APPENDICES

APPENDIX A

Calculation of Compositions in Liposome Formulations

For the 7:2:1(+)AmB liposomes, the following information were used.

- The final volume of liposome formulations was 40 ml.
- The total lipid was 5 mg lipid in 1 ml of liposome formulation.
- The concentration of AmB was 0.05 mg in 1 mg of total lipid.

Thus, the total lipid in 40 ml = (5 mg/ml) x 40 ml

= *200 mg*

The contents of AmB in this formulation were :

= (0.05 mg/mg lipid) x 200 mg

= *10 mg*

From the label amount, one vial of 111.2 mg of the product Fungizone® contained of AmB 50 mg, sodium deoxycholate 41 mg and sodium phosphate 20.2 mg.

AmB 50 mg from the Fungizone® product = 111.2 mg

Then, AmB 10 mg from the Fungizone® product = (111.2 mg / 50 mg) x 10 mg

= *22.24 mg*

- The molar ratio of lipids in this formulation was HSC / CHL / SA = 7:2:1.
  HSC, CHL and SA have the molecular weights (MW) of 762, 386.67 and 269.5 respectively.

Thus, the mass ratio was :

= (7x762) : (2x386.67) : (1x269.5)

= 5334 : 773.34 : 269.5 g

The total mass was = 5334 + 773.34 + 269.5

= 6376.84 g

Thus, from the total lipid of 6376.84 g containing HSC 5334 g

For the total lipid of 200 mg

containing HSC = (5334 g/ 6376.84 g) x 200 mg

= *167.29 mg* or *0.167 g* of HSC

containing CHL = (773.34 g/ 6376.84 g) x 200 mg

= *24.25 mg* or *0.024 g* of CHL

containing SA = (269.5 g/ 6376.84 g) x 200 mg

= *8.45 mg* or *0.009 g* of SA

Therefore, for the preparation of 40 ml of 7:2:1(+)AmB liposomes, the following compounds was prepared :

- Fungizone® 0.0222 g
- HSC 0.1670 g
- CHL 0.0240 g
- SA 0.0090 g

APPENDIX B

Calculation of the Contents of Amphotericin B in Fungizone®

Form the standard curve : $y = 354.34x - 8.01$ (Figure 3.26)

When x = concentration of AmB (µg/ml)

y = peak area

if $y = 69.98$ (Table 3.21)

- then, $69.98 = (354.34x) - 8.01$

$x = 0.22$ µg/ml

- This concentration was converted to the initial concentration by multiply with the dilution factor (1,000) as follows :

= 0.22 µg/ml x 1,000

= 220 µg/ml

= 0.220 mg/ml

- The contents of AmB in 50 ml

= (0.220 mg/ml) x 50 ml

= *11 mg*

- From 27.7 mg of the Fungizone® product, the contents of AmB = 11 mg

For 1 mg of the product, the contents of AmB = (11 mg / 27.7 mg) x 1 mg

= *0.40 mg*

Thus, the contents of AmB in 1 mg of Fungizone® = 0.40 mg

APPENDIX C

Calculation of the Percentages of Entrapment of Amphotericin B in Liposomes

From the Table 3.36 for the 7:2:1(+)AmB liposome lot 1, the total drug and the entrapped drug had the mean peak areas of 66.25 and 128.51 respectively.

The inject concentration of the total and the entrapped drug when substituted the peak areas to the standard curve were 0.21 and 0.39 μg/ml respectively.

- The initial concentrations were obtained by multiplying with the dilution factor

The initial concentration of the total drug = (0.21 μg/ml) x 1,000

= *210 μg/ml = 0.21 mg/ml*

The initial concentration of the entrapped drug = (0.39 μg/ml) x 500

= *192.63 μg/ml = 0.193 mg/ml*

Then, the total drug and the entrapped drug were 0.21 and 0.19 mg in 1 ml of liposome formulation respectively.

- The percentages of the entrapment of AmB in liposome

= (the amount of the entrapped AmB / the total amount of AmB) x 100%

= (0.19 mg/ 0.21 mg) x 100%

= 91.91%

Thus, the percentage of the entrapment of AmB in 7:2:1(+) liposome (lot 1) was 91.91%.

APPENDIX D

Calculation of Degradation Rate and Shelf life of Liposome Formulations

For the 1:1AmB liposome, Table 3.47 showed the percentages of the remaining amphotericin B in liposomes at 4°C, 30°C and 45°C.

Day (s)	4°C	30°C	45°C
0	100.00	100.00	100.00
5	100.31	102.27	78.42
20	95.91	98.57	51.97
40	95.48	95.14	39.95
90	95.03	95.46	25.70

The percentages of the remaining amphotericin B in liposomes at the given time in 0, 5, 20, 40 and 90 days were used for the linear regression with the equation of zero and first order and the Higuchi model (Table 3.48).

Types of equation	Equation	Term descriptions
Zero order	$C = C_0 + kt$	C_0 = initial concentration
First order	$\ln C = \ln C_0 + kt$	C = concentration at given t
Higuchi model	$C = C_0 + kt^{0.5}$	t = time k = rate constant
Arrhenius	$\ln k = \ln A - (Ea / RT)$	ln A = frequency factor Ea = activation energy R = gas constant T = absolute temperature
Shelf life (only for Higuchi model)	$(t_{90})^{0.5} = (C - C_0) / \text{antiln } k_s$ $t_{90} = ((t_{90})^{0.5})^2$	k_s = rate constant at the specific temperature

The C_0, k and r^2 values were showed in Table 3.49.

