{max}	maximum concentration
C_0	initial concentration
C_p	plasma concentration
$C_{perfusate}$	concentration of analyte in the perfusate
C_{tissue}	concentration of analyte in the extracellular fluid of tissue
C_{ECF}	concentration of analyte in the extracellular fluid

LIST OF ABBREVIATIONS (cont.)

C_t	unbound concentration
C_u	total concentration
CL	Clearance
cm	centimeter (s)
cm^2	square centimeter (s)
cm^3	cubic centimeter (s)
EDTA	ethylenediaminetetraacetic acid
Eq.	equation
Et al.	et alli, and others
Etc.	et celera, and other things
eV	electron volt
F	perfusion flow rate
FDA	Food and Drug Administration
f_u	fraction of unbound drug
g	gram
GC-MS	gas chromatography mass spectrometry
h	hour (s)
ICH	International Conference on Harmonisation
i.d.	inner diameter (s)
i.e.	id est, that is
i.v.	intravenous injection
IS	internal standard
kDa	kilo Dalton (s)
kg	kilogram (s)
L	liter (s)
lb	the position variable along the probe tubing
Lb	length of tubing
LLOQ	lower limit of quantification

LIST OF ABBREVIATIONS (cont.)

MF	matrix factor
mg	milligram (s)
min	minute (s)
ml	milliliter (s)
mm	millimeter (s)
$MRT_{0-\infty}$	mean residence time
MWCO	molecular weight cut off
m/z	mass-to-charge ratio
ng	nanogram (s)
NIH	National Institutes of Health
NSAID	Nonsteroidal anti-inflammatory drug (s)
o/w	oil in water
pH	the negative logarithm of the hydrogen ion concentration
QC	quality control
r	the mass transport coefficient
r^2	coefficient of determination
R.E.	relative error
R.S.D.	relative standard deviation
rpm	rounds per minute
SC	Schwarz Criterion
S.D.	standard deviation
sec	second (s)
SIM	selected ion monitoring
t	time
$t_{1/2}$	elimination half-life
t_{max}	time to maximum concentration
μg	microgram (s)

LIST OF ABBREVIATIONS (cont.)

µl	microliter (s)
µm	micrometer (s)
V_z	volume of distribution
V_{ss}	volume of distribution at steady state ()
w/w	weight by weight

CHAPTER I
INTRODUCTION

Plai oil is an essential oil derived from steam distillation of rhizomes of *Zingiber cassumunar* Roxb., (Zingiberaceae). The oil alone or in combination with other oils has traditionally been used to treat inflammatory conditions such as muscle and joints pain by direct application to affected areas. In addition to topical anti-inflammatory creams, gels and ointments, the oil is incorporated as the main active ingredients in many massage oils, hygiene products including soaps, shampoos as well as in products for aromatherapy.

The chemical composition of plai oil was identified, consisting of a complex mixture of monoterpenes and terpinen-4-ol is considered to be one of the main active ingredients responsible for the anti-inflammatory activity of the oil (1, 2). Furthermore, terpinen-4-ol has been shown to possess diverse biological activities, including antiviral, antibacterial, antifungal, and insecticidal effects as well as antioxidant and anti-inflammatory activities (3-7). Recent reports have demonstrated that terpinen-4-ol could be developed as new therapies against melanoma either systemic administration or targeted drug delivery (8-11).

Since the skin acts as the protective barrier against the external environment, the permeation of active components of plai oil into and through the skin is important for the efficacy of the oil. 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 (12-19). The results showed that terpinen-4-ol was highly 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. Nevertheless, the knowledge on the *in vivo* pharmacokinetics following topical application of plai oil has not been established.

Although the *in vitro* diffusion cell is considered as a robust screening method for estimation of topical drug delivery, the system does not adequately mimic the *in vivo* situation. Furthermore, since the unbound fraction is responsible for pharmacological activity, the determination of free active concentrations underlying the site of application, particularly the skin, is more clinically relevant than the measurement of total plasma concentrations. 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 (20) and has been applied to dermatological research including drug pharmacokinetics in dermis (21-23). With the use of microdialysis, it is possible to obtain full local pharmacokinetic profiles of dermal drug penetration from each sampling site. This technique also minimizes the sampling burden on a patient in comparison to conventional tissue sampling methods, e.g. skin stripping, skin biopsy and skin blister (22, 24, 25).

Measurement of terpinen-4-ol concentrations in biological matrices is crucial for many studies including pharmacokinetic and bioequivalence study. Various analytical methods have been reported to estimate terpinen-4-ol concentrations in cosmeceutical formulations (14, 19, 26), follicular casts (27), plant extracts (15, 16, 18, 28) and skin tissues (12, 13). Therefore, to carry out preliminary pharmacokinetic parameters, the dermal and plasma pharmacokinetic studies of terpinen-4-ol using dermal microdialysis with simultaneous conventional blood sampling, an efficient analytical method was required. In the present study 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 plai oil, human plasma, human blood, rat plasma and dermal microdialysis samples.

For topical use of plai oil, detailed knowledge on dermal absorption of active components of the oil is essential for improving its topical application. Therefore, the purpose of this study was to investigate the skin pharmacokinetics of terpinen-4-ol following topical administration of plai at three different doses (2, 4 and 8 mg/cm^2) using microdialysis. Furthermore, the distribution of terpinen-4-ol in

dermis and plasma of rat following intravenous (i.v.) bolus injection at a dose of 2 mg/kg was also investigated by using dermal microdialysis with simultaneous conventional blood sampling.

The ultimate goals of this study were:

- To develop and validate the analytical methods for determination of terpinen-4-ol in rat dermal microdialysates, rat plasma, human plasma, human whole blood and plai oil.

- To study the preliminary pharmacokinetic parameters of terpinen-4-ol in its pure form and plai oil.

- To explore the permeability of the dialysis membrane for terpinen-4-ol and optimise the microdialysis parameters for the subsequent *in vivo* studies.

- To investigate the influence of perfusion flow rate and terpinen-4-ol concentration on *in vitro* recovery and inlet probe binding.

- To determined the distribution of terpinen-4-ol in dermis and plasma of rat following intravenous (i.v.) bolus injection at a dose of 2 mg/kg.

- To assess the skin pharmacokinetics of terpinen-4-ol following topical administration of plai at three different doses (2, 4 and 8 mg/cm^2) using microdialysis.

CHAPTER II
LITERATURE REVIEW

2.1 *Zingiber cassumunar* Roxb.

Zingiber cassumunar Roxb., commonly known as plai, belongs to the botanical family of, as its scientific name signifies, Zingiberaceae. The plant is a perennial herb, consisting of underground rhizome which is bright yellow inside. The rhizome of plai has been traditionally used in Thai and Southeast Asian folklore remedies as a single plant or in combination with other plants for treatment of conditions such as relieving dysmenorrheal, inflammation and asthma. The essential oil derived from steam distillation of rhizomes of plai has traditionally been used to treat inflammatory conditions such as arthritis, muscle and joints pain by direct application to affected areas. Terpinen-4-ol is considered to be one of the main active ingredients responsible for the anti-inflammatory activity of the oil (1, 2). The chemical structure of terpinen-4-ol was shown in Figure 2.1. Furthermore, terpinen-4-ol has been shown to possess diverse biological activities, including antiviral, antibacterial, antifungal, and insecticidal effects (3-6). Employed largely for its anti-inflammatory activity, plai oil is incorporated as the active ingredient in many topical anti-inflammatory creams, gels and ointments which are widely available over the counter in Thailand.

2.1.1 Composition and chemistry of plai oil

Plai oil is composed of terpene hydrocarbons, mainly monoterpenes and their associated alcohols. Terpenes are volatile, aromatic hydrocarbons and may be considered polymers of isoprene, which has the formula C_5H_8. The oil has a pale amber color, cool scent and a green pepery flavor with a touch of a bite. The recent report on the composition of plai oil determined by gas chromatography-mass spectrometry described 22 components and their ranges of concentrations (Table 2.1).

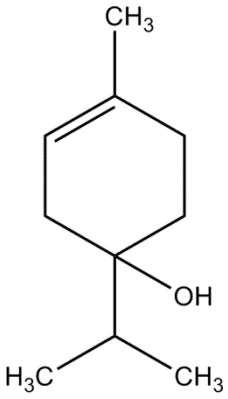

Figure 2.1 Chemical structure of terpinen-4-ol.

Table 2.1. Percentage of constituents of plai oil identified by GC-MS analysis (1).

Retention time (min)	Possible compound [a]	% Peak area					
		Hexane extracted plai oil			Distilled plai oil		
		S1	S2	S3	S1	S2	S3
14.05	α-thujene	0.70	-	-		0.44	0.60
14.53	α-pinene	0.58	0.69	0.34	1.15	1.53	2
17.42	sabinene	39.11	30.39	24.05	36.71	41.39	53.5
17.68	β-pinene	2.43	1.96	1.36	2.25	2.77	3.78
18.77	β-myrcene	2.13	2.03	1.63	1.83	2.15	2.65
21.03	α-terpinene	3.18	2.16	1.83	2.04	2.6	3.39
21.76	p-cymene	0.87	1.66	0.77	0.74	1.63	1.71
22.16	β-phellandrene	0.84	2.34	-	0.74	1.69	1.24
24.99	γ-terpinene	7.54	7.74	6.68	5.27	6.31	7.25
25.83	(Z)-sabinene hydrate	1.25	0.67	0.61	-	-	-
27.50	terpinolene	1.11	0.87	1	0.84	0.94	1.09
28.39	(E)-sabinene hydrate	1.07	0.56	0.54	-	0.49	-
30.14	(Z)-p-menth-2-en-1-ol	-	0.54	0.62	-	0.41	-
34.09	terpinen-4-ol	33.11	42.23	49.36	27	29.96	21.85
34.90	α-terpineol	-	-	0.76	0.29	-	-

Table 2.1 Percentage of constituents of plai oil identified by GC-MS analysis (1) (continued)

Retention time (min)	Possible compound [a]	% Peak area					
		Hexane extracted plai oil			Distilled plai oil		
		S1	S2	S3	S1	S2	S3
35.96	myrtenol	-	-	0.27	-	-	-
43.57	β-terpinyl acetate	0.21	0.31	0.46	0.29	-	-
51.35	β-sesquiphellandrene	-	0.53	0.27	1.03	0.83	-
54.11	Unknown1	-	-	0.26	0.54	0.32	-
55.61	(E)-1-(3,4-dimethoxyphenyl) butadiene	5.52	5.31	8.28	16.16	6.54	0.95
62.31	4-(2,4,5-trimethoxyphenyl)but-1,3-diene	0.34	-	0.28	0.21	-	-
68.00	Unknown2	-	-	0.64	1.91	-	-
	Total oxygenated compounds	40.25	48.95	60.57	43.66	37.4	22.8
	Total non-oxygenated compounds	59.75	51.05	38.53	52.6	62.28	77.2
	Unknown	-	-	0.9	2.54	0.32	-
	Total	100	100	100	100	100	100

[a] Compounds are listed in order of their elution time from a DB-5 column

S1, S2 and S3 are the essential oils obtained from Sa Kaeo, Chiang Mai and Prachuap Khiri Khan, respectively.

The main active chemical of plai oil obtained from hydro distillation have been identified as sabinene (36.71-53.50%), γ-terpinene (5.27-7.25%), terpinen-4-ol (21.85-29.96%), and (E)-1-(3', 4'-dimethoxyphenyl) buta-diene (DMPBD, 0.95-16.16%). For the oil obtained by hexane extraction, the main composition were sabinene (24.05-39.11%), γ-terpinene (6.68-7.74%), terpinen-4-ol (33.11-49.36%), and DMPBD (5.31-8.28%). The yield of oil obtained from steam distillation of fresh rhizome was 1.14-1.37% (w/w) while the yield from hexane extraction was 0.86-0.98% (w/w). The specific gravity of the oil obtained by hydro distillation and hexane extraction ranged from 0.9360 to 0.9743 g/cm^3 and 1.0243 to 1.0450 g/cm^3, respectively (1).

2.1.2 Anti-inflammatory activity of plai oil and terpinen-4-ol

Many studies now support the anecdotal evidence attributing anti-inflammatory activity to both plai oil and terpinen-4-ol. Plai oil exhibits a dose-dependent topical anti-inflammatory effect in the model of carrageenan-induced hind paw edema in rats. Individual assessment of topical anti-inflammatory activity of the five major components of plai oil demonstrated that DMPBD, terpinen-4-ol and, to a lesser degree, α-terpinene significantly inhibited edema formation, whereas sabinene and γ-terpinene were inactive. Therefore, DMPBD and terpinen-4-ol are apparently responsible for the anti-inflammatory activity of the oil. Nevertheless, although DMPBD is more potent that terpinen-4-ol, the relative content of terpinen-4-ol in the oil is four times higher (2).

The *in vitro* study over the last decade has demonstrated that terpinen-4-ol can suppress the production of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-8, IL-10 and prostaglandin E2 (PGE2) by lipopolysaccharide (LPS)-activated human blood monocytes (9). Brand C. et al examined the effect of topically applied tea tree oil and terpinen-4-ol on histamine-induced oedema in the ears of mice. This study reported that topical application of tea tree oil, and in particular terpinen-4-ol, may be effective in controlling histamine-induced skin oedema which is often associated with immediate-type allergic hypersensitivities (29). Also, terpinen-4-ol significantly reduced both the wheal and flare following histamine injection in rodent and human skin (30).

Pharmaceuticals and Natural Products Department of Thailand Institute of Scientific and Technological Research (TISTR) has conducted research regarding medicinal properties of plai and its topical product since 1975. Plai oil was obtained from hydro-distillation and developed as a cream, Plygesal®. The cream has been manufactured under license of The Government Pharmaceutical Organization and used topically for relief of muscular pains, bruises and sprains. The preliminary study on the use of Plygesal® in 89 subjects in different places during 1978-1989 revealed 77.7-90% active anti-inflammatory effect. Nowadays, a cream containing 14% phlai oil is one of the List of National Essential Medicine AD 2006 (List of Herbal Medicinal Products) of Thailand. However, the better controlled clinical trials are needed in future studies.

2.1.3 The *in vitro* skin penetration studies of terpinen-4-ol

The *in vitro* skin absorption and elimination kinetics of terpinen-4-ol in its pure form, essential oils and topical formulations was extensively investigated by Cal et al. using skin stripping and Franz diffusion cell system (12, 13, 17, 18). Pure terpinen-4-ol, pure tea tree oil, terpinen-4-ol incorporated in concentration of 5% in grape seeds oil, carbomer hydrogel and oil in water (o/w) emulsion were applied on the skin. After application time, the skin was separated into layers using tape-stripping technique: three fractions of stratum corneum and epidermis with dermis. The results demonstrated that when pure terpinen-4-ol was applied for 1 h, its absorption in stratum corneum and epidermis with dermis were 170 and 130 μg/cm^2, respectively. Extension of application time to 4 h caused increase in skin accumulation to 590 and 780 μg/cm^2 in stratum corneum and epidermis with dermis, respectively. About 50% of terpinen-4-ol absorbed during 1 h was eliminated from epidermis and dermis (13). Eight hours application time of tea tree oil caused significant increase in skin accumulation of terpinen-4-ol to 910 and 1500 μg/cm^2 in stratum corneum and epidermis with dermis, respectively (18).

To avoid influence of other components of tea tree oil on skin penetration of terpinen-4-ol, pure terpinen-4-ol was incorporated in a concentration of 5% into dermatological vehicles: oily solution, hydrogel and o/w emulsion. The greatest absorption of terpinen-4-ol in the skin layers was determined for hydrogel (175 μg/cm^2

in stratum corneum and 530 μg/cm^2 in epidermis with dermis after 4 h application). Significantly lower absorption of terpinen-4-ol was observed after application of oily solution and o/w emulsion (60 μg/cm2 in stratum corneum and 140 μg/cm^2 in epidermis with dermis for oily solution, and 80 μg/cm^2 in stratum corneum and 100 μg/cm^2 in epidermis with dermis for o/w emulsion). The elimination rate of terpinen-4-ol from the skin layers depended on its amount absorbed initially (12).

The same oily solution and hydrogel were further tested on human skin under non-occlusive conditions with an infinite dose (17). After 1 h application of oily solution and hydrogel, terpinen-4-ol contents in the stratum corneum were 20 and 110 μg/cm^2, respectively. After 2 h of elimination process, the fast elimination rate was observed and the amount of terpinen-4-ol decreased to about 5 μg/cm^2 independently of the vehicle used. In contrast to studies by Reichling et al. and by (14) Cross et al. (15), these experiments showed that terpinen-4-ol was not detected in the acceptor fluid of Franz diffusion cells but extensive accumulated in the skin tissue. It was assumed that dermal skin tissue served as a natural acceptor for terpinen-4-ol permeating through human skin. Reichling et al. investigated the *in vitro* human skin permeation of terpinen-4-ol with pure tea tree oil and 3 preparations (o/w emulsion, white petrolatum, ambiphilic cream) containing 5 % tea tree oil. The results showed that the absorption of terpinen-4-ol from the 5 % preparations was 4 to 12 times lower compared with the neat oil (14). Cross et al. demonstrated *in vitro* that following application of tea tree oil in its pure form and 20 % solution in ethanol, a small quantity of terpinen-4-ol penetrated into or through human skin (15). Nevertheless, the knowledge on the *in vivo* pharmacokinetics following topical application of plai oil has not been established.

2.2 The skin

The skin is the largest organ of the human body, with a surface area of approximately 1.8 m^2 and a total weight estimated, for a typical adult of 70 kg, to be 4 kg (31). In normothermic conditions, the cutaneous circulation comprises 5–10% of the total cardiac output (32). For 70-kg human males, the skin blood flow is

approximately 4.64 cm^3/s or 16,700 cm^3/h (33). Thus, the ratio of the total capillary flow to the corresponding skin surface area is approximately 0.93 cm^3/h.

The skin is a heterogeneous organ, containing a number of layers as well as appendages, such as sweat glands, hair follicles, and sebaceous glands. The thickness of the skin and composition of the stratum corneum vary according to body region. Until the beginning of the 20^{th} century, the skin was thought to be completely inert and impermeable to chemicals that might otherwise enter the body. While the skin does act as a barrier, it is not a complete barrier. Many chemicals do penetrate the skin, either intentionally or unintentionally, and cutaneous metabolism does occur. Because of its large surface area, the skin may be a major route of entry into the body in some exposure situations (34).

2.2.1 Skin structure

The skin is composed of three readily distinguishable layers: (a) epidermis, and (b) the dermis, penetrated by a highly complex network of capillaries involved in the removal of drugs from the skin into the systemic circulation (Figure 2.2). In addition, several pilosebaceous and sweat glands are dispersed throughout the skin, in various numbers and size, depending on body site.

2.2.1.1 Epidermis

The epidermis comprises about 5% of full-thickness skin and is divided into five or six layers, based on cellular characteristics (Figure 2.3). The majority of cells in the epidermis are called keratinocytes, which are formed by differentiation from one layer of mitotic basal cells. The number of distinguishable layers is dependent upon the anatomical site.

Basal layer (stratum germinativum) keratinocytes are metabolically active cells with the capacity to divide. Some of the resulting daughter cells of the basal layer move outward and differentiate. The cells adjacent to the basal layer start to produce lamellar granules, intracellular organelles that later fuse with the cell membrane to release the neutral lipids that are thought to form a barrier to penetration through the epidermis. The desmosomes (intercellular adhesive junctions) and other bridges connecting the adjacent cells have the appearance of spines, giving the name stratum spinosum to this three- to four-cell-thick layer above the basal layer

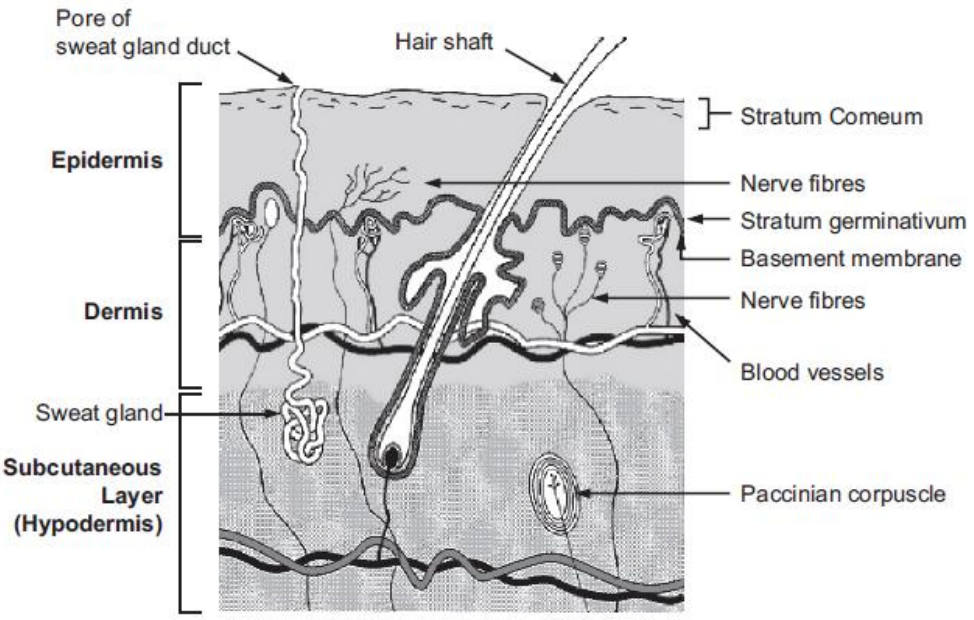

Figure 2.2 Structure of the skin (34).

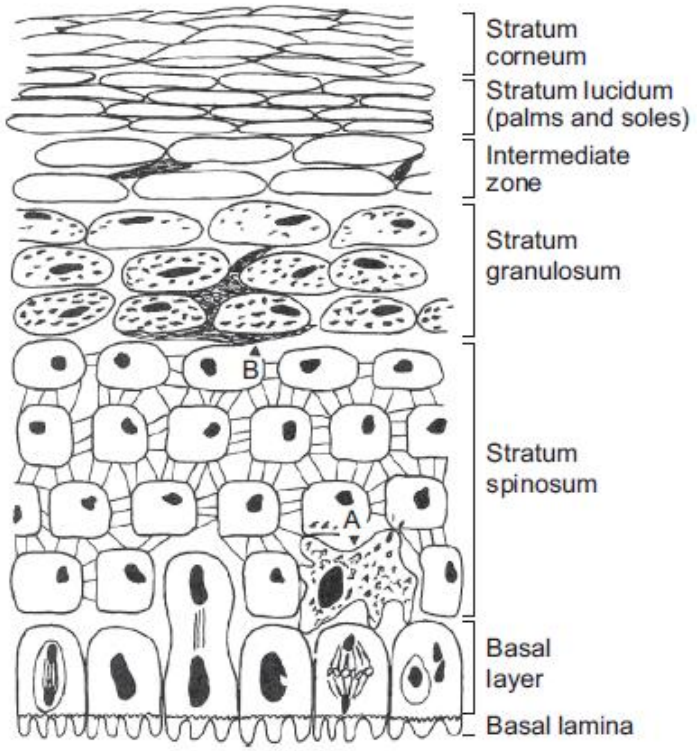

Figure 2.3 The epidermis. All cell layers and locations of the two dendritic cell types, melanocytes (A) and Langerhans cells (B), are shown (34).

the name stratum spinosum to this three- to four-cell-thick layer above the basal layer (35). The keratinocytes migrate outwards to the third epidermal layer, called the stratum granulosum, which is characterized by the presence of keratohyalin granules, polyribosomes, large Golgi bodies, and rough endoplasmic reticulum. These are the outermost viable cells of the epidermis.

The major barrier to permeation within the skin is the nonviable stratum corneum which is well-recognized as a heterogeneous two-compartment system of flattened keratinized cells embedded in a multilamellar lipid matrix mainly composed of neutral lipids and ceramides (36-39). It has a thickness of about10 μm (40-42) with about 20 cell layers (41). Its main homeostatic functions are restriction of excessive water loss to the external environment, moisture retention, cell cohesion and desquamation. In addition, stratum corneum is considered to be the rate-controlling membrane for transport of xenobiotics across the skin (37, 43). Therefore, the permeation of pharmacological active drugs into and through the skin is important for their efficacy.

2.2.1.2 Dermis

The dermis provides the nutritional support for the avascular epidermis. The thickness of dermis is a 0.2 to 0.3 cm that comprises a fibrous protein matrix, mainly collagen, elastin, and reticulum, embedded in an amorphous colloidal ground substance. The physical behaviour of the dermis, including elasticity, is determined by the fiber bundles and ground substance. The dermis is the locus of blood vessels, sensory nerves (pressure, temperature, and pain), and lymphatics. It contains the inner segments of the sweat glands and pilosebaceous units. The dermis provides flexibility with strength, serves as a barrier to infection, and functions as a water storage organ (44).

2.2.1.3 Skin appendages

The skin appendages originate in the subpapillary dermis and consist of eccrine sweat glands, apocrine sweat glands, sebaceous glands, and hair follicles, with their associated erector muscles. Appendages are found in most anatomical sites, although the number of each varies significantly by site. An average human skin surface contains 40-70 hair follicles and 200-250 sweat ducts per square centimeter. Sebaceous glands are most numerous and largest on the face, especially

the forehead, in the ear, on the midline of the back, and on anogenital surfaces. They secrete sebum, the principal components being glycerides, free fatty acids, cholesterol, cholesterol esters, wax esters, and squalene. Sebum acts as a skin lubricant and a source of stratum corneum plasticizing lipid and maintains acidic conditions (pH 5) on the skin's outer surface.

The eccrine glands are epidermal structures that are simple coiled tubes arising from a coiled ball of approximately 100 μm in diameter located in the lower dermis. These glands secrete a dilute salt solution with a pH of about 5. This secretion is stimulated by temperature-controlling determinants, such as exercise and high environmental temperature, as well as emotional stress, through the autonomic (sympathetic) nervous system. The apocrine glands are limited to specific body regions (e.g. armpit, the breast areola, and the perianal region) and are about 10 times larger than eccrine ducts. The apocrine glands, present after puberty, are sweat ducts in axillary skin (34).

2.2.2 Drug permeation routes

Penetration of a substance can occur via three main routes. (a) The intercellular pathway along the lamellar lipids between the corneocytes. (b) The intracellular route, which means the passage from corneocyte to corneocyte. (c) Passage through skin appendages such as hair follicles with their associated sebaceous glands, and through the sweat ducts.

The permeation of drugs through the skin includes the diffusion through the intact epidermis and through the appendages, i.e. hair follicles and sweat glands, which form shunt pathways through the intact epidermis. However, these skin appendages occupy only 0.1% of the total human skin surface and the contribution of this pathway is usually considered to be small (45). As mentioned above, drug permeation through the skin is usually limited by the stratum corneum. Two pathways through the intact barrier may be identified (Figure 2.4): the intercellular lipid route between the corneocytes and the transcellular route crossing through the corneocytes and the intervening lipids; that is, in both cases the drug must diffuse at some point through the intercellular lipid matrix, which is now recognized as the major determinant of percutaneous transport rate (46).

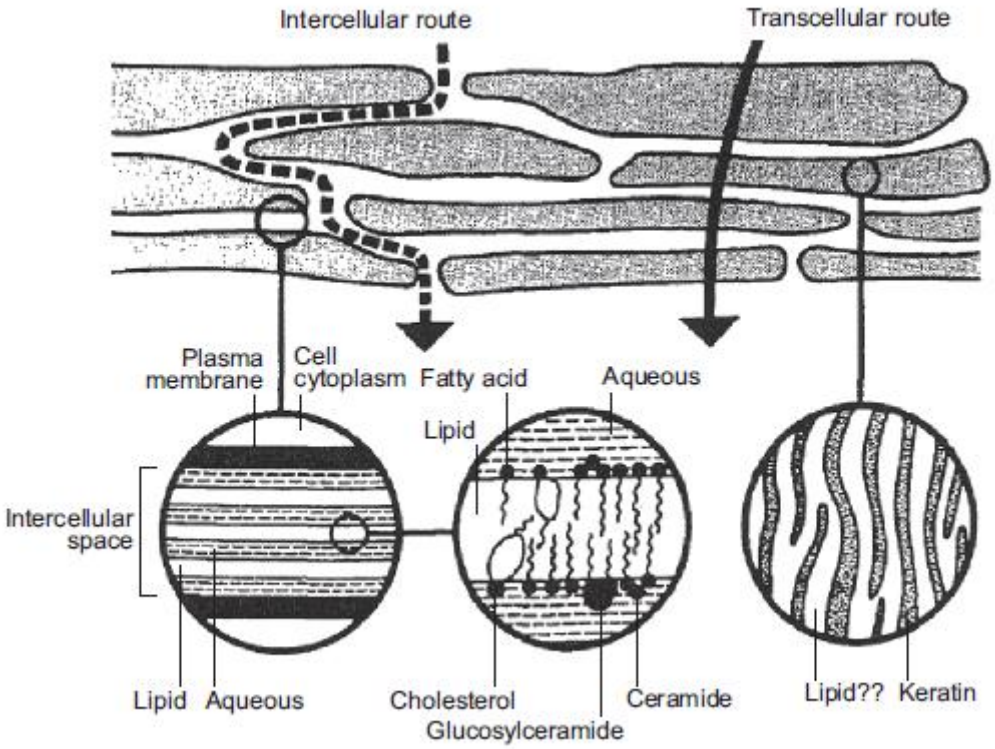

Figure 2.4 Permeation routes through the stratum corneum: (i) via the lipid matrix between the corneocytes (intercellular route) and (ii) across the corneocytes and the intercellular lipid matrix (transcellular route) (34).

2.3 Microdialysis

Microdialysis is a membrane-based sampling technique used for continuous measurement of unbound analyte concentrations in the extracellular fluid of many tissues. This technique was originally developed for sampling of neurotransmitters in rat brain studies (20) and has been applied to dermatological research including pharmacokinetics in dermis (21-23). The basic microdialysis system consists of a microdialysis probe, a subject (an animal or human), a perfusion pump, inlet and outlet tubing, and sample collection vial in which the microdialysis sample is collected (Figure 2.5).

The principle of this method is to mimic the passive function of a small blood vessel by perfusing a thin dialysis tube implanted into the tissue. The probe consists of a small semi-permeable hollow fiber membrane, permeable to water and small solutes, connected to an inlet and outlet tubing with a small diameter (typically 0.2-0.5 mm diameter). Depending on the probe design, the probe has one end connected to an afferent impermeable tube, which leads to a microdialysis pump, and the other end to an efferent sampling tube.

2.3.1 Probe design

Two kinds of probe are in common use for cutaneous microdialysis: the linear and concentric probes (Figure 2.6). The former is the most prevalent design used because it is simple, thinner and inexpensive to manufacture from artificial kidney fibres (24, 47, 48). Both types are available in a variety of membrane materials and pore sizes (molecular weight cut-off). Although there are probes with a pore size from 6 to 3000 kDa, the majority of experiments have been performed using membranes with a cut-off of 20 kDa. These membranes have been chosen since they are small enough to allow free diffusion of a great variety of solutes, but restrict the passage of proteins and macromolecules. The recovery of molecules declines very fast with an increase of the molecular weight (51). As there is increasing interest in soluble macromolecules, membranes with a cut-off of 100 kDa and even 3000 kDa are of interest in microdialysis studies (52, 53).

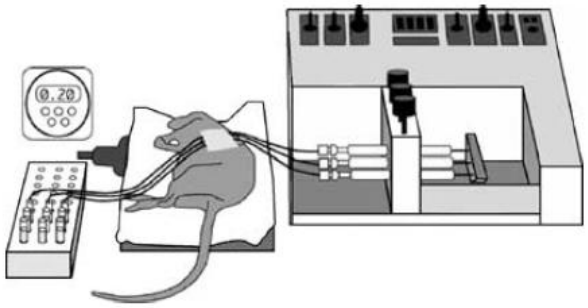

Figure 2.5 Experimental set-up for *in vivo* dermal microdialysis. The rat is anaesthetised and placed on a temperature controlled heating pad. Three microdialysis probes were inserted in the dermis and perfused at a constant flow rate by the microdialysis pump. After topical application of formulation to the demarcated area, microdialysis samples are collected at specific time interval (49).

