CHAPTER III

MATERIALS AND METHODS

Materials

1. Ascorbic acid (fine powder, minimum 98% through sieve No. 100, Batch No. 507362, Roche, Switzerland).
2. Uric acid (Sodium salt) (Batch No. 53H5031, Sigma Chemical Co., USA).
3. Dehydroascorbic acid (Batch No. 09624JN, Aldrich Chemical Co., USA).
4. Ethylcellulose (ethoxy content 48.6%, viscosity 10 cP (5% w/w solution in toluene-ethanol 80:20), Batch No. MM950213-1, Dow Chemical Co., USA).
5. Triacetin (Lot No. 326952/1 993, Fluka, Switzerland).
6. Triethyl citrate (Lot No. 328224/1 1293, Fluka, Switzerland).
7. Dibutyl sebacate (Batch No. 85H1327, Sigma Chemical Co., USA).
8. Span80 (Lot No. S6002, supplied by Srichand United Dispensary Co., LTD., Thailand).
9. Tween80 (Batch No. 91H0685, Sigma Chemical Co., USA).
10. Disodium edetate (Batch No. K22995418 623, Merck, Germany).
11. Sodium dihydrogenphosphate (Batch No. K22051045 530, Merck, Germany).
12. Orthophosphoric acid (Batch No. 4D050294G, Carlo Erba, Italy).
13. Cyclohexane (Batch No. 3L576233M, Carlo Erba, Italy).

14. Acetone (Batch No. K22097106 531, BDH, England).

15. Hexanes (Lot No. K26587, J.T. Baker, USA).

16. Chloroform (Batch No. K20693941 410, BDH, England).

17. Methanol HPLC grade (Lot No. 3041 KPDE, Mallinckrodt, USA).

18. Light liquid paraffin (Lot No. LDB74, supplied by Srichand United Dispensary Co., LTD., Thailand).

19. Deionized water.

All chemicals were of analytical or pharmaceutical grades and were used as received.

Equipment

1. Vessel (capacity 1 liter) with a flanged 4 port cover.

2. A variable-speed stirring motor fitted with a four-blade stirring shaft (Model R25, GmbH&Co., France).

3. Vacuum Pump (Model DOA-V130-BN, Waters, USA).

4. High performance liquid chromatography (HPLC) equipped with

System Controller (Waters 600E, USA),

Intelligent Sample Processor (WISP Model 712, Waters, USA),

Tunable Absorbance Detector (Model 484, Waters, USA),

Data Module (Model 746, Waters, USA),

C 18 column, Sphereclone 5μ ODS2, 250x4.60 mm (OOG-4144-EO S/N 152851, Phenomenex, USA).

6. Ultrasonicator (Model T900/H, Elma, Germany).

7. Centrifuge (Model 4206, ALC, Italy).

8. Optical microscope (Model KHC 211409, Olympus, Japan).

9. Ocular piece (Model Ocular P7X Micro, Olympus, Japan).

10. Objective micrometer (Olympus, Japan).

11. Scanning electron microscope (JEOL JSM-T220A, Model 5785, Japan).

12. UV/Visible spectrophotometer (Model 7800, printer Model PTL-3965, Jasco, Japan).

13. Dissolution apparatus (VK 7000, heater/circulator VK650A, Vankel Industries Inc., USA).

14. Electronic precision balance (Model RC219P, Sartorius, Germany).

15. pH meter (Model 420A, ORION, USA).

16. Simultaneous thermal analysis (Model 4094, Netzsch, Germany).

Methods

1. Preparation of Microcapsules

1.1 Temperature Induced Coacervation Technique

The microcapsules of ascorbic acid were prepared by temperature induced coacervation technique modified from a method described by Samejima et al. (1982) and Chemtob, Gruber, and Chaumeil (1989). The coating vessel used had 1 liter capacity fitted with a flanged 4 port cover. A stirrer was mounted through the center port and connected to the chuck of a variable speed motor. The remaining ports were used to provide the coating vessel with a reflux condenser, a thermometer, and an entry point. The lower part of the vessel was heated by immersion in a water bath.

