CHAPTER IV

RESULTS AND DISSCUSSION

A. Doxycycline Hyclate Loaded Chitosan Microspheres

1. Preparation of Doxycycline Hyclate Loaded Chitosan Microspheres

Doxycycline hyclate loaded chitosan microspheres were successfully prepared by using water in oil emulsification and ionotropic gelation method. Sunflower oil and span®85 were chosen to be an external phase and emulsifying agent with the fixed amounts of 142.5 and 7.5 ml, respectively. The volumes of internal and external phases were kept constant since the variation of the phase volume had a strong influence on the size of the microspheres (Sansdrap and Moës, 1993). In the preparation process, the geometry of the manufacturing or preparing systems (e.g. reactor or container, stirrer, etc.) was kept unchanged to prevent any uncontrolled influences on the production of microparticles.

Many studies involving the preparation of chitosan microspheres using emulsification and ionotropic gelation technique have been reported (Berthold et al., 1996; Lim, Wan and Thai, 1997; Ko et al., 2002). STPP was a non-toxic and multivalent anion that could form cross-links by ionic interaction between positive charged amino groups of chitosan and multivalent negatively charged STPP molecules (Shu and Zhu, 2000). The STPP treatment of chitosan microspheres was expected to improve their stability and their applicability in controlled drug delivery.

2. Morphology and Particle Size

Microspheres were formed in the oil phase. After filtration and drying, the shape and surface morphology of chitosan microspheres were observed by scanning electron microscopy. The chitosan microspheres were found to be spherical. Some aggregated particles were found (Figure 16). This might be due to weakness of chitosan microspheres prepared by tripolyphosphate ion (Lim, Wan and Thai, 1997).

From the preliminary study, chitosan microspheres which prepared by other crosslinking agent such as glutaraldehyde showed a spherical shape and less aggregation, these might be due to a stronger bond of chemical crosslink between chitosan and glutaraldehyde. However, the microspheres obtained from this method showed a dark-brown surface which might be affect dentists compliance. Moreover, according to previous studies (Lim, Wan and Thai, 1997, Ko et al., 2002) reported that chemically synthesized materials such as glutaraldehyde and formaldehyde which frequently used as a crosslinking agent could cause irritation to mucosal membranes due to its toxicity.

(a) (b)

Figure 16 Scanning electron microphotographs of doxycycline hyclate loaded chitosan microspheres comprising of doxycycline hyclate, chitosan, STPP of 30:3:10 (a) x 85 (b) x 1000

Particle size analysis done by laser light scattering revealed that the average sizes of microspheres prepared by this method were in the range 17.41-130.97 μm as shown in Table 5. However, the larger average sizes were obtained from formulation 13 and 16. The causes could not be clearly explained. The only one observation on the formulation was that both formulations contained high concentration (4%) of chitosan.

The result demonstrated that, at the same amount of chitosan, the formulations of which drug : polymer ratio showed highly part of polymer (formulation 4, 9, 12, 16) tended to give larger average sizes of chitosan microspheres. It might be possible that the

formulations with high amount of drug load may decrease viscosity of chitosan solution and it was easily to form small droplet in external phase when sheared with simple paddle at the same speed.

Table 5 The mean particle size of doxycycline hyclate loaded chitosan microspheres from various formulations.

Formulation	Factor combinations at different levels			Core : wall ratio	Size (μm) [a]
	DC (% w/w)[+]	CC (% w/w)	STPPC (% w/v)		
1	75	2	10	1:1.3	19.51 ± 0.01
2	30	2	5	1:3.3	23.11 ± 0.39
3	30	2	15	1:3.3	28.02 ± 0.33
4	10	2	10	1:10	31.64 ± 0.54
5	75	3	5	1:1.3	17.41 ± 0.10
6*	30	3	10	1:3.3	20.42 ± 0.15
7*	30	3	10	1:3.3	18.36 ± 0.24
8	75	3	15	1:1.3	16.16 ± 0.12
9	10	3	5	1:10	26.29 ± 0.24
10*	30	3	10	1:3.3	18.53 ± 0.11
11*	30	3	10	1:3.3	26.35 ± 0.66
12	10	3	15	1:10	35.14 ± 0.58
13	30	4	15	1:3.3	127.26 ± 9.87
14	30	4	5	1:3.3	34.91 ± 0.68
15	75	4	10	1:1.3	38.71 ± 0.40
16	10	4	10	1:10	130.97 ± 1.66

* = The center points of the design
[+] = Based on chitosan concentration
a = mean ± SD, n = 3

3. The Percentage Yield of Doxycycline Hyclate Loaded Chitosan Microspheres

The percentage yields obtained from various factors are exhibited in Table 6. The values were in the wide range 27.72-91.27%. The high value of yield percentage was obtained from formulation 9 which had the core : wall ratio of 1:10 and 5% STPP.

From the percentage yields obtained depicted in Table 6, a scattered plot of the percentage yields of the formulations with 1:1.3, 1:3.3 and 1:10 core to wall ratios and

with different (5, 10 and 15 %) concentrations of STPP are demonstrated in Figures 17 and 18.

Table 6 The percentage yield of doxycycline hyclate loaded chitosan microspheres from various formulations.

Formulation	Factor combinations at different levels			Core : wall ratio	Yield (%)
	DC (% w/w)$^{+}$	CC (% w/w)	STPPC (% w/v)		
1	75	2	10	1:1.3	52.89
2	30	2	5	1:3.3	79.62
3	30	2	15	1:3.3	56.06
4	10	2	10	1:10	84.23
5	75	3	5	1:1.3	71.83
6*	30	3	10	1:3.3	60.66
7*	30	3	10	1:3.3	69.84
8	75	3	15	1:1.3	56.82
9	10	3	5	1:10	91.27
10*	30	3	10	1:3.3	71.73
11*	30	3	10	1:3.3	74.01
12	10	3	15	1:10	50.88
13	30	4	15	1:3.3	27.72
14	30	4	5	1:3.3	52.34
15	75	4	10	1:1.3	55.57
16	10	4	10	1:10	43.42

* = The center points of the design
$^{+}$ = Based on chitosan concentration
a = mean $\pm$ SD, n = 3

From Figures 17 and 18, it revealed that the formulations with consisted of the lowest concentration (5%) of STPP in every core to wall ratio (1:1.3, 1:3.3 and 1:10) had high percentages yields (formulation 2, 5 9 and 14). Moreover, comparison between formulation 2 and 14, it demonstrated that the lower chitosan concentration, the higher the percentage yield. At the same concentration of STPP of 5%, it also demonstrated that the lower core to wall ratio (1:10) or lower drug load (10%), the higher the percentage yield (Figure 18).

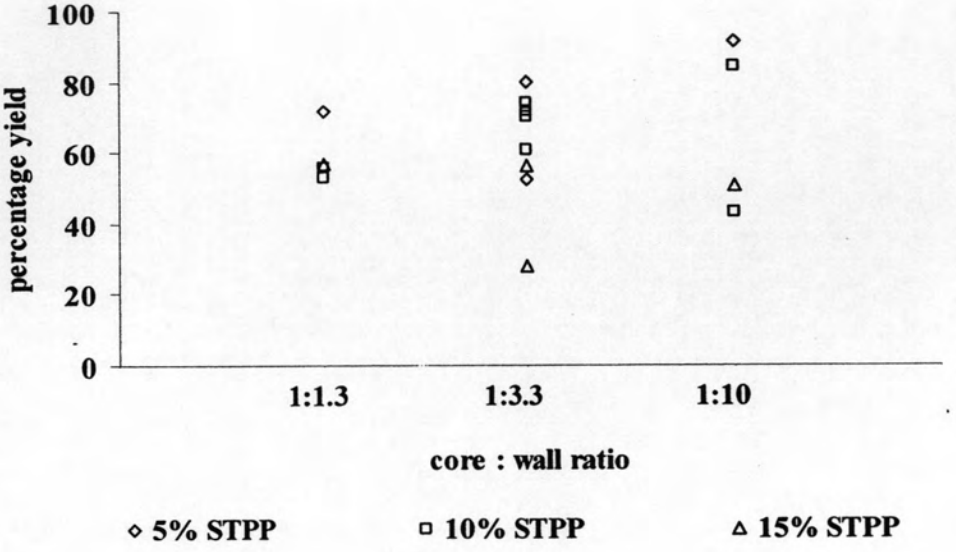


Figure 17 Scattered plot of percentage yield and core to wall ratio

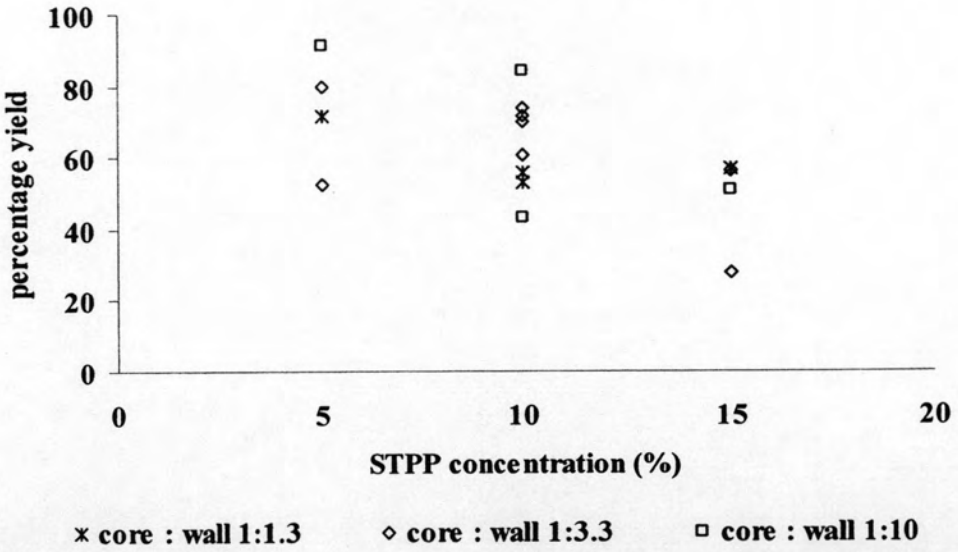


Figure 18 Scattered plot of percentage yield and STPP concentration

It is surprising that the high concentration of STPP that was used as a crosslinking agent brought to low percentage yield. However, the results were also dependent on chitosan concentration.

4. Determination of Drug Content of Chitosan Microspheres

4.1 Assay of Encapsulation Efficiency by UV Spectrophotometric Method

The validation of analytical method is the process for evaluation that the method is suitable and reliable for the intended analytical applications. The analytical parameters used for the UV spectrophotometric assay validation were specificity, linearity, accuracy and precision.

4.1.1 Specificity

The UV absorption spectrum of doxycycline hyclate is shown in Figure 19. The maximum absorbance was found at the wavelength of 268 nm. Therefore, the detection of doxycycline hyclate was performed at this wavelength. Furthermore, under the condition selected for assay of encapsulation efficiency, the peak of other components in the formulations was not interfering with the peak of doxycyline hyclate as shown in Figure 20. This validation was made by comparing the peak scan from UV spectrophotometer between 1 N hydrochloric acid with the peak scan of doxycycline hyclate.

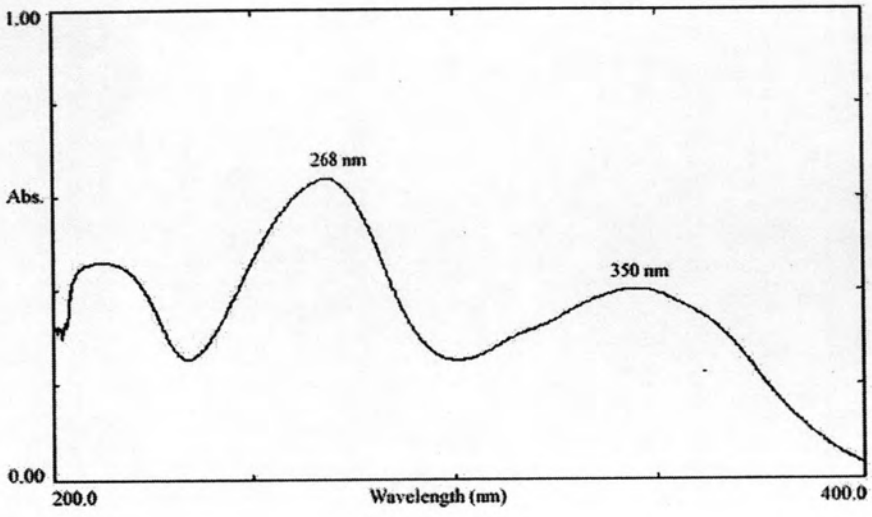


Figure 19 UV spectrum of doxycycline hyclate in 1 N hydrochloric acid

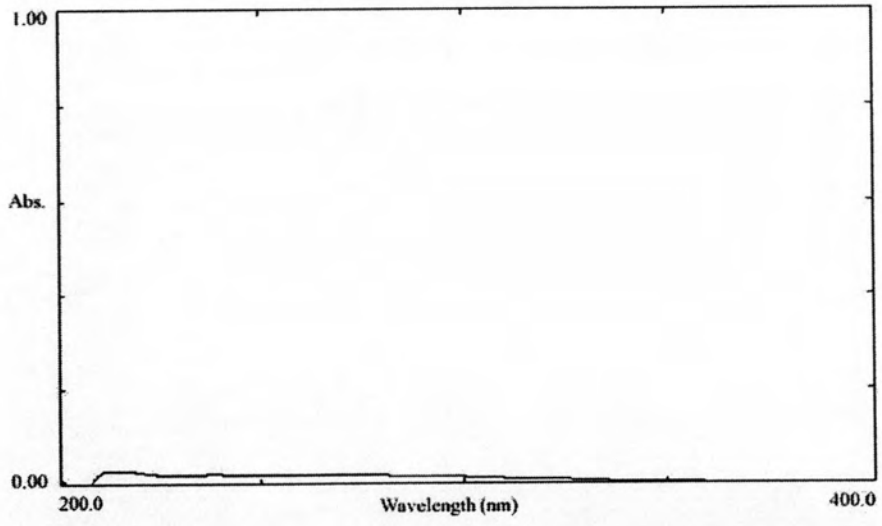


Figure 20 UV spectrum of blank microspheres in 1 N hydrochloric acid

4.1.2 Linearity

The linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of the analyte in samples within a given range. The linearity is usually expressed in terms of the variance around the slope of the regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of the analyte. The calibration curve of doxycycline hyclate in 1N hydrochloric acid is shown in Figure 21. Linear regression analysis of the absorbances versus the corresponding concentrations was performed and the coefficient of determination (R^2) was calculated as 0.9999. The calibration data were found to be linear with excellent coefficient of determination. These results indicated that UV spectrophotometric method was acceptable for quantitative analysis of doxycycline hyclate in the range of studied.

Table 7 Data for calibration curve of doxycycline hyclate by UV spectrophotometric method.

Concentration (µg/ml)	Absorbance			Mean	SD	%CV
	Set1	Set2	Set3			
6	0.240	0.240	0.241	0.240	0.001	0.26
9	0.344	0.363	0.357	0.355	0.010	2.79
12	0.476	0.465	0.472	0.471	0.006	1.24
15	0.585	0.589	0.595	0.589	0.005	0.86
18	0.710	0.704	0.705	0.706	0.003	0.45
21	0.813	0.814	0.823	0.817	0.006	0.72
24	0.932	0.924	0.933	0.930	0.005	0.51
R^2	0.9994	0.9996	0.9999	0.9999	-	-

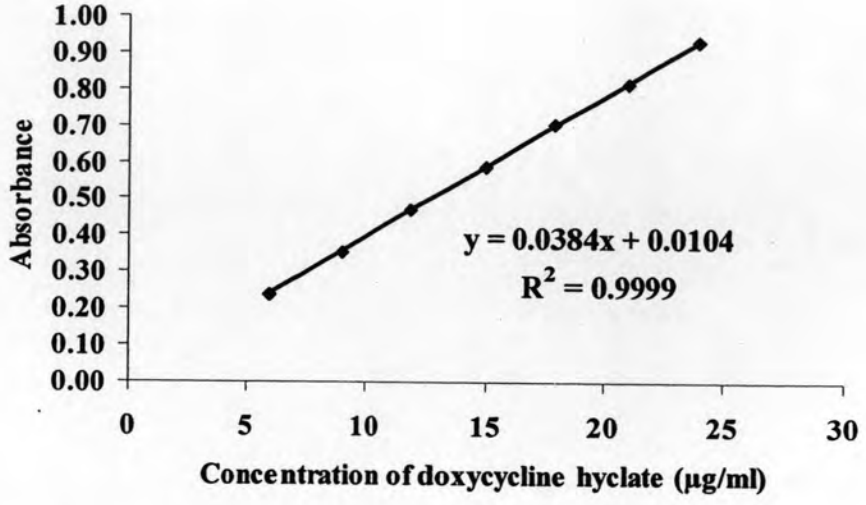


Figure 21 Calibration curve of doxycycline hyclate by UV spectrophotometric method

4.1.3 Accuracy

Doxycycline hyclate solutions were prepared at the concentration of 10.5, 16.5 and 22.5 µg/ml in five sets. Each individual sample was analyzed by UV spectrophotometer. The inversely estimated concentrations and percentages of analytical recovery of each drug concentration are shown in Table 8 and Table 9, respectively. All percentages of analytical recovery were in the range of 98.45-99.68%, which indicated the high accuracy of this method. Thus, it could be used for analysis of doxycycline hyclate in all concentrations studied.

Table 8 The inversely estimated concentrations of doxycycline hyclate by UV spectrophotometric method.

Concentration (µg/ml)	Inversely estimated concentration(µg/ml)					Mean±SD
	Set1	Set2	Set3	Set4	Set5	
10.5	10.3869	10.3392	10.3216	10.3392	10.3015	10.3377±0.03
16.5	16.6256	16.3945	16.3794	16.3643	16.3090	16.4146±0.12
22.5	22.5352	22.3317	22.4623	22.3668	22.4472	22.4286±0.08

Table 9 The percentage of analytical recovery of doxycycline hyclate by UV spectrophotometric method.

Concentration (μg/ml)	Percent analytical recovery					Mean±SD
	Set1	Set2	Set3	Set4	Set5	
10.5	98.92	98.47	98.30	98.47	98.11	98.45±0.30
16.5	100.76	99.36	99.27	99.18	98.84	99.48±0.74
22.5	100.16	99.25	99.83	99.41	99.77	99.68±0.36

4.1.4 Precision

The precision of doxycycline hyclate analyzed by UV spectrophotometric method were determined both within run precision and between run precision as illustrated in Table 10 and Table 11, respectively. All coefficients of variation values were very low, as 0.95-1.26% and 1.39-1.44 % respectively. The coefficient of variation of an analytical method should generally be less than 2%. Therefore, The UV spectrophotometric method was precise for quantitative analysis of doxycycline hyclate in the range studied.

Table 10 Data of within run precision by UV spectrophotometric method.

Concentration (μg/ml)	Absorbance					Mean	SD	%CV
	Set1	Set2	Set3	Set4	Set5			
10.5	0.414	0.417	0.406	0.407	0.407	0.410	0.005	1.26
16.5	0.640	0.638	0.644	0.643	0.654	0.643	0.006	0.95
22.5	0.872	0.876	0.876	0.884	0.895	0.880	0.009	1.05

Table 11 Data of between run precision by UV spectrophotometric method.

Concentration (μg/ml)	Absorbance					Mean	SD	%CV
	Set1	Set2	Set3	Set4	Set5			
10.5	0.420	0.417	0.407	0.408	0.413	0.413	0.006	1.39
16.5	0.659	0.638	0.640	0.648	0.637	0.644	0.009	1.44
22.5	0.903	0.876	0.877	0.880	0.897	0.886	0.013	1.43

In conclusion, the analysis of doxycyclline hyclate in 1 N HCl by UV spectrophotometric method developed in this study showed good specificity, linearity, accuracy and precision. Thus this method was used for the determination of the content of doxycycline hyclate in the assay of encapsulation efficiency.