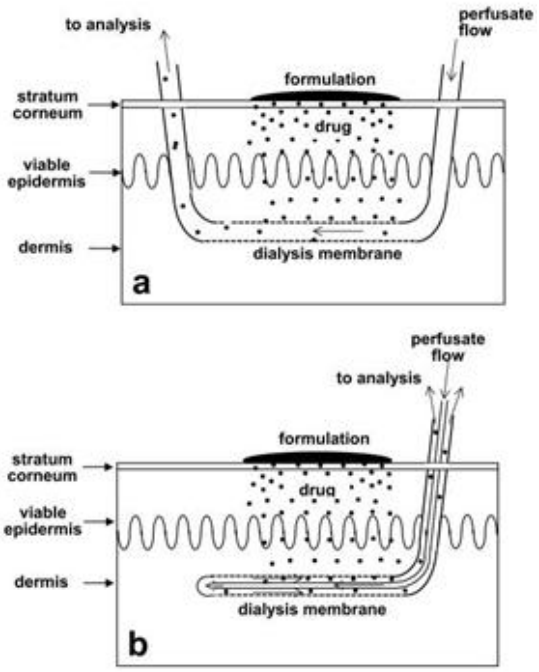

Figure 2.6 Schematic diagram of two currently used microdialysis probes for dermal pharmacokinetic experiments. In both cases, (a) Linear probe: the membrane is located in the middle of the probe, and the perfusate flow is mono-directional. (b) Concentric probe: the membrane is on the tip of the probe, and the perfusate flow is bi-directional (50).

2.3.2 Probe implantation

For cutaneous microdialysis, the probe is implanted superficially into the dermis, parallel to the skin surface via a guide cannula (Figure 2.7a). Generally, the probe was inserted through the skin for an appropriate distance (15-20 mm) leaving the skin through an exit puncture (Figure 2.7b-d). The guide is then withdrawn leaving the dialysis membrane in the dermis. The probe is continuously perfused with a physiological solution, which equilibrates with the extracellular fluid of the surrounding tissue, exchanging substances smaller than the cut-off value of the membrane. The exchange of compounds is driven by passive diffusion (54), and this technique can be applied either to remove or to deliver substances to the tissue, since the direction of the flux is dependent on the concentration gradient.

2.3.3 Features of microdialysis

The main feature of microdialysis is the possibility of assessing drug concentrations directly in the target tissue. The technique enables estimation of both endogenous and exogenous substances in many tissues and organs, and can be used for delivery of drugs to tissues. Due to the typically low cut-off values of microdialysis membranes, microdialysis samples are protein free and readily analyzable without the need for further analytical purification. The free protein unbound drug fraction is, therefore, determined directly. Since the free unbound drug generally responsible for the pharmacodynamic response in the tissues, this feature further adds to the pharmacological relevance (25). With the use of microdialysis, full pharmacokinetic profiles of drug in the target tissue can be obtained with high temporal resolution. This reduces the number of subjects needed for pharmacokinetic investigations. Since the probe is present at a certain location within the targeted tissue, dialysate concentrations will reflect extracellular concentrations in a distinct region (55). This technique is minimally invasive, and only implied a minor reversible trauma by insertion of the guide cannula used for the implantation of the probe (24, 56-59).

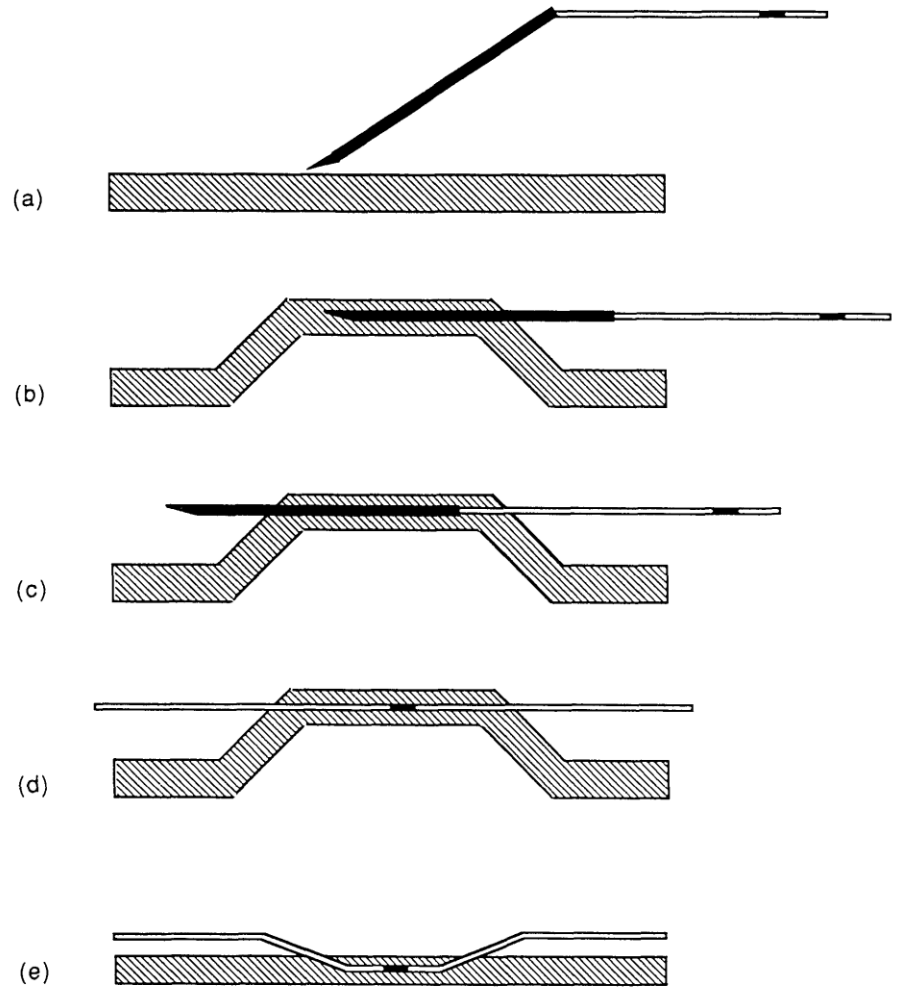

Figure 2.7 Diagram of the dermal microdialysis probe implantation scheme. The shaded region represents the skin while the black section along the probe represents the exposed dialysis membrane (24).

2.3.4 Relative recovery

Since the microdialysis probe is continuously perfused with the perfusate, an equilibrium between the concentration of the analyte in the extracellular fluid and in the dialysate will not be reached. The concentration of analyte in the dialysate ($C_{dialysate}$) is a fraction of the actual analyte concentration in the extracellular fluid surrounding the probe (C_{tissue}). The ratio between these concentrations is defined as relative recovery, which is an important parameter to describe the efficacy of microdialysis:

$$\text{Relative recovery} = \frac{C_{dialysate} - C_{perfusate}}{C_{tissue} - C_{perfusate}} \quad (2.1)$$

In most cases the concentration of analyte of interest will be zero in the perfusate ($C_{perfusate}$) before passage through the skin. Under this condition, equation 2.1 can be simplified:

$$\text{Relative recovery} = \frac{C_{dialysate}}{C_{tissue}} \quad (2.2)$$

Relative recovery is theoretically independent of the concentration since the concentration gradient and partition coefficient is proportional to the amount, which diffuses into the perfusate. This is a prerequisite for application of the microdialysis technique to estimate true unbound extracellular levels of a compound. However, technical problems i.e., adhesion of the compound of interest to the microdialysis probe, can render the concentration independency obsolete, and should hence be examined *in vitro* prior to onset of a microdialysis study (22).

Usually, a high recovery is preferred from an analytical point of view as higher concentrations of the analyte make detection more practical. However, high recovery is typically achieved either by using larger membrane surface area (longer probe) or very slow perfusion flow rates. Whereas, longer dialysis membranes may result in a loss of spatial resolution, using a too slow perfusion rate may cause loss of

temporal resolution. Thus a compromise between perfusion flow rate, length of dialysis membrane and collection time is often made to obtain a satisfactory result in microdialysis analyses (60).

The relative recovery is influenced by numerous parameters. Most important are environmental related parameters (such as temperature), probe related parameters (such as length of the dialysis membrane or pore size), the physicochemical properties of the compound of interest, interactions between the substance of interest and the probe (rejection due to charge, or adhesion to the probe material, the conditions in the tissue, and the experimental setup (such as location of the probe in the tissue, the analytical conditions or the experimental time schedule (22, 23, 61). It is obvious that, due to the complex nature of the tissue, the *in vitro* recovery is not the same as the *in vivo* recovery. Therefore, quantitative microdialysis studies require the determination of the *in vivo* recovery of a substance (59).

2.3.5 Probe calibration

Calibration of microdialysis probes has been a major concern in microdialysis sampling. As the sampling is performed under nonequilibrium conditions the drug concentration in the dialysate is lower than that in the extracellular fluid of the studied tissue. Thus, in order to obtain quantitative data that represents *in vivo* events, relative recovery of the probe needs to be determined. There are many methods have been proposed to determine the relative recovery which yield results of diverse quality.

2.3.5.1 Flow rate method

This method is also known as the extrapolation to zero flow method where *in vivo* recovery is calibrated by varying the perfusion flow rate (62). Based on the observations that probe recovery is dependent upon perfusion flow rate, membrane area and an average mass transfer coefficient, the following equation was proposed to estimate the extracellular concentration using a regression analysis:

$$C_{\text{dialysate}} = C_0 \left(1 - e^{-rA/F}\right) \qquad (2.3)$$

where F is the perfusion flow rate, r is the mass transport coefficient, A is the surface area of the dialysis membrane, C_0 is the concentration of analyte in the external medium, and $C_{dialysate}$ is the concentration of analyte in the dialysate at flow rate F.

As recovery is a function of flow rate, increasing as the flow rate is decreased, the change in the analyte concentration is plotted as a function of perfusion flow rate with the actual sample concentration determined by extrapolating to zero flow. With no flow, the dialysis is in equilibrium with the extracellular fluid, and thus the analyte concentration at zero flow should represent the *in vivo* concentration. The main drawback of this method is a poor temporal resolution as a rather long sampling time (to collect enough dialysate) is required.

2.3.5.2 Method of no-net-flux

Lonnroth et al. (1987) developed a method to estimate recovery *in vivo* based on determining mass transport of analyte across the microdialysis membrane as a function of perfusate concentration while maintaining the extracellular concentration of analyte *in vivo* at steady state (47). This is referred to as zero-net flux or point of no-net flux method, and makes no assumption regarding mass transfer within the tissue.

The method involves consecutive perfusion of the probe with different known concentrations of analyte ($C_{perfusate}$) that are expected to bracket those anticipated in the tissue**.** If the concentration in the perfusate is lower than the concentration in the extracellular fluid (C_{ECF}) the analyte will diffuse through the membrane into the perfusate, resulting in a higher microdialysate concentration ($C_{dialysate}$) than that of the perfusate. If concentrations are vice versa, then $C_{perfusate} > C_{ECF}$, and compared to the perfusate $C_{dialysate}$ will be decreased. A special situation occurs in case of $C_{perfusate}$ being equal to $C_{dialysate}$, thus resulting in a net flow of zero. The different in the amount of the analyte in the perfusion medium before ($C_{perfusate}$) and after dialysis $C_{dialysate}$ is measured as a function of initial perfusate concentration ($C_{perfusate}$), to determine probe recovery *in vivo*. The intercept of the y-axis corresponds to the actual tissue concentration *in vivo* because at this point there is no net loss or gain of the analye from or into the probe (Figure 2.8). The slope of the regression line

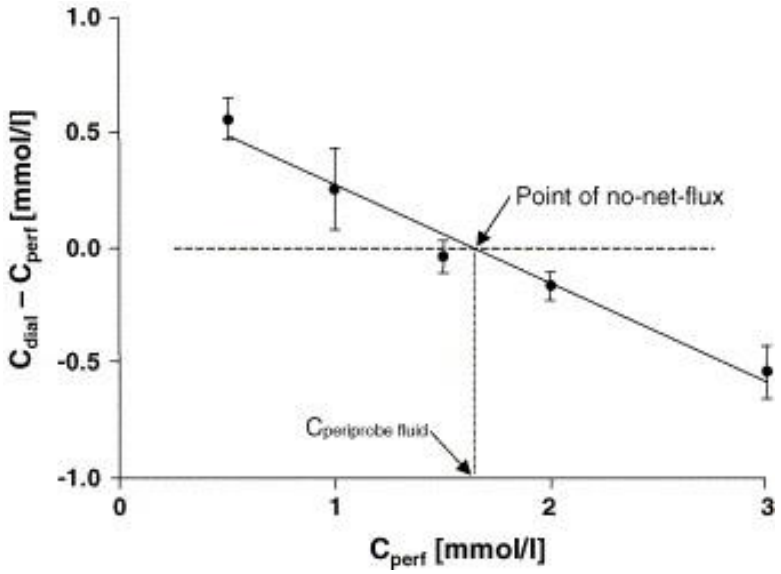

Figure 2.8 Method of no-net-flux. The net loss or gain of a substance in the dialysate in relation to the perfusate ($C_{dialysate}$ - $C_{perfusate}$) is described as a function of the perfusate concentration. The condition of no-net-flux ($C_{perfusate}$ = $C_{dialysate}$) is met at the cross-section of the line with the x-axis, when $C_{perfusate}$ = C_{ECF} (65).

represents the *in vivo* probe recovery of the compound (63, 64). However, this method is rather time-consuming to collect sufficient data at various concentrations of analyte added to the perfusate in order to provide an accurate estimate of the equilibrium state. The requirement of a constant *in vivo* concentration for several hours limits the use of this method in the determination of basal levels of endogenous compounds, as well as in the determination of steady state concentration of drugs during constant infusion experiment (66). Furthermore, the method is inadequate for monitoring C_{ECF} and probe recovery as a function of time. Thus this method has little practicality for pharmacokinetic experiments, as the sustained steady-state concentrations during the calibration procedure would potentially alter pharmacokinetic parameters during later experiments.

2.3.5.3 Dynamic no-net-flux

To estimate *in vivo* recovery as a function of time, Olson and Justice (66) presented an extended or modified version of the no-net-flux method, which is called the dynamic no-net-flux method. Instead of serial perfusion of individual animals with different concentrations via the probe, a group of animals are continuously perfused with one selected perfusion concentration. Different groups receive different concentrations and the results are combined at each time points. Regression of the mean data points of the different groups at a particular point in time will give the actual C_{ECF} with the associated *in vivo* concentration recovery value at that time. Although this is a powerful experimental setup, more experimental animals are required for calibration procedures, which in part reduce the advantage of minimizing the use of living experimental animals by the microdialysis technique.

2.3.5.4 Retrodialysis method

The methods previously described are either time-consuming or require the use of different animals/study subjects and are therefore not applicable for clinical studies. The clinical approach requires a calibration procedure that allows probe calibration *in situ* and will not expose the patient to avoidable stress. A technique that meets these criteria is retrodialysis, also called delivery method (67). It operates by using a perfusate spiked with the analyte in a known concentration. As the diffusion process is assumed to be quantitatively equal in both directions, the

substance loss through the membrane is the same as its *in vivo* recovery. The relative loss can be calculated by the following equation:

$$\text{Relative loss (\%)} = 100 - \left(\frac{C_{\text{dialysate}}}{C_{\text{perfusate}}}\right) \times 100 \qquad (2.4)$$

Essential for obtaining correct results is the following consideration: the diffusion process will only be equal if the tissue does not contain any analyte before the actual calibration process. If retrodialysis is used for measuring drug concentrations the determination of relative recovery should be carried out before the first administration of the drug.

2.3.5.5 Internal standard methods

Another approach to determine *in vivo* recovery for every dialysate sample during the experiment is the use of internal standard, which is added to the perfusate during the course of the experiment. In this method, recovery of the analyte is considered to be equivalent to the delivery of the internal standard. The method is based on the assumption that the internal standard and analyte of interest exhibit a similarity in their physical properties (such as diffusion characteristics and method of analysis) and biological behavior (such as metabolism, protein binding, receptor uptake and release, etc.) (68). This method would be suited to determined changes in recovery if brought about by factors that decrease probe efficiency, such as the formation of air bubbles in the semi-permeable membrane or occlusion of membrane pores by cells or sticky drugs (69-72). However, the *in vivo* diffusion of the internal standard and drug is assumed to be equal. This may not be realistic as demonstrated by Stahle (73) for theophylline and caffeine, for which a difference in the *in vivo* recovery was found using the no-net-flux method. Moreover, the difference found was dependent on the tissue, with the highest deviation in the brain. Also interaction of the internal standard with the drug should be rule out. This stresses prudence to be exercised in the use of internal standards.

2.3.5.6 Endogenous reference substance

There have been approaches to use an endogenous substance as a reference for the determination of relative recovery. They originated from the idea

to fine a calibration procedure that is less time-consuming and would not require steady state conditions. As urea is a freely diffusible molecule and investigators have proposed that the relative recovery ratios of two substances should be similar both *in vitro* and *in vivo* (74). Based on these considerations the use of urea as an endogenous recovery marker has been examined under *in vivo* conditions. The results, however, have been inconsistent. While some authors could not confirm the equivalence between established calibration techniques and the use of urea (75) others conclude it to be suitable as a recovery marker (76). Further studies have to be conducted before this calibration technique can be applied in a standard setting.

2.3.5.7 Other calibration techniques

A calibration method that gives a first rough idea about the relative recovery of an analyte is the assessment of *in vitro* recovery. Both dialysis and retrodialysis can be used for the assessment, as the diffusion process is considered to be equal in both directions (70). However, it is not possible to derive *in vivo* recovery from *in vitro* results. In general the relative recovery *in vitro* is higher than *in vivo* (77) because, as described in more detail earlier, under *in vivo* conditions the relative recovery depends on additional factors such as the volume fraction and the tortuosity of the extracellular space or release, uptake and clearance processes (78). The only possibility to directly use *in vitro* results is its use for semiquantitative experiments as it is possible to detect changes in analyte concentrations in the tissue surrounding the probe (51). Another possibility to calculate true tissue concentrations is by the use of mathematical models. A detailed review concerning this issue has already been published (54).

2.3.6 Invasiveness

The skin traumas induced by the probe implantation procedure, includes an increase in blood flow and erythema, wheal of the skin, and histamine release (56-59, 79-81). As drug recovery is much affected by alterations in blood flow and other parameters which influences elimination, the minor trauma, which is inflicted by implantation of the microdialysis probe, should be diminished before onset of the experiment. Studies have shown that probe design, species and anaesthesia are important factors, which determines the duration of the trauma. Groth et al. studied

skin traumas in both rats (57, 81) and humans (58) following insertion of linear microdialysis probes with a 21-gauge (0.8 mm inner diameter (i.d.)) cannula. The studies showed an initial increase in blood flow, skin thickness and histamine levels in rats, which were normalised approximately 30 min after probe insertion. Skin wheal (30% of normal thickness) was, however, not significantly reduced during the experiments. Similar observations were made in humans; however, the vascular effects required a minimum of 90 min to normalise. Concurrent injection of lidocaine reduced the vascular effects of the trauma. Anderson et al. found a normalisation of the increased blood flow (56) in humans 60 min after insertion of a 0.5-mm outer diameter (o.d.) concentric probe, and a normalisation of the increased histamine levels (80) after 40 min. A decrease in vascular effect if the subjects received local anaesthesia (mepivacaine) prior to the insertion was also demonstrated by these studies. By initial application of EMLA cream (lidocaine, prilocaine), Petersen (59) observed a return to blood flow baseline levels after 40 min, following insertion of a linear probe with a 23-gauge guide cannula (0.6 mm i.d.) in humans. Generally it appears that smaller traumas develop from implantation of probes (or more correctly, guide cannula used for the implantation) with smaller diameter, and the increase in blood flow subsides faster in rats compared to humans and by concurrent application of local anaesthetics.

A histological study of the cell layers of rat skin following implantation of a linear probe (using a 25-gauge cannula) over 32 h, have indicated that no significant oedema or blood accumulation occurs around the probe after implantation (24). However, infiltration of lymphocytes after 6 h and development of scar tissue after 24 h was observed. In another study, Ault et al. demonstrated an increase in transdermal flux *in vitro* of 5-fluorouracil when a concentric probe (via a 21-gauge cannula) was implanted (82) compared to a linear probe (via a 25-gauge cannula) (24), indicating that the tissue disruption was greater by insertion of a concentric probe. The observation was most likely due to the smaller diameter of the guide cannula used for insertion of the linear probe.

2.3.7 Limitations and challenges

The microdialysis technique has been demonstrated to be applicable to multiple tissues and organs for sampling of numerous different substances. The most

substantial challenge for this technique is sampling of lipophilic substances, due to the low recovery (22, 83). The limitations are related to the hydrophilic nature of the perfusate used for most microdialysis experiments, and possibilities of adherence of the drug to the microdialysis equipment. Presently, an isotonic aqueous buffer, e.g., Ringers solution, is often used as perfusate, in which lipophilic compounds have a low solubility and hence low relative recovery. However, this limitation can be solved by the addition of solvents (e.g., polyethylene glycol, cyclodextrins, lipids or proteins) to perfusate (84-87). Furthermore, there are many studies have shown that some moderately or highly lipophilic compounds bind to the microdialysis probe leading to overestimation of recovery and would therefore underestimation of the tissue concentration (22, 88-90). There are several methods have been proposed to eliminate the effect of non-specific binding on the recovery, such as the addition of albumin (91, 92) and cyclodextrin (93) in the perfusion fluid. Another approach is the correction of recovery by using the degree of analyte binding to the probe (89, 90).

Previously, sampling of substances with high molecular volume has also been considered a limitation for the microdialysis technique. However, with the introduction of microdialysis membranes with cut-off values around 3000 kDa (52, 94). The challenge has instead shifted to the analytical methods where sample preparation may be required due to the subsequent introduction of macromolecules and protein in the dialysate.

Another limitation is associated with the small sample volumes (10-50 μl) which requires sensitive analytical methods. Recent introduction of the microbore/capillary liquid chromatography methods and more sensitive detectors, e.g., mass spectrometers, biosensors, etc., to analyse microdialysis samples are methods which has extensively broaden the range of substances that can be sampled and analysed by the microdialysis technique (25).

2.3.8 Application of microdialysis in dermatological research

Topical application of drugs, mostly of Nonsteroidal anti-inflammatory drugs (NSAIDs) aims at achieving high local drug concentrations in connective tissues underneath the application site. Although this concept theoretically provides the advantage of achieving a therapeutic effect without the risks of potentially severe

systemic side effects associated with systemic administration, the validity of this approach has hardly been documented in a convincing fashion (95). To date, information on transdermal drug transport was mostly obtained from *in vitro* studies or *ex vivo* measurements in skin biopsies, since, until recently no method has been available for the direct characterization of time versus concentration profiles of *in vivo* drug release at the site of administration, the human skin, and for providing information on processes in tissue layers deeper than the stratum corneum (22).

Addressing this issue has become possible by employing microdialysis and ultrasound (21) and some recent studies provided, for the first time, *in vivo* pharmacokinetic data on the penetration characteristics of various compounds into subepidermal layers. In particular, formulations and dose regimens could be identified where topical administration of non-steroidal antiinflammatory drugs (NSAIDs) leads to effective (96-98) or ineffective (99) target site concentrations. To date, Microdialysis has provided important information on the degree of direct penetration of ethanol (21), nicotine (100, 101), estradiol (101), salicylic acid (102), acetylsalicylic acid (103), salicylate esters (96, 97), local anaesthetics (104, 105), ibuprofen (106), ketoproten (107), lipophilic analytes (83), organic solvents (108), methylnicotinate (109) and diclofenac following single (99) and multiple-dose administration (98). In addition attempts were made to study the enhancement of topical penetration by iontophoresis (110, 111), tape stripping (102) and by local administration of chemicals like sodium lauryl sulfate (102). Some studies have also addressed the penetration of dermatologically active compounds like antihistamines (112) or NSAIDS (103, 106) following systemic administration.

Microdialysis may also address the issue of transdermal bioequivalence of various new formulations like gels or foams, a fact which was also acknowledged at a recent workshop of the US-FDA (113). The application of microdialysis in topical drug research may thus lead to a critical reappraisal of cost/benefit ratios of topically administered drugs in clinical drug development.

2.3.9 Strategy for determining experimental design

The challenge in designing and implementing a microdialysis experiment is to collect a sample with both sufficient volume and concentration of analyte to

permit separation and detection by the analytical technique employed, while satisfying the experimental design specified by the research question. Therefore, the first step in designing a dialysis experiment is to obtain information regarding the analytical sensitivity and minimum volume of microdialysate necessary to isolate and quantify the analyte(s) of interest. Familiarity with the physicochemical properties of the analyte of interest (e.g., molecular weight, lipophilicity) and its distribution, concentration, and clearance from the tissue of interest will greatly facilitate subsequent experimental design.

The volume of the collected dialysis sample is determined by the flow rate (μl/min) of the fluid that is perfused through the probe (perfusate) and the collection period (min). The concentration of analyte in the collected sample is dependent on the relative recovery of the analyte by the microdialysis probe (also referred to as probe efficiency) and the analyte concentration in tissue. An estimate of tissue concentration can generally be obtained from the literature. Relative recovery is dependent on a number of factors that affect diffusion of the analyte from the tissue to the perfusate, including perfusate composition, membrane composition, active area (or length) of the membrane, flow rate, temperature, and, in many cases, diffusion characteristics of the analyte(s) in tissue. Small changes in these factors may produce significant changes in relative recovery of the analyte(s). In addition, other factors such as the material and surface area of the outflow tubing employed, tubing length, and method of sample storage may diminish the actual concentration of an analyte in the sample. For example, some analytes are particularly prone to adsorption to materials such as the surface of the outflow tubing or the inner surface of the collection vial. Others may have low thermal stability (e.g., monoamines that are oxidized) and degrade quickly following collection. Fortunately, many of these problems have been identified in the literature or can be resolved using *in vitro* assays.

The following strategy is recommended for investigators first setting up microdialysis, or for those establishing procedures for the collection of substances not already described in the literature (114).

2.3.9.1 Determine the sensitivity of the analytical equipment and the minimum sample volume required for the handling of physiologically relevant levels of analyte.

2.3.9.2 Determine if any loss of the analyte might occur following its diffusion into the microdialysis probe. This can be assessed by perfusing a known concentration of the analyte of interest through the inflow/outflow tubing into a collection vial. The concentration of analyte in the collection vial should be the same as the starting concentration. A physiologically relevant concentration(s) of the analyte of interest should be used, and any additional equipment, such as a liquid switch, that the analyte might contact should be tested in this way. If significant loss of analyte is found, then the source of the loss can be identified by systematically testing each component of the dialysis setup. Loss of analyte to the tubing, collection vial, or any other piece of equipment through the which the perfusate flows is often the result of surface adsorption. However, degradation of the sample by enzymes (from a bacterial source) or physicochemical interactions (e.g., oxidation) with metal or tubing may also contribute to sample loss.

2.3.9.3 Evaluate the sample storage method. Two issues should be considered. First, some analytes will degrade rapidly at room temperature (e.g., monoamines). Therefore, optimal collection and storage conditions must be determined. Frequently, reducing the temperature of the sample once it is collected or including a protective agent in the collection vial or perfusate will increase analyte stability. For example, the addition of ascorbic acid to the perfusate or perchloric acid to the collection vial will protect catecholamines from oxidative degradation. Second, because microdialysis samples tend to be very small (in the microliter range), evaporation of the sample may occur over long collection periods. In this case, it would be advisable to seal the collection vials during sample collection.

2.3.9.4 Consider the length and diameter of the inflow/outflow tubing. The combination of long, narrow tubing and higher flow rates may produce considerable back pressure in the probe, resulting in ultrafiltration (“sweating”) of the perfusate. The net impact on sample collection will be a reduction in relative recovery. In addition, it is important to know the “dead volume” for each dialysis setup, as this will affect the calculated time course of a given neurochemical response. For example, if the capacity of the outflow tubing is 10 μl and the perfusion rate is 2.0 μl/min, there will be a 5-min lag in measuring the response to a given manipulation.

2.3.9.5 Choose a probe membrane. The molecular weight cutoff should be considerably larger than the analyte of interest yet small enough to maintain the semipermeable nature of the membrane. Several types of membranes are used commercially, and at least one report has shown that some membrane materials may be better suited for the collection of analytes that prove to be sticky, e.g., hydrophobic neuropeptides (115).

2.3.9.6 Determine the maximum length of active membrane accessible to the tissue under study. The goal here is to increase the surface area through which dialysis occurs. The surface area of a microdialysis probe is increased by increasing the length, and not the diameter, of the probe. In tissue, the maximum length of the probe is generally dictated by the size of the region in which the probe will be implanted. When probe length is not limited by tissue size, the length should be great enough to produce maximum or near maximum recovery. The effect of probe length on analyte recovery can be easily determined *in vitro*.

2.3.9.7 Choose a perfusate that is compatible with the organ system and analyte being measured. As a rule, perfusion fluids should be isoosmotic. Remember that diffusion during microdialysis is bidirectional, so that low-molecular-weight solutes in the perfusate will diffuse out of the probe and into tissue.

2.3.9.8 Determine the longest collection period that will still permit hypothesis testing. Typically, the longer the collection period the slower is the flow rate necessary to yield a sufficient volume for analytical detection.

2.3.9.9 Choose a flow rate that will yield sufficient volume for the desired collection period. Slower flow rates will increase the relative recovery of the analyte of interest.

2.3.9.10 Use an *in vitro* assay to determine if the flow rate and collection period selected yield a sample with a detectable concentration of analyte (use an estimate of actual extracellular tissue concentration).

2.3.9.11 Consider an *in vivo* pilot procedure to assure adequate sample recovery before investing in a larger experiment.

2.4 The other *in vivo* methods for the assessment of topical drug delivery

The percutaneous route may be an attractive solution for systemic delivery of potent drugs with low oral bioavailability, low systemic clearance and narrow therapeutic window, due to the avoidance of hepatic first-pass metabolism and potential of long-term controlled release. However, the greatest potential for the topical administration route is targeted drug delivery to the skin and underlying tissues, where dramatically higher skin-to-plasma ratios can be obtained compared to systemic drug delivery, and thereby maintain therapeutically effective drug concentrations in the organ without the risk of inducing side-effects due to high systemic exposure (116).

Since the skin acts as the main barrier for drug penetration, detailed knowledge on dermal absorption of pharmacological active drug is essential for improvement of the topical drug products. Although the skin is the most accessible organ of the body to superficial investigations, the direct measurements of penetrating substances have long posed major hurdles for detailed mechanistic studies (117). To date, the most prevalent method for estimation of topical drug delivery is still diffusion through excised animal or human skin or artificial membranes in the classical two-compartment Franz-type diffusion cell (118). Although this method has been proven as a robust screening system for estimation of topical drug delivery, the system does not adequately mimic the *in vivo* situation. Among the most critical is the lack of elimination routes in terms of the vascular system and viable metabolizing enzymes, alterations in the stratum corneum structure due to water uptake, and this method actually determines percutaneous permeation instead of cutaneous penetration (25). In order to obtain clinically relevant information about pharmacokinetic profiles in the skin, the in *vivo* techniques must be applied. Many *in vivo* techniques have been proposed to investigate dermal pharmacokinetics of topical formulations. Amongst these, tape stripping and microdialysis are the most widely used for cutaneous drug delivery assessments.

2.4.1 The indirect radiochemical method

This is the classable method, introduced by Feldmann and Maibach (119-121) by which most human *in vivo* percutaneous penetration experiments have been perfomed. Typically, a known quantity of a ^{14}C labelled substance is applied topically in a volatile solvent vehicle and penetration is evaluated from the excretion of the ^{14}C radiolabel over the next 5-10 days. A correction for incomplete elimination is made by performing an identical protocol after intravenous administration of the same ^{14}C labelled material. This method has some limitations, since it does not discriminate between the parent drug and its metabolites, and the time resolution is bound to be coarse due to a combination of analytical sensitivity and practical factor (urinary and faecal collections). Furthermore, the elimination process following topical and intravenous drug administration must be assumed to be identical. It has been suggested to add tape stripping of the application site to the protocol in order to obtain an improved mass balance, i.e. account for all radiolabelled drug (122). This method is not applicable in humans for compounds, which are toxic or either poorly absorbed or excreted, and it is relatively time-consuming, expensive and unsuitable for routine studies (123).

2.4.2 Skin stripping

Skin stripping of the skin has been a frequent technique to assess topical drug delivery. This method involves sequentially removing microscopic layers (typically 0.5-1.0 μm) of stratum corneum by consecutive adhesion of adhesive tape pieces to the skin surface and stripping of the top cell layers (Figure 2.9). The procedure is relatively painless and non-invasive, given that only dead cells (corneocytes) embedded in their lipid matrix are removed. However, the technique only determines the concentration of drug in the stratum corneum which is usually not the therapeutic target of topical drug delivery and can only determine a single concentration-time point per administration site (25, 50). The limitation of this method is that the quantify of stratum corneum cells removed by tape stripping is not linearly proportional with the number of tape strips, and several factors can influence the quantity of stratum corneum removed by each piece of tape, i.e. the manner of tape stripping, the hydration of the skin, anatomical site and interindividual variation (124).

