CHAPTER III

MATERIALS AND METHODS

Materials

1. Dacarbazine (Sigma, USA)
2. Sodium alginate (Carlo Erba, France)
3. Chitosan MW 15000 dalton, 90% deacetylation and MW 100000 dalton, 95% deacetylation (Seafresh Co., Ltd, Thailand)
4. Calcium chloride dihydrate (Carlo Erba, France)
5. Glacial acetic acid (Lab-Scan Co., Ltd, Ireland)
6. Acetonitrile HPLC grade (Lab-Scan Co., Ltd., Thailand)
7. Sodium dihydrogen phosphate dihydrate (Merck, Germany)
8. tri-sodium citrate dihydrate (Merck, Germany)
9. Citric acid (Ajax Finechem, Australia)
10. Normal saline solution (A.N.B. Laboratories Co., Ltd., Thailand)

Equipment

1. Analytical balance (Mettler Toledo XP205, Switzerland)
2. Ultracentrifuge (Beckman Coulter Optima™ LE-80K, USA)
3. High performance liquid chromatography (HPLC, Dionex, Germany) instrument equipped with the following:
   - System controller (Chromeleon 6.4)
   - Pump (P580A LPG)
   - Diode array detector (UVD340U)
   - Automated sample injector (ASI-100)
4. Column : Reverse phase column C18, Vertisep UPS, 5 μm, 4.6 × 250 mm (Vertical Chromatography Co., LTD, Thailand)

5. Freeze-dryer (Dura-Dry™, Science Engineer International Co., LTD, Thailand)
6. Transmission Electron Microscope (H-7000, Hitachi, Japan)
7. Zetasizer Nano ZS (Malvern, UK)
8. pH meter (Mettler Toledo MPC227, Switzerland)
9. Ultrasonic bath (TRU-SWEEP™ model 690D, USA)

Methodology

1. Preparation of chitosan-coated alginate nanoparticles containing dacarbazine

1.1 Preparation of sodium alginate solution

The sodium alginate solution was prepared in the concentration of 0.6 mg/mL. The solution was filtered through 0.45 μm membrane filter to remove particulates.

1.2 Preparation of calcium chloride solution

The calcium chloride solution was prepared in the concentration of 0.6 mg/ml. The solution was filtered through 0.45 μm filter disc to remove particulates.

1.3 Preparation of chitosan solution

The solution of 0.3 mg/ml chitosan was prepared in 2.0% v/v acetic acid. The solution was filtered through 0.45 μm filter disc to remove particulates.

1.4 Preparation of chitosan-coated alginate nanoparticles containing dacarbazine

The method of preparation was modified from De and Robinson (2003). In their study, the mass ratio of sodium alginate, calcium chloride and chitosan was 10:2:1. This mass ratio ensured that the calcium alginate was maintained in the pre-gel phase and sufficient cationic polymer was present to form nanoparticles (De and Robinson, 2003). Briefly, an accurate weight of dacarbazine was added to 10 mL of sodium alginate solution (0.6 mg/mL). The mixture was stirred under magnetic stirring at 100 rpm for 10 min. Then, an aqueous calcium chloride solution (2 mL of 0.6 mg/mL) was added dropwise to the agitated mixture of sodium alginate and

dacarbazine. The mixture was continuously sonicated for 30 min. Then, 2 mL of 0.3 mg/mL chitosan was added and stirred with a magnetic stirrer at 100 rpm for additional 30 min. The suspensions were left overnight in a refrigerator to allow nanoparticles to form. Then, the nanoparticles were isolated by ultracentrifugation at 50,000 rpm for 60 min at 4°C. The precipitates were redispersed in deionized water by sonicating for 30 sec. The nanoparticles were washed twice in deionized water by ultracentrifugation.

In this research, six formulations of dacarbazine chitosan-coated alginate nanoparticles were prepared by using three concentrations of dacarbazine (1, 2 and 5 mg) and two different molecular weight and percent deacetylation of chitosans (MW15000, 90% deacetylation and MW 100000, 95% deacetylation).

2. Characterization of nanoparticles

2.1 Determination of particle size and surface charge

The particle size, size distribution and zeta potential were measured by Zetasizer Nano ZS (Malvern, UK, facilitated by Nanotech). Samples were sonicated for 30 sec before measurement to ensure that the particles were well dispersed and the dispersion was homogeneous. The measurements were repeated three times for each sample.

2.2 Determination of particle morphology

The morphology of nanoparticles were determined by transmission electron microscopy (TEM) (Model Hitachi-7000, Japan) facilitated by Faculty of Tropical Medicine, Mahidol University.

3. Determination of entrapment efficiency

3.1 Unentrapped dacarbazine from nanoparticle suspension

The nanoparticle suspensions were ultracentrifuged at 50,000 rpm for 60 min at 4°C. The supernatants (unencapsulated dacarbazine) were collected for analysis. Then, the precipitates were washed twice in deionized water and sonicated for 30 sec before ultracentrifuged at the same condition. The supernatants from washing were collected for analysis. All fractions were analyzed by HPLC with suitable condition.

3.2 Entrapped dacarbazine in chitosan-coated alginate nanoparticles

The nanoparticles (precipitates) were lysed by addition of 5 mL of 0.5 M sodium citrate, followed by vortex mixing for 15 min. The suspensions were filtered through 0.45 μm filter disc and prepared for HPLC.