4.2 Encapsulation Efficiency

The effect of 1 N HCl to the degradation of doxycycline hyclate (1mg/ml) was determined at 25 °C for 24 hrs. A small decrease of doxycycline hyclate of 2% was detected. This confirmed that 1 N HCl had a little effect to the stability of doxycycline hyclate. Percentages of encapsulation efficiency of doxycycline hyclate loaded chitosan microspheres prepared with varied concentrations are demonstrated in Table 12. The values varied with a wide range of 1.40-105.11 % encapsulation efficiency. Many factors affected the entrapment efficiency of the drug into the chitosan microspheres, e.g. nature of the drug, chitosan concentration, drug polymer ratio, stirring speed, etc. Generally a low concentration of chitosan shows low encapsulation efficiency (Sinha et al., 2004).This may be explained on the basis that an increase in viscosity of the chitosan solution with increase in concentration prevents drug crystals from leaving the droplet (Nishioka et al., 1990). However, at higher concentrations of chitosan forms highly viscous solutions, which are difficult to process.

Table 12 Percentage encapsulation efficiency of microspheres prepared by factor combinations at different levels.

Formulation	Factor combinations at different levels			Core : wall ratio	% encapsulation efficiency[a]
	DC (% w/w)[+]	CC (% w/w)	STPP (% w/v)		
1	75	2	10	1:1.3	2.08 ± 0.1
2	30	2	5	1:3.3	3.06 ± 0.32
3	30	2	15	1:3.3	4.90 ± 0.06
4	10	2	10	1:10	1.40 ± 0.26
5	75	3	5	1:1.3	6.51 ± 0.18
6*	30	3	10	1:3.3	28.03 ± 0.47
7*	30	3	10	1:3.3	42.44 ± 1.08
8	75	3	15	1:1.3	105.11± 8.49
9	10	3	5	1:10	1.58 ± 0.34
10*	30	3	10	1:3.3	45.85 ± 0.86
11*	30	3	10	1:3.3	37.91 ± 0.15
12	10	3	15	1:10	6.29 ± 0.38
13	30	4	15	1:3.3	11.23 ± 0.75
14	30	4	5	1:3.3	6.33 ± 0.21
15	75	4	10	1:1.3	81.97 ± 1.53
16	10	4	10	1:10	4.90 ± 0.29

* = The center points of the design
[+] = Based on chitosan concentration
a = mean ± SD, n = 3

A scattered plot between the percentages encapsulation efficiency of chitosan microspheres with different core to wall ratios (1:1.3, 1:3.3 and 1:10) and varied concentrations of STPP are shown in Figure 22.

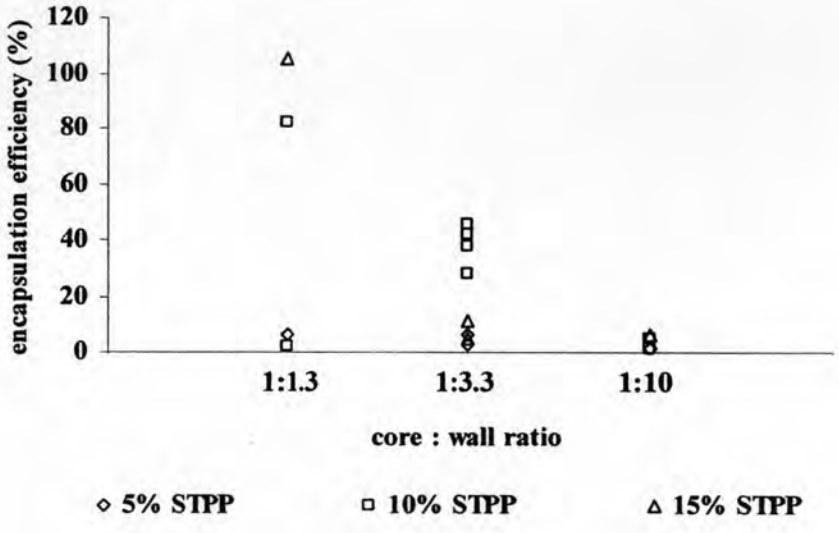


Figure 22 Scattered plot of core to wall ratio and encapsulation efficiency

From Figure 22, it demonstrated that as the core to wall drug ratio was 1:1.3, the percentage encapsulation efficiency increased as shown in formulations 8 and 15. In contrast, formulation 1 which also had the core to wall ratio of 1:1.3, gave a low encapsulation efficiency of 2.08%. Comparison between formulation 1 and 15, it showed that the higher chitosan concentration gave higher encapsulation efficiency.

From Table 12, it revealed that in the same chitosan concentration higher concentration of STPP might increase encapsulation efficiency as could be seen in formulation 3, 8, 12 and 13. This result might be caused by higher concentration of STPP that could form strong walls of chitosan microspheres and the drug could not leak in the washing process. This finding was inconsistent with previous research in that lower loading was found at higher concentration (12-16 %) of STPP (Anal, Stevens and Remuñán-López, 2006). Moreover, it was shown that lower core to wall ratio tended to result in the lower encapsulation efficiency.

In another words, it was found that when the formulation combined with higher levels of drug loading and STPP may result higher encapsulation efficiency as

illustrated in formulation 8 and 15 while encapsulation efficiency quite low in lower STPP concentration though the high drug load was obtained in formulation 5 and 14. This may be due to the higher concentration of doxycycline hyclate can incorporated into the microspheres after that STPP would react with positive charge of chitosan and then the drug could not be loss from the spheres as described above. This finding was similar to results from previous investigations by Jameela and Jayakrishnan (1995).

B. Experimental Design

From the preliminary study, drug loaded concentration (core), chitosan concentration (wall) and crosslinking agent concentration had influences on the preparation of chitosan microspheres. The result was consistent with that reported by Govender et al. (2005). Thus, in this study from the Box-Behnken experimental design, sixteen formulations of doxycycline hyclate loaded chitosan microspheres were obtained.

1. Fitting of encapsulation data to the model

Based on the experimental design, Table 4 summarises the experimental runs. In order to determine the levels of factors which yielded optimal encapsulation efficiency, mathematical relationships were generated between the dependent and independent variables. Using software described earlier, 2 FI model was selected and repeated backward elimination regression was used to eliminate the insignificant effect and to generate the equation for the response parameter (encapsulation efficiency). The regression equation and the significant coefficient before and after eliminate the insignificant effect were presented in Tables 13 and 14, respectively. The initial model was refined to include in those terms for which the level of significance was below or equal to $P < 0.05$. Statistical testing (ANOVA) indicated that the model obtained was statistical significant ($P = 0.0352$).

Table 13 Coefficient, estimation and significant value of the encapsulation efficiency before elimination the insignificant effect.

Term	Coefficient	Value	*P*-value
	b_0	+24.35	
Doxycycline hyclate (A)	b_1	+22.69	0.0170
Chitosan (B)	b_2	+11.62	0.1687
STPP (C)	b_3	+13.76	0.1103
AB	b_4	+19.10	0.1161
AC	b_5	+23.47	0.0613
BC	b_6	+0.77	0.9460
Model significance			0.0425

Table 14 Regression equation and significant coefficients term of the encapsulation efficiency after elimination the insignificant effect.

Term	Coefficient	Value	*P*-value
	b_0	+24.35	
Doxycycline hyclate (A)	b_1	+22.69	0.0352
Model significance			0.0352

$$\text{Response} = b_0 + b_1 (DC) \quad (6)$$

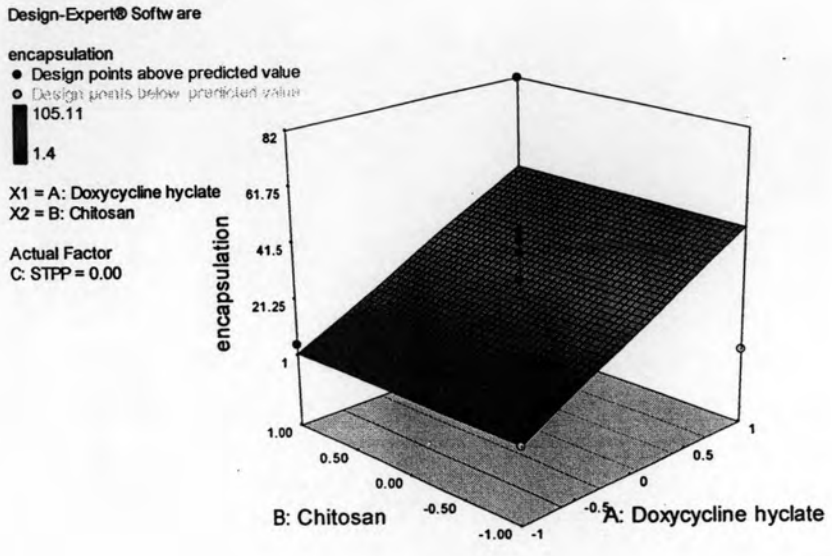
2. Examining the coefficient for maximum encapsulation efficiency and response surface plots.

The resultant equation for maximum encapsulation efficiency is given below in equation (7)

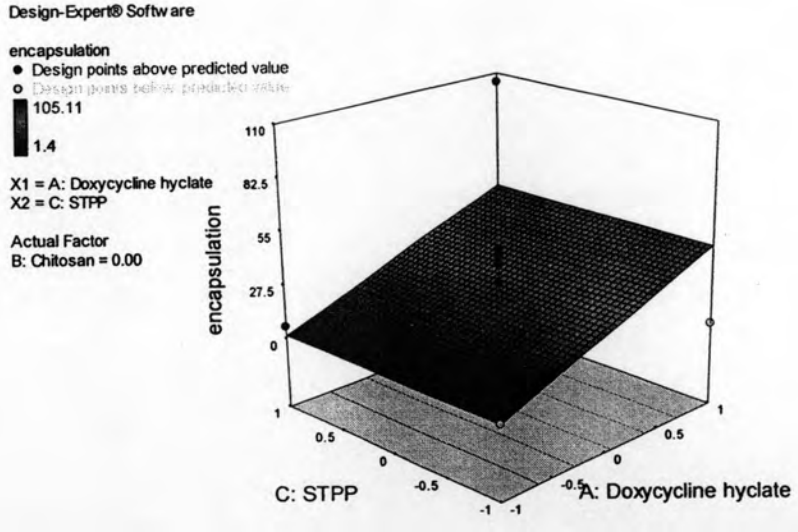
$$\text{Encapsulation efficiency} = 24.35 + 22.69 (DC) \quad (7)$$

A positive value represents an effect that favors the optimization, while a negative value indicates an inverse relationship between the factor and the response. Following the

resultant linear equation (7), it demonstrated that only doxycycline hyclate concentration had a positive effect to on encapsulation efficiency.



(a)



(b)

Figure 23 (a) Response surface plot illustrating the influence of doxycycline hyclate and chitosan concentrations on encapsulation efficiency. (b) Response surface plot illustrating the influence of doxycycline hyclate and sodium tripolyphosphate (STPP) concentration on encapsulation efficiency.

3. Fitting of yield data to the model

In order to determine the levels of factors which yielded are optimal, mathematical relationships were generated between the dependent and independent variables. Using software described earlier, quadratic model was selected and repeated backward stepwise regression was used to eliminate the insignificant effect and to generate the equation for the response parameter (% yield). The regression equation and the significant coefficient before and after eliminate the insignificant effect were presented in Tables 15 and 16, respectively. The initial model was refined to include in those terms for which the level of significance was below or equal to $P < 0.05$. Statistical testing (ANOVA) indicated that the model obtained was statistical significance ($P < 0.0001$).

Table 15 Coefficient, estimation and significant value of the yield before elimination the insignificant effect

Term	Coefficient	Value	*P*-value
	b_0	+69.06	
Doxycycline hyclate (A)	b_1	-4.40	0.0631
Chitosan (B)	b_2	-12.78	0.0006
STPP (C)	b_3	-13.61	0.0004
AB	b_4	+12.28	0.0042
AC	b_5	+8.38	0.0221
BC	b_6	+0.45	0.8747
A^2	b_7	+2.54	0.3889
B^2	b_8	-13.97	0.0022
C^2	b_9	-1.87	0.5208
Model significance			0.0013

Table 16 Regression equation and significant coefficients term of the yield after elimination the insignificant effect

Term	Coefficient	Value	*P-value*
	b_0	+69.40	
Doxycycline hyclate (A)	b_1	-4.40	0.0329
Chitosan (B)	b_2	-12.78	<0.0001
STPP (C)	b_3	-13.61	<0.0001
AB	b_4	+12.28	0.0008
AC	b_5	+8.38	0.0080
B^2	b_6	-13.98	0.0003
Model significance			<0.0001

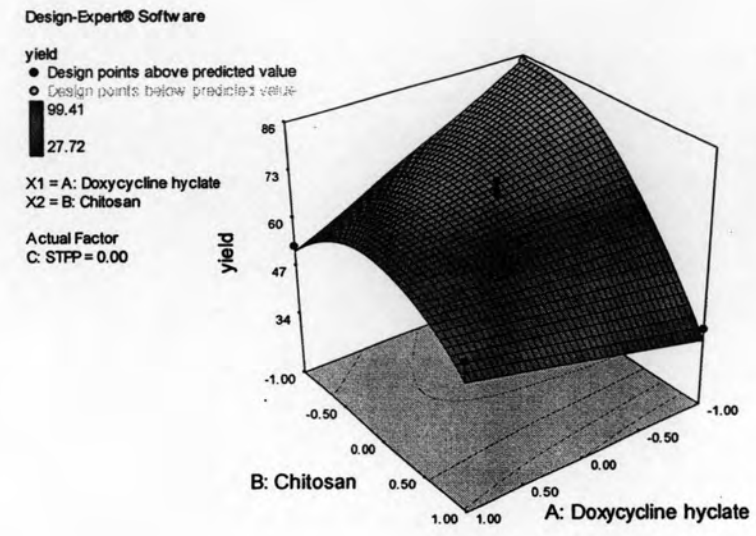
$$\text{Response} = 69.40 - b_1\,(\text{DC}) - b_2\,(\text{CC}) - b_3\,(\text{STPP}) + b_4\,(\text{DC x CC}) + b_5\,(\text{DC x STPP}) - b_6\,(\text{CC}^2) \quad (8)$$

4. Examining the coefficient for maximum yield and response surface plots

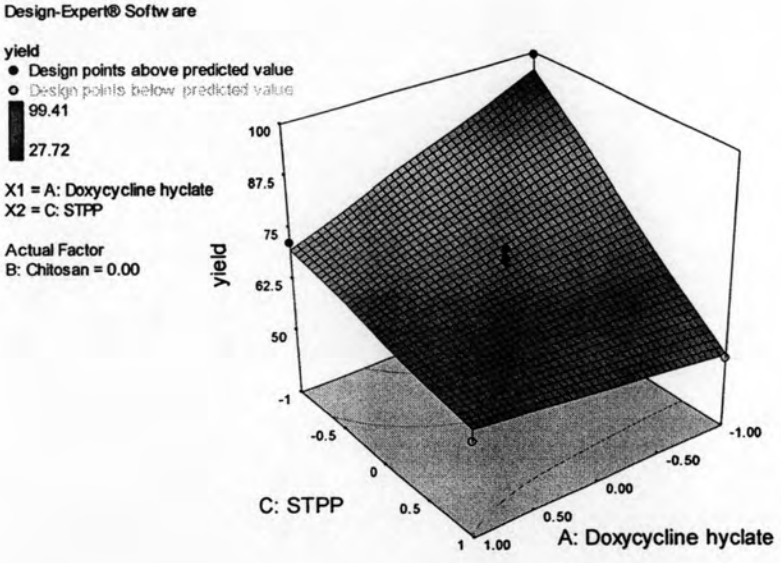
The mechanical relationship generated for the response variable (yield) was expressed as the polynomial equation:

$$\text{yield} = 69.40 - 4.40\,(\text{DC}) - 12.78\,(\text{CC}) - 13.61\,(\text{STPP}) + 12.28\,(\text{DC x CC}) + 8.38\,(\text{DC x STPP}) - 13.98\,(\text{CC}^2) \quad (9)$$

Equation (9) indicated that doxycycline hyclate, chitosan and sodium tripolyphosphate concentration had a negative main effect on yield, at the high level concentrations the yield decreased. In addition, the interaction effect between doxycycline hyclate-chitosan concentration and doxycycline hyclate-sodium tripolyphosphate concentration affected the yield positively.



(a)



(b)

Figure 24 (a) Response surface plot illustrating the influence of doxycycline hyclate and chitosan concentration on yield. (b) Response surface plot illustrating the influence of doxycycline hyclate and sodium tripolyphosphate (STPP) concentration on yield.

5. Selection of an optimal formulation for encapsulation and yield

Using the Design Expert Statiatical Software, the contour plot by optimization of the percentage encapsulation efficiency of 1.40-105.11%, the percentage yield of 50.00-99.41% and the criteria of selection were chosen as maximize of all responses was performed as shown in Figure 25. Eleven optimized formulations were demonstrated with different desirability values and shown in Table 17. It was found that one of the optimized formulation (solution no. 2) had the same compositions of DC, CC and STPPC with the formulation 8. However, the predicted responses were slightly different to the obtained values of formulation 8 as encapsulation efficiency 105.11% and yield of 56.82%. The predicted of encapsulation efficiency was 84.27% and the predicted yield was 59.77%. Moreover, another formulations which showed higher encapsulation efficiency and optimal percentage yield (formulation 15; 81.97 % encapsulation efficiency, 55.57 % yield, formulation 11; 37.91 % encapsulation efficiency, 74.01 % yield) were also selected to the further studies (Table 6, 12).

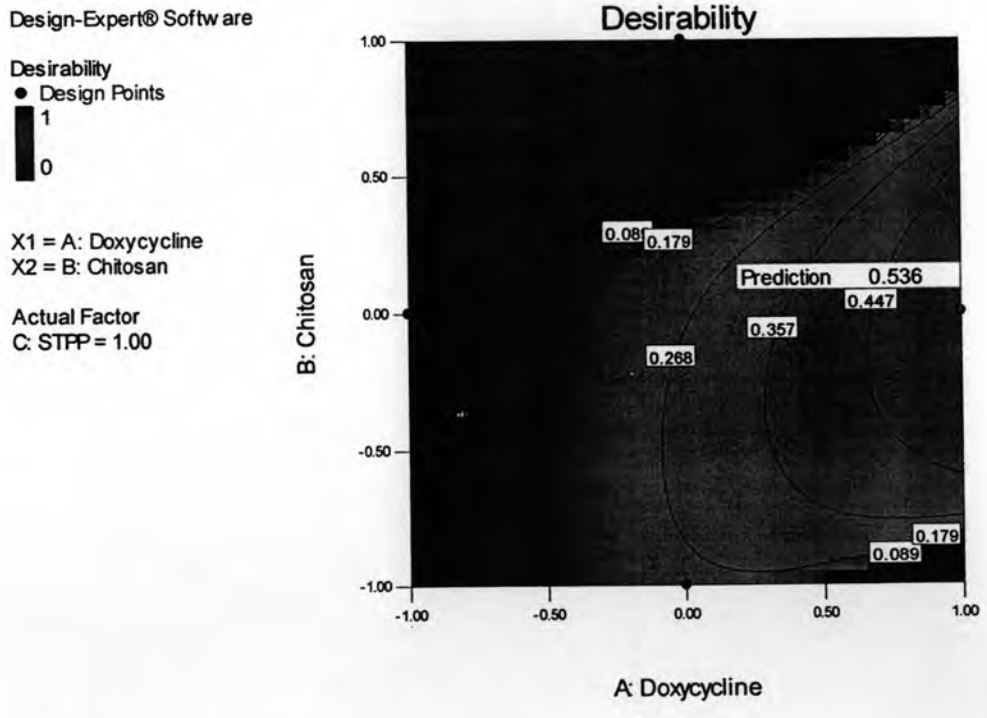


Figure 25 Contour plot showing the optimization of the formulation with 84.27% encapsulation efficiency and 59.77% yield and desirability values.