2.4.3 The suction blister technique

The development of a defined suction blister technique that separates the skin strata subepidermally was first described by Kiistala et al. (125-127) and uses a dome-shaped cap with several small holes of 4-6 mm diameter. In a typical blister induction a consistent vacuum of about 200 mm Hg (2.66 Pa) is employed for a 2-3 h period, after which 50-150 μl of suction blister fluid and small stratum corneum-epidermal sheets can be harvested from the induced vesicles. The blister fluid is approximately of the same composition as the interstitial fluid. Blisters cannot be raised uniformly on diseased skin and, hence, standardization of methodology has not been possible. Multiple small blisters are formed for pharmacokinetic studies, drug is administered systemically or topically (either before, during or after blister raising) and the blister fluid and vesicle roof are harvested at various time intervals for subsequent analysis. Each blister results in a small erosion, which heals over 5-10 days, often with post-inflammatory hyperpigmentation during some months, so the number of blisters that can be raised in subject is limited. In diseased skin, particularly if epidermal changes are pronounced, blisters can often not be raised and the technique has not been standardized for use in diseased skin.

2.4.4 Skin biopsy

The most invasive method to access skin compartments is the direct excision of skin tissue. In contrast to the other methods, the punch and shave biopsies allow a direct ingression into the compartment of interest. After removal of the stratum corneum from skin (optional) with an appropriate technique (tape stripping or adhesive) the punch biopsy will contain parts of the subcutaneous tissues, dermis, and epidermis and the shave biopsy will mainly contain epidermis and some dermis. Parts of the stratum corneum may remain on the epidermis depending on the method used for stratum corneum removal. Subcutaneous tissue can mechanically be separated from the dermis and the latter can be separated from the epidermis by heating techniques (128). Human skin samples larger than 100 mg are difficult to obtain and the usual amount that can be harvested is less than 50 mg. Despite skilled investigators, sophisticated sampling techniques, and instrumentation, the information gained from these tissue samples is probably only an estimate of the chemical

distribution within the skin. Accurate and specific information on drug localization within a particular skin compartment following both routes of administration is not obtained by these methods (129).

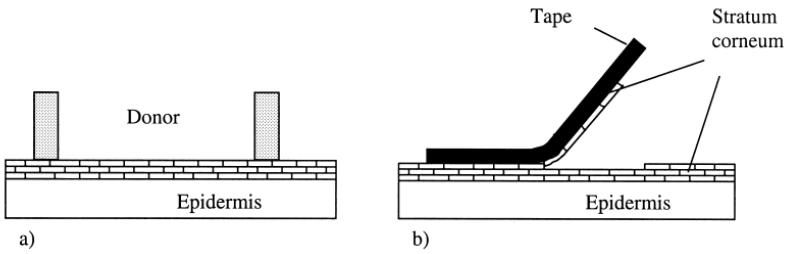

Figure 2.9 Schematic representation of the method of tape stripping for determination of the amount of drug penetrated into the skin. After application of the formulation at the donor site (a) and removal of the formulation, the stratum corneum is progressively removed by tape stripping (b).

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

The reagents and substances used in the present study were listed as the following:

3.1.1 (+)-Terpinen-4-ol (≥ 98.5%) (Sigma-Aldrich (Buchs, Switzerland)

3.1.2 Methyl salicylate (≥ 99.5%) (Sigma-Aldrich (Buchs, Switzerland)

3.1.3 Water HPLC grade (Fisher Scientific, Fairlawn, NJ, USA)

3.1.4 Hexane Optima® H303-1 (Fisher Scientific, Fairlawn, NJ, USA)

3.1.5 Methanol HPLC grade (Fisher Scientific, Fairlawn, NJ, USA)

3.1.6 Plai oil (Kovic Kate International Co., Ltd Bangkok, Thailand)

3.1.7 Heparin sodium injection, USP (Hospira, Inc., Lake forest, IL, USA)

3.1.8 Sodium chloride (Fisher Scientific, Fairlawn, NJ, USA)

3.1.9 Isoflurane USP (Webster Veterinary Supply, Charlotte, NC, USA)

3.1.10 Sterile water for injection (Thai Nakorn Patana Co., Ltd Nonthaburi, Thailand)

3.2 Equipment

3.2.1 Balance AB104 (Mettler, Toledo, Hightstown, NJ, USA)

3.2.2 Vortex (Kraft Apparatus model PV-5, Fisher, Pittsburgh, PA, USA)

3.2.3 Pipette tips 1-200 µl (Fisher Scientific, Fairlawn, NJ, USA)

3.2.4 Pipette tips 200-1000 µl (Fisher Scientific, Fairlawn, NJ, USA)

3.2.5 Micropipettes (Eppendorf AG, Hamburg, Germany)

3.2.6 Gas chromatography Mass spectrometry Shimadzu GCMS-QP-2010
gas chromatograph (Shimadzu, Kyoto, Japan).

3.2.7 BPX5 capillary column (SGE Analytical Science, Ringwood, Australia)

3.2.8 Gas chromatography Mass spectrometry Trace GC 2000 (Thermo Finnigan, San Jose, CA, USA)

3.2.9 AT^{TM}-WAX capillary column (Alltech, State College, PA, USA)

3.2.10 Centrifuge model 235V (Fisher Scientific, Fairlawn, NJ, USA)

3.2.11 Heating pad for animal (Peco Services, Cumbria, CA, USA)

3.2.12 BD Microtainer® tubes with EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ, USA)

3.2.13 Autosampler vial (SUN Sri, Rockwood, TN, USA)

3.2.14 Conical glass insert (Fisher Scientific, Fairlawn, NJ, USA)

3.2.15 Glass vial 4 ml (Fisher Scientific, Fairlawn, NJ, USA)

3.2.16 Graduated Syringes 5 ml (Popper & Sons, Inc., New Hyde Park, NY, USA)

3.2.17 Heated Stir Plate (Fisher Scientific, Fairlawn, NJ, USA)

3.2.18 Animal hair clipper (Andis Company, Sturtevant, WI, USA)

3.2.19 Isotec-4 isoflurane vaporizer (SurgiVet/ Smiths Medical, Waukesha, WI, USA)

3.2.20 Sonicator (Fisher Scientific, Fairlawn, NJ, USA)

3.2.21 Microcon® Ultracel YM-30 ultrafiltration units (Millipore Corporation, Billerica, MA, USA)

3.2.22 Paraffin film (Eppendorf AG, Hamburg, Germany)

3.2.23 Microdialysis probe CMA 30 (CMA microdialysis AB, Stockholm, Sweden)

3.2.24 Microdialysis pump model 55-4150 (Harvard Apparatus, Holliston, MA, USA)

3.3 Biological samples

3.3.1 Human plasma (Blood Bank, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Bangkok, Thailand.)

3.3.2 Human blood (Blood Bank, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Bangkok, Thailand.)

3.3.3 Drug-free Wistar rat plasma with EDTA (Innovative Research, Inc., Novi, MI, USA)

3.4 Animals

3.4.1 Wistar rats weighing between 300-350 g (Harlan Laboratories, Tampa, FL, USA).

3.5 Methods

3.5.1 Validation of a bioanalytical method for determination of terpinen-4-ol in dermal microdialysate.

A simple, sensitive and reproducible GC-MS method was developed and validated for the quantitative analysis of terpinen-4-ol in dermal microdialysate. The method validation was carried out according to the FDA guidance on bioanalytical method validation (130).

3.5.1.1 Preparation of standard stock solutions

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

3.5.1.2 Preparation of calibration and quality control samples

Microdialysate calibration samples were prepared by diluting the stock solution of terpinen-4-ol with normal saline to obtain final concentrations of 0.005, 0.01, 0.02, 0.05, 0.125, 0.25, 0.50, 0.75 and 1.00 µg/ml.

Microdialysate QC samples were prepared in the same manner as the calibration standards at three concentration levels of terpinen-4-ol (0.01, 0.50 and 0.75 µg/ml).

3.5.1.3 Sample preparation

A 30 µl aliquot of microdialysis sample was added to 4 ml glass vials followed by the addition of 20 µl of IS solution (0.5 µg/ml). The samples are extracted by the addition of 200 µl of hexane followed by vortexing for 30 sec. The upper organic layer (2 µl) was injected into the GC-MS system.

3.5.1.4 GC-MS condition

GC-MS analysis was performed using a Thermo Finnigan Trace GC 2000 gas chromatograph/quadrupole ion trap mass spectrometer (San Jose, CA, USA) equipped with an AT^{TM}-WAX capillary column (0.25 mm i.d. x 30 m, 0.25 µm film thickness; Alltech, State College, PA, USA). 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.

3.5.1.5 Specificity

The specificity was evaluated by analyzing blank microdialysis 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.

3.5.1.6 Sensitivity

Sensitivity was achieved by determining the lower limit of quantification (LLOQ) of terpinen-4-ol which 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.

3.5.1.7 Linearity

Linearity was assessed by plotting terpinen-4-ol: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.

3.5.1.8 Precision and accuracy

Precision and accuracy were evaluated by injecting QC samples (0.01, 0.50 and 0.75 µg/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.

$$\% \text{ R.S.D.} = \frac{(\text{S.D.} \times 100)}{\bar{X}} \quad (3.1)$$

$$\% \text{ Bias} = \frac{(\text{theoretical concentration} - \text{mean observed concentration})}{\text{theoretical concentration}} \times 100 \quad (3.2)$$

where S.D. is standard deviation and $\bar{X}$ is mean value of analyzed drug concentration in spiked sample.

3.5.1.9 carryover

The potential for carryover was investigated by injecting blank solvent immediately after analysis of the highest concentration point of the calibration curve.

3.5.1.10 Extraction efficiency

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 solution containing the same concentration in hexane (*n*=3).

3.5.1.11 Stability

The stability of terpinen-4-ol in microdialysate at concentrations of 0.01, 0.50 and 0.75 µg/ml was evaluated by determining the short-term stability (12 h at room temperature, 25 ± 5°C), freeze and thaw stability (three freeze-thaw cycles) and long-term stability (1 month at -20°C). The post-preparative stability of the analyte (36 h in the autosampler of the GC-MS) was also studied. The concentrations were compared to the corresponding results before storage.

a. Short-term temperature stability

Short-term temperature stability at room temperature was evaluated by spiking plasma with terpinen-4-ol at the three QC concentration levels (0.01, 0.50 and 0.75 µg/ml). The samples were kept at room temperature for 12 h. After that, the sample were spiked with IS, extracted and analyzed.

b. Freeze and thaw stability

Freeze and thaw stability was evaluated by spiking microdialysates with terpinen-4-ol at the three QC concentration levels (0.01, 0.50 and 0.75 µg/ml). The samples were frozen for 24 h at -20°C then allowed to thaw unassisted at room temperature. This process was repeated two more times, and after the third cycle, samples were spiked with IS, extracted and analyzed. The samples were analyzed together with a freshly prepared calibration curve.

c. Frozen stability

Long-term stability of terpinen-4-ol in plasma was evaluated by spiking microdialysates with terpinen-4-ol at the three QC concentration levels (0.01, 0.50 and 0.75 µg/ml). The samples were kept at -20°C for 1 month. After that, the sample were spiked with IS, extracted and analyzed. The amount of terpinen-4-ol in the microdialysis samples after three freeze–thaw cycles was determined using a freshly prepared calibration curve.

d. The post-preparative stability

The post-preparative stability was also studied. QC samples from the first day were kept on the autosampler of the GC-MS at room temperature (25°C) for 36 h and injected again. Terpinen-4-ol was quantified using the calibration curve constructed on the first day of analysis.

3.5.2 Validation of a bioanalytical method for determination of terpinen-4-ol in rat plasma

A simple, sensitive and reproducible GC-MS method was developed and validated for the quantitative analysis of terpinen-4-ol in rat plasma. The method validation was carried out according to the FDA guidance on bioanalytical method validation (130).

3.5.2.1 Preparation of stock solutions

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

3.5.2.2 Preparation of calibration and quality control samples

Matrix-based calibration standards in rat plasma were prepared by spiking equal volumes (20 µl) of terpinen-4-ol working solutions into blank rat plasma (100 µl) to yield terpinen-4-ol concentrations of 0.005, 0.01, 0.50, 0.10, 0.25, 0.50, 0.75 and 1.00 µg/ml.

Rat plasma QC samples were prepared in the same manner as the calibration standards at three concentration levels of terpinen-4-ol (0.01, 0.5 and 0.75 µg/ml).

3.5.2.3 Sample preparation

A 100 µl aliquot of rat plasma sample was transferred to 4 ml glass vials followed by the addition of a 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 sec and centrifuged at 855 × g for 15 min. The upper organic layer (1 µl) was injected into the GC-MS system for analysis.

3.5.2.4 GC-MS condition

GC-MS analysis was performed using a Thermo Finnigan Trace GC 2000 gas chromatograph/quadrupole ion trap mass spectrometer (San Jose, CA, USA) equipped with an AT^{TM}-WAX capillary column (0.25 mm i.d. x 30 m, 0.25 µm film thickness; Alltech, State College, PA, USA). 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 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.

3.5.2.5 Specificity

The specificity was evaluated by analyzing blank rat 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.

3.5.2.6 Sensitivity

Sensitivity was achieved by determining the LLOQ of terpinen-4-ol which 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.

3.5.2.7 Linearity

Linearity was assessed by plotting terpinen-4-ol:IS peak area ratios versus concentrations of calibration standards. All calibration curves were required to have a correlation value of at least 0.995.

3.5.2.8 Precision and accuracy

Precision and accuracy were evaluated by injecting QC samples (0.01, 0.50 and 0.75 µg/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.

3.5.2.9 Carryover

The potential for carryover was investigated by injecting blank hexane immediately after analysis of the highest concentration point of the calibration curve.

3.5.2.10 Extraction efficiency

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 solution containing the same concentration in hexane (n=3).

3.5.2.11 Stability

The stability of terpinen-4-ol in rat plasma at concentrations of 0.01, 0.50 and 0.75 µg/ml was evaluated by determining the short-term stability (12 h at room temperature, 25 ± 5°C), freeze and thaw stability (three freeze-thaw cycles) and long-term stability (1 month at -20°C). The post-preparative stability of the analyte (36 h in the autosampler of the GC-MS) was also studied. The concentrations were compared to the corresponding results before storage.

a. Short-term temperature stability

Short-term temperature stability at room temperature was evaluated by spiking rat plasma with terpinen-4-ol at the three QC concentration levels (0.01, 0.50 and 0.75 µg/ml). The samples were kept at room temperature for 12 h. After that, the sample were spiked with IS, extracted and analyzed.

b. Freeze and thaw stability

Freeze and thaw stability was evaluated by spiking rat plasma with terpinen-4-ol at the three QC concentration levels (0.01, 0.50 and 0.75 µg/ml). The samples were frozen for 24 h at -20°C then allowed to thaw unassisted at room temperature. This process was repeated two more times, and after the third cycle,

samples were spiked with IS, extracted and analyzed. The samples were analyzed together with a freshly prepared calibration curve.

c. Frozen stability

Long-term stability of terpinen-4-ol in rat plasma was evaluated by spiking plasma with terpinen-4-ol at the three QC concentration levels (0.01, 0.50 and 0.75 µg/ml). The samples were kept at -20°C for 1 month. After that, the sample were spiked with IS, extracted and analyzed. The amount of terpinen-4-ol in the rat plasma samples after three freeze–thaw cycles was determined using a freshly prepared calibration curve.

d. The post-preparative stability

The post-preparative stability was also studied. QC samples from the first day were kept on the autosampler of the GC-MS at room temperature (25°C) for 36 h and injected again. Terpinen-4-ol was quantified using the calibration curve constructed on the first day of analysis.

3.5.3 Validation of the analytical method for determination of terpinen-4-ol in human plasma

A simple, sensitive and reproducible GC-MS method was developed and validated for the quantitative analysis of terpinen-4-ol in human plasma. The method validation was carried out according to the FDA guidance on bioanalytical method validation (130).

3.5.3.1 Preparation of stock solutions

Stock solutions of terpinen-4-ol and methyl salicylate which was used as internal standard were prepared in hexane at concentrations of 0.10 and 0.72 mg/ml, respectively and stored at 4°C. The stock solutions were further diluted in hexane to give appropriate working solutions used for preparing the calibration standards and QC samples.

3.5.3.2 Preparation of calibration and quality control samples

Matrix-based calibration standards and QC samples were prepared by spiking equal volumes (20 µl) of terpinen-4-ol working solutions into drug-free human plasma (500 µl). The concentration ranges of terpinen-4-ol used in the assay were 0.03, 0.09, 0.20, 0.40, 0.80 and 1.20 µg/ml. QC samples were prepared

by spiking blank samples to obtain concentrations of 0.09, 0.60 and 1.00 µg/ml. Both the calibration standards and QC samples of plasma were spiked with the IS working solution (20 µl) to yield concentrations of 0.40 µg/ml.

3.5.3.3 Sample preparation

Accurately measured 500 µl aliquots of human plasma samples were each added to 4 ml glass vials followed by the addition of a 20-µl of the IS working solution (equivalent to 0.2 µg IS). Then, 2 ml of hexane was added into the vial. The mixture was vortexed for 30 sec and centrifuged at 855 × g for 15 min. The upper organic layer (1 µl) was injected into the GC-MS system for analysis.

3.5.3.4 GC-MS analysis

Analysis was carried out on a GC system coupled with quadrupole mass spectrometer (GCMS-QP2010, Shimadzu, Kyoto, Japan). The compounds were separated on BPX5 capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm, SGE Analytical Science, Ringwood, Australia) with helium as the carrier gas (1.0 ml/min). Sample injection (1 µl) was performed in the splitless mode. The initial oven temperature was set at 60°C, and increased by 10°C/min to 115°C, which was maintained for 5 min, then to 220°C at the rate of 80°C/min and held for 4 min (total run time 16 min). The temperature of the injection port, interface and ion source were set at 230, 230 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. A full-scan MS method (*m/z* 30-400) was used to select three appropriate ions for identification in the SIM method (Figure 2).The ions monitored 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 71/120 was used for quantification, whereas the other ions were used as qualifiers.

3.5.3.5 Selectivity

Pooled-blank plasma samples were analyzed according to the procedure previously described in order to evaluate method specificity. The absence of interfering compounds, characterized by ions at *m/z* values equal to those of the investigated analytes and eluting at the same analytes retention time, was verified.

In order to predict the variability of matrix effects in samples from individual subjects, internal standard (IS)-normalized matrix factor (MF) were determined for 7 individual lots of the matrix at concentration of 0.9 and 1 μg/ml. Each sample was analyzed in triplicate. The variability in matrix factors, as measured by the coefficient of variation should be less than 15 %. IS-normalized MF can be defined as a ratio of the peak response (analyte/IS) in the presence of matrix ions to the analyte peak response in the absence of matrix ions (Eq. 3.1).

$$\text{Matrix Factor} = \frac{\text{Peak response (analyte/IS) in presence of matrix ions}}{\text{Peak response (analyte/IS) in absence of matrix ions}} \qquad (3.3)$$

3.5.3.6 Sensitivity

Sensitivity was assessed by determining the LLOQ of terpinen-4-ol which was established as the lowest concentration of terpinen-4-ol used in the calibration curve with accuracy and precision of 100 ± 20 % and signal to noise ratio of > 5. Bias and relative standard deviation were used as measures of accuracy and precision respectively.

3.5.3.7 Linearity

Calibration curves were constructed by linear regression of the peak area ratio of terpinen-4-ol to IS (Y-axis) against the nominal standard T4 concentration (X-axis). Concentrations of QC samples were calculated using the equation of the calibration curve. The calibration curve with the coefficient of determination of at least 0.995 was acceptable. Back-calculated calibration concentrations were also determined, as well as the accuracy and precision of the calibration standards, i.e. the percentage deviation of the so-determined concentrations from nominal ones (%R.E.) and the relative standard deviation, respectively.

3.5.3.8 Precision and accuracy

Precision of the assay procedure was assessed from the percent relative standard deviation values of analyzed drug concentration in spiked plasma samples. Three difference concentrations of terpinen-4-ol in plasma were prepared and used to determine within (intraday) and between-run (inter-day) precision. For within-run precision, five replicates of each concentration were assayed within on day

whereas between-run precision was performed by assaying the samples of each concentration for three consecutive days. The accuracy of the analytical method was calculated as the percent relative error in the calculated mean concentration relative to the nominal terpinen-4-ol concentration (%R.E.). For the assay to be considered acceptable, the precision and accuracy determined at each QC concentration level was required to be within 15 %.

3.5.3.9 Carryover

The possibility of carry-over was evaluated by analysing blank hexane at the highest calibration level.

3.5.3.10 Extraction efficiency

The extraction recovery of the assay was evaluated for terpinen-4-ol at low, medium and high concentrations (n=3). The recovery at each concentration was evaluated by comparing the peak area ratio of terpinen-4-ol to IS in plasma after extraction to the unextracted recovery standards containing the same concentration of terpinen-4-ol and IS in hexane. The percentage of recovery was calculated as the ratio of the peak area ratio of terpinen-4-ol to IS in plasma after extraction to the peak area ratio of terpinen-4-ol to IS in unextracted standard. A good recovery should be more than 90 %.

3.5.3.11 Stability

The stability of terpinen-4-ol in human plasma at concentrations of 0.90 and 1.00 μg/ml was evaluated by determining the short-term stability (12 h at room temperature, 25 ± 5°C), freeze and thaw stability (three freeze-thaw cycles) and long-term stability (1 month at -20°C). The post-preparative stability of the analyte (36 h in the autosampler of the GC-MS) was also studied. The concentrations were compared to the corresponding results before storage.

a. Short-term temperature stability

Short-term temperature stability at room temperatute was evaluated by spiking plasma with terpinen-4-ol at two different concentrations (0.90 and 1.00 μg/ml). The samples were kept at room temperature for 12 h. After that, the sample were spiked with IS, extracted and analyzed.

b. Freeze and thaw stability

Freeze and thaw stability was evaluated by spiking plasma with terpinen-4-ol at two different concentrations (0.90 and 1.00 μg/ml). The samples were frozen for 24 h at -20°C then allowed to thaw unassisted at room temperature. This process was repeated two more times, and after the third cycle, samples were spiked with IS, extracted and analyzed. The samples were analyzed together with a freshly prepared calibration curve.

c. Frozen stability

Long-term stability of terpinen-4-ol in plasma was evaluated by spiking plasma with terpinen-4-ol at two different concentrations (0.90 and 1.00 μg/ml). The samples were kept at -20°C for 1 month. Then, the sample were spiked with IS, extracted and analyzed. The amount of terpinen-4-ol in the plasma samples after three freeze–thaw cycles was determined using a newly prepared calibration curve.

d. The post-preparative stability

The post-preparative stability was also studied. QC samples from the first day were kept on the autosampler of the GC-MS at room temperature for 36 h and injected again. Terpinen-4-ol was quantified using the calibration curve constructed on the first day of analysis.

3.5.4 Validation of the analytical method for determination of terpinen-4-ol in human whole blood

A simple, sensitive and reproducible GC-MS method was developed and validated for the quantitative analysis of terpinen-4-ol in human whole blood. The method validation was carried out according to the FDA guidance on bioanalytical method validation (130).

3.5.4.1 Preparation of stock solution

Stock solutions of terpinen-4-ol and methyl salicylate which was used as internal standard were prepared in hexane at concentrations of 0.10 and 0.72 mg/ml, respectively and stored at 4°C. The stock solutions were further diluted in hexane to give appropriate working solutions used for preparing the QC samples.

3.5.4.2 Preparation of calibration and quality control samples

Calibrators and QC samples were prepared by spiking equal volumes (20 µl) of the above working solutions of terpinen-4-ol into 250 µl of drug-free whole blood to yield the final blood concentrations of 0.045, 0.13, 0.20, 0.40, 0.60, 0.80, 1.00 and 1.20 µg/ml. For validation, QC samples were prepared by spiking blank whole blood to obtain three levels of the final concentration at 0.13, 0.60 and 1.00 µg/ml. Both the calibration standards and QC samples of plasma were spiked with the IS working solution (20 µl) to yield concentrations of 0.32 µg/ml.

3.5.4.3 Sample preparation

Accurately measured 250 µl aliquots of human blood samples were each added to 4 ml glass vials followed by the addition of a 20-µl of the IS working solution (equivalent to 0.08 µg IS). Then, 2 ml of hexane was added into the vial. The mixture was vortexed for 30 sec and centrifuged at 855 × g for 15 min. The upper organic layer (1 µl) was injected into the GC-MS system for analysis.

3.5.4.4 GC-MS analysis

Analysis was carried out on a GC system coupled with quadrupole mass spectrometer (GCMS-QP2010, Shimadzu, Kyoto, Japan). The compounds were separated on BPX5 capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm, SGE Analytical Science, Ringwood, Australia) with helium as the carrier gas (1.0 ml/min). Sample injection (1 µl) was performed in the splitless mode. The initial oven temperature was set at 60°C, and increased by 10°C/min to 115°C, which was maintained for 5 min, then to 220°C at the rate of 80°C/min and held for 4 min (total run time 16 min). The temperature of the injection port, interface and ion source were set at 230, 230 and 200°C, respectively. Electron impact ionization was used with an ionization energy of 70 eV. The MS was operated in the SIM mode in order to maximize the sensitivity and robustness of the method. A full-scan MS method (*m/z* 30-400) was used to select three appropriate ions for identification in the SIM method.The ions monitored 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 71/120 was used for quantification, whereas the other ions were used as qualifiers.

3.5.4.5 Selectivity

Whole blood samples from six healthy volunteers were analyzed according to the procedure previously described in order to evaluate method specificity. The absence of interfering compounds, characterized by ions at *m/z* values equal to those of the investigated analytes and eluting at the same analytes retention time, was verified.

In order to predict the variability of matrix effects in samples from individual subjects, IS-normalized MF were determined for 7 individual lots of the matrix at concentration of 0.90 and 1.00 μg/ml. Each sample was analyzed in triplicate. The variability in matrix factors, as measured by the coefficient of variation should be less than 15 %. IS-normalized MF can be defined as a ratio of the peak response (analyte/IS) in the presence of matrix ions to the analyte peak response in the absence of matrix ions.

3.5.4.6 Sensitivity

Sensitivity was assessed by determining the LLOQ of terpinen-4-ol which was established as the lowest concentration of terpinen-4-ol used in the calibration curve with accuracy and precision of 100 ± 20 % and signal to noise ratio of > 5. Bias and relative standard deviation were used as measures of accuracy and precision respectively.

3.5.4.7 Linearity

Calibration curves were constructed by linear regression of the peak area ratio of terpinen-4-ol to IS (Y-axis) against the nominal standard terpinen-4-ol concentration (X-axis). Concentrations of QC samples were calculated using the equation of the calibration curve. The calibration curve with the coefficient of determination of at least 0.995 was acceptable. Back-calculated calibration concentrations were also determined, as well as the accuracy and precision of the calibration standards, i.e. the percentage deviation of the so-determined concentrations from nominal ones (%R.E.) and the relative standard deviation, respectively.

3.5.4.8 Precision and accuracy

Precision of the assay procedure was assessed from the percent relative standard deviation values of analyzed drug concentration in spiked whole blood samples. Three difference concentrations of terpinen-4-ol in whole blood were

prepared and used to determine within (intra-day) and between-run (inter-day) precision. For within-run precision, five replicates of each concentration were assayed within on day whereas between-run precision was performed by assaying the samples of each concentration for five consecutive days. The accuracy of the analytical method was calculated as the percent relative error in the calculated mean concentration relative to the nominal terpinen-4-ol concentration. For the assay to be considered acceptable, the precision and accuracy determined at each QC concentration level was required to be within 15 %.

3.5.4.9 Carryover

The possibility of carry-over was evaluated by analysing blank hexane at the highest calibration level.

3.5.4.10 Extraction efficiency

The extraction recovery of the assay was evaluated for terpinen-4-ol at low, medium and high concentrations (n=3). The recovery at each concentration was evaluated by comparing the peak area ratio of terpinen-4-ol to IS in whole blood after extraction to the unextracted recovery standards containing the same concentration of terpinen-4-ol and IS in hexane. The percentage of recovery was calculated as the ratio of the peak area ratio of terpinen-4-ol to IS in whole blood after extraction to the peak area ratio of terpinen-4-ol to IS in unextracted recovery standard. A good recovery should be more than 90 %.

3.5.4.11 Stability

The stability of terpinen-4-ol in whole blood at concentrations of 0.13 and 1.00 μg/ml was evaluated by determining the short-term stability (12 h at room temperature, 25 ± 5°C) and long-term stability (1 week at -20°C). The post-preparative stability of the analyte (36 h in the autosampler of the GC-MS) was also studied. The concentrations were compared to the corresponding results before storage.

a. Short-term temperature stability

Short-term temperature stability was evaluated by spiking whole blood with terpinen-4-ol at two different concentrations (0.13 and 1.00 μg/ml). The samples were kept at room temperature for 12 h. After that, the sample were spiked with IS, extracted and analyzed.

b. Frozen matrix stability

Frozen matrix stability was evaluated by spiking whole blood with terpinen-4-ol at two different concentrations (0.13 and 1.00 μg/ml). The samples were kept at -20°C for 1 week. After that, the sample were spiked with IS, extracted and analyzed. The amount of terpinen-4-ol in the whole blood samples after three freeze–thaw cycles was determined using a freshly prepared calibration curve.

c. The post-preparative stability

The post-preparative stability was also studied. QC samples from the first day were kept on the autosampler of the GC-MS at room temperature for 36 h and injected again. Terpinen-4-ol was quantified using the calibration curve constructed on the day of analysis.

3.5.5 Partial validation of an analytical method for determination of terpinen-4-ol in plai oil

A simple, reproducible analytical method was developed and validated for the determination of terpinen-4-ol in plai oil. The method validation was done according to ICH guidelines Q2B Guideline on validation of analytical procedures-methodology, 1996.

3.5.5.1 Preparation of stock solutions and calibration samples

The standard stock solutions of terpinen-4-ol and methyl salicylate were prepared in hexane at 1 μg/ml and stored at 4°C. The stock solutions were then diluted with hexane to obtain working solutions of terpinen-4-ol and IS at 625 and 1250 ng/ml, respectively. From the working solution, suitably diluted standard solutions were prepared to contain 25, 62.5, 125, 187.5 and 250 ng/ml of terpinen-4-ol, containing 62.5 ng/ml of IS.

3.5.5.2 Sample preparation

A 10 mg of plai oil was weighted and transferred to a 10 ml volumetric flask and diluted with hexane (theoretical concentration of 1 mg/ml). The solution was further diluted with hexane and mixed with 50 μl of internal standard solution to give a final concentration of 200 ng/ml of the oil and 62.5 ng/ml of IS.

3.5.5.3 GC-MS condition

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

3.5.5.4 Linearity

The linearity of the method was determined using terpinen-4-ol as a reference substance at five concentration levels. Calibration curves were constructed by linear regression of the peak area ratio of terpinen-4-ol to methyl salicylate (Y-axis) and the nominal standard terpinen-4-ol concentration (X-axis).

3.5.5.5 Precision

Method's precision was studied by repeatability and intermediary precision. The repeatability of the method was evaluated by six replicate injections, during the same day under the same experimental conditions. The intermediary precision was demonstrated by assaying six samples of the oil solution, at same concentrations in three consecutive days. The terpinen-4-ol content of the oil was calculated using the equation of the calibration curve.

3.5.5.6 Accuracy

Accuracy was determined by recovery, in which known amounts of terpinen-4-ol were added to the oil solutions, the recovery was evaluated at

three different concentrations. Aliquots of 25, 125 and 250 µl of the terpinen-4-ol standard solution (625 ng/ml) were added to the oil solutions (corresponding to 15.6, 78.1 and 156.3 ng/ml added, respectively). The experiment was done in triplicate.

3.5.6 Determination of aqueous solubility of terpinen-4-ol in its pure form and plai oil

3.5.6.1 Experimental procedure

An accurately weighed amount of pure terpinen-4-ol and plai oil (21.29 % w/w of terpinen-4-ol) was separately added to 2 ml distilled water in the corresponded to the amount of terpinen-4-ol of 0.18, 0.36, 0.71 and 1.06 g. The mixtures were placed in an incubator shaker at 25 ± 1°C and 200 rpm for 72 h. After centrifugation (855 × g for 15 min), the oil layer (upper) was carefully removed by a micropipette. The experiment was done in duplicate.

