การตั้งตำรับยาเตรียมเฉพาะครีมแอมโฟเทอริซินบีที่มีไดเมทิลซัลฟอกไซด์ปริมาณสูงเพื่อใช้เฉพาะที่สำหรับรักษาโรคเชื้อราที่เล็บ

นางสาวพจนา โกเมศมุนีบริรักษ์

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FORMULATION OF EXTEMPORANEOUS AMPHOTERICIN B TOPICAL PREPARATIONS CONTAINING HIGH AMOUNT OF DIMETHYL SULFOXIDE FOR THE TREATMENT OF ONYCHOMYCOSIS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Department of Pharmaceutics and Industrial Pharmacy
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Thesis Title	FORMULATION OF EXTEMPORANEOUS AMPHOTERICIN B TOPICAL PREPARATIONS CONTAINING HIGH AMOUNT OF DIMETHYL SULFOXIDE FOR THE TREATMENT OF ONYCHOMYCOSIS
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พจนา โกเมศมุนีบริรักษ์ : การตั้งตำรับยาเตรียมเฉพาะคราวแอมโฟเทอริซินบีที่มีไดเมทิลซัลฟอกไซด์ปริมาณสูงเพื่อใช้เฉพาะที่สำหรับรักษาโรคเชื้อราที่เล็บ (FORMULATION OF EXTEMPORANEOUS AMPHOTERICIN B TOPICAL PREPARATIONS CONTAINING HIGH AMOUNT OF DIMETHYL SULFOXIDE FOR THE TREATMENT OF ONYCHOMYCOSIS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ร.ต.ท.หญิง วลัยศิริ ม่วงศิริ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. พรเพ็ญ วีระวัฒกานนท์, 102 หน้า.

การศึกษาวิจัยนี้มีวัตถุประสงค์เพื่อตั้งตำรับยาเตรียมเฉพาะคราวแอมโฟเทอริซินบีที่มีความคงตัวสำหรับใช้เฉพาะที่เพื่อรักษาโรคเชื้อราที่เล็บ และสามารถกำหนดอายุของผลิตภัณฑ์ที่เตรียมขึ้นได้ ซึ่งปัจจุบันยังไม่มีผลิตภัณฑ์ยาแอมโฟเทอริซินบีสำหรับใช้เฉพาะที่วางจำหน่ายทั่วไปในประเทศไทย อีกทั้งยังไม่มีข้อมูลสำหรับการศึกษาความคงตัวของผลิตภัณฑ์รูปแบบนี้ออกมาเผยแพร่ ยาเตรียมเฉพาะคราวแอมโฟเทอริซินบีในงานวิจัยนี้ได้เตรียมขึ้นจากยาแอมโฟเทอริซินบีในรูปแบบยาฉีดที่มีจำหน่ายในปัจจุบัน โดยตำรับที่ได้พัฒนาขึ้นมี 3 รูปแบบดังนี้คือ ยาเตรียมเฉพาะคราวแอมโฟเทอริซินบีในรูปแบบอิมัลชันน้ำมันในน้ำ รูปแบบขี้ผึ้ง และรูปแบบเจล ซึ่งในการเตรียมตำรับจะใช้ไดเมทิลซัลฟอกไซด์ปริมาณสูงเพื่อเป็นสารช่วยเพิ่มการดูดซึมตัวยาเข้าสู่เล็บ และใช้ทริสไฮโดรคลอไรด์เป็นบัฟเฟอร์เพื่อควบคุมพีเอชของผลิตภัณฑ์ให้เท่ากับ 7 ทำการทดสอบคุณสมบัติทางกายภาพของตำรับที่เตรียมได้โดยการเก็บผลิตภัณฑ์ที่อุณหภูมิ 4 องศาเซลเซียสนาน 48 ชั่วโมงสลับกับอุณหภูมิ 45 องศาเซลเซียสนาน 48 ชั่วโมงเป็นเวลา 6 รอบ จากนั้นทำการประเมินการเปลี่ยนแปลงที่เกิดขึ้น จากการทดลองพบความไม่คงตัวในยาเตรียมเฉพาะคราวแอมโฟเทอริซินบีรูปแบบเจล โดยพบการตกตะกอนของตัวยาเมื่อผ่านการทดสอบทางกายภาพ ผลิตภัณฑ์ที่เตรียมขึ้นจะถูกเก็บไว้ที่อุณหภูมิ 30±2 องศาเซลเซียส เป็นเวลา 60 วัน ซึ่งในระหว่างนี้จะมีการนำผลิตภัณฑ์ที่ได้มาตรวจวัดปริมาณแอมโฟเทอริซินบีด้วยเครื่องไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทรกราฟี ผลการทดลองพบว่ายาเตรียมเฉพาะคราวแอมโฟเทอริซินบีในรูปแบบครีมและขี้ผึ้งที่ผสมไดเมทิลซัลฟอกไซด์ร้อยละ 30 ในตำรับ มีปริมาณแอมโฟเทอริซินบีอยู่ในเกณฑ์ ตามที่กำหนดเป็นเวลา 60 วัน นอกจากนี้ในงานวิจัยยังได้ทดสอบการซึมผ่านแบบนอกกายของผลิตภัณฑ์กับเล็บที่ตัดจากอาสาสมัครสุขภาพดีซึ่งพบว่า ยาเตรียมเฉพาะคราวแอมโฟเทอริซินบีในรูปแบบครีมและขี้ผึ้งที่เตรียมได้สามารถผ่านเข้าเล็บได้เมื่อเปรียบเทียบกับการทาผลิตภัณฑ์ที่ไม่มีตัวยาและตำรับยาเตรียมในรูปแบบขี้ผึ้งสามารถผ่านเข้าสู่เล็บได้มากกว่าตำรับยาเตรียมในรูปแบบครีมอย่างมีนัยสำคัญทางสถิติ การศึกษาในอนาคตจะได้นำตำรับยาเตรียมที่มีแอมโฟเทอริซินบีเข้มข้นร้อยละ 3 ในรูปแบบครีมและขี้ผึ้งไปศึกษาต่อทางคลินิกเพื่อประเมินประสิทธิภาพและผลข้างเคียงที่เกิดขึ้นในการรักษา รวมทั้งความพึงพอใจในการใช้ผลิตภัณฑ์ต่อไป

ภาควิชา	วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม	ลายมือชื่อนิสิต .....
		ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
สาขาวิชา	เภสัชกรรม	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
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PHOJANA KOMESMUNEEBORIRAK: FORMULATION OF EXTEMPORANEOUS AMPHOTERICIN B TOPICAL PREPARATIONS CONTAINING HIGH AMOUNT OF DIMETHYL SULFOXIDE FOR THE TREATMENT OF ONYCHOMYCOSIS. ADVISOR: ASST. PROF. POL.LT. WALAISIRI MUANGSIRI, Ph.D., CO-ADVISOR: ASSOC. PROF. PORNPEN WERAWATGANONE, Ph.D., 102 pp.

The main purpose of this research was to formulate the stable extemporaneous Amphotericin B topical preparation in a treatment onychomycosis and to determine beyond-use date of the product. To date, there is no marketed topical Amphotericin B product available in Thailand and no data available on stability of these preparations. The formulations in this study were prepared from a commercial Amphotericin B for injection. Three Amphotericin B topical preparations; i.e. o/w emulsion, hydrophilic ointment and gel, were developed. The preparations were formulated with high amount of dimethyl sulfoxide (DMSO) as a nail permeation enhancer and use Tris-hydrochloride buffer for controlling pH value of the preparation at pH 7. The physical stabilities of these formulations were tested by heating-cooling method (4°C for 48 hours – 45°C for 48 hours) and also monitored for appearance changes. The amphotericin B gel lost its viscosity and showed visible non-uniformity due to precipitation of Amphotericin B after stability testing. The preparations were storage at 30±2°C up to 60 days. During that time, Amphotericin B was quantified by high-performance liquid chromatography (HPLC). The extemporaneous Amphotericin B cream and ointment containing 30% concentrations of DMSO were stable for 60 days. Moreover, the *In vitro* permeation to clipping nail plates from healthy human volunteer showed that Amphotericin B in cream and ointment could permeate into nail plate when compared with the self-base formulation. The ointment formulation was better than cream ($p < 0.05$). The stable cream and ointment containing 3% Amphotericin B were obtained for further clinical study to evaluate the therapeutic and adverse effects, including the patient's satisfaction.

Department:	Pharmaceutics and Industrial Pharmacy	Student's Signature	.....
		Advisor's Signature	.....
Field of Study:	Pharmaceutics	Co-Advisor's Signature	.....
Academic Year:	2015		

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LIST OF ABBREVIATIONS

%	=	percentage
ºC	=	degree Celsius
µ	=	micron
µg	=	microgram
µl	=	microliter
µm	=	micrometer
cm^2	=	square centimeter
DMSO	=	dimethyl sulfoxide
EDTA	=	ethylenediaminetetra acetic acid
et al.	=	*et alii*, and other
g	=	gram
HPLC	=	High Performance Liquid Chromatography
M	=	Molar
ml	=	milliliter
mm	=	millimeter
min	=	minute
nm	=	nanometer
o/w	=	oil in water
pH	=	the negative logarithm of the hydrogen ion concentration
R^2	=	coefficient of determination
RH	=	relative humidity
rpm	=	round per minute
RSD	=	relative standard deviation
UV	=	ultraviolet
v/v	=	volume by volume
w/w	=	weight by weight

CHAPTER I

INTRODUCTION

Onychomycosis is a fungal infection of nail that is caused by dermatophytes, yeasts and nondermatophyte molds. In Thailand, nondermatophyte molds such as *Fusarium* spp. and *Scytalidium* spp. are leading cause of onychomycosis (Ungpakorn, 2005). Standard treatment of onychomycosis is oral administration of antifungal agents for example terbinafine, itraconazole, or fluconazole. Treatment failure in onychomyosis is due to low availability of drugs at the infection site, high hepatotoxicity of the antifungal drugs, and low patient compliance (Rodgers, and Bassler, 2001; Welsh, Vera-Cabrera, and Welsh, 2010). The nail plate contains several layers of dead and compacted keratin supported by nail bed, viable dermis with capillaries. Delivering therapeutic quantity of orally administered drugs to the nail plate requires high oral dose and long therapeutic regimen. Amphotericin B is shown to effectively cure onychomycosis caused by nondermatophyte molds (Lurati et al., 2011). Nail preparation containing antifungal drugs is not commercially available in Thailand. In order to overcome the above drawbacks, the ultimate goal of this research is to develop a topical nail formulation containing Amphotericin B and high concentration of permeation enhancer to be used as a monotherapy or in a combination with the oral antifungal drugs. Since the nail plate contains water more than lipid structure, the nail formulations should be an o/w emulsion or hydrophilic formula (Murdan, 2002). In addition, the topical nail formulations must adhere to the nail plate for a reasonable time so that permeation enhancer can facilitate drug permeation into the nail plate. Dimethyl sulfoxide (DMSO) is known to enhance the nail permeability (Vejnovic, Simmler, and Betz, 2010). DMSO is slightly hazard to human. DMSO can cause formulation problems since it is an organic solvent by

nature. Thus, development of formulations containing high concentration of DMSO is quite a challenge.

In this study, the extemporaneous Amphotericin B cream, hydrophilic ointment and gel containing high amount of DMSO were formulated and tested physicochemical stability. Then, the stable formulations were investigated to compare permeation to human nail plate.

The purposes of this study are as follows:

1. To formulate the extemporaneous Amphotericin B cream, hydrophilic ointment and gel containing high amount of DMSO.
2. To evaluate the physical and chemical stability of extemporaneous preparations for specification beyond-use date.
3. To study the *In vitro* permeation to compare of each stable formulation on the nail clippings from healthy human volunteer.

CHAPTER II

LITERATURE REVIEWS

1. Nail

The human nails protect the tips of fingers and toes. The structure of nail, shown in Figure 1, is composed of the nail plate, nail fold, nail bed, nail matrix and hyponychium. The nail plate is stemmed from the nail matrix and overlays the nail bed which separated at the hyponichium. The nail consists of tightly keratinized dead cells which is nonvascular with average growth rates of 3 mm per month for fingernails and 1 mm per month for toenails (Murdan, 2002).

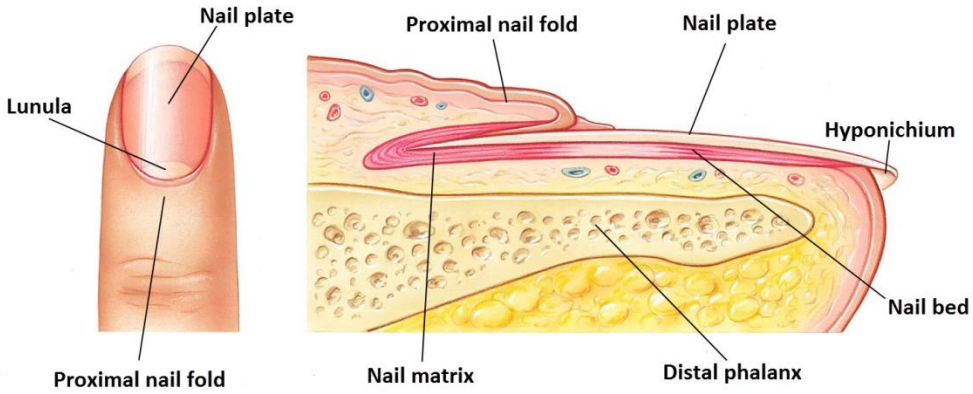


Figure 1 Structure of the finger nail

(Reproduced from:
http://web.uaccb.edu/AcademicDivisions/MathScience/Science/BWheeler/Ess/figs/04_09Figure-U.jpg)

The nail plate can be divided into three layers; dorsal, intermediate and ventral which thickness ratio is 3:5:2. The ventral layer connects the nail plate to the nail bed. Mainly of keratins in the nail plate is hard-type keratin that linked with disulfide, peptide, hydrogen and polar bonds. The keratin is a barrier to drug permeation into the nail plate. Unlike other body membrane, the total lipid content of the nail plate is between 0.1-1% while the stratum corneum's lipid content is about 10% (Walters, Abdalghafor, and Lane, 2012). Water content in nails is around 10-30% which is associated with the relative humidity. The nail

water content is responsible for nail elasticity and flexibility (Murdan, 2002). Based on the chemical composition, the nature of human nail plate shows more hydrophilic characteristic than lipophilic characteristic.

2. Onychomycosis

Onychomycosis is the most common nail disorders that caused by a fungal infection. Onychomycosis affect toenails more often than fingernails that probably due to the slower growth rate of toenails. The incidence increases in older adults because aging changes immune system and almost 30% of patients are older than 60 years. Many risk factors of onychomycosis include occlusive footwear, humidity, repeated nail trauma and in persons with immunosuppressive conditions such as poor peripheral circulation, diabetes mellitus or HIV infection (Welsh et al., 2010).

Onychomycosis is classified into several groups based on clinical patterns such as distal and lateral subungual onychomycosis, proximal subungual onychomycosis and superficial onychomycosis. The distal and lateral subungual onychomycosis (Figure 2) is the most common form that begins at the hyponychium and spreads to the nail plate and bed. The nail becomes thickened and discoloured until results in onycholysis (separation of the nail plate from the nail bed) (Westerberg, and Voyack, 2013; Ameen et al., 2014).

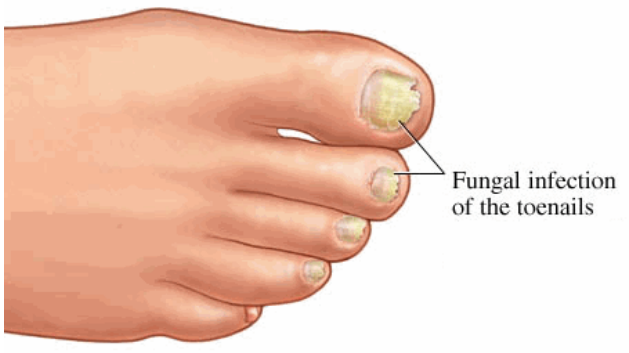


Figure 2 Distal and lateral subungual onychomycosis

(Reproduced from: http://pharmacylands.blogspot.com/2013/07/fungal-nail-infection-onychomycosis.html)

Onychomycosis caused by dermatophytes, yeasts and nondermatophyte molds. *Trichophyton rubrum* and *Trichophyton mentagrophytes* are the dermatophytes that the main caused of onychomycosis worldwide, 60% and 20% of cases, respectively (Welsh et al., 2010). In contrast, the majority of onychomycosis in Thailand are caused by nondermatophyte molds. Figure 3 show the percentage of onychomycosis causes, *Scytalidium dimidiatum* and *Fusarium* species are the main fungal pathogens in Thailand (Ungpakorn, 2005).

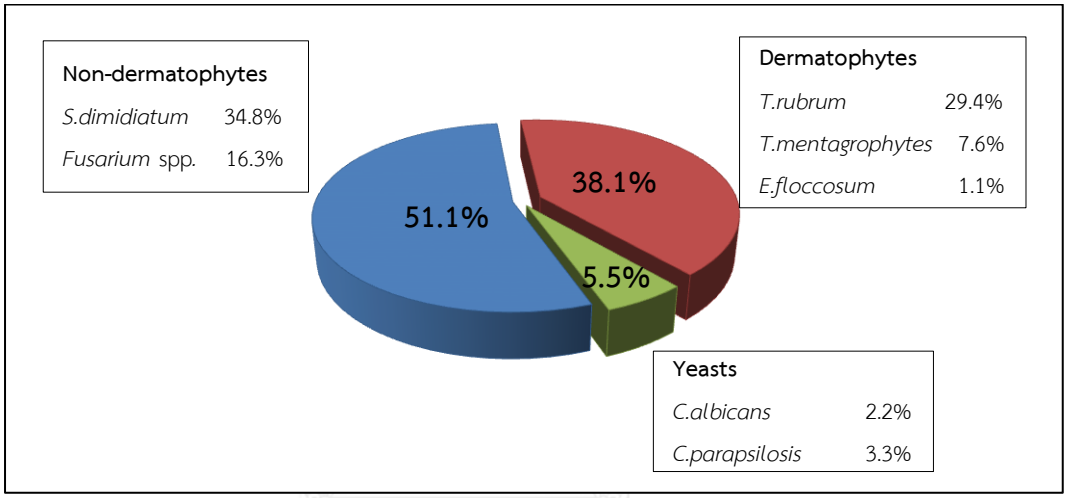


Figure 3 Prevalence of onychomycosis in Thailand from epidemiological survey of 10,000 patients
(Reproduced from: Ungpakorn, 2005)

3. Treatment of onychomycosis

The diagnosis is an important step for successful treatment. Although more than 50% of nail problems are caused by onychomycosis, the diagnosis by physical examination alone can be inaccurate. So, laboratory analysis should be confirmed (Westerberg, and Voyack, 2013). Therapeutic outcomes are including "mycological cure" (negative cultures) and "clinical cure" (normal nail morphology).

3.1 Systemic treatment

Antifungals, the allylamine and azole classes, are available oral drugs that used to treat onychomycosis. Terbinafine is an allylamine antifungal agent that is the first-line drug for dermatophytes. The azole class is represented by itraconazole, fluconazole and ketoconazole; but ketoconazole is rarely used as first-line treatment because of drug interactions and hepatotoxicity. The medications and their dosing regiments are shown in Table 1. Itraconazole is a board antifungal spectrum including nondermatophyte molds that is the main caused of onychomycosis in Thailand. The significant adverse effect of these drugs is the hepatotoxicity so the monitoring the liver function at the beginning of the treatment and during the therapy is recommended. Moreover, drug-drug interactions are also concerned, especially in the elderly and chronic disease patients. Drug interaction between oral antifungal and other medications is shown in Table 2.