Table 17 Optimal formulations obtained from Design Expert Statistical software

Solution No.	DC	CC	STPPC	Desirability	Predicted %encapsulation efficiency	Predicted %yield
1	1.00	-0.02	1.00	0.536	84.2655	59.7720
2	1.00	0.00	1.00	0.536	84.2656	59.7690
3	1.00	-0.03	1.00	0.536	84.2616	59.7701
4	1.00	-0.05	1.00	0.536	84.2655	59.7568
5	1.00	-0.08	1.00	0.535	84.2652	59.7214
6	1.00	0.07	1.00	0.535	84.2651	59.6720
7	0.99	-0.04	1.00	0.532	83.5926	59.7158
8	0.99	-0.08	1.00	0.532	83.5925	59.6799
9	0.95	0.11	1.00	0.518	82.0553	57.2705
10	-1.00	-1.00	-1.00	0.188	11.3780	106.869
11	-1.00	-0.16	-1.00	0.188	11.3776	99.4106

C. Characterization of Doxycycline Hyclate Loaded Chitosan Microspheres

1. The Release of Doxycycline Hyclate from Chitosan Microspheres

1.1 Validation of UV Spectrophotometric Method for the Release Study

The validation of analytical method is the process for evaluation that the method is suitable and reliable for the intended analytical applications. The analytical parameters used for the UV spectrophotometric assay validation were specificity, linearity, accuracy and precision.

1.1.1 Specificity

The UV absorption spectrum of doxycycline hyclate in phosphate buffer saline pH 6.8 is shown in Figure 26. The maximum absorbance was found at the wavelength of 268 nm. Therefore, the detection of doxycycline hyclate was performed at this wavelength. The UV spectrophotometry would be used for analysis of doxycycline hyclate in the release studies both from the chitosan microspheres and from the glyceryl monooleate-based drug delivery system containing doxycycline hyclate loaded chitosan

microspheres. Furthermore, under the condition selected for *in vitro* release study, the peak of other components in the formulations was not interfering with the peak of doxycyline hyclate (Figures 27 and 28). From the absorption spectrum of the solution taken from blank microspheres (without doxycycline hyclate) depicted in figure 27, it showed that there was no interference from the blank microspheres with the drug absorption spectra. Similarly, from the absorption spectrum of the recepter fluid taken from the Franz diffusion cell at 120 hr, glyceryl monooleate-based drug delivery system also demonstrated no interfering absorption peak.

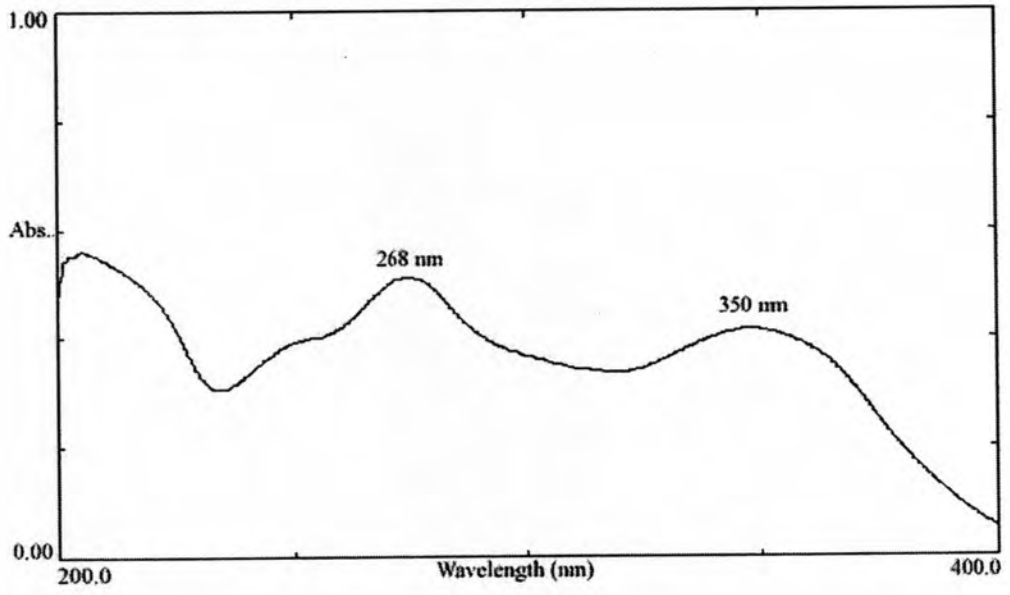


Figure 26 UV spectrum of doxycycline hyclate in phosphate buffer saline pH 6.8

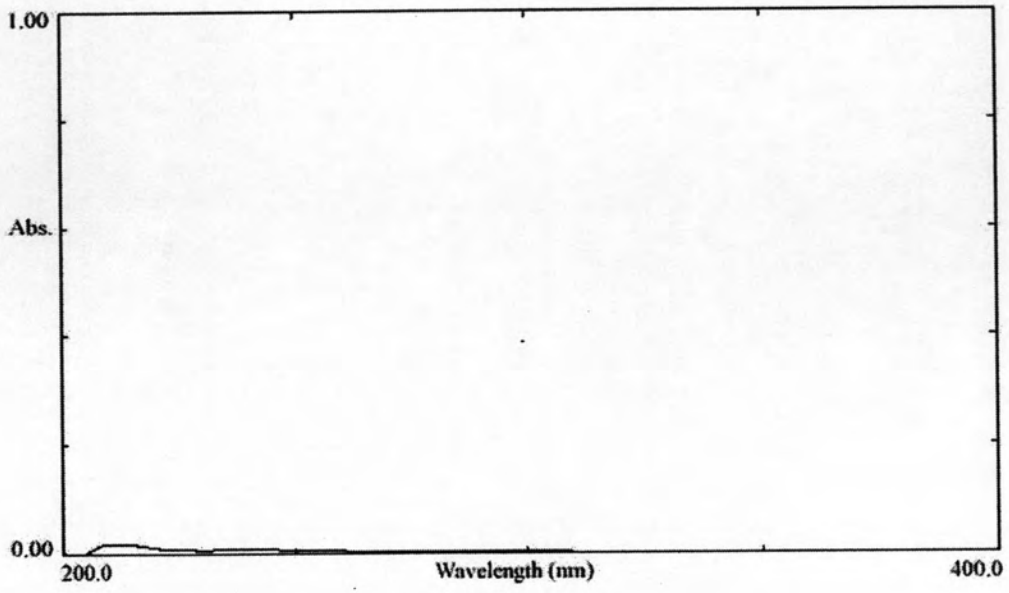


Figure 27 UV spectrum of blank microspheres in phosphate buffer saline pH 6.8

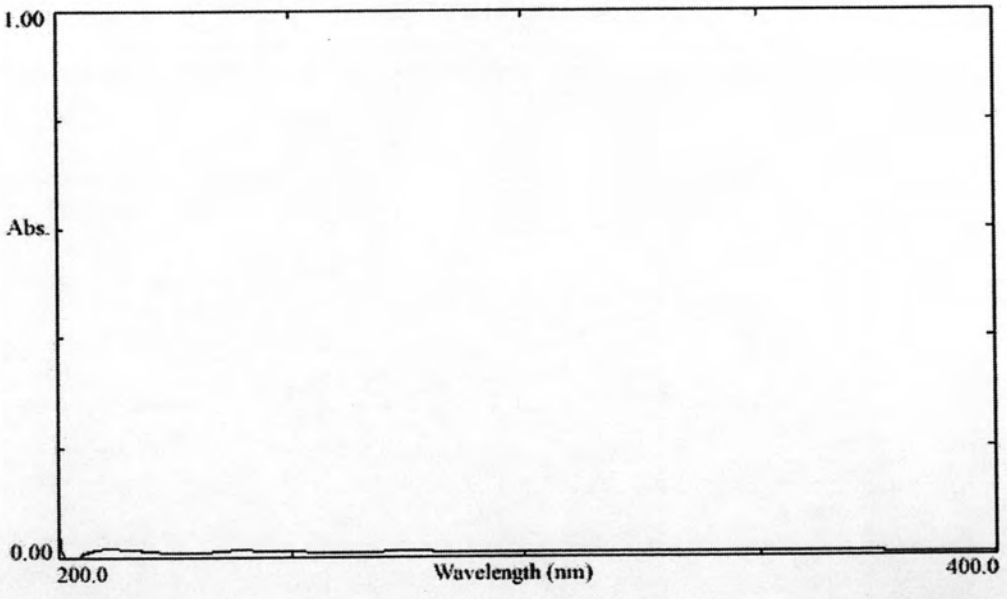


Figure 28 UV spectrum of the blank microspheres incorporated in glyceryl monooleate-based drug delivery system in phosphate buffer saline pH 6.8 from the receptor fluid at 120 hr.

1.1.2 Linearity

The calibration curve of doxycycline hyclate in phosphate buffer saline pH 6.8 is shown in Figure 29. Linear regression analysis of the absorbances versus the corresponding concentrations was performed and the coefficient of determination (R^2) was calculated as 1.0000. The calibration data were found to be linear with excellent coefficient of determination. These results indicated that UV spectrophotometric method was acceptable for quantitative analysis of doxycycline hyclate in phosphate buffer saline pH 6.8 in the range of studied.

Table 18 Data for calibration curve of doxycycline hyclate in phosphate buffer saline pH 6.8 by UV spectrophotometric method

Concentration (µg/ml)	Absorbance			Mean	SD	%CV
	Set1	Set2	Set3			
8	0.236	0.234	0.238	0.236	0.002	0.84
12	0.354	0.348	0.357	0.353	0.005	1.28
16	0.469	0.471	0.470	0.470	0.001	0.16
20	0.588	0.587	0.588	0.588	0.000	0.05
24	0.703	0.708	0.702	0.704	0.004	0.50
28	0.820	0.827	0.821	0.822	0.004	0.47
32	0.934	0.931	0.942	0.936	0.006	0.60
R^2	1.0000	0.9997	0.9999	1.0000	-	-

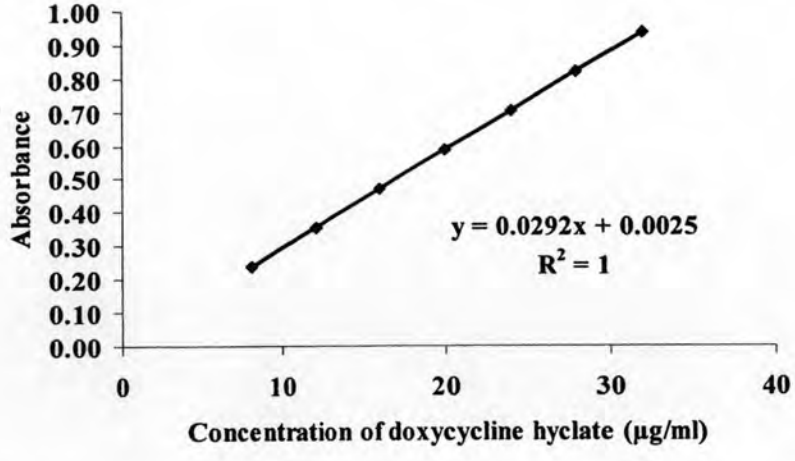


Figure 29 Calibration curve of doxycycline hyclate in phosphate buffer saline pH 6.8 by UV spectrophotometric method

1.1.3 Accuracy

Doxycycline hyclate solutions were prepared at the concentrations of 14, 22 and 30 μg/ml in five sets. Each individual sample was analyzed by UV spectrophotometer. The inversely estimated concentrations and percentages of analytical recovery of each drug concentration are shown in Table 19 and Table 20, respectively. All percentages of analytical recovery were in the range of 99.99-100.77%, which indicated the high accuracy of this method. Thus, it could be used for analysis of doxycycline hyclate in all concentrations studied.

Table 19 The inversely estimated concentrations of doxycycline hyclate in phosphate buffer saline pH 6.8 by UV spectrophotometric method

Concentration (μg/ml)	Inversely estimated concentration(μg/ml)					Mean±SD
	Set1	Set2	Set3	Set4	Set5	
14	14.0274	14.0205	13.9384	14.0651	13.9384	13.9979±0.06
22	22.0308	22.1644	22.0308	22.2260	21.9932	22.0890±0.10
30	30.3904	30.2260	30.2021	30.1747	30.1678	30.2322±0.09

Table 20 The percentage of analytical recovery of doxycycline hyclate in phosphate buffer saline pH 6.8 by UV spectrophotometric method

Concentration (μg/ml)	Percent analytical recovery					Mean±SD
	Set1	Set2	Set3	Set4	Set5	
14	100.20	100.15	99.56	100.46	99.56	99.99±0.41
22	100.14	100.75	100.14	101.03	99.97	100.40±0.46
30	101.30	100.75	100.67	100.58	100.56	100.77±0.30

1.1.4 Precision

The precision of doxycycline hyclate analyzed by UV spectrophotometric method were determined both within run precision and between run precision as illustrated in Tables 21 and 22, respectively. All coefficient of variation values were very low, as 0.25-1.06% and 1.71-1.87% respectively. The coefficient of variation of an analytical method should generally be less than 2%. Therefore, The UV spectrophotometric method was precise for quantitative analysis of doxycycline hyclate in phosphate buffer saline pH 6.8 in the range studied.

Table 21 Data of within run precision by UV spectrophotometric method

Concentration (μg/ml)	Absorbance					Mean	SD	%CV
	Set1	Set2	Set3	Set4	Set5			
14	0.434	0.441	0.433	0.429	0.431	0.433	0.005	1.06
22	0.681	0.681	0.680	0.680	0.669	0.678	0.005	0.74
30	0.931	0.931	0.932	0.931	0.926	0.930	0.002	0.25

Table 22 Data of between run precision by UV spectrophotometric method

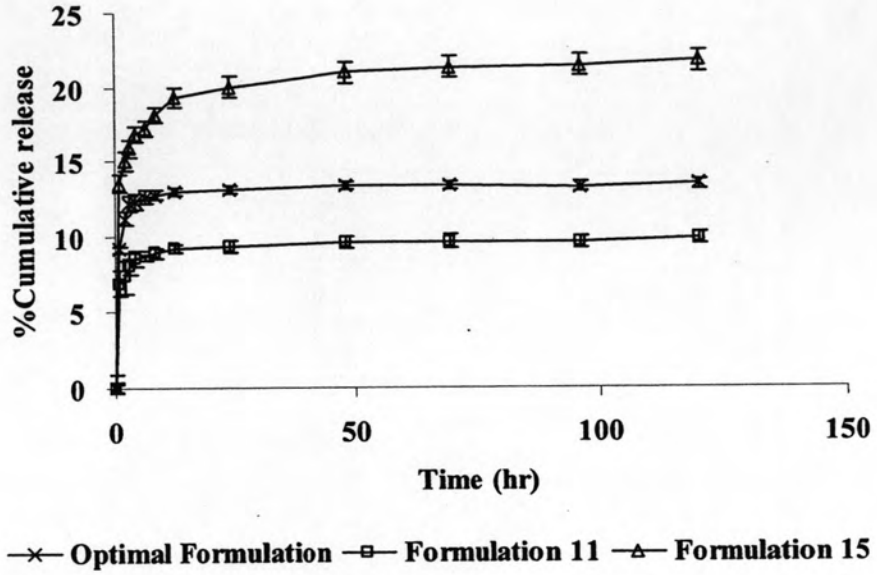
Concentration (μg/ml)	Absorbance					Mean	SD	%CV
	Set1	Set2	Set3	Set4	Set5			
14	0.413	0.429	0.422	0.429	0.416	0.422	0.007	1.71
22	0.652	0.680	0.656	0.672	0.659	0.664	0.012	1.81
30	0.884	0.931	0.903	0.910	0.902	0.906	0.017	1.87

In conclusion, The analysis of doxycycline hyclate in phosphate buffer saline pH 6.8 by UV spectrophotometric method developed in this study showed good specificity, linearity, accuracy and precision. Thus this method was used for the determination of the content of doxycycline hyclate in the *in vitro* drug release study both from doxycycline hyclate loaded chitosan microspheres and from the glyceryl monooleate-based drug delivery system containing doxycycline hyclate loaded chitosan microspheres (in the section D).

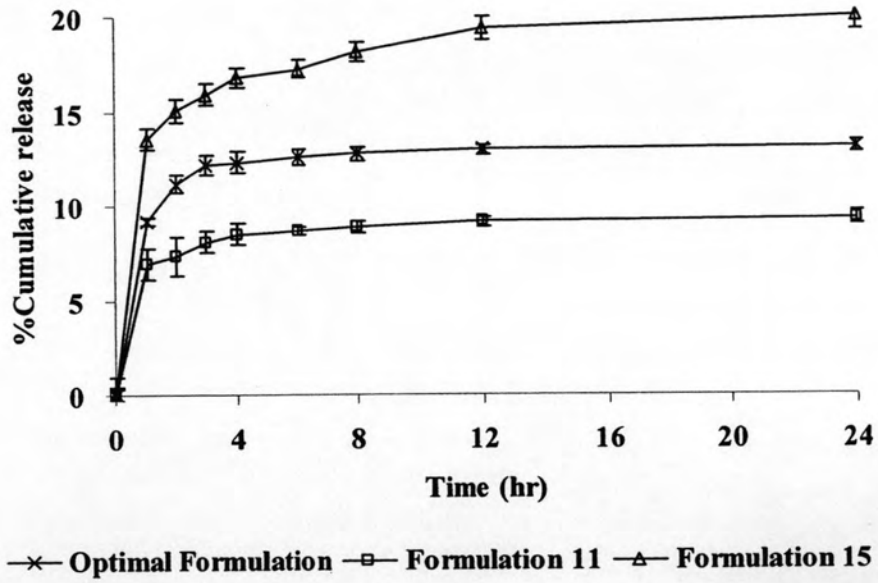
1.2 *In vitro* Drug Release Study

In vitro drug release methods were frequently used to gain information about the release profiles of the active ingredients in the microspheres. In this study, conical centrifuge tubes mounted on the shaking water bath that controlled the temperature at 37±0.5 °C were applied for the experiments. The amounts of drug release were analyzed using UV spectrophotometer.

From the previous section, three formulations (optimal formulation, formulation 11 and formulation 15) that possessed high loading efficiency and yield were selected to the in vitro release study. The release profiles plotted between the cumulative amounts of drug release versus time are shown in Figure 30. The release data are presented in Appendix B.



(a)



(b)

Figure 30 Release profiles of doxycycline hyclate from chitosan microspheres (a) over 120 hr (b) over 24 hr

From the data obtained in Figure 30 it was found that the incorporated drug was slowly released from the crosslinked microspheres over 24 hr. This finding was consistent with Shu and Zhu (2000). However, only 9.32-19.96 % of the drug was released from various chitosan microspheres formulations. This might be caused from the effect of pH of the dissolution medium. Since the properties of ionically crosslinked chitosan microspheres were influenced by the electrostatic interactions between tripolyphosphate and chitosan, therefore, it should exhibit pH-responsive properties. According to the previous study (Shu and Zhu, 2002) in a higher pH condition such as stimulated intestinal fluid (SIF) the microspheres remained in a shrinkage state and only 49% of drug was released in 24 hr. While in an acidic condition such as stimulated gastric fluid (SGF), part of the charge of tripolyphosphate was protonated and hence, electrostatic interaction weakened or disappeared, then microspheres swelled and dissociated, resulting the drug release up to 59% in 24 hr.

In addition, with increasing load of the drug in the microspheres matrix, there is an increase release, as shown by the data in Figure 30a at the interval time 120 hr, the optimal formulation and formulation 15 which contained drug load 75% w/w released the drug of 13.45% and 21.73% respectively. Whereas formulation 11 which contained 30% w/w drug load, released about 9.83%. This might be due to drug diffused from the matrix produces more pores and channels through which the release occurred at a faster rate (Jameela and Jayakrishnan., 1995, Filipović-Grčić et al., 1996). However, the optimal formulation which contained higher crosslinking agent concentration of 15% showed much lesser drug release than formulation 15 (10% crosslinking agent concentration). This might be the effect of the crosslinking agent concentration used. The more crosslink density, the stronger retard the release of drug from the microspheres. This finding was consistent with Jameela et al. (1998).

Several mathematical models have been used to describe the release of the drug. The release data obtained from this study were plotted according to the following models to describe the mechanism of drug release: zero-order kinetics, first-order kinetics (Figure 31), and Higuchi diffusion model (Figure 32) where the cumulative amount of drug release is directly proportional to square root of time.