3.5.6.2 Sample analysis

An aliquot of aqueous was extracted 2 ml of hexane by vortexing for 30 sec and analysed by GC-MS.

3.5.6.3 Statistical analysis

All data were expressed as mean value ± SD. The significances ($p < 0.05$) among the corresponding mean values were determined by using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison test. SPSS 14.0 software for windows was used for statistical analysis.

3.5.7 Determination of plasma protein binding rate of terpinen-4-ol

3.5.7.1 Preparation of stock solution

The stock solution of terpinen-4-ol was prepared in normal saline at concentration of 1 µg/ml. The solution was diluted with normal saline to obtain appropriate working solutions for preparing rat and human plasma samples containing various concentrations of terpinen-4-ol.

3.5.7.2 Preparation of plasma samples

Aliquots of rat plasma containing various concentrations of terpinen-4-ol were prepared by spiking equal volumes (20 µl) of terpinen-4-ol working

solutions into blank plasma (2 ml) to yield terpinen-4-ol concentrations of 0.01, 0.25, 0.50, 0.75, 1.00 and 10.00 µg/ml.

Aliquots of human plasma containing various concentrations of terpinen-4-ol were prepared by spiking equal volumes (20 µl) of terpinen-4-ol working solutions into blank plasma (2 ml) to yield terpinen-4-ol concentrations of 0.25, 0.50, 2.50 and 5.00 µg/ml.

3.5.7.3 Plasma protein binding

The human and rat plasma protein binding of terpinen-4-ol was determined using the ultrafiltration method. Aliquots of plasma containing various concentrations of terpinen-4-ol 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, USA) and centrifuged at 2,000 g for 8 min. The ultrafiltrate concentration represents the free plasma concentration. The fraction of unbound drug was determined by the following equation:

$$f_u = C_u/C_t \quad (3.4)$$

where f_u is the fraction of unbound drug in plasma and C_u and C_t are the unbound and total concentration of the analyte in plasma, respectively.

3.5.7.4 Sample analysis

Aliquots of 30 µl of rat plasma and 50 µl of human plasma ultrafiltrates were extracted with hexane by vortexing for 30 sec. An aliquot of clear supernatant was analysed by GC-MS.

A 100 µl aliquot of rat plasma sample was transferred to 4 ml glass vials followed by the addition of a 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 sec and centrifuged at 855 × g for 15 min. The upper organic layer (1 µl) was injected into the GC-MS system for analysis.

A 500 µl aliquot of human plasma samples was added to 4 ml glass vials followed by the addition of a 20-µl of the IS working solution (equivalent to 0.2 µg IS). Then, 2 ml of hexane was added into the vial. The mixture was vortexed

for 30 sec and centrifuged at 855 × g for 15 min. The upper organic layer (1 µl) was injected into the GC-MS system for analysis.

3.5.7.5 Statistical analysis

All data were expressed as mean value ± SD. The significances ($p < 0.05$) among the corresponding mean values were determined by using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison test. SPSS 14.0 software for windows was used for statistical analysis.

3.5.8 Determination of human blood to plasma ratio of terpinen-4-ol in its pure form and plai oil

3.5.8.1 Preparation of stock solution

The stock solutions of terpinen-4-ol and plai oil were prepared in hexane at concentration of 1 µg/ml. The solution was diluted with hexane to obtain appropriate working solutions for preparing human blood samples containing various concentrations of terpinen-4-ol.

3.5.8.2 Human whole blood

Fresh blood samples were obtained by venepuncture from 3 healthy subjects aged between 20 and 40 years. Whole blood was collected using Vacutainer blood collection tubes containing ethylenediaminetetraacetic acid and used within 1 h after collection. The hematocrit was determined for each subject using a micro capillary centrifuge at Alpha Laboratory, Phyathai Hospital, Bangkok, Thailand. The study protocol was approved by the local Ethics Committee, Mahidol University, Thailand. Each volunteer provided informed consent before entering the study.

3.5.8.3 Blood to plasma ratio study

Aliquouts of pure terpinen-4-ol and plai oil solutions were evaporated to dryness in screw-cap centrifuge tubes (n=2) under nitrogen at 25 ºC. An aliquot of blood was transferred to the tubes to obtain final terpinen-4-ol concentrations of 0.06 and 0.60 µg/ml. The tubes were gently shaken and then incubated at 37°C for 30 min. After incubation, duplicate aliquots of 250 µl of whole blood were transferred to glass vials and kept at -20°C until analyzed. Plasma was separated from the remaining blood by centrifugation at 6,000 g at 4°C for 10 min. Aliquots (n=2) of plasma were transferred to glass vials and stored at -20°C until

analyzed. The whole blood-to-plasma ratio was calculated as the total concentration in whole blood divided by the total concentration in plasma.

3.5.8.4 Sample analysis

A 500 µl aliquot of human plasma samples was added to 4 ml glass vials followed by the addition of a 20-µl of the IS working solution (equivalent to 0.2 µg IS). Then, 2 ml of hexane was added into the vial. The mixture was vortexed for 30 sec and centrifuged at 855 × g for 15 min. The upper organic layer (1 µl) was injected into the GC-MS system for analysis.

Accurately measured 250 µl aliquots of human blood samples were each added to 4 ml glass vials followed by the addition of a 20-µl of the IS working solution (equivalent to 0.08 µg IS). Then, 2 ml of hexane was added into the vial. The mixture was vortexed for 30 sec and centrifuged at 855 × g for 15 min. The upper organic layer (1 µl) was injected into the GC-MS system for analysis.

3.5.8.5 Statistical analysis

All data were expressed as mean value ± SD. The significances ($p < 0.05$) among the corresponding mean values were determined by using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison test. SPSS 14.0 software for windows was used for statistical analysis.

3.5.9 Influence of perfusion flow rate on *in vitro* recovery

3.5.9.1 Microdialysis system

The microdialysis probes (CMA 30, CMA microdialysis AB, Stockholm, Sweden) with a molecular weight cut-off value of 6 k Da, outer membrane diameter of 0.38 mm, dialysis membrane length of 10 mm, an inlet and outlet tubing length of 245 mm were used in this study. The inlet tube of the probe was connected to a microinjection pump (Harvard Apparatus 22 injection pump, model 55-4150). The experimental setup was shown in Figure 3.1.

3.5.9.2 Preparation of stock and working solutions

The stock solution of terpinen-4-ol was prepared in methanol at concentration of 1 mg/ml. The solution was diluted with normal saline to yield a terpinen-4-ol concentration of 1 µg/ml.

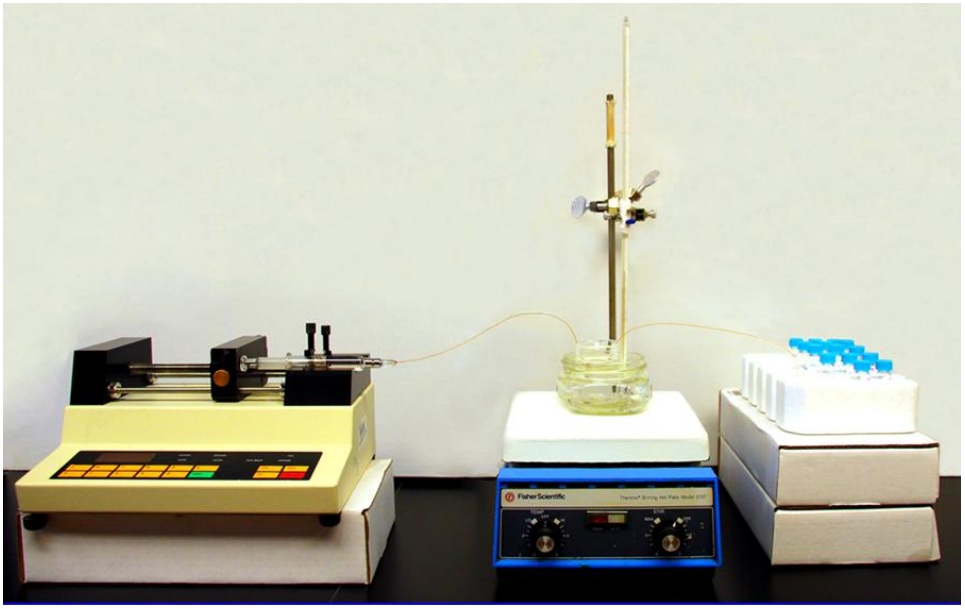

Figure 3.1 The *in vitro* microdialysis system.

3.5.9.3 Experimental procedure

The influence of perfusion flow rate on recoveries determined by dialysis and retrodialysis methods was investigated. The dialysis method was performed by placing two microdialysis probes in normal saline containing terpinen-4-ol at concentration of 1 µg/ml which was magnetically stirred at 350 rpm at 37 ^{0}C. The probes were continuously perfused with normal saline at flow rates of 2, 3, 4, 5 and 6 µl/min. After an equilibration period of 30 min, three consecutive microdialysis samples were collected every 20 min from each probe. The relative recovery was calculated as

$$\text{Relative recovery} = C_{\text{dialysate}}/C_{\text{medium}} \quad (3.5)$$

where $C_{\text{dialysate}}$ and C_{medium} are the analyte concentrations in the microdialysate and the medium, respectively.

For the retrodialysis studies, the dialysis process was reversed by adding terpinen-4-ol to the perfusate instead of the medium. The probes were placed in normal saline and perfused with terpinen-4-ol solution (1 µg/ml) at five different flow rates (2, 3, 4, 5, and 6 µl/min). After an equilibration period of 30 min, three consecutive microdialysis samples were collected every 20 min from each probe. The relative loss during perfusion was calculated as

$$\text{Relative loss} = (C_{\text{perfusate}}-C_{\text{dialysate}})/C_{\text{perfusate}} \quad (3.6)$$

where $C_{\text{perfusate}}$ is the analyte concentration in the perfusate.

3.5.9.4 Sample preparation

A 30 µl aliquot of 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 sec. The upper organic layer (2 µl) was injected into the GC-MS system.

3.5.9.5 Statistical analysis

All data were expressed as mean value ± SD. The significances ($p < 0.05$) among the corresponding mean values were determined by using one-way

analysis of variance (ANOVA) followed by Tukey's pairwise comparison test. SPSS 14.0 software for windows was used for statistical analysis.

3.5.10 Influence of perfusion flow rate on inlet probe binding

3.5.10.1 Microdialysis system

The microdialysis probes (CMA 30, CMA microdialysis AB, Stockholm, Sweden) with a molecular weight cut-off value of 6 kDa, outer membrane diameter of 0.38 mm, dialysis membrane length of 10 mm, an inlet and outlet tubing length of 245 mm were used in this study. The inlet tube of the probe was connected to a microinjection pump (Harvard Apparatus 22 injection pump, model 55-4150).

3.5.10.2 Preparation of stock and working solutions

The stock solution of terpinen-4-ol was prepared in methanol at concentration of 1 mg/ml. The solution was diluted with normal saline to yield terpinen-4-ol concentrations of 1 µg/ml.

3.5.10.3 Experimental procedure

To investigate the effect of perfusion flow rate on inlet probe binding, two polyimide inlet tubings connected to polyurethane tubing connectors with a length of 257 mm were separated and used in this study. The inlet tubings were perfused with normal saline containing terpinen-4-ol at concentrations of 1 µg/ml at five different flow rates (2, 3, 4, 5, and 6 µl/min). After an equilibration period of 30 minutes, two samples were collected from each tubing.

Binding to the inlet tubing was calculated as the proportion of drug lost during perfusion through the tube (89).

$$B = 1\text{-}C_{(lb=Lb)}/C_{(lb=0)} \quad (3.7)$$

where C is the analyte concentration, lb is the position variable along the probe tubing (mm), and Lb is the length of tubing (mm).

The binding to the inlet tubing (B) was used to calculate the perfusion concentration ($C_{perfusate}$) before entering the dialysis membrane which was then used to calculate both *in vitro* and *in vivo* recoveries by retrodialysis. Terpinen-4-

ol binding to the outlet tubing was considered negligible and no correction was used for the recovery determined by dialysis.

3.5.10.4 Sample preparation

A 30 µl aliquot of 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 sec. The upper organic layer (2 µl) was injected into the GC-MS system.

3.5.10.5 Statistical analysis

All data were expressed as mean value ± SD. The significances ($p < 0.05$) among the corresponding mean values were determined by using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison test. SPSS 14.0 software for windows was used for statistical analysis.

3.5.11 Influence of terpinen-4-ol concentrations on *in vitro* recovery

3.5.11.1 Microdialysis system

The microdialysis probes (CMA 30, CMA microdialysis AB, Stockholm, Sweden) with a molecular weight cut-off value of 6 k Da, outer membrane diameter of 0.38 mm, dialysis membrane length of 10 mm, an inlet and outlet tubing length of 245 mm were used in this study. The inlet tube of the probe was connected to a microinjection pump (Harvard Apparatus 22 injection pump, model 55-4150).

3.5.11.2 Preparation of stock and working solutions

The stock solution of terpinen-4-ol was prepared in methanol at concentration of 1 mg/ml. The solution was diluted with normal saline to yield terpinen-4-ol concentrations of 0.01, 0.25, 0.50, 0.75, 1.00, 10.00 and 20.00 µg/ml.

3.5.11.3 Experimental procedure

To investigate the influence of terpinen-4-ol concentrations on recoveries, the flow rate was fixed at 2 µl/min and seven different terpinen-4-ol concentrations were used to estimate relative recovery and loss: 0.01, 0.25, 0.50, 0.75, 1.00, 10.00 and 20.00 µg/ml. The dialysis method was performed by placing two microdialysis probes in normal saline containing terpinen-4-ol solution which was magnetically stirred at 350 rpm at 37 ^{0}C. The probes were continuously perfused with normal saline at a flow rate of 2 µl/min. After an equilibration period of 30 min, three

consecutive microdialysate samples were collected every 20 min from each probe. The relative recovery was calculated using equation 3.5.

For the retrodialysis studies, the dialysis process was reversed by adding terpinen-4-ol to the perfusate instead of the medium. The probes were placed in normal saline and perfused with terpinen-4-ol solution (0.01, 0.25, 0.50, 0.75, 1.00, 10.00 and 20.00 µg/ml) at a flow rate of 2 µl/min. After an equilibration period of 30 min, three consecutive microdialysate samples were collected every 20 min from each probe. The relative loss during perfusion was calculated using equation 3.6.

3.5.11.4 Sample preparation

A 30 µl aliquot of 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 sec. The upper organic layer (2 µl) was injected into the GC-MS system.

3.5.11.5 Statistical analysis

All data were expressed as mean value ± SD. The significances ($p < 0.05$) among the corresponding mean values were determined by using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison test. SPSS 14.0 software for windows was used for statistical analysis.

3.5.12 Influence of terpinen-4-ol concentrations on inlet probe binding

3.5.12.1 Microdialysis system

The microdialysis probes (CMA 30, CMA microdialysis AB, Stockholm, Sweden) with a molecular weight cut-off value of 6 k Da, outer membrane diameter of 0.38 mm, dialysis membrane length of 10 mm, an inlet and outlet tubing length of 245 mm were used in this study. The inlet tube of the probe was connected to a microinjection pump (Harvard Apparatus 22 injection pump, model 55-4150).

3.5.12.2 Preparation of stock and working solutions

The stock solution of terpinen-4-ol was prepared in methanol at concentration of 1 mg/ml. The solution was diluted with normal saline to yield terpinen-4-ol concentrations of 0.01, 0.25, 0.5, 0.75, 1.00, 10.00 and 20.00 µg/ml.

3.5.12.3 Experimental procedure

To investigate the effect of perfusion flow rate on inlet probe binding, two polyimide inlet tubings connected to polyurethane tubing connectors with a length of 257 mm were separated and used in this study. The inlet tubings were perfused with normal saline containing terpinen-4-ol at concentrations of 0.01, 0.25, 0.50, 0.75, 1.00, 10.00 and 20.00 µg/ml at a flow rate of 2 µl/min. After an equilibration period of 30 minutes, two samples were collected from each tubing. Binding to the inlet tubing was calculated using equation 3.7.

3.5.12.4 Sample preparation

A 30 µl aliquot of 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 sec. The upper organic layer (2 µl) was injected into the GC-MS system.

3.5.12.5 Statistical analysis

All data were expressed as mean value ± SD. The significances ($p < 0.05$) among the corresponding mean values were determined by using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison test. SPSS 14.0 software for windows was used for statistical analysis.

3.5.13 Dermal and plasma pharmacokinetics following intravenous bolus injection of terpinen-4-ol

3.5.13.1 Preparation of terpinen-4-ol solution for injection

On the day of the experiment, the solution of terpinen-4-ol (2 mg/ml) was prepared in 0.9% Normal saline and subsequently filter-sterilized using a 0.22 µm filter membrane.

3.5.13.2 Animal

The experiment was performed in 4 male Wistar rats weighing between 300-350 g obtained from Harlan Laboratories (Tampa, FL). All rats were acclimatized to standard acclimatization housing in a 12-hour light-dark cycle for a minimum of 5 days before the beginning of the experiment to allow them to adjust to the new environment. Animals were socially housed (2 per cage) in a temperature- and

humidity controlled vivarium. During this period, they had free access to food and water. All experiments were performed during the light phase of the cycle.

All subjects were treated in accordance with the National Institutes of Health guidelines regarding the principles of animal care. Animal facilities and experimental protocols were in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). 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).

3.5.13.3 Animal preparation

One day before the experiment, the abdominal fur of the rat was carefully shaved with an animal hair clipper. Prior to conduct the experiment, the rat was anesthetized by an isoflurane vaporizer. Isoflurane inhalation was performed under the fume hood in the following way: The rat was placed in an induction chamber supplied with an air-isoflurane (4%) mixture at a flow rate of 500 ml/min for 5 min. When the animal was under anesthesia, which was checked by a loss of reflexes (tail pinch, or ear pinch), a nose cone was placed on the nose, and the concentration of isoflurane decreased to 1.5-2.0% during the experiment. The rat was then placed on a temperature-controlled heating-pad to maintain its body temperature at 37-38°C in the dorsal position with the tail toward the investigator. It was then restrained with straps of adhesive tape for the whole period of study. The rat was remain anesthetized on the heating pad for the whole period of study and was not allowed to recover from anesthesia between sample collection.

3.5.13.4 Experimental procedure

The distribution of terpinen-4-ol in rat dermis and plasma after i.v. injection at a dose of 2 mg/kg was investigated using dermal microdialysis with simultaneous conventional blood sampling.

A 22-guage cannulas was implanted in the dermis, at a length of 20 mm, and resurfacing through exit puncture. A microdialysis probe was inserted through the tip of the cannula and the needle was then retracted, leaving the dialysis membrane implanted in the dermal tissue. The actual depth of the probe could not be measured since the appropriate instrument was not available. However, the needle was

carefully inserted into the skin as superficially as possible and the needle was clearly visible through the skin layer. In these conditions, the depth of the probe was not greater than approximately 2 mm, which corresponds to the deep dermis (131).

The probe was connected to the microdialysis pump and perfused with normal saline at a flow rate of 2 µl/min. After equilibration period of 30 min, the probe was calibrated using the retrodialysis technique (132) by perfusing with terpinen-4-ol (10 ng/ml) at a flow rate of 2 µl/min 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. Microdialysis samples 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 6000 rpm for 15 min. Then, the plasma was immediately separated and stored at -20°C until analysis. The experiment was done in four rats and the animals were sacrificed at the end of the study.

3.5.13.5 Sample analysis

A 100 µl aliquot of rat plasma sample was transferred to 4 ml glass vials followed by the addition of a 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 sec and centrifuged at 855 × g for 15 min. The upper organic layer (1 µl) was injected into the GC-MS system for analysis.

For microdialysis samples, a 30 µl aliquot of 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 sec. The upper organic layer (2 µl) was injected into the GC-MS system.

3.5.14 Dermal pharmacokinetics of terpinen-4-ol following topical application of plai oil

3.5.14.1 Animal

The experiment was performed in 12 male Wistar rats weighing between 300-350 g obtained from Harlan Laboratories (Tampa, FL). All rats were acclimatized to standard acclimatization housing in a 12-hour light-dark cycle for a minimum of 5 days before the begining of the experiment to allow them to adjust to the new environment. Animals were socially housed (2 per cage) in a temperature- and humidity controlled vivarium. During this period, they had free access to food and water. All experiments were performed during the light phase of the cycle.

All subjects were treated in accordance with the National Institutes of Health guidelines regarding the principles of animal care. Animal facilities and experimental protocols were in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). 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).

3.5.14.2 Animal preparation

One day before the experiment, the abdominal fur of the rat was carefully shaved with an animal hair clipper. Prior to conduct the experiment, the rat was anesthetized by an isoflurane vaporizer. Isoflurane inhalation was performed under the fume hood in the following way: The rat was placed in an induction chamber supplied with an air-isoflurane (4%) mixture at a flow rate of 500 ml/min for 5 min. When the animal was under anesthesia, which was checked by a loss of reflexes (tail pinch, or ear pinch), a nose cone was placed on the nose, and the concentration of isoflurane decreased to 1.5-2.0% during the experiment. The rat was then placed on a temperature-controlled heating-pad to maintain its body temperature at 37-38°C in the dorsal position with the tail toward the investigator. It was then restrained with straps of adhesive tape for the whole period of study. The rat was remain anesthetized on the heating pad for the whole period of study and was not allowed to recover from anesthesia between sample collection.

3.5.14.3 Experimental procedure

The dermal pharmacokinetic study of terpinen-4-ol following topical application plai oil was performed under non-occlusive conditions. The oil was topically applied at a dose of 2, 4 and 8 mg/cm^2 plai oil correspond to the amount of 1.2, 2.4 and 4.8 mg/cm^2 terpinen-4-ol, respectively.

A 22-guage cannulas was implanted in the dermis, at a length of 20 mm, and resurfacing through exit puncture. A microdialysis probe was inserted through the tip of the cannula and the needle was then retracted, leaving the dialysis membrane implanted in the dermal tissue. At the center of the implanted probe, a plastic application chamber (1.4 cm ID) was glued to the skin with cyanoacrylate glue. The actual depth of the probe could not be measured since the appropriate instrument was not available. However, the needle was carefully inserted into the skin as superficially as possible and the needle was clearly visible through the skin layer. In these conditions, the depth of the probe was not greater than approximately 2 mm, which corresponds to the deep dermis (131).

The probe was connected to the microdialysis pump and perfused with normal saline at a flow rate of 2 µl/min. After equilibration period of 30 min, the probe was calibrated using the retrodialysis technique (132) by perfusing with terpinen-4-ol (10 ng/ml) at a flow rate of 2 µl/min 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, an accurate amount of plai oil was applied into the application chamber at a dose of 2, 4 and 8 mg/cm^2, under non-occlusive conditions. The microdialysis samples were collected every 20 min for 6 h. Time-points were calculated as the mid-point between sampling intervals. The collected microdialysis samples were stored in a -20°C until analysis. The sample measurement represents the average concentration for each time interval.

3.5.14.4 Sample analysis

A 30 µl aliquot of 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 sec. The upper organic layer (2 µl) was injected into the GC-MS system.

3.5.15 Non-compartmental analysis

Noncompartmental analysis was performed using WinNonlin version 5.3 (Pharsight Corporation, Mountain View, CA). The following parameters were calculated for each rat, and the mean and S.D. of each parameter were determined.

3.5.15.1 Plasma sample

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 volume of distribution at steady state (V_{ss}) was calculated as the mean residence time ($MRT_{0-\infty}$) × CL, where $MRT_{0-\infty}$ is the area under the first moment curve ($AUMC_{0-\infty}$)/$AUC_{0-\infty}$.

3.5.15.2 Microdialysis sample

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 by retrodialysis. The maximum observed concentration (C_{max}) and time to reach C_{max} (t_{max}) after topical administration of plai oil were determined directly from the concentration-time curves. Dose linearity was determined by comparison of the dose-normalized $AUC_{0-\infty}$. The others 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 between tissue the area under the free plasma concentration-time curve and free tissue/free plasma (fAUC$_{tissue}$/fAUC$_{plasma}$) ratios from zero to infinity. The percentage of free AUC of terpinen-4-ol per amount of administered in dermis was calculated as 100 × fAUC$_{tissue}$/absolute amount of terpinen-4-ol administered.

3.5.15.3 Statistical analysis

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

3.5.16 Compartmental analysis

Compartmental analysis was performed using WinNonlin version 5.3 (Pharsight Corporation, Mountain View, CA). The plasma concentration-time data following i.v. injection were subject to compartmental model analysis. The pharmacokinetic 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. The model with the smallest values for AIC and SC was chosen as the best model. Plasma concentration-time data for each rat were fitted using a two-compartment open body model described by the following equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \quad (3.8)$$

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.

CHAPTER IV
RESULTS

4.1 Optimization of the GC-MS operating conditions

The gas chromatography-mass spectrometric operating conditions were optimized to assess the concentrations of terpinen-4-ol in rat plasma, dermal dialysate samples, plai oil, human plasma and blood. GC-MS analyses were necessarily carried out at different times (hours, weeks or months), during which instrumental conditions (as far as regards tuning and cleaning) could vary, leading to incomparable responses. In order to be sure that the difference of signals was actually due to the stability of the analyte and not to instrumental variations, an internal standard was added before the measurement. The relative analytical responses were measured as area ratios of terpinen-4-ol to the internal standard.

Terpinen-4-ol and methyl salicylate which was used as internal standard are liquids at ambient temperature with a boiling point around 200 ºC. As a result, these compounds were separated using gas chromatography, and then identified and confirmed by mass spectrometry. Acetonitrile, dichloromethane, hexane and methanol were tried for dissolving terpinen-4-ol, methyl salicylate and plai oil. The test substances are well soluble in hexane and dichlromethane. In the present study, hexane was used as solvent because of its low baseline noise and the absence of interfering peaks on the chromatogram when compare to dichloromethane.

In order to maximize the sensitivity and robustness of the method, dynamic mass calibration was performed to define the exact *m/z* values monitored in the SIM mode. A full-scan MS method (*m/z* 30-400) was used to select three appropriate ions for identification in the SIM method (Figure 4.1). The most abundant massed (*m/z* 71, 93 and 111 for terpinen-4-ol and *m/z* 92, 120 and 152 for methyl salicylate) were then selected for SIM detection. The chromatographic conditions (temperature programming) were optimized to achieve sufficient resolution of the analytes while maintaining the run-time as short as possible.

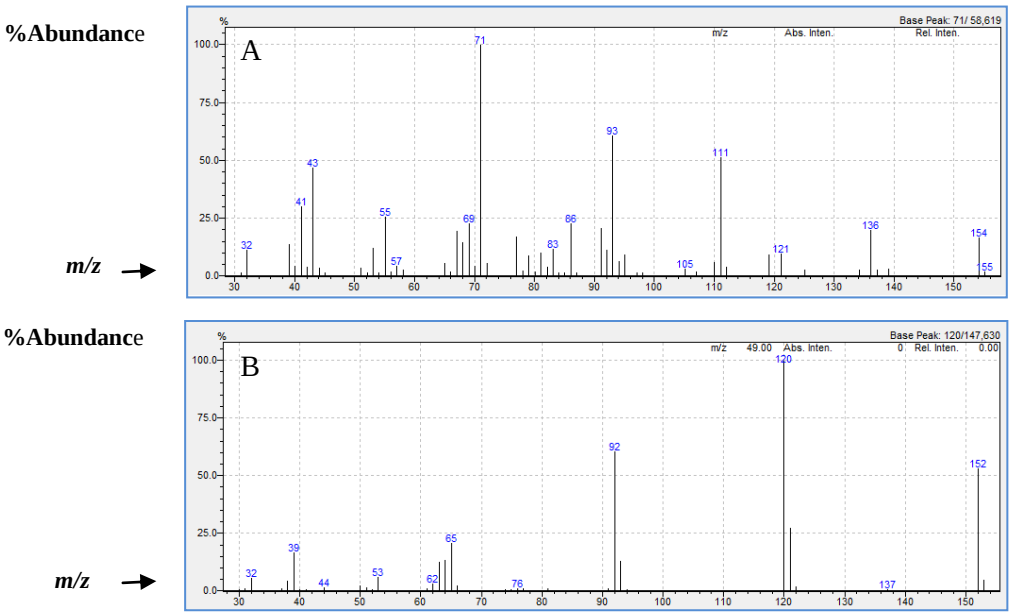

Figure 4.1 Full scan mass spectrum of terpinen-4-ol (A) and Methyl salicylate (B).

4.2 Validation of a bioanalytical method for determination of terpinen-4-ol in dermal microdialysis sample

4.2.1 Specificity

The specificity of the method was evaluated as lack of endogenous interference by analyzing blank microdialysis samples before terpinen-4-ol administration. No interfering peaks from blank samples were observed. The representative of GC-MS chromatogram of blank microdialysate is shown in Figure 4.2.

4.2.2 Sensitivity

The limit of quantification of terpinen-4-ol in dermal microdialysate was established as 0.005 μg/ml. Accuracy and precision of LLOQ in microdialysate were 1.2 and 17.4 %, respectively. A representative chromatogram of blank microdialysate spiked with IS and terpinen-4-ol at the limit of quantification is shown in Figure 4.3. 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.

4.2.3 Linearity

The calibration curve for the quantitative determination of terpinen-4-ol in dermal microdialysate 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 calibration curves showed good linearity within the range of 0.005-1 μg/ml. The correlation coefficient was > 0.995 for all validation batches. A typical calibration curve of terpinen-4-ol for microdialysis samples is shown in Figure 4.4.

4.2.4 Accuracy and precision

The inter-day accuracy and precision of the method were determined at three different QC concentrations (0.01, 0.50 and 0.75 μg/ml) in three different days. Table 4.1 summarizes the intra-day, as well as the inter-day accuracy and precision of the method. Intra- and inter-day precision ranged from 2.0 to 6.5 % and 3.6 to 7.1 %,

respectively. Intra- and inter-day relative errors (%bias) were less than 1.6 and 3.3 %, respectively Precision and accuracy were within the acceptable ranges for bio-analytical purposes.

4.2.5 Carryover

Carryover was studied by analyzing a blank hexane after the analysis of a standard sample at the highest calibration level (1 μg/ml). The result showed that there was no evidence of carry-over effect for this matrix.

4.2.6 Extraction recoveries

Terpinen-4-ol extraction recoveries from microdialysis samples at three concentration levels are reported in Table 4.2. The mean recovery of terpinen-4-ol for all microdialysis samples was 98.6 % with R.S.D. of < 6.4 %.

4.2.7 Stability

Stability of stored QC samples was determined with freshly spiked calibration curve standards. The analyte was found to be stable at ambient temperature for at least 24 h and at -20°C for one month. The post-processed samples were shown to be stable at approximately 25°C for at least 36 h (autosampler stability). Furthermore, microdialysis 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. Table 4.3 and table 4.4 summarize the result of stability of terpinen-4-ol in microdialysates carried out under various conditions and post-processed samples, respectively.

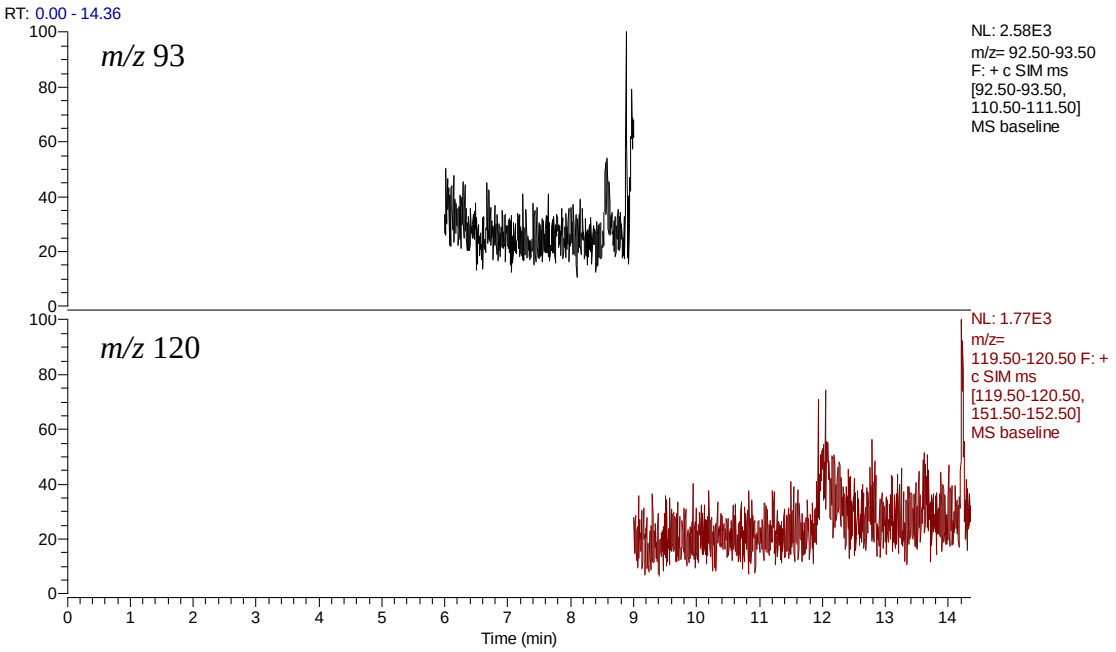

Figure 4.2 A representative GC-MS chromatogram of blank microdialysis sample.

