

TABLE OF CONTENTS

	Page
Acknowledgement	iii
Abstract (English)	iv
Abstract (Thai)	vi
List of tables	xi
List of illustrations	xv
Abbreviations and symbols	xix
Chapter 1 Introduction	1
1.1 Statement and significance of the problem	1
1.2 Objective	2
1.3 Scope of study	2
1.4 Literature reviews	3
1.4.1 Liposomes	4
1.4.2 Amphotericin B	28
1.4.3 Percutaneous absorption	36
Chapter 2 Experimental	53
2.1 Materials and equipments	53
2.1.1 Chemicals	53
2.1.2 Skin	53
2.1.3 Equipments	54
2.2 Methods	55
2.2.1 Preparation	55
2.2.2 Physical properties study of liposome sample	57
2.2.3 Analysis of amphotericin B	58

2.2.4	Determination of the percentages of entrapment of amphotericin B in liposomes	60
2.2.5	Stability study of amphotericin B in liposome formulations comparing to Fungizone® solution and Fungizone® powder	61
2.2.6	Transdermal absorption of amphotericin B through the full-thickness of rat skin	61
Chapter 3 Results		64
3.1	Characteristics of liposome formulations	64
3.1.1	The physical appearances of liposome formulations	64
3.1.2	pH measurement of liposome formulations	66
3.1.3	Charges and zeta potential	67
3.1.4	Size determination by SEM	68
3.1.5	Lamellarity determined by TEM	79
3.1.6	Investigation of the transition temperature and the enthalpy of transition by DSC	80
3.2	Qualitative and quantitative analysis of amphotericin B, Fungizone® and the drug in liposome formulations by HPLC	87
3.2.1	UV absorption spectra	87
3.2.2	Chromatogram of amphotericin B and Fungizone® by HPLC with UV detection at 382 nm	89
3.2.3	The amphotericin B standard curve preparation	90
3.2.4	Contents (mg) determination of amphotericin B in Fungizone® powder (1mg)	92
3.3	Determination of the percentages of entrapment of amphotericin B in liposomes	93
3.4	Stability study of amphotericin B entrapped in liposome formulations	103
3.4.1	Physical stability	103
3.4.2	Chemical stability	108
3.5	The transdermal absorption of amphotericin B liposome formulations Through rat skin, by the vertical Franz diffusion cells	126

3.5.1 Validation of the experiment	126
Chapter 4 Discussion	134
4.1 Physical properties of the prepared liposome formulations	134
4.1.1 pH measurement	134
4.1.2 Charges	134
4.1.3 Sizes	135
4.1.4 Lamellarity	135
4.1.5 Physical appearances	135
4.1.6 The DSC study	136
4.2 Qualitative and quantitative analysis of amphotericin B, Fungizone® and the drug in liposome formulations by HPLC	138
4.3 The percentages of the entrapment of amphotericin B in liposomes	140
4.4 The stability study of various liposome formulations, Fungizone® solution and Fungizone® powder	141
4.5 The transdermal absorption of amphotericin B liposome formulations through the full-thickness rat skin, by the vertical Franz diffusion cells	143
Chapter 5 Conclusion	146
References	148
Appendices	157
Appendix A Calculation of Compositions in Liposome Formulations	158
Appendix B Calculation of the Contents of Amphotericin B in Fungizone®	160
Appendix C Calculation of the Percentages of Entrapment of Amphotericin B in Liposomes	161
Appendix D Calculation of Degradation Rate and Shelf life of Liposome Formulations	162
Appendix E Calculation of the Flux (ng/cm ² per hr) through the Wistar Rat Skin	164
Curriculum vitae	166