Zero-order equation: $Q_t = Q_0 - k_0 t$

First-order equation: $\ln Q_t = Q_0 - k_1 t$

Higuchi's equation: $Q_t = Q_0 - k_H t^{1/2}$

where Q_t is the amount of drug release at time t, k_0 is the zero-order release rate constant, k_1 is the first-order release rate constant, and k_H is the diffusion rate constant.

The kinetic parameters for zero-order, first-order and Higuchi model during the initial 4 hr were calculated and are presented in Table 23. The release data of doxycycline hyclate from chitosan microspheres in all formulations tended to follow Higuchi model rather than the other two models, because the highest coefficients of determination (R^2) were obtained with the Higuchi model. This result presented that the mechanism of drug release would probably be through the dissolution of drug in microspheres and followed by diffusion-controlled release. Moreover, analysis of variance by regression showed a significant difference ($P<0.05$) in coefficients of determination of the Higuchi model, which indicated the correlation of % cumulative release and square root of time.

Analysis of variance indicated that the Higuchi release rate constant of all formulations were statistically significant different ($P<0.05$). The highest Higuchi release rate constant was obtained from the formulation 15 (75% w/w doxycycline hyclate, 4% w/w chitosan solution and 10 % w/v sodium tripolyphosphate), followed by the optimal formulation (75% w/w doxycycline hyclate, 3% w/w chitosan solution and 15 % w/v sodium tripolyphosphate) and formulation 11 (30% w/w doxycycline hyclate, 3% w/w chitosan solution and 10 % w/v sodium tripolyphosphate) respectively.

Table 23 Kinetic parameters of doxycycline hyclate from chitosan microspheres

Formulation	Zero-order plot		First-order plot		Higuchi plot	
	$k_o(\%h^{-1})$	R^2	$k_1(h^{-1})$	R^2	$k_H(\%h^{-1/2})$	R^2
Optimal formulation	2.7581	0.7138	0.0296	0.7243	6.3618	0.9285
11	1.8158	0.6684	0.0190	0.6746	4.2461	0.8934
15	3.5987	0.6691	0.0396	0.6824	8.4380	0.8985

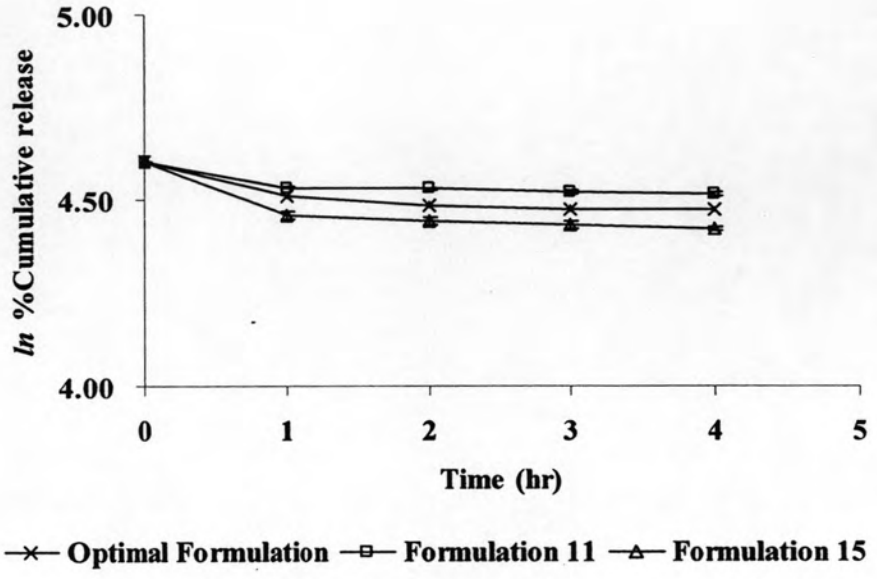


Figure 31 First-order plot of doxycycline hyclate from chitosan microspheres

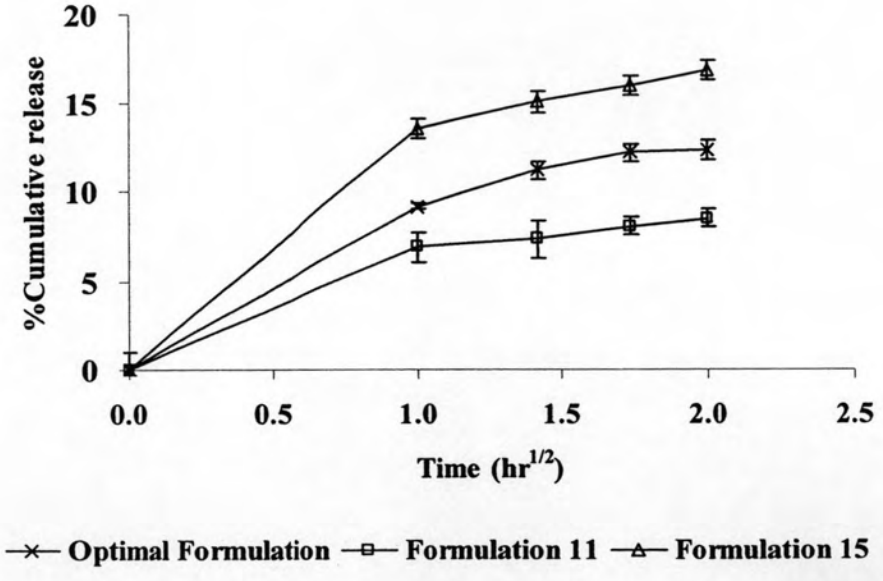


Figure 32 Higuchi plot of doxycycline hyclate from chitosan microspheres

2. Differential Scanning Calorimetric (DSC) Method

DSC is a thermal analysis method for detecting changes in physical or chemical properties of materials as a function of temperature. In this study, DSC analysis of the drug, drug-free and drug loaded microspheres were performed in order to determine the thermal change of chitosan and doxycycline hyclate before and after microspheres preparation. In Figure 33 shows the DSC thermograms obtained during the heating stage of doxycycline hyclate (a), drug free chitosan microspheres (b), doxycycline hyclate and chitosan microspheres physical mixture (c), drug load chitosan microspheres optimal optimal formulation (d), formulation 11 (e) and formulation 15 (f). The DSC thermograms of doxycycline hyclate showed an endothermic melting peak with an onset from 125 °C to reach a maximum peak at 172 °C and showed an exothermic peak at 225 °C which might be related to decomposition peak. This revealed that doxycycline hyclate was present in a crystalline state (Figure 33). From previous report, doxycycline hydrochloride exhibited melting peak at approximate 200 °C. The difference in melting point occurred might be caused of the different in salt form. Drug-free chitosan microspheres showed a large broad endotherm that related to evaporation of water. In chitosan the bound water molecules were associated with hydrophilic hydroxyl groups. The endothermic peak thermogram of chitosan microspheres showed at 95 °C. The endothermic peak of chitosan changed with related to the crosslinking of chitosan with STPP and thus to the capacity of water holding of crosslinked chitosan in the microspheres (Bhumkar and Pokharkar, 2006). As shown in Figure 33 D, E and F different formulations of drug loaded chitosan microspheres, which had different degrees of crosslinkages, demonstrated endothermic peak of water evaporation of different temperatures. It was observed that there was a small endothermic peak at 132.3, 137.5, 144.8 °C in the thermograms of drug loaded chitosan microspheres of optimal formulation, formulation 11 and formulation 15, respectively. These small peaks might be due to the melting of doxycycline hyclate in a crystalline state, but in a lesser degree of crystallinity than intact drug. It could be seen that formulation 11 had the smallest melting peak of doxycycline hyclate. The endothermic peak at temperatures over 240 °C of chitosan microspheres referred to the degradation.

The decrease of the doxycycline hyclate melting peak in drug loaded chitosan microspheres might indicate that some interaction between chitosan and doxycycline hyclate occurred. The DSC curve for physical mixture of chitosan microspheres and doxycycline hyclate (C) exhibited peak that combined between doxycycline hyclate and drug free chitosan microspheres.

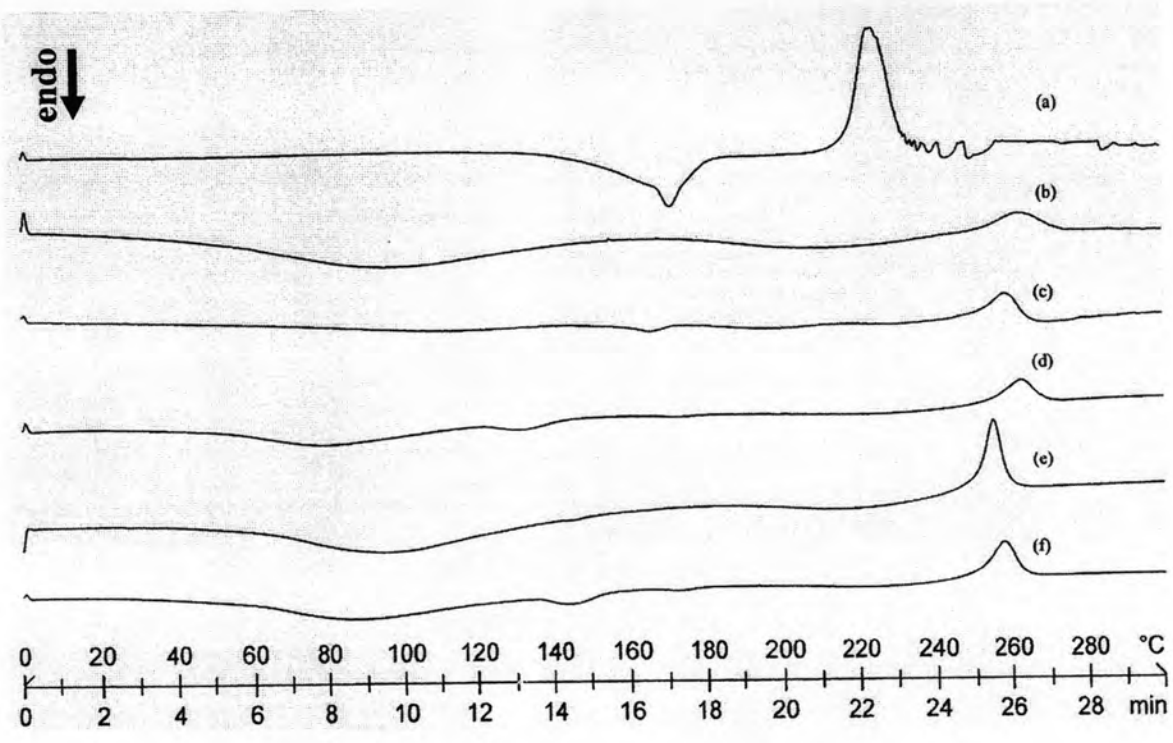


Figure 33 The DSC thermograms of doxycycline hyclate (a), drug free chitosan microspheres (b), doxycycline hyclate and chitosan microspheres physical mixture (c), drug load chitosan microspheres optimal formulation (d), formulation 11 (e) and formulation 15 (f)

3. Powder X-ray Diffractometry

Powder X-ray diffractometry may be used to detect crystallinity in the solid state. Figure 34 showed the X-ray diffractograms of doxycycline hyclate (a), doxycycline hyclate-chitosan microspheres physical mixture 1:1 (b), drug free chitosan microspheres (c), drug load chitosan microspheres formulation of optimal formulation (d), formulation 11 (f) and 15 (h) and drug load chitosan microspheres of optimal optimal formulation (e), formulation 11 (g) and 15 (i) after kept in 60°C for two weeks. Doxycycline hyclate gave a sharp and intense diffraction peaks due to its crystalline character at 11.03, 14.65 and 24.74°. This was contrast to the case of drug free chitosan microspheres that gave a diffuse diffraction pattern due to its amorphous character.

In the case of drug loaded chitosan microspheres of optimal formulation, formulation 11 and 15 before stability showed small intense diffraction peaks of doxycycline hyclate. The corresponding formulations of doxycycline hyclate loaded chitosan microspheres after stability showed larger intensities of diffraction peaks. This might be due to the crystallinity change of doxycycline hyclate or the decomposition of the drug after storage at high temperature. However, formulation 11 showed diffused diffraction pattern that revealed that doxycycline hyclate present in the microspheres in an amorphous state. After exposure to heat, a very small diffraction peak due to doxycycline hyclate crystallinity occurred.

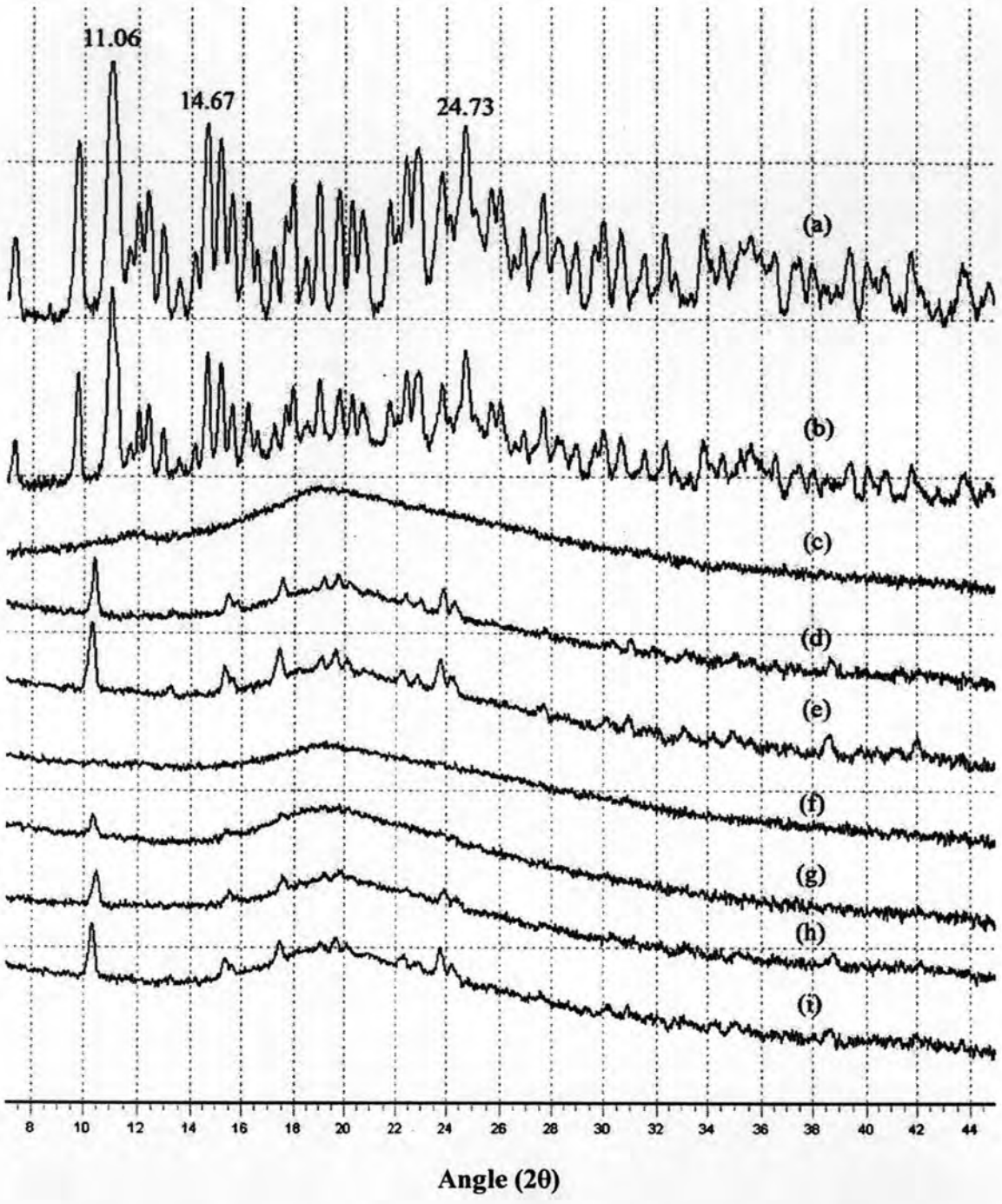


Figure 34 X-ray diffractogram of doxycycline hyclate (a), the physical mixture of doxycycline hyclate-chitosan microspheres (b), drug free chitosan microspheres (c), chitosan microspheres of optimal formulation, formulation 11 and 15 before (d, f ,h) and after (e, g, i) kept at 60° C for two weeks

4. Stability Studies of Doxycycline Hyclate Loaded Chitosan Microspheres

The doxycycline hyclate loaded chitosan microspheres optimal formulation, formulation, 11 and 15 which showed highest encapsulation efficiency were chosen for stability study. Accelerated stability testing are based on obtaining a more rapid rate of decomposition by applying to the product a storage condition that placed a higher stress when compared with normal storage conditions. In this study, the best formulation was the one that exhibited the least amount of decomposition in given time. Moreover, the results obtained from the accelerated test would enable to predict the amount of decomposition in the microspheres under normal conditions. The shelf-lives of the doxycycline hyclate loaded chitosan microspheres would be predicted (Alfonso, 1995).

4.1 Analysis of Doxycycline Hyclate by High Performance Liquid Chromatographic (HPLC) Method

In the stability study of doxycycline hyclate loaded chitosan microspheres, the HPLC method was performed due to high specificity. The validation of the analytical method is the process by which performance characteristics of the method are established to meet the requirements for the intended analytical applications. The performance characteristics were expressed in terms of analytical parameters. For HPLC assay validation, these included specificity, linearity, accuracy and precision.

4.1.1 Specificity

The specificity of an analytical method is its ability to measure the analyte accurately and specificity in the presence of other components in the sample. The internal standard technique was performed by determining the peak area ratio of doxycycline hyclate to prednisolone (internal standard) to give the complete separation, appropriate resolution and sharp peaks of all components. The acetronitrile-water and perchloric acid mixture with the ratio of 26: 74: 0.25 v/v was developed used as the mobile phase. The chromatograms of the extracted solution of blank chitosan microspheres, internal standard solution, doxycycline hyclate standard solution are shown in Figures 35-38. It was demonstrated that there was no interfering peak of the blank solution and internal standard to the peak of doxycycline hyclate. The chromatogram of doxycycline hyclate

showed peaks at the retention time of 4.55, 10.21 and 11.51 min. The peak at the retention time of 11.51 min was reported to refer to doxycycline, and used for the quantitative analyses of doxycycline. The other two peaks at 4.55 and 10.21 min were not clearly specified. However they were reported in Skúlason, Ingólfsson and Kristmundsdóttir (2003) as methacycline and 6-epidoxycycline, respectively.

Moreover, to ensure that the degraded product of doxycycline did not interfere with the intact drug, the 5 samples of doxycycline hyclate solution were prepared by adding 2-3 drops of water, hydrogen peroxide, 3N hydrochloric acid, 5N sodium hydroxide and dry powder. The samples were kept under storage temperature at 80°C for 3 hr and diluted with mobile phase (จุไรรัตน์ รักวาทิน, 2004). The solutions were examined for the chromatogram as shown in figures 39-43. It demonstrated the decrease of peak intensity of doxycycline and showed no other peak at this wavelength.

Figure 44 also shows the chromatogram of extracted solution of doxycycline hyclate chitosan microspheres optimal formulation after storage in incubator chamber temperature 70 °C for 6 weeks for stability study. There are no more other peaks occur to interfere the peak of doxycycline.