Three hundred milliliters of cyclohexane was placed in the reaction vessel. With stirring at a suitable speed[1], an amount[2] of ethylcellulose used as a wall material was added at room temperature. The system was heated to the boiling point of cyclohexane (80-81°C) to generate a homogeneous polymer solution. An accurate amount of ascorbic acid was then dispersed in the solution and held for 10 min at this temperature. With continued stirring at the controlled rate, the system was allowed to cool within 100 min to 40°C. The mixture was then cooled quickly on an ice bath to 20°C. The stirring was continued further for 15 min. The microcapsules formed were recovered by decantation. The microcapsules were washed twice with 100 ml of organic solvent[3] at room temperature to remove any empty wall polymer coacervate droplets. The microcapsules were then separated by vacuum filtration, air dried at room temperature overnight and stored in a desiccator for further studies. The schematic of the method of preparation is illustrated in figure 14.

The following microcapsules were prepared using the above procedure to investigate the effects of formulation variables on properties of ascorbic acid microcapsules.

1.1.1 Effect of Core to Wall Ratios

The microcapsules of ascorbic acid were prepared with 1:2, 1:1, and 3:2 weight ratios of ascorbic acid (core material) to ethylcellulose (wall material). Three grams of ethylcellulose was weighed and transferred to the vessel containing 300 ml of cyclohexane and 1.5, 3 or 4.5 g of ascorbic acid was added depending on core to wall

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[1] Preliminary study at 400, 600, and 800 rpm.
[2] Preliminary study at ethylcellulose concentrations of 1 and 2 %w/v in cyclohexane.
[3] Organic solvents that were preliminarily studied were cyclohexane and hexanes.

300 ml of cyclohexane (+ 20 or 30 %w/w plasticizer)

+ Ethylcellulose

↓ continued stirring

Heated to 80°C to form a homogeneous solution

↓

+ Ascorbic acid

↓ 80°C 10 min

Slowly cooled to 40°C within 100 min

↓

Quickly cooled to 20°C

↓ continued stirring further for 15 min

Microcapsules recovered by decantation

↓

Washed twice with 100 ml organic solvent

↓

Vacuum filtration

↓

Air dried at room temperature overnight

↓

Ascorbic acid microcapsules

Figure 14. Typical steps of temperature induced coacervation technique for the microencapsulation of ascorbic acid.

ratio studied while other factors were controlled. The microencapsulation was performed in the same manner as 1.1. The core to wall ratio providing the highest yield of ascorbic acid microcapsules was selected to evaluate the effect of type and amount of plasticizer in 1.1.2.

1.1.2 Effect of Type and Amount of Plasticizer

The microcapsules were prepared using triacetin (TA), triethyl citrate (TEC), and dibutyl sebacate (DBS) as plasticizers. The plasticizer was added to cyclohexane in the preparation in an amount of 20 or 30 %w/w of the ethylcellulose weight. Then ethylcellulose was added and the procedure was continued in the same manner as 1.1.

1.2 Solvent Evaporation Technique

The microcapsules of ascorbic acid were prepared by solvent evaporation technique modified from a method described by Amperiadou and Georgarakis (1995) and Zinutti et al. (1996). Acetone was used as the polymer solvent; light liquid paraffin, the microencapsulation vehicle; and hexanes, the decanter of light liquid paraffin. An amount of ethylcellulose (5 %w/v) was dissolved in acetone[4]. A weighed amount of ascorbic acid, depending on the desired core to wall ratio, was dispersed in this solution. The dispersion was poured into light liquid paraffin[4] containing 1%w/v Span80 at a controlled speed[5] at room temperature (30±1°C) under ambient pressure. The stirring was continued for 5 h. Acetone was completely removed by evaporation.

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[4] Volumes of acetone and light liquid paraffin studied were 60-90 ml, and 200-240 ml, respectively.
[5] Preliminary study at 800, 900, and 1000 rpm.

Light liquid paraffin was decanted off. The collected microcapsules were washed twice with 100 ml of hexanes to remove oil from the surface of the microcapsules. The microcapsules were then separated by vacuum filtration, air dried overnight and stored in a desiccator for further studies. The schematic of the method of preparation is illustrated in figure 15.

The following microcapsules were prepared using the above procedure to investigate the effects of formulation variables on properties of ascorbic acid microcapsules.

1.2.1 Effect of Ethylcellulose Concentration

Microcapsules of ascorbic acid were prepared using 4, 5, and 6 %w/v ethylcellulose in acetone. The other factors controlled were the volumes of acetone and light liquid paraffin, 1:2 core to wall ratio, and 1 %w/v Span80. The concentration of ethylcellulose providing the highest yield of ascorbic acid microcapsules was chosen to investigate the effect of core to wall ratio in 1.2.2.