Table 1 Oral antifungals for the treatment of Onychomycosis (Reproduced from Rodgers, and Bassler, 2001)

Antifungal agent	*Indication*	*Dosage*	*Monitoring*
Terbinafine	First-line therapy for dermatophytic infections (most cases of onychomycosis)	250 mg per day for 6 weeks to treat fingernails and for 12 weeks to treat toenails*	Complete blood count and ALT and AST levels at baseline, then every 4 to 6 weeks during therapy
Itraconazole	Alternative first-line therapy for dermatophytic infections Preferred therapy for nondermatophytic and candidal infections	Continuous therapy: 200 mg per day for 6 weeks to treat fingernails and for 12 weeks to treat toenails*	ALT and AST levels at baseline, then every 4 to 6 weeks during therapy
		Pulse therapy: 200 mg twice daily for 7 days per month, with the treatment repeated for 2 to 3 months ("pulses") to treat fingernails* and for 3 to 4 months to treat toenails†	None recommended
Fluconazole	First-line therapy for candidal infections but also active against dermatophytes Consider for use in patients with complicated medication regimens	150 mg once weekly until nail is normal or acceptably improved (treatment often requires 6 to 9 months)†	None recommended

ALT = alanine transaminase; AST = aspartate transaminase; FDA = U.S. Food and Drug Administration.

*—*Labeled by FDA.*
†—*Not labeled by FDA.*

Table 2 Drug interactions with oral antifungals (Iorizzo et al., 2005)

Oral antifungals	Drug interactions
Terbinafine	Dosage adjustment requested: tricyclic antidepressants; cimetidine; cyclosporin; rifampin; theophyilline; warfarin
Itraconazole	Contraindicated: alprazolam; astemizole; cisapride; HMG-Co reductase inhibitors; midazolam; terfenadine; triazolam; vincristine Itraconazole efficacy reduced: carbamazepine; isoniazid; phenytoin; phenobarbital; rifampin; rifabutin Dosage adjustment requested: buspirone; busulfan; cyclosporin; digoxin; dihydropiridines; HIV protease inhibitors; hypoglicemic agents; metylprednisolone; quinidine; sidenafil citrate; tacrolimus; warfarin
Fluconazole	Controindicated: astemizole; terfenadine Dosage adjustment requested: tricyclic antidepressants; cisapride; cyclosporin; hydrochlorothiazide; phenytoin; rifampicin; oral contraceptives; sulfonylurea; theophylline; tolbutamide; warfarin; zidovudine

HMG-Co: 3-Hydroxy-3-methylglutaril coenzyme.

3.2 Topical treatment

Many topical agents are developed for the treatment of onychomycosis because of an antifungal directly to the nail. So, there are no drug interactions and have few contraindications. Ciclopirox 8% solution and amorolfine 5% lacquer are the topical preparation for treatment of onychomycosis. The topical drugs can be used alone in patient that has severe adverse effects from systemic therapy. Nevertheless, the combination used with oral agents brings the better results of mycological and clinical cure rate (Baran, and Kaoukhov, 2005; Avner, Nir, and Henri, 2005)

Currently, no effectively topical antifungal drugs are the standard treatment of onychomycosis, especially nondernatophyte molds that are difficult to eradicate. And the standard systemic therapy with itraconazole and terbinafine often fails (Tosti, Piraccini, and Lorenzi, 2000; Baudraz-Rosselet et al., 2010). So, the study to formulate the stable topical preparation for nondernatophyte molds is important for added to the treatment regimens.

Lacroix, and De Chauvin, 2008) studied the *in vitro* activities of Amphotericin B, itraconazole, voriconazole, posaconazole, caspofungin and terbinafine against clinical isolates of *Scytalidium dimidiatum* and *Scytalidium hyalinum*. The result showed that Amphotericin B and voriconazole has the lowest Minimum Inhibitory Concentration (MIC) for *Scytalidium* spp. (Table 3). Similarly, Amphotericin B was the most effective drug to against *Fusarium* spp. (Alastruey-Izquierdo et al., 2008). Table 4 presented the result of susceptibility test of *Fusarium* spp. to Amphotericin B, miconazole, itraconazole, fluconazole, ketoconazole and flucytosine (Pujol et al., 1997). On the whole, Amphotericin B is selected to study owing to efficacy. Moreover, Amphotericin B has a suitable cost for develop to topical preparation.

Lurati et al., 2011) reported the efficacy of a topical Amphotericin B solution on nondernatophyte molds onychomycosis. The results showed the

clinical cure in all 8 patients, who had previously been treated with antifungal regimens to no avail, after 12-month treatment and the mycological cure in 7 patients. This report reveals the opportunity to develop the suitable topical preparation for treatment of nondernatophyte molds onychomycosis.

Table 3 Antifungal susceptibility of *S. dimidiatum* and *S. hyalinum* isolates (Lacroix, and De Chauvin, 2008)

Species (no. of isolates tested)	Antifungal agent	MIC (mg/L)		
		range	50%[a]	90%[b]
S. dimidiatum (17)	amphotericin B	0.06–1	0.5	0.5
	itraconazole	<0.03 to >16	4	>16
	voriconazole	<0.03 to 0.5	0.125	0.25
	posaconazole	0.125–2	2	2
	terbinafine	0.125–0.5	0.25	0.5
	caspofungin	0.06–8	0.5	8
S. hyalinum (15)	amphotericin B	0.06–1	0.25	0.5
	itraconazole	0.125–4	1	1
	voriconazole	<0.03 to 0.25	0.06	0.125
	posaconazole	0.06–1	0.5	1
	terbinafine	0.06–2	0.5	1
	caspofungin	0.125–8	0.5	1

[a]MIC at which at least 50% of isolates are inhibited.
[b]MIC at which at least 90% of isolates are inhibited.

Table 4 Antifungal susceptibility of 58 isolates of *Fusarium* spp. (Pujol et al., 1997)

Antifungal	MIC (mg/L)					
	48 h			72 h		
	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
Amphotericin B	0.14–9.24	2.31	4.62	0.58–>36.94	4.62	18.47
Miconazole	0.60–>40	>40	>40	0.60–>40	>40	>40
Itraconazole	1.25–>10	>10	>10	5–>10	>10	>10
Fluconazole	80–>80	>80	>80	>80	>80	>80
Ketoconazole	1.60–>51.20	51.20	>51.20	>51.20	51.20	>51.20
Flucytosine	>322.7	>322.7	>322.7	>322.7	>322.7	>322.7

The topical preparation for treatment of onychomycosis should adhere to the nail plate; such as cream, ointment, gel or lacquer for increase contact time to permeation. Thus, the topical Amphotericin B solution is not suitable for nail disease. Besides the formulating, the enhancer is notable for improve poor drug permeation to the nail plate.

4. Enhancement of nail plate permeability

Topical therapy of nail disease has clear advantages as elimination of systemic adverse events and drug interactions and minimized cost of treatment. But the limited success is due to the poor permeability to keratinized cells in the human nail plate. Physical and chemical method have been used to disrupt the structure of nail plate for enhance the permeation (Elkeeb et al., 2010)

4.1 Physical method

The methods of physical enhancer, such as abrasion, avulsion, microporation, phonophoresis and iontophoresis are used to improve permeability efficacy. However, most of methods must perform by dermatologists or specialists with specific equipment. As a result, the low patient compliance from technical complexity and high cost will be the problems to the treatment. The abrasion with abrasive stick is a simplest method that removes the layer of the nail plate, thus reducing the barrier can permeate drug to the deeper nail layer.

4.2 Chemical method

The chemical enhancement focuses on breaking the bond linkage of the nail plate keratin. Figure 4 shows the type of bonds in the nail keratin including disulfide, peptide, hydrogen and polar bonds that could potentially be targeted by chemical enhancer. The disulfide bond is the main target, due to its critical role in protein stability. The mechanisms to cleavage of nail disulfide bond are the reduction and oxidation (Murdan, 2008). The reducing and oxidizing agents have functional groups to cleave the disulfide bond and increase nail permeability. However, the property of these agents often affects stability of the active drug in the formulation. For this reason, nail pretreatment with these enhancers is the way to solve incompatibility problem. But a possible

disadvantage of separate drug and enhancer is the difficult using which may reduce patient compliance.

Urea and salicylic acid are the keratolytic agents which are widely used in skin preparation. The previous experiments show uncertain results of these agents. They do not seem to benefit in increasing of drug uptake to nail plate (Quintanar-Guerrero et al., 1998) but they are mostly used for the chemical avulsion of disease nail plates (Murdan, 2002).

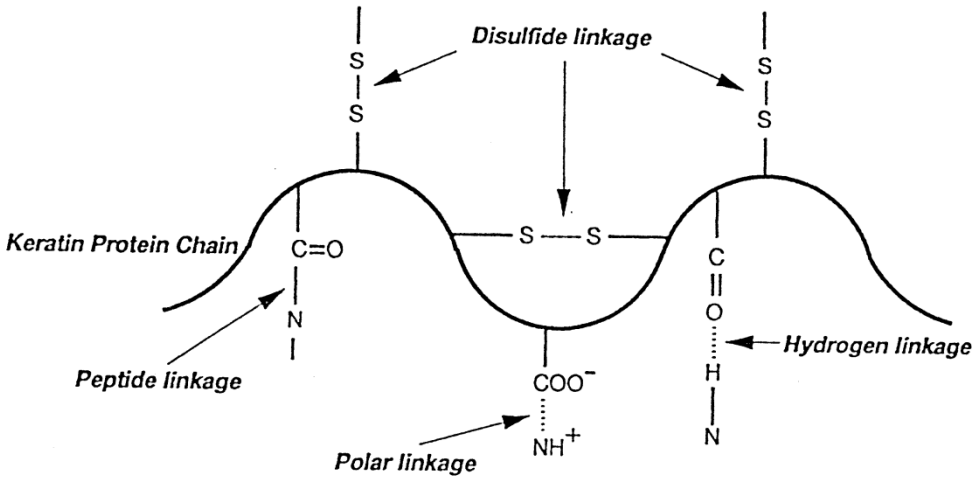


Figure 4 The types of bonds in the nail keratin proteins (Murdan, 2002)

In addition, dimethyl sulfoxide (DMSO) and water have evidence bases to increase drug permeation through the nail plate. Stüttgen, and Bauer, 1982) claimed that high content of DMSO in lotion could increase the permeation of econazole in lotion through the deeper human nail layer. DMSO has been shown to increase the permeation rate of amorolfine across the human nail plate from a nail lacquer formulation (Franz, 1992). However, the mechanism of DMSO to improve nail permeation is not clear. In the literature, DMSO did not change the protein structure, but it reasonably formed hydrogen bonds with the OH or NH

groups of the keratin side chain (Myoung, and Choi, 2003). So, this effect may increase drug permeation through the nail plate.

Water is the principle plasticizer for the nail. The water hydration of the nail plate makes it to swell. Since the nail plate is described as hydrogel, swelling results in the separation of keratin fibers and the formation of pores which molecules can transverse (Murdan, 2002). Gunt, and Kasting, 2007) demonstrated that increasing of the water content in the human nails can enhance the permeation of ketoconazole. Consequently, formulations that have more hydrophilic property which increase the nail hydration may improve topical nail permeation.

5. Factors of topical nail formulation

5.1 Size of drug molecules

The molecular size of drug has an inverse relationship with permeation. The larger the molecular size, the harder it is for molecules to diffuse through the keratin network and lower the drug permeation. In this study, Amphotericin B is an active drug for treatment of nondermatophyte molds onychomycosis. Figure 5 shows the structure of Amphotericin B that is a large molecule, so the enhancer is an important role to drug permeation.

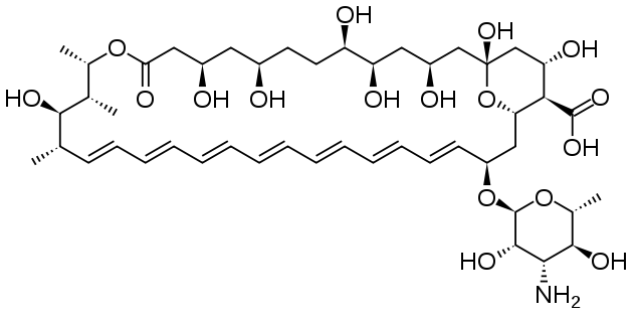


Figure 5 Structure of Amphotericin B

(From: http://commons.wikimedia.org/wiki/File:Amphotericin_B_structure.svg)

Amphotericin B is a macrocyclic, polyene antifungal that is a yellow to orange, odorless or almost odorless powder. Its molecular formula is $C_{47}H_{73}NO_{17}$, with a molecular weight of 924.1 (Q. Alan Xu, 2011). Amphotericin B is insoluble

in water and ethanol. Solubility in DMSO is 30-40 mg/ml. The chemical stability of amphotericin B is greatest at a neutral pH, where it exists as a zwitterion; the pH of the commercial product typically ranges from 5 to 8. Amphotericin B is subject to photolytic degradation so it should be kept in light-resistant containers in the refrigerator (2–8 °C). Amphotericin B is incompatible with chloride-containing solution (Trissel, 2009).

5.2 Hydrophilicity of preparation

As mentioned above, the nail plate is characterized as a hydrophilic gel membrane when hydrated with water. So, the topical hydrophilic formulation such as o/w emulsion, hydrophilic ointment and hydrogel is suitable for drug delivery through the nail plate,

Many gelling agents are used in pharmaceutical formulations. In this study, the significant property of gelling agent is that it can produce high viscosity at pH about 7, which is a stable condition of Amphotericin B.

5.3 pH of formulation

The isoelectric point of nail keratin is around 5 (Murdan, 2002). So, keratin is negatively charged when higher than pH 5 and positively charged in lower than pH 5. Due to electrostatic repulsion causing decrease of nail permeation, the charge of active compound should not be the same as that of keratin. For this reason, the suitable pH of formulation is brought to uncharged drug species to get rid of this problem.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Raw material

1.1 Amphotericin B from *Streptomyces sp.* - ~80% (HPLC), powder (Lot. 082M4012V Sigma-Aldrich)

1.2 Amphotericin B for injection U.S.P (Lot. 3K001 Asence, India)

2. Chemicals

2.1 Acetronitrile (HPLC grade, Lot. 14060025 RCI Labscan Limited)

2.2 Dimethyl sulfoxide (HPLC grade, Lot. 13060279 RCI Labscan Limited)

2.3 Ethylenediaminetetra acetic acid calcium disodium salt (Lot 2G160212G Farmitalia Carlo Erba)

2.4 Hexane (AR grade, Lot. 12080015 RCI Labscan Limited)

2.5 Hydrochloric acid (AR grade, Lot. LG056410 Lobachemie)

2.6 Methanol (HPLC grade, Lot. 14060105 RCI Labscan Limited)

2.7 Tris-hydroxymethyl-methylamine (Lot. 1302135499 Ajax Finechem Pty Ltd)

2.8 Poloxamer 407 (Lot. WPWG5938 BASF)

2.9 Glyceryl monostearate SE (Lot. 66529 S.Tong chemicals)

2.10 Cetyl alcohol (Lot. M13239003O Emery)

2.11 Mineral oil (Lot. 2015031330 S.Tong chemicals)

2.12 Propylene glycol (Lot. C815E69TDI S.Tong chemicals)

2.13 Sodium lauryl sulfate (Lot. 11305 Srichand)

2.14 Span 60 (Lot. 29621 S.Tong chemicals)

2.15 Stearyl alcohol (Lot. 0210830 Srichand)

2.16 Tween 60 (Lot. 24853 S.Tong chemicals)

2.17 White petrolatum (Lot. 802330 S.Tong chemicals)

3. Instruments and apparatus

3.1 High Performance Liquid Chromatography (LC-20 AD, Shimadzu, Japan)

- Auto sampler (SIL-20 HT, Shimadzu, Japan)
- UV detector (SPD-M20A, Shimadzu, Japan)
- Pumps (LC-20AD, Shimadzu, Japan)
- Communications Bus Module (RF-10AXL, Shimadzu, Japan)
- Column (Phenomenex C18, 5µ, 250 x 4.6 mm)
- Guard cartridges (KJO-4282, Phenomenex)

3.2 Ultrapure water (Model Elgastat Maxima UF, ELGA Ltd., England)

3.3 Micropipettes (50-200 µL), (100-1000 µL), (1-5 mL) (BIOHIT)

3.4 pH meter (Orion Model 420A, USA)

3.5 Analytical balances (MUT2, Mettler Toledo), (AX105 Delta Range, Mettler Toledo)

3.6 Micropipettes tips (BIOHIT)

3.7 Microscope (ECLIPSE E200,Nikon)

3.8 Disposable syringe 3 and 5 mL without needle (NIPRO)

3.9 High speed refrigerated micro centrifuge (MX-305, Tomy)

3.10 Incubator (KBF 720, Binder), (Memmert, Thailand)

3.11 Refrigerator (Kelvinator, Australia)

3.12 Vacuum pump (DOA-V130-BN, Waters division of Millipore)

3.13 Vernier (MOD S235, Sylvac)

Methodology

In this research, a series of experiments were designed to formulate suitable extemporaneous Amphotericin B topical preparations in a treatment of onychomycosis. Dimethyl sulfoxide (DMSO) was chosen as a nail permeation enhancer. Stability studies of the extemporaneous preparation were performed. Remaining concentrations of Amphotericin B over 60 days were determined using a HPLC method. Beyond-use date was predicted based on the obtained physical and chemical stability data. *In vitro* study was tested to compare the permeation of each stable formulation on the nail clippings from healthy human volunteers.

Methods

The experiments were divided into four parts:

1. HPLC method for an analysis of Amphotericin B
2. Development of three formulation bases (cream, ointment and gel) containing high amount of DMSO
3. Formulation and stability evaluation of extemporaneous Amphotericin B topical preparations
4. Evaluation of permeation of extemporaneous Amphotericin B topical preparations in human nail plate

Part 1: HPLC method for an analysis of Amphotericin B

The HPLC method was selected for quantitative analysis of Amphotericin B in extemporaneous preparations due to its specificity and high sensitivity.

1.1 HPLC conditions

The HPLC conditions were adapted from Wilkinson et al., 1998)

Column	:	Phenomenex C18 (5µ, 250 x 4.6 mm)
Precolumn	:	Phenomenex HPLC SecurityGuard (KJO-4282)
Mobile phase	:	methanol : acetonitrile : 0.0025 M EDTA (50:35:20 v/v/v)
Injection volume	:	20 µl
Flow rate	:	1 ml/min
Detector	:	UV detector 405 nm
Temperature	:	30 °C
Run time	:	20 min

The mobile phase was prepared by mixing methanol, acetonitrile and 0.0025 M EDTA in water with the ratio of 50:35:20 v/v/v. The mixture solution was thoroughly mixed, filtered through 0.45 µm membrane filter and degassed by sonication for 30 minutes prior use. Every sample was filtered through 0.45 µm membrane filter prior to injection into the HPLC column.

1.2 Preparation of standard solutions

Amphotericin B purchased from Sigma-Aldrich was used as a reference standard. A standard stock solution of Amphotericin B was prepared by weighing 1 mg of Amphotericin B standard into a 10 ml volumetric flask. Dimethyl sulfoxide (DMSO) was used to dissolve the Amphotericin B and to adjust the final volume. The final concentration of the standard stock solution was 0.1 mg/ml.

Six concentrations of Amphotericin B standard solutions; i.e. 1, 2, 5, 10, 20 and 30 µg/ml were prepared by pipetting appropriate volume of the standard stock solution, transferring to 5 or 10 ml volumetric flask and diluting with the mobile phase to the final volume. These standard solutions were freshly prepared for each

HPLC run. As a result, the standard curve of Amphotericin B between concentrations and peak areas were plotted.

1.3 Validation of HPLC method

1.3.1 Validation of HPLC method for drug molecule

The analytical parameters used in this section were selectivity, limit of detection, limit of quantitation, linearity, accuracy and precision.

a. Selectivity

Under the employed chromatographic conditions, the selectivity of HPLC method was evaluated by comparing the assay result of Amphotericin B in the standard solution with the solvent and with Amphotericin B standard which were stressed in 0.1N hydrochloric acid, 0.01N sodium hydroxide, 0.5% hydrogen peroxide and water. The peak of Amphotericin B reference must be completely separated from interference, other components in the solvent or degradation products by examination of peak purity and peak purity index of Amphotericin B peak obtained from stressed samples against that of Amphotericin B standard.

