CHAPTER IV

RESULTS AND DISCUSSIONS

1. Preparation of chitosan-coated alginate nanoparticles containing dacarbazine

The value of percent yield of dacarbazine chitosan-coated alginate nanoparticles which were coated with chitosan 15000 dalton were in the range of 9.8 % – 14.6 % (Table1) and of those coated with chitosan 100000 dalton were in the range of 13.3 % - 17.9 % (Table2). The percent yield between two groups was significantly different ($p < 0.05$). The percent yield also significantly increased when the drug used in formulation increased ($p < 0.05$). In the same amount of dacarbazine used, the obtained nanoparticles which were coated with chitosan 100000 provided more product yield than that which was coated with chitosan 15000.

Table 1 The percent yield of dacarbazine chitosan-coated alginate nanoparticles coated with chitosan 15000 dalton (n = 3)

Formulation	% yield [a]
DTIC 1 mg in NP with chitosan 15000	9.80 ± 0.12
DTIC 2 mg in NP with chitosan 15000	10.43 ± 1.05
DTIC 5 mg in NP with chitosan 15000	14.57 ± 0.42

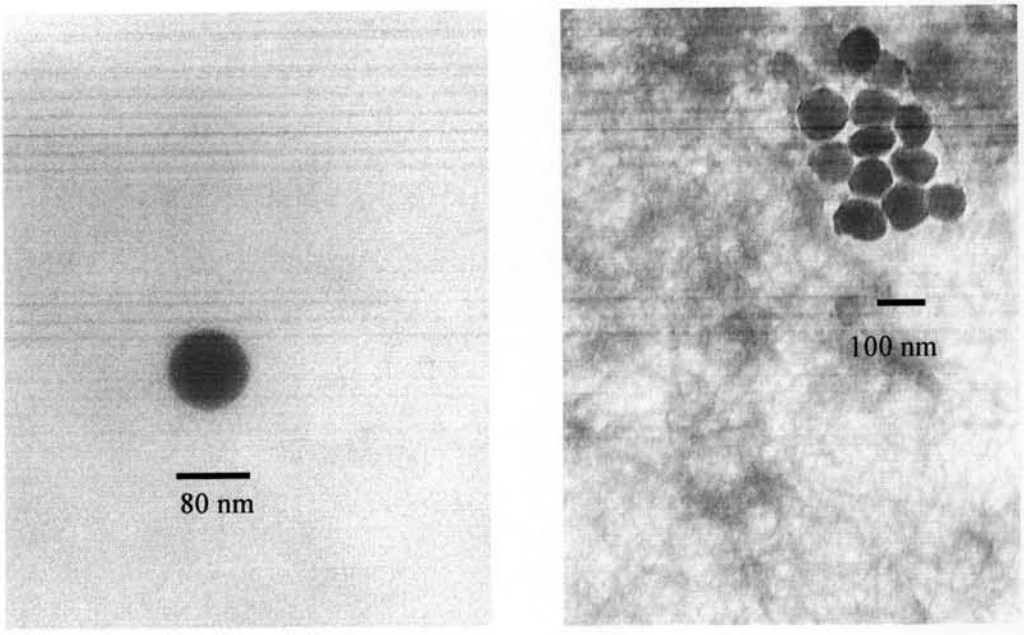
[a] compared to the polymer used

Table 2 The percent yield of dacarbazine chitosan-coated alginate nanoparticles coated with chitosan 100000 dalton (n = 3)

Formulation	% yield [a]
DTIC 1 mg in NP with chitosan 100000	13.26 ± 0.35
DTIC 2 mg in NP with chitosan 100000	14.55 ± 0.46
DTIC 5 mg in NP with chitosan 100000	17.93 ± 1.30

[a] compared to the polymer used

TEM images in Figure 6A reveal the presence of dacarbazine chitosan-coated alginate nanoparticles in the supernatant. Thus, the ultracentrifugation of 50,000 rpm could not precipitate these nanoparticles that smaller than 100 nm, then nanoparticles were not completely separated from the suspensions. This is the reason that provided low percent yield.



A B

Figure 6 Transmission electron micrographs of dacarbazine chitosan-coated alginate nanoparticles after ultracentrifugation

A : 2 mg dacarbazine nanoparticles in supernatant (× 100,000 magnification)

B : 2 mg dacarbazine nanoparticles as precipitates (× 50,000 magnification)

2. Characterization of nanoparticles

2.1 Size measurement and surface charge

The particle size, size distribution and zeta potential were measured by Zetasizer Nano ZS (Malvern, UK, facilitated by Nanotech). The principle of Zetasizer Nano ZS for size measurement is dynamic light scattering also known as photon correlation spectroscopy which is the simplest and the only routine method presently applicable (Kreuter, 1994).

In this study, the mean particle size of dacarbazine alginate nanoparticles which were coated with chitosan 15000 dalton were in range of 489.03 - 556.90 nm (Table 3) and that which was coated with chitosan 100000 dalton were in the range of 548.67 – 584.77 (Table 4). The mean particle size of obtained nanoparticles which were coated with chitosan 15000 dalton increased significantly when the amount of dacarbazine increased ($p < 0.0001$). The mean particle size of obtained nanoparticles which were coated with chitosan 100000 dalton also increased significantly when the amount of dacarbazine increased ($p = 0.02$). In the case of obtained nanoparticles which contained 1 mg and 2 mg of dacarbazine, the mean particle size increased significantly when coated with chitosan 100000 dalton ($p = 0.0002$ and 0.01, respectively). In the case of obtained nanoparticles which contained 5 mg of dacarbazine, the mean particle size of dacarbazine alginate nanoparticles coated with the higher molecular weight chitosan were not different from those coated with the lower molecular weight chitosan ($p = 0.07$).

Table 3 Mean particle size, polydispersity index and zeta potential of dacarbazine chitosan-coated alginate nanoparticles coated with chitosan 15000 dalton (n =3)

Formulation	Mean particle size (nm)	Polydispersity index	Zeta potential (mV)
Blank alginate NP coated with chitosan 15000	522.53 ± 7.29	0.42 ± 0.03	-24.83 ± 0.47
DTIC 1 mg in NP coated with chitosan 15000	489.03 ± 2.39	0.57 ± 0.03	-28.13 ± 0.40
DTIC 2 mg in NP coated with chitosan 15000	538.33 ± 5.69	0.30 ± 0.01	-28.30 ± 0.53
DTIC 5 mg in NP coated with chitosan 15000	556.90 ± 10.55	0.54 ± 0.03	-28.97 ± 0.15

Table 4 Mean particle size, polydispersity index and zeta potential of dacarbazine chitosan-coated alginate nanoparticles coated with chitosan 100000 dalton (n =3)

Formulation	Mean particle size (nm)	Polydispersity index	Zeta potential (mV)
Blank alginate NP coated with chitosan 100000	534.70 ± 7.83	0.55 ± 0.03	-24.30 ± 0.78
DTIC 1 mg in NP coated with chitosan 100000	548.67 ± 7.51	0.38 ± 0.01	-28.03 ± 0.29
DTIC 2 mg in NP coated with chitosan 100000	559.00 ± 6.56	0.42 ± 0.06	-28.03 ± 0.47
DTIC 5 mg in NP coated with chitosan 100000	584.77 ± 17.18	0.41 ± 0.03	-28.07± 0.21