1.2.2 Effect of Core to Wall Ratio

The microcapsules were prepared with 1:2, 1:1, and 3:2 weight ratios of ascorbic acid (core material) to ethylcellulose (wall material) by varying amount of ascorbic acid and keeping amount of ethylcellulose constant. The other factors were controlled. The emulsifier used was 1.0 %w/v Span80. The core to wall ratio providing the highest yield of ascorbic acid microcapsules was chosen to evaluate the effect of type and amount of emulsifier in 1.2.3.

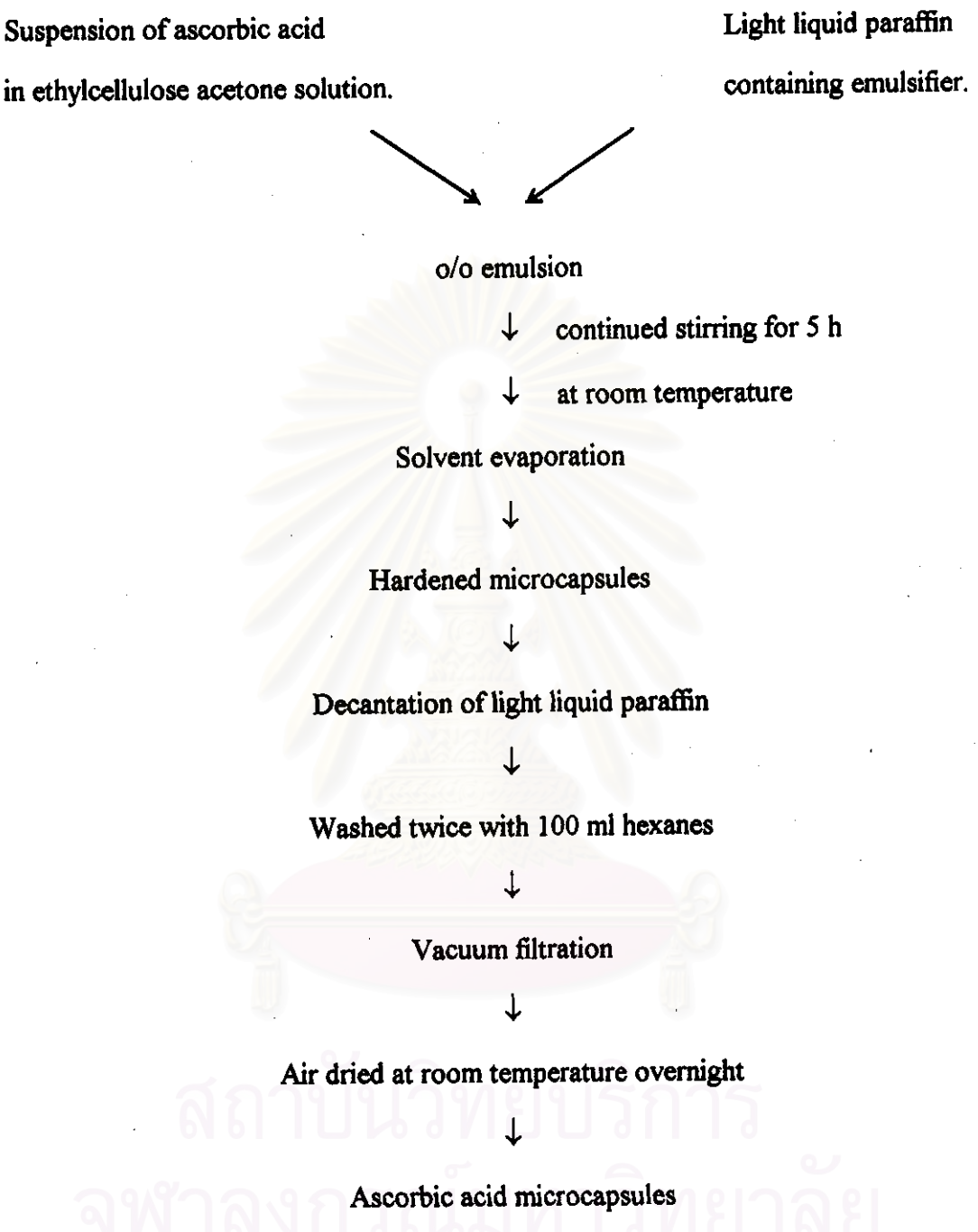

Figure 15. Typical steps of solvent evaporation technique for the microencapsulation of ascorbic acid.