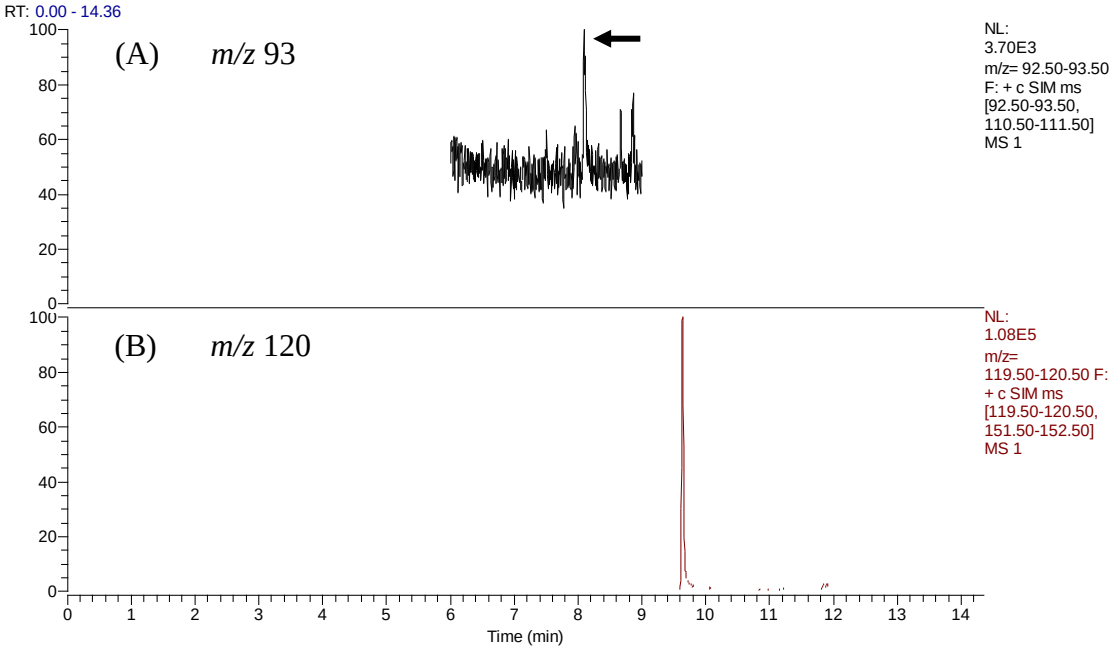

Figure 4.3 Representative GC-MS chromatograms of blank microdialysate spiked with terpinen-4-ol at 5 ng/ml (A) and IS (B). Arrows indicate the signal of terpinen-4-ol (8.1 min).

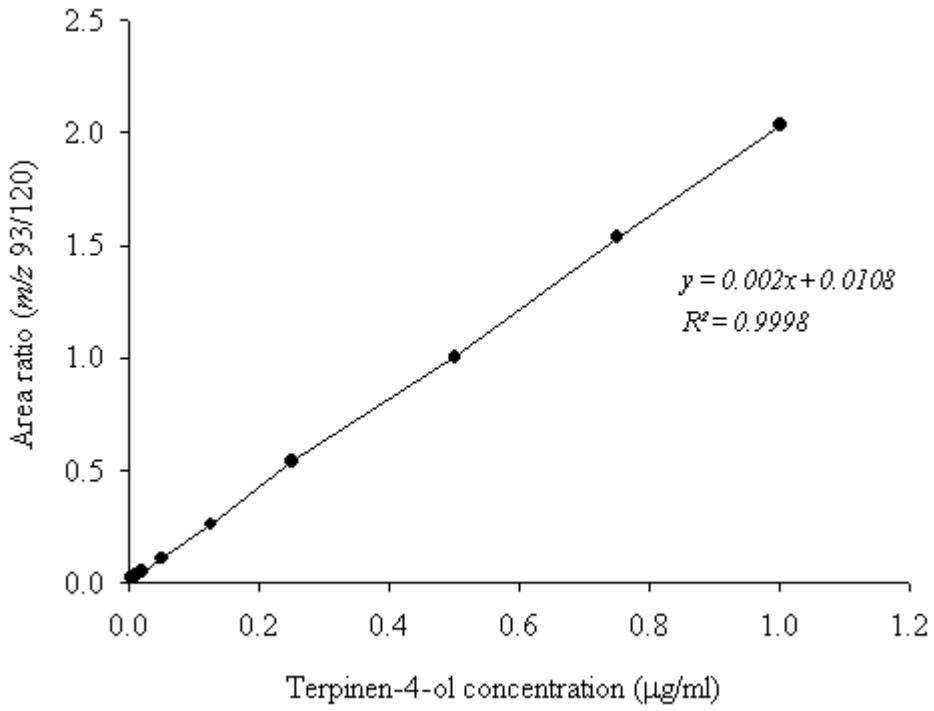

Figure 4.4 A typical calibration curve for microdialysis samples.

Table 4.1 Precision and accuracy of the GC-MS assay for terpinen-4-ol in microdialysis sample

Concentration added (µg/ml)	Sample	Intra-day			Inter-day		
		Batch 1	Batch 2	Batch 3	Founded (µg/ml)	%R.S.D.	%R.E.
0.01	1	0.0107	0.0100	0.0113			
	2	0.0097	0.0108	0.0110			
	3	0.0099	0.0100	0.0112			
	4	0.0100	0.0091	0.0097			
	5	0.0089	0.0096	0.0103			
	Founded (µg/ml)	0.0098	0.0099	0.0107	0.0102	7.12	-1.52
	%R.S.D.	6.47	6.24	6.39			
	%R.E.	1.56	0.92	-7.05			
0.50	1	0.4867	0.4379	0.5478			
	2	0.5174	0.5043	0.5423			
	3	0.4817	0.5169	0.5451			
	4	0.5083	0.5062	0.5334			
	5	0.4983	0.5152	0.5281			
	Founded (µg/ml)	0.4985	0.4961	0.5393	0.5113	5.58	-2.26
	%R.S.D.	2.96	6.65	1.54			
	%R.E.	0.30	0.78	-7.87			

Table 4.1 Precision and accuracy of the GC-MS assay for terpinen-4-ol in microdialysis sample (continued)

Concentration added (µg/ml)	Sample	Intra-day			Inter-day		
		Batch 1	Batch 2	Batch 3	Founded (µg/ml)	%R.S.D.	%R.E.
0.75	1	0.7552	0.7510	0.8126			
	2	0.7374	0.7619	0.8020			
	3	0.7501	0.7514	0.7827			
	4	0.7628	0.8177	0.8147			
	5	0.7786	0.7464	0.7990			
	Founded (µg/ml)	0.7568	0.7657	0.8022	0.7749	3.60	-3.32
	%R.S.D.	2.02	3.87	1.59			
	%R.E.	-0.91	-2.09	-6.96			

Table 4.2 Extraction recoveries of the GC-MS assay for terpinen-4-ol in microdialysate

Concentration added (μg/ml)	Sample	Extraction efficiency (%)
0.01	1	104.20
	2	112.02
	3	103.22
	4	94.54
	5	99.78
	Average (%)	102.75
	S.D.	6.4
	%R.S.D.	6.2
0.50	1	86.81
	2	99.99
	3	102.48
	4	100.37
	5	102.14
	Average (%)	98.36
	S.D.	6.5
	%R.S.D.	6.7
0.75	1	92.91
	2	94.27
	3	92.97
	4	101.18
	5	92.35
	Average (%)	94.74
	S.D.	3.7
	%R.S.D.	3.9

Table 4.3 Stability results of terpinen-4-ol in microdialysate sample

Condition	Added (µg/ml)	Sample	Found (µg/ml)	Average (µg/ml)	%R.S.D.	%Bias
Room temperature (25°C, 24 h)		1	0.0093			
	0.01	2	0.0096	0.0095	1.50	5.30
		3	0.0095			
		1	0.4736			
	0.50	2	0.4672	0.4699	0.70	6.03
		3	0.4688			
		1	0.7108			
	0.75	2	0.7372	0.7246	1.82	3.39
		3	0.7257			
Frozen-matrix (-20°C, 1 month)		1	0.0093			
	0.01	2	0.0088	0.0096	9.21	4.39
		3	0.0105			
		1	0.4523			
	0.50	2	0.5290	0.4866	8.01	2.68
		3	0.4784			

Table 4.3 Stability results of terpinen-4-ol in microdialysate sample (continued)

Condition	Added (µg/ml)	Sample	Found (µg/ml)	Average (µg/ml)	%R.S.D.	%Bias
Frozen-matrix (-20°C, 1 month)		1	0.7309			
	0.75	2	0.7023	0.7065	3.20	5.80
		3	0.6863			
Freeze-thaw (-20°C, 3 cycles)		1	0.0107			
	0.01	2	0.0113	0.0107	6.36	-6.50
		3	0.0099			
		1	0.4505			
	0.50	2	0.4575	0.4561	1.12	8.77
		3	0.4604			
		1	0.7310			
	0.75	2	0.6911	0.7135	2.86	4.86
		3	0.7185			

Table 4.4 Auto-sampler stability of terpinen-4-ol in microdialysis sample

Condition	Concentration added (μg/ml)	Sample	Found (μg/ml)	Average (μg/ml)	%R.S.D.	%Bias
Autosampler stability (-25°C, 36 h)		1	0.0091			
		2	0.0090			
	0.01	3	0.0093	0.0091	3.40	9.41
		4	0.0093			
		5	0.0086			
		1	0.5243			
		2	0.4919			
	0.50	3	0.4723	0.4989	3.85	0.23
		4	0.5076			
		5	0.4982			
		1	0.7201			
		2	0.7242			
	0.75	3	0.6877	0.7134	2.19	4.88
		4	0.7252			
		5	0.7099			

4.3 Validation of a bioanalytical method for determination of terpinen-4-ol in rat plasma

4.3.1 Specificity

The specificity of the method was evaluated as lack of endogenous interference by analyzing blank rat plasma samples before terpinen-4-ol administration. No interfering peaks from blank samples were observed. The representative of GC-MS chromatogram of blank rat plasma was shown in Figure 4.5.

4.3.2 Sensitivity

The limit of quantification in rat plasma was established as 0.005 μg/ml. Accuracy and precision of LLOQ were 2.9 and 11.6%, respectively. A representative chromatogram of blank samples spiked with IS and terpinen-4-ol at the limit of quantification was shown in Figure 4.6. 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.

4.3.3 Linearity

The calibration curve for the quantitative determination of terpinen-4-ol in rat plasma 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 calibration curves showed good linearity within the range 0.005-1 μg/ml. The correlation coefficient was > 0.995 for all validation batches. A typical calibration curve of terpinen-4-ol for rat plasma samples was shown in Figure 4.7.

4.3.4 Carryover

Carryover was studied by analyzing a blank hexane after the analysis of a standard sample at the highest calibration level (1 μg/ml). The result showed that there was no evidence of carry-over effect for this matrix.

4.3.5 Accuracy and precision

The inter-day accuracy and precison of the method were determined at three different QC concentrations (0.01, 0.50 and 0.75 μg/ml) in three different days. Table 4.5 summarizes the intra-day, as well as the inter-day accuracy and precision of the method. Precision and accuracy were within the acceptable ranges for bio-analytical purposes. Intra- and inter-day precision ranged from 4.0 to 8.7 % and 5.9 to 7.3 %, respectively. Intra- and inter-day relative errors (%bias) were less than 4.2 and 1.5 %, respectively.

4.3.6 Extraction recoveries

Terpinen-4-ol extraction recoveries from rat plasma at three concentration levels are reported in Table 4.6. The mean recovery of terpinen-4-ol for all plasma sample was 90.3 % with R.S.D. of < 7.3 %.

4.3.7 Stability

Stability of stored QC samples was determined with freshly spiked calibration curve standards. The analyte was found to be stable at ambient temperature for at least 24 h and at -20°C for one month. The post-processed samples were shown to be stable at approximately 25°C for at least 36 h (autosampler stability). Furthermore, rat 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. Table 4.7 and table 4.8 summarize the result of stability of terpinen-4-ol in rat plasma carried out under various conditions and post-processed samples, respectively.

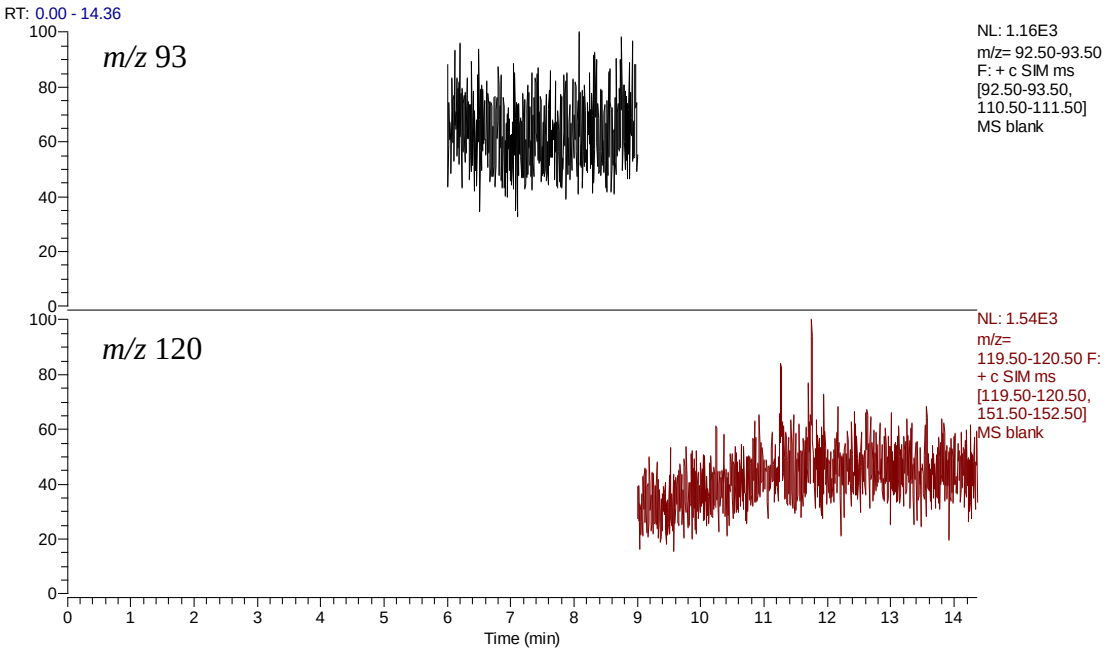

Figure 4.5 A representative GC-MS chromatograms of blank rat plasma.

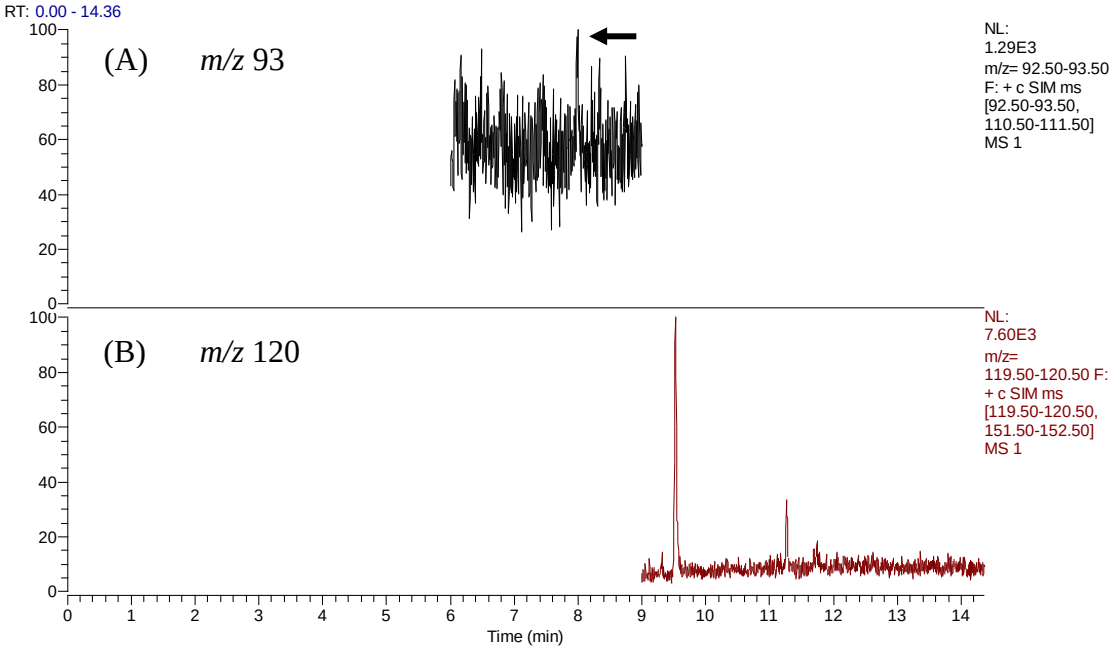

Figure 4.6 Representative GC-MS chromatograms of blank rat plasma spiked with terpinen-4-ol at 5 ng/ml (A) and IS (B). Arrows indicate the signal of terpinen-4-ol (8.1 min).

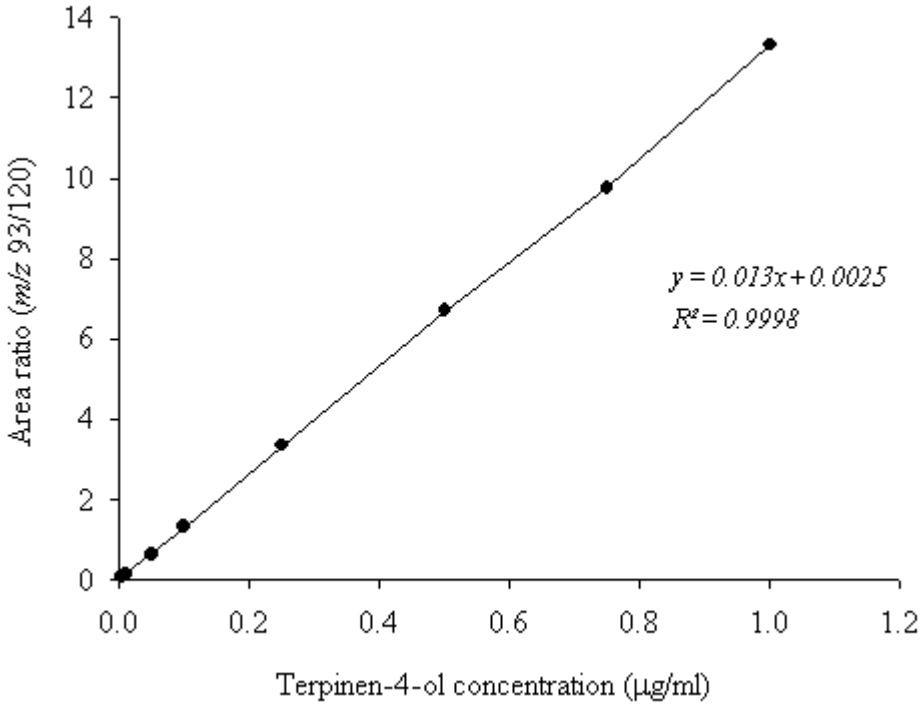

Figure 4.7 Typical calibration curves for rat plasma samples.

Table 4.5 Precision and accuracy of the GC-MS assay for terpinen-4-ol in rat plasma sample

Concentration added (µg/ml)	Sample	Intra-day			Inter-day		
		Batch 1	Batch 2	Batch 3	Founded (µg/ml)	%R.S.D.	%R.E.
0.01	1	0.0113	0.0105	0.0092			
	2	0.0105	0.0099	0.0095			
	3	0.0113	0.0103	0.0102			
	4	0.0098	0.0104	0.0103			
	5	0.0093	0.0109	0.0089			
	Founded (µg/ml)	0.0104	0.0104	0.0096	0.0102	7.26	-1.52
	%R.S.D.	8.69	3.58	6.61			
	%R.E.	-4.17	-4.24	3.85			
0.50	1	0.5212	0.5012	0.4540			
	2	0.4741	0.5041	0.4566			
	3	0.5218	0.5428	0.4921			
	4	0.5015	0.5212	0.4723			
	5	0.4916	0.5733	0.5199			
	Founded (µg/ml)	0.5020	0.5285	0.4790	0.5113	6.37	-0.63
	%R.S.D.	4.04	5.68	5.73			
	%R.E.	-0.41	-5.70	4.20			

Table 4.5 Precision and accuracy of the GC-MS assay for terpinen-4-ol in rat plasma sample (continued)

Concentration added (μg/ml)	Sample	Intra-day			Inter-day		
		Batch 1	Batch 2	Batch 3	Founded (μg/ml)	%R.S.D.	%R.E.
0.75	1	0.7695	0.7671	0.6971			
	2	0.7113	0.7749	0.7042			
	3	0.7969	0.8278	0.7526			
	4	0.7448	0.7626	0.6930			
	5	0.8091	0.7502	0.6816			
	Founded (μg/ml)	0.7663	0.7765	0.7057	0.7749	5.92	0.06
	%R.S.D.	5.17	3.87	3.89			
	%R.E.	-2.18	-3.54	5.90			

Table 4.6 Extraction recoveries of the GC-MS assay for terpinen-4-ol in rat plasma

Concentration added (μg/ml)	Sample	Extraction efficiency (%)
0.01	1	86.08
	2	80.83
	3	84.52
	4	85.36
	5	89.30
	Average (%)	85.22
	S.D.	3.0
	%R.S.D.	3.6
0.50	1	92.33
	2	92.86
	3	100.00
	4	96.02
	5	105.61
	Average (%)	97.36
	S.D.	5.5
	%R.S.D.	5.7
0.75	1	87.10
	2	87.98
	3	93.99
	4	86.59
	5	85.17
	Average (%)	88.17
	S.D.	3.4
	%R.S.D.	3.9

Table 4.7 Stability results of terpinen-4-ol in rat plasma

Condition	Added (µg/ml)	Sample	Found (µg/ml)	Average (µg/ml)	%R.S.D.	%Bias
Room temperature (25°C, 24 h)	0.01	1	0.0100	0.0097	8.62	2.52
		2	0.0088			
		3	0.0104			
	0.50	1	0.5002	0.5325	5.29	-6.50
		2	0.5521			
		3	0.5451			
	0.75	1	0.7317	0.7537	5.15	-0.49
		2	0.7309			
		3	0.7986			
Frozen-matrix (-20°C, 1 month)	0.01	1	0.0087	0.0088	1.75	11.71
		2	0.0089			
		3	0.0089			
	0.50	1	0.4387	0.4607	4.14	7.86
		2	0.4719			
		3	0.4715			

Table 4.7 Stability results of terpinen-4-ol in rat plasma (continued)

Condition	Added (µg/ml)	Sample	Found (µg/ml)	Average (µg/ml)	%R.S.D.	%Bias
Frozen-matrix (-20°C, 1 month)		1	0.7176			
	0.75	2	0.7133	0.6838	8.02	8.83
		3	0.6205			
Freeze-thaw (-20°C, 3 cycles)		1	0.0093			
	0.01	2	0.0093	0.0095	2.96	5.21
		3	0.0098			
		1	0.4635			
	0.50	2	0.5409	0.5065	7.78	-1.30
		3	0.5150			
		1	0.7314			
	0.75	2	0.7224	0.7383	2.75	1.56
		3	0.7612			

Table 4.8 Auto-sampler stability of terpinen-4-ol in rat plasma

Condition	Concentration added (µg/ml)	Sample	Found (µg/ml)	Average (µg/ml)	%R.S.D.	%Bias
Autosampler stability (-25°C, 36 h)		1	0.0091			
		2	0.0090			
	0.01	3	0.0093	0.0091	3.40	9.41
		4	0.0093			
		5	0.0086			
		1	0.5243			
		2	0.4919			
	0.50	3	0.4723	0.4989	3.85	0.23
		4	0.5076			
		5	0.4982			
		1	0.7201			
		2	0.7242			
	0.75	3	0.6877	0.7134	2.19	4.88
		4	0.7252			
		5	0.7099			

4.4 Validation of the analytical method for determination of terpinen-4-ol in human plasma

4.4.1 Selectivity

The selectivity of the method was studied using drug-free human plasma. Mass chromatograms of pooled blank plasma sample are presented in Figure 4.8. It was shown that the chromatograms were free of interfering peaks at the retention times of terpinen-4-ol (9.3 min) and methyl salicylate (9.9 min).

The matrix effect was evaluated by determination of IS-normalized matrix factor from 7 independent sources of plasma. The mean matrix effects of terpinen-4-ol in human plasma at low and high QC samples were 0.88 ± 0.02 and 0.88 ± 0.01, respectively with %R.S.D. of 1.8 and 1.6 %, respectively. Since the value is close to 1, indicating that the matrix effect was low and could be ignored in the quantitative analysis (Table 4.9).

4.4.2 Sensitivity

The LLOQ for terpinen-4-ol in human plasma samples was established as 0.03 μg/ml with a precision (%R.S.D.) and accuracy (% bias) of 1.7 and -9.6 %, respectively (Figure 4.8).

4.4.3 Linearity

The calibration curve for the quantitative determination of terpinen-4-ol in human plasma was constructed using the peak area of terpinen-4-ol and methyl salicylate at *m/z* 71 and 120, respectively. The calibration curve was linear over the range of 0.030-1.20 μg/ml with the correlation coefficient > 0.995 for all validation batches (Figure 4.9).

4.4.4 Carryover

Carryover was studied by analyzing a blank hexane after the analysis of a standard sample at the highest calibration level (1.20 μg/ml). No detectable carryover was observed.

4.4.5 Precision and accuracy

The accuracy and precision of the analytical method were evaluated by analyzing quality control samples at three concentration levels (0.09, 0.60 and 1.0 μg/ml). Intra- and Inter-day accuracy (percent difference between mean and nominal concentrations) were less than 4.2 and 7.4 %, respectively, while intra- and interday precision was less than 5.6 and 4.5 %, respectively (Table 4.10).

4.4.6 Extraction recovery

The extraction recovery of the method for terpinen-4-ol in quality control samples are presented in Table 4.11 as percent recovery. The mean extraction recoveries for terpinen-4-ol (*n*=3) were 100.3, 98.0 and 96.3% at concentration of 0.09, 0.60 and 1.00 μg/ml, respectively.

4.4.7 Stability

The storage stability of terpinen-4-ol in the human plasma matrix was found to be stable at intended storage temperatures for different periods (12 h at 25°C, 1 month at -20°C) and the samples could be thawed and refrozen for without compromising the integrity of the samples. The post-processed samples were shown to be stable at approximately 25°C for at least 36 h (autosampler stability). Table 4.12 and table 4.13 summarize the result of stability of terpinen-4-ol in human plasma carried out under various conditions and post-processed samples, respectively.

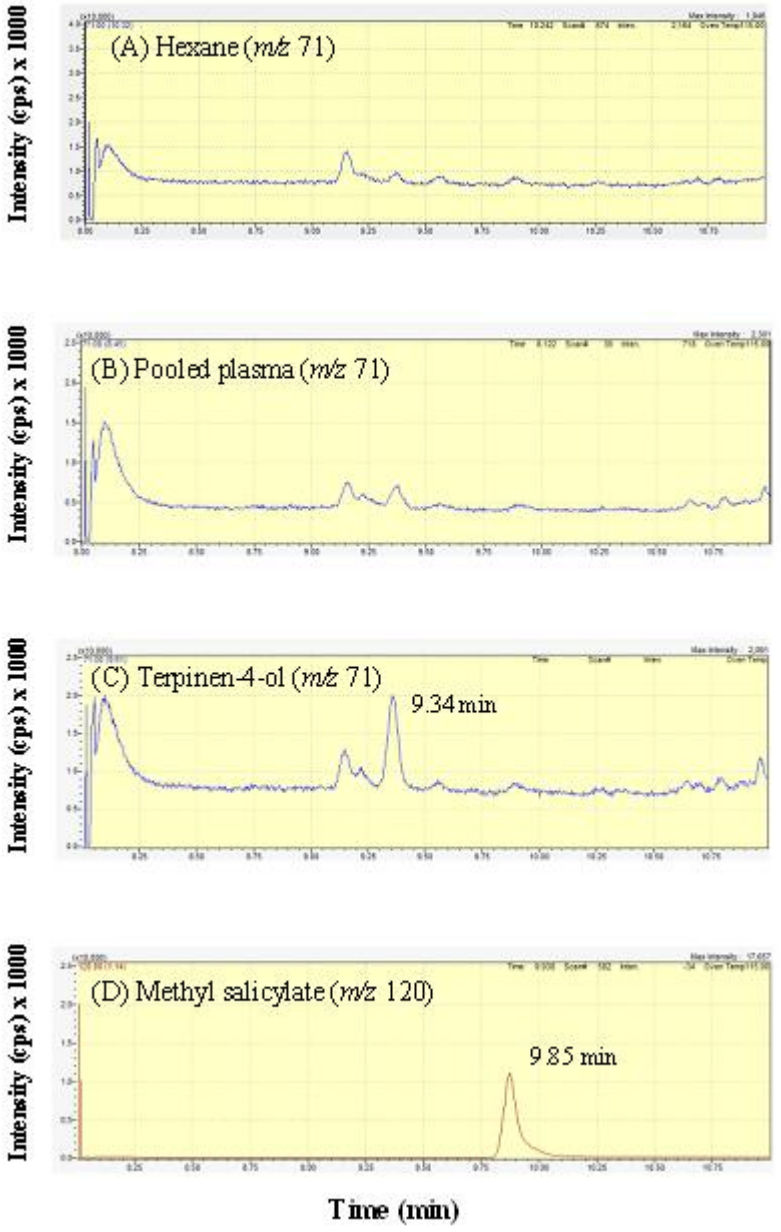

Figure 4.8 Typical GC-MS chromatograms of blank hexane (A), analyte-free plasma (B), analyte-free plasma spiked with terpinen-4-ol (30 ng/ml, LLOQ) (C) and methyl salicylate (720 ng/ml) (D).

Table 4.9 Matrix factor data of terpinen-4-ol in human plasma

Concentration added (μg/ml)	Subject	Matrix factor			Average	S.D.	%R.S.D.
		1	2	3			
0.90	1	0.86	0.88	0.97	0.90	0.05	6.03
	2	0.88	0.90	0.90	0.89	0.01	0.90
	3	0.90	0.90	0.87	0.89	0.02	1.96
	4	0.85	0.94	0.87	0.89	0.05	5.25
	5	0.87	0.82	0.91	0.87	0.04	5.14
	6	0.85	0.84	0.86	0.85	0.01	1.23
	7	0.85	0.89	0.85	0.86	0.03	3.07
					0.88	**0.02**	**2.13**
1.00	1	0.86	0.90	0.89	0.88	0.02	1.97
	2	0.89	0.87	0.91	0.89	0.02	2.08
	3	0.87	0.85	0.90	0.87	0.02	2.39
	4	0.85	0.87	0.88	0.87	0.02	2.25
	5	0.84	0.89	0.88	0.87	0.02	2.69
	6	0.86	0.89	0.91	0.89	0.02	2.52
	7	0.91	0.91	0.90	0.91	0.00	0.22
					0.88	**0.01**	**1.60**

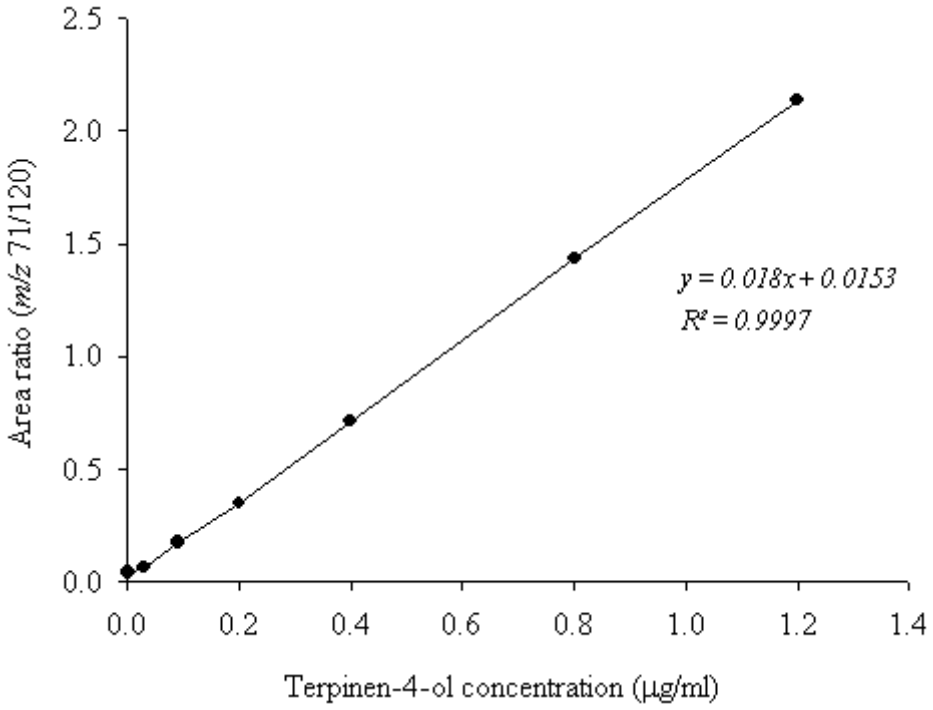

Figure 4.9 A typical calibration standard curve of terpinen-4-ol in human plasma.