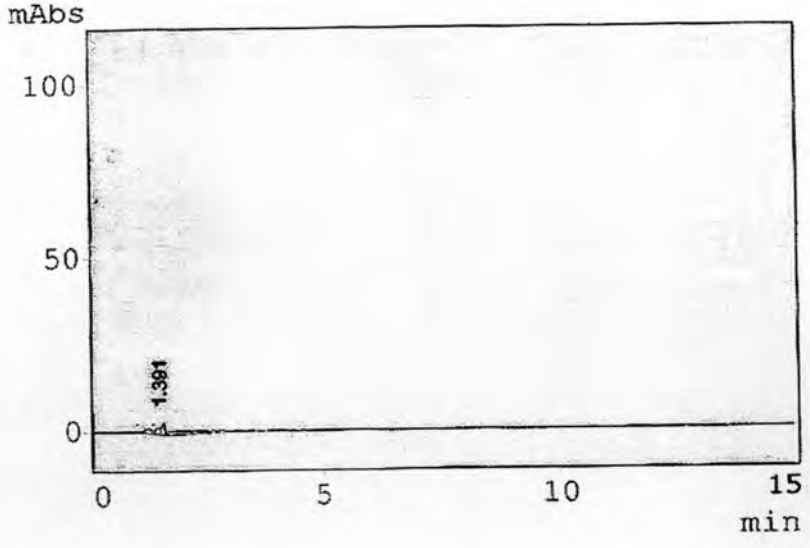


Figure 35 HPLC Chromatogram of extracted solution of blank chitosan microspheres

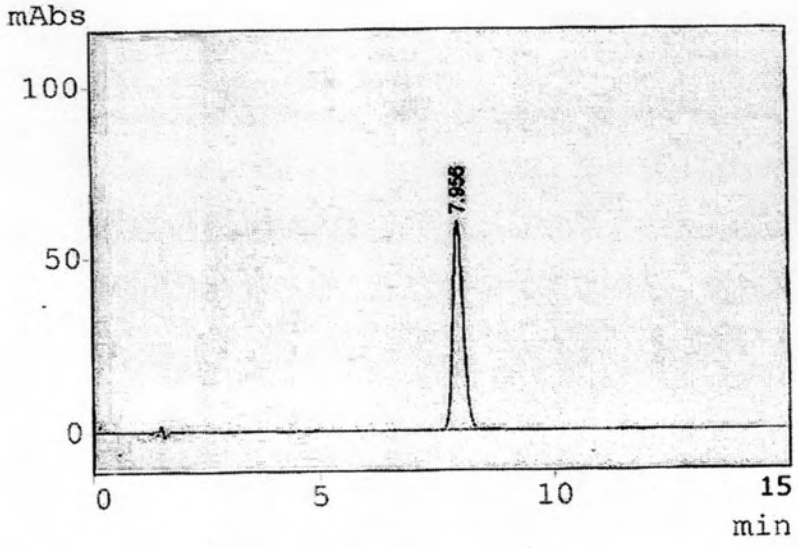


Figure 36 HPLC Chromatogram of internal standard solution (prednisolone)

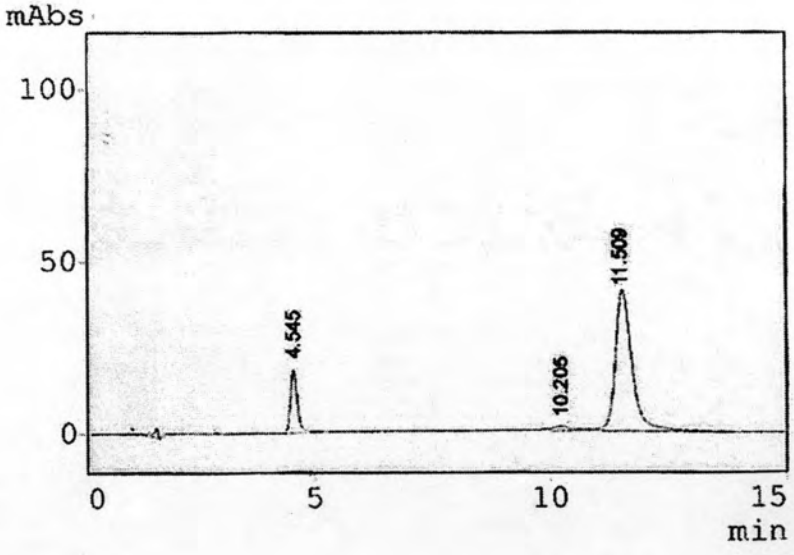


Figure 37 HPLC Chromatogram of doxycycline hyclate standard solution

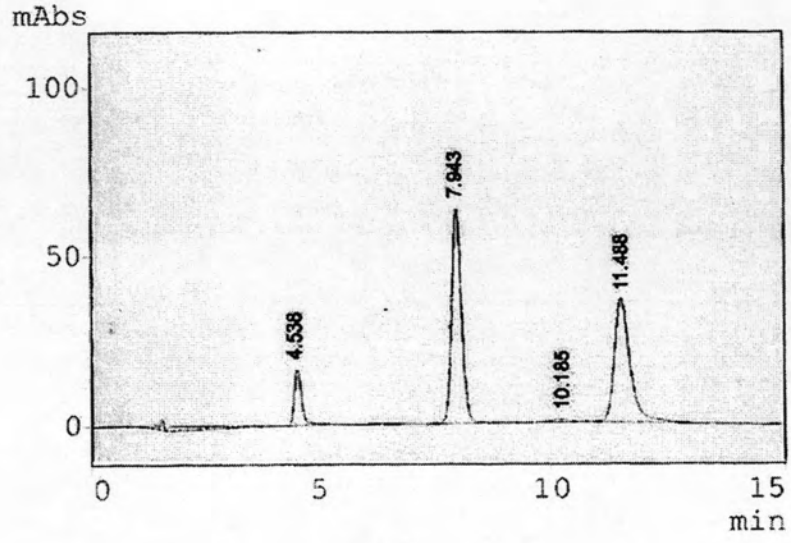


Figure 38 Chromatogram of mixture of doxycycline hyclate and internal standard

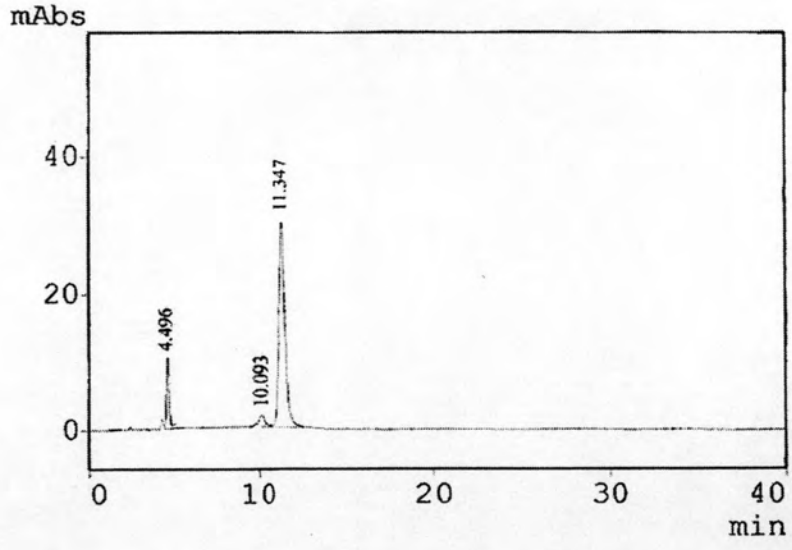


Figure 39 Chromatogram of doxycycline hyclate in water and storage at temperature 80 °C for 3 hr

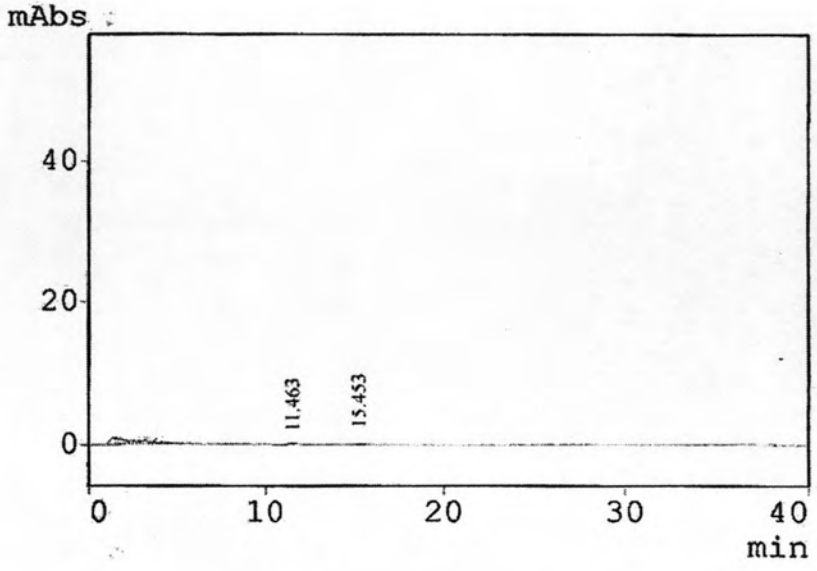


Figure 40 Chromatogram of doxycycline hyclate in hydrogen peroxide and storage at temperature 80 °C for 3 hr

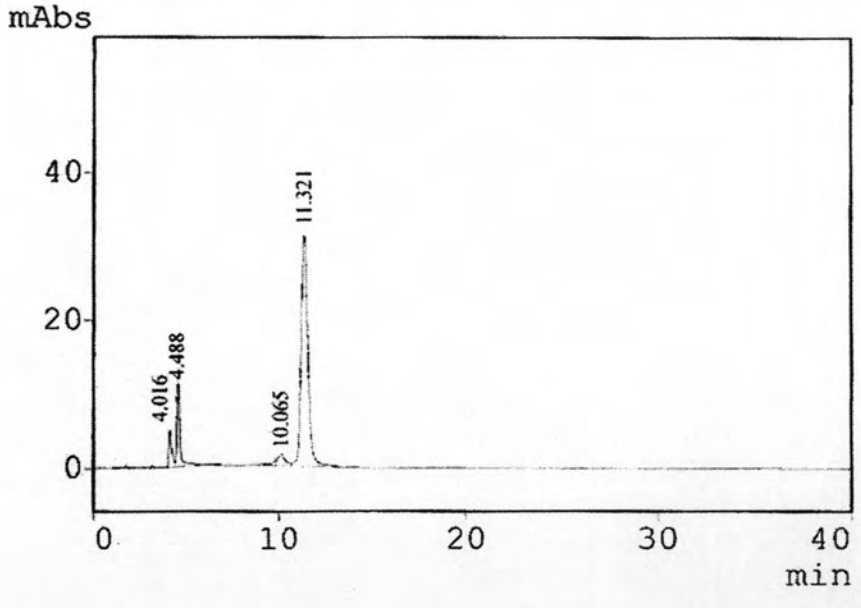


Figure 41 Chromatogram of doxycycline hyclate in 3N hydrochloric acid and storage at temperature 80 °C for 3 hr

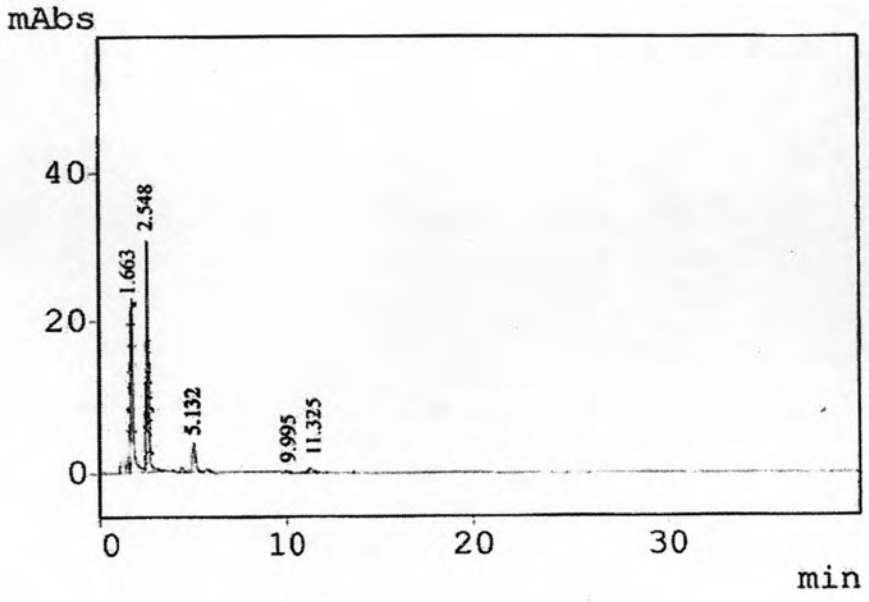


Figure 42 Chromatogram of doxycycline hyclate in 5N sodium hydroxide and storage at temperature 80 °C for 3 hr

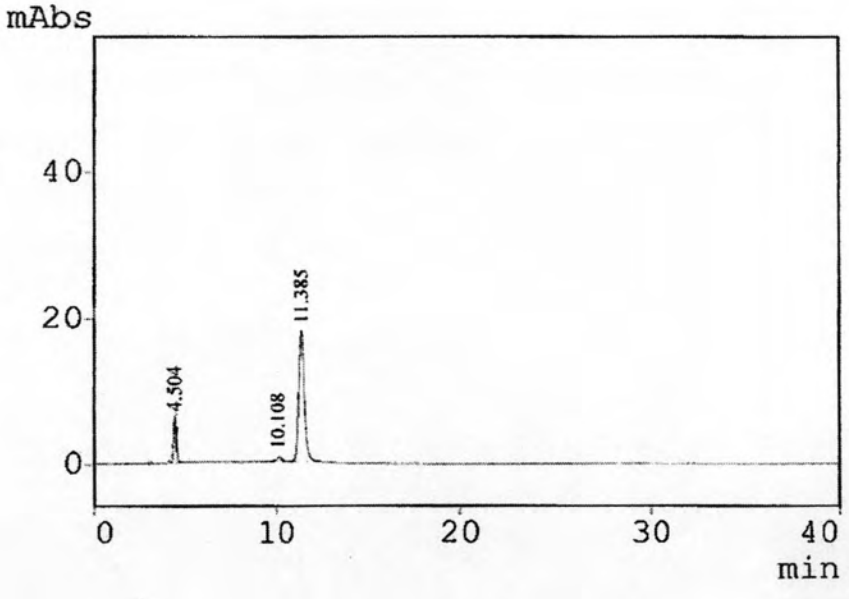


Figure 43 Chromatogram of doxycycline hyclate dry powder storage at temperature 80 °C for 3 hr

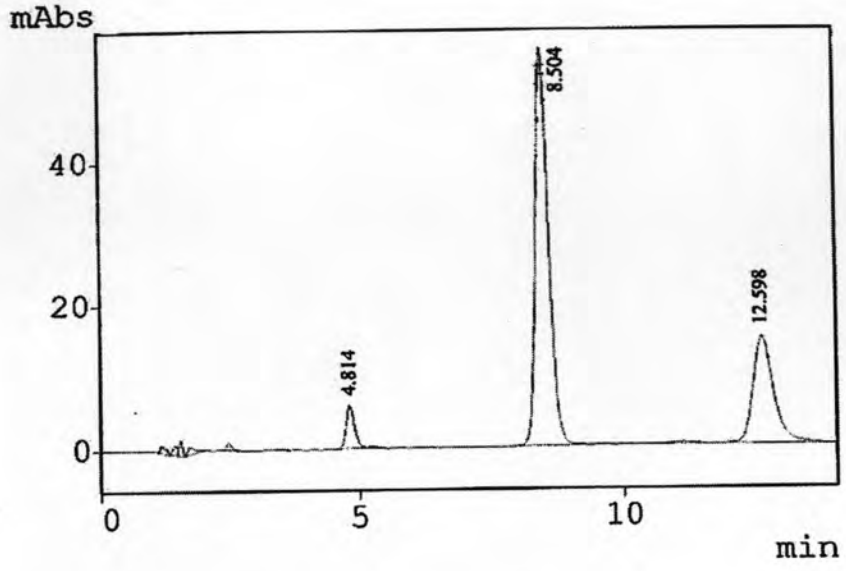


Figure 44 Chromatogram of doxycycline hyclate dry powder after storage at temperature 60 °C for 6 weeks

4.1.2 Linearity

The calibration curve data of doxycycline hyclate are shown in Table 24. The plot of doxycycline hyclate concentrations versus the peak area ratios of doxycycline and its internal standard (Figure 45) illustrated the linear correlation in the concentration range studied of 20-70 µg/ml. The coefficient of determination (R^2) of this line was 0.9997. These results indicated that HPLC method was acceptable for quantitative analysis of doxycycline hyclate in the range studied.

Table 24 Data for calibration curve of doxycycline hyclate by HPLC method

Concentration (µg/ml)	Peak area ratio			Mean	SD	%CV
	Set1	Set2	Set3			
20	0.346	0.344	0.352	0.347	0.004	1.22
30	0.522	0.535	0.525	0.527	0.007	1.28
40	0.694	0.708	0.711	0.704	0.009	1.27
50	0.882	0.898	0.893	0.891	0.008	0.89
60	1.081	1.070	1.070	1.074	0.006	0.60
70	1.270	1.245	1.245	1.254	0.015	1.16
R^2	0.9993	0.9997	0.9999	1.0000	-	-

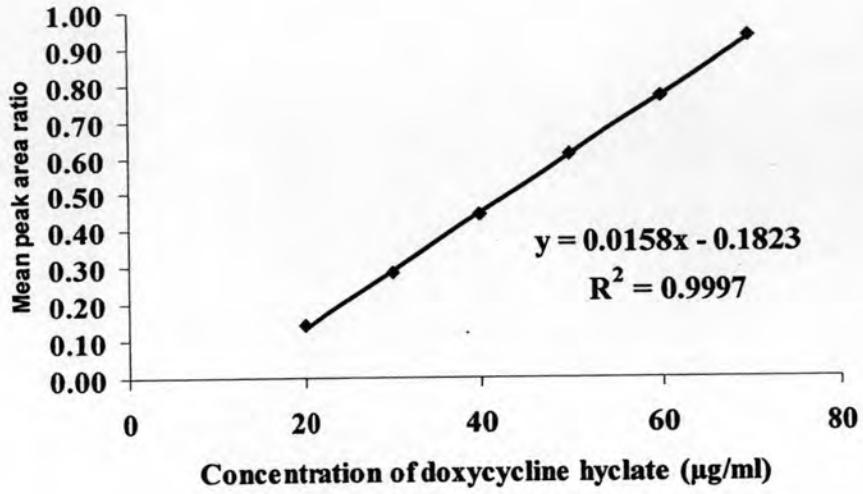


Figure 45 Calibration curve of doxycycline hyclate by HPLC method

4.1.3 Accuracy

Doxycycline hyclate solutions were prepared at the concentration of 35, 45 and 55 µg/ml in five sets. The inversely estimated concentrations and percentages of analytical recovery of each drug concentration are shown in Table 25 and Table 26, respectively. All percentages of analytical recovery were in the range of 99.21-100.03%, which indicated the high accuracy of this method. Thus, it could be used for analysis of doxycycline hyclate in all concentration studied with high accuracy.

Table 25 The inversely estimated concentrations of doxycycline hyclate by HPLC method

Concentration (µg/ml)	Inversely estimated concentration(µg/ml)					Mean±SD
	Set1	Set2	Set3	Set4	Set5	
35	34.6860	35.0711	34.8136	35.2139	35.2742	35.0117±0.25
45	44.5806	45.4684	44.9690	44.3465	44.3899	44.7509±0.47
55	54.4802	54.7833	54.1227	54.4802	54.9522	54.5637±0.32

Table 26 The percentage of analytical recovery of doxycycline hyclate HPLC method

Concentration (μg/ml)	Percent analytical recovery					Mean±SD
	Set1	Set2	Set3	Set4	Set5	
35	99.10	100.20	99.47	100.61	100.78	100.03±0.73
45	99.07	101.04	99.93	98.55	98.64	99.45±1.05
55	99.05	99.61	98.40	99.05	99.91	99.21±0.58

4.1.4 Precision

The precision of doxycycline hyclate analyzed by HPLC method were determined both within run precision and between run precision as illustrated in Tables 27 and 28. All coefficient of variation values were small, as 0.68-1.45% and 0.58-1.79% respectively. The coefficient of variation of an analytical method should generally be less than 2%. Therefore, The HPLC method was precise for quantitative analysis of doxycycline hyclate in the range studied.