1.2.3 Effect of Type and Amount of Emulsifier

Ascorbic acid microcapsules were prepared using Span80 and Tween80 as emulsifiers. The amounts of emulsifier studied were 0.5, 1.0, and 1.5 %w/v of the light liquid paraffin volume.

Summaries of the formulations of ascorbic acid microcapsules prepared according to 1.1.1, 1.1.2, 1.2.1, 1.2.2, and 1.2.3 are shown in tables 8 and 9 for temperature induced coacervation and solvent evaporation techniques, respectively.

2. Evaluation of Ascorbic Acid Microcapsules

2.1 Preparation of Standard Curve of Ascorbic Acid

2.1.1 UV/Visible Spectrophotometer

An aqueous solution of 5 mM EDTA was used for preparing standard ascorbic acid solutions as well as samples. This vehicle was found to stabilize ascorbic acid over the entire period of assay. About 10-11 mg of ascorbic acid was accurately weighed in a 100-ml volumetric flask. The drug was dissolved and adjusted to volume using 5 mM EDTA solution. Six appropriate dilutions were then made with the same vehicle to obtain standard solutions with various concentrations ranging from 2 to 14 µg/ml. The absorbances of these solutions were determined at a wavelength of 265 nm, which was the λ_{max} of ascorbic acid in this vehicle, using UV/Visible spectrophotometer and using 5 mM EDTA solution as a blank. The relationship

Table 8. Formulations of ascorbic acid microcapsules prepared by temperature induced coacervation technique.

Formulation no.	Variables	
	Core to wall ratio	%w/w Plasticizer
1	1:2	-
2	1:1	-
3	3:2	-
4	1:1	20% Triacetin
5	1:1	30% Triacetin
6	1:1	20% Triethyl citrate
7	1:1	30% Triethyl citrate
8	1:1	20% Dibutyl sebacate
9	1:1	30% Dibutyl sebacate

Table 9. Formulations of ascorbic acid microcapsules prepared by solvent evaporation technique.

Formulation no.	Variables		
	%w/v Ethylcellulose	Core to wall ratio	%w/v Surfactant
10	4	1:2	1.0% Span80
11	5	1:2	1.0% Span80
12	6	1:2	1.0% Span80
13	6	1:1	1.0% Span80
14	6	3:2	1.0% Span80
15	6	1:2	-
16	6	1:2	0.5% Span80
17	6	1:2	1.5% Span80
18	6	1:2	0.5% Tween80
19	6	1:2	1.0% Tween80
20	6	1:2	1.5% Tween80

between absorbances and concentrations was fitted using linear regression analysis. Specification of this method was done by spectrum scanning of each ingredient in the formulation.

2.1.2 HPLC Method

The determination of ascorbic acid by the modified reverse phase HPLC assay with UV detection was developed from a method described by Haralpanhalli, Howell, and Rao (1993). A mobile phase was a buffer consisting of 0.01 M sodium dihydrogenphosphate (NaH_2PO_4) and 0.2 mM EDTA; the solution pH was adjusted to 3.1 with orthophosphoric acid. The buffer was filtered through a Millipore HA 0.45 μm filter and degassed using an ultrasonicator prior to use. A column used was Sphereclone 5μ ODS2 and was maintained at ambient temperature. A constant flow rate of the mobile phase was 1.0 ml/min. An aqueous solution of 5 mM EDTA was used for preparing standard ascorbic acid solutions as well as samples. About 2 mg of ascorbic acid was accurately weighed into a 100-ml volumetric flask; the drug was then dissolved and diluted with the vehicle. Six appropriate dilutions were then made with the same vehicle in six 10-ml volumetric flasks, each containing 1 ml of internal standard solution (12 μg/ml uric acid (sodium salt) in 5 mM EDTA solution), to obtain standard solutions with various concentrations ranging from 2 to 14 μg/ml. Fifty microliters of these solutions was injected. The peak area was integrated by Waters 746 data module at a wavelength of 243 nm, which was the λ_{max} of ascorbic acid in the mobile phase, and at a sensitivity of 0.001 a.u.f.s. (Absorbance Units Full Scale). The relationship between peak area ratios of ascorbic acid to internal standard and concentrations (μg/ml) of standard solutions was fitted using linear regression analysis.

The HPLC method used to determine amount of ascorbic acid was validated under the following conditions.

2.1.2.1 Specificity

The specificity of the HPLC method used to determine ascorbic acid content in the prepared microcapsules was evaluated by comparing the chromatograms of vehicle used to prepare standard solutions and samples, ascorbic acid, internal standard, degradation products (dehydroascorbic acid and oxalic acid), ascorbic acid-internal standard (sodium urate), ascorbic acid-internal standard-dehydroascorbic acid, and other ingredients. The peak area of drug and internal standard must not be interfered by those of other compounds.