The chromatograms of Amphotericin B standard solution would be examined peak purity with peak purity index of the HPLC LC-20 program before testing the similarity with all of chromatograms of Amphotericin B in every condition.

b. Limit of detection (LOD)

In this validation, the LOD of HPLC method would be evaluated with the Signal-to-Noise ratio (S/N). The lowest concentration of Amphotericin B standard solution that chromatograms showed S/N in 2:1 or 3:1 was the limit of detection of system.

c. Limit of quantitation (LOQ)

The LOQ of HPLC method would use the Signal-to-Noise ratio (S/N), as the same as the LOD, but the criteria was different. The lowest concentration of Amphotericin B standard solution that chromatograms showed S/N in 10:1 was the limit of quantitation of system.

d. Linearity

Six concentrations of Amphotericin B standard solutions (1-30 µg/ml) were prepared and analyzed. Linear regression analysis of peak area and their concentrations were performed. The coefficient of determination (R^2) must be more than 0.9990.

e. Accuracy

The accuracy of the analytical method was determined from percentage of analytical recovery. Five sets of three concentrations at 1.5, 15, 25 µg/ml were prepared and analyzed. The percentage of analytical recovery of each concentration was calculated which that must be within 95-105% of each nominal concentration.

f. Precision

1) Within-run precision

The within-run precision was determined by analyzing five sets of three standard concentrations of Amphotericin B at 1.5, 15, 25 µg/ml in the same day. The relative standard deviation (%RSD) of the peak area responses at each concentration was determined.

2) Between-run precision

The between-run precision was determined by analyzing three standard concentrations of Amphotericin B at 1.5, 15, 25 µg/ml on five different days. The relative standard deviation (%RSD) of the peak area responses at each concentration was determined.

The percentage of relative standard deviation (%RSD) for both within-run and between-run precision must be less than 2%.

1.3.2 Partial validation of HPLC method for drug products

The analytical parameters used in this validation were selectivity and accuracy. In this step, the sample preparation procedure would be described later in topic 3.2.1 and 3.2.2.

a. Selectivity

The selectivity of HPLC method was evaluated by comparing the chromatograms of Amphotericin B obtained from the extemporaneous formulations with that in the standard solution. The peak of Amphotericin B must be completely separated from interference and other components in the sample formulation. Moreover, peak purity and peak purity index of the Amphotericin B peak obtained from the extemporaneous preparations were also determined

b. Accuracy

The accuracy of the analytical method was determined from percentage of analytical recovery. The samples were prepared by addition of known amount of Amphotericin B into base preparations. Four sets of each freshly extemporaneous preparation were analyzed to determine the amount of Amphotericin B compare with the theoretical concentration. The percentage of analytical recovery of each preparation was calculated which that must be within 95-105% of each theoretical concentration.

Part 2: Development of three formulation bases (cream, ointment, and gel) containing high amount of DMSO

Cream, ointment and gel formulations were modified from Handbook of Pharmaceutical Manufacturing Formulations: Semisolid Products (Niazi, 2009), USP36, 2013 and Handbook of pharmaceutical excipients, 2009, respectively.

2.1 Formulation of cream base

The o/w emulsions were modified by varying concentration of various ingredients as shown in Table 5. The cream base was prepared using the beaker method. Oil phase consisted of glyceryl monostearate SE, cetyl alcohol, stearyl alcohol, mineral oil, and Span 60 were heated to 75^{o}C. Water phase consisted of purified water, Tween 60, and DMSO were heated to 80^{o}C. The oil phase was added into the water phase and gently stirred until it was congealed.

Table 5 The formulation of cream base

Ingredients	Functions	% w/w
Glyceryl monostearate SE	Emollient, emulsifying agent	1-5
Cetyl alcohol	Stiffening agent	2-10
Stearyl alcohol	Stiffening agent	1-5
Mineral oil	Emollient	5-10
Tween 60	Emulsifying agent	1-10
Span 60	Emulsifying agent	1-10
Dimethyl sulfoxide	Permeation enhancer	5-50
Purified water	Vehicle	q.s. to 100

2.2 Formulation of hydrophilic ointment base

The hydrophilic ointment bases were prepared by varying concentration of ingredients as shown in Table 6. In short, stearyl alcohol and white petrolatum were fused at 75°C and the mixture was stirred until it became homogeneous. The aqueous phase composed of sodium lauryl sulfate, propylene glycol, DMSO and

purified water was heated to 75°C. The oil phase was added to the aqueous phase. The hydrophilic ointment base was stirred gently until it was congealed.

Table 6 The formulation of hydrophilic ointment base

Ingredients	Functions	% w/w
Sodium lauryl sulfate	Emulsifying agent	0.5-2.5
Stearyl alcohol	Stiffening agent	5-25
White petrolatum	Emollient	5-50
Dimethyl sulfoxide	Permeation enhancer	5-30
Purified water	Vehicle	q.s. to 100

2.3 Formulation of gel base

The formulation of gel base was shown in Table 7. Poloxamer 407 was chosen as a gelling agent because it formed non-ionic gel. Poloxamer 407 was slowly dispersed in cold mixture (5^{o}C) of DMSO and purified water. The mixture was gently stirred until the gel was formed.

Table 7 The formulation of gel base

Ingredients	Functions	% w/w
Poloxamer 407	Gelling agent	12.5-20
Dimethyl sulfoxide	Permeation enhancer	5-30
Purified water	Solubilizer	q.s. to 100

2.4 Physical stability evaluation of base formulations

Physical stability of selected preparations was evaluated using the heating-cooling method. Under stress conditions, samples were stored in a refrigerator at 4^{o}C for 48 hours, and then kept in an incubator at 45^{o}C for 48 hours. The preparations were gone through all steps mentioned above for 6 cycles prior to organoleptic evaluations such as color, viscosity and appearance of formulations.

Part 3: Formulation and stability evaluation of extemporaneous Amphotericin B topical preparations

3.1 Formulation of extemporaneous Amphotericin B topical preparations

In this study, the extemporaneous Amphotericin B topical preparations were prepared by an addition of Amphotericin B for injection dissolved in DMSO into the base formulations. Briefly, base formulations were prepared in the absence of DMSO. Then, the 50 mg of Amphotericin B in Amphotericin B for injection powder was dissolved in 5 ml of DMSO. Finally, the Amphotericin B solution was added and well mixed with the base preparation. After mixing well, these extemporaneous Amphotericin B topical preparations were kept in amber glass vials for further physical and chemical stability studies.

3.2 Physical stability evaluation

Physical stability was evaluated by heating-cooling method same as topic 2.4. Moreover, the finished products were investigated under a microscope for checking homogeneity of preparation.

3.3 Chemical stability evaluation

Three batches of each formulation were prepared for chemical stability study. The extemporaneous Amphotericin B preparations were investigated for content of active drug by HPLC method. After the extemporaneous Amphotericin B topical preparations were freshly prepared, they were kept in each amber glass vials before storage. The storage condition was at 30±2^{o}C. Samples were withdrawn and determined Amphotericin B content on day 0, 3, 7, 14, 21, 28, 35, 42, 49, 56 and 60. The sample preparation procedures for each formulation were described in topic 3.2.1 and 3.2.2. The peak areas of samples were calculated and the concentrations of Amphotericin B in samples were determined from the standard curve.

3.3.1 Cream and ointment

The samples were dissolved in 5 ml of hexane. Then, 10 ml of DMSO was added. The mixture was shaken in a centrifugal tube. After centrifugation at 10,000 rpm for 10 min, the Amphotericin B was separated into the lower DMSO layer. Then

1 ml of the lower layer was pipetted and transferred in to a 10 ml volumetric flask and adjusted to volume with the mobile phase. Finally, the amount of Amphotericin B in the preparation was investigated by HPLC method as mentioned in 1.1.

3.3.2 Gel

For gel formulation, Poloxamer 407 was a gelling agent which was soluble in mobile phase of this study. The sample was mixed with 5 ml of DMSO and adjusted to 10 ml with the mobile phase in a volumetric flask. Then 1 ml of solution was further diluted with the mobile phase in a 10 ml volumetric flask before the determination amount of Amphotericin B by HPLC method.

Part 4: Evaluation of permeation of extemporaneous Amphotericin B topical preparations in human nail plate

4.1 Study design and subject selection

The protocol was approved by The Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University. The certificate of approval number was 120/2014 (shown in Appendix A).

The fifteen healthy volunteers with age ranging from 18-60 years participated in this study. The volunteers were allowed to drop out from study at any time when they were uncomfortable.

Subject selection

Inclusion criteria

- Healthy volunteer
- Age from 18-60 years old
- Permission to participating in the study

Exclusion criteria

- History of fungal nail disease
- Manicure or nail varnish

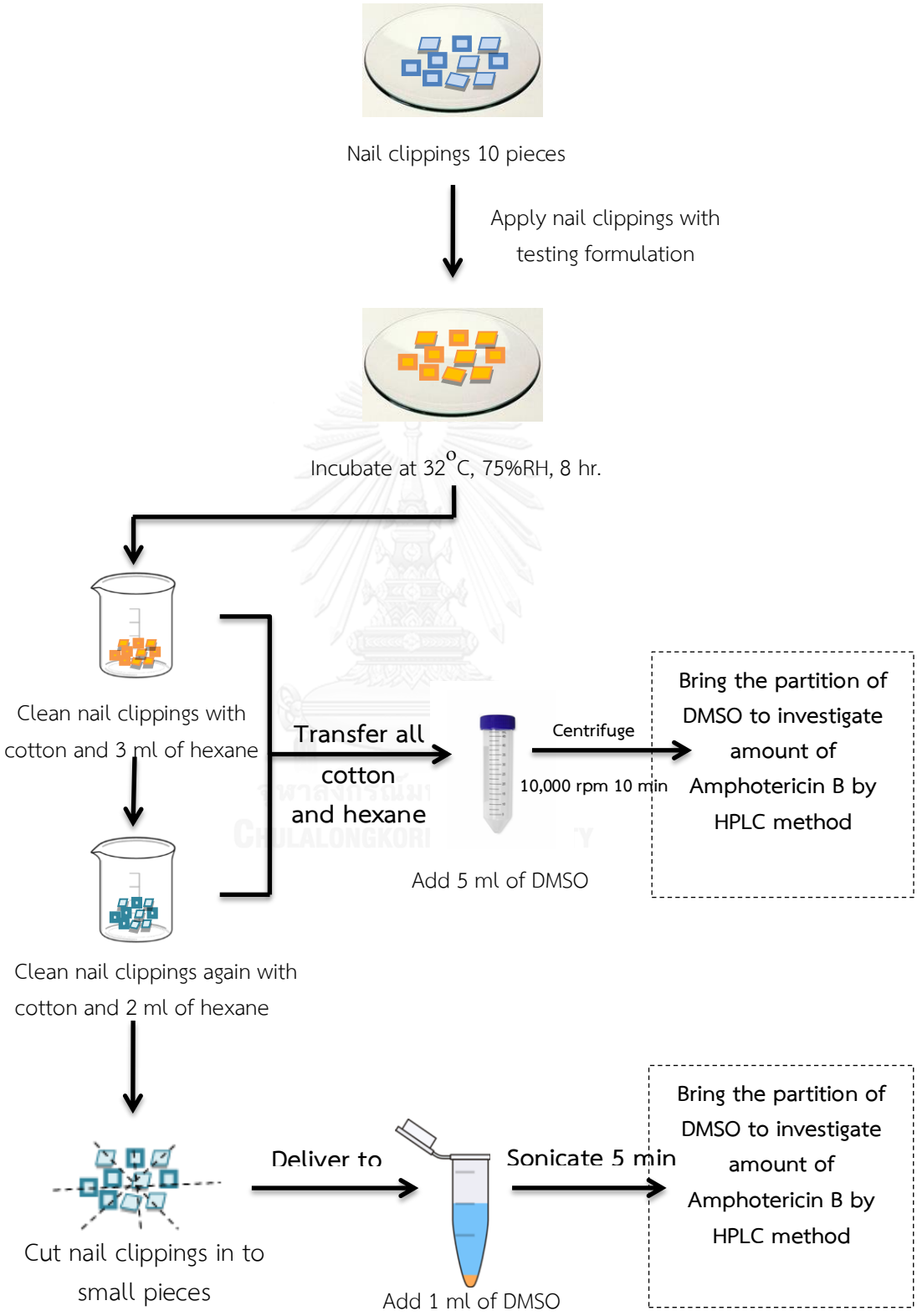
The clipping nail plates were used in this study should be long at least 3 mm. Moreover, the subjects should cut the nail plate in each finger in the whole pieces.

4.2 Permeation studies

The nail clippings from each healthy volunteer were well cleaned with the gentle soap and then allowed to dry overnight on watch glass at room temperature. Nail clippings were cut again in the square shape giving surface area around 20 mm^2. Before the experiment, weight and surface area of each piece were recorded. Ten nail clippings from the same subject were used in each round of the experiment. Each formulation was tested in triplicate. The experiment started by applying of the tested formulation about 3.5 mg/mm^2 on the nail clippings. Then each set of nail clipping was kept in an incubator (KBF 720, Binder) at 32±2^oC and 75±5% humidity

for 8 hours. After 8 hours the nail clippings were cleaned twice times using 3 ml and 2 ml of hexane and rubbed with cotton. The cotton and the hexane were pooled and transferred to a 15 ml centrifuge tube and 5 ml of DMSO was added to dissolve Amphotericin B. The mixture was centrifuged at 10,000 rpm for 10 min. Then 2 ml of the DMSO layer was transferred into a 5 ml volumetric flask and adjusted to volume with the mobile phase. The remaining nail clippings were cut in to small pieces and transferred to an eppendorf. A 1 ml of DMSO was added to dissolve Amphotericin B in the nail clippings. Finally, the amount of Amphotericin B in the nail clippings and the cleansing mixture were analyzed by HPLC method (Figure 6).

Figure 6 Method of permeation study



CHAPTER IV
RESULTS AND DISCUSSION

Part 1: HPLC method for an analysis of Amphotericin B

The modified HPLC condition was employed to analyze Amphotericin B content in the extemporaneous preparations. The partial validation of analytical method showed that the analytical method was suitable for determination of Amphotericin B free molecules and Amphotericin B in the extemporaneous preparation according to ASEAN guidelines for validation of analytical procedures.

1.1 Validation of HPLC method in reference standard

a. Selectivity

The selectivity of analytical method is the ability to separate the active ingredient or Amphotericin B from its degradation products and the other components. The typical chromatograms of mobile phase, Amphotericin B standard solution and Amphotericin B in extemporaneous formulations are shown in Figures 7-11. The peaks with a retention time around 5 minutes were Amphotericin B. All chromatograms are presented under the same attenuation and scale.

Moreover, the method was determined to be stability indicating by accelerated degradation of Amphotericin B. Standard solutions were diluted with 0.1N hydrochloric acid, 0.01N sodium hydroxide, 0.5% hydrogen peroxide and water. The water dilution was kept under storage temperature at 45^{o}C for 30 minutes while the others were stored at room temperature for 10 minutes before analysis. The chromatograms were shown in Appendix B. The degradation of Amphotericin B in 0.1N hydrochloric acid, 0.01N sodium hydroxide, 0.5% hydrogen peroxide and water were 39.8%, 30.38%, 12.61% and 12.54%, respectively. The result showed that the acid and alkaline conditions were the main causes of Amphotericin B degradation.

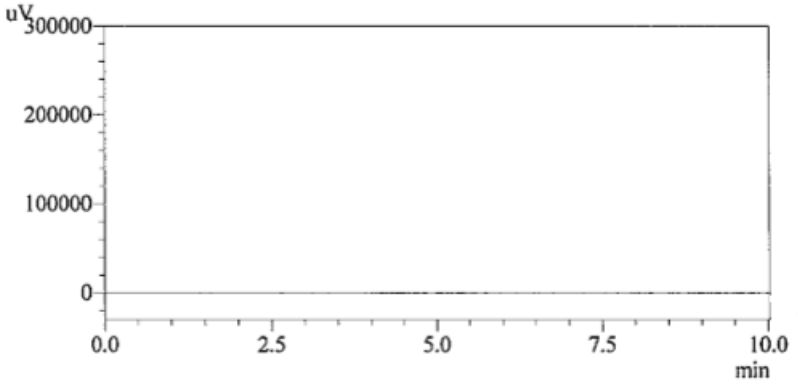


Figure 7 HPLC chromatogram of mobile phase

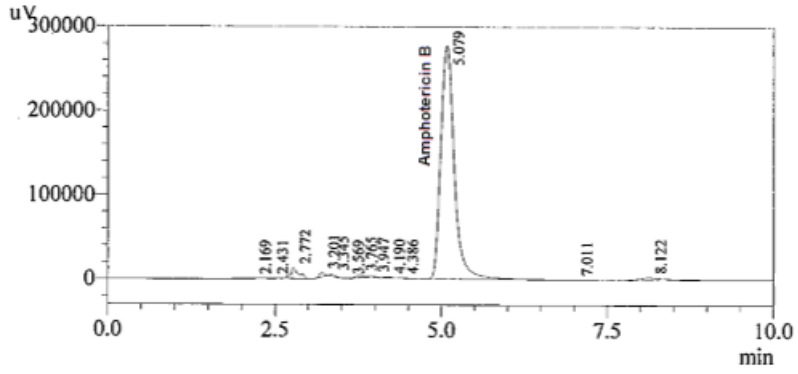


Figure 8 HPLC chromatogram of Amphotericin B reference standard

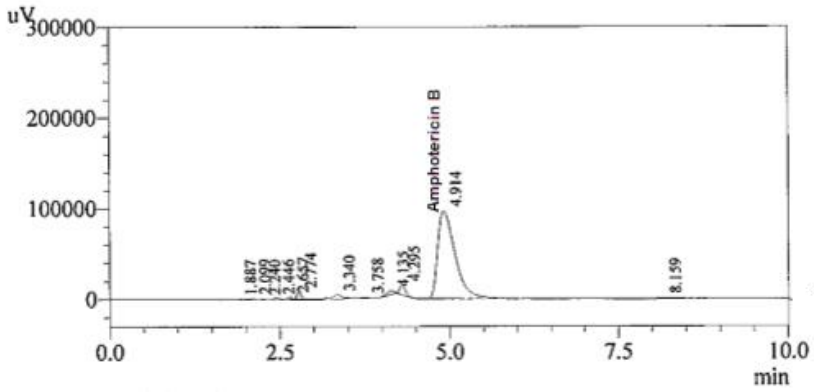


Figure 9 HPLC chromatogram of Amphotericin B in cream preparation

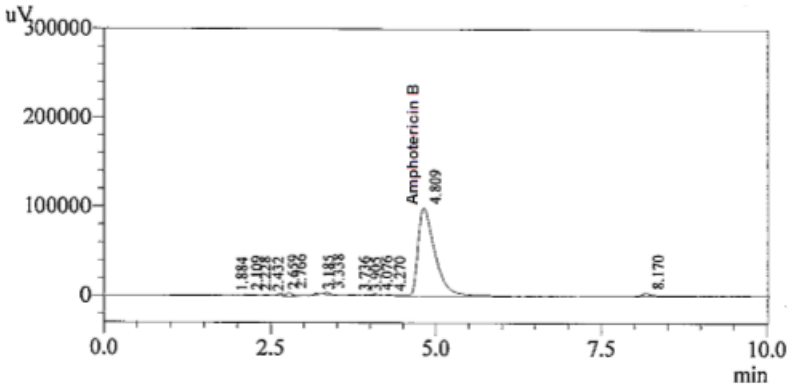


Figure 10 HPLC chromatogram of Amphotericin B in gel preparation

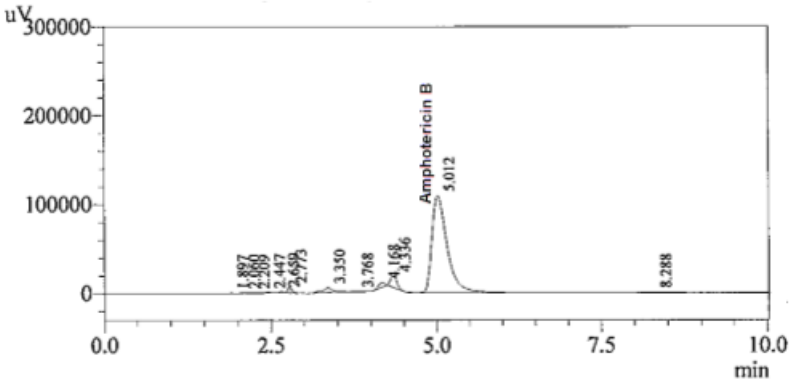


Figure 11 HPLC chromatogram of Amphotericin B in ointment preparation

Peak purity indexes were calculated in order to show selectivity of the employed HPLC method. UV spectra of Amphotericin B reference standard was obtained by scanning the main peak of Amphotericin B reference standard solution using the SPD-M20A PDA detector. The obtained UV spectrum was used as a reference spectrum. Amphotericin B peaks from stressed sample and formulations were also scanned for its corresponding UV spectrum. The latter UV spectrums were compared with the reference spectrum and calculated for peak purity indexes. The result showed that peak purity indexes of all samples were more than 0.9999. The peak purity method from LC solution program was shown in Appendix B (topic II). Neither degraded products nor other component in the formulation was co-eluted with the main peak of amphotericin B. In other words, the HPLC method was shown to process selectivity.

b. Limit of detection (LOD)

The limit of detection (LOD) of an analytical method is the minimum concentration of analyte in a sample that the method can detect but cannot identify true quantity.