LIST OF TABLES

Table	Page
1.1 Liposome classification (Swarbrick and Boylan, 1994)	17
2.1 The contents (mg) of eight different liposome dispersion samples (40 ml)	55
3.1 The appearances of liposome formulations	64
3.2 The pH's of liposome formulations comparing to phosphate buffer(pH 7.4)solution	66
3.3 Charges and zeta potential values of eight liposome formulations	67
3.4 Sizes (μm) of the 1:1 liposome formulation (100 particles)	73
3.5 Sizes (μm) of the 7:2 liposome formulation (100 particles)	73
3.6 Sizes (μm) of the 7:2:1(+) liposome formulation (100 particles)	74
3.7 Sizes (μm) of the 7:2:1(-) liposome formulation (100 particles)	74
3.8 Sizes (μm) of the 1:1AmB liposome formulation (100 particles)	75
3.9 Sizes (μm) of the 7:2AmB liposome formulation (100 particles)	75
3.10 Sizes (μm) of the 7:2:1(-)AmB liposome formulation (100 particles)	76
3.11 Sizes (μm) of the 7:2:1(+)AmB liposome formulation (8 particles)	76
3.12 Mode, mean, standard deviation and variation coefficient of particle sizes (μm) of liposome formulations	77
3.13 Sizes frequency distributions of 100 particles of each liposome formulation	77
3.14 The transition temperatures of FGZ, HSC, CHL, DCP, SA and the eight liposome formulations calculated from the DSC curves	84
3.15 The enthalpy of transition (ΔH , J/g) of FGZ, HSC, CHL, DCP, SA and the eight liposome formulations calculated from the DSC curves	85
3.16 Conclusion of the mean (\pm SD) of transition temperature and enthalpy of transition of the compositions in liposome	86
3.17 Conclusion of the mean (\pm SD) of transition temperature of liposome formulations	86

3.18 Conclusion of the mean(\pm SD)of enthalpy of transition(J/g) of liposome formulations	86
3.19 Peak areas in various concentrations of the reference standard amphotericin B	90
3.20 The peak areas from duplicate analysis of the standard amphotericin B at various concentrations	91
3.21 The contents of amphotericin B in 1 mg of Fungizone [®] powder determined by HPLC	92
3.22 Peak areas from HPLC analysis of the total amount of AmB in 1:1AmB liposomes	96
3.23 Peak areas from HPLC analysis of the entrapped AmB in 1:1AmB liposomes	96
3.24 Peak areas from HPLC analysis of the unentrapped AmB in 1:1AmB liposomes	96
3.25 Peak areas from HPLC analysis of the total amount of AmB in 7:2AmB liposomes	97
3.26 Peak areas from HPLC analysis of the entrapped AmB in 7:2AmB liposomes	97
3.27 Peak areas from HPLC analysis of the unentrapped AmB in 7:2AmB liposomes	97
3.28 Peak areas from HPLC analysis of the total amount of AmB in 7:2:1(+)AmB liposomes	98
3.29 Peak areas from HPLC analysis of the entrapped AmB in 7:2:1(+)AmB liposomes	98
3.30 Peak areas from HPLC analysis of the unentrapped AmB in 7:2:1(+)AmB liposomes	98
3.31 Peak areas from HPLC analysis of the total amount of AmB in 7:2:1(-)AmB liposomes	99
3.32 Peak areas from HPLC analysis of the entrapped AmB in 7:2:1(-)AmB liposomes	99
3.33 Peak areas from HPLC analysis of the unentrapped AmB in 7:2:1(-)AmB liposomes	99
3.34 Mean peak areas, concentrations, percentages of drug (total, entrapped and unentrapped AmB) of 1:1AmB liposome formulation	100
3.35 Mean peak areas, concentrations, percentages of drug (total, entrapped and unentrapped AmB) of 7:2AmB liposome formulation	100
3.36 Mean peak areas, concentrations, percentages of drug (total, entrapped and unentrapped AmB) of 7:2:1(+)AmB liposome formulation	101
3.37 Mean peak areas, concentrations, percentages of drug (total, entrapped and unentrapped AmB) of 7:2:1(-)AmB liposome formulation	101