Table 27 Data of within run precision by HPLC method

Concentration (μg/ml)	PAR					Mean	SD	%CV
	Set1	Set2	Set3	Set4	Set5			
35	0.635	0.634	0.638	0.617	0.640	0.633	0.009	1.45
45	0.810	0.816	0.809	0.805	0.818	0.812	0.006	0.68
55	1.004	0.998	1.028	0.997	1.020	1.009	0.014	1.38

Table 28 Data of between run precision by HPLC method

Concentration (μg/ml)	PAR					Mean	SD	%CV
	Set1	Set2	Set3	Set4	Set5			
35	0.611	0.617	0.611	0.601	0.631	0.614	0.011	1.79
45	0.783	0.756	0.783	0.764	0.757	0.768	0.013	1.74
55	0.942	0.938	0.942	0.937	0.951	0.942	0.006	0.58

In conclusion, the analysis of doxycycline hyclate by HPLC method developed in this study showed good specificity, linearity, accuracy and precision. Thus this method was used for the determination of the content of doxycycline hyclate in the stability study.

4.2 Accelerated Stability Studies

The stability study of doxycycline hyclate loaded chitosan microsphres were determined by accelerated stability testing method. The selected formulations were incubated at 40, 50, 60 and 70±1°C. The first sample taken at time zero was referred to 100 percent of initial concentration. The concentrations of doxycycline hyclate remaining in subsequent samples were calculated as percentage of initial concentration. The percent of doxycycline hyclate remaining after incubation at different temperatures are shown in Figures 46-48. The natural logarithmic plot of the results are also demonstrated (Figures 49-51)

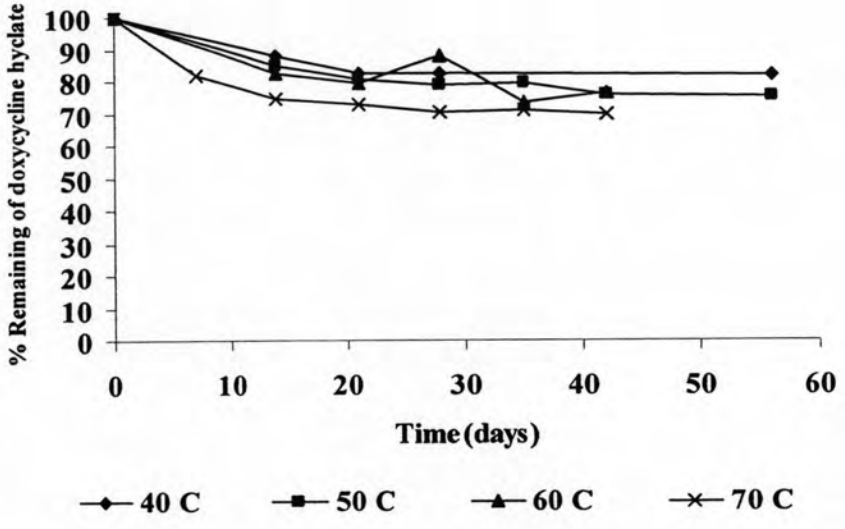


Figure 46 Percents remaining of doxycycline hyclate in chitosan microspheres optimal formulation

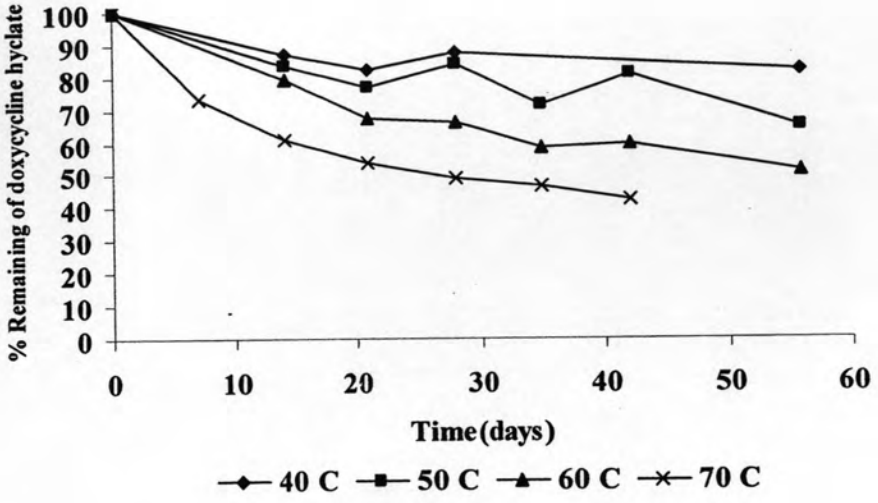


Figure 47 Percents remaining of doxycycline hyclate in chitosan microspheres formulation 11

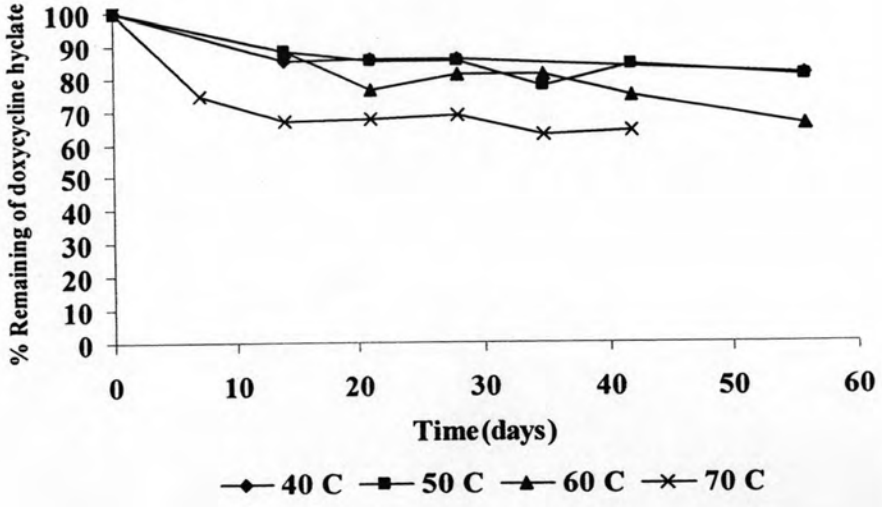


Figure 48 Percents remaining of doxycycline hyclate in chitosan microspheres formulation 15

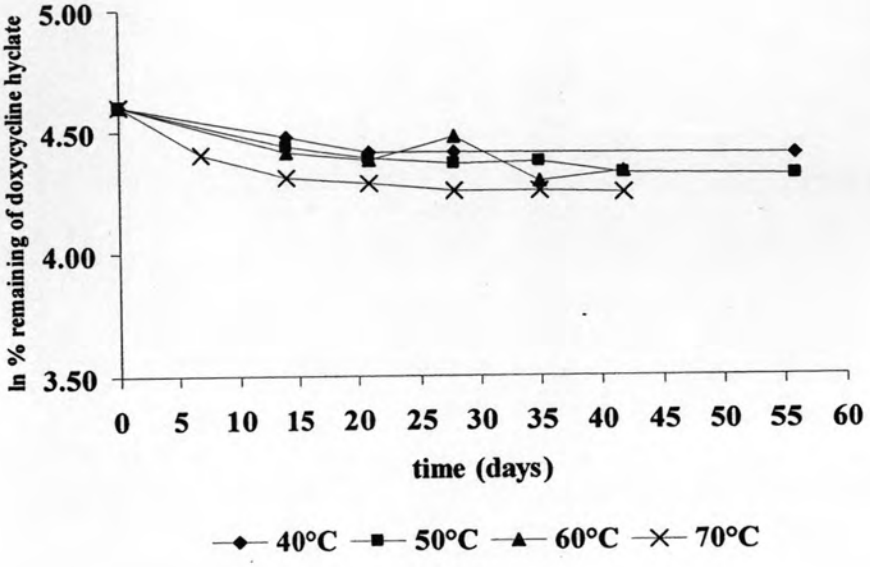


Figure 49 The natural logarithmic plot of percents remaining of doxycycline hyclate in chitosan microspheres optimal formulation

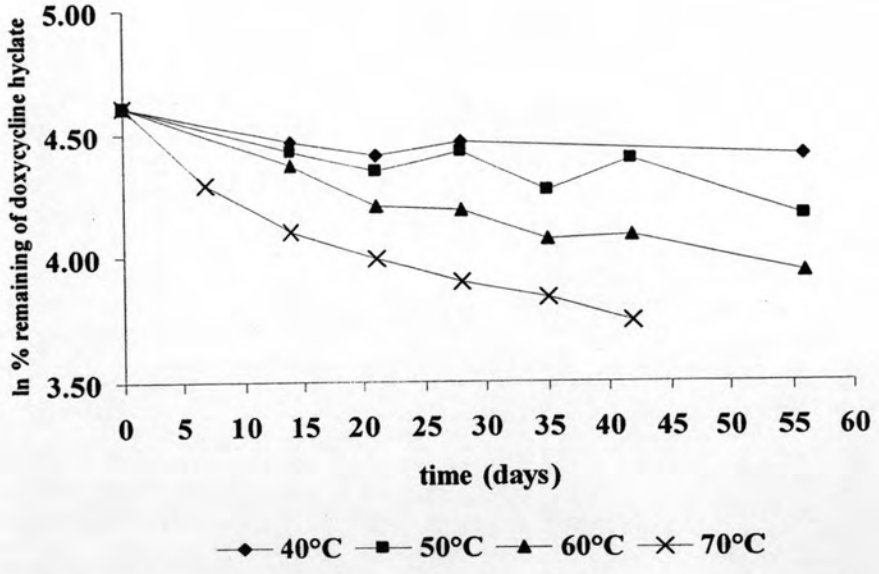


Figure 50 The natural logarithmic plot of percents remaining of doxycycline hyclate in chitosan microspheres formulation 11

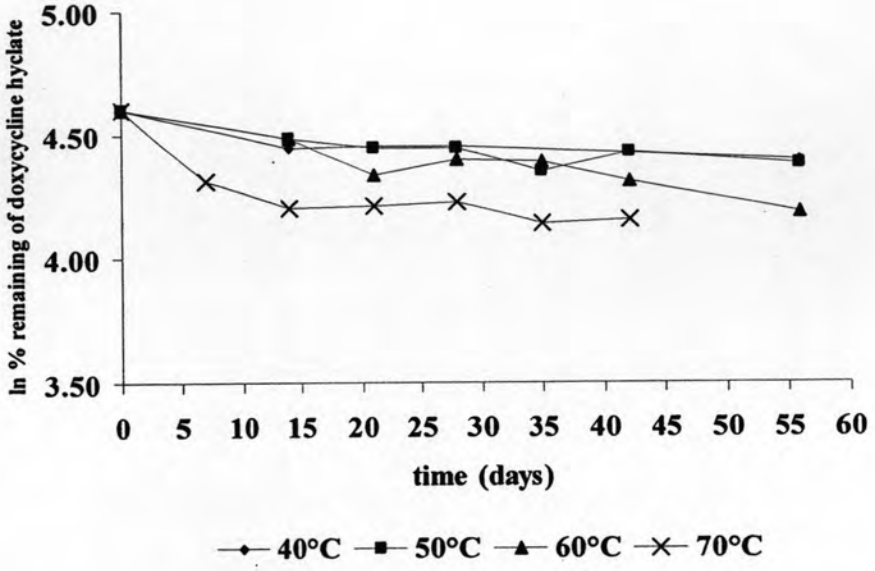


Figure 51 The natural logarithmic plot of percents remaining of doxycycline hyclate in chitosan microspheres formulation 15

The correlation coefficients between the concentration-time and log concentration-time of the selected formulas are shown in Table 29. After simulating the data for zero and first order kinetics, most of the correlation coefficients obtained from the first order kinetic were higher than those obtained from the zero order kinetic. Therefore, it could be concluded that the kinetic reaction of doxycycline hyclate loaded chitosan microspheres were first order kinetic. The result obtained was consistent with that reported by Dihuidi et al. (1982). This meant that the degradation proceeded dependent on the concentration of the drug present.

Table 29 The degradation rate constants and correlation coefficient (r) obtained from the concentration vs time profiles (zero-order reaction) and log concentration vs time profiles (first-order reaction) of optimal formulation, formulation 11 and 15 at various temperature.

Formulation No	Temp(°C)	Zero order[a]		First order[a]	
		K_0 (% day^{-1})	r	K_1 (day^{-1})	R
Optimal formulation	40	-0.2919	0.7823	-0.0014	0.7889
	50	-0.4075	0.8805	-0.0021	0.8966
	60	-0.5185	0.8189	-0.0026	0.8186
	70	-0.5969	0.8300	-0.0032	0.8502
11	40	-0.2621	0.7407	-0.0013	0.7466
	50	-0.5163	0.8594	-0.0028	0.8622
	60	-0.9789	0.9492	-0.0056	0.9619
	70	-1.2130	0.9152	-0.0082	0.9573
15	40	-0.2794	0.8053	-0.0013	0.8131
	50	-0.3250	0.8317	-0.0016	0.8294
	60	-0.5436	0.8796	-0.0027	0.8776
	70	-0.6625	0.7779	-0.0037	0.8045

a = mean $\pm$ SD, n = 3

Like all tetracyclines, doxycycline undergoes epimerization at carbon 4 in solutions between pH 4 and 8, and these isomers are called 4-epidoxycycline. Under acidic conditions, few of the epidoxycyclines are present, but at neutral pH they may be present with an amount to 90% of the mixture. The epidoxycyclines have about 5 % of the antimicrobial activity of doxycycline and are undesirable components of product (Foye, Lemke and Williams, 1995). From the chromatograms of the sample solutions prepared from doxycycline hyclate loaded chitosan microspheres after accelerated test, the peak refered to the 4-epidoxycycline could not be observed. Additionally, the small peak refered to 6-epidoxycycline was detected (Skúlason, Ingólfsson and Kristmundsdóttir, 2003). However, this peak did not increased while the doxycycline degraded. This might be meant that in this study, the degraded product of doxycycline was not mainly 6-epidoxycycline.

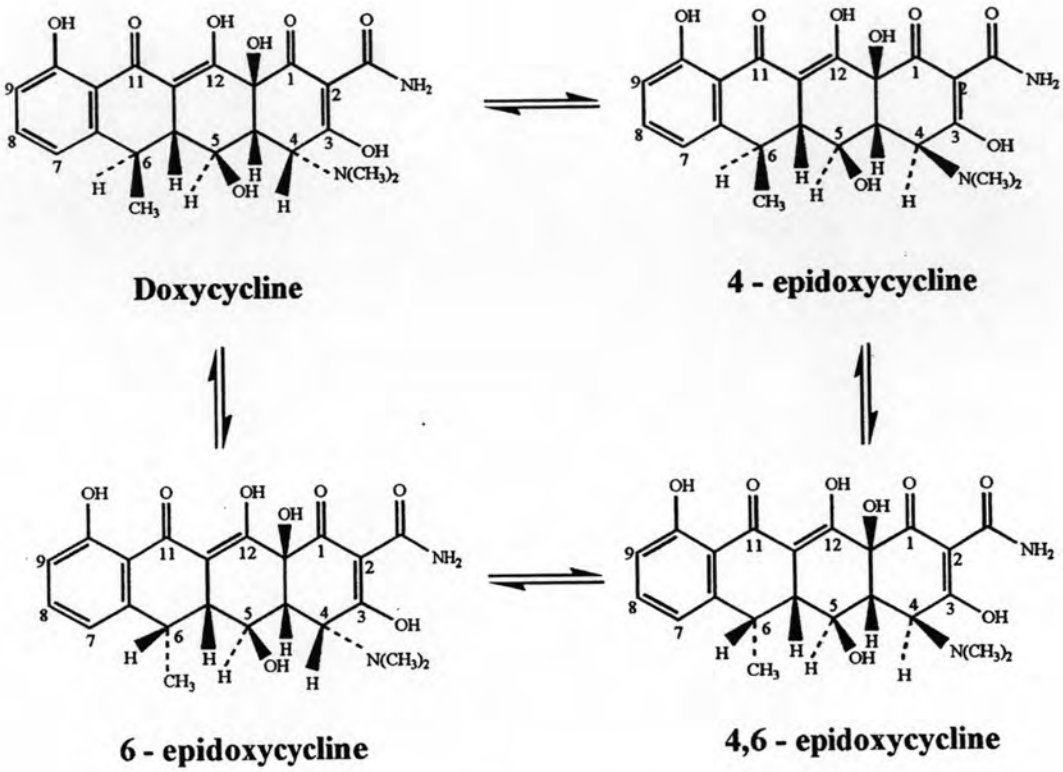


Figure 52 Schematic of epimerization of doxycycline

In this study, the specific rate constants of doxycycline hyclate in chitosan microsphers optimal formulation, formulation 11 and 15 at temperatures 40, 50, 60 and 70 °C were plotted according to Arrhenius relationship. The Arrhenius plot of natural logarithm of specific rate constants (ln k) versus the corresponding reciprocal of degree kelvin (1/T) were shown in Figures 53-55 and possible prediction of degradation rate at lower temperature could be obtained by extrapolation.

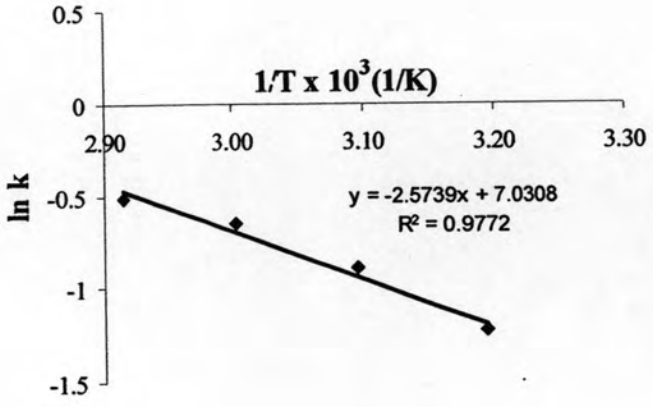


Figure 53 The Arrhenius plot of natural logarithm of specific rate constants (ln k) versus the corresponding reciprocal of degree kelvin (1/T) of doxycycline hyclate loaded chitosan microspheres optimal formulation

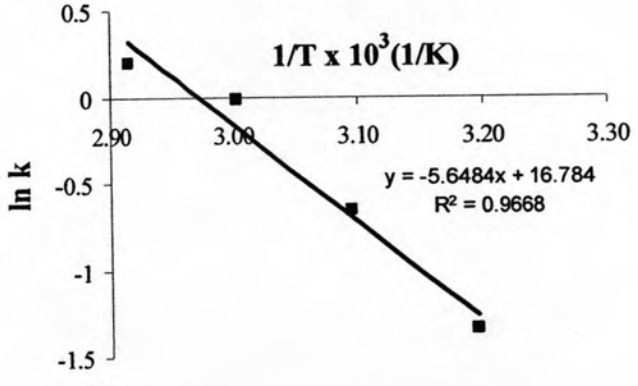


Figure 54 The Arrhenius plot of natural logarithm of specific rate constants (ln k) versus the corresponding reciprocal of degree kelvin (1/T) of doxycycline hyclate loaded chitosan microspheres formulation 11

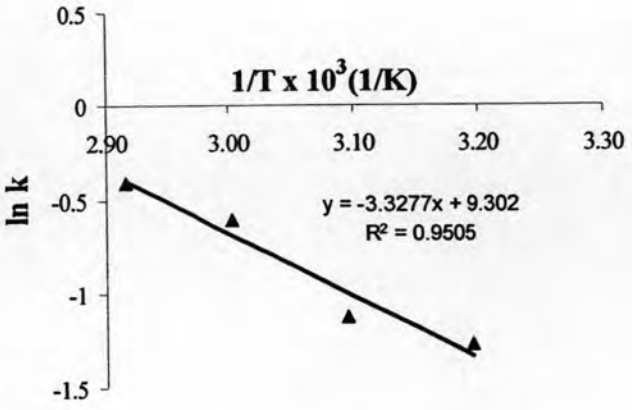


Figure 55 The Arrhenius plot of natural logarithm of specific rate constants (ln k) versus the corresponding reciprocal of degree kelvin (1/T) of doxycycline hyclate loaded chitosan microspheres formulation 15

Arrhenius equation and kinetic parameters of the degradation of doxycycline hyclate were calculated by linear regression analysis and summarized in Table 30. From the slopes of Arrhenius equation, the activation energy (Ea) values of doxycycline hyclate loaded chitosan microspheres optimal formulation, formulation 11 and 15 were calculated as 5.11, 11.22 and 6.61 kcal/mol, respectively. Theoretically, before the degradation reaction occurred, the free energy of the system must overcome the activation energy, Ea. Thus the lower Ea revealed the faster rate of reaction. The apparent activation energies or heats of activation obtained in this study were rather lower than the usual range of the Ea about 10 to 30 kcal/mol. In this study, As shown in Table 30, formulation 11 exhibited the highest Ea of 11.22 kcal/mole, that it needed the highest amount of energy needed to start the reaction. Thus, formulation 11 seemed to be the most stable formulation. This might be due to the effect of drug: polymer ratio (Table 31). Because the polymer could play an important role to protect the drug in the microspheres, hence the more polymer part, the drug would more stable.