Signal obtained from standard solution injection was utilized in calculation of Signal-to-Noise ratio (S/N). The calculation method and the results were shown in topic IV in Appendix B. The limit of detection of this HPLC method was calculated to be 0.50 µg/ml.

c. Limit of quantitation (LOQ)

The limit of quantitation (LOQ) of an analytical method is the minimum concentration of analyte in a sample that the method can determine with acceptable accuracy and precision.

The limit of quantitation (LOQ) was calculated based on Signal-to-Noise ratio (S/N) of 10:1. The limit of quantitation of this HPLC method was estimated to be 0.75 µg/ml and (topic V at Appendix B).

d. Linearity

The linearity of an analytical method is the ability of the method to obtain test results proportional to the concentration of analyte.

The calibration curve data of Amphotericin B standard solutions are shown in Table B2 (Appendix B). The plot between concentration of Amphotericin B solutions and their peak areas demonstrated a linear correlation in the concentration range of 1-30 µg/ml (Figure 12). The coefficient of determination (R^2) of this line was 1.0000.

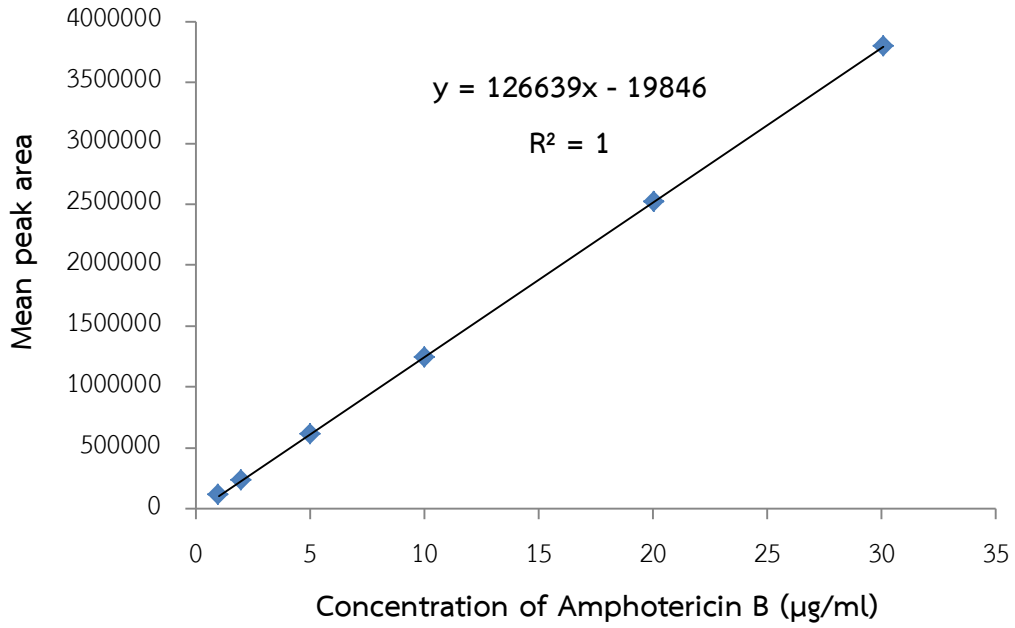


Figure 12 Typical calibration curve of Amphotericin B by HPLC method

e. Accuracy

The accuracy of an analytical method is the closeness of agreement between the added concentration values and the observed value.

The determination of accuracy was performed by analyzing five sets of three concentrations at 1.5, 15, 25 µg/ml (n=5). The full data were showed in topic VII (Appendix B). The estimated concentration and percentage of analytical recovery for each concentration are shown in Table 8 and Table 9, respectively. The percentages of analytical recovery of Amphotericin B were in the range of 98.12 – 102.49%, which was acceptable according to the criteria of AOAC International guidelines for standard method performance requirements, 2012. The result indicated that this HPLC method could be used to determine Amphotericin B with high accuracy in the concentration range (1-30 µg/ml).

Table 8 The estimated concentration of Amphotericin B by HPLC method

Nominal concentration of Amphotericin B (µg/ml)	Estimated concentration (µg/ml)					Mean ± SD
	set 1	set 2	set 3	set 4	set 5	
1.5	1.54	1.51	1.52	1.53	1.52	1.53 ± 0.01
15	14.83	14.83	14.90	14.68	14.83	14.81 ± 0.08
25	24.92	24.87	24.94	24.96	25.05	24.95 ± 0.07

Table 9 The percentage of analytical recovery of Amphotericin B by HPLC method

Nominal concentration of Amphotericin B (µg/ml)	%Analytical recovery					Mean ± SD
	set 1	set 2	set 3	set 4	set 5	
1.5	102.49	100.24	100.24	102.12	101.06	101.23 ± 1.05
15	98.63	98.14	98.14	98.12	98.49	98.30 ± 0.24
25	99.45	98.72	98.72	100.14	99.80	99.37 ± 0.64

f. Precision

The precision of an analytical method is the closeness of agreement between individual test results when the method is applied repeatedly from the same sample. The precision of an analytical method is usually expresses as the variance, standard deviation or relative standard deviation (%RSD).

Table 10 and 11 showed the analytical data of within-run precision and between-run precision, respectively. The full data were showed in topic VIII (Appendix B). All range of percent relative standard deviation values were small, 0.41-1.60% and 0.86-1.50%, respectively. The relative standard deviation of the analytical method should be less than 2%, therefore this method was precise for the quantitative analysis of Amphotericin B in the studied range.

Table 10 Data of within-run precision by HPLC method

Nominal concentration of Amphotericin B (µg/ml)	Estimated concentration (µg/ml)					Mean ± SD	%RSD
	set 1	set 2	set 3	set 4	set 5		
1.5	1.49	1.45	1.49	1.50	1.52	1.49 ± 0.02	1.60
15	14.53	14.34	14.74	14.74	14.79	14.63 ± 0.19	1.30
25	24.46	24.20	24.27	24.23	24.30	24.29 ± 0.10	0.41

Table 11 Data of between-run precision by HPLC method

Nominal concentration of Amphotericin B (µg/ml)	Estimated concentration (µg/ml)					Mean ± SD	%RSD
	set 1	set 2	set 3	set 4	set 5		
1.5	1.54	1.49	1.54	1.53	1.52	1.52 ± 0.02	1.50
15	14.83	14.53	14.79	14.68	14.83	14.73 ± 0.13	0.86
25	24.92	24.46	24.52	24.96	25.05	24.78 ± 0.27	1.10

1.2 Validation of HPLC method in sample preparation

a. Selectivity

The result in topic 1.1 (a. selectivity) showed the selectivity of the HPLC method in sample preparations. The Amphotericin B in all extemporaneous preparations could separate from the other components because all of samples showed peak purity more than 0.9999.

b. Accuracy

For each formulation, the percentage of recovery was based on the theoretical concentration of Amphotericin B in the preparations and concentration found after analysis. The average percentage of analytical recovery in cream,

ointment and gel were 98.18, 93.57 and 108.85%, respectively, (Table 12). The cream and ointment formulations showed the average percentage of analytical recovery less than the gel formulation because Amphotericin B was lost during the extraction. For gel formulation, the high average percentage of analytical recovery showed non-homogeneous nature of gel preparation which would explain later on.

The data in the table 12 indicated that the relative standard deviation of the extraction method was more than 2%. It showed the error of the method which might be came up with the technique of transferring Amphotericin B solution in the extraction step. The analysis of Amphotericin B in gel formulation did not use the extraction so the relative standard deviation of gel was less than cream and ointment. However, the result represented all errors that occurring in the analytical method. The extraction procedure was applied to separate Amphotericin B from the formulation. In the future, the method of extraction should be further modified for correcting the error which would lower the %RSD.

Table 12 The percentage of analytical recovery of Amphotericin B in the formulation

Formulation		Spike concentration of Amphotericin B (mg/ml)	Start concentration of Amphotericin B found in formulation (mg/ml)	%Analytical recovery	Mean ± SD	%RSD
Cream	1	11.70	11.90	101.74	98.18 ± 6.74	6.86
	2	12.73	11.37	89.31		
	3	11.47	12.01	104.76		
	4	11.33	10.98	96.93		
Ointment	1	12.21	11.62	95.15	93.57 ± 3.82	4.09
	2	12.10	11.62	96.03		
	3	12.10	10.64	87.86		
	4	12.68	12.08	95.21		
Gel	1	12.36	13.67	110.53	108.85 ± 2.93	2.69
	2	11.20	11.71	104.50		
	3	9.88	10.94	110.66		
	4	11.16	12.24	109.70		

In conclusion, the analytical method of Amphotericin B in this study showed good selectivity which this method could separate the of Amphotericin B from the other components with the limit of detection (LOD) and the limit of quantitation (LOQ), 0.50 µg/ml and 0.75 µg/ml, respectively. The method indicated the suitable linearity that showed the coefficient of determination (R^2) of equation more than 0.9999. Moreover, the result demonstrated good accuracy and precision by showing the percentage of analytical recovery within range of 95-105% and the percentage of

relative standard deviation (%RSD) of this method less than 2%. Thus, this method was used for determination amount of Amphotericin B.

Furthermore, this analytical method was used to analyze Amphotericin B in the extemporaneous preparations to evaluate their stabilities. Although this partial validation showed the percentage of analytical recovery over range of 95-105%, this analytical method presented the selectivity that could separate Amphotericin B from the other components in the extemporaneous preparations.

Part 2: Development of three formulation bases (cream, ointment and gel) containing high amount of DMSO

Since the nail plate contained water more than lipid structure, the nail formulations should be an o/w emulsion or a hydrophilic formula (Murdan, 2002). In addition, the topical nail formulations must adhere to the nail plate for a reasonable time so that permeation enhancer could facilitate drug permeation into the nail plate. Dimethyl sulfoxide (DMSO) was reported to enhance the nail permeability (Vejnovic et al., 2010). DMSO is considered as safe for human; however it can cause formulation problems since it is an organic solvent by nature. Thus, development of formulations containing high concentration of DMSO was quite a challenge. An objective of this present study is to develop three stable based formulations, i.e. o/w cream, hydrophilic ointment and gel, containing high amount of DMSO to be used as nail formulations.

The three formulation bases, i.e. o/w cream, hydrophilic ointment and gel were developed for containing high amount of DMSO, a nail permeation enhancer. The percentage of DMSO in the formulation in a range of 5-50% is safe according to Canada FDA criterion, 2009. USFDA approves employing up to 45.5% of DMSO in topical drug products (Fda Database, 2015). In this study, the aqueous part of preparations was buffer solution (pH 7) because acidic and alkaline condition was the main cause of Amphotericin B degradation. Primarily, phosphate buffer (pH 7) had been selected for all preparations. However, in gel base formulation, phosphate

buffer was precipitated after an addition of gelling agent. Thus, Tris-hydrochloride buffer pH 7.00 was used instead to deal with this problem. In order to evaluate physical stabilities, the freshly prepared Amphotericin B preparations of all base formulations were divided into two groups, control and sample groups. They were stored in tightly closed glass vials. The control group was kept at room temperature and the test sample was kept in heating-cooling conditions. In the study, each formulation was coded as follows:

CB = cream base formulation

OB = hydrophilic ointment base formulation

GB = gel base formulation

D = DMSO

And the numbers 5-50 represented percentage of DMSO in the formulations.

2.1 Formulation of cream base

The cream base formulation was modified from a formulation presented in Handbook of Pharmaceutical Manufacturing Formulations: Semisolid Products (Niazi, 2009). During the formulation process, the compatibility of cream base ingredient with DMSO and Amphotericin B was in concern. In this study, non-ionic surfactants, Tween 60 and Span 60 were used as emulsifying agents to make o/w emulsion. Selection criteria included a formulation containing a large volume of water phase for supporting high concentration of DMSO and a formulation with physical stability.

The freshly prepared o/w cream bases containing 5-50% DMSO were opaque thick cream (Figure 13A). After passing through 6 heating-cooling cycles, o/w cream based containing DMSO less than 30% showed no any significant physical changes (Figure 13B). However, o/w cream based containing DMSO more than 35% showed a translucent layer at the bottom of the cream. The translucent layer was due to high concentration of DMSO that could dissolve wax and oil in the formulation.

After that, hydrophilic ointment and gel bases were prepared in a percent of DMSO in the range of 5-30% because this range was the maximum amount of DMSO

in stability cream base formulation. These based formulations which contained equal highest amount of DMSO would be selected for incorporation of Amphotericin B.

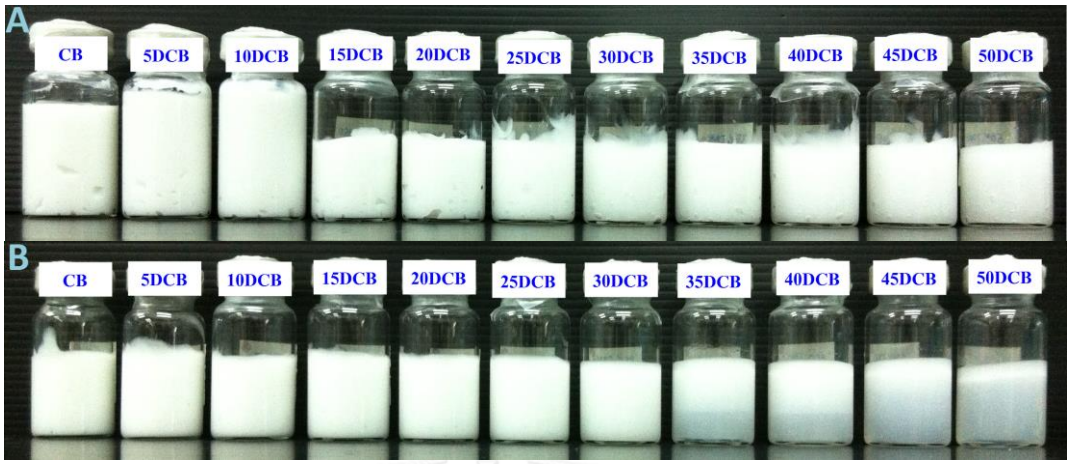


Figure 13 Physical appearance of freshly prepared o/w cream base (A) and cream base after 6 cycles of heating-cooling cycle (B).

2.2 Formulation of hydrophilic ointment base

The hydrophilic ointment base formulation was followed hydrophilic ointment in USP36. Sodium lauryl sulfate was used as an anionic emulsifier for hydrophilic ointment base to make an anionic o/w emulsion. The hydrophilic ointment base was formulated in the presence of 5-30% DMSO. The hydrophilic ointments were thick opaque cream (Figure 14A) with no significant changes in physical appearance after keeping at heating-cooling for 6 cycles (Figure 14B).

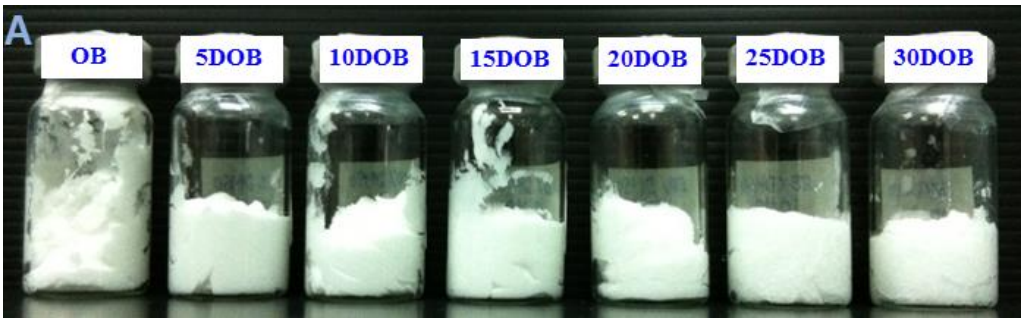


Figure 14 Physical appearance of freshly prepared hydrophilic ointment base (A) and hydrophilic ointment base after 6 cycles of heating-cooling cycle (B).

2.3 Formulation of gel base

The formulation of gel base was modified from a formulation shown in Handbook of Pharmaceutical Manufacturing Formulations: Semisolid Products (Niazi, 2009). Poloxamer 407 was selected as the gelling agent because it is generally regarded as nontoxic and nonirritant material. Moreover, Poloxamer 407 is used in a variety of pharmaceutical formulations (Fda Database, 2015). It does not need to be neutralized for producing high viscosity as carbomer and is compatible with preservative, paraben. The gel bases were prepared by addition of 5-30%DMSO into 12.5% of Poloxamer gel (Figure 15A). The obtained gels were clear viscous gels. The surface active properties of Poloxamer 407 gave rise to formation of air bubbles after gently stirring. As a result, the air bubbles were entrapped inside the viscous gels. At low concentration of DMSO, elimination of air bubbles could be done by keeping the gels in a refrigerator since the Poloxamer 407 reverts to solution when the temperature falls below the critical gelation temperature (CGT). However, at high concentration of DMSO, the air bubbles were still present even after keeping the gels in the refrigerator overnight. It was conjectured that the problem could be alleviated by mixing the mixture in a vacuum tank. Every 5% DMSO increasing could cause the reduction in the critical micellization temperature (CMT) of Poloxamer 407 about 1.5-3.5°C (Ur-Rehman, Tavelin, and Gröbner, 2010). The reducing CMT was referred to decrease of the CGT. When concentration of DMSO increased, gel bases could not revert to solution at the temperature of the refrigerator temperature (4-8 °C) so the bubbles in the gel bases at 25-30% DMSO were still presented in the formulation.

Furthermore, the gels showed no significant changes in their physical appearance after passing through 6 heating-cooling cycles (Figure 15A).