Table 4.10 Precision and accuracy of the GC-MS assay for terpinen-4-ol in human plasma sample

Concentration added (µg/ml)	Sample	Intra-day			Inter-day		
		Batch 1	Batch 2	Batch 3	Founded (µg/ml)	%R.S.D.	%R.E.
0.09	1	0.0880	0.1009	0.1023			
	2	0.0911	0.0945	0.0998			
	3	0.0883	0.0989	0.0966			
	4	0.0957	0.1021	0.0979			
	5	0.0879	0.0985	0.1018			
	Founded (µg/ml)	0.0902	0.0990	0.0997	0.0963	5.5	-7.0
	%R.S.D.	3.72	2.92	2.46			
	%R.E.	-0.21	-9.96	-10.73			
0.60	1	0.6434	0.5396	0.6434			
	2	0.5893	0.6138	0.6246			
	3	0.6072	0.6564	0.6530			
	4	0.6077	0.6009	0.5919			
	5	0.6403	0.6635	0.6429			
	Founded (µg/ml)	0.6176	0.6148	0.6312	0.6212	1.4	-3.53
	%R.S.D.	3.79	8.11	3.84			
	%R.E.	-2.93	-2.47	-5.19			

Table 4.10 Precision and accuracy of the GC-MS assay for terpinen-4-ol in human plasma sample (continued)

Concentration added (µg/ml)	Sample	Intra-day			Inter-day		
		Batch 1	Batch 2	Batch 3	Founded (µg/ml)	%R.S.D.	%R.E.
1.00	1	1.0549	1.1064	1.0570			
	2	1.0851	1.0731	1.0629			
	3	1.0518	1.1169	1.0296			
	4	0.9411	1.1488	1.0180			
	5	1.0791	1.1395	1.0372			
	Founded (µg/ml)	1.0424	1.1169	1.0410	1.0668	4.1	-6.68
	%R.S.D.	5.61	2.67	1.80			
	%R.E.	-4.24	-11.69	-4.10			

Table 4.11 Extraction efficiency of terpinen-4-ol in human plasma

Concentration added (μg/mL)	%Recovery			Average	S.D.	%R.S.D.
	1	2	3			
0.90	103.52	101.06	96.36	100.31	3.64	3.62
0.60	101.23	98.53	94.38	98.04	3.45	3.52
1.00	100.40	93.24	95.14	96.26	3.70	3.85

Table 4.12 Stability results of terpinen-4-ol in human plasma

Condition	Concentration added (µg/ml)	Sample	Found (µg/ml)	Average (µg/ml)	%R.S.D.	%Bias
Room temperature (25°C, 12 h)	0.09	1	0.0863	0.0890	2.66	1.14
		2	0.0899			
		3	0.0907			
	1.00	1	1.0667	1.0458	1.97	-4.58
		2	1.0453			
		3	1.0255			
Frozen-matrix (-20°C, 2 month)	0.09	1	0.0936	0.0935	2.34	-3.83
		2	0.0912			
		3	0.0955			
	1.00	1	1.0646	1.0683	0.30	-6.83
		2	1.0702			
		3	1.0701			
Freeze-thaw (-20°C, 3 cycles)	0.09	1	0.0825	0.0892	6.54	0.88
		2	0.0918			
		3	0.0933			
	1.00	1	1.0326	1.0100	6.92	-1.00
		2	0.9317			
		3	1.0658			

Table 4.13 Auto-sampler stability of terpinen-4-ol in human plasma

Condition	Concentration added (µg/ml)	Sample	Found (µg/ml)	Average (µg/ml)	%R.S.D.	%Bias
Autosampler stability (-25°C, 36 h)		1	0.1036			
		2	0.1010			
	0.09	3	0.0949	0.0988	4.00	-9.73
		4	0.0997			
		5	0.0946			
		1	0.6291			
		2	0.6438			
	0.60	3	0.6445	0.6256	3.35	-4.27
		4	0.6163			
		5	0.5944			
		1	1.0006			
		2	0.9882			
	1.00	3	1.0531	1.0359	3.73	-3.59
		4	1.0705			
		5	1.0670			

4.5 Validation of the analytical method for determination of terpinen-4-ol in human blood

4.5.1 Selectivity

The selectivity of the method was studied using drug-free human whole blood. Mass chromatograms of pooled blank sample are presented in Figure 4.10. It was shown that there was no significant interference at the retention time of terpinen-4-ol and methyl salicylate in the blank matrix. The chromatograms were free of interfering peaks at the retention times of terpinen-4-ol (9.3 min) and methyl salicylate (9.9 min).

The matrix effect was evaluated by determination of IS-normalized matrix factor from 6 independent sources of whole blood. The mean matrix effects at low and high QC levels were 1.12 ± 0.04 and 1.03 ± 0.03, respectively with %R.S.D. of 3.3 and 3.2 %, respectively. Since the value is close to 1, indicating that the matrix effect was low and could be ignored in the quantitative analysis (Table 4.14).

4.5.2 Sensitivity

The lower limit of quantification of terpinen-4-ol in human whole blood was established as 0.45 μg/ml with a precision (%R.S.D.) and accuracy (%bias) of 9.0 and 0.4 %, respectively (Figure 4.10).

4.5.3 Linearity

The calibration curve for the quantitative determination of terpinen-4-ol in human whole blood was constructed using the peak area of terpinen-4-ol and methyl salicylate at *m/z* 71 and 120, respectively. The calibration curve was linear over the range of 0.45-1.20 μg/ml with the correlation coefficient > 0.995 for all validation batches (Figure 4.11).

4.5.4 Carryover

Carryover was studied by analyzing a blank hexane after the analysis of a standard sample at the highest calibration level (1.2 μg/ml). No detectable carryover was observed.

4.5.5 Precision and accuracy

The accuracy and precision of the analytical method were evaluated by analyzing quality control samples at three concentration levels (0.13, 0.60 and 1.00 μg/ml). Intra- and Interday accuracy were less than 2.8 and 4.1 %, respectively, while intra- and inter-day precision was less than 3.4 and 4.2 %, respectively (Table 4.15).

4.5.6 Extraction recovery

The extraction recovery of the method for terpinen-4-ol in quality control samples are presented in Table 4.16 as percent recovery. The mean extraction recoveries were 99.2, 98.7 and 99.4 % at concentration of 0.13, 0.60 and 1.00 μg/ml, respectively.

4.5.7 Stability

The storage stability of terpinen-4-ol in the human plasma matrix was found to be stable at intended storage temperatures for different periods (12 h at 25°C, 1 month at -20°C). The post-processed samples were shown to be stable at approximately 25°C for at least 36 h (autosampler stability). Table 4.17 and table 4.18 summarize the result of stability of terpinen-4-ol in human plasma carried out under various conditions and post-processed samples, respectively.

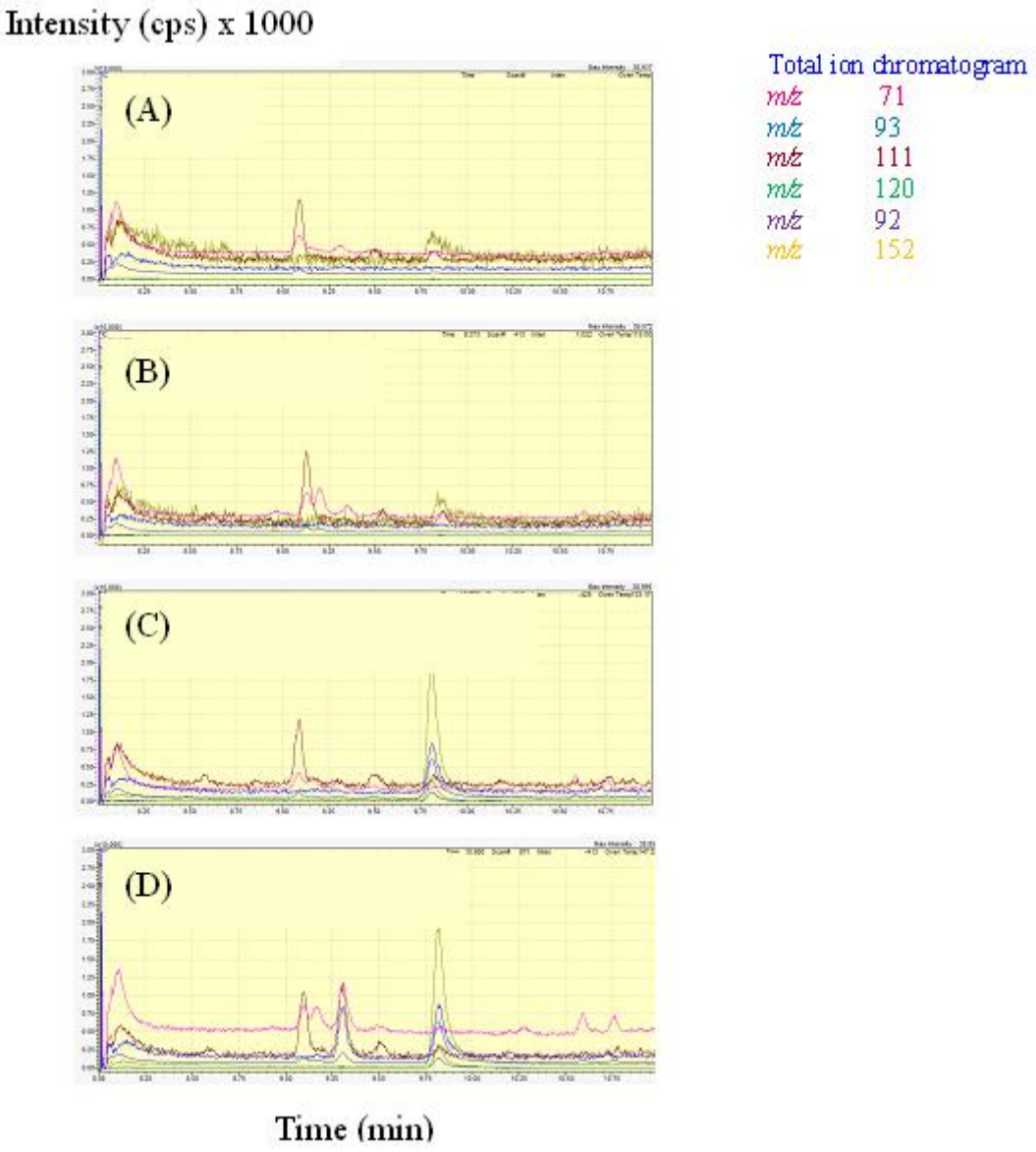

Figure 4.10 Typical chromatograms of hexane (A), analyte-free whole blood (B), analyte-free whole blood spiked with IS (C) and spiked with IS and terpinen-4-ol at concentration of 0.45 μg/mL (LLOQ) (D).

Table 4.14 Matrix factor data of terpinen-4-ol in human whole blood

Concentration added (μg/ml)	Subject	Matrix factor			Average	S.D.	%R.S.D.
		1	2	3			
0.90	1	1.13	1.14	1.01	1.10	0.08	7.01
	2	1.10	1.16	1.13	1.13	0.03	2.76
	3	1.18	1.20	1.17	1.19	0.02	1.45
	4	1.04	1.04	1.18	1.09	0.08	7.15
	5	1.16	1.04	1.13	1.11	0.06	5.40
	6	1.14	1.16	1.07	1.12	0.05	4.11
					1.12	**0.04**	**3.18**
1.00	1	1.01	0.95	1.02	1.00	0.04	3.57
	2	1.05	1.06	1.07	1.06	0.01	1.09
	3	1.03	1.09	1.07	1.06	0.03	3.09
	4	1.02	1.04	1.00	1.02	0.02	1.91
	5	1.01	0.98	1.00	0.99	0.02	1.62
	6	1.05	1.04	1.08	1.05	0.02	1.91
					1.03	**0.03**	**3.17**

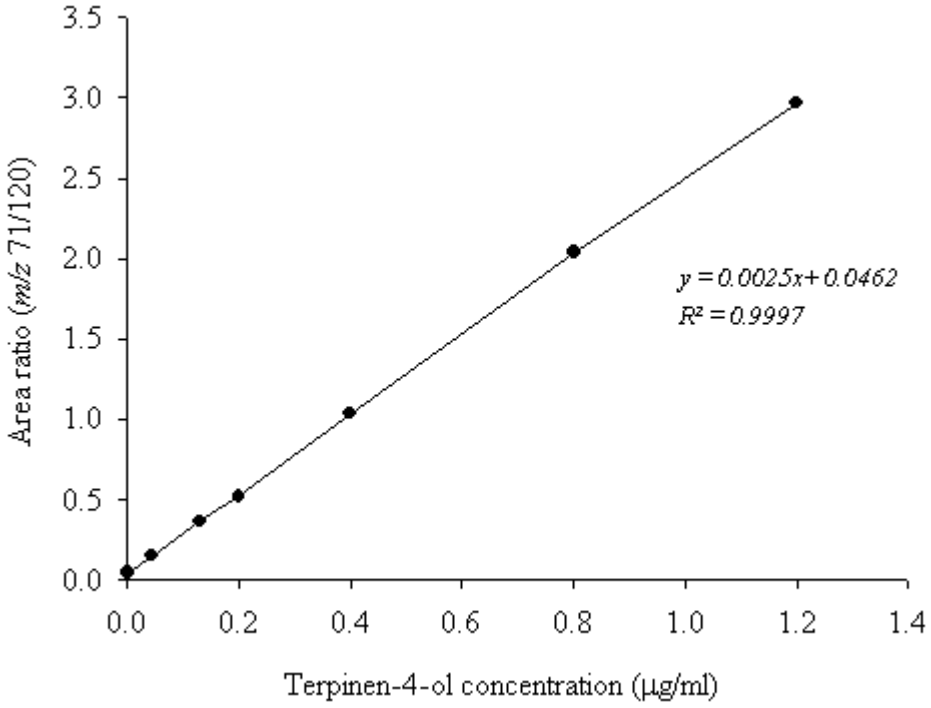

Figure 4.11 A typical calibration standard curve of terpinen-4-ol in human whole blood.

Table 4.15 Precision and accuracy of the GC-MS assay for terpinen-4-ol in human whole blood

Concentration added (μg/ml)	Sample	Intra-day			Inter-day		
		Batch 1	Batch 2	Batch 3	Founded (μg/ml)	%R.S.D.	%R.E.
0.13	1	0.1347	0.1436	0.1319			
	2	0.1380	0.1314	0.1314			
	3	0.1268	0.1476	0.1322			
	4	0.1322	0.1372	0.1258			
	5	0.1368	0.1293	0.1293			
	Founded (μg/ml)	0.1337	0.1378	0.1301	0.1339	2.9	-3.0
	%R.S.D.	3.35	5.66	2.06			
	%R.E.	-2.84	-6.02	-0.09			
0.60	1	0.6012	0.6589	0.6279			
	2	0.6000	0.6245	0.6061			
	3	0.6021	0.6560	0.6278			
	4	0.6139	0.6650	0.6114			
	5	0.5868	0.6609	0.6231			
	Founded (μg/ml)	0.6008	0.6530	0.6193	0.6244	4.2	-4.06
	%R.S.D.	1.60	2.50	1.61			
	%R.E.	-0.13	-8.84	-3.21			

Table 4.15 Precision and accuracy of the GC-MS assay for terpinen-4-ol in human whole blood (continued)

Concentration added (μg/ml)	Sample	Intra-day			Inter-day		
		Batch 1	Batch 2	Batch 3	Founded (μg/ml)	%R.S.D.	%R.E.
1.00	1	1.0342	1.1141	1.0723			
	2	1.0322	1.1148	1.0379			
	3	0.9705	1.0737	1.0061			
	4	0.9706	0.9425	1.0385			
	5	1.0198	1.0795	1.0815			
	Founded (μg/ml)	1.0055	1.0649	1.0473	1.0392	2.9	-3.92
	%R.S.D.	3.22	6.67	2.89			
	%R.E.	-0.55	-6.49	-4.73			

Table 4.16 Extraction efficiency of terpinen-4-ol in human whole blood

Concentration added (ng/mL)	%Recovery			Average	S.D.	%R.S.D.
	1	2	3			
0.13	102.12	97.94	97.45	99.17	2.57	2.59
0.60	100.50	99.54	96.06	98.70	2.33	2.36
1.00	99.57	99.83	98.66	99.35	0.61	0.62

Table 4.17 Stability results of terpinen-4-ol in human whole blood

Condition	Concentration added (µg/ml)	Sample	Found (µg/ml)	Average (µg/ml)	%R.S.D.	%Bias
Room temperature (25°C, 12 h)	0.13	1	0.1460	0.1349	0.0174	12.90
		2	0.1238			
		3	0.1117			
	1.00	1	1.0239	0.9455	0.0852	9.01
		2	0.8671			
		3	0.8877			
Frozen-matrix (-20°C, 1 week)	0.13	1	0.1132	0.1228	0.0098	8.02
		2	0.1323			
		3	0.1186			
	1.00	1	0.9216	0.8985	0.0914	10.17
		2	0.8754			
		3	1.0516			

Table 4.18 Auto-sampler stability of terpinen-4-ol in human whole blood

Condition	Concentration added (µg/ml)	Sample	Found (µg/ml)	Average (µg/ml)	%R.S.D.	%Bias
Autosampler stability (-25°C, 36 h)	0.13	1	0.1360	0.1291	3.97	0.69
		2	0.1285			
		3	0.1224			
		4	0.1268			
		5	0.1318			
	0.60	1	0.6082	0.6182	1.69	-3.03
		2	0.6141			
		3	0.6354			
		4	0.6196			
		5	0.6136			
	1.00	1	1.0601	1.0463	1.19	-4.63
		2	1.0446			
		3	1.0558			
		4	1.0427			
		5	1.0284			

4.6 Partial validation of the analytical method for determination of terpinen-4-ol in plai oil

4.6.1 Linearity

The calibration curve for the quantitative determination of terpinen-4-ol in 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 linearity of peak area ratio response versus concentration for terpinen-4-ol was studied from 25-250 ng/ml. A linear simple regression by the least squares was applied. The calibration curves showed good linearity in the investigated range, with excellent correlation coefficient ($r^2 > 0.995$) (Figure 4.12).

4.6.2 Precision

The intra-day precision (repeatability) was studied by assaying six different samples (*n*=6) by only one operator, at same concentration (200 ng/ml), during the same day. The concentration of terpinen-4-ol in the oil obtained in this assay was 48.1 % (Table 4.19). The inter-day precision (reproducibility) was demonstrated by assaying six samples in three different days (Table 4.20). The R.S.D. values for intra- and inter-day precision were 0.8 and 1.2 %, respectively.

4.6.3 Accuracy/recovery studies

The accuracy of the method was determined by fortifying sample with known amounts of terpinen-4-ol. The mean recovery was 100.01 % of with low R.S.D. (less than 0.5 %) as shown in Table 4.21.

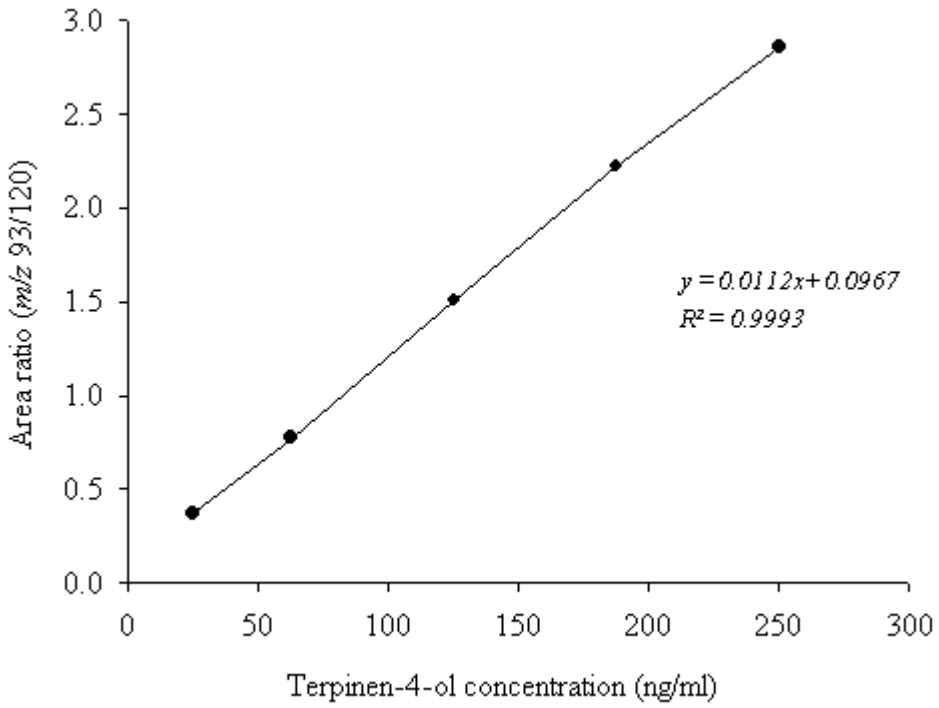

Figure 4.12 A typical calibration standard curve of terpinen-4-ol in plai oil.

Table 4.19 Results of quantitative determination of terpinen-4-ol in plai oil solution (200 ng/ml) by GC/MS, repeatability test

Sample	Found (ng/ml)	Content (%)	Average (%)	%R.S.D.
1	97.12	48.56	48.10	0.78
2	96.01	48.01		
3	94.91	47.45		
4	96.19	48.09		
5	96.72	48.36		
6	96.29	48.15		

Table 4.20 Data obtained from intermediary precision of plai oil samples by GC/MS analysis

Sample	Terpinen-4-ol content (%)			Average	S.D.	%R.S.D.
	Day 1	Day 2	Day 3			
1	47.33	48.47	48.56	47.78	0.76	1.58
2	47.57	47.66	48.01			
3	49.15	47.16	47.45			
4	49.02	46.96	48.09			
5	47.39	47.40	48.36			
6	47.01	46.26	48.15			

Table 4.21 The recovery of terpinen-4-ol in plai oil

Concentration added (ng/ml)	Sample	Recovery (%)
16	1	99.85
	2	101.27
	3	100.62
	Average (%)	100.58
	S.D.	0.70
	%R.S.D.	0.70
78	1	100.55
	2	98.51
	3	99.97
	Average (%)	99.68
	S.D.	1.10
	%R.S.D.	1.10
156	1	100.35
	2	99.32
	3	99.69
	Average (%)	99.78
	S.D.	0.52
	%R.S.D.	0.52

4.7 Solubility study

The average aqueous solubility of terpinen-4-ol in its pure form and plai oil were 3.53 ± 0.07 and 1.26 ± 0.14 mg/ml (mean ± S.D.), respectively which independent of the amount added to the aqueous phases (Table 4.22). The aqueous solubility of terpinen-4-ol in its pure form was significantly higher than the solubility of terpinen-4-ol in plai oil (p<0.05).

4.8 Plasma protein binding

The average protein binding of terpinen-4-ol in rat plasma was 76.67 ± 2.11 % (mean ± S.D.), with no dependence on concentrations in the range 0.01-10 μg/ml (Table 4.23). The value of 76.67 % was used as the average protein binding of terpinen-4-ol in rat plasma for calculations of tissue distribution factor.

The average human plasma protein binding of terpinen-4-ol in plai oil and its pure form were 78.50 ± 3.40 and 79.11 ± 3.74 % (mean ± S.D.), respectively, with no dependence on concentrations in the range 0.25-5.0 μg/ml (Table 4.24).

4.9 Blood to plasma ratio

The blood to plasma ratios of pure terpinen-4-ol at blood concentrations of 0.06 and 0.60 μg/ml were 0.88 ± 0.05 and 0.67 ± 0.03, respectively, indicating that the distribution of pure terpinen-4-ol within blood seemed to be concentration-dependent. For plai oil, the mean blood to plasma ratio was 0.68 ± 0.01 with no concentration dependence of the oil. The results were shown in Table 4.24.

Table 4.22 Aqueous solubility results of terpinen-4-ol

Substance	Amount of terpinen-4-ol added (g)	Average (mg/ml)
Terpinen-4-ol	0.04	3.45 ± 0.16
	0.08	3.49 ± 0.13
	0.15	3.57 ± 0.14
	0.23	3.59 ± 0.09
		3.53 ± 0.07
Plai oil	0.04	1.11 ± 0.02
	0.08	1.25 ± 0.02
	0.15	1.33 ± 0.05
	0.23	1.43 ± 0.01
		1.28 ± 0.14

Table 4.23 Protein binding data of terpinen-4-ol in rat plasma

Concentration added (μg/ml)	Plasma protein binding (%)	Average (%)	S.D.	%R.S.D.
0.01	69.52 81.88 73.20	74.87	6.35	8.48
0.03	72.65 72.83 74.02	73.17	0.74	1.02
0.05	79.25 75.01 77.18	77.15	2.12	2.75
0.10	78.86 77.36 79.13	78.45	0.95	1.22
0.25	76.40 78.43 76.09	76.98	1.27	1.65
0.50	80.81 78.80 79.24	79.62	1.06	1.33
1.00	77.45 79.03 77.21	77.90	0.99	1.27
10.00	74.37 75.24 76.05	75.22	0.84	1.12
		76.67	**2.11**	**2.76**

Table 4.24 Protein binding data of terpinen-4-ol in human plasma

	Concentration added (µg/mL)	% Protein binding (%), (n = 2)		Average	S.D.	%R.S.D.
		Subject 1	Subject 2			
Pure terpinen-4-ol	0.25	66.70	67.33	67.01	0.44	0.66
	0.50	71.94	77.67	74.81	4.06	5.42
	2.50	79.53	82.63	81.08	2.19	2.70
	5.00	80.35	82.57	81.46	1.57	1.92
				79.11	**3.74**	**4.72**
Plai oil	0.25	73.76	76.38	75.07	1.85	2.47
	0.50	75.32	76.88	76.10	1.10	1.45
	2.50	81.59	80.75	81.17	0.59	0.73
	5.00	81.69	81.61	81.65	0.06	0.07
				78.50	**3.40**	**4.33**

Table 4.25 Blood to plasma ratio of terpinen-4-ol in its pure form and plai oil

Substance	Concentration added (µg/ml)	Subject	Hematocrit (%)	Observed concentration (µg/ml)		Blood/Plasma ratio	Average	S.D.	%R.S.D.
				Blood	Plasma				
Terpinen-4-ol	0.06	1	45	0.0558	0.0636	0.88	0.88	0.05	5.23
		2	44	0.0564	0.0671	0.84			
		3	49	0.0516	0.0553	0.93			
	0.60	1	45	0.5969	0.9074	0.66	0.67	0.05	7.44
		2	44	0.6109	0.9810	0.62			
		3	49	0.5674	0.7872	0.72			
Plai oil	0.06	1	45	0.0560	0.0847	0.66	0.69	0.06	8.80
		2	44	0.0571	0.0869	0.66			
		3	49	0.0568	0.0743	0.77			
	0.60	1	45	0.6058	1.0165	0.60	0.67	0.07	10.91
		2	44	0.5744	0.8376	0.69			
		3	49	0.5704	0.7691	0.74			

4.10 *In vitro* microdialysis

4.10.1 Influence of perfusion flow rate on *in vitro* recovery and inlet probe binding

The investigation of perfusion flow rate influence on *in vitro* recovery and inlet probe binding showed that the non-specific binding of terpinen-4-ol to the inlet tubing and relative recovery and loss depended on the perfusion flow rate. Table 4.26 and 4.27 summarize the results of *in vitro* recovery of terpinen-4-ol and inlet probe binding at different flow rates, respectively. Reducing the flow rate from 6 to 2 µl/min resulted in an increase in apparent recovery and relative loss from 12.82 to 32.37 % and 19.86 to 49.88 %, respectively, and the percentage of probe binding increased from 7.24 to 27.44 %. At each perfusion flow rate, recoveries determined by retrodialysis were significantly higher than those determined by dialysis method. Nevertheless, as shown in Figure 4.13, after correction for probe binding determined at each flow rate, the relative loss was not significantly different from recovery. In order to characterize the pharmacokinetic profile with adequate recovery and manageable sampling volumes, a flow rate of 2 µl/min was chosen as optimal flow rate for the subsequent *in vitro* and *in vivo* studies.

4.10.2 Influence of terpinen-4-ol concentrations on *in vitro* recovery and inlet probe binding

Changing the concentration of terpinen-4-ol surrounding or perfusing a probe did not affect the relative recovery and loss and percentage of terpinen-4-ol binding to the inlet tubing, indicating that these parameters were independent of terpinen-4-ol concentrations. The average recoveries by dialysis and retrodialysis were 32.76 ± 2.00 % and 50.67 ± 2.18 %, respectively and the mean percentage binding was 28.41 ± 0.91 % (mean ± S.D.). Table 4.28 and 4.29 summarize the results of *in vitro* recovery and inlet probe binding at different concentrations of terpinen-4-ol, respectively. The average inlet probe binding value was applied to estimate the actual concentration of perfusate in the retrodialysis studies. After correction for probe binding, the mean recovery by retrodialysis was 30.99 ± 3.05 % (mean ± S.D.) which was not significantly different to that of dialysis method.

Table 4.26 Results of *in vitro* microdialysis of terpinen-4-ol (1 μg/ml) at different perfusion flow rates

	Flow rate	Probe1			Probe2			Average	S.D.	%R.S.D.
	(μl/min)	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3			
Relative	2	34.23	31.22	35.43	30.28	32.36	30.72	32.37	2.06	6.37
recovery	3	22.60	23.89	25.49	23.41	21.61	23.02	23.34	1.31	5.61
	4	19.87	17.16	19.18	17.56	19.20	15.94	18.15	1.51	8.31
	5	16.46	15.52	17.09	14.33	16.52	13.73	15.61	1.34	8.56
	6	15.22	12.98	13.66	11.38	11.81	11.88	12.82	1.45	11.30
Relative	2	53.68	48.92	50.51	48.62	47.53	50.03	49.88	2.14	4.29
loss	3	38.84	41.96	36.89	38.18	39.52	36.75	38.69	1.93	4.99
	4	31.86	28.46	31.26	31.07	30.37	32.72	30.96	1.46	4.70
	5	24.89	26.69	26.65	25.82	25.40	25.09	25.75	0.77	3.00
	6	15.55	22.73	21.27	20.39	19.04	20.17	19.86	2.44	12.30
Corrected	2	36.16	29.59	31.79	29.18	27.69	31.13	30.92	2.95	9.54
relative loss	3	24.46	28.31	22.04	23.64	25.30	21.88	24.27	2.39	9.83
	4	21.19	17.26	20.50	20.27	19.47	22.19	20.15	1.68	8.36
	5	16.03	18.04	17.99	17.06	16.60	16.25	17.00	0.87	5.09
	6	8.03	15.85	14.26	13.30	11.83	13.06	12.72	2.66	20.91

Table 4.27 Results of *in vitro* binding experiments of terpinen-4-ol (1 μg/ml) at different perfusion flow rates

Flow rate (μl/min)	Probe 1		Probe 2		Average	S.D.	%R.S.D.
	Sample 1	Sample 2	Sample 1	Sample 2			
2	24.59	26.65	29.34	29.19	27.44	2.27	8.27
3	19.84	18.37	19.19	18.77	19.04	0.63	3.30
4	14.83	13.39	13.72	12.20	13.54	1.08	7.98
5	10.22	10.09	10.44	11.46	10.55	0.62	5.87
6	5.94	6.79	9.77	6.47	7.24	1.72	23.77

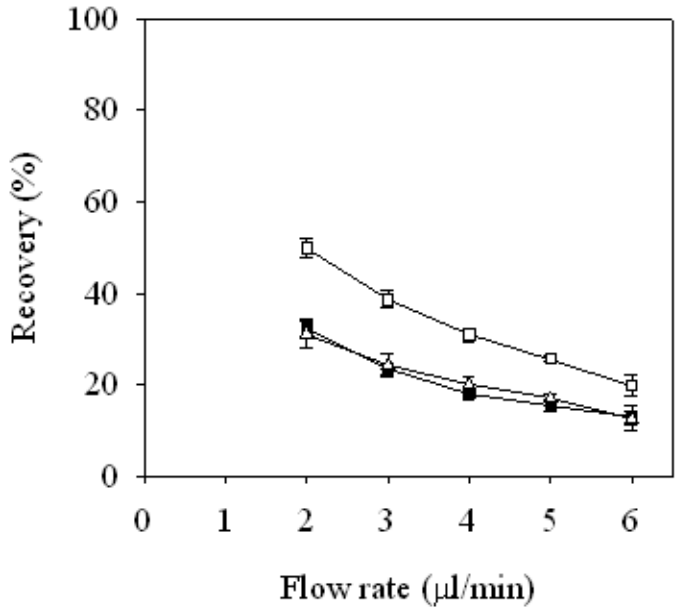

Figure 4.13 *In vitro* relative recovery (■), relative loss (□) and the corrected relative loss (Δ) (mean ± S.D.) at different flow rates. The samples were collected from each tubing at each flow rate (2 probes, *n* =6).