Entrapment efficiency was calculated by the following equation:

$$\text{Entrapment efficiency} = \frac{\text{Total amount of drug entrapped in nanoparticles}}{\text{Total amount of drug used}} \times 100$$

3.3 HPLC analysis

3.3.1 Preparation of standard solutions

An accurate weight of 1 mg dacarbazine was dissolved in 25 mL mobile phase solution by using 25 mL volumetric flask to obtain a stock standard solution having a concentration of 40 μg/mL. The stock standard solution of 5, 2.5, 2, 1.25, 0.5, 0.25, 0.125, 0.0625 mL were pipetted and transferred into 10 mL volumetric flask and adjusted to volume with mobile phase solution so that the final dacarbazine concentrations were 20, 10, 8, 5, 2, 1, 0.5 and 0.25 μg/mL, respectively.

3.3.2 Preparation of samples for analysis

An aliquot from each sample was diluted and adjust to volume with the mobile phase solution in a 10-ml volumetric flask and ready for HPLC analysis.

3.3.3 HPLC condition

The HPLC condition was used as follow

Column	: Reverse phase column C18, Vertisep UPS, 5 μm 4.6 × 250 mm
Mobile phase	: Acetonitrile : 10 mM monobasic sodium phosphate dihydrate (20:80) adjust to pH 4.2 with phosphoric acid
Flow rate	: 1 mL/min
Detector:	: UV-visible detector at wavelength 327 nm.
Injection volumn	: 20 μL
Run time	: 5 min
Retention time	: 3.4 min

3.3.4 HPLC Method Validation

1) Accuracy

The International Conference on Harmonization (ICH) documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels (three concentrations and three replicates of each concentration).

In this study, the accuracy was established from the standard solution. The known amount of dacarbazine were spiked into the mobile phase solution. Each sample was analyzed in three replicates. Accuracy in the terms of percent recovery was calculated from the drug found in standard solution.

2) Precision

The precision is expresses as the relative standard deviation of the measurements.

2.1) Repeatability (intra-assay precision)

The ICH recommends that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (three concentrations and three replicates of each concentration) within a laboratory over a short period of time using the same analyst with the same equipment.

In this study, three sample solutions with different concentration of dacarbazine were prepared. Each of them was analyzed in three replicates on the same day. The relative standard deviation (RSD) should not more than 2%.

2.2) Intermediate precision

According to the ICH documents, intermediate precision should be assessed as intra-assay precision but on the different days, or with different analysts or equipment within the same laboratory.

In this study, three sample solutions with different concentration of dacarbazine were prepared. Each sample was analyzed in three replicates on three different days. The relative standard deviation (RSD) should not more than 2%.

3) Linearity

The ICH recommends that a minimum of five concentrations normally be used for establishment of linearity.

In this study, five concentrations of sample solution were prepared and injected for 3 times. Three linearity with different stock solutions were generated. A linearity correlation coefficient above 0.999 was acceptable. Three linearity obtained from dacarbazine standard solution were also generated.

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.

The lower and upper concentrations on the calibration curve which the analytical method has adequate accuracy, precision were used as range.

5) System suitability

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 tailing factor is a measurement of peak symmetry obtained from peak area of five replicate injections of a standard solution.

In this study, a standard solution was injected for five replications. The % relative standard deviation was calculated from five peak areas. The obtained result should be less than 2%. The tailing factor of each five peaks from five replicate injections of a standard solution should be less than 2.

4. Freeze-drying process

The obtained dacarbazine chitosan-coated alginate nanoparticles with the highest entrapment efficiency were freeze-dried for further study. The dacarbazine chitosan-coated alginate nanoparticles in vials were freezed at -20°C and then were in the process under -60°C, 400 mTorr for 48 hours.

5. Stability evaluation

The obtained dacarbazine chitosan-coated alginate nanoparticles with the highest entrapment efficiency were chosen for stability evaluation. The chosen dacarbazine nanoparticle suspension was freeze-dried and characterized for the particle size, size distribution, zeta potential and particle morphology.

The stability of dacarbazine chitosan-coated alginate nanoparticles in aqueous solution were determined and compared with the stability of free dacarbazine in aqueous solution. The pH 3-4 citric acid solution and normal saline solution were used as aqueous solution in this study.

5.1 Preparation of dacarbazine solution and dacarbazine nanoparticle suspension

An aqueous dacarbazine solution was prepared by dissolving an accurate weight of 1 mg dacarbazine in 10 mL of pH 3-4 citric acid solution to obtain 100 μg/mL of free dacarbazine. An aqueous dacarbazine nanoparticle suspension was prepared by suspending freeze dried dacarbazine nanoparticles, equivalent to 500 μg of free dacarbazine in 10 mL of pH 3-4 citric acid solution to obtain 50 μg/mL of dacarbazine. Each solution and suspension was divided into two parts, one was diluted with the pH 3-4 citric acid solution to obtain 5 μg/mL of dacarbazine, and the other was diluted with normal saline solution to obtain 5 μg/mL of dacarbazine.

5.2 The stability in aqueous solution of dacarbazine chitosan-coated alginate nanoparticles

Each preparation was filled in 2 sets of six tubes for stability evaluation at two storage conditions, one set was stored at room temperature and the other set was stored at 2-8°C. All samples were protected from light. Free dacarbazine in solution or total dacarbazine in nanoparticle suspensions were analyzed by HPLC at the 6, 12, 24, 48, 72 hours and 10 days.

6. Statistical study

Mean comparisons were made by analysis of variance (ANOVA). Contrasts were considered significant at $P < 0.05$. Data were reported as means ± standard deviation (SD).