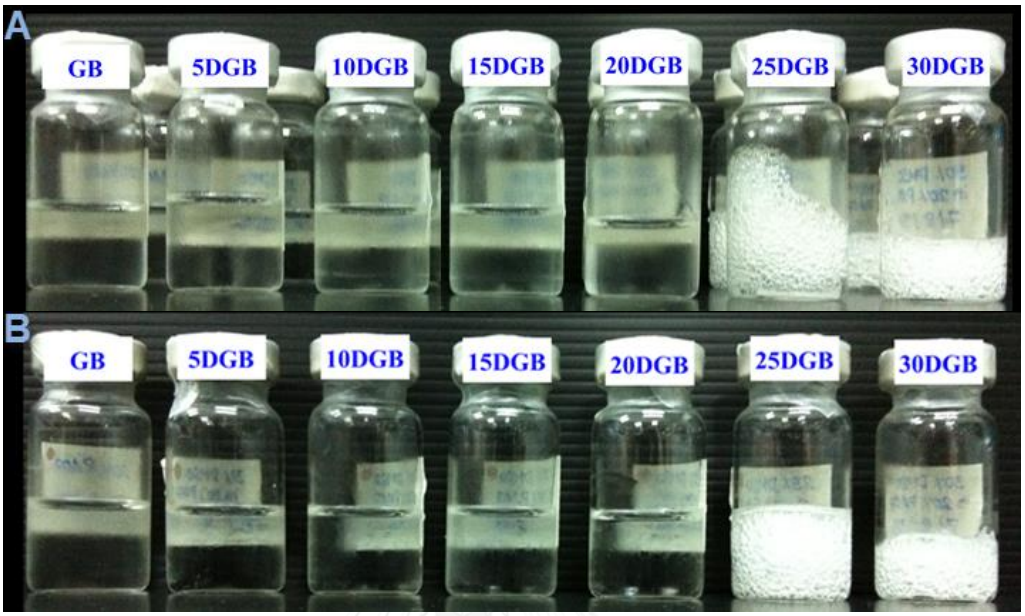


Figure 15 Physical appearance of freshly prepared gel base (A) and gel base after 6 cycles of heating-cooling cycle (B).

In this present work, three based formulations; i.e. o/w emulsion, hydrophilic ointment and gel, containing 30%DMSO were developed. All of them showed reasonable physical stability after passing through 6 heating-cooling cycles. In the further studies, Amphotericin B would be incorporated into these based formulations. The formulations were subject to both physical and chemical tests prior to determination of the nail permeability.

Part 3: Formulation and stability evaluation of extemporaneous Amphotericin B topical preparations

3.1 Formulation of extemporaneous Amphotericin B topical preparations

In this section, the extemporaneous Amphotericin B topical preparations; i.e. o/w emulsion, hydrophilic ointment and gel, containing 30%DMSO were formulated by incorporating Amphotericin B for injection into the base formulations. The Amphotericin B for injection is supplied in vials as a sterile lyophilized powder

providing 50 mg Amphotericin B in 41 mg sodium desoxycholate and 25.2 mg sodium phosphate. The concentration of topical preparation depended on the dissolution of Amphotericin B for injection in DMSO. Although the solubility of Amphotericin B in DMSO is 30-40 mg/ml (Asher, and Schwartzman, 1977), in this study, the highest concentration of Amphotericin B for injection in DMSO was about 10 mg/ml giving a clear solution. This is because the Amphotericin B for injection composes of not only Amphotericin B but also other excipients and the buffer may incompletely dissolve in DMSO resulting in a suspension which is not suitable for preparation of topical preparations. To obtain a clear solution, 5 ml of DMSO was used to dissolve content in a vial of Amphotericin B for injection. The Amphotericin B for injections before and after dissolving were shown in Figure 16. Since the base formulations containing 30%DMSO showed good physical stabilities, the drug loading in these topical preparations was limited to 3 mg/ml or 0.3% Amphotericin B.

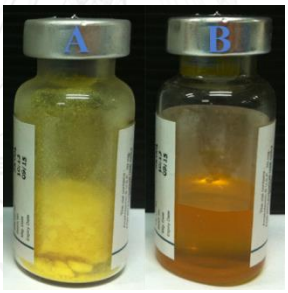


Figure 16 Amphotericin B for injection (A) and Amphotericin B solution mixed with DMSO (B)

3.2 Physical stability evaluation

In terms of physical stability evaluation, the extemporaneous Amphotericin B topical preparations; i.e. o/w emulsion, hydrophilic ointment and gel, were evaluated by heating-cooling method. Figure 17-19 showed three batches of the physical appearance of the extemporaneous Amphotericin B cream, ointment and gel, respectively, after passing through 6 heating-cooling cycles. Color of cream and ointment products changed from pale yellow to yellow after 6 heating-cooling cycles. The color change was expected to be due to Amphotericin B degradation at high temperature of heating cycles (45^{o}C). But phase separation was not observed.

The microscope image of Amphotericin B standard reference powder, Amphotericin B cream and Amphotericin B ointment were shown in topic I of Appendix C. The crystallization of Amphotericin B was not observed in preparations after storage at room temperature for 1 year.

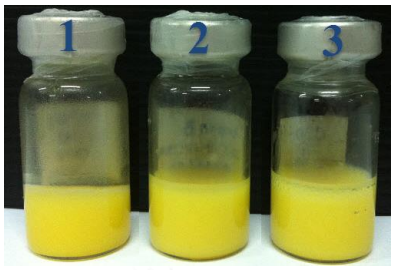


Figure 17 Physical appearance of extemporaneous Amphotericin B cream after passing through 6 heating-cooling cycles

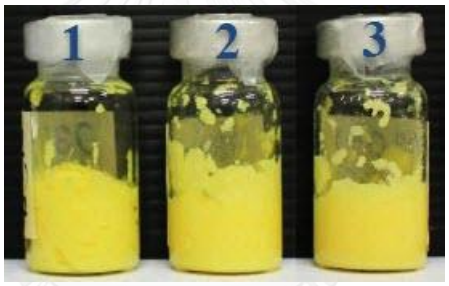


Figure 18 Physical appearance of extemporaneous Amphotericin B ointment after passing through 6 heating-cooling cycles

For three batches of Amphotericin B gel formulation, after through 6 cycles of heating-cooling method, the Amphotericin B precipitated in to the bottom of vial. In this study, the extemporaneous Amphotericin B gel reverted to solution when cooling phase that contrast with what was observed in the gel base. Therefore, the precipitation was expected. The result showed that Poloxamer 407 might be sensitive to the concentration of ingredient in the system. A sol-gel transition temperature of Poloxamer 407 in the presence of different solutes is unpredictable until the experiment is actually performed (Dumortier et al., 2006).

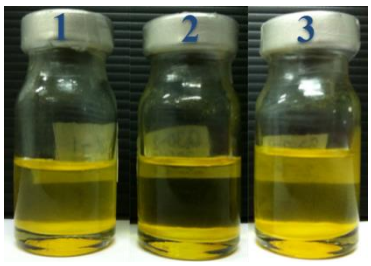


Figure 19 Physical appearance of extemporaneous Amphotericin B gel after passing through 6 heating-cooling cycles

There was a partially physical testing of cream formulation. The freshly prepared of Amphotericin B cream was separated in to two vials. Frist vial was kept in incubator at 30ºC and the other was kept in a refrigerator. After three month, the physical appearance of the 2 samples was determined. The Amphotericin B cream was kept in a refrigerator showed no color change but the cream in an incubator turned yellow (Figure 20). The Amphotericin B ointment kept in a refrigerator showed no color change but the ointment in an incubator turned yellow as well (the picture was not shown). As mention earlier the color change was expected to be due to the instability of Amphotericin B at high temperature. Thus, the Amphotericin B product was recommended to store in a cool (Groeschke et al., 2006).

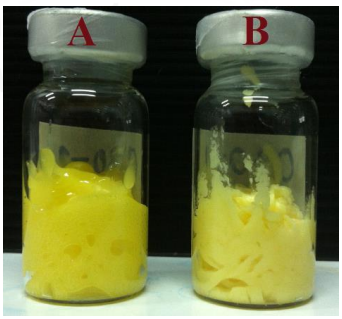


Figure 20 Physical appearance of cream after passing three month (A) in incubator, 30ºC and (B) in refrigerator

In conclusion, only extemporaneous Amphotericin B cream and ointment showed reasonable physical stability after passing through 6 heating-cooling cycles. However, they had darker yellow than the beginning when keeping in incubator at 30ºC.

3.3 Chemical stability evaluation

The chemical stability of each extemporaneous Amphotericin B preparation was studied according to the ASEAN guideline on stability study of drug product. Previous stability studies of Amphotericin B (Dentinger, Swenson, and Anaizi, 2001, Manosroi, Kongkaneramit, and Manosroi, 2004, Groeschke et al., 2006) reported that shelf-lives of Amphotericin B preparation depend on formulations. The purpose of this part was to explore beyond-used dates of the preparation up to 1 month. The extemporaneous Amphotericin B cream was brought to this preliminary experiment. The preliminary results showed that the extemporaneous Amphotericin B cream was chemically stable for more than one month so the test period was extended to 2 months. The linear regression analysis of the concentration remaining at each time point was established to determine the shelf life of drug product. The shelf life was estimated by finding the first time at which the 95 percent confidence limit of the mean around the linear regression curve intersected the proposed acceptance criterion.

The 95 percent confidence interval was constructed from the true value of amount of Amphotericin B (Y) in each time point (X) by Equation 1 (Bolton, and Bon, 2010).

$$Y \pm t(S_{Y,x})\sqrt{\frac{1}{N} + \frac{(X - \overline{X})^2}{\sum (X - \overline{X})^2}} \qquad \textbf{Equation 1}$$

Where Y = the predicted amount of Amphotericin B from the least squares line

t = *t* value of 95% confidence interval, *N*-2 d.f.

$S_{Y,x}$ = variance from least squares line

N = number of data

X = time (days)

$\overline{X}$ = mean of time

3.3.1 The extemporaneous Amphotericin B cream

Three lots of Amphotericin B cream were prepared for the chemical stability evaluation. The first lot was the pilot lot so the sampling time points were different from the others. The acceptance criteria in USP36 were not less than 90 percent and not more than 125 percent of labeled amount of Amphotericin B, so the range in this study was 2.70-3.75 mg/ml. The result of stability of Amphotericin B in extemporaneous Amphotericin B cream was shown in Table 13. The data was indicated in the Amphotericin B concentration at the sampling time point. The full data were showed in topic II of Appendix C.

Table 13 Stability of Amphotericin B 3 mg/mL in extemporaneous Amphotericin B cream at 30 ± 2°C

Lot.	Initial Concentration (mg/mL)	Amphotericin B Concentration Remaining (mg/ml)*				
	T = 0	3 Days	11 Days	36 Days	59 Days	
C-1	3.37 ± 0.02	3.22 ± 0.03	3.23 ± 0.03	3.30 ± 0.02	3.31 ± 0.06	
	T = 0	7 Days	14 Days	23 Days	28 Days	35 Days	48 Days	62 Days
C-2	3.57 ± 0.01	3.58 ± 0.01	3.35 ± 0.01	3.47 ± 0.05	3.56 ± 0.01	3.49 ± 0.03	2.92 ± 0.04	2.48 ± 0.04
C-3	3.26 ± 0.01	3.26 ± 0.01	2.94 ± 0.01	2.88 ± 0.03	2.68 ± 0.01	2.81 ± 0.01	3.35 ± 0.03	3.57 ± 0.00

*Mean ± S.D. (n = 3)

All data of remaining amount of Amphotericin B in the extemporaneous cream were plotted for the assessment of the Amphotericin B stability. The amount of Amphotericin B in each time interval was converted to the predicted amount of Amphotericin B from the least squares regression analysis. Then, 95 percent confidence interval was constructed from Equation 1. The Figure 21 showed the trend of 95 percent confidence interval of the amount of Amphotericin B that remained above 90 percent of labeled amount until 62 days. In the future, if there was repeating the result of stability, the data of remaining amount of Amphotericin B should be extended to cover the real shelf-life that was the time point at the lower of 95 percent confidence interval intersects with the lower acceptance criterion. Moreover, the pH of all lots was in 6.9-7.1 and the physical appearance of the sample showed no significant changes throughout the experiment period.

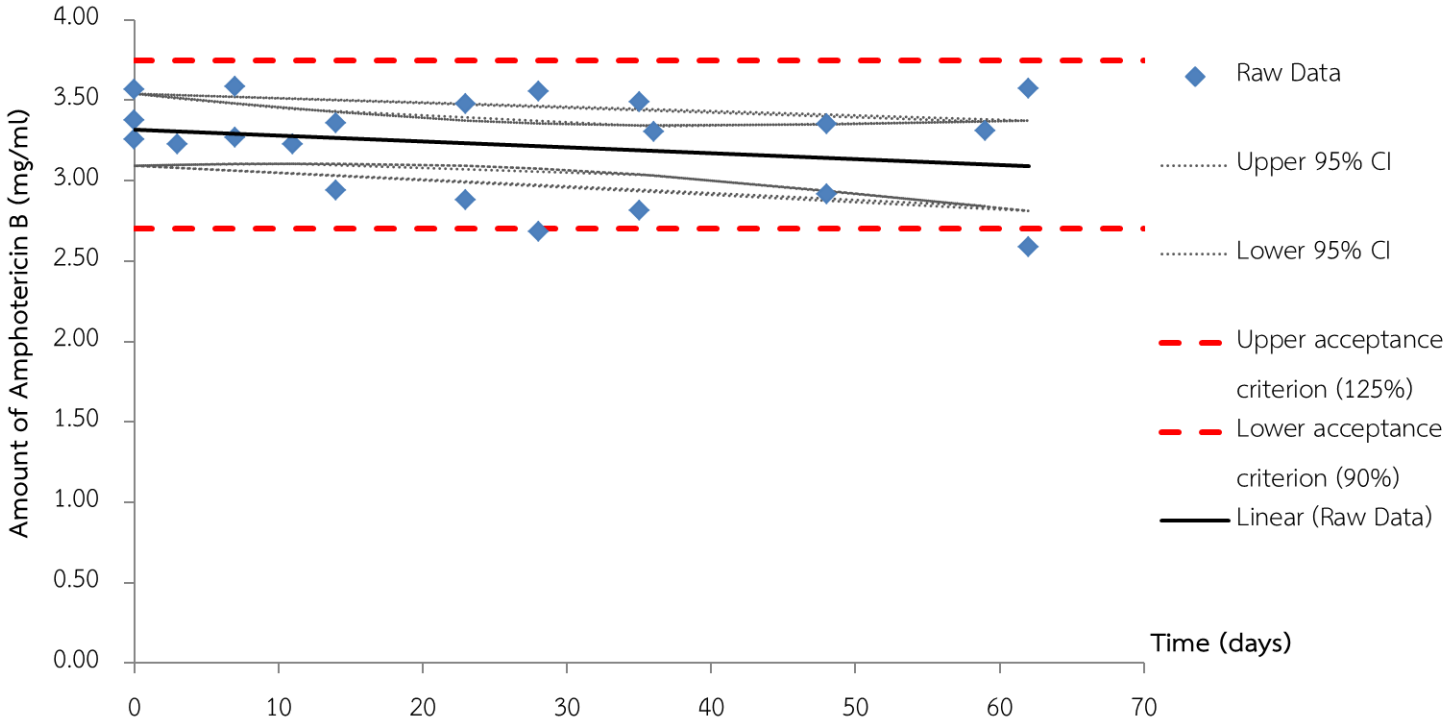


Figure 21 Shelf life estimation of extemporaneous Amphotericin B cream with upper and lower acceptance criteria based on assay at 30±2 °C

3.3.2 The extemporaneous Amphotericin B ointment

The result of the chemical stability of Amphotericin B in the extemporaneous ointment was represented in Table 14 and Figure 22 which were similar to the other formulations. The Figure 22 showed the trend of 95 percent confidence interval of the amount of Amphotericin B that remained above 90 percent of labeled amount and the pH of all lots was the same in cream formulation, pH= 6.9-7.1, throughout 62-day study period.

Table 14 Stability of Amphotericin B 3 mg/mL in extemporaneous Amphotericin B ointment at 30 ± 2°C

Lot.	Initial Concentration (mg/mL)	Amphotericin B Concentration Remaining (mg/ml)*							
	T = 0	7 Days	16 Days	21 Days	28 Days	35 Days	48 Days	55 Days	62 Days
O-1	3.61 ± 0.01	3.40 ± 0.00	3.70 ± 0.01	3.06 ± 0.01	3.69 ± 0.03	3.84 ± 0.01	3.52 ± 0.00	2.76 ± 0.01	3.29 ± 0.01
O-2	3.63 ± 0.01	3.32 ± 0.02	3.63 ± 0.01	3.71 ± 0.02	3.50 ± 0.00	3.56 ± 0.01	3.32 ± 0.01	3.30 ± 0.01	3.12 ± 0.01
O-3	3.32 ± 0.01	3.51 ± 0.00	3.55 ± 0.01	3.19 ± 0.01	3.55 ± 0.01	3.64 ± 0.10	3.68 ± 0.01	3.24 ± 0.01	2.89 ± 0.03

*Mean ± S.D. (n = 3)

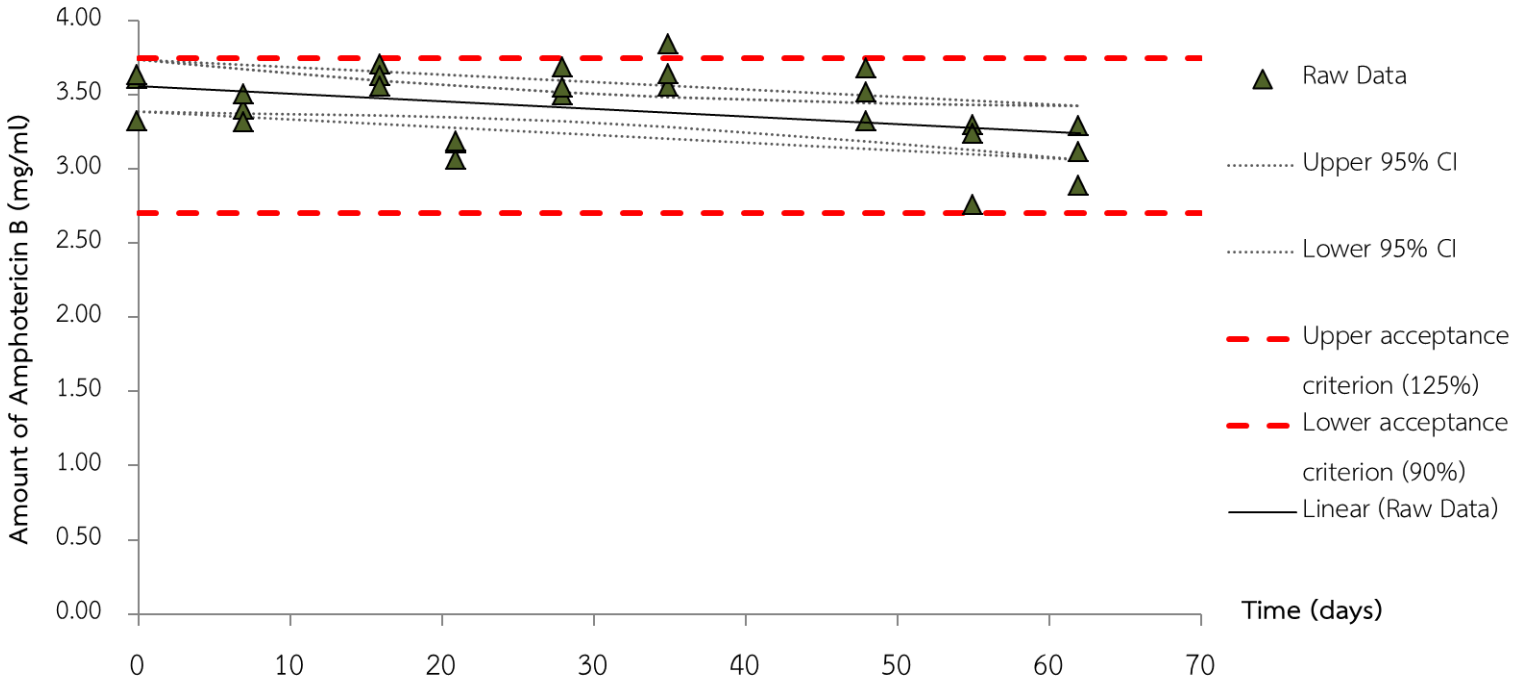


Figure 22 Shelf life estimation of extemporaneous Amphotericin B ointment with upper and lower acceptance criteria based on assay at 30±2 °C

3.3.3 The extemporaneous Amphotericin B gel

The chemical stability of the extemporaneous Amphotericin B gel was performed in the same approach of other formulations. The result in Table 15 indicated the Amphotericin B concentration at each time interval and Figure 23 showed the continuous tendency of Amphotericin B in the extemporaneous gel. The decrease amount of Amphotericin B in gel formulation happened faster than in other formulations so the extemporaneous gel would be stable about 50 days after mixing. The range of pH in gel formulation was 7.4-7.7 which was the most valuable pH of all formulation. Although it was a stable range of Amphotericin B, the ability of buffer might be influenced by solubility of Poloxamer 407. This would be one factor that affected the less stability of gel formulation than the others. Graph in Figure 23 showed the intersection of lower 95 percent confidence limit and lower acceptance criterion that was the estimated the real shelf life.