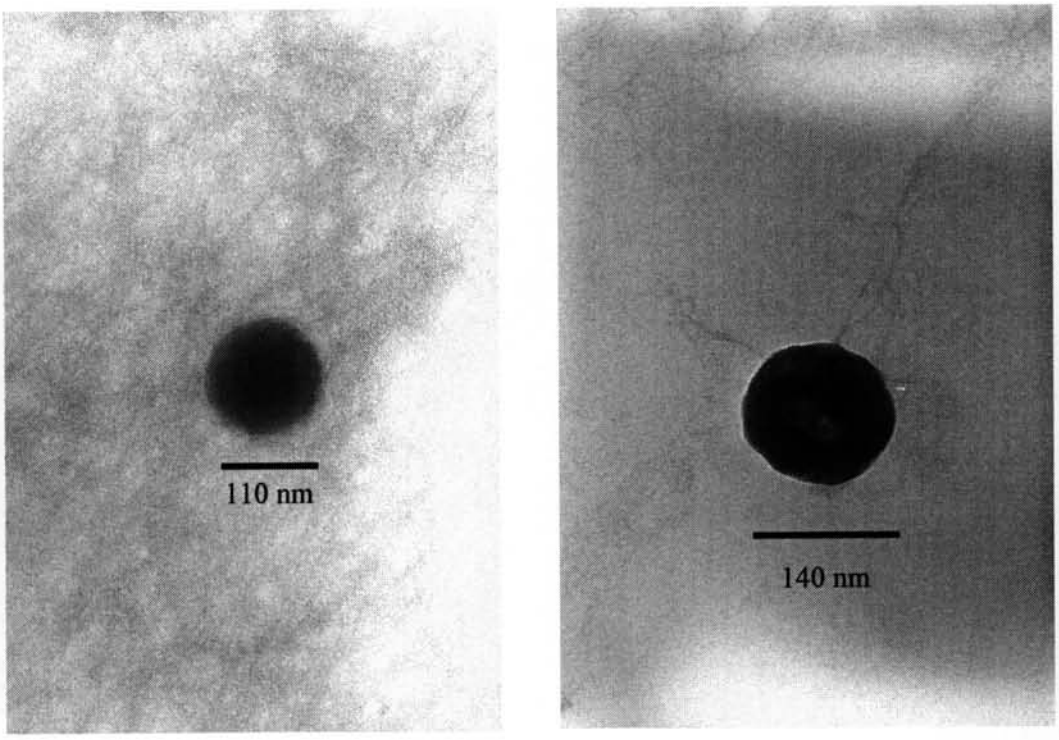
The polydispersity index of dacarbazine alginate nanoparticles which were coated with chitosan 15000 were in the range of 0.30 - 0.57 (Table 3) and those were coated with chitosan 100000 dalton were in the range of 0.38 – 0.42 (Table 4). The higher polydispersity index indicated a wider particle size distribution. The zeta potential of obtained dacarbazine nanoparticles which were coated with chitosan 15000 dalton

were in range of -29.97 to -28.13 mV (Table 3) and those were coated with chitosan 100000 dalton were in the range of -29.30 to -28.03 mV (Table 4). Dacarbazine-loaded nanoparticles showed more negative value of zeta potential compared to the blank nanoparticles. Thus, dacarbazine itself influenced to the zeta potential of obtained nanoparticles ($p < 0.0001$). However, the amount of dacarbazine did not influence to this property ($p > 0.05$). The molecular weight of chitosan also did not influence to the zeta potential of obtained nanoparticles.

2.2 Particle morphology

The morphology of dacarbazine chitosan-coated alginate nanoparticles were determined by transmission electron microscopy (TEM). TEM was used to verify the presence and size of obtained nanoparticles. It did not clearly show various particle sizes when evaluated with TEM because of field limitation. In this study, the micrograph from TEM did not show difference between alginate nanoparticles which contained different amount of drug (see Appendix).

TEM micrographs in Figure 7 reveal the presence of dacarbazine chitosan-coated alginate nanoparticles and illustrated the spherical shape. Dacarbazine chitosan-coated alginate nanoparticles were considerably smaller when viewed with TEM than when measured by nanosizer. TEM images showed particle size between 100 and 200 nm, whereas nanosizer indicated the mean particle size of obtained nanoparticles in the range of 400 - 600 nm. This apparent discrepancy can be explained by the dehydration of the hydrogel particles during sample preparation for TEM imaging. Additionally, nanosizer measured the hydrodynamic diameter of the particle leading to an overestimation of particle size (Kreuter, 1994).



A B

Figure 7 Transmission electron micrographs of dacarbazine chitosan-coated alginate nanoparticles. Nanoparticle morphology related to a generic spherical shape.
A : 2 mg dacarbazine alginate nanoparticles coated with chitosan 15000 dalton
(× 100,000 magnification)
B : 2 mg dacarbazine alginate nanoparticles coated with chitosan 100000 dalton
(× 100,000 magnification)

3. Determination of entrapment efficiency

The drug entrapment efficiency was calculated as the percentage of drug entrapped in chitosan-coated alginate nanoparticles compared with the initial amount of drug. In order to separate dacarbazine chitosan-coated alginate nanoparticles from the suspensions, the suspensions were ultracentrifuged at 50,000 rpm for 60 min. Then, both the supernatant and the precipitate were analyzed for dacarbazine content. The entrapped dacarbazine was determined by breaking nanoparticles with sodium citrate. Chitosan-coated alginate nanoparticles were destabilized when treated with a

sequestrant agent such as sodium citrate. This ionic exchange leads to swelling of the gel, resulting in leakage of entrapped drug (Raj and Sharma, 2003). Because the ultracentrifugation of 50,000 rpm of these nanoparticles preparations was not completely separated nanoparticles from the suspensions, the encapsulated dacarbazine in chitosan-coated alginate nanoparticles could be calculated by estimating the amount of entrapped and unentrapped drug recovered from the supernatant and precipitate after ultracentrifugation.

The drug entrapment efficiency of dacarbazine alginate nanoparticles which coated with chitosan 15000 dalton were 31.36 % to 40.14 % (Table 5) and of those coated with chitosan 100000 dalton were 33.34 % to 41.55 % (Table 6). The percentage of entrapment efficiency of dacarbazine nanoparticles increase significantly when the amount of drug increased from 1 mg to 2 mg ($p < 0.05$) and decrease significantly when the amount of drug increased to 5 mg ($p < 0.05$). At the same amount of dacarbazine, the percentage of entrapment efficiency of dacarbazine alginate nanoparticles coated with the higher molecular weight chitosan were not different from that coated with the lower molecular weight chitosan ($p > 0.05$). Thus, the molecular weight was not affected to the entrapment efficiency of dacarbazine nanoparticles.

Table 5 The percentage of entrapment efficiency of dacarbazine in alginate nanoparticles coated with chitosan 15000 dalton (n = 3)

Formulation	**Amount of entrapped dacarbazine (mg)**	**% Entrapment efficiency**
DTIC 1 mg in NP coated with chitosan 15000	0.38 ± 0.01	38.19 ± 0.84
DTIC 2 mg in NP coated with chitosan 15000	0.80 ± 0.01	40.14 ± 0.35
DTIC 5 mg in NP coated with chitosan 15000	1.56 ± 0.05	31.36 ± 1.14

Table 6 The percentage of entrapment efficiency of dacarbazine in alginate nanoparticles coated with chitosan 100000 dalton (n = 3)

Formulation	Amount of entrapped dacarbazine (mg)	% Entrapment efficiency
DTIC 1 mg in NP coated with chitosan 100000	0.40 ± 0.01	39.21 ± 0.56
DTIC 2 mg in NP coated with chitosan 100000	0.83 ± 0.01	41.55 ± 0.49
DTIC 5 mg in NP coated with chitosan 100000	1.68 ± 0.09	33.34 ± 1.46

The obtained nanoparticles which contained 2 mg of dacarbazine either coated with chitosan 15000 dalton or 100000 dalton showed the highest percentage of entrapment efficiency. This may be concluded that the highest interaction between dacarbazine and alginate/chitosan occurred with the 2 mg of dacarbazine. The entrapment efficiency of the drug to the nanoparticles depends on the type of drug, polymer and the preparation condition.