3.38 The average percentages of the entrapment of AmB and the free AmB in liposome formulations	102
3.39 The amount of AmB per total lipid (ug/mg) in liposome formulations	102
3.40 The physical changes of the 1:1, 7:2, 7:2:1(+), 7:2:1(-) liposome formulations with and without entrapped drug, Fungizone® solution and Fungizone® powder right after preparation and after stored at 4±1°C, 30±1°C and 45±1°C	103
3.41 The remaining amounts of amphotericin B in the 1:1AmB liposome sampling at 0, 5, 20, 40 and 90 days when kept at 4±1°C, 30±1°C and 45±1°C	109
3.42 The remaining amounts of amphotericin B in the 7:2AmB liposome sampling at 0, 5, 20, 40 and 90 days when kept at 4±1°C, 30±1°C and 45±1°C	110
3.43 The remaining amounts of amphotericin B in 7:2:1(+)AmB liposome sampling at 0, 5, 20, 40 and 90 days when kept at 4±1°C, 30±1°C and 45±1°C	111
3.44 The remaining amounts of amphotericin B in 7:2:1(-)AmB liposome sampling at 0, 5, 20, 40 and 90 days when kept at 4±1°C, 30±1°C and 45±1°C	112
3.45 The remaining amounts of amphotericin B in Fungizone® solution sampling at 0, 5, 20, 40 and 90 days when kept at 4±1°C, 30±1°C and 45±1°C	113
3.46 The remaining amounts of amphotericin B in Fungizone® powder sampling at 0, 5, 20, 40 and 90 days when kept at 4±1°C, 30±1°C and 45±1°C	114
3.47 Comparison of the percentages of the remaining amphotericin B in liposome formulations, Fungizone® solution and Fungizone® powder sampling at 0, 5, 20, 40 and 90 days when kept at 4±1°C, 30±1°C and 45±1°C	115
3.48 The equations used to calculation the degradation rate of AmB in various formulations	117
3.49 Calculation of the degradation rate (slope) and the shelf life of the 1:1AmB liposome formulation	118
3.50 Calculation of the degradation rate (slope) and the shelf life of the 7:2AmB liposome formulation	119
3.51 Calculation of the degradation rate (slope) and the shelf life of the 7:2:1(+)AmB liposome formulation	120
3.52 Calculation of the degradation rate (slope) and the shelf life of 7:2:1(-)AmB liposome formulation	121

3.53 Calculation of the degradation rate (slope) and the shelf life of the Fungizone [®] solution	122
3.54 Calculation of the degradation rate (slope) and the shelf life of the Fungizone [®] powder	123
3.55 Conclusion of the degradation rate of liposome formulations, Fungizone [®] solution and Fungizone [®] powder	124
3.56 Predicted shelf life of the liposome formulations, Fungizone [®] solution and Fungizone [®] powder	124
3.57 The peak areas from transdermal absorption study of amphotericin B in various formulations	130
3.58 The amounts (μg) of amphotericin B in various formulations in different strata of the rat skin at $37\pm 1^\circ\text{C}$ for 24 hrs	131
3.59 The flux (ng/cm^2 per h) of amphotericin B of various formulations in different strata of the rat skin at $37\pm 1^\circ\text{C}$ for 24 hrs	132

LIST OF ILLUSTRATIONS

Figure	Page
1.1 Structures of Liposome (A) and sectional view of liposome (B) (Lucas Meyer GmbH, 1991)	4
1.2 The assembly conformations of amphiphiles. (Swarbrick and Boylan, 1994)	6
1.3 Schematic representation of the lipid polymorphism, dashed symbols correspond to the phases with titled chains ; L_c , crystalline lamellar phase ; L_p , ordered or gel lamellar phase ; P_β , periodical gel phase ; L_α , disordered or fluid lamellar phase (Cevc, 1993)	7
1.4 Disposition of phospholipid diacyl chains by rotation about one C-C single bond from a trans- to a gauche- conformation. (New, 1990)	8
1.5 Structures of phospholipids used in liposome formation (New, 1990)	10
1.6 Structural formula of some synthetic, for the formation of nonionic surfactant vesicles (Swarbrick and Boylan, 1994)	13
1.7 The structure of cholesterol and position occupied by cholesterol in the membrane bilayer (New, 1990)	14
1.8 The structure of lysolecithin (New, 1990)	16
1.9 Schematic two-dimensional representation of types of liposomes (Swarbrick and Boylan, 1994)	18
1.10 Schematic diagram of regularly used methods for liposome preparation (Crommelin and Schreier, 1994)	19
1.11 The structure of amphotericin B (Budavari, 1996)	28
1.12 The structure of the skin (Potts et al., 1992)	36
1.13 The structure of the epidermis (Eckert, 1992)	37
1.14 The sequential steps involved in percutaneous absorption (Potts et al., 1992)	44