Table 15 Stability of Amphotericin B 3 mg/mL in extemporaneous Amphotericin B gel at 30 ± 2°C

Lot.	Initial Concentration (mg/mL)	Amphotericin B Concentration Remaining (mg/ml)*						
	T = 0	7 Days	14 Days	21 Days	30 Days	42 Days	49 Days	62 Days
G-1	3.12 ± 0.00	3.15 ± 0.01	3.09 ± 0.00	3.08 ± 0.00	3.10 ± 0.02	2.98 ± 0.01	3.14 ± 0.01	3.03 ± 0.00
G-2	2.93 ± 0.00	2.75 ± 0.01	2.69 ± 0.01	2.60 ± 0.01	2.70 ± 0.01	2.59 ± 0.01	2.66 ± 0.01	2.57 ± 0.01
G-3	3.26 ± 0.01	3.12 ± 0.01	3.20 ± 0.01	3.08 ± 0.00	3.04 ± 0.01	2.79 ± 0.01	3.09 ± 0.00	3.07 ± 0.01

*Mean ± S.D. (n = 3)

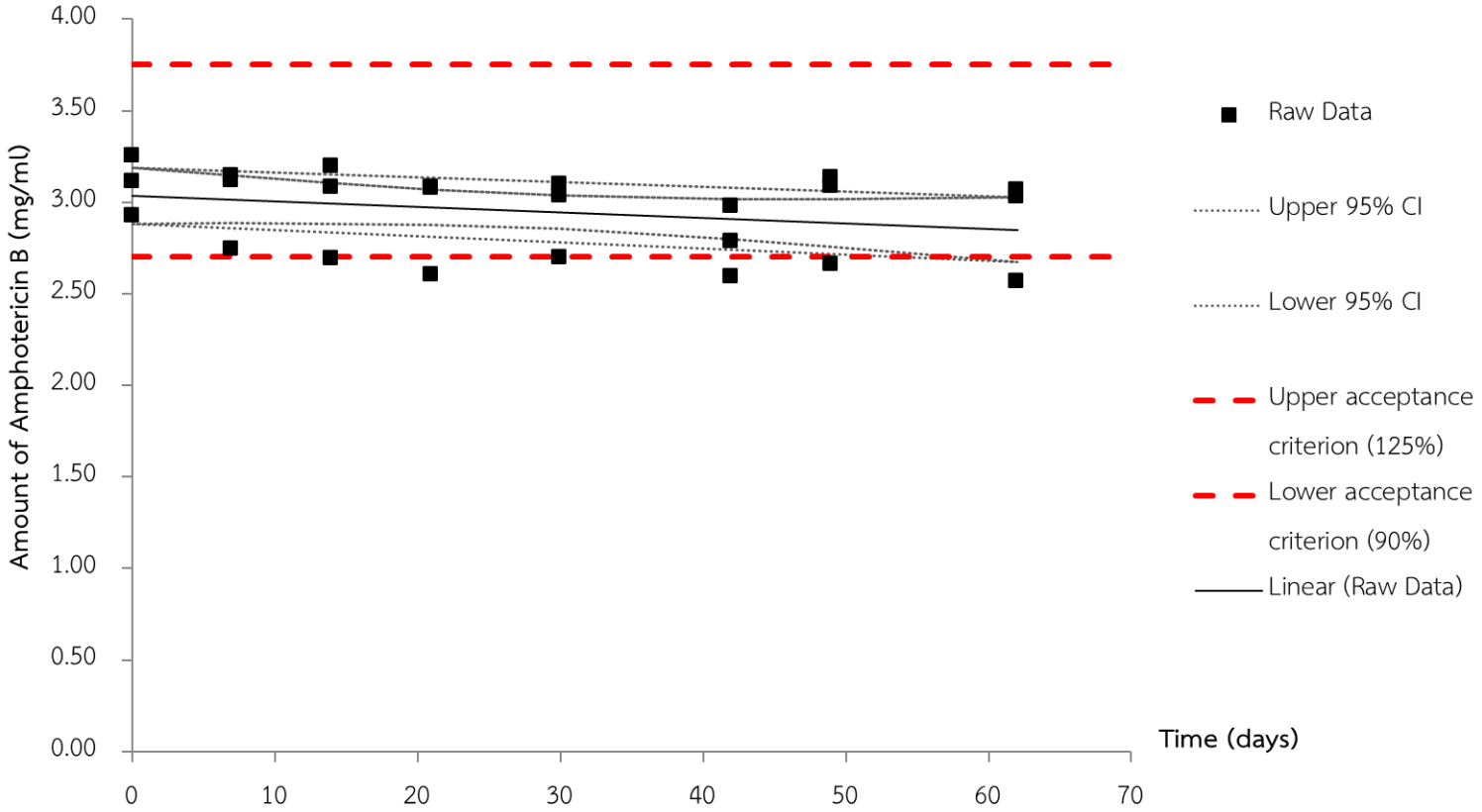


Figure 23 Shelf life estimation of extemporaneous Amphotericin B gel with upper and lower acceptance criteria based on assay at 30±2 °C

In the experiment, the extemporaneous Amphotericin B formulation was prepared from commercial drug instead of pure drug so the variation of the initial concentration was to be expected. When compared with three formulations, the gel formulation would be the smallest amount of Amphotericin B in initial concentration. This might occur from the precipitated of Amphotericin B in the unstable gel preparation so the initial concentration was smaller than the true concentration.

The result from the three studies showed the shelf life of the extemporaneous Amphotericin B cream, ointment and gel which depended on the chemical stability was 62, 62 and 50 days, respectively. When consideration of chemical stability and organoleptic characteristics in physical stability, the suitable formulation was used in further *in vitro* study were the cream and ointment formulation.

Part 4: Evaluation of permeation of extemporaneous Amphotericin B topical preparations in human nail plate

The purpose of this part was to compare the permeation of Amphotericin B in each preparation in the healthy human nail plate. The cream and ointment formulations which passed stability test were chosen for further test in this *in vitro* study. The formulation with the highest permeation would be selected for clinical study in the future.

In this permeation study, only finger clipping nail plates were used because the fingernail grew about 3 mm per month while the toenail used about 3 months for the same length so this method was convenient for subjects. In this experiment, the clipping human nail plates from the healthy volunteers were used because the permeation through healthy and fungal nail plates was not significantly different (Kobayashi et al., 2004). The clipping nail plates were cleaned, cut and dried at room temperature before the experiment. Ten pieces of nail clippings form the same subject were used in each set of experiment. There was recorded weight and area of nail clippings in order to limit the variation that influence amount of Amphotericin B in the nail plate. In this experiment, the pool of sample was selected because there

was a little of Amphotericin B that would permeate into the nail plate. If the spiking was done, there was more error than the pooling method. So ten pieces of nail clippings were verified that could present the amount of Amphotericin B in the nail plate higher than the limit of quantity of HPLC method.

After apply the testing formulation on the clipping nail plates, they was kept in an incubator at 32±2^{o}C and 75±5% humidity for 8 hours. This storage conditions was simulated a real patient using. The practical use was after patient applied formulation on onychomycosis, the nail would be wrapped with a bandage for occlusion effect and dirty protection before bed time. The surface skin temperature was around 32 ºC so the clipping nail plates were incubated in this temperature. Moreover, Thailand was in zone IV climatic condition so the relative humidity in this experiment was 75%.

4.1 Development of nail permeation procedure

The pooling ten pieces of nail clippings was used in this study because this quantity could show the amount of Amphotericin B that permeated into the nail plate at a concentration more than the limit of quantity of HPLC method. Hexane was used to clean the preparation that was mostly oil and wax from the surface of the nail plates.

4.1.1 Verification of cleansing procedure

The cleansing method was divided into two steps. Initially, the amount of Amphotericin B which from the second cleansing step by 2 ml of hexane would be analyzed by HPLC method separated from first step. The result in table 16 showed the amount of Amphotericin B in each step of cleansing and the percentages of analytical recovery of Amphotericin B in cleansing process. This method ensured the cleansing process because the amount of Amphotericin B that was analyzed in the second step was lower than the limit of quantity of HPLC method (0.75 µg/ml).

Table 16 The percentage of analytical recovery of Amphotericin B in cleansing process

Set	Adjusted concentration of cream (mg/ml)	Apply on the nail plates (mg)	Total amount of Amphotericin B in cream (µg)	Amount of Amphotericin B (µg)				%Analytical recovery
				1st clean	2nd clean	Nail plate	Total	
1	3.59	27.51	98.80	102.11	< 0.75	4.16	106.38	107.67
2	3.59	36.27	130.26	134.56	< 0.75	4.40	139.08	106.77
3	3.59	35.59	127.82	130.63	< 0.75	6.56	137.30	107.42

After that, two steps of cleansing process would be pooled for analysis the amount of Amphotericin B, that could not permeate to the nail plate, in only one time as same as the present method. The cotton was used to wipe the clipping nail plate to make sure that the cleansing would be complete process.

4.2 Nail permeation studies

In general, a Franz diffusion cells was the equipment that used to evaluate the permeation study. But the limitation in this study was the model for nail plate which be made for specific size. Moreover, very limited amount of Amphotericin B is expected to permeate through the nail plate so the Franz diffusion method was not chosen in this study.

4.2.1 Nail permeation after application of cream preparation

In this section, three sets of ten nail clippings that immediately cleaned after applying cream were compared the amount of Amphotericin B with the nail clippings that cleaned after applying 24 hours. There was no statistically significant difference between groups (p=0.499) when evaluated by parametric statistic in SPSS program version 17.0. The amount of Amphotericin B in the nail clippings of two groups showed in table 17 and the comparison of them was computed by ANOVA that in topic I (Appendix D).

Table 17 The amount of Amphotericin B in the nail clippings at 0 hr. and 24 hr. after applying Amphotericin B cream preparation

Set	Time to apply = 0 hr.		Time to apply = 24 hr.	
	Nail area (mm^2)	Amphotericin B in nail plates (µg)	Nail area (mm^2)	Amphotericin B in nail plate (µg)
1	148.37	7.00	146.88	7.29
2	155.89	5.85	159.39	8.04
3	123.54	2.41	125.49	4.04

The nail clippings were matching in the nearly same area. The result showed that Amphotericin B cream could not permeate to the nail plate. However, the percentage of analytical recovery of Amphotericin B from nail clippings (table 16) were more than 100%, this meant the method of the analysis can examine the amount of Amphotericin B in nail plate more than the actual amount. This problem would be proved in clinical trial.

Moreover, the filed nail clipping was examined for the amount of Amphotericin B that could permeate to the nail plate compared with the normal nail clipping. The experiment was tested in six sets of ten nail clippings. After apply cream preparation, they was kept in an incubator at 32±2^{o}C and 75±5% humidity for 24 hours. The amount of Amphotericin B in the two groups of nail clippings showed in table 18 and the statistical result that computed by ANOVA was shown in topic II (Appendix D). The two groups of nail were not significantly different (p=0.559) and the filing on the nail plate would not increase the permeation. The nail clippings in each couple of this experiment a section brought from one healthy volunteer for minimize the variation. The controls were the normal nail clippings which were applied cream base but the tested nail clippings were taken from the other healthy volunteers.

Table 18 The amount of Amphotericin B in the normal and filed nail clippings after applying Amphotericin B cream preparation about 24 hr.

Set	Amphotericin B in nail plate (µg)		
	Control	Non-filed	Filed
1	< 0.75	3.83	6.43
2	< 0.75	4.04	5.70
3	< 0.75	5.95	7.19
4	< 0.75	8.04	7.86
5	< 0.75	6.42	6.69
6	< 0.75	7.29	7.31

4.2.2 Nail permeation studies to compare cream and ointment preparations

The study design was mimicked the practical use of the patient which would apply a topical antifungal drug in every day. In this experiment, the permeation of Amphotericin B in the nail clippings were compared the cream and ointment formulations. The study was done in triplicates in nail plates from 3 different volunteers nail. Nails from each volunteer were separately exposed to both cream and ointment. The nail clippings were selected for the testing with the same area and weight which this method tried to eliminate the extraneous variables that could affect the result. Moreover, the nail clippings would be applied with almost the same amount of formulation.

At first trial method, the nail clippings were applied with the same amount of cream or ointment and then each set of nail clippings would be kept at room temperature for 8 hours. After 8 hours, they were washed with tap water until clean. The cream or ointment was applied with the same process for 5 consecutive days. After that, each set of nail clippings were cut and mixed with 1 ml of DMSO to

dissolving Amphotericin B in the nail clippings. The amount of Amphotericin B in each set of nail clippings showed in table 19.

Table 19 The amount of Amphotericin B in the nail clippings (after 8 hr. application)

Formulation	Cream			Ointment		
	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
Nail clipping area (mm^2)	204.48	193.74	236.37	206.12	182.10	206.92
Nail clipping weight (mg)	98.12	83.51	117.08	103.49	82.83	110.72
Amphotericin B in nail (µg)	< 0.75	< 0.75	0.82*	< 0.75	< 0.75	4.81*

The result from the first method shown that there was only one set of nail clippings in each formulation (*) that could correctly examine the amount of Amphotericin B because the amount was more over the limit of quantitation of the HPLC system. However, the total results were tested the difference of permeation with Independent Samples Test in SPSS program version 17.0. The result in topic III (Appendix D) shown that there was no significant differences between two formulations (p=0.499). The probable cause of this was Amphotericin B can dissolve in a lot of water during daily cleansing step. The solubility of Amphotericin B in basic water is 0.1 mg/ml (Asher, and Schwartzman, 1977) so a small amount of Amphotericin B that permeated in to nail clipping could dissolve back to tab water. The proper instruction was patients should apply before bedtime because Amphotericin B would has enough time contacting the nail and convenient to use.

At second method, the process of cleansing was improved to use hexane instead of water and the amount of hexane was strictly limited to 5 ml for cleansing. The hexane had property to dissolve wax and oil; in addition, hexane could dissolve Amphotericin B less than water. The solubility of Amphotericin B in hexane is 0.02 mg/ml (Asher, and Schwartzman, 1977). The method was conducted as same as the first. The nail clippings in the second method not only had the same weight and area but also brought from the same volunteer in each set of comparison. The result shown in Table 20 revealed the dissimilar amount of Amphotericin B when

compared with the first process. In the second cleansing method could find Amphotericin B from the nail clipping. After comparison with Independent Samples Test in SPSS program, the result showed significantly different among the formulations (p=0.007). Both of formulation could permeate to the nail plate when compared with the self-base formulation (Appendix D, topic IV b and c), but the ointment formulation was better than cream. The result of was shown in topic IV a (Appendix D).

Table 20 The amount of Amphotericin B in the nail clippings (at second method)

Formulation	Cream			Ointment		
	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
Nail clipping area (mm^2)	195.62	217.11	195.66	192.48	224.91	199.54
Nail clipping weight (mg)	85.00	90.40	80.30	84.30	94.70	88.10
Amphotericin B in nail (µg)	12.85	8.39	14.63	24.35	34.40	29.04

The better permeation of Amphotericin B observed from the ointment formulation was probably due to the presence of sodium lauryl sulfate (SLS) in the ointment formulation. The SLS, the anionic emulsifier in this hydrophilic ointment preparation, could increase solubility of Amphotericin B in the water (Asher, and Schwartzman, 1977). SLS was also a human skin permeation enhancer. When the solubility of Amphotericin B in preparation was improved, the permeation was increased. Although the Amphotericin B ointment would permeate to the nail clipping more than cream, both formulations showed that the concentrations of Amphotericin B presented in the nail plate were higher than the minimum inhibitory concentrations (MICs) of the interesting fungi (Lacroix, and De Chauvin, 2008, Pujol et al., 1997).

In the next step, the extemporaneous Amphotericin B topical preparations those are formulated and examined the correct shelf life will be brought to clinical study in the onychomycosis patient for evaluation of the efficacy. If this clinical

research is successful, the extemporaneous Amphotericin B topical preparations will be a choice for treatment of onychomycosis in Thailand.

CHAPTER V

CONCLUSIONS

The present study was aimed to formulate the stable extemporaneous Amphotericin B topical preparation from an amphotericin B for injection product for treatment of onychomycosis. Three extemporaneous Amphotericin B topical preparations; i.e. o/w emulsion, hydrophilic ointment and gel, were developed. The preparations contain high amount of dimethyl sulfoxide (DMSO) as a nail permeation enhancer. Concentration of DMSO in the three base formulations was limited to 30% based on their physical stability and solubility of Amphotericin B. This concentration of DMSO was shown to be safe in human. Tris-hydrochloride buffer was employed to control pH at 7 where Amphotericin B is the most stable.

After stability test, only Amphotericin B cream and hydrophilic ointment showed acceptable stabilities with beyond-use date around 60 days. Amphotericin B ointment preparation showed the higher permeability than cream preparation. However, both formulations gave rise to amount of Amphotericin B in nail plate higher than the MICs of Amphotericin B against *Scytalidium dimidiatum* and *Fusarium* spp, the main causes of nondermatophyts onychomycosis in Thailand. In the further clinical study, these extemporaneous Amphotericin B topical preparations will be evaluated for the therapeutic and adverse effects, including the patient's satisfaction of using.

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APPENDICES

APPENDIX A

The Ethics certificate of approval for researching involve human subjects

AF 02-12

The Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University
Institute Building 2, 4 Floor, Soi Chulalongkorn 62, Phyat hai Rd., Bangkok 10330, Thailand,
Tel: 0-2218-8147 Fax: 0-2218-8147 E-mail: eccu@chula.ac.th

COA No. 120/2014

Certificate of Approval

Study Title No.079.1/57 : **FORMULATION OF EXTEMPORANEOUS AMPHOTERICIN B TOPICAL PREPARATIONS CONTAINING HIGH AMOUNT OF DIMETHYL SULFOXIDE FOR THE TREATMENT OF ONYCHOMYCOSIS**

Principal Investigator : MISS PHOJANA KOMESMUNEEBORIRAK

Place of Proposed Study/Institution : Faculty of Pharmaceutical Sciences, Chulalongkorn University

The Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University, Thailand, has approved constituted in accordance with the International Conference on Harmonization – Good Clinical Practice (ICH-GCP) and/or Code of Conduct in Animal Use of NRCT version 2000.