Table 7 The amount of entrapped, unentrapped and % recovery of dacarbazine in alginate nanoparticles coated with chitosan 15000 dalton (n = 3)

Formulation	Entrapped (mg)	Unentrapped (mg)	Total dacarbazine recovery (mg)	% Recovery
DTIC 1 mg in NP coated with chitosan 15000	0.38 ± 0.01	0.03 ± 0.00	0.42 ± 0.01	41.46 ± 0.86
DTIC 2 mg in NP coated with chitosan 15000	0.80 ± 0.01	0.06 ± 0.00	0.86 ± 0.01	43.24 ± 0.24
DTIC 5 mg in NP coated with chitosan 15000	1.56 ± 0.05	0.31 ± 0.01	1.87 ± 0.04	37.60 ± 1.07

Table 8 The amount of entrapped, unentrapped and % recovery of dacarbazine in alginate nanoparticles coated with chitosan 100000 dalton (n = 3)

Formulation	Entrapped (mg)	Unentrapped (mg)	Total dacarbazine recovery (mg)	% Recovery
DTIC 1 mg in NP coated with chitosan 100000	0.40 ± 0.01	0.03 ± 0.01	0.43 ± 0.01	42.66 ± 0.65
DTIC 2 mg in NP coated with chitosan 100000	0.83 ± 0.01	0.06 ± 0.01	0.89 ± 0.01	44.59 ± 0.59
DTIC 5 mg in NP coated with chitosan 100000	1.68 ± 0.09	0.31 ± 0.01	1.99 ± 0.09	39.47 ± 1.55

The percentage of recovery of dacarbazine in chitosan alginate nanoparticles coated with chitosan 15000 dalton were 37.60 % to 43.24 % (Table 7) and those coated with chitosan 100000 dalton were 39.47 % to 44.59 % (Table 8).

The percentage of recovery of dacarbazine nanoparticles increase significantly when the amount of drug increased from 1 mg to 2 mg ($p < 0.05$) and decrease significantly when the amount of drug increased to 5 mg ($p < 0.05$). At the same amount of dacarbazine, the percentage of recovery of dacarbazine alginate nanoparticles coated with the higher molecular weight chitosan were not different from those coated with the lower molecular weight chitosan ($p > 0.05$). The low percentage of recovery may due to the incompletely extraction of the drug from nanoparticles and/or the drug degradation may occurred during the study.

HPLC analysis

The High Performance Liquid Chromatography (HPLC) was used to analyze the amount of dacarbazine in this study. In this study, the analysis was achieved with isocratic reverse phase HPLC. The mobile phase composed of acetonitrile and 10 mM monobasic sodium phosphate dihydrate in the ratio of 20:80, adjust to pH 4.2 with phosphoric acid. The flow rate was 1.0 mL/min. The injection volume was 20 µl,

and the retention time was found to be 3.4 min (Figure 8). The column effluent was detected at 327 nm. Before sample analysis, system suitability which composes of reproducibility and tailing factor were established (Table 9). The calibration curve for the quantification of dacarbazine was linear over the range of concentration of 0.25– 20.0 μg/mL (Table 10, Figure 9).

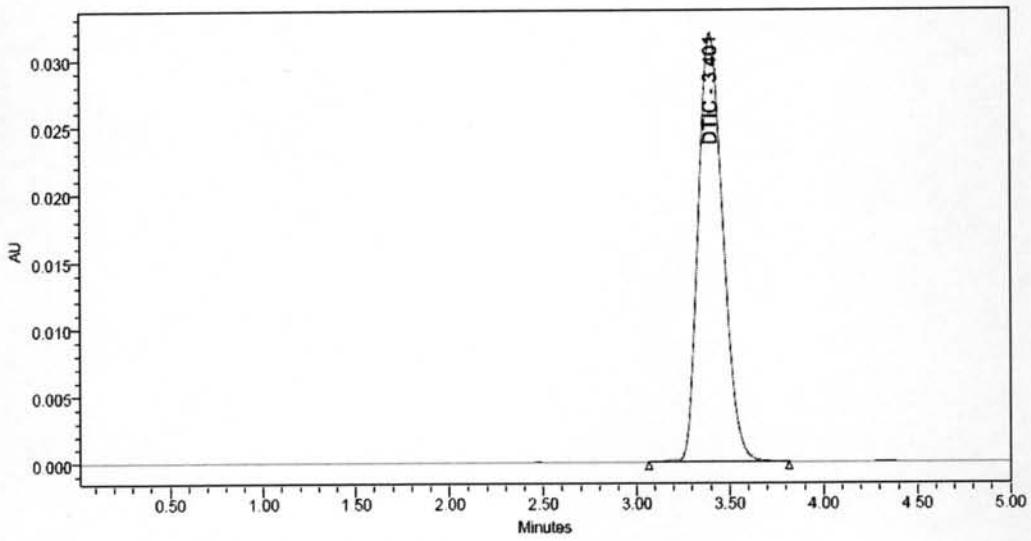


Figure 8 The chromatogram of dacarbazine analysis

Table 9 Reproducibility (% RSD) and tailing factor of five replicate injections of the standard solution.

Number of Replication	Concentration (μg/mL)	Peak Area (mAU)	Tailing Factor
1	5.00	9.9908	1.36
2	5.00	10.066	1.34
3	5.00	10.0363	1.34
4	5.00	10.0306	1.35
5	5.00	10.0479	1.36
Mean		10.0343	1.35
SD		0.0278	0.01
% RSD		0.28	0.74

The % RSD obtained from this method was 0.28%. This result was within acceptable range (< 2.0%). The tailing factor, a measurement of peak symmetry, was 1.35 ± 0.01 which was in acceptable range (< 2). In conclusion, the system of HPLC was suitable for quantitative analysis of dacarbazine chitosan-coated alginate nanoparticles.

Table 10 The concentration and peak area of dacarbazine standard solutions for analysis.

Concentration (μg/mL)	Peak area (mAU)
0.25	0.18
0.50	0.48
1.00	0.94
2.00	1.84
5.00	4.77
8.00	7.81
10.00	9.81
20.00	19.83

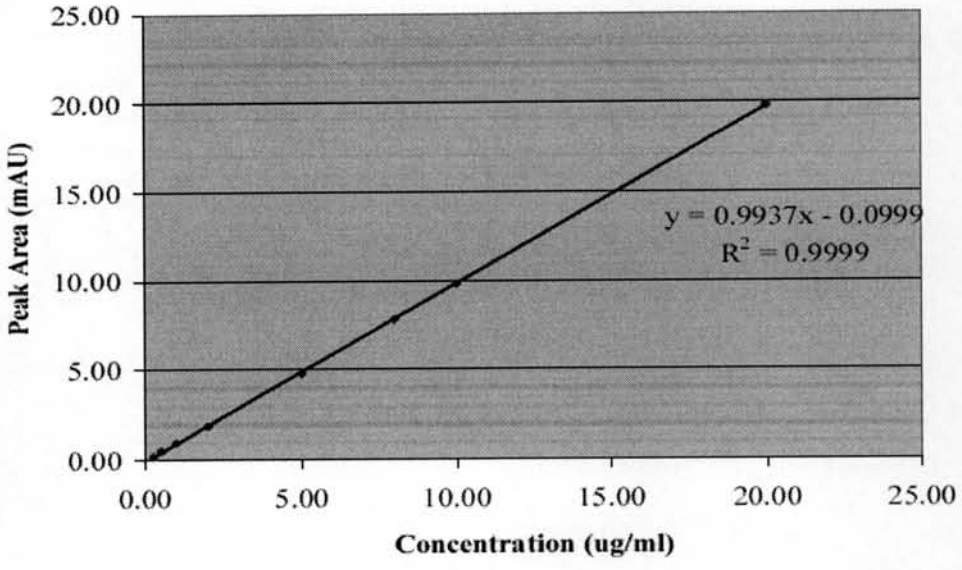


Figure 9 The calibration curve of dacarbazine

HPLC Method Validation

1) Accuracy

In this study, the percent recovery of analytical method for dacarbazine nanoparticles obtained from dacarbazine standard solutions was 83.97 % - 98.03 % (Table 11).