1.15	The putative pathways of penetration across the SC (Potts et al.,1992)	45
1.16	Proposed mechanisms for the interaction of liposomes with the skin (Mezei, 1994)	52
3.1	The physical appearance of liposome formulations from left to right 1:1, 7:2, 7:2:1(+) and 7:2:1(-) kept at 4±1°C for one day	65
3.2	The physical appearance of liposome formulations from left to right 1:1AmB, 7:2AmB, 7:2:1(+)AmB and 7:2:1(-)AmB kept at 4±1°C for one day	65
3.3	Histograms of zeta potential of liposome formulations with and without the entrapped AmB	68
3.4	Scanning electron micrograph of the 1:1 liposome formulation, 15000x	69
3.5	Scanning electron micrograph of the 7:2 liposome formulation, 15000x	69
3.6	Scanning electron micrograph of the 7:2:1(+) liposome formulation, 15000x	70
3.7	Scanning electron micrograph of the 7:2:1(-) liposome formulation, 30000x	70
3.8	Scanning electron micrograph of the 1:1AmB liposome formulation, 15000x	71
3.9	Scanning electron micrograph of the 7:2AmB liposome formulation, 15000x	71
3.10	Scanning electron micrograph of the 7:2:1(+)AmB liposome formulation, 12000x	72
3.11	Scanning electron micrograph of the 7:2:1(-)AmB liposome formulation, 15000x	72
3.12	Size distribution of liposome formulations without the entrapped amphotericin B	78
3.13	Size distribution of liposome formulations with the entrapped amphotericin B	78
3.14	The lamellarity of the 7:2AmB liposome formulation	79
3.15	The DSC curve of amphotericin B powder at rate 5°C/min	80
3.16	The DSC curve of Fungizone® powder at rate 5°C/min	80
3.17	The DSC curve of hydrogenated soya phosphatidylcholine (Emulmetik950®) powder at rate 5°C/min	81
3.18	The DSC curves of cholesterol (—) , dicetyl phosphate (-.-) and stearylamine (-.-) powder at rate 5°C/min	81
3.19	The DSC curves of 1:1 (—) and 1:1AmB (-.-) lyophilized liposome formulations at rate 5°C/min	82
3.20	The DSC curves of 7:2 (—) and 7:2AmB (-.-) lyophilized liposome formulations at rate 5°C/min	82
3.21	The DSC curves of 7:2:1(+) (—) and 7:2:1(+)AmB (-.-) lyophilized liposome formulations at rate 5°C/min	83