Table 4.28 Results of *in vitro* microdialysis of terpinen-4-ol at different concentrations

	Concentration	Probe1			Probe2			Average	S.D.	%R.S.D.
	(μg/ml)	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3			
Relative	0.01	39.20	34.38	39.95	30.89	34.60	34.12	35.52	3.43	9.65
recovery	0.25	34.92	34.51	34.17	28.22	30.05	29.66	31.92	2.94	9.20
	0.50	37.62	38.93	35.10	32.58	34.57	32.57	35.23	2.60	7.39
	0.75	34.03	34.42	33.48	29.57	29.45	29.55	31.75	2.46	7.74
	1	35.38	35.32	35.17	30.26	31.17	32.35	33.28	2.31	6.93
	10	35.23	34.31	35.45	28.71	26.79	27.91	31.40	4.00	12.75
	20	31.63	32.11	32.30	27.87	28.68	28.90	30.25	1.97	6.52
								32.76	**2.00**	**6.09**
Relative	0.01	57.97	48.70	50.62	57.33	52.60	50.22	52.90	3.88	7.34
loss	0.25	56.02	46.71	54.18	54.59	53.46	52.38	52.89	3.26	6.17
	0.50	50.70	49.37	52.88	49.12	51.65	50.06	50.63	1.44	2.84
	0.75	51.68	49.07	49.30	53.36	53.64	52.81	51.65	2.02	3.91
	1	50.64	50.12	49.31	53.41	52.31	48.76	50.76	1.79	3.52
	10	47.73	47.14	47.64	50.29	49.82	51.61	49.04	1.79	3.66
	20	48.36	47.28	49.16	43.84	46.13	46.09	46.81	1.89	4.05
								50.67	**2.18**	**4.30**

Table 4.28 Results of *in vitro* microdialysis of terpinen-4-ol at different concentrations (continued)

	Concentration (μg/ml)	Probe1			Probe2			Average	S.D.	%R.S.D.
		Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3			
Corrected relative loss	0.01	41.19	28.23	30.92	40.30	33.68	30.35	34.11	5.43	15.93
	0.25	38.48	25.44	35.90	36.48	34.89	33.38	34.10	4.56	13.39
	0.50	31.02	29.16	34.08	28.82	32.37	30.14	30.93	2.01	6.50
	0.75	32.41	28.76	29.08	34.76	35.14	33.98	32.35	2.82	8.73
	1	30.95	30.22	29.08	34.82	33.28	28.32	31.11	2.50	8.03
	10	26.88	26.05	26.75	30.46	29.80	32.31	28.71	2.51	8.74
	20	27.75	26.24	28.87	21.43	24.63	24.58	25.59	2.65	10.36
								30.99	**3.05**	**9.83**

Table 4.29 Results of *in vitro* inlet probe binding of terpinen-4-ol at different concentrations

Concentration (μg/ml)	Probe 1		Probe 2		Average	S.D.	%R.S.D.
	Sample 1	Sample 2	Sample 1	Sample 2			
0.01	26.58	25.49	28.97	27.90	27.24	1.52	5.57
0.25	33.13	28.17	27.22	29.53	29.51	2.59	8.78
0.50	27.57	29.47	27.66	26.63	27.83	1.19	4.27
0.75	25.80	30.46	31.43	31.13	29.70	2.64	8.87
1	26.04	28.00	28.03	29.13	27.80	1.29	4.63
10	26.43	29.33	28.02	29.44	28.31	1.41	4.97
20	27.49	30.92	27.38	28.06	28.46	1.67	5.86
					28.41	**0.91**	**3.21**

4.11 Terpinen-4-ol distribution studies following i.v. bolus injection

To study the distribution of terpinen-4-ol in plasma and dermal tissue of the rat, terpinen-4-ol was administered to rats by intravenous bolus injection at a dose of 2 mg/kg.

The individual plasma and dermal concentration time data were summarized in Table 4.30 and 4.31, respectiverly. The individual total and free terpinen-4-ol concentration-time profiles in plasma and in dermal tissue were shown in Figure 4.14 and 4.15, respectively. The mean concentration-time profiles of terpinen-4-ol in plasma and dermal tissue were shown in Figure 4.16 with free plasma concentrations based on 76.7 % plasma protein binding.

4.11.1 Probe recovery

The individual probe recovery for each animal was used to determined the actual free terpinen-4-ol concentration in the extracellular fluid of dermal tissue and all the recovery values were summarized in Table 4.30. The average relative loss was 52.06 ± 4.28 % (mean ± S.D.). After correction for probe binding, the mean recovery was 33.23 ± 6.0 % (mean ± S.D.) which was similar to the *in vitro* results.

4.11.2 Noncompartmental pharmacokinetic analysis

The experimental data terpinen-4-ol from 2 mg/kg intravenous dose were analyzed by both noncompartmental pharmacokinetic analysis and compartmental pharmacokinetic analysis.

4.11.2.1 Plasma

The results of the non-compartmental of terpinen-4-ol in plasma after i.v. administration were summarized in Table 4.32. The mean maximum concentration (C_{max}) was 6.30 ± 1.90 μg/ml, which declined with a mean terminal half-life of 1.80 ± 0.39 h. The mean area under the curve ($AUC_{0-\infty}$) was 4.25 ± 0.59 μg.hr/ml. The mean total body clearance is 0.48 ± 0.06 L/hr/kg. The mean residence time (MRT_{inf}) was 1.16 ± 0.25 h. The volume of distribution (V_z) was 1.25 ± 0.29 L/kg. The mean unbound area under the curve in plasma (AUC_f) was 0.99 ± 0.14 μg.hr/ml based on a protein binding of 76.67 ± 2.11 %. Data were expressed as mean ± S.D.

4.11.2.2 Dermal tissue

The results of the non-compartmental of terpinen-4-ol in dermal tissue after i.v. administration were summarized in Table 4.33. 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 shown in Figure 4.16, 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. The free dermal concentration declined with a mean terminal half-life of 1.17 ± 0.25 h. The mean area under the curve ($AUC_{0-\infty}$) was 0.20 ± 0.04 μg.hr/ml. The mean residence time (MRT_{inf}) was 1.84 ± 0.65 h. Based on the AUC ratios, terpinen-4-ol penetrates into dermal tissue resulting in $fAUC_{dermal}/fAUC_{plasma}$ of 0.20 ± 0.06. Data were expressed as mean ± S.D.

4.11.3 Compartmental analysis

All the plasma concentration-time profiles showed a remarkable distribution phase as shown in Figure 4.14. A two-compartmental model was able to produce a good curve fit of the average plasma concentrations. The coefficient of determination (r^2) was 0.987, which indicates reasonable curve fits. The respective parameters were α = 16.37 ± 5.36 and β = 0.75 ± 0.20 h^{-1} (mean ± S.D).

4.11.4 Skin trauma

The microdialysis probes were easily inserted in the dermis and rat skin responses to microdialysis and terpinen-4-ol were evaluated by visual observation during the experiment. All animals remained in good health throughout the study periods and no visual terpinen-4-ol and microdialysis probe related adverse effects such as rash, erythma, and edema were observed. Therefore, the experimental conditions used in this study were well tolerated by the rat.

Table 4.30 Individual plasma concentration-time data of terpinen-4-ol following intravenous injection (2 mg/kg)

Time (min)	Concentration (µg/ml)				Average	S.D.	% R.S.D.
	Rat 1	Rat 2	Rat 3	Rat 4			
5	7.66	7.44	missing	6.55	7.22	0.59	8.12
10	2.18	2.26	3.55	4.11	3.02	0.96	31.64
20	1.29	1.36	1.64	1.61	1.47	0.17	11.81
30	0.99	1.01	1.35	1.59	1.23	0.29	23.43
45	0.68	0.76	0.94	1.06	0.86	0.17	19.85
60	0.41	0.54	0.88	0.83	0.66	0.23	34.04
120	0.28	0.31	0.55	0.41	0.39	0.13	32.20
180	0.14	0.16	0.31	0.19	0.20	0.08	38.58
240	0.09	0.10	0.20	0.11	0.13	0.05	37.42
300	0.07	0.06	0.17	0.08	0.09	0.05	51.82
360	0.05	0.05	0.12	0.06	0.07	0.03	47.43
Recovery	54.96	55.95	50.68	46.64	52.06	4.28	8.21
Corrected recovery	37.28	38.66	31.32	25.69	33.23	5.95	17.91

Table 4.31 Individual dermal concentration-time data of terpinen-4-ol following intravenous injection (2 mg/kg)

Time (min)	Concentration (ng/ml)				Average	S.D.	% R.S.D.
	Rat 1	Rat 2	Rat 3	Rat 4			
10	89.15	92.90	33.80	178.69	98.63	59.82	60.65
30	78.20	84.57	37.61	126.00	81.59	36.18	44.34
50	62.64	78.48	34.25	100.91	69.07	28.02	40.57
70	55.49	76.74	34.21	82.64	62.27	22.04	35.40
90	41.47	53.30	37.79	52.81	46.34	7.90	17.04
110	32.21	42.62	41.72	40.45	39.25	4.78	12.18
130	29.37	38.97	28.52	39.63	34.12	5.99	17.56
150	21.56	33.59	38.10	23.87	29.28	7.86	26.84
170	20.89	26.67	24.03	> LLOQ	23.86	2.89	12.13
190	> LLOQ	23.09	15.42	> LLOQ	19.26	5.43	28.17
210	> LLOQ	14.05	21.59	> LLOQ	17.82	5.34	29.94
230	> LLOQ	> LLOQ	> LLOQ	> LLOQ	-	-	-
250	> LLOQ	> LLOQ	> LLOQ	> LLOQ	-	-	-
270	> LLOQ	> LLOQ	> LLOQ	> LLOQ	-	-	-
290	> LLOQ	> LLOQ	> LLOQ	> LLOQ	-	-	-
310	> LLOQ	> LLOQ	> LLOQ	> LLOQ	-	-	-
330	> LLOQ	> LLOQ	> LLOQ	> LLOQ	-	-	-
350	> LLOQ	> LLOQ	> LLOQ	> LLOQ	-	-	-
Recovery	54.96	55.95	50.68	46.64	52.06	4.27	8.21
Corrected recovery	37.28	38.66	38.66	25.69	35.07	6.29	17.93

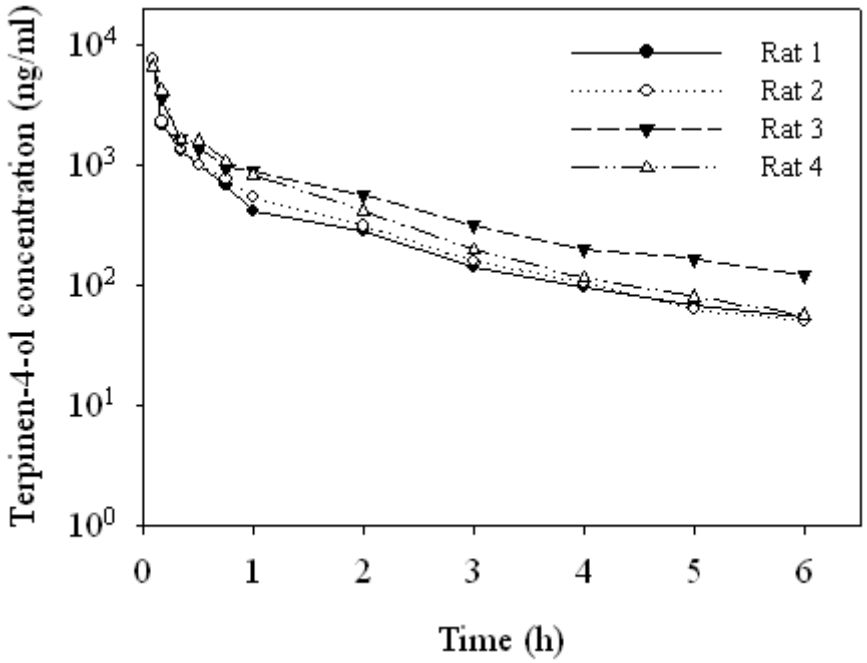

Figure 4.14 Individual plasma concentration-time profile of terpinen-4-ol following i.v. injection of terpinen-4-ol (2 mg/kg) in male Wistar rats (n = 4).

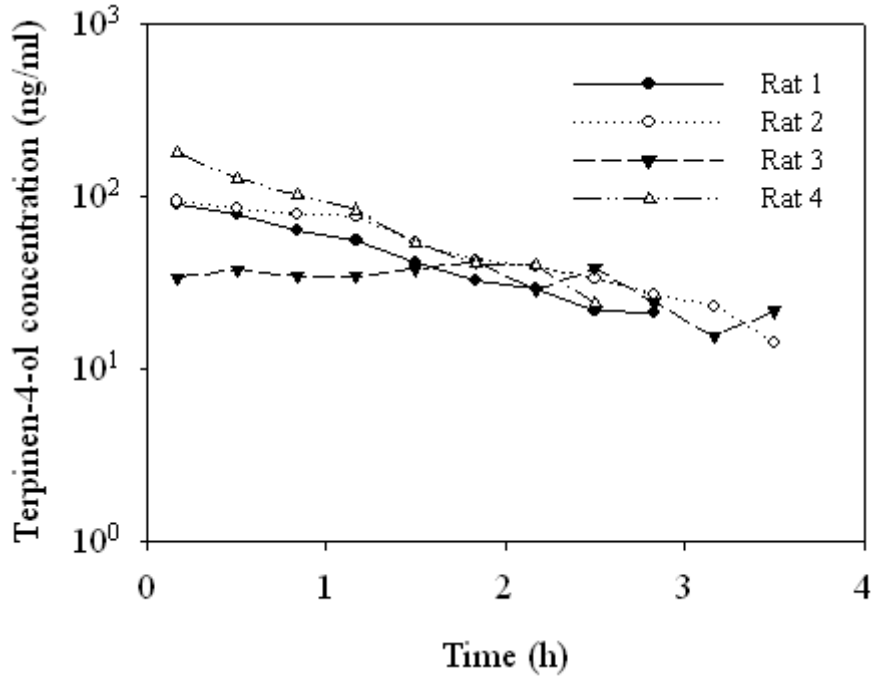

Figure 4.15 Individual free terpinen-4-ol concentration-time profile in dermal fluid following i.v. injection of terpinen-4-ol (2 mg/kg) in male Wistar rats (n=4).

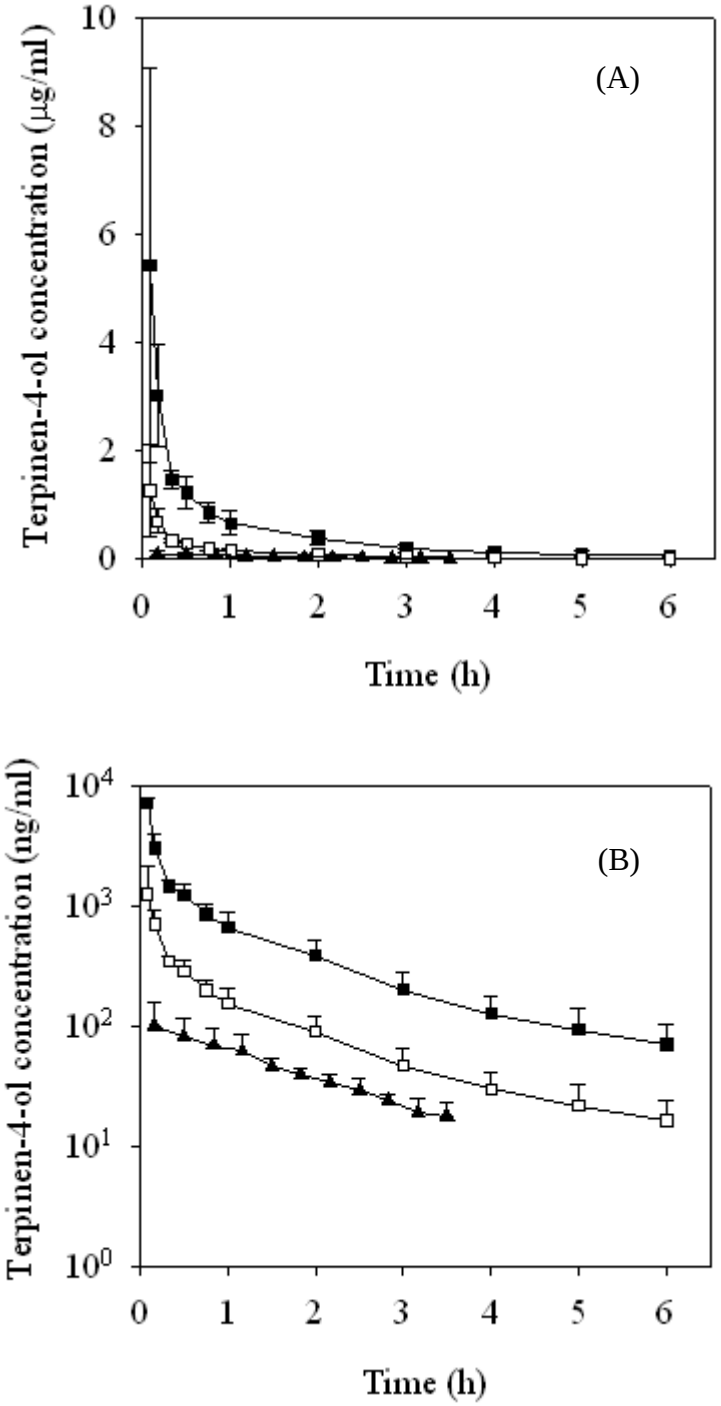

Figure 4.16 Normal (A) and semilogarithmic (B) scales of mean concentration-time profile in plasma (■), free plasma (□) and in the interstitial space fluid (free) of dermal tissue (▲) of terpinen-4-ol following i.v. injection (2 mg/kg) in male Wistar rats (mean ± S.D., *n*=4.).

Table 4.32 Pharmacokinetic parameters of terpinen-4-ol in plasma after i.v. bolus administration in rats (2 mg/kg)

Pharmacokinetic paramter	Rat 1	Rat 2	Rat 3	Rat 4	Average	S.D.	Median
λ_z (1/h)	0.32	0.49	0.41	0.35	0.39	0.07	0.38
$t_{1/2}$ (h)	2.14	1.42	1.69	1.97	1.80	0.32	1.83
t_{max} (h)	0.10	0.10	0.20	0.10	0.13	0.05	0.10
C_o (µg/ml)	26.91	24.56	16.72	10.46	19.66	7.52	20.64
C_{max} (µg/ml)	7.66	7.44	3.55	6.55	6.30	1.90	7.00
$AUC_{0\text{-}last}$ (µg.hr/ml)	3.79	3.82	4.84	3.82	4.07	0.52	3.82
$AUC_{0\text{-}\infty}$ (µg.hr/ml)	3.96	3.93	5.14	3.98	4.25	0.59	3.97
AUC_f (µg.hr/ml)	0.92	0.92	1.20	0.93	0.99	0.14	0.93
V_z (L/kg)	1.56	1.05	0.95	1.43	1.25	0.29	1.24
Clearance (L/hr/kg)	0.51	0.51	0.39	0.50	0.48	0.06	0.50
$AUMC_{last}$ (h.h.µg/ml)	2.54	2.75	5.10	3.56	3.48	1.16	3.15
$AUMC_{inf}$ (h.h.µg/ml)	4.03	3.58	7.55	4.98	5.04	1.78	4.51
MRT_{last} (h)	0.67	0.72	1.05	0.93	0.84	0.18	0.83
MRT_{inf} (h)	1.02	0.91	1.47	1.25	1.16	0.25	1.14
α (1/h)	18.99	17.88	20.17	8.45	1.16	0.25	18.43
β (1/h)	1.00	0.83	0.54	0.64	1.16	0.25	0.74

Table 4.33 Pharmacokinetic parameters of terpinen-4-ol in dermal tissue after i.v. bolus administration in rats (2 mg/kg)

Pharmacokinetic paramter	Rat 1	Rat 2	Rat 3	Rat 4	Average	S.D.	Median
λ_z (1/h)	0.59	0.57	0.48	0.82	0.62	0.15	0.58
$t_{1/2}$ (h)	1.17	1.22	1.44	0.84	1.17	0.25	1.20
t_{max} (h)	0.20	0.20	1.80	0.20	0.60	0.80	0.20
C_o (µg/ml)	0.10	0.10	0.03	0.23	0.11	0.08	0.10
C_{max} (µg/ml)	0.09	0.09	0.04	0.18	0.10	0.06	0.09
$AUC_{0\text{-}last}$ (µg.hr/ml)	0.14	0.19	0.11	0.22	0.16	0.05	0.16
$AUC_{0\text{-}\infty}$ (µg.hr/ml)	0.18	0.21	0.16	0.25	0.20	0.04	0.19
V_z (L/kg)	19.26	16.61	26.56	9.89	18.08	6.89	17.93
Clearance (L/hr/kg)	11.38	9.45	12.80	8.14	10.44	2.06	10.42
$AUMC_{last}$ (h.h.µg/ml)	0.14	0.24	0.18	0.18	0.18	0.04	0.18
$AUMC_{inf}$ (h.h.µg/ml)	0.30	0.37	0.43	0.29	0.35	0.06	0.33
MRT_{last} (h)	1.02	1.27	1.59	0.83	1.18	0.33	1.15
MRT_{inf} (h)	1.72	1.73	2.73	1.17	1.84	0.65	1.73
$AUC_{ISF}/fAUC_{plasma}$	0.09	0.03	0.05	0.05	0.06	0.02	0.05
%Free terpinen-4-ol in dermis	0.03	0.03	0.02	0.04	0.03	0.01	0.03

4.12 Dermal pharmacokinetics of terpinen-4-ol following topical application of plai oil

The dermal pharmacokinetics of terpinen-4-ol following topical administration of plai oil was performed under normal use and human exposure conditions to plai oil. Finite doses of the oil (2, 4 and 8 mg/cm^2) were applied on rat skin and dermal concentrations of terpinen-4-ol were continuously monitored by an implanted microdialysis probe.

The individual recovery and dermal concentration time data following topical application of plai oil at the doses of 2, 4 and 8 mg/cm^2 were summarized in Table 4.34, 4.35 and 4.36, respectiverly. The individual and mean free terpinen-4-ol concentration-time profiles in the interstitial space fluid of dermal tissue were shown in Figure 4.17, and 4.18, respectively.

4.12.1 Probe recovery

The individual probe recovery for each animal at each dose level was used to determined the actual free terpinen-4-ol concentration in the extracellular fluid of dermal tissue. The average *in vivo* recovery determined by retrodialysis was 51.6 ± 1.6 %, (mean ± S.D). After correction for probe binding (28.4 %), the recovery was 32.33 ± 2.28 %, (mean ± S.D) which is similar to the *in vitro* results.

4.12.2 Noncompartmental pharmacokinetic analysis

Results of the non-compartmental analysis of the dermal tissue data following topical application of plai oil at 2, 4 and 8 mg/cm^2 are summarized in Table 4.37, 4.38 and 4.39, respectively. The mean maximum concentration (C_{max}) of terpinen-4-ol in dermal fluid was 4.90 ± 0.93, 8.09 ± 0.76 and 10.68 ± 1.33 μg/ml with a median time to maximum concentration (t_{max}) of 0.65, 0.80 and 0.80 h for 2, 4 and 8 mg/cm^2 dose, respectively. The half-life ($t_{1/2}$) of terpinen-4-ol was 0.64 ± 0.22, 0.67 ± 0.19 and 0.71 ± 0.04 h following 2, 4 and 8 mg/cm^2 dose, respectively. The area under the concentration-time curve ($AUC_{0-\infty}$) was 5.31 ± 0.87, 11.23 ± 2.08 and 16.23 ± 1.35 μg.hr/ml. The mean residence time ($MRT_{0-\infty}$) was 0.97 ± 0.09, 1.20 ± 0.10 and 1.37 ± 0.07 h for 2, 4 and 8 mg/cm^2 dose, respectively. A one-way ANOVA found no

significant different between the doses for dose-normalized area under the curve ($AUC_{0-\infty}/D$). Therefore, terpinen-4-ol demonstrated linear pharmacokinetics in dermal tissue across the investigated dosage range. Data were expressed as mean ± S.D.

4.12.3 Skin trauma

The microdialysis probes were easily inserted in the dermis and rat skin responses to microdialysis, plai oil and terpinen-4-ol were evaluated by visual observation during the experiment. All animals remained in good health throughout the study periods and no visual plai oil and microdialysis probe related adverse effects such as rash, erythma, and edema were observed. Therefore, the experimental conditions used in this study were well tolerated by the rat.

Table 4.34 Individual dermal concentration-time data of terpinen-4-ol topical application of plai oil (2 mg/cm^2)

Time (min)	Concentration (μg/ml), Dose: 2 mg/cm^2				Average	S.D.	Median
	Rat 1	Rat 2	Rat 3	Rat 4			
0	0.00	0.00	0.00	0.00	0.0000	0.00	0.00
10	4.29	1.22	0.76	0.73	1.7495	1.71	0.99
30	6.00	5.36	3.95	4.00	4.8269	1.02	4.68
50	4.96	4.63	4.08	4.17	4.4629	0.41	4.40
70	2.69	2.69	2.36	2.56	2.5737	0.16	2.62
90	1.35	1.35	1.48	1.25	1.3573	0.09	1.35
110	0.56	0.75	0.79	0.63	0.6827	0.11	0.69
130	0.27	0.37	0.40	0.28	0.3293	0.06	0.33
150	0.17	0.20	0.28	0.14	0.1974	0.06	0.19
170	0.11	0.14	0.17	0.08	0.1224	0.04	0.12
190	0.07	0.09	0.11	0.04	0.0773	0.03	0.08
210	0.05	0.06	0.07	0.02	0.0528	0.02	0.06
230	0.05	0.04	0.04	0.02	0.0375	0.01	0.04
250	0.03	0.04	0.03	0.01	0.0286	0.01	0.03
270	0.03	0.03	0.03	0.01	0.0273	0.01	0.03
290	0.02	0.03	0.02	0.01	0.0188	0.01	0.02
310	0.01	0.02	>LLOQ	>LLOQ	0.0162	0.00	0.02
330	0.02	0.02	>LLOQ	>LLOQ	0.0197	0.01	0.02
350	0.02	0.02	>LLOQ	>LLOQ	0.0162	0.00	0.02
Recovery	54.37	49.78	51.40	50.29			
Corrected recovery	36.27	29.86	32.12	30.57			

Table 4.35 Individual dermal concentration-time data of terpinen-4-ol topical application of plai oil (4 mg/cm^2)

Time (min)	Concentration (μg/ml), Dose: 2 mg/cm^2				Average	S.D.	Median
	Rat 1	Rat 2	Rat 3	Rat 4			
0	0.00	0.00	0.00	0.00	0.0000	0.00	0.00
10	2.01	2.17	1.01	1.18	1.5900	0.58	1.59
30	7.21	7.58	7.50	7.72	7.5030	0.22	7.54
50	7.65	9.22	7.76	7.55	8.0441	0.79	7.70
70	5.23	8.82	6.32	5.92	6.5698	1.57	6.12
90	3.30	6.19	3.93	4.07	4.3724	1.26	4.00
110	1.74	3.68	2.28	2.07	2.4402	0.85	2.17
130	0.89	2.16	1.04	1.54	1.4079	0.57	1.29
150	0.52	1.23	0.63	0.85	0.8087	0.31	0.74
170	0.28	0.78	0.35	0.59	0.4986	0.23	0.47
190	0.21	0.44	0.19	0.44	0.3189	0.14	0.32
210	0.15	0.28	0.13	0.25	0.2043	0.07	0.20
230	0.11	0.20	0.03	0.17	0.1277	0.08	0.14
250	0.09	0.16	0.03	0.15	0.1090	0.06	0.12
270	0.07	0.14	0.02	0.07	0.0746	0.05	0.07
290	0.06	0.11	0.01	0.05	0.0548	0.04	0.05
310	0.04	0.11	0.02	0.09	0.0650	0.04	0.07
330	0.03	0.08	0.01	0.02	0.0363	0.03	0.03
350	0.03	0.08	0.00	0.09	0.0519	0.04	0.06
Recovery	52.32	52.81	53.82	48.78			
Corrected recovery	33.40	34.09	35.49	28.45			

Table 4.36 Individual dermal concentration-time data of terpinen-4-ol topical application of plai oil (8 mg/cm^2)

Time (min)	Concentration (μg/ml), Dose: 2 mg/cm^2				Average	S.D.	Median
	Rat 1	Rat 2	Rat 3	Rat 4			
0	0.00	0.00	0.00	0.00	0.0000	0.00	0.00
10	1.89	1.60	1.59	2.26	1.8373	0.31	1.75
30	12.43	7.09	9.49	9.31	9.5822	2.19	9.40
50	11.50	9.35	10.94	10.00	10.4490	0.96	10.47
70	10.40	8.62	9.10	8.72	9.2085	0.82	8.91
90	6.88	5.80	6.19	6.07	6.2348	0.46	6.13
110	3.87	3.41	4.25	3.77	3.8239	0.34	3.82
130	2.49	2.16	2.63	2.79	2.5181	0.27	2.56
150	1.55	1.54	1.82	1.84	1.6870	0.16	1.69
170	0.93	1.00	1.14	1.29	1.0902	0.16	1.07
190	0.57	0.69	0.85	1.05	0.7905	0.21	0.77
210	0.37	0.54	0.64	0.63	0.5445	0.13	0.58
230	0.30	0.26	0.48	0.52	0.3875	0.13	0.39
250	0.23	0.22	0.37	0.42	0.3096	0.10	0.30
270	0.18	0.18	0.27	0.31	0.2349	0.06	0.23
290	0.15	0.16	0.23	0.24	0.1962	0.05	0.19
310	0.11	0.11	0.18	0.15	0.1378	0.03	0.13
330	0.12	0.09	0.18	0.12	0.1255	0.04	0.12
350	0.09	0.10	0.13	0.10	0.1064	0.01	0.10
Recovery	52.12	50.51	51.00	51.41			
Corrected recovery	33.12	30.88	31.56	32.13			

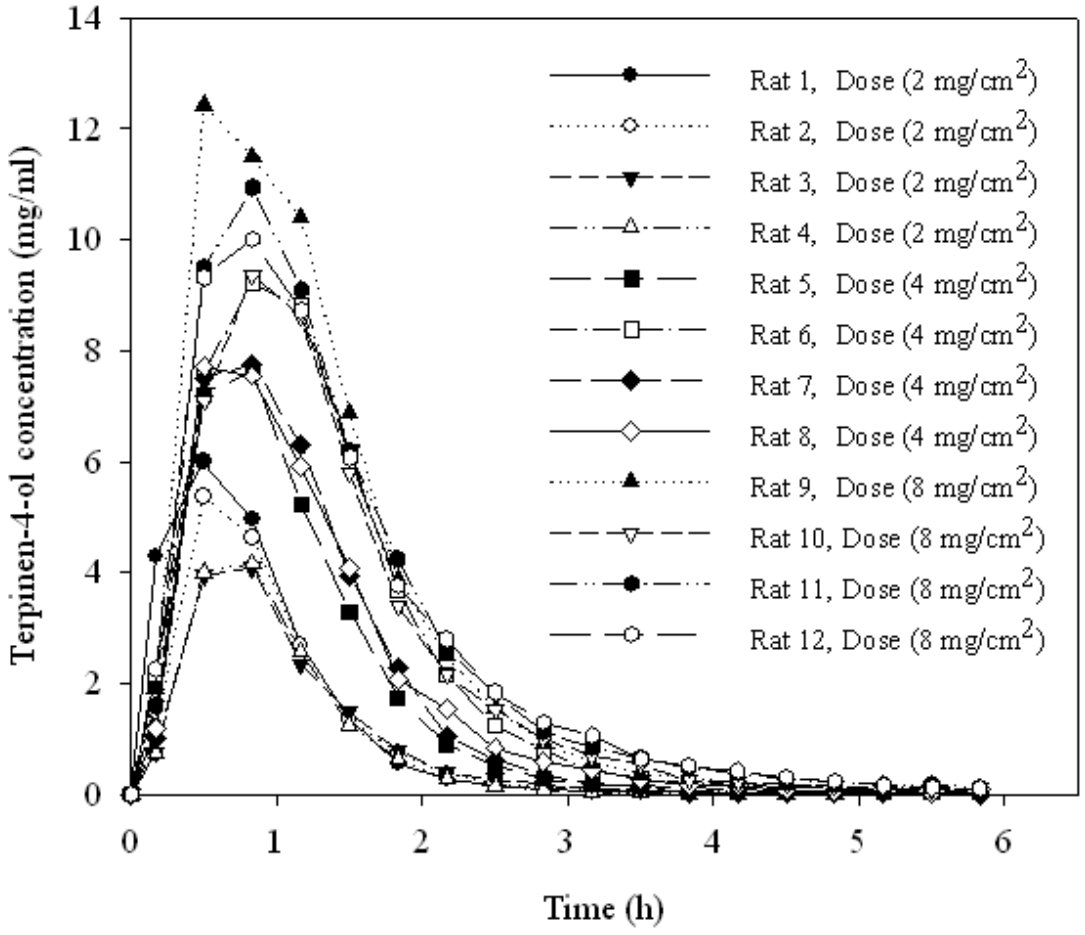

Figure 4.17 Individual dermal fluid free concentration-time profile of terpinen-4-ol following topical application of three different doses of plai oil (2, 4 and 8 mg/cm^2).