Table 11 Accuracy of analytical method for dacarbazine nanoparticles obtained from dacarbazine standard solutions

Replication	Known amount (μg)	Found amount (μg) [a]	SD	% RSD	%Recovery
1	27.00	22.67	0.06	0.28	83.97
	67.50	63.13	0.11	0.17	93.52
	108.00	100.89	0.10	0.10	93.42
2	27.00	25.42	0.04	0.14	94.15
	67.50	65.24	0.21	0.33	96.65
	108.00	101.81	0.27	0.27	94.27
3	27.00	23.45	0.04	0.19	86.83
	67.50	61.79	0.17	0.27	91.54
	108.00	105.87	0.25	0.24	98.03

[a] : results were mean of three injections

2) Precision

The relative standard deviation (%RSD) obtained from repeatability (intra-assay precision) were in the range of 0.28 % - 0.56 % (Table 12) and that from intermediate precision were in the range of 0.76 % - 0.92 % (Table 13). The precision of analytical method for dacarbazine nanoparticles was acceptable (%RSD < 2.0%).

Table 12 Repeatability (intra-assay precision) of analytical method obtained from sample solutions

Concentration	Dacarbazine concentration (µg/mL) [a]			Mean	SD	% RSD
	Injection 1	Injection 2	Injection 3			
1	2.71	2.70	2.68	2.70	0.02	0.56
2	3.44	3.42	3.43	3.43	0.01	0.28
3	3.93	3.96	3.94	3.94	0.01	0.34

[a] : results were mean of three injections

Table 13 Intermediate precision of analytical method obtained from sample solutions

Day	Dacarbazine concentration (µg/mL) [a]		
	Concentration 1	Concentration 2	Concentration 3
1	2.75	3.48	4.00
2	2.73	3.43	3.97
3	2.70	3.43	3.94
Mean	2.73	3.45	3.97
SD	0.03	0.03	0.03
% RSD	0.92	0.84	0.76

[a] : results were mean of three injections

3) Linearity

In this study, the linearity curves were obtained both from standard solutions (Table 14) and sample solutions (Table 15).

Table 14 Linearity of analytical method obtained from dacarbazine standard solutions

Linearity	Concentration (µg/mL)	Peak Area (mAU)	Coefficient of determination (r^2)
1	0.30	0.4997	1.0000
	0.51	0.8612	
	1.06	1.757	
	2.27	3.7815	
	6.32	10.5212	
	10.10	16.8185	
	13.18	21.9452	
	27.61	45.9802	
2	0.33	0.5547	1.0000
	0.60	1.0029	
	1.23	2.0683	
	2.54	4.2643	
	6.53	10.9502	
	10.20	17.1027	
	13.64	22.8747	
	27.25	45.6792	
3	0.34	0.5589	1.0000
	0.60	0.9751	
	1.14	1.8725	
	2.35	3.8417	
	6.19	10.1239	
	10.61	17.3528	
	12.48	20.4262	
	27.77	45.4355	

The coefficient of determination (r^2) from all the linearity curves of analytical method for dacarbazine nanoparticles were acceptable ($r^2 > 0.999$).

Table 15 Linearity of analytical method obtained from sample solutions

Linearity	Concentration (μg/mL)	Peak Area (mAU)	Coefficient of determination (r^2)
1	0.85	1.4164	1.0000
	1.58	2.6361	
	3.33	5.5413	
	6.79	11.3139	
	13.58	22.6167	
2	1.22	2.0441	0.9999
	1.71	2.8708	
	3.35	5.4720	
	6.70	11.2406	
	13.30	22.3049	
3	0.90	1.4673	1.0000
	1.40	2.2849	
	2.69	4.3961	
	5.24	8.5684	
	10.94	17.9030	

4) Range

The range of a method can be defined as the lower and upper concentrations for which the analytical method has adequate accuracy, precision, and linearity.

In this study, the concentration range obtained from dacarbazine standard solution was 0.30-27.77 μg/mL (Table 14) and the concentration range obtained from samples was about 0.85-13.58 μg/mL (Table 15).

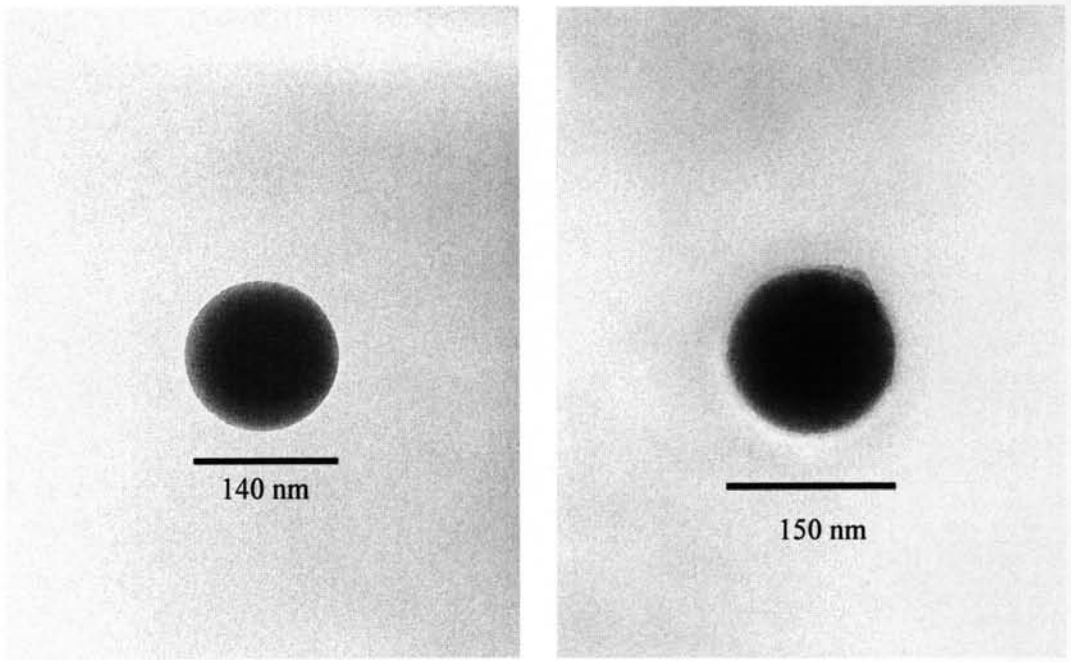
5) System suitability

System suitability also used in analytical method to verify that the detection sensitivity, resolution, and reproducibility of the chromatographic system is adequate for the analysis to be done. This must be established before sample analysis. The parameters used in this study are reproducibility and tailing factor. Reproducibility is the % relative standard deviation calculated from peak area of five replicate injections of a standard solution. The obtained result should be less than 2%. The tailing factor is a measurement of peak symmetry. The accuracy of quantitation decreases if the peak tailing increases. The tailing factor of each five peaks from five replicate injections of a standard solution should be less than 2. One of system suitability in this study is shown in table 9.