Signature: Prida Tasanapradit Signature: Nuntaree Chaichanawongsaroj

(Associate Professor Prida Tasanapradit, M.D.) Chairman

(Assistant Professor Dr. Nuntaree Chaichanawongsaroj) Secretary

Date of Approval : 15 August 2014 Approval Expire date : 14 August 2015

The approval documents including

1) Research proposal
2) Patient/Participant Information Sheet and Informed Consent Form
3) Researcher

Protocol No. 079.1/57
Date of Approval 15 AUG 2014
Approval Expire Date 14 AUG 2015

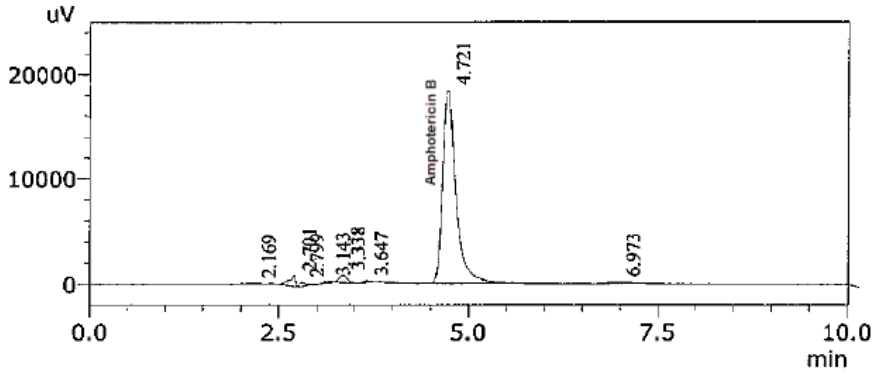
The approved investigator must comply with the following conditions:

1. *The research/project activities must end on the approval expired date of the Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University (ECCU). In case the research/project is unable to complete within that date, the project extension can be applied one month prior to the ECCU approval expired date.*
2. *Strictly conduct the research/project activities as written in the proposal.*
3. *Using only the documents that bearing the ECCU's seal of approval with the subjects/volunteers (including subject information sheet, consent form, invitation letter for project/research participation (if available).*
4. *Report to the ECCU for any serious adverse events within 5 working days*
5. *Report to the ECCU for any change of the research/project activities prior to conduct the activities.*
6. *Final report (AF 03-12) and abstract is required for a one year (or less) research/project and report within 30 days after the completion of the research/project. For thesis, abstract is required and report within 30 days after the completion of the research/project.*
7. *Annual progress report is needed for a two- year (or more) research/project and submit the progress report before the expire date of certificate. After the completion of the research/project processes as No. 6.*

APPENDIX B

Validation of HPLC method

I. The chromatogram of stability indicating assay of Amphotericin B

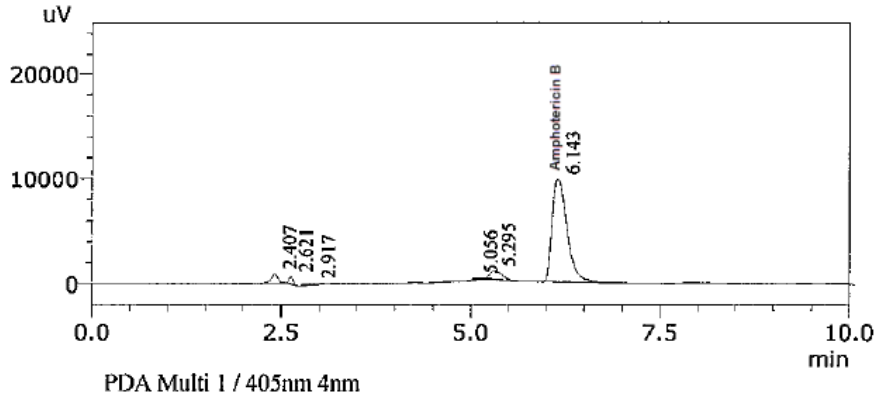


PDA Multi 1 / 405nm 4nm

PeakTable
PDA Ch1 405nm 4nm

Peak#	Ret. Time	Area	Height	ailing Facto	Theoretical Plate#	Resolution
1	2.169	1052	85	0.872	610.847	0.000
2	2.701	6066	1051	0.615	2373.676	1.856
3	2.799	2434	389	2.226	4208.418	0.497
4	3.143	1263	201	0.844	5600.461	2.024
5	3.338	4690	657	1.209	4178.425	1.037
6	3.647	1182	188	1.330	5845.884	1.559
7	4.721	220360	18411	1.634	3798.201	4.317
8	6.973	2337	142	0.847	3598.073	5.839
Total		239385	21125			

Figure B1 HPLC chromatogram of 2 µg/ml Amphotericin B standard solution

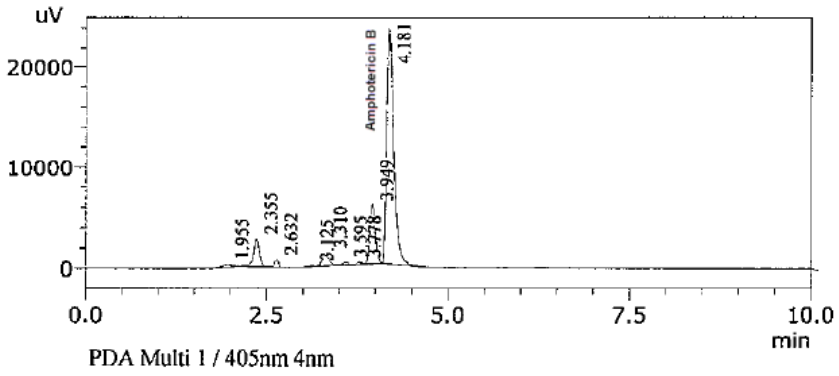


PDA Multi 1 / 405nm 4nm

PeakTable
PDA Ch1 405nm 4nm

Peak#	Ret. Time	Area	Height	ailing Facto	Theoretical Plate#	Resolution
1	2.407	5492	845	0.907	2880.015	0.000
2	2.621	2589	679	1.181	7822.884	1.438
3	2.917	1731	130	0.662	2233.629	1.620
4	5.056	1046	145	1.223	10464.555	9.620
5	5.295	8662	740	1.795	4756.133	0.946
6	6.143	132666	9749	1.619	4750.040	2.557
Total		152186	12288			

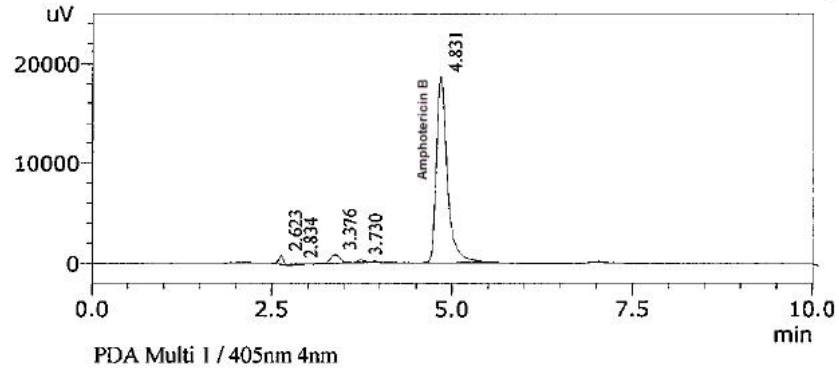
Figure B2 HPLC chromatogram of 2 µg/ml Amphotericin B in 0.1N hydrochloric acid



PeakTable
PDA Ch1 405nm 4nm

Peak#	Ret. Time	Area	Height	Tailing Factor	Theoretical Plate#	Resolution
1	1.955	2762	268	0.971	598.651	0.000
2	2.355	14854	2735	0.923	3784.814	1.690
3	2.632	3116	750	0.883	7518.633	2.016
4	3.125	550	97	0.718	13637.643	4.322
5	3.310	7926	1281	1.108	5545.767	1.294
6	3.595	1458	266	0.938	7807.000	1.675
7	3.778	1083	226	0.933	11823.915	1.214
8	3.949	30583	5891	1.275	10405.142	1.168
9	4.181	153409	23522	1.853	8430.762	1.374

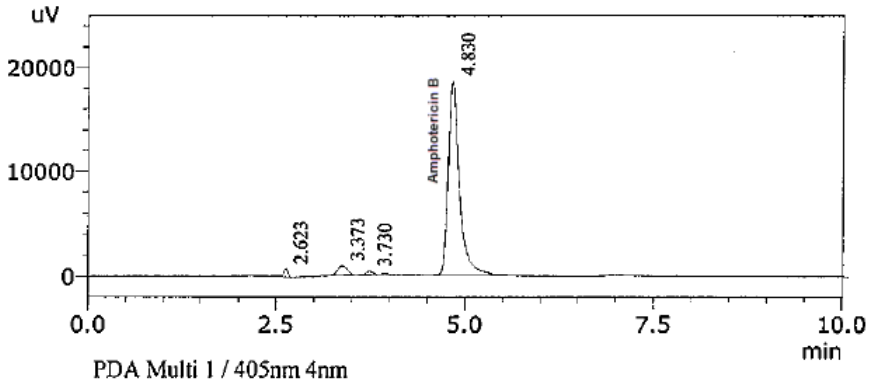
Figure B3 HPLC chromatogram of 2 µg/ml Amphotericin B in 0.01N sodium hydroxide



PeakTable
PDA Ch1 405nm 4nm

Peak#	Ret. Time	Area	Height	Tailing Factor	Theoretical Plate#	Resolution
1	2.623	3746	808	0.924	6138.321	0.000
2	2.834	1064	132	1.253	3618.405	1.307
3	3.376	7511	815	0.956	2935.575	2.477
4	3.730	1361	233	1.128	6521.597	1.629
5	4.831	192737	18651	1.673	5267.266	4.883
Total		206419	20638			

Figure B4 HPLC chromatogram of 2 µg/ml Amphotericin B in 0.5% hydrogen peroxide



PeakTable
PDA Ch1 405nm 4nm

Peak#	Ret. Time	Area	Height	Tailing Factor	Theoretical Plate#	Resolution
1	2.623	3158	732	0.913	6622.656	0.000
2	3.373	7575	852	1.048	2954.938	3.981
3	3.730	1750	277	1.140	6020.157	1.618
4	4.830	192565	18671	1.675	5317.699	4.811
Total		205048	20532			

Figure B5 HPLC chromatogram of 2 µg/ml Amphotericin B in water

II. Total peak purity method from LC solution program

Total peak purity method

The total peak purity index method compares every point with a relatively close peak selected as the reference.

In this method, the spectrum similarity index can be determined with partially excluding the pattern variation from the absorbance variation, in such case that the signal linearity declines as absorbance increases. The method is applicable to data around 1 AU or less.

In addition, around the foot of the peak where the drift and the peak integration destabilize the evaluation, the method mitigates their influence in some degrees.

Total Peak Purity Algorithms

Given:

- The number of Nspec spectra are assumed to exist in the chromatogram's peak, and the number of NPts absorbances are assumed to exist in each spectrum.
- The noise spectrum is assumed to be Noi (I=~NPts). In general, the standard deviation is specified at each wavelength for this noise spectrum.
- Each spectrum in the peak is compared with the reference spectrum.

<Reference Spectrum>

Obtain the reference spectrum by selecting the reference point with a higher absorbance, slightly inner in relation to the spectrum at an inspected point in the peak.
The similarity index is calculated as the inspected spectrum is compared with the reference peak.
The point of reference varies with the absorbance at each data point, specifying the point to allow some mAbs to some 10 mAbs of absorbance difference in terms of the spectrum's maximum absorbance (Max Plot in the designated wavelength range).

Sref = Reference Spectrum. Consists of the factors of the absorbance value B1, B2, BNPts.

S = Calculated spectrum. Consists of the factors of the absorbance value A1, A2, ANPts.

$|S|$ = Size of the spectrum S

$$= \sqrt{\sum_{i=1}^{NPts} A_i^{\,2}}$$

$|S_{ref}|$ = Size of the spectrum S_{ref}

$$= \sqrt{\sum_{i=1}^{NPts} B_i^{\,2}}$$

In this case, the total peak purity is calculated with the following procedure.

1. Calculation of the size of the noise spectrum (N)

$$N = \sqrt{\sum_{i=1}^{NPts} No_i^{\,2}}$$

2. Evaluation of the purity for one spectrum

Similarity Index : SI= Size of the spectrum S_{ref}

$$= \frac{\sum_{i=1}^{NPts} Ai \times Bi}{|S| \times |Sref|}$$

$$\text{Threshold: } t = \sqrt{\left(1 - \frac{N^2}{|S|^2}\right) \bullet \left(1 - \frac{N^2}{|S_{ref}|^2}\right)} - \frac{N^2}{|S| \times |S_{ref}|}$$

Peak Purity Index: R = (SI-t) x 10,000,000

When R is negative, it will be determined that the spectrum S is different from Sref and the impurity is present.

For the purpose of facilitating the display, the peak purity is multiplied by a million.

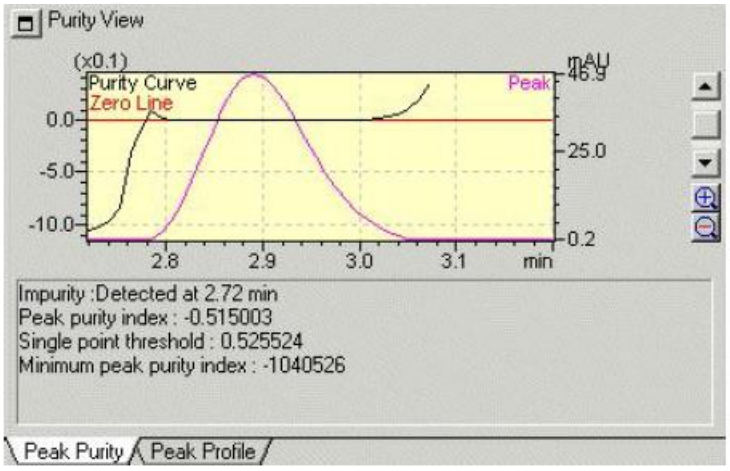
3. Minimum peak purity coefficient

The purity index is calculated with the corresponding threshold subtracted from the similarity index of each inspected point in the peak.
The data point that indicates the purity index to be the minimum value is searched for outputting the retention time, Peak purity index, Single point threshold, and Minimum peak purity index.

[Reference]

The points for examining the uniformity of peaks are described as follows.

1) Spectra at each time in the peak and the similarity indices with the corresponding reference spectra are calculated at first. The purity is calculated by subtracting the threshold from that value. As the noise spectrum is used to calculate the threshold, make sure to measure it at the baseline of the data. The graph where each calculated value is plotted in the time direction can be viewed by changing Graph Type to Similarity at [Display Setting] on the purity window.



2) The purity is calculated in the interval between the peak detection start point and end point. If the value is apparently incorrect, check the position of the detection point. In addition, modifying peak integration commands for analysis changes the position of the detection point and eventually varies the shape of the purity curve and the purity index.

3) The wavelength range also influences the value of the purity and must be specified appropriately.

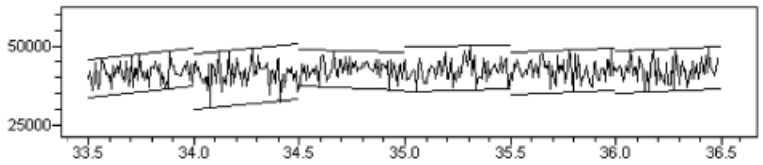
4) The purity is not calculated where the spectrum size is smaller than the registered noise spectrum. The purity is calculated in the area where the purity curve is displayed.

5) When the background compensation is executed, Spectra at each time in the peak are compensated with the line between the peak detection start point and end point being the compensation line. Therefore, the correction is always required when the baseline drifts. In addition, when adjacent peaks overlap at their skirts, the compensation somewhat offsets the influence of overlapping.

III. The calibration for S/N ratio, limit of detection (LOD) and limit of quantitation (LOQ) from LC solution program

- S/N ratio

The S/N ratio denotes the ratio between signals and noises.
The noises in this system is obtained with the ASTM method as follows.



The figure above shows the image of obtaining the noise level for every 0.5 minute from 33.5 minutes to 36.5 minutes.
The data is divided with 0.5 minute intervals and parallel lines are obtained for each section as shown in the figure.

Procedure: For each section, the slope of the approximate line is obtained with the least square method for the data in each section. Two lines with the same slope are parallel shifted upward and downward so as to completely contain the data of the section.

The noise level is the mean value of the Y-axis distance between the parallel lines for all sections.
The divided section can be specified on [S/N Ratio Detailed Settings]. When [Calculate on processing time of each compound] is specified, the data is automatically divided into 0.5 minute intervals and calculated.

- Limit of detection (LOD)

The detection limit is obtained by the following equation.

$$DL = S_B \times \alpha / f$$

DL	: Detection limit
S_B	: Standard deviation of signal for zero concentration
α	: Coefficient (normally 3 to 3.3)
f	: Slope of calibration curve

The value of S_B is manually selected from the $S_{y/x}$, S_y and the noises(when calculating from the S/N ratio).
When an internal standard method is used, the signal ratio and the concentration ratio are used for the calculation and the result is indicated as the concentration ratio.
When the detection limit is calculated based on the noise, the result can be obtained only when the quantitative parameter "Calc. by" is set to "Height" and "X Axis" to "Conc."

- Limit of quantitation (LOQ)

The quantitation limit is obtained by the following equation.

$$QL = S_B \times \alpha / f$$

QL	: Quantitation limit
S_B	: Standard deviation of signal for zero concentration
α	: Coefficient (normally 10)
f	: Slope of calibration curve

IV. The calculation results of limit of detection (LOD) and limit of quantitation (LOQ) of Amphotericin B

Calculation Results

Report for Sample Type "Unknown"

- <Method Filename> D:\Phojana\26042013\Method 26042013.lcm

PDA

- Index

Calibration

Calibration

- Level 1 Count 1

Data	Data File Path	Sample Name	Sample ID	Analysis Date	Data File Status
Data 1:	D:\Phojana\26042013\0.0004 mgml.lcd	0.0004 mg/ml		26/4/2556 15:55:17	Normal

- Level 2 Count 1

Data	Data File Path	Sample Name	Sample ID	Analysis Date	Data File Status
Data 1:	D:\Phojana\26042013\0.0005 mgml.lcd	0.0005 mg/ml		26/4/2556 14:43:15	Normal

- Level3 Count 1

Data	Data File Path	Sample Name	Sample ID	Analysis Date	Data File Status
Data 1:	D:\Phojana\26042013\0.0006 mgml.lcd	0.0006 mg/ml		26/4/2556 14:59:43	Normal

- Level4 Count 1

Data	Data File Path	Sample Name	Sample ID	Analysis Date	Data File Status
Data 1:	D:\Phojana\26042013\0.0007 mgml.lcd	0.0007 mg/ml		26/4/2556 15:10:11	Normal

- Level5 Count 1

Data	Data File Path	Sample Name	Sample ID	Analysis Date	Data File Status
Data 1:	D:\Phojana\26042013\0.00075 mgml.lcd	0.000075 mg/ml		26/4/2556 16:06:18	Normal

- Level6 Count 1

Data	Data File Path	Sample Name	Sample ID	Analysis Date	Data File Status
Data 1:	D:\Phojana\26042013\0.0008mg ml.lcd	0.0008 mg/ml		26/4/2556 15:21:50	Normal

- Noise (ASTM)

Method	Calculate on processing time of each compound

- [Result]

ID	Compound Name	Level1 S/N	Level2 S/N	Level3 S/N	Level4 S/N	Level5 S/N	Level6 S/N
1	Ampho	-	3.680006	5.629314	9.1546238	11.209855	93.170793

- [Result](S/N)
  - Level1

ID	Compound Name	Data1 Signal	Data1 Noise	Data1 S/N
1	Ampho	-	48.108930	-

  - Level2

ID	Compound Name	Data1 Signal	Data1 Noise	Data1 S/N
1	Ampho	171.428491	46.583754	3.680006

  - Level3

ID	Compound Name	Data1 Signal	Data1 Noise	Data1 S/N
1	Ampho	241.878923	42.967744	5.629314

- Level4

ID	Compound Name	Data1 Signal	Data1 Noise	Data1 S/N
1	Ampho	369.592990	40.372275	9.1546238

- Level5

ID	Compound Name	Data1 Signal	Data1 Noise	Data1 S/N
1	Ampho	448.473857	40.007106	11.209855

- Level6

ID	Compound Name	Data1 Signal	Data1 Noise	Data1 S/N
1	Ampho	3769.307525	40.455892	93.170793