Table 30 Arrhenius equation and kinetic parameters of the degradation of doxycycline hyclate loaded chitosan microspheres optimal formulation, formulation 11 and 15

	Formulation		
	Optimal formulation	11	15
Arrhenius equation	ln k=-2.5739(1/T) + 7.0308	ln k=-5.6484(1/T) + 16.784	ln k=-3.3277(1/T) + 9.302
Coefficient of determination (R^2)	0.9772	0.9688	0.9505
Activation Energy, Ea(kcal/mol)	5.11	11.22	6.61
K extrapolate at 30°C(%day^{-1})	2.23×10^{-3}	4.73×10^{-6}	0.43×10^{-3}
T 90%(day) First order	47.04	22208.38	245.65

The predicted degradation rate constants were obtained from extrapolation of the Arrhenius plot to room temperature at 30°C (1/T = 3.3003×10^{-3} K^{-1}) as shown in a typical plot in Figures 53-55. The predicted degradation rates at 30°C of three formulations are also shown in Table 30. Similarly, formulation 11 showed the lowest degradation rate constant, followed by formulations 8 and 15, respectively.

Table 31 Drug: Polymer ratio of the selected formulations

Formulation	Drug (%w/w)	Chitosan Concentration(%w/w)	Drug: polymer ratio
Optimal formulation	75	3	1 : 1.3
11	30	3	1 : 3.3
15	75	4	1 : 1.3

4.3 Shelf-life

Shelf-life of doxycycline hyclate in chitosan microspheres was the time required for doxycycline hyclate degraded from 100% concentration to reach 90%. As the degradation of doxycycline hyclate was previously indicated to be a first order kinetic. Therefore, the shelf-life (t_{90}) of doxycycline hyclate in chitosan microspheres at ambient temperature could be calculated from the first order reaction rate constant (equation 8).

$$t_{90} = 0.105/k_1 \qquad (8)$$

In this study, shelf lives (t_{90}) of optimal formulation, formulation 11 and 15 were calculated by using predicted rate constants in Table 30. The predicted shelf-lives calculated from Arrhenius plot were 47.04 days, 22208.38 days (61 years) and 245.65 days, respectively as demonstrated in Table 30. Formulation 11 could be indicated that it was very stable.

D. Monoglyceride-Based Drug Delivery System

1. Preparation of Monoglyceride-Based Drug Delivery System

1.1 Ternary Phase diagram

The monoglyceride-based drug delivery systems were developed by the basis properties of glyceryl monooleate (GMO) or monoolein to form liquid crystals upon contact with water. A triglyceride with a low melting point (sesame oil) was added to improve the flow characteristics of the composition upon release from the dosing device and induced the formation of a reversed hexagonal liquid crystalline phase upon contact with an aqueous fluid. Generally, without addition of triglyceride to GMO, the liquid crystal structure of the gel would be cubic instead of reverse hexagonal.

In this study the partial ternary phase diagram of GMO: sesame oil: water was constructed. The data of ternary system of varied proportions between the components were collected in Appendix C. The partial ternary phase diagram is displayed in Figure 56. The reason of ternary diagram was constructed partially because the outer area could not give the 1-phase liquid crystals. The phase behavior of these systems was similar to the system in the previous studies (Norling et al., 1992 and Tan, 2004). However, the precise locations of the phase boundaries in the diagram had to be investigated and found differed slightly from the previous studies.

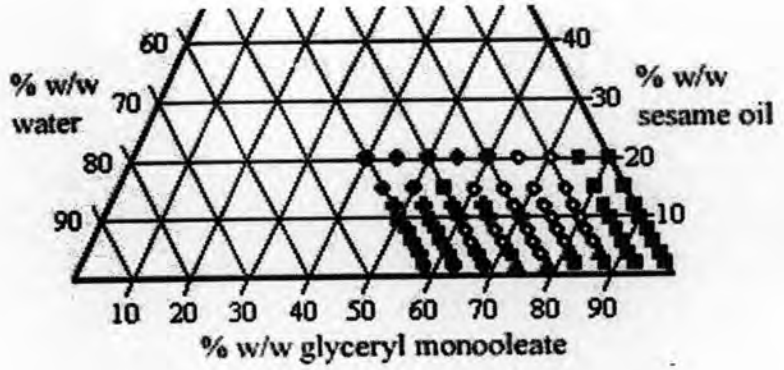


Figure 56 Ternary phase diagram of glyceryl monooleate-sesame oil-water system;(▲) lamellar phase (L_α); (✪)reverse hexagonal phase (H_{II}); (✚) reverse hexagonal phase(H_{II}) + aqueous; (■) isotropic, 1-phase; and (●) isotropic, 2-phase

1.2 Polarized Light Microscopy

The formation and structure of liquid crystals obtained from glyceryl monoleate-base drug delivery system were identified under polarized light microscopy. The structure of reverse hexagonal and lamellar looked radiant when viewed between crossed polarizers. The lamellar phase (L_α) had a pattern of oily streak and maltese crosses; while angular fan-like textures were observed for the reversed hexagonal phase as can be seen in Figures 57 and 58, respectively. The cubic phase was identified as being very viscous gel and isotropic when examined under polarizing microscope. These observations were in agreement with Komsri (1997) and Sallam et al. (2002).

From the data obtained it was found that liquid crystals formed with triglyceride content not more than 4% had liquid crystal structures conformable to a lamellar structure. When increasing the content of triglyceride from 6% to 20%, liquid crystal structures were changed to close similar to a reversed hexagonal phase. This finding was consistent with the previous study by Tan (2004). From the results, the formation and structure of liquid crystalline phase were demonstrated to be dependent on the ratio of the components in the phase diagram. Regarding the data of ternary phase diagram (Appendix C) and the physical appearances of the systems, formulations 22, 31, 39 and 48 were selected to the further study. Moreover, these formulations of reversed hexagonal liquid crystals contained different amounts of sesame oil of 6, 8, 10 and 12 % w/w, respectively.

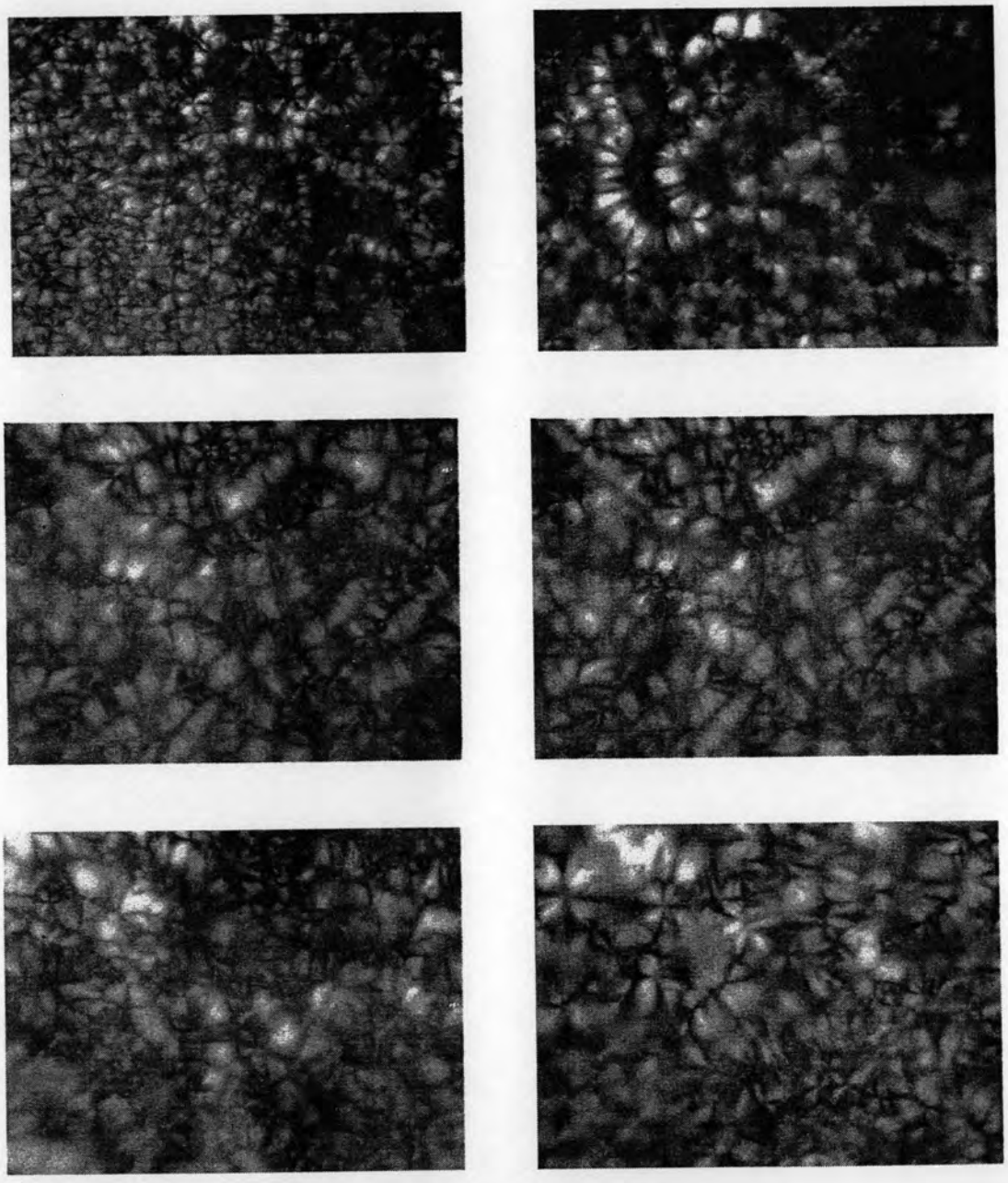


Figure 57 Polarizing microscopic images of the lamellar phases, as observed at x 40 magnification

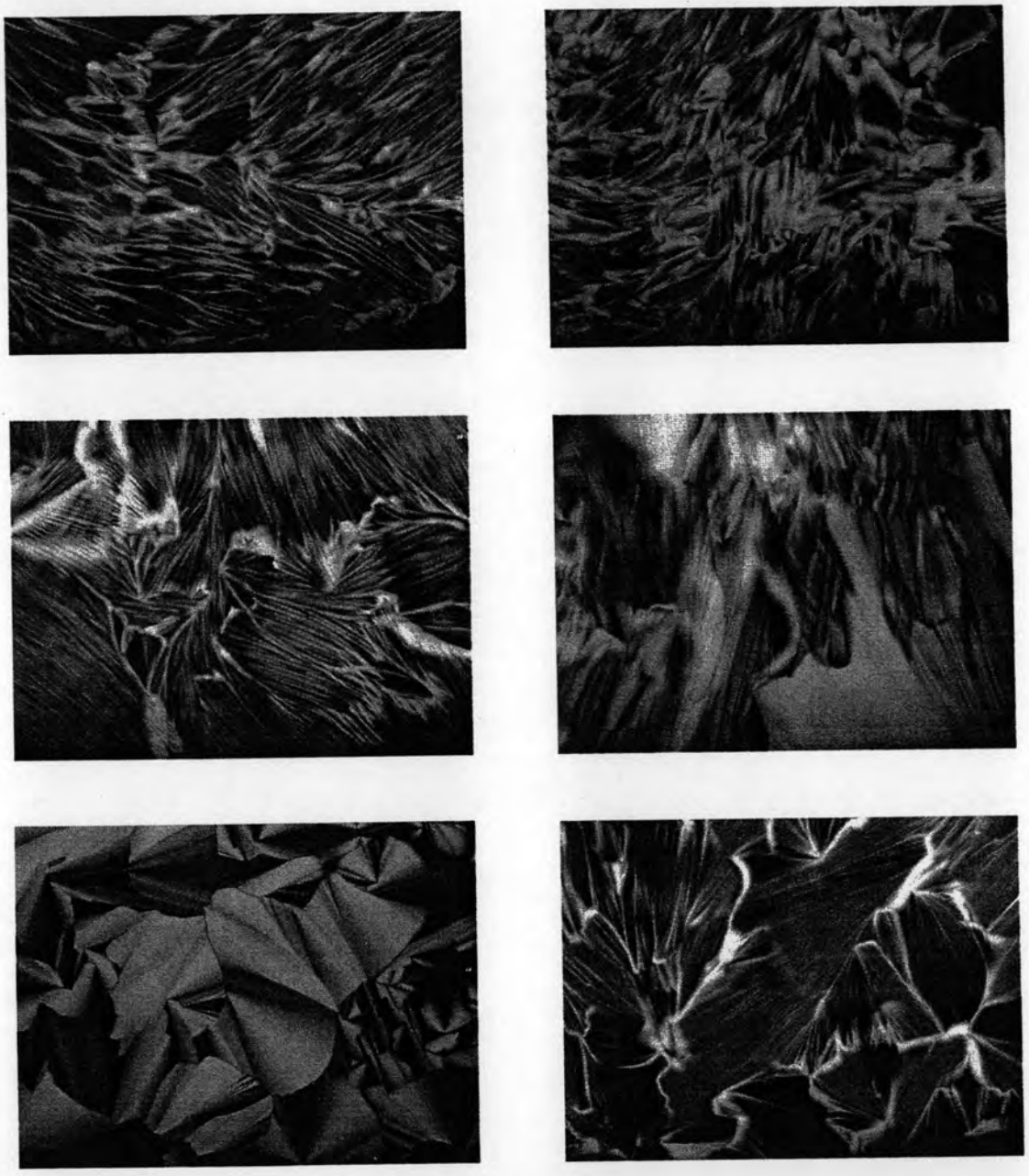


Figure 58 Polarizing microscopic images of the reversed hexagonal phases, as observed at x 40 magnification

1.3 *In vitro* Liquid Crystalline Phase Formation Study

The *in situ* transition from a low-viscous state to the required high-viscous liquid crystalline phase after administration was important to the use of liquid crystalline phase for drug delivery. The triggering parameter used in glyceryl monooleate to form liquid crystalline phases was water (Ganem-Quitanar, Quintarnar-Guerrero and Buri, 2000).

In the current study, low viscous state of glyceryl monooleate was added into excessed amount of water, the liquid crystalline phase was detected by polarized light microscopy. As the result shown in Table 32, all formulations were transformed to the high-viscous liquid crystalline phase. The changes were found within 10 min after addition of water. The *in vitro* study showed that the low-viscous state formulation could be transformed to high-viscous liquid crystalline states upon dilution with water. Therefore it was possible to form this state upon *in vivo* application. From the results obtained, formulation 22 was selected as a glyceryl monooleate-based drug delivery system due to its ability to form liquid crystal at the shortest setting time.

Table 32 Liquid crystals setting time of the selected formulation

Formulation	Sesame oil	Water	Glyceryl monooleate	Setting Time(min^{-1})
22	6	15	79	0:31
31	8	15	77	2:56
39	10	10	80	3:14
48	12	10	78	8:22

2. Preparation and Characterization of Monoglyceride-Based Drug Delivery System Containing Doxycycline Hyclate Loaded Chitosan Microspheres

From the ternary phase diagram, it demonstrated that liquid crystals as in lamellar phase and reverse hexagonal phase as examined under the polarized light microscope could be obtained. The glyceryl monooleate-based drug delivery systems were constructed by formulation 22 at the ratio of 6:15:79 (triglyceride: monoglyceride: water) because of the ability to formed liquid crystal at the most rapid time upon contact with water when compared to other formulations. The doxycycline hyclate loaded

chitosan microspheres 10 % was incorporated into the glyceryl monooleate-based drug delivery system, in order to determined the in vitro release and antimicrobial activity.

2.1 Determination of physicochemical properties

2.1.1 Physical Appearances

The physical appearances of the selected formulation liquid crystals such as color, clarity and phase separation were observed. The color of monoglyceride-based drug delivery systems was light yellow, according to the color of glyceryl monooleate. After incorporation of doxycycline hyclate microspheres, the color of formulations changed to dark yellow due to the color of chitosan microspheres.

2.1.2 Viscosity Measurement

The viscosity values of monoglyceride-based drug delivery systems before and after incorporation of chitosan microspheres were in the range of 315.19-671.74 cps (Table 33). The viscosity of formulations with chitosan microspheres was slightly higher than the formulation without chitosan microspheres. Analysis of data indicated that there was statistically significant difference in all formulations ($P<0.05$).

Table 33 Viscosities of glyceryl monooleate-based drug delivery systems optimal formulation, formulation 11 and 15 before and after incorporation of chitosan microspheres various formulations

Formulation	Viscosity (cps) [a]
Liquid crystalline base	315.19 ± 1.84
Liquid crystalline base with optimal formulation	401.20 ± 2.13
Liquid crystalline base with formulation 11	671.74 ± 6.06
Liquid crystalline base with formulation 15	458.75 ± 24.15

a = mean ± SD (n = 3)

3. *In vitro* Release Study

The *In vitro* drug release study of the liquid crystalline phase were carried out using two compartment Franz diffusion cells and isotonic phosphate buffer pH 6.8 as a medium. The amounts of drug release were analyzed using UV spectrophotometer at 268 nm. The validation methods were as same as in the topic C 1.1

To compare the effect of drug loaded chitosan microspheres the formulation of monoglyceride-based drug delivery system containing 10 % w/w doxycycline hyclate in the same amount as in microspheres, was included as control in the release study. Doxycycline hyclate could dissolve in the system homogenously.

The release profiles plotted between the cumulative amounts of drug release versus time are exhibited in Figure 59. The release data are presented in Appendix B. From the data obtained it was found that in each formulation the release profiles of the drug from liquid crystalline phase were prolonged over a period of 48 hr and consistent with those from microspheres, although the amounts of drug release were smaller. These could be due to the more obstructive diffusion pathway of the reversed hexagonal form in glyceryl monooleate-based drug delivery system. These findings agreed with the previous study by Norling et al. (1992) in that the reversed hexagonal form could give a sustained release of drug over 48 hr while the cubic form showed a complete release in 24 hr. Moreover, In vivo results from Esposito et al. (1996) showed that glyceryl monooleate-based drug delivery system was persistent in the periodontal pockets with 80% of the initial level after 8 hr of application, whereas poloxamer system disappeared in 1 hr.

The prolonged effect of monoglyceride based system for drug release was demonstrated from the release profile of the system containing doxycycline hyclate (Figure 59). The prolonged release over 120 hr of doxycycline hyclate was exhibited. The cumulative amounts release of doxycycline hyclate from the microspheres incorporated in the glyceryl monooleate-based drug delivery systems were ranged from 3.17-18.67 %. The explanation of the low drug release from the system might be due to the high affinity of drug in the complex structure of reverse hexagonal liquid crystals.

To elucidate the mechanism of drug release from the system, the zero-order plot, the first-order plot and the Higuchi plot are shown in Figures 59, 60 and 61, respectively. The kinetic parameters for zero-order, first-order and Higuchi model during

the initial 24 hr were calculated and are presented in Table 34. The release of doxycycline hyclate from glyceryl monooleate-based drug delivery system in all formulations tended to follow Higuchi model rather than the other two models, because the highest coefficient of determination (R^2) was obtained with the Higuchi model. Analysis of data showed that there were statistically significance differences ($P<0.05$) in coefficients of determination between the three models. Moreover, analysis of variance by regression showed a significant difference ($P<0.05$) in coefficients of determination of the Higuchi model, which indicated the correlation of % cumulative release and square root of time. As can be expected the control sample gave the highest Higuchi release rate constant because it did not comprise of a protective wall to obstruct the diffusion pathway, following by formulation 15, optimal formulation and formulation 11, respectively. It was possible that the release rate from the drug delivery system may depend on the drug release from chitosan microspheres.