4. Freeze-drying process

The major limit of the use of nanoparticles when they are stored as aqueous suspensions is due to the physical instability such as aggregation and particle fusion and/or to the chemical instability such as hydrolysis of polymer materials forming the nanoparticles, drug leakage of nanoparticles and chemical reactivity of drug during the storage. The time of contact with water also influence the amount of drug incorporated into the nanoparticles, especially the drug which degrades in an aqueous environment. In order to improve the physical and chemical stability of dacarbazine chitosan-coated alginate nanoparticles, water has to be removed in this system. In this study, freeze-drying process was used to keep the obtained dacarbazine chitosan-coated alginate nanoparticles in dried powder. TEM micrographs in Figure 10 show the morphology of freeze-dried chitosan-coated alginate nanoparticles containing 2 mg dacarbazine after reconstituted which were still in the spherical shape and were in nano-size. However, the mean particle size which were measured by Zetasizer Nano ZS showed that the mean particle size of reconstituted dacarbazine alginate nanoparticles which were coated with chitosan 15000 dalton was 916.5 ± 138.5 with the polydispersity index of 0.8 ± 0.2 and that which was coated with chitosan 100000 dalton was 878.8 ± 133.7 with the polydispersity index of 0.6 ± 0.0 (Table 16).

The increased particle size and wide particle size distribution of dacarbazine chitosan-coated alginate nanoparticles may result from the aggregation and fusion to from large particle of nanoparticles during the preparation process. The zeta potential of reconstituted dacarbazine nanoparticles which were coated with chitosan 15000 and that which was coated with chitosan 100000 dalton was -36.8 ± 4.9 and -45.8 ± 6.4, respectively (Table 16).



A B

Figure 10 Transmission electron micrographs of freeze-dried chitosan-coated alginate nanoparticles containing 2 mg dacarbazine after reconstituted

A : coated with chitosan 15000 dalton (x 100,000 magnification)

B : coated with chitosan 100000 dalton (x 100,000 magnification)

Table 16 Mean particle size, polydispersity index and zeta potential of freeze-dried dacarbazine chitosan-coated alginate nanoparticles after reconstituted (n =3)

Formulation	Mean particle size (nm)	Polydispersity index	Zeta potential (mV)
DTIC 2 mg in NP coated with chitosan 15000	916.5 ± 138.5	0.8 ± 0.2	-36.8 ± 4.9
DTIC 2 mg in NP coated with chitosan 100000	878.8 ± 133.7	0.6 ± 0.0	-45.8 ± 6.4

5. Stability evaluation

The obtained alginate nanoparticles containing 2 mg of dacarbazine coating with either chitosan at 15000 dalton or that at 100000 dalton were chosen to study the stability in aqueous solution. The solutions used in this study were pH 3-4 citric acid solution and normal saline solution (NSS) and the conditions used in this study were at room temperature and at 2-8°C. The stability of dacarbazine formulated as chitosan-coated alginate nanoparticles was presented in terms of dacarbazine content. The amounts of undegraded dacarbazine, including a sum of encapsulated drug in nanoparticles and released drug in dacarbazine nanoparticles suspension or total dacarbazine in the case of drug solution, were measured as a function of time. The dacarbazine content analyzed at the day of preparation (time 0 hr) was quoted as 100% remaining. The percentage remaining of dacarbazine at different conditions and at the specific time was shown in Table 17.

Table 17 The stability of formula 1 and formula 2 dacarbazine chitosan-coated alginate nanoparticles compared with free dacarbazine (n=3)

Condition	hours	Dacarbazine Nanoparticles (Formula 1)[a]		Dacarbazine Nanoparticles (Formula 2)[b]		Free dacarbazine	
		DTIC content (μg/mL)	% remaining	DTIC content (μg/mL)	% remaining	DTIC content (μg/mL)	% remaining
In citric acid solution at room temperature	0	4.92 ± 0.10	100.00 ± 0.0	4.93 ± 0.08	100.00 ± 0.0	5.03 ± 0.15	100.00 ± 0.0
	6	4.65 ± 0.06	94.51 ± 0.62	4.49 ± 0.12	91.10 ± 2.83	4.41 ± 0.08	87.77 ± 4.20
	12	4.06 ± 0.09	82.50 ± 0.14	4.05 ± 0.11	82.12 ± 3.07	3.78 ± 0.10	75.08 ± 0.46
	24	3.75 ± 0.13	76.25 ± 3.77	3.82 ± 0.13	77.37 ± 2.78	3.58 ± 0.06	71.25 ± 1.15
	48	3.52 ± 0.09	71.70 ± 3.10	3.60 ± 0.13	72.91 ± 2.40	3.27 ± 0.05	65.10± 1.10
	72	3.20 ± 0.07	65.09 ± 0.98	3.22 ± 0.10	65.21 ± 2.29	2.61 ± 0.08	51.99 ± 3.08
	240 (10 days)	2.23 ± 0.06	45.31 ± 2.13	2.28 ± 0.09	46.16 ± 2.17	2.40 ± 0.12	47.76 ± 2.54
In citric acid solution at 2-8°C	0	4.93 ± 0.04	100.00 ± 0.0	4.98 ± 0.09	100.00 ± 0.0	5.04 ± 0.07	100.00 ± 0.0
	6	4.75 ± 0.05	96.35 ± 0.93	4.62 ± 0.08	92.73 ± 2.89	4.47 ± 0.09	88.78 ± 2.94
	12	4.26 ± 0.09	86.42 ± 1.77	4.17 ± 0.13	83.84 ± 3.84	3.89 ± 0.08	77.25 ± 0.61
	24	3.95 ± 0.07	80.13 ± 1.00	3.95 ± 0.12	79.42 ± 3.45	3.73 ± 0.06	74.08 ± 0.56
	48	3.72 ± 0.13	75.40 ± 2.00	3.71 ± 0.09	74.53 ± 2.85	3.55 ± 0.10	70.57 ± 2.43
	72	3.46 ± 0.05	70.21 ± 1.40	3.49 ± 0.08	70.11 ± 2.74	2.93 ± 0.09	58.20 ± 2.39
	240 (10 days)	2.53 ± 0.08	51.29 ± 1.74	2.44 ± 0.12	49.09 ± 2.83	2.57 ± 0.13	50.99 ± 3.34
In NSS solution at room temperature	0	4.95 ± 0.08	100.00 ± 0.0	4.90 ± 0.08	100.00 ± 0.0	4.93 ± 0.07	100.00 ± 0.0
	6	4.77 ± 0.08	96.30 ± 0.25	4.74 ± 0.08	96.81 ± 1.14	4.34 ± 0.06	87.88 ± 0.52
	12	4.43 ± 0.09	89.49 ± 0.52	4.56 ± 0.12	93.14 ± 2.66	3.93 ± 0.07	79.69 ± 0.26
	24	4.18 ± 0.16	84.36 ± 1.93	4.14 ± 0.08	84.49 ± 2.00	3.63 ± 0.08	73.69 ± 1.45
	48	3.76 ± 0.09	75.95 ± 0.69	3.67 ± 0.10	74.92 ± 3.21	3.42 ± 0.06	69.24 ± 0.61
	72	3.31 ± 0.07	66.87 ± 0.77	3.33 ± 0.05	68.01 ± 0.39	2.82 ± 0.13	57.17 ± 2.12
	240 (10 days)	2.76 ± 0.26	55.66 ± 4.79	2.75 ± 0.08	56.12 ± 2.50	2.69 ± 0.08	54.61 ± 1.70