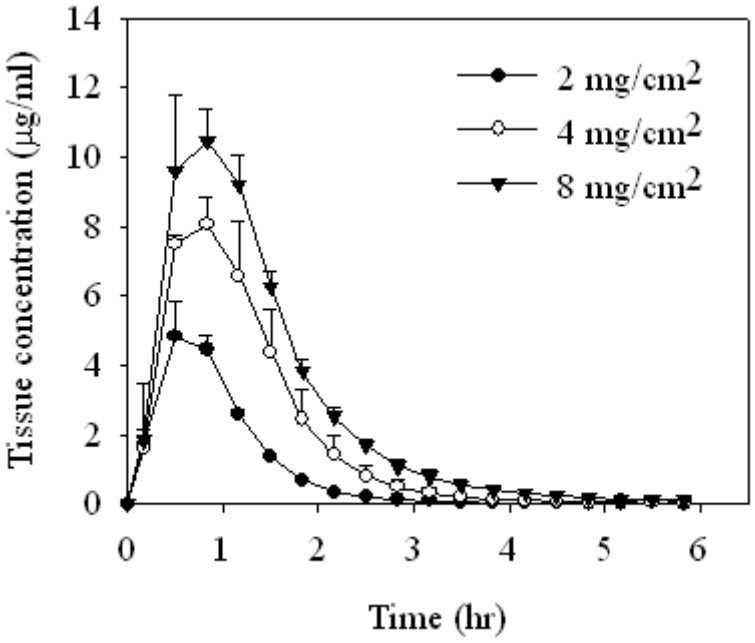

Figure 4.18 Mean unbound concentration-time profiles (mean ± S.D.) for terpinen-4-ol levels in dermal tissue after topical administration of 2 mg/cm^2 (●), 4 mg/cm^2 (○) and 8 mg/cm^2 (▼) of plai oil.

Table 4.37 Pharmacokinetic parameters of terpinen-4-ol in dermal tissue after topical application of plai oil (2 mg/cm^2)

Pharmacokinetic paramter	Rat 1	Rat 2	Rat 3	Rat 4	Average	S.D.	Median
C_{max} (µg/ml)	6.00	5.36	4.08	4.17	4.90	0.93	4.77
C_{max}/Dose (µg/ml/D)	3.00	2.68	2.04	2.08	2.45	0.47	2.38
t_{max} (h)[a]	0.50	0.50	0.80	0.80	0.65	0.17	0.65
$AUC_{0\text{-last}}$ (µg.hr/ml)	6.43	5.50	4.73	4.54	5.30	0.86	5.11
$AUC_{0\text{-}\infty}$ (µg.hr/ml)	6.45	5.52	4.74	4.54	5.31	0.87	5.13
$AUC_{0\text{-}\infty}$ /Dose (µg.hr/ml/D)	3.22	2.76	2.37	2.27	2.66	0.43	2.57
$t_{1/2}$ (h)	0.58	0.97	0.51	0.51	0.64	0.22	0.55
MRT_{last} (h)	0.84	0.98	1.05	0.96	0.96	0.09	0.97
MRT_{inf} (h)	0.85	1.01	1.06	0.97	0.97	0.09	0.99
%Free terpinen-4-ol in dermis	0.47	0.40	0.35	0.33	0.39	0.06	0.37

Table 4.38 Pharmacokinetic parameters of terpinen-4-ol in dermal tissue after topical application of plai oil (4 mg/cm^2)

Pharmacokinetic paramter	Rat 1	Rat 2	Rat 3	Rat 4	Average	S.D.	Median
C_{max} (µg/ml)	7.65	9.22	7.76	7.72	8.09	0.76	7.74
C_{max}/Dose (µg/ml/D)	1.91	2.30	1.94	1.93	2.02	0.19	1.93
t_{max} (h)[a]	0.80	0.80	0.80	0.50	0.73	0.15	0.80
$AUC_{0\text{-}last}$ (µg.hr/ml)	9.59	14.19	10.23	10.70	11.18	2.06	10.46
$AUC_{0\text{-}\infty}$ (µg.hr/ml)	9.63	14.27	10.23	10.78	11.23	2.08	10.50
$AUC_{0\text{-}\infty}$ /Dose (µg.hr/ml/D)	2.41	3.57	2.56	2.69	2.81	0.52	2.63
$t_{1/2}$ (h)	0.93	0.65	0.48	0.63	0.67	0.19	0.64
MRT_{last} (h)	1.09	1.27	1.11	1.22	1.17	0.09	1.16
MRT_{inf} (h)	1.12	1.30	1.11	1.26	1.20	0.10	1.19
%Free terpinen-4-ol in dermis	0.35	0.52	0.37	0.39	0.41	0.08	0.38

Table 4.39 Pharmacokinetic parameters of terpinen-4-ol in dermal tissue after topical application of plai oil (8 mg/cm^2)

Pharmacokinetic paramter	Rat 1	Rat 2	Rat 3	Rat 4	Average	S.D.	Median
C_{max} (µg/ml)	12.43	9.35	10.94	10.00	10.68	1.33	10.47
C_{max}/Dose (µg/ml/D)	1.55	1.17	1.37	1.25	1.34	0.17	1.31
t_{max} (h)[a]	0.50	0.80	0.80	0.80	0.73	0.15	0.80
$AUC_{0\text{-}last}$ (µg.hr/ml)	17.64	14.08	16.55	16.20	16.12	1.49	16.37
$AUC_{0\text{-}\infty}$ (µg.hr/ml)	17.72	14.19	16.69	16.31	16.23	1.49	16.50
$AUC_{0\text{-}\infty}$ /Dose (µg.hr/ml/D)	2.22	1.77	2.09	2.04	2.03	0.19	2.06
$t_{1/2}$ (h)	0.67	0.68	0.75	0.73	0.71	0.04	0.71
MRT_{last} (h)	1.24	1.34	1.37	1.38	1.33	0.07	1.35
MRT_{inf} (h)	1.26	1.38	1.41	1.42	1.37	0.07	1.40
%Free terpinen-4-ol in dermis	0.32	0.26	0.30	0.30	0.30	0.03	0.30

CHAPTER V
DISCUSSION

Since the protein binding plays an important role in drug distribution, many techniques have been developed to determine the plasma protein binding of the compounds. Terpinen-4-ol binding to rat and human plasma was determined by ultrafiltration method at different concentrations of terpinen-4-ol to investigate its binding characteristics. The results of this study demonstrate that the human plasma protein binding rates of terpinen-4-ol in its pure form (78.50 %) was close to those of plai oil (79.11 %) suggesting that the protein binding was not interfered by other compounds in the oil. Furthermore, there was no significantly different between the protein binding of terpinen-4-ol in rat plasma (76.67 %) and human plasma. Theoretically, at steady state, free drug levels in plasma and tissues should be equal assuming that tissue distribution of drug molecules only depends on passive diffusion. Based on these assumptions, the total plasma concentrations and plasma protein binding values can be used to predict the free tissue concentrations. However, this statement does not always hold true because many studies have shown the discrepancy between free drug levels in plasma versus tissue (133-136).

In addition to plasma protein binding, the blood to plasma ratio is also the essential parameter in interpreting pharmacokinetics data. The different distribution of drug in blood fractions may have great effect on its pharmacokinetic behaviors. Our present study was designed to study the characteristics of terpinen-4-ol distribution in human blood by measuring blood to plasma ratio of terpinen-4-ol in its pure form and plai oil *in vitro*. The distributions of terpinen-4-ol in its pure form and plai oil were concentration-dependent and concentration-independence, respectively. Therefore, the forms of terpinen-4-ol should be taken into consideration when its pharmacokinetic characteristics are evaluated. Furthermore, for practical reasons, analysis of plasma or serum should be preferred over analysis of whole blood due to higher analyte concentrations, an easier handling during sample processing cleaner extracts.

The conventional pharmacokinetic profiles of active compounds are described mainly through the plasma concentration-time curves which is clearly appropriate for most of the orally and intravenously administered drugs. However, many topical formulations, such as local anesthetics and topical anti-inflammatory products, are designed to target local tissues which limited systemic absorption. Therefore, the pharmacokinetic measurements in the local tissue are more clinically relevant than the systemic measurement.

Many *in vivo* techniques have been proposed to investigate dermal pharmacokinetics of topical formulations. Amongst these, microdialysis and tape stripping are the most widely used for cutaneous drug delivery assessments. Nevertheless, the skin stripping technique only assesses the penetration of drug into the stratum corneum which is usually not the target tissue and can only determine a single concentration-time point per administration site. Furthermore, it is important to realize that only the free tissue concentrations of active compounds at the target site are responsible for therapeutic effect. With the use of microdialysis technique, sampling can be performed continuously and complete unbound concentration-time profiles can be obtained from each sampling site.

The main purpose of this study was to investigate pharmacokinetics of terpinen-4-ol following intravenous (i.v.) bolus injection and topical administration. The concentrations of terpinen-4-ol in plasma and microdialysate were measured by conventional blood sampling and microdialysis, respectively. Microdialysis sampling of the dermis was accomplished using a linear microdialysis probe. Since the probe is continuously perfused with fresh perfusate, a total equilibrium across the membrane cannot be established. Consequently, the concentration in the dialysate will always represent a fraction of the actual concentration in the tissue. Therefore, calibration of the probe is very important to determine how much analyte of interest can be recovered by the microdialysis probe. In the present study, the recovery of terpinen-4-ol was assessed according to the retrodialysis technique (132). The principle of this method is based on the assumption that diffusion process is quantitatively equal in both directions through the semipermeable membrane.

Before using the microdialysis technique in vivo, the in vitro studies were investigated to find the optimal experimental conditions for terpinen-4-ol.

Furthermore, since the analyte concentration is expected to change overtime, it is important to verify if the probe recovery remains constant over different analyte concentrations. The *in vitro* microdialysis studies results also clearly demonstrate that recoveries determine by dialysis and retrodialysis were independent of concentration but inversely dependent on perfusion flow rate. Furthermore, at each flow rate, the relative loss was significantly higher than relative recovery as a result of terpinen-4-ol binding to the inlet of microdialysis catheter. Previous studies have shown that some moderately or highly lipophilic compounds bind to the microdialysis probe leading to overestimation of recovery and would therefore underestimation of the tissue concentration (22, 88-90). There are several methods have been proposed to eliminate the effect of non-specific binding on the recovery, such as the addition of albumin (91, 92) and cyclodextrin (93) in the perfusion fluid. In the present study, the correction of recovery by using the degree of analyte binding to the probe (89, 90) was used to minimize this limitation. The results demonstrate that percent binding to the inlet probe was independent of concentration but influenced by the perfusion flow rate. With increasing perfusion flow rate, the binding effect is less pronounced since the contact time between the analyte and the tubing decrease. After correction for inlet probe binding, the relative loss was not statistically different from the relative recovery. Therefore, this correction method is considered efficient in terms of eliminating nonspecific binding issue for terpinen-4-ol.

Following i.v. injection, terpinen-4-ol rapidly distributed into the dermis and its concentrations in the dermal tissue was lower than the unbound and total plasma concentrations for the entire length of the experiment. The concentration-time profiles of terpinen-4-ol in plasma can be described by a two-compartmental model. These finding indicates that plasma level 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 levels is necessary.

The dermal pharmacokinetics of terpinen-4-ol following topical administration of plai oil was performed under normal use and human exposure conditions to plai oil. Finite doses of the oil (2, 4 and 8 mg/cm^2) were applied on rat skin and dermal concentrations of terpinen-4-ol were continuously monitored by an

implanted microdialysis probe. After topical application of the oil, terpinen-4-ol rapidly distributed into the dermis which is in good agreement with the *in vitro* skin penetration results obtained from diffustion cells and skin stripping method (12-15, 17, 18). The elimination half-lives were not significantly different between dosing groups. The mean dose-normalized $AUC_{0-\infty}$ ($AUC_{0-\infty}$/Dose) between the doses of 2 and 8 mg/cm^2 showed no statistically significant different suggesting a dose proportional increase in $AUC_{0-\infty}$. However, dose-normalized $AUC_{0-\infty}$ tends to decrease with increasing dose from 4 to 8 mg/cm^2 which may result from the loss of terpinen-4-ol due to evaporation from the skin surface. In this present study, the oil was applied under non-occlusive conditions which allow any volatile component of the test preparation to evaporate and the normal drying process to occur (137). The loss of active compounds by surface evaporation would thereby reduce the topical dose available for absorption, leading to lower dose-normalized $AUC_{0-\infty}$. This may have implications in the assessment of skin absorption, particularly when absorption is assessed in terms of percent of applied dose (138)**.** The extent of evaporation from the skin surface is a function of the dose applied, airflow, and temperature of the skin surface which could have a major impact on the results of *in vivo* skin absorption studies (139). In addition, anatomic conditions, the mode of application (e.g., repetitive administration), or massaging may also potentially influence the amount of terpinen-4-ol penetration. So, these variables should be taken into consideration and strictly controlled during the skin penetration study.

The principal finding of this study was that the topical administration of plai oil led to greater percentage of unbound AUC of terpinen-4-ol per amount of administered in dermis compared to that of i.v. injection of terpinen-4-ol. The mean percentage of free terpinen-4-ol in dermis following i.v. injection (0.03 ± 0.03%) was about 10-14 times lower than those of topical application of plai oil (0.39 ± 0.06%, 0.41 ± 0.08% and 0.30 ± 0.03% for 2, 4 and 8 mg/cm^2 doses, respectively). From a pharmacokinetic point of view, these results clearly suggest that topical application of plai oil could achieve higher concentrations in the tissues subjacent to the site of application which may offer the advantages of local anti-inflammatory effect.

CHAPTER VI
CONCLUSION

6.1 Development and validation of GC-MS methods for the determination of terpinen-4-ol in biological samples and plai oil

This study reports the development and validation of quantification methods for terpinen-4-ol in rat dermal microdialysate, rat plasma, human plasma, human blood and plai oil by a gas chromatographic-mass spectrometric method using methyl salicylate as internal standard. Extraction of terpinen-4-ol and IS from biosamples was carried out with a simple liquid-liquid extraction using hexane. The LLOQ for all matrices was sufficient for the purpose of conducting pharmacokinetic studies. Accuracy and precision were good for all matrices and all conditions investigated, with %R.E. and %R.S.D. values below 15%. Terpinen-4-ol was considered to be stable in biological matrices under several conditions investigated during sample collection, preparation and determination. The methods met the acceptance criteria according to the FDA guidance for bioanalytical method validation and were shown to be applicable for quantification of terpinen-4-ol in biological samples and plai oil. Furthermore, the methods were sensitive, selective, precise, and accurate for the determination of terpinen-4-ol in all matrices and were successfully applied to rat and human plasma protein binding, human blood to plasma ratio *in vitro* and *in vivo* pharmacokinetic studies.

6.2 The investigation of aqueous solubility and basic pharmacokinetic parameters

From the aqueous solubility study of terpinne-4-ol, the results demonstrate that the solubility of pure form was significantly higher than those of the oil ($p<0.05$). This indicates that terpinen-4-ol in plai oil predominantly partitions in the oil as compared to the reference standard. The protein binding and blood to plasma ratio of

terpinen-4-ol were investigated *in vitro* at different concentrations. The human plasma protein binding rates of terpinen-4-ol in its pure form was not significantly different from those of plai oil ($p<0.05$), suggesting that the protein binding was not interfered by other compounds in the oil. Furthermore, at the same concentrations of terpinne-4-ol in rat and human plasma, there was no significantly different between the protein binding of terpinen-4-ol in rat plasma and human plasma. For the blood to plasma ratio studies, it was demonstrated that terpinen-4-ol obviously tend to accumulate in human plasma and the blood to plasma ratio of pure terpinen-4-ol were different from those of terpinen-4-ol contained in plai oil. The distributions of terpinen-4-ol in its pure form and plai oil were concentration-dependent and concentration-independence, respectively. Therefore, the forms of terpinen-4-ol should be taken into consideration when its pharmacokinetic characteristics are evaluated. The results provide a basis for a further understanding of the behavior of terpinen-4-ol *in vivo*.

6.3 The *in vitro* microdialysis and probe binding

The *in vitro* microdialysis studies clearly demonstrate that the percentage of inlet probe binding, relative recovery and loss were independent of concentration but inversely dependent on perfusion flow rate. At each flow rate, the relative loss was significantly higher than relative recovery as a result of terpinen-4-ol binding to the inlet of microdialysis catheter. The correction of recovery by using the degree of analyte binding to the probe was used to minimize this limitation. The results demonstrate that after correction for inlet probe binding, the relative loss was not statistically different from the relative recovery. Therefore, this correction method is considered efficient in terms of eliminating nonspecific probe binding issue for terpinen-4-ol.

6.4 The *in vivo* pharmacokinetics of terpinen-4-ol

This study demonstrates that cutaneous microdialysis is an effective and minimally invasive tool to evaluate the dermal pharmacokinetics of terpinen-4-ol

following systemic or topical administration**.** Following i.v. bolus injection, terpinen-4-ol rapidly distributed into the dermis and its concentrations in the plasma exceed free concentrations in dermal tissue for the entire length of the experiment. Therefore using plasma concentrations would overestimate the dermal concentrations and probably clinical efficacy. After topical application of plai oil at three different doses (2, 4 and 8 mg/cm^2), terpinen-4-ol demonstrated linear pharmacokinetics in dermal tissue with no changes in dose-normalized area under the concentration-time curves across the investigated dosage range. The mean percentage of free AUC of terpinen-4-ol per amount of administered in dermis following i.v. injection was about 10-14 times lower than those of topical application of plai oil suggesting that topical application of plai oil may offer the advantages of local anti-inflammatory effect. The plasma and dermal pharmacokinetics of terpinen-4-ol would provide information for its further formulation development and therapy schedules. In addition, rat skin is typically two to ten times more permeable than human skin. Thus, to obtain more clinically relevant details, dermal pharmacokinetics of terpinen-4-ol should be further investigated in human.

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APPENDICES

APPENDIX A

DOCUMENTARY PROOF OF ETHICAL CLEARANCE

COA. No. MU-IRB 2009/008.1702

Documentary Proof of Mahidol University Institutional Review Board

Title of Project: Novel Pharmaceutical Formulations of Phlai Oil (Zingiber cassumunar Roxb): Preformulation, Formulation Development and Formulation Evaluation (Thesis for Ph.D.)

Principle Investigator: Miss Kotchaphan Chooluck

Name of Institution: Faculty of Pharmacy

Approval includes: 1) MU-IRB Submission form version received date 16 February 2009
2) Participant Information sheet version date 16 February 2009
3) Informed Consent form version date 16 February 2009

Mahidol University Institutional Review Board is in full compliance with International Guidelines for Human Research Protection such as Declaration of Helsinki, The Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Date of Approval: 17 February 2009

Date of Expiration: 16 February 2010

Signature of Chairman: .....
(Professor Shusee Visalyaputra)

Signature of Head of the Institute: .....
(Associate Professor Sansanee Chaiyaroj)
Vice President for Research and Academic Affairs

Office of the President, Mahidol University, 999 Phuttamonthon 4 Rd., Salaya, Phuttamonthon District, Nakhon Pathom 73170. Tel. (662) 8496223-5 Fax. (662) 8496223

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The Foundation for The Gator Nation

Notification of Initial Approval

From:	Michael Katovich
To:	Hartmut Derendorf
CC:	Kotchaphan Chooluck
Re:	Continuing Review ID: IC00001499 2011 Review for 201004187
Title:	Investigation of dermal penetration of terpinen-4-ol using microdialysis.

I am pleased to inform you that the continuation for study 201004187 has been approved. You are required to return to this site at least 60 days prior to 6/7/2012 and file a Continuation or a Triennial.

If this IACUC protocol pertains to a sponsored research project it is the responsibility of the PI to forward a copy of IACUC approval and associated PeopleSoft Project number to the Office of Award Administration via Fax at (352)392-4522 or email at ufawards@rgp.ufl.edu

Sincerely,

Michael Katovich

IACUC Chair

Institutional Animal Care & Use Committee
PO Box 100142
Gainesville, Florida 32610-0142
Tele: (352) 273-9535 Fax: (352) 273-9538

APPENDIX B
INTERNATIONAL CONFERENCE MEETING ABSTRACT
(THE 3rd ASIAN PACIFIC REGIONAL ISSX)

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139. Method Development and Validation for Determination of 3,4-Methylenedioxymethamphetamine, Methylenedioxyamphetamine, and Methamphetamine in Human Urine Using GC-FID

Benjamas Janchawee,[1] Apichai Phonchai,[2] Sathaporn Prutipanlai,[1] and Sittipoom Thainchaiwattana[3]

[1]*Department of Pharmacology, Faculty of Science, Prince of Songkla University, Songkhla, Thailand*
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A gas chromatography with a flame ionization detection (GC-FID) method was developed and validated for the simultaneous determination of 3,4-methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), and methamphetamine (MA) in human urine. Alkalinized (pH 12) samples were prepared by solid-phase extraction, using an Oasis® HLB cartridge (Oasis catridge, Waters, Milford, MA, USA). The cartridge was washed with a 5% methanol-water mixture containing 2% ammonium hydroxide and eluted with a 70% methanol-water mixture containing 2% acetic acid. One microliter of the reconstituted sample was injected into the GC system, using a CP-Sil 24 CB WCOT fused silica capillary column (30 m × 0. 32 mm i.d.; 0.25-μm film thickness). Flow rate of the carrier gas (N_2) was 2.6 mL min^{-1}. Flow rates of both fuel gas (H_2) and make-up gas (N_2) were 30 mL min^{-1}. Oxidant gas (O_2) flow rate was 300 mL min^{-1}. Temperatures of the injector and the detector were 290 and 300°C, respectively. Column temperature was initially 80°C (0 minutes) and increased with a ramp rate of 20°C min^{-1} to a final temperature of 270°C, where it was held for 1 minute. The analytes were eluted at 4.2, 6.8, 7.1, and 9.1 minutes for MA, MDA, MDMA, and DPA (internal standard), respectively. Calibration curves were linear over the concentration range of 1–20 μg mL^{-1} for MDMA (r = 1.0000), MDA (r = 0.9971), and MA (r = 0.9998). The intra- and interday precisions (%RSD) were ranged from 2.97 to 17.31% for MDMA, 6.28 to 16.54% for MDA, and 6.86 to 17.18% for MA, respectively. The accuracy (%DEV) ranged from (–) 16.68 to (+) 4.28% for MDMA, (–) 18.10 to (+) 14.94% for MDA, and (–) 18.47 to (+) 0.60 for MA, respectively. The extraction recoveries ranged from 84.17 to 95.01% for MDMA, 92.56 to 107.60% for MDA, and 80.35 to 90.74% for MA. The lower limit of quantification (LLOQ) of all analytes was 1 μg mL^{-1}.

140. Physicochemical Characteristics Related to Decomposition of Quercetin

Saengrawee Sutthiparinyanont,[1] Aroonsri Priprem,[1] and Malyn Chulasiri[2]

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[2]*Reseach and Development, S&J International Enterprises PCL, Bangkok, Thailand*

Quercetin (MW 338.3 g/mol), a flavonol compound, is generally found in fruits, vegetables, flowers, botanicals, and beverages. It is a potent antioxidant that could be potentially used as for antiaging and photoprotection. Its physicochemical properties in relevance to stability in aqueous environments provide better product development, particularly for external use. Various techniques were used to elaborate its properties and interactions with solvents at 25°C. Its crystallographic structure by XRD suggests a crystalline form of initial and stored quercetin dihydrate powder. NMR showed that C3-OH is involved in intramolecular bonding; also, FT-IR spectrophotometry revealed the attacking at C3-OH of quercetin in storage aqueous conditions, toward protocatechuic acid (MW 154.1 g/mol), when it was accelerated by light and elevated temperature, as confirmed by UV spectrophotometry. Thermal analysis by DSC and TGA indicates physicochemical changes of quercetin with three major steps of phase-transition temperature at about 116, 320, and 350°C. Its zero-order decomposition at 25°C was also shown by a series of stability profiles of a range of solvents and the effect of some additives. In the aqueous system, quercetin presents as almost insoluble in water (0.2 and 0.7 μg/mL at 25 and 37°C), and the solubility was a little bit increased when adding phosphate ions (1.1 and 4.3 μg/mL at 25 and 37°C), which also enhanced protocatechuic acid production and reduced activation energy (Ea). Solubilities and decomposition profiles in some alcohols and polyols, polymers, surfactants, and albumin will be discussed. Partition coefficient, log koctanol/water of quercetin of 2.48 at 25°C suggests a concentration-dependent permeability. It could be concluded that quercetin is a hydrophobic compound with planar structure, but its affinity with certain aqueous moieties could be induced via C3-OH, which is the most active site and is prone to oxidative degradation and complex formation of this compound.

141. Preliminary Evaluation of GC-MS for the determination of Terpinen-4-Ol in Cutaneous Microdialysis Samples

Kotchaphan Chooluck,[1] Kittisak Sripha,[2] Hartmut Derendorf,[3] and Korbtham Sathirakul[4]

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Terpinen-4-ol (T4) is the main active ingredient in Phlai oil, obtained from steam distillation of the rhizome of *Zingiber cassumunar* Roxb., which was commonly used as topical anti-inflammatory herbal medicine. The aim of this work was to develop an analytical method for the assessment T4 in cutaneous microdialysis samples by means of gas chromatography-mass spectrometry (GC-MS). In order to confirm a suitable method for intended use of the procedure employed for this determination, our developed analytical method needs to be firstlvalidated. The calibration curve demonstrated a linear relationship between the peak-area ratio of T4 and methyl salicylate (MeS), which was used as an internal standard, over a range of T4 concentrations (0.36–1.79 ppm). The intra- and interday precisions at all concentrations tested were less than 1.5 and 4.0% RSD, respectively. The recoveries of T4 were in the range of 101.22–111.44%. The analyte was shown to be stable in working standard solutions after 40 hours at room temperature and in standard stock solutions after 3 days at –20°C without a relevant loss of signal. The limit of detection and quantification were 0.0294 and 0.0883 ppm, respectively. According to the results, our analytical method met the requirement of method validation followed by the International Conference on Harmonisation guideline. However, based on this preliminary evaluation, further method testing of this developed approach in the conditions of cutaneous microdialysis should be performed. Thereafter, it can be applied to the determination of T4 in topical formulations by using a microdialysis model for the dermatopharmacokinetic study. This study will be Thailand's first pharmacokinetic study of Thai herbal medicine.

142. Solid-Phase Extraction and Method Validation of Mitragynine in Urine by HPLC Technique

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Mitragynine is an indole alkaloid extracted from the leaves of *Mitragyna speciosa* Korth. (Rubiaceae). The mitragynine level in serum is developed, but its level in urine was not conducted. Therefore, an extraction procedure for mitragynine from urine was implemented in this study. The analyses were extraction from rat urine by solid-phase extraction on an Oasis® HLB SPE column (Oasis, Waters, Milford, MA, USA) and quantitative analysis by high-performance liquid chromatography with ultraviolet detection. The separation system consisted of a C18 column heated to 35°C, a methanol-water (80:20, v/v) mobile phase, and a flow rate of 0.8 mL/min. Mitragynine, with a retention time of 9.2 minutes, was well resolved from any interference in rat urine. The calibration curve was linear from 0.1 to 10 μg/mL ($r = 0.9992$). The method of mitragynine extraction from urine, using HLB®, shows good validation parameters with a rate of recovery rate over 94%. The intra- and interday precision rates of the method were 0.67–3.28% RSD and 0.74–3.49% RSD, respectively. The accuracy ranged from –2.69 to +13.72% DEV. The lower limit of quantification was 0.1 μg/mL.

143. Validated HPLC for Analysis of Flavonoid Content in Siamese Neem Young Flowers

Worarat Chaisawangwong and Wandee
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The Siamese neem tree (*Azadirachta indica* A. Juss. var. *siamensis* Valeton) is a medicinal plant found in every part of Thailand. The young flower is consumed as a bitter tonic vegetable. Moreover, it has been used as an element tonic and for the treatment of fever. The flower extract was reported to exhibit *in vitro* free radical–scavenging activity and can inhibit lipid peroxidation of the bronchogenic cancer cell line. Active compounds in the flowers are flavonoids, such as rutin and quercetin. To evaluate the quantity of active components in Siamese neem young flowers, a high-performance liquid chromatographic (HPLC) method was developed for the assessment of two bioactive flavonoids: rutin and quercetin in the aqueous extract of this plant. A Hypersil® BDS C18 column (Hypersil column, Thermo Fisher Scientific, Waltham, MA, USA) (250 × 4.6 mm, 5-μm particle size) was used. The elution was carried out with isocratic solvent systems with a flow rate of 1 mL/min at an ambient temperature. The mobile phase consisted of 0.5% formic acid in methanol:water (3:7) (A) and methanol (B). Quantitaitve analysis was programmed at A:B (8.5:1.5). The wavelength of the UV-vis detector was set at 254 nm. Separation of the two

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PUBLICATIONS

Kotchaphan Chooluck , Kittisak Sripha, Hartmut Derendorf, Korbtham Sathirakul. Preliminary evaluation of GC-MS for the determination of Terpinen-4-ol in cutaneous microdialysis samples. Drug Metabolism Reviews. 2009 August; 41 (Suppl 2): S64-5. (Meeting Abstract)

POSTER PRESENTATIONS

Kotchaphan Chooluck , Kittisak Sripha, Hartmut Derendorf, Korbtham Sathirakul. Preliminary evaluation of GC-MS for the determination of Terpinen-4-ol in cutaneous microdialysis samples, The 3rd Asian Pacific Regional ISSX, The Imperial Queen's Park Hotel, Bangkok, Thailand, May 10–12, 2009.

ORAL PRESENTATION

Kotchaphan Chooluck, Hartmut Derendorf, Korbtham Sathirakul. Dermal pharmacokinetics of terpinen-4-ol following topical application of Plai oil: A microdialysis study, The RGJ-Ph.D. Congress XIII, Jomtien Palm Beach Resort, Pattaya, Chonburi, Thailand, April 1-3, 2010.