3.22	The DSC curves of 7:2:1(-) (—) and 7:2:1(-)AmB (-.-) lyophilized liposome formulations at rate 5°C/min	83
3.23	Ultraviolet absorption spectra of amphotericin B in DMSO/methanol solution (A) and Fungizone® in methanol (B) (5 µg/ml)	87
3.24	Ultraviolet absorption spectra of liposomes with the drug in methanol (A) (about 5 µg/ml), liposome without the drug in methanol (B) methanol (C) and DMSO/methanol solution (D)	88
3.25	Chromatograms of methanol (A), amphotericin B in methanol (B) and Fungizone® in methanol (C)	89
3.26	The standard curve of standard amphotericin B with $r^2 = 0.9999$ and $y = 354.34x - 8.01$ from the linear regression analysis	91
3.27	The HPLC chromatogram of the untrapped amphotericin B in the supernatant of the 1:1AmB liposome formulation	93
3.28	The HPLC chromatograms of the liposome formulation without the entrapped drug when assay for the total amount of drug (liposome dispersion) (A), the entrapped drug (pellets) (B) and the free drug (supernatant) (C)	94
3.29	The physical appearance of freshly prepared 1:1 (No.1), 7:2 (No.2), 7:2:1(+) (No.3), 7:2:1(-) (No.4), 1:1AmB (No.5), 7:2AmB (No.6), 7:2:1(+)AmB (No.7) and 7:2:1(-)AmB (No.8)	104
3.30	The physical appearance of 1:1 (No.1), 7:2 (No.2), 7:2:1(+) (No.3), 7:2:1(-) (No.4), 1:1AmB (No.5), 7:2AmB (No.6), 7:2:1(+)AmB (No.7) and 7:2:1(-)AmB (No.8) when stored at $4\pm 1^\circ\text{C}$ (A), $30\pm 1^\circ\text{C}$ (B) and $45\pm 1^\circ\text{C}$ (C) for 90 days	105
3.31	Fungizone® solution (A) and Fungizone® powder (B) at time 0 day and at 90 days when stored at $4\pm 1^\circ\text{C}$, $30\pm 1^\circ\text{C}$ and $45\pm 1^\circ\text{C}$ (from left to right)	107
3.32	The comparison of the percentages of the remaining amphotericin B in liposome formulations, Fungizone® solution and Fungizone® powder sampling at 0, 5, 20, 40 and 90 days when kept at $4\pm 1^\circ\text{C}$, $30\pm 1^\circ\text{C}$ and $45\pm 1^\circ\text{C}$	116
3.33	The Arrhenius plot of the 1:1AmB liposome formulation	118
3.34	The Arrhenius plot of the 7:2AmB liposome formulation	119
3.35	The Arrhenius plot of the 7:2:1(+)AmB liposome formulation	120
3.36	The Arrhenius plot of the 7:2:1(-)AmB liposome formulation	121

3.37 The Arrhenius plot of the Fungizone® solution	122
3.38 The Arrhenius plot of the Fungizone® powder	123
3.39 The histograms of the predicted shelf life (days) at $4\pm 1^\circ\text{C}$, $30\pm 1^\circ\text{C}$ and $45\pm 1^\circ\text{C}$ of liposome formulations, Fungizone® solution and Fungizone® powder	125
3.40 The HPLC chromatogram of 50:50 v/v of ethanol/water solution	126
3.41 The HPLC chromatogram of the viable epidermis and dermis without amphotericin B	127
3.42 The HPLC chromatogram of stratum corneum in striped-tape extracted by methanol	127
3.43 The HPLC chromatogram of unstripped tape extracted by methanol	128
3.44 The HPLC chromatogram of water	128
3.45 The HPLC chromatogram of phosphate buffer pH 7.4	129
3.46 The HPLC chromatogram of 1:9 v/v of DMSO/methanol solution	129
3.47 The flux of amphotericin B from various formulations in different strata of the rat skin	133

ABBREVIATIONS AND SYMBOLS

1:1 liposome formulation	Liposome with 1:1 molar ratio of lipid and without amphotericin B
7:2 liposome formulation	Liposome with 7:2 molar ratio of lipid and without amphotericin B
7:2:1(+) liposome formulation	Liposome with 7:2:1 molar ratio of lipid, positively charged and without amphotericin B
7:2:1(-) liposome formulation	Liposome with 7:2:1(-) molar ratio of lipid, negatively charged and without amphotericin B
1:1AmB liposome formulation	1:1 liposome with amphotericin B
7:2AmB liposome formulation	7:2 liposome with amphotericin B
7:2:1(+)AmB liposome formulation	7:2:1(+) liposome with amphotericin B
7:2:1(-)AmB liposome formulation	7:2:1(-) liposome with amphotericin B
AmB	Amphotericin B
AUC	Area under the curve
CHL	Cholesterol
DCP	Dicetyl phosphate
DSC	Differential scanning calorimeter
HPLC	High performance liquid chromatography
HSC	Hydrogenated soya phosphatidylcholine
SA	Stearylamine
SC	Stratum corneum
SEM	Scanning electron microscope
TEM	Transmission electron microscope
VED/D	Viable epidermis and dermis