V. HPLC data at limit of quantitation (LOQ)

Table B1

Set	Theoretical concentration of Amphotericin B (µg/ml)	Estimated concentration (µg/ml)			Mean	SD	%Analytical recovery	%RSD
		n=1	n=2	n=3				
1	0.7518	0.7246	0.7362	0.7138	0.7249	0.0112	96.42	1.55
2	0.7557	0.7233	0.7437	0.7376	0.7349	0.0105	97.24	1.43
3	0.7479	0.7123	0.7356	0.7139	0.7206	0.0130	96.35	1.81

VI. Data for calibration curve of Amphotericin B by HPLC method

Table B2

Concentration of Amphotericin B (µg/ml)	Peak area			Mean	SD
	n=1	n=2	n=3		
1.00	113121	113542	112956	113206	302
2.01	235085	235085	234889	235020	113
5.02	612960	612795	613004	612920	110
10.03	1248562	1248562	1241137	1246087	4287
20.06	2516245	2518749	2514768	2516587	2012
30.10	3796631	3796631	3795038	3796100	920

VII. Data for validation in the accuracy of HPLC method

Table B3

Set	Theoretical concentration of Amphotericin B (µg/ml)	Estimated concentration (µg/ml)			Mean	SD	%Analytical recovery
		n=1	n=2	n=3			
1	1.50	1.54	1.55	1.53	1.54	0.01	102.49
	15.04	14.77	14.92	14.79	14.83	0.08	98.63
	25.06	24.90	24.89	24.98	24.92	0.05	99.45
2	1.51	1.53	1.50	1.52	1.51	0.01	100.24
	15.11	14.81	14.82	14.87	14.83	0.03	98.14
	25.19	24.79	24.97	24.85	24.87	0.09	98.72
3	1.52	1.52	1.53	1.52	1.52	0.01	100.33
	15.17	14.89	14.91	14.90	14.90	0.01	98.24
	25.28	25.07	24.89	24.85	24.94	0.11	98.65
4	1.50	1.52	1.53	1.53	1.53	0.00	102.12
	14.96	14.66	14.68	14.69	14.68	0.01	98.12
	24.93	24.94	24.99	24.96	24.96	0.02	100.14
5	1.51	1.53	1.51	1.52	1.52	0.01	101.06
	15.06	14.91	14.81	14.77	14.83	0.07	98.49
	25.10	25.11	24.95	25.08	25.05	0.09	99.80

VIII. Data for validation in the precision of HPLC method

Table B4 Data of within-run precision

Set	Theoretical concentration of Amphotericin B (µg/ml)	Estimated concentration (µg/ml)			Mean	SD	%RSD
		n=1	n=2	n=3			
1	1.50	1.49	1.48	1.49	1.49	0.00	0.15
	15.01	14.54	14.51	14.55	14.53	0.02	0.14
	25.01	24.44	24.44	24.49	24.46	0.03	0.12
2	1.50	1.45	1.46	1.45	1.45	0.01	0.38
	14.99	14.28	14.33	14.41	14.34	0.07	0.47
	24.98	24.34	24.14	24.12	24.20	0.12	0.51
3	1.51	1.49	1.49	1.49	1.49	0.00	0.06
	15.07	14.85	14.69	14.68	14.74	0.09	0.64
	24.98	24.20	24.23	24.37	24.27	0.09	0.36
4	1.51	1.50	1.50	1.51	1.50	0.00	0.30
	15.07	14.74	14.76	14.72	14.74	0.02	0.16
	24.98	24.29	24.29	24.13	24.23	0.09	0.39
5	1.51	1.52	1.52	1.52	1.52	0.00	0.06
	15.07	14.75	14.85	14.77	14.79	0.05	0.35
	24.98	24.33	24.33	24.24	24.30	0.05	0.21

Table B5 Data of between-run precision

Set	Theoretical concentration of Amphotericin B (µg/ml)	Estimated concentration (µg/ml)			Mean	SD	%RSD
		n=1	n=2	n=3			
1	1.50	1.54	1.55	1.53	1.54	0.01	0.72
	15.04	14.77	14.92	14.79	14.83	0.08	0.55
	25.06	24.90	24.89	24.98	24.92	0.05	0.18
2	1.50	1.49	1.48	1.49	1.49	0.00	0.15
	15.01	14.54	14.51	14.55	14.53	0.02	0.14
	25.01	24.44	24.44	24.49	24.46	0.03	0.12
3	1.50	1.52	1.57	1.53	1.54	0.02	1.52
	15.03	14.89	14.70	14.77	14.79	0.10	0.65
	25.06	24.45	24.48	24.64	24.52	0.10	0.40
4	1.50	1.52	1.53	1.53	1.53	0.00	0.20
	14.96	14.66	14.68	14.69	14.68	0.01	0.07
	24.93	24.94	24.99	24.96	24.96	0.02	0.09
5	1.51	1.53	1.51	1.52	1.52	0.01	0.68
	15.06	14.91	14.81	14.77	14.83	0.07	0.49
	25.10	25.11	24.95	25.08	25.05	0.09	0.34

APPENDIX C

Stability of the extemporaneous Amphotericin B topical preparations

I. Microscope Images

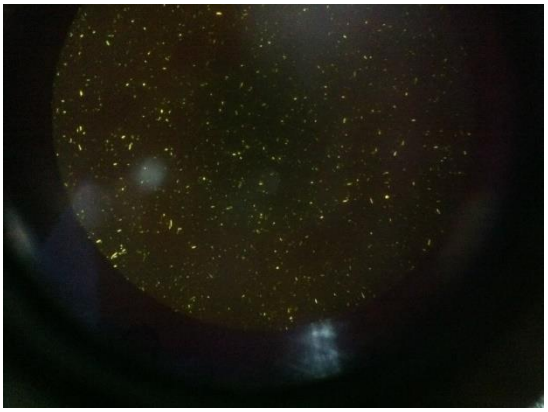


Figure C1 Amphotericin B reference standard powders (x40)

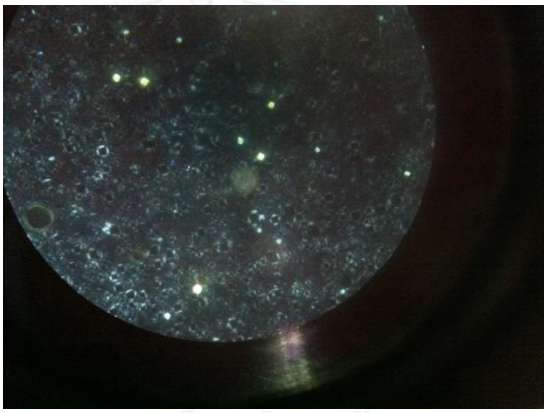


Figure C2 Amphotericin B cream (x40)

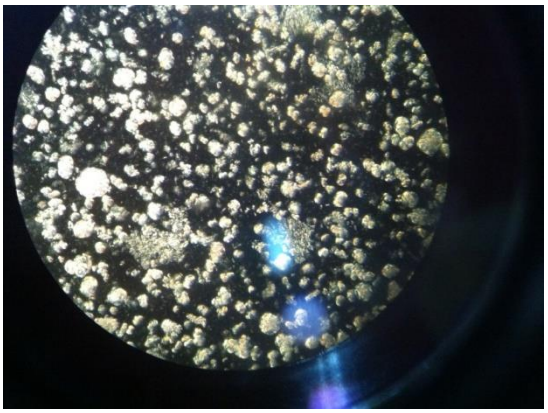


Figure C3 Amphotericin B ointment (x40)

II. Data stability of the extemporaneous Amphotericin B Cream

Table C1

Stability of Amphotericin B 3 mg/mL in extemporaneous Amphotericin B Cream at 30 ± 2°C

Cream	Day	Adjusted concentration (mg/ml)			Average Concentration (mg/ml)	SD	% Initial Amphotericin B concentration Remainning				
		n=1	n=2	n=3			n=1	n=2	n=3	Average	SD
C-1	0	3.36	3.38	3.39	3.37	0.02	99.47	100.09	100.43	100.00	0.49
(Lot.17022014)	3	3.26	3.20	3.21	3.22	0.03	96.72	94.90	95.08	95.57	1.00
	11	3.21	3.21	3.27	3.23	0.03	95.17	95.00	96.78	95.65	0.98
	36	3.32	3.27	3.31	3.30	0.02	98.38	97.05	98.13	97.85	0.71
	59	3.25	3.30	3.37	3.31	0.06	96.48	97.80	100.03	98.10	1.79
C-2	0	3.56	3.58	3.56	3.57	0.01	99.75	100.34	99.91	100.00	0.30
(Lot.25032014)	7	3.57	3.59	3.59	3.58	0.01	100.13	100.55	100.79	100.49	0.34
	14	3.35	3.36	3.35	3.35	0.01	93.81	94.23	94.08	94.04	0.21
	23	3.47	3.52	3.43	3.47	0.05	97.28	98.79	96.18	97.42	1.31
	28	3.56	3.56	3.54	3.56	0.01	99.97	99.84	99.31	99.71	0.35
	35	3.48	3.52	3.46	3.49	0.03	97.68	98.79	96.98	97.81	0.91
	48	2.92	2.95	2.87	2.92	0.04	82.01	82.70	80.59	81.77	1.07
	62	2.62	2.55	2.58	2.58	0.04	73.59	71.37	72.38	72.45	1.11
C-3	0	3.26	3.25	3.26	3.26	0.01	100.14	99.80	100.06	100.00	0.18
(Lot.25032014)	7	3.26	3.27	3.27	3.26	0.01	100.00	100.41	100.35	100.25	0.22
	14	2.93	2.94	2.94	2.94	0.01	89.85	90.40	90.24	90.17	0.28
	23	2.90	2.88	2.85	2.88	0.03	88.96	88.55	87.47	88.33	0.77
	28	2.68	2.68	2.69	2.68	0.01	82.31	82.26	82.64	82.40	0.21
	35	2.80	2.81	2.82	2.81	0.01	85.99	86.39	86.63	86.34	0.32
	48	3.32	3.34	3.38	3.35	0.03	101.96	102.61	103.92	102.83	1.00
	62	3.58	3.57	3.57	3.57	0.00	109.88	109.63	109.77	109.76	0.13

III. Data stability of the extemporaneous Amphotericin B Ointment

Table C2

Stability of Amphotericin B 3 mg/mL in extemporaneous Amphotericin B ointment at 30 ± 2°C

Ointment	Day	Adjusted concentration (mg/ml)			Average Concentration (mg/ml)	SD	% Initial Amphotericin B concentration Remainning				
		n=1	n=2	n=3			n=1	n=2	n=3	Average	SD
O-1	0	3.60	3.61	3.61	3.61	0.01	99.82	100.08	100.10	100.00	0.16
(Lot.01042014)	7	3.40	3.40	3.40	3.40	0.00	94.14	94.37	94.24	94.25	0.11
	16	3.70	3.71	3.71	3.70	0.01	102.43	102.78	102.77	102.66	0.20
	21	3.07	3.06	3.06	3.06	0.01	84.99	84.70	84.93	84.87	0.16
	28	3.72	3.69	3.66	3.69	0.03	102.98	102.23	101.35	102.19	0.82
	35	3.83	3.83	3.85	3.84	0.01	106.26	106.03	106.81	106.37	0.40
	48	3.51	3.52	3.52	3.52	0.00	97.36	97.52	97.45	97.44	0.08
	55	2.77	2.76	2.74	2.76	0.01	76.73	76.60	76.04	76.46	0.37
	62	3.30	3.28	3.28	3.29	0.01	91.36	90.91	90.97	91.08	0.24
O-2	0	3.65	3.62	3.63	3.63	0.01	100.44	99.67	99.89	100.00	0.40
(Lot.01042014)	7	3.31	3.34	3.29	3.32	0.02	91.24	91.94	90.73	91.30	0.61
	16	3.62	3.62	3.64	3.63	0.01	99.70	99.80	100.21	99.90	0.27
	21	3.15	3.16	3.20	3.17	0.02	86.77	87.09	88.05	87.30	0.67
	28	3.49	3.50	3.50	3.50	0.00	96.23	96.50	96.37	96.36	0.14
	35	3.56	3.56	3.55	3.56	0.01	98.10	98.03	97.83	97.99	0.14
	48	3.33	3.31	3.32	3.32	0.01	91.60	91.26	91.47	91.44	0.17
	55	3.31	3.29	3.30	3.30	0.01	91.19	90.64	90.75	90.86	0.29
	62	3.12	3.11	3.12	3.12	0.01	86.05	85.56	85.83	85.81	0.25
O-3	0	3.33	3.33	3.30	3.32	0.01	100.25	100.24	99.51	100.00	0.43
(Lot.01042014)	7	3.51	3.50	3.51	3.51	0.00	105.58	105.46	105.64	105.56	0.09
	16	3.56	3.56	3.54	3.55	0.01	107.34	107.21	106.58	107.04	0.41
	21	3.18	3.20	3.19	3.19	0.01	95.71	96.22	95.93	95.95	0.26
	28	3.55	3.55	3.56	3.55	0.01	106.84	106.81	107.14	106.93	0.18
	35	3.74	3.64	3.54	3.64	0.10	112.68	109.64	106.74	109.69	2.97
	48	3.67	3.68	3.67	3.68	0.01	110.65	110.91	110.63	110.73	0.16
	55	3.24	3.23	3.24	3.24	0.01	97.66	97.25	97.46	97.45	0.21
	62	2.86	2.87	2.92	2.89	0.03	86.08	86.55	88.04	86.89	1.02

IV. Data stability of the extemporaneous Amphotericin B gel

Table C3

Stability of Amphotericin B 3 mg/mL in extemporaneous Amphotericin B gel at 30 ± 2°C

Gel	Day	Adjusted concentration (mg/ml)			Average Concentration (mg/ml)	SD	% Initial Amphotericin B concentration Remainning				
		n=1	n=2	n=3			n=1	n=2	n=3	Average	SD
G30-1-1	0	3.11	3.12	3.12	3.12	0.00	99.87	100.14	99.99	100.00	0.13
(Lot.18032014)	7	3.16	3.14	3.14	3.15	0.01	101.37	100.79	100.75	100.97	0.35
	14	3.09	3.09	3.09	3.09	0.00	99.02	98.97	98.99	98.99	0.03
	21	3.08	3.08	3.09	3.08	0.00	98.85	98.94	98.95	98.92	0.05
	30	3.11	3.10	3.08	3.10	0.02	99.86	99.47	98.89	99.41	0.49
	42	2.99	2.98	2.98	2.98	0.01	95.82	95.47	95.69	95.66	0.18
	49	3.14	3.14	3.13	3.14	0.01	100.78	100.73	100.36	100.62	0.23
	62	3.03	3.03	3.03	3.03	0.00	97.08	97.11	97.34	97.18	0.14
G30-1-2	0	2.93	2.93	2.93	2.93	0.00	100.09	99.91	100.00	100.00	0.09
(Lot.18032014)	7	2.76	2.75	2.73	2.75	0.01	94.14	93.96	93.36	93.82	0.41
	14	2.70	2.70	2.68	2.69	0.01	92.27	92.09	91.49	91.95	0.41
	21	2.61	2.61	2.59	2.60	0.01	89.26	89.02	88.45	88.91	0.42
	30	2.70	2.71	2.68	2.70	0.01	92.34	92.54	91.67	92.18	0.46
	42	2.60	2.60	2.58	2.59	0.01	88.78	88.83	88.16	88.59	0.37
	49	2.65	2.66	2.67	2.66	0.01	90.57	90.95	91.00	90.84	0.23
	62	2.58	2.56	2.57	2.57	0.01	88.01	87.44	87.73	87.73	0.29
G30-1-3	0	3.24	3.26	3.27	3.26	0.01	99.54	100.13	100.33	100.00	0.41
(Lot.18032014)	7	3.11	3.12	3.13	3.12	0.01	95.53	95.70	96.10	95.78	0.29
	14	3.19	3.20	3.20	3.20	0.01	98.03	98.31	98.34	98.22	0.17
	21	3.08	3.08	3.09	3.08	0.00	94.56	94.48	94.76	94.60	0.15
	30	3.05	3.04	3.03	3.04	0.01	93.49	93.43	93.05	93.33	0.24
	42	2.78	2.80	2.78	2.79	0.01	85.41	85.85	85.43	85.56	0.25
	49	3.10	3.09	3.09	3.09	0.00	95.04	94.87	94.77	94.89	0.13
	62	3.08	3.08	3.06	3.07	0.01	94.52	94.43	93.92	94.29	0.33

APPENDIX D

The permeation of extemporaneous Amphotericin B topical preparations in human nail plate

I. Analysis the permeation of Amphotericin B in the nail clippings at 0 hr. and 24 hr. after applying Amphotericin B cream preparation assay by ANOVA

Test of Homogeneity of Variances

Amphotericin B

Levene Statistic	df1	df2	Sig.
.058	1	4	.822

ANOVA

Amphotericin B

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.815	1	2.815	.551	.499
Within Groups	20.450	4	5.112		
Total	23.265	5			

II. Analysis the permeation of Amphotericin B in the normal and filed nail clippings after applying Amphotericin B cream preparation about 24 hr. assay by ANOVA

Test of Homogeneity of Variances

Amphotericin B

Levene Statistic	df1	df2	Sig.
8.348	2	15	.004

ANOVA

Amphotericin B

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	158.415	2	79.207	68.299	.000
Within Groups	17.396	15	1.160		
Total	175.810	17			

Post Hoc Tests

Multiple Comparisons

Dependent Variable : Amphotericin B

	(I) Nail	(J) Nail	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Dunnett T3	Control	Non-filed	-5.77333*	.69582	.001	-8.1385	-3.4082
		Filed	-6.70833*	.30938	.000	-7.7598	-5.6569
	Non-filed	Control	5.77333*	.69582	.001	3.4082	8.1385
		Filed	-.93500	.76147	.559	-3.2703	1.4003
	Filed	Control	6.70833*	.30938	.000	5.6569	7.7598
		Non-filed	.93500	.76147	.559	-1.4003	3.2703

*. The mean difference is significant at the 0.05 level.

III. Analysis the permeation of Amphotericin B in the first method from different formulations assay by Independent Samples T Test

Group Statistics

	Formulation	N	Mean	Std. Deviation	Std. Error Mean
Amphotericin B	Cream	3	.6500	.15133	.08737
	Ointment	3	1.8600	2.56010	1.47807

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Amphotericin B	Equal variances assumed	13.629	.021	-.817	4	.460	-1.21000	1.48065	-5.32095	2.90095
	Equal variances not assumed			-.817	2.014	.499	-1.21000	1.48065	-7.53856	5.11856

IV. Analysis the permeation of Amphotericin B in the second method from different formulations assay by Independent Samples T Test

a. Amphotericin B **cream and ointment formulations**

Group Statistics

	Formulation	N	Mean	Std. Deviation	Std. Error Mean
Amphotericin B	Cream	3	11.9567	3.21449	1.85589
	Ointment	3	29.2633	5.02872	2.90333

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Ampho Tericin B	Equal variances assumed	.344	.589	-5.023	4	.007	-17.30667	3.44582	-26.87379	-7.73954
	Equal variances not assumed			-5.023	3.401	.011	-17.30667	3.44582	-27.57700	-7.03633

b. Amphotericin B cream and cream base formulations

Group Statistics

	Formulation	N	Mean	Std. Deviation	Std. Error Mean
Amphotericin B	Cream	3	11.9567	3.21449	1.85589
	Cream base	3	.1533	.01528	.00882

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Amphotericin B	Equal variances assumed	9.072	.039	6.360	4	.003	11.80333	1.85591	6.65051	16.95616
	Equal variances not assumed			6.360	2.000	.024	11.80333	1.85591	3.81836	19.78831

c. Amphotericin B ointment and ointment base formulations

Group Statistics

	Formulation	N	Mean	Std. Deviation	Std. Error Mean
Amphotericin B	Ointment	3	29.2633	5.02872	2.90333
	Ointment base	3	.1833	.01528	.00882

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Amphotericin B	Equal variances assumed	4.541	.100	10.016	4	.001	29.08000	2.90335	21.01902	37.14098
	Equal variances not assumed			10.016	2.000	.010	29.08000	2.90335	16.58813	41.57187

VITA

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