Condition	hours	Dacarbazine Nanoparticles (Formula 1)[a]		Dacarbazine Nanoparticles (Formula 2)[b]		Free dacarbazine	
		DTIC content (µg/mL)	% remaining	DTIC content (µg/mL)	% remaining	DTIC content (µg/mL)	% remaining
In NSS solution at 2-8°C	0	5.04 ± 0.08	100.00 ± 0.0	4.95 ± 0.11	100.00 ± 0.0	4.90 ± 0.11	100.00 ± 0.0
	6	4.86 ± 0.06	96.50 ± 0.56	4.82 ± 0. 09	97.52 ± 1.48	4.48 ± 0.10	91.44 ± 0.87
	12	4.64 ± 0.10	92.13 ± 1.69	4.64 ± 0.11	93.87 ± 1.39	4.03 ± 0.10	82.14 ± 0.38
	24	4.37 ± 0.08	86.70 ± 1.22	4.30 ± 0.02	86.96 ± 2.24	3.74 ± 0.11	76.26 ± 2.40
	48	3.86 ± 0.11	76.57 ± 1.31	3.74 ± 0.12	75.73 ± 4.00	3.55 ± 0.12	72.46 ± 1.76
	72	3.67 ± 0.09	72.93 ± 0.66	3.65 ± 0.07	73.79 ± 0.36	2.90 ± 0.16	59.17 ± 2.80
	240 (10 days)	2.96 ± 0.17	58.68 ± 2.76	2.84 ± 0.13	57.54 ± 3.80	2.73 ± 0.15	55.58 ± 1.72

[a] : Dacarbazine Nanoparticles (Formula 1) was 2 mg dacarbazine nanoparticles coated with chitosan 15000

[b] : Dacarbazine Nanoparticles (Formula 2) was 2 mg dacarbazine nanoparticles coated with chitosan 100000

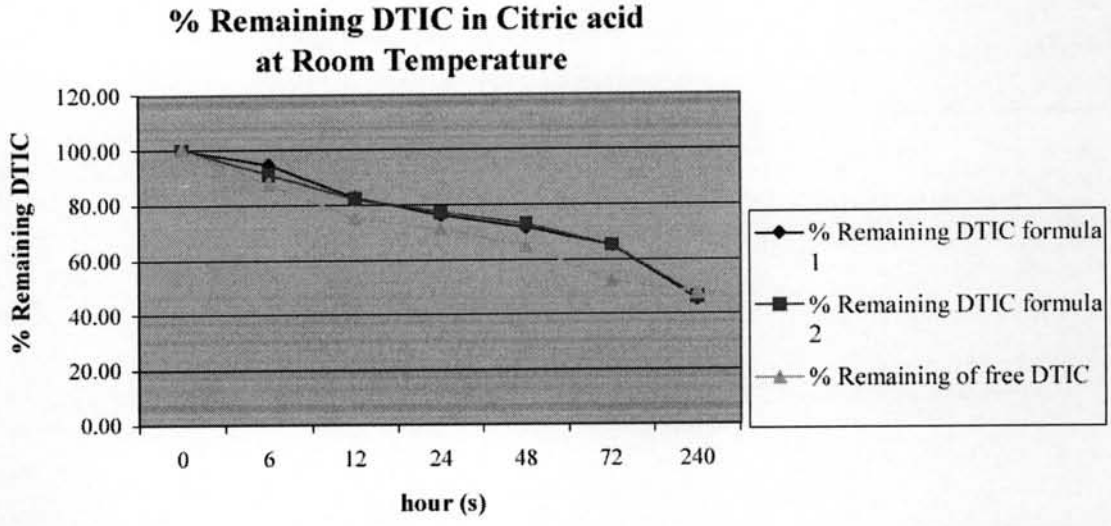


Figure 11 The percentage of remaining dacarbazine in citric acid at room temperature

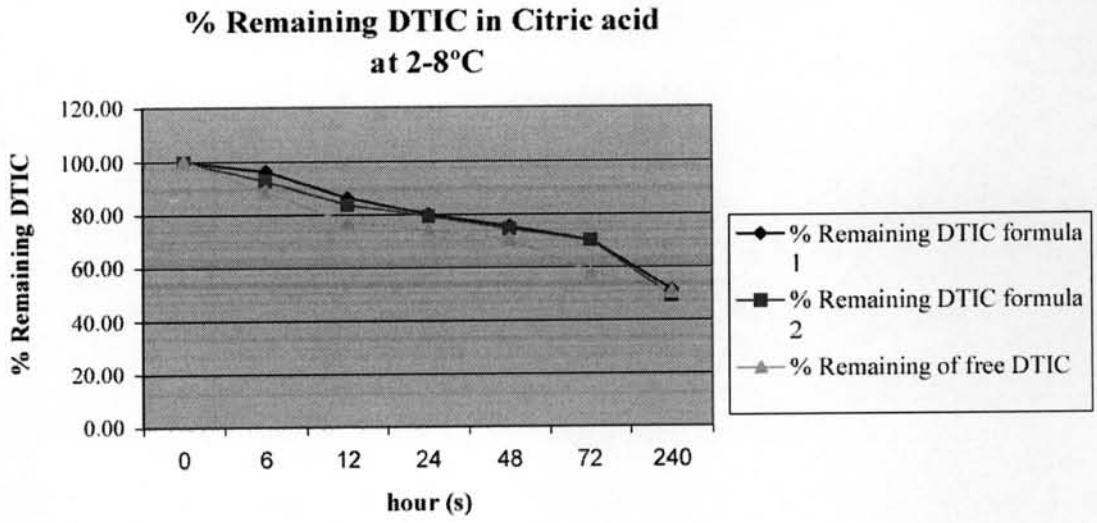


Figure 12 The percentage of remaining dacarbazine in citric acid at 2-8°C

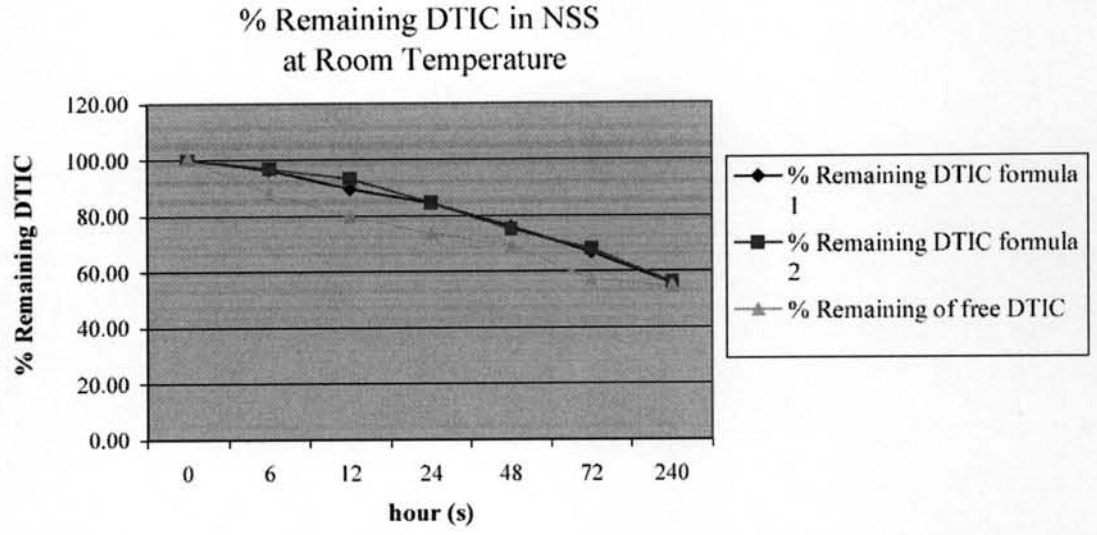


Figure 13 The percentage of remaining dacarbazine in normal saline solution (NSS) at room temperature