1:1 AmB	Zero order			First order			Higuchi model		
	Slope (-)	Inter-cept	R square	Slope (-)	Inter-cept	R square	Slope (-)	Inter-cept	R square
4°C	0.056	99.07	0.6196	0.0006	4.60	0.6225	0.6264	100.17	0.7866
30°C	0.0667	100.36	0.6435	0.0007	4.61	0.6470	0.6828	101.36	0.6788
45°C	0.7208	81.55	0.7723	0.0141	4.41	0.9085	8.0095	95.28	0.9602

The r^2 values from the Higuchi model were higher than those from the zero and first order equation. Thus, the degradation rate (k) from Higuchi model at the temperatures of 4°C, 30°C and 45°C were used for the calculation of linear regression in the Arrhenius equation. This equation was as the following :

$$\ln k = 16.29 - 4738.45 \left(1/T \right)$$

The degradation rate (k) and shelf life (t_{90}) at any temperature can be predicted by the substitution the interested temperatures (absolute temperature, T)

For example, prediction at 30°C ;

$\ln k_{303}$ = 16.29 – 4738.45 (1/(273+30))

= 0.65

antiln k_{303} 1.92

k = degradation rate = 1.92 (% / day $^{0.5}$)

$(t_{90})^{0.5}$ = (90 – 100) / (-1.92) = 5.21

shelf life (t_{90}) = $(5.21)^2$

= 27.13 days

Thus, the 1:1AmB liposome gave the predicted degradation rate and shelf life of 1.92 (% / day $^{0.5}$) and 27.13 days, respectively.

APPENDIX E

Calculation of the Flux (ng/cm^2 per hr) through the Wistar Rat Skin

For the 7:2:1(+)AmB liposome from Table 3.57, the peak area of AmB at initial (in 2 ml of sample) was 72.53.

From the standard curve, the inject concentration = (72.53+8.01) / 354.34
= 0.227 µg/ml

Then, the initial concentration (dilution factor 1,000) = (0.227 µg/ml) x 1,000
= 227 µg/ml or 0.227 mg/ml

Amounts of AmB in 2 ml of sample = (0.227 mg/ml) x 2 ml
= *0.454 mg.*

For, the peak areas of the assayed AmB in SC, it was 462.82.

From the standard curve, the inject concentration = (462.82+8.01) / 354.34
= 1.33 µg/ml

This inject concentration was from the injection volume of 100 µl. This amount was divided by 2 because the injection volume of the standard curve was 50 µl.

So, the inject concentration = (1.33 µg/ml) / 2
= 0.665 µg/ml

Amounts of AmB in SC (by extraction from tape strips in 5 ml methanol)
= (0.665 µg/ml) x 5 ml
= *3.32 µg*

For the peak areas of the assayed AmB in VED/D, it was 71.51.

From the standard curve, the inject concentration = (71.51+8.01) / 354.34

= 0.224 µg/ml

This inject concentration was from the injection volume of 100 µl. This amount was divided by 2 because the injection volume of the standard curve was 50 µl.

So, the inject concentration = (0.224 µg/ml) / 2

= 0.112 µg/ml

Amounts of AmB in VED/D (by extraction of the VED/D in 5 ml methanol)

= (0.112 µg/ml) x 5 ml

= *0.56 µg*

For the amount of AmB in the receiver medium, please see AmB in DMSO/methanol solution in Table 3.57

The peak area was 2.78.

From the standard curve, the inject concentration = (2.78+8.01) / 354.34

= 0.030 µg/ml

This inject concentration was from the injection volume of 100 µl. This amount was divided by 2 because the injection volume of the standard curve was 50 µl.

So, the inject concentration = (0.030 µg/ml) / 2

= 0.015 µg/ml

Amounts of AmB in 12 ml of the receiver medium = (0.015 µg/ml) x 12 ml

= *0.18 µg*

The equation of the flux(ng/cm^2 per hr) in this experiment used in this experiment was :

Flux (ng/cm^2 per hr) = amount of AmB (ng) / { 1.77 (cm^2) x 24 (hrs) }

For the flux of the 7:2:1(+)AmB in SC when 3.32 µg of AmB was found in SC, it can be calculated as

= (3.32 x 1000) ng / { 1.77 (cm^2) x 24 (hrs) }

= *78.15 ng/cm^2 per hr*

Therefore, the flux of AmB in the skin strata can be calculated from this equation when the amounts of AmB is known.

CURRICULUM VITAE

Name	Miss Lalana Kongkaneramit
Date of birth	5th August 1972
Birth Place	Songkhla, Thailand.
Education	High school degree from Waranareechaleam School, Songkhla, Thailand. B. Pharm. Degree from Songkhla University, Songkhla, Thailand.
Address	6/4, Moo 5, Soi 3, Tumbol Pungla, Amphur Sadao, Songkhla, Thailand.
Further Plan	Working as a researcher in the area of research and development of pharmaceuticals and cosmetics
Presentation	Lalana Kongkaneramit, Jiradej Manosroi, Boonsom Liawruangrath and Arunya Manosroi "Stability Study of Amphotericin B Entrapped in Liposomes". Poster B-079, the 25th Congress on Science and Technology of Thailand, Amarin Lagoon Hotel, Pitsanuloke, Thailand, 20-22 October 1999.