The result from this study confirmed to many reports which suggested that the release mechanism from glyceryl monooleate-based drug delivery system followed square root of time kinetics indicating that the rate of release was diffusion controlled (Garaghty et al., 1996; Chang and Bodmeier, 1997; Komsri 1997; Helledi and Schubert, 2001; Tan, 2004). However, other studies showed that glyceryl monooleate-based drug delivery system could be followed zero-order and first-order kinetics (Burrows,Collett and Attwood, 1994). These data indicated that the similar liquid crystalline system could demonstrate different release profiles. These depended on several factors including a wide range of drug, solubility and concentration of the incorporated drug substances.

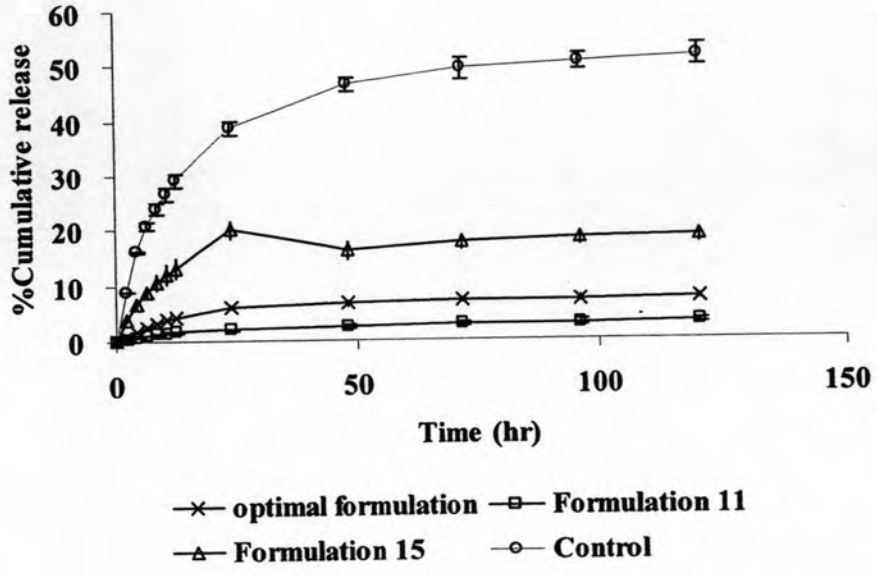


Figure 59 Release profiles of doxycycline hyclate loaded chitosan microspheres compared to doxycycline hyclate (non microspheres) in glyceryl monooleate-based drug delivery system

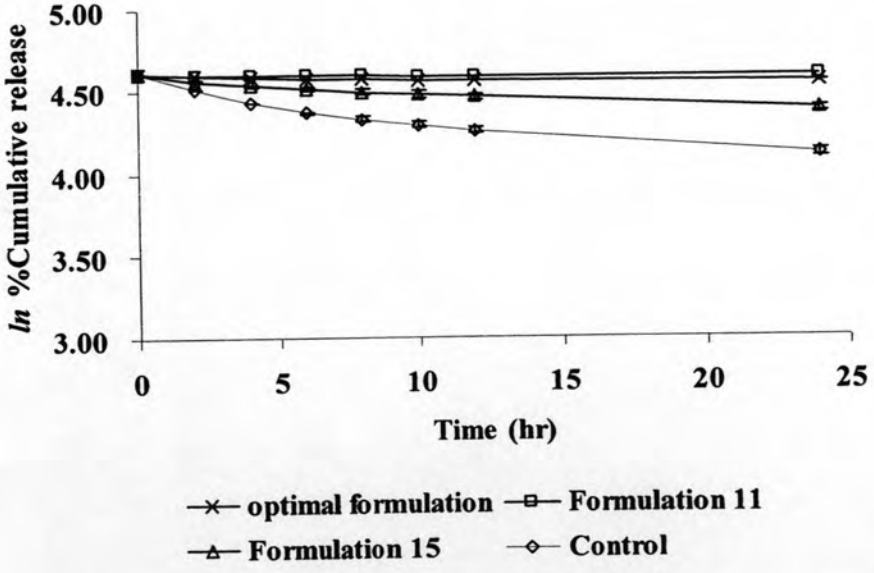


Figure 60 First-order plot of doxycycline hyclate loaded chitosan microspheres compared to doxycycline hyclate (non microspheres) in glyceryl monooleate-based drug delivery system

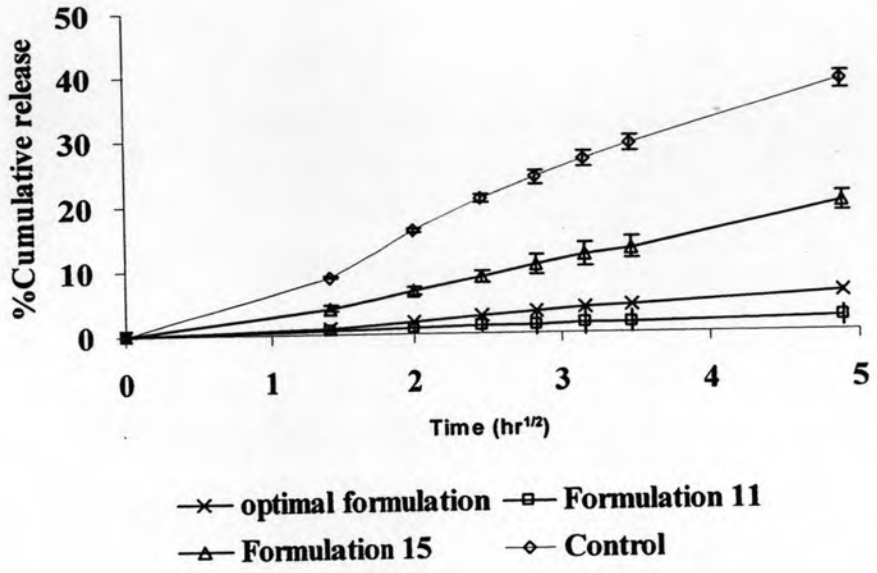


Figure 61 Higuchi plot of doxycycline hyclate loaded chitosan microspheres compared to doxycycline hyclate (non microspheres) in glyceryl monooleate-based drug delivery system

Table 34 Kinetic parameters of doxycycline hyclate loaded chitosan microspheres release from glyceryl monooleate-based drug delivery system

Formulation	Zero-order plot		First-order plot		Higuchi plot	
	$k_o(\%h^{-1})$	R^2	$k_1(h^{-1})$	R^2	$k_H(\%h^{-1/2})$	R^2
control	1.4925	0.8502	0.0195	0.9055	8.2894	0.9884
Optimal formulation	0.2446	0.9039	0.0025	0.8980	1.3037	0.9682
11	0.0792	0.8233	0.0008	0.8272	0.4449	0.9813
15	0.7813	0.9291	0.0088	0.9472	4.1462	0.9852

4. Differential Scanning Calorimetric (DSC) Method

The change of physicochemical property of glyceryl monooleate-based drug delivery system as a function of temperature was measured by means of DSC. The hydrocarbon chains of amphiphilic molecules were subjected to undergoing a transformation from an order (gel) state to a more disorder (liquid crystalline) state. These changes have been characterized by DSC which requires the input addition thermal energy show up as endothermic peaks on heating.

In this study, DSC thermograms of liquid crystalline reverse hexagonal form (Figure 62 (b)) showed two endothermic peaks at temperature range 10 to 20 °C and 60-80 °C. This finding was consistent with that reported by Norling et al. (1992). The presence of the first endothermic peak was possible that a phase transition occurred. The second endothermic peak at higher temperature approximately at 60-80 °C might be due to the phase transition of the liquid crystalline. It was reported previously that phase transition might occur when the temperature rised. However, the phase transition by the elevated temperature might not affect the presence of the drug delivery system in the oral cavity since the transition temperature was higher than normal body temperature. The DSC thermogram of the mixture between glyceryl monooleate and sesame oil (Figure 62 (a)) indicated that phase transformation of glyceryl monooleate-based drug delivery system might occur at temperature below 30 °C. This suggested that the condition that should be kept the glycerylmonooleate-based drug delivery system must over 30 °C to protect the transformation of the base. It was notable that the melting endothermic peak due to doxycycline hyclate could not be observed in all drug delivery systems. This might be due to the interaction of the drug delivery system with doxycycline hyclate.

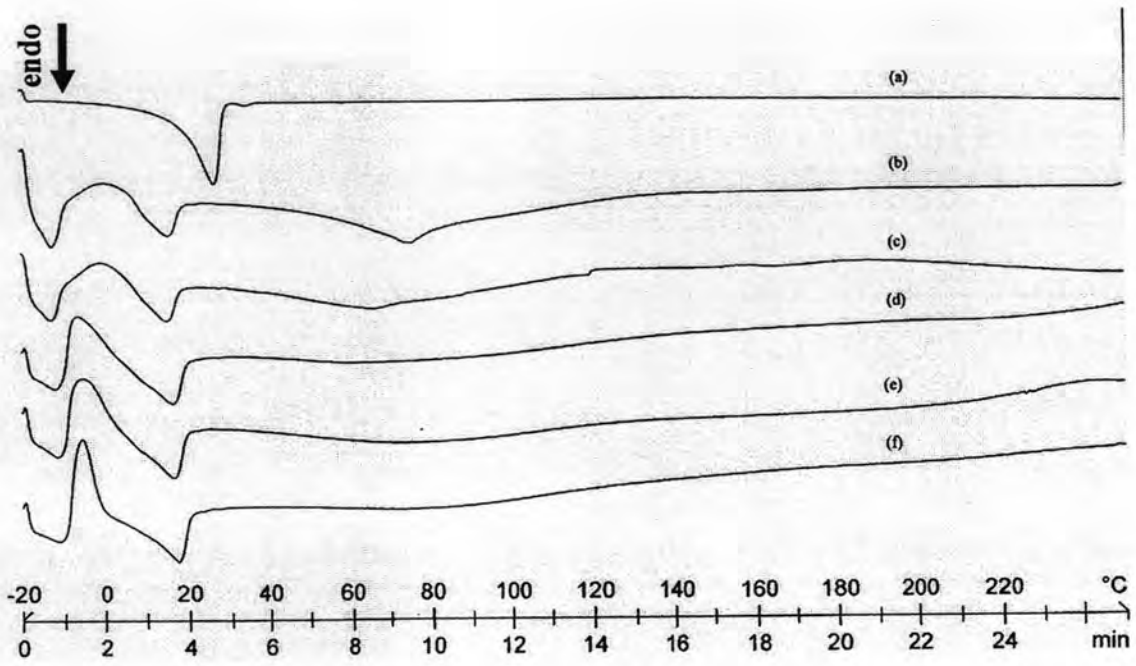


Figure 62 DSC thermograms of a mixture of 79% glyceryl monooleateand sesame oil (a) and liquid crystalline reversed hexagonal form (b), the glyceryl monooleate-based drug delivery system incorporated with -doxycycline hyclate(c), -optimal formulation (d), -formulation 11 (e), -formulation 15 (f)

5. Determination of Antimicrobial Activity of Doxycycline Hyclate Loaded Chitosan Microspheres in Glyceryl Monooleate-Based Drug Delivery System

The *in vitro* antimicrobial activities of the drug loaded microspheres, blank microspheres (negative control) and doxycycline hyclate (positive control) which were suspended in PBS pH 6.8 and glyceryl monooleate-based drug delivery system were evaluated against *Staphylococcus aureus* ATCC 6538P, The microbe was reported to be found in the periodontal pockets of patients with periodontitis (Parthasarathy et al., 2002), using agar diffusion method.

The agar diffusion method was used as the method for determining the antimicrobial susceptibility pattern of bacterial strain in this study. A filter paper disk, a hole, a porous cup or an open-ended cylinder containing measured quantities of drug might be used to place on a solid medium that has been heavily seeded with the test organisms. In this study, the antimicrobial activity against *Staphylococcus aureus* ATCC

6538P was examined by using a cylinder cup for placing the samples. When bacterial multiplication proceeded more rapidly than the drug diffused, the bacterial cells that were not inhibited by the antimicrobial agent would continue to multiply until a lawn of growth was visible and no zone of inhibition appeared around the hole. When the antimicrobial was present in inhibitory concentrations, no growth would appear in the zone around the hole.

As a result, all of the drug load microsphere formulations exhibited inhibition zones. The diameters of clear zone of the formulations were as follows: formulation 15 > optimal formulation > formulation 11. This result was consistent with the results obtained from the release study in amounts of drug release from microspheres in each formulation, but in a statistical view the inhibition zone of three formulations were not significantly different ($P>0.05$). The microsphere formulations that suspended in PBS pH 6.8 gave a larger clear zone than the formulations suspended in glyceryl monooleate-based drug delivery system (Figures 63, 64 and 65), this may be due to viscosity and complex structure of the liquid crystalline that could obstruct the diffusion of drug from the microspheres. Over the expectation, the blank chitosan microspheres in PBS pH 6.8 showed antibacterial activity, the possibility that chitosan has an antimicrobial activities needed to be considered. This finding agreed with previous research by Rhoades and Roller (2000). However, the blank chitosan microspheres in glyceyl monooleate-based could not inhibited bacterial growth. It is possible that glyceyl monooleate-based is a barrier of chitosan activities.

From the result of the current study, it would be concluded that the antimicrobial activities of doxycycline hyclate loaded chitosan microspheres against *Staphylococcus aureus* ATCC 6538P might be the synergistic effect of doxycycline hyclate and chitosan. However, after incorporate microspheres in glyceryl monooleate-based drug delivery system, the antimicrobial activities would be exerted from only doxycycline hyclate.

Table 35 Inhibition zones of the test compounds on *Staphylococcus aureus* ATCC 6538P

Formulation	Inhibition zone diameter (mm)	
	in GMO[a]	in PBS pH 6.8[a]
Doxycycline hyclate	40.53 ± 0.35	47.63 ± 1.42
Optimal formulation	34.93 ± 1.62	44.10 ± 1.65
Formulation 11	32.83 ± 1.27	41.40 ± 1.65
Formulation 15	35.63 ± 0.84	44.73 ± 0.65
Blank microspheres of optimal formulation	0.00	18.27 ± 0.55
Blank microspheres of formulation 11	0.00	14.67 ± 1.25
Blank microspheres of formulation 15	0.00	15.03 ± 4.24
without sample	0.00	0.00

a = mean ± SD, n = 3

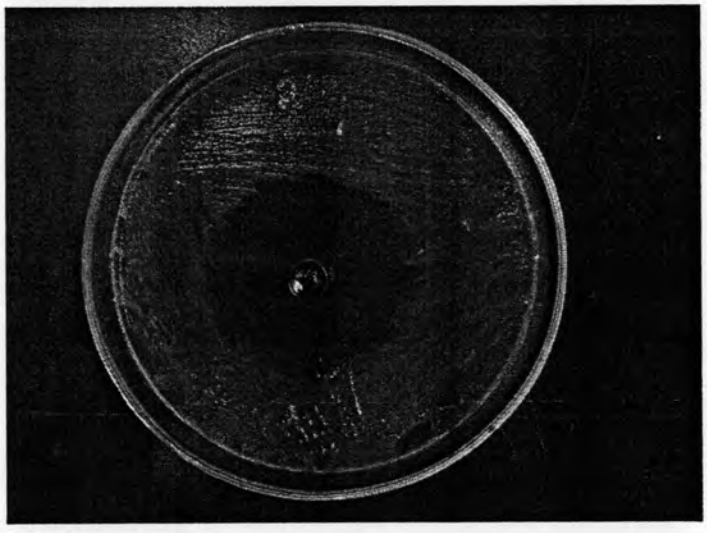


Figure 63 Agar diffusion test of glyceryl monooleate-based drug delivery system incorporated with doxycycline hyclate equivalent to 10 % of the system

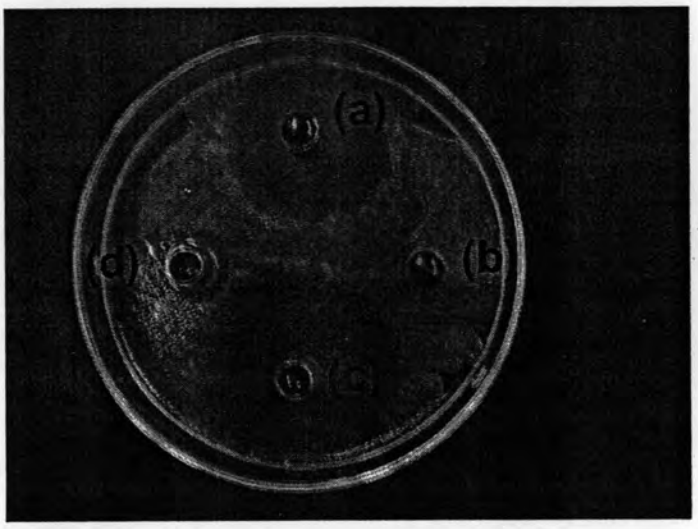


Figure 64 Agar diffusion test of glyceryl monooleate-based drug delivery system; (a) microspheres optimal formulation in glyceryl monooleate-based; (b) glyceryl monooleate-based; (c) microspheres optimal formulation in PBS pH 6.8 and (d) PBS pH 6.8

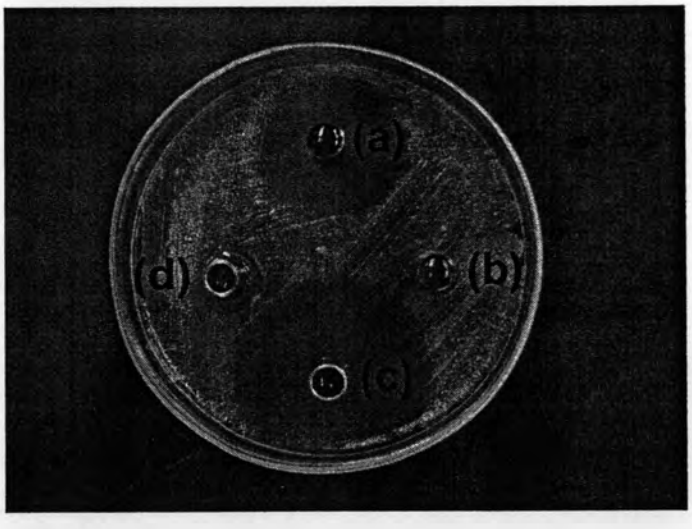


Figure 65 Agar diffusion test of glyceryl monooleate-based drug delivery system; (a) microspheres formulation 11 in glyceryl monooleate-based; (b) glyceryl monooleate-based; (c) microspheres formulation 11 in PBS pH 6.8 and (d) PBS pH 6.8

Figure 66 Agar diffusion test of glyceryl monooleate-based drug delivery system; (a) microspheres formulation 15 in glyceryl monooleate-based; (b) glyceryl monooleate-based; (c) microspheres formulation 15 in PBS pH 6.8 and (d) PBS pH 6.8

6. Determination of Injectability through the Syringe Needle

From above section (topic D 1.3) glyceryl monooleate-based drug delivery system formulation 22 was chosen as the drug delivery system due to its ability to form liquid crystal at the shortest time and it could administered through the syringe with 23-guage tip needle. However, after mixing the chitosan microspheres with the glycerylmonooleate-based drug delivery system, the viscosity was highly increased. Due to their high viscosity and stiffness, liquid crystalline phases were found difficult to be administered through a syringe needle.

To resolve this problem, glyceryl monooleate-based drug delivery system formulations 31, 39 and 48 were tested. The result exhibited that glyceryl monooleate-based drug delivery system formulation 48 was the only one that could be administered through the syringe needle gauged 23. The reason might be explained that the formulation 48 had the highest amount of sesame oil (12%) that improved the flowability of the fluid (Norling, 1992).

In conclusion, glyceryl monooleate-based drug delivery system formulation 48 might be the proper formulation in clinical used inspite of having the in vitro liquid crystalline formation of 8:22 minutes.