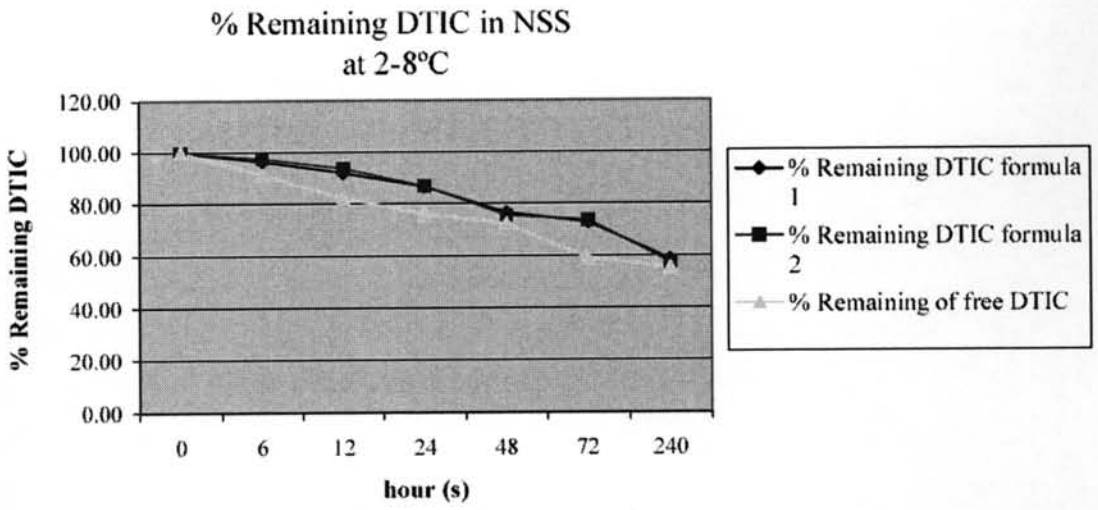


Figure 14 The percentage of remaining dacarbazine in normal saline solution (NSS) at 2-8°C

Figure 11 show the degradation profile of dacarbazine alginate nanoparticles coated with chitosan 15000 dalton (formula 1), coated with chitosan 100000 dalton (formula 2) and free dacarbazine as a function of storage time in pH 3-4 citric acid solution at room temperature. Dacarbazine chitosan-coated alginate nanoparticles, both formula 1 and 2, presented more stability within 72 hours than free dacarbazine ($p < 0.05$). After 10 days of storage, the stability of dacarbazine chitosan-coated alginate nanoparticles was not different from that of free dacarbazine insignificantly ($p > 0.05$). The percentage of remaining dacarbazine from chitosan-coated alginate nanoparticles formula 1, formula 2 and free dacarbazine at 10 days were 45.31%, 46.16% and 47.76%, respectively.

Figure 12 show the degradation profile of dacarbazine alginate nanoparticles coated with chitosan 15000 dalton (formula 1), coated with chitosan 100000 dalton (formula 2) and free dacarbazine as a function of storage time in pH 3-4 citric acid solution at 2-8°C. In the same way, dacarbazine nanoparticles, both formula 1 and 2, presented more stability within 72 hours than free dacarbazine ($p < 0.05$). After 10 days of storage, the stability of dacarbazine chitosan-coated alginate nanoparticles was not different from that of free dacarbazine ($p > 0.05$). The percentage of remaining dacarbazine from chitosan-coated alginate nanoparticles formula 1,

formula 2 and free dacarbazine at 10 days were 51.29%, 49.09% and 50.99%, respectively.

Figure 13 show the degradation profile of dacarbazine alginate nanoparticles coated with chitosan 15000 dalton (formula 1), coated with chitosan 100000 dalton (formula 2) and free dacarbazine as a function of storage time in normal saline solution (NSS) at room temperature. Dacarbazine chitosan-coated alginate nanoparticles, both formula 1 and 2, presented more stability within 72 hours than free dacarbazine ($p < 0.05$). After 10 days of storage, the stability of dacarbazine chitosan-coated alginate nanoparticles was not different from that of free dacarbazine ($p > 0.05$). The percentage of remaining dacarbazine from chitosan-coated alginate nanoparticles formula 1, formula 2 and free dacarbazine at 10 days were 55.66%, 56.12% and 54.61%, respectively.

Figure 14 show the degradation profile of dacarbazine alginate nanoparticles coated with chitosan 15000 dalton (formula 1), coated with chitosan 100000 dalton (formula 2) and free dacarbazine as a function of storage time in normal saline solution (NSS) at 2-8°C. Dacarbazine chitosan-coated alginate nanoparticles, both formula 1 and 2, presented more stability within 72 hours than free dacarbazine ($p < 0.05$). After 10 days of storage, the stability of dacarbazine chitosan-coated alginate nanoparticles was not different from that of free dacarbazine ($p > 0.05$). The percentage of remaining dacarbazine from chitosan-coated alginate nanoparticles formula 1, formula 2 and free dacarbazine at 10 days were 58.68%, 57.54% and 55.58%, respectively.

It can concluded that the stability in pH 3-4 citric acid solution and normal saline solution (NSS), either at room temperature or at 2-8°C of dacarbazine in chitosan-coated alginate nanoparticles better than that of free dacarbazine up to 72 hours and showed no difference from free dacarbazine after 10 days. Dacarbazine showed better stability because the drug was entrapped within the alginate matrix which protects them from environments. However, the stability of dacarbazine entrapped in chitosan-coated alginate nanoparticles was still not good because the remaining dacarbazine was less than 90% within short period of time. Thus, the dacarbazine nanoparticles must be developed for better stability.

In the same conditions, the stability of dacarbazine chitosan-coated alginate nanoparticles formula 1 showed no difference from formula 2 (Figures 11-14, $p > 0.05$). Thus, the difference of molecular weight did not influence to the stability of dacarbazine nanoparticles.

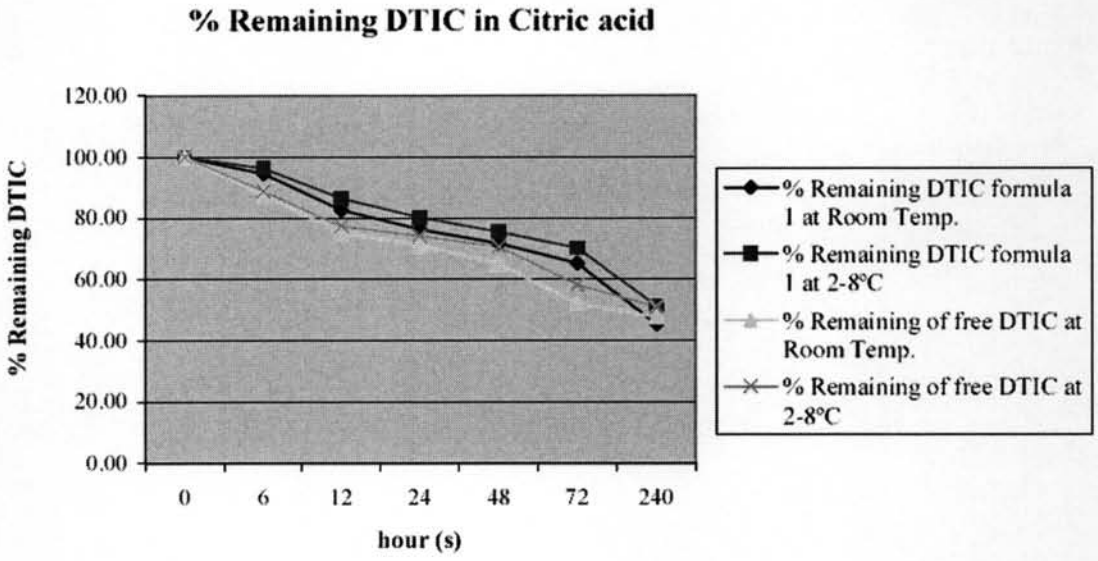


Figure 15 The percentage of remaining dacarbazine from dacarbazine chitosan-coated alginate nanoparticles formula 1 compared with free dacarbazine in citric acid solution pH 3-4

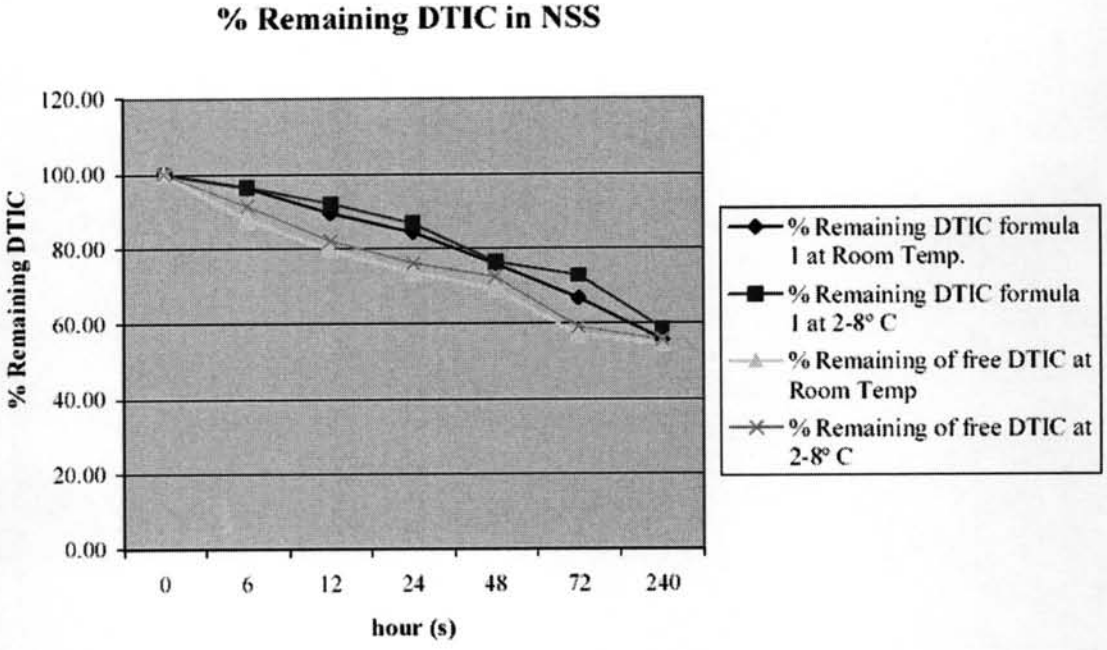


Figure 16 The percentage of remaining dacarbazine from dacarbazine chitosan-coated alginate nanoparticles formula 1 compared with free dacarbazine in normal saline solution (NSS)

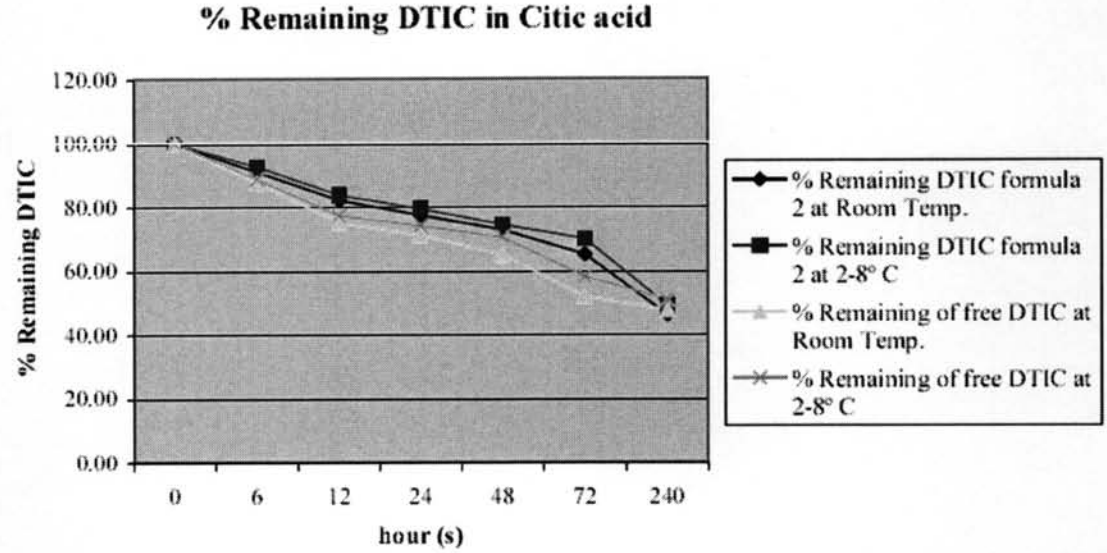


Figure 17 The percentage of remaining dacarbazine from dacarbazine chitosan-coated alginate nanoparticles formula 2 compared with free dacarbazine in citric acid solution pH 3-4

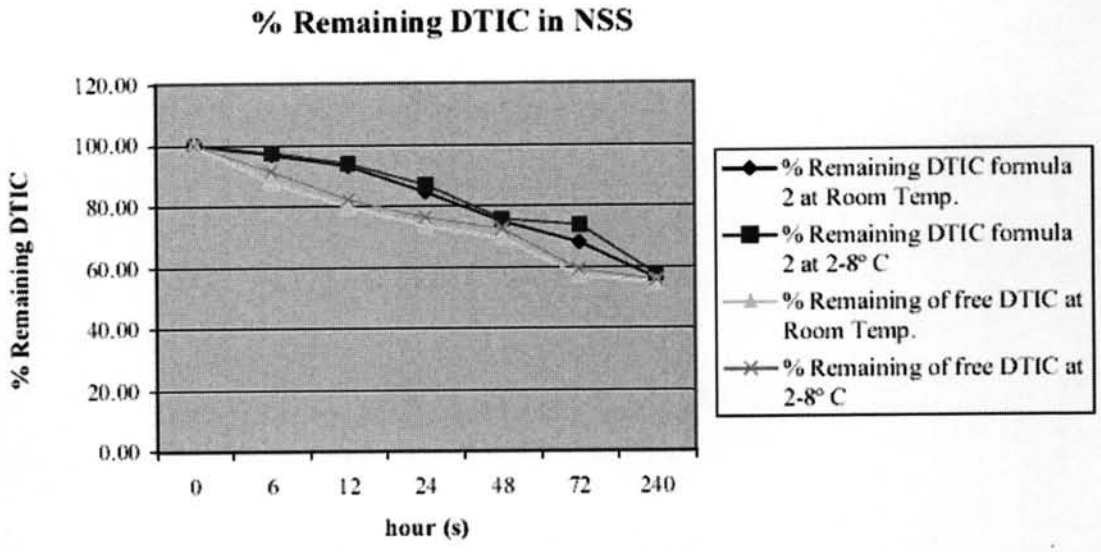


Figure 18 The percentage of remaining dacarbazine from dacarbazine chitosan-coated alginate nanoparticles formula 2 compared with free dacarbazine in normal saline solution (NSS)

Figures 15-18 show that the stability of both formulation of dacarbazine nanoparticles at 2-8 °C better than that at room temperature. This is because temperature greatly influences the stability of dacarbazine in solution (Williams and Lokich, 1992). At the higher temperature, the degradation of dacarbazine was faster. However, the stability of both formulation of dacarbazine nanoparticles stored at room temperature was still better than the stability of free dacarbazine stored at 2-8 °C.

The further study should be established to compare the stability of obtained dacarbazine nanoparticles with the stability of dacarbazine from commercial products. The obtained dacarbazine nanoparticles must be formulated using the same amount of dacarbazine and the excipients as the commercial products.