2.1.2.2 Linearity

The linearity was determined by plotting the standard curve between the peak area ratios of ascorbic acid to sodium urate and the concentrations of ascorbic acid (μg/ml). Then the standard curve was fitted using linear regression analysis. The coefficient of correlation (r) and the equation for the line were calculated.

2.1.2.3 Accuracy

The evaluation of accuracy of ascorbic acid assayed by HPLC method was done by analyzing percent recoveries of three injections of 4.34, 8.68, and 13.02 μg/ml ascorbic acid solution. Percent recovery of each injection was calculated

by dividing the concentration fitted from a calibration curve by the known concentration. The mean, standard deviation and percent coefficient of variation (%CV) were determined.

2.1.2.4 Precision

2.1.2.4.1 Within-run precision

The within-run precision was evaluated by analyzing peak area ratios of ascorbic acid to sodium urate of three injections of each concentration injected within the same day. The mean, standard deviation (SD) and percent coefficient of variation (%CV) of each concentration were determined.

2.1.2.4.2 Between-run precision

The between-run precision was evaluated by analyzing peak area ratios of ascorbic acid to sodium urate of three sets of calibration curves injected on different days. The mean, standard deviation and percent coefficient of variation (%CV) of each concentration were determined.

2.2 Scanning Electron Microscope (SEM)

The morphology of the surface of microcapsules was observed by scanning electron microscope (SEM). The physical appearance of microcapsules was visually observed. The color, shape, and aggregation of particles were recorded.

2.3 Microcapsule Size and Size Distribution

A sample of the produced microcapsules was dispersed in light liquid paraffin on a glass slide. The longest diameter of each microcapsule was determined using an optical microscope of which ocular scale had been already calibrated with an objective micrometer and the diameters of 625 particles were measured for each sample. Arithmetic mean diameter and standard deviation were calculated from frequency distribution table. The sizes (μm) were plotted against the cumulative percentage frequency undersize.

2.4 Yield of Microcapsules

An amount of the produced microcapsules was weighed and % yield was calculated according to equation (7).

$$\% \text{ Yield} = \frac{\text{Wt of produced microcapsules (g) X 100}}{\text{Theoretical Wt of microcapsules (g)}} \quad (7)$$

where

$$\text{Theoretical wt (g)} = \text{Wt of ascorbic acid (g) + Wt of ethylcellulose (g)} \quad (8)$$

2.5 Drug Content and Drug Entrapment

Drug content of ascorbic acid microcapsules was determined using the HPLC method developed in 2.1.2. Quadruplicate samples of microcapsules equivalent to

about 2.5-3.5 mg of ascorbic acid were accurately weighed and dissolved off all the coating materials with 5 ml of chloroform. Ascorbic acid in ethylcellulose-chloroform solution was then extracted twice, each time with 5 ml of 5 mM EDTA aqueous solution and the pooled aqueous solution was diluted with 5 mM EDTA solution in a 25-ml volumetric flask. The solution was adjusted to volume. One ml of this solution was pipetted and transferred into a 10-ml volumetric flask containing 1 ml of internal standard solution and assayed by the HPLC method. The amount of ascorbic acid was determined from the standard curve. The drug content and drug entrapment were calculated using equations (9) and (10), respectively. The mean value and standard deviation of drug content and drug entrapment were reported.

$$\% \text{ Observed content} = \frac{\text{Assayed amount of ascorbic acid X 100}}{\text{Amount of produced microcapsules}} \quad (9)$$

$$\% \text{ Entrapment} = \frac{\% \text{ Observed content X 100}}{\% \text{ Theoretical content}} \quad (10)$$

where

$$\% \text{ Theoretical content} = \frac{\text{Wt of ascorbic acid X 100}}{\text{Wt of ascorbic acid + Wt of ethylcellulose}} \quad (11)$$

2.6 Release Characteristics

Release characteristics of ascorbic acid from the microcapsules were undertaken using the USP XXIII rotating basket dissolution apparatus. An aqueous solution of 5 mM EDTA was used as the dissolution medium. From preliminary

studies, ascorbic acid was stable in this medium over the entire period of dissolution test (8 h). About 200, 120, and 100 mg of microcapsules having the core to wall ratios of 1:2 (Formulation no. 10-12 and 15-20), 1:1 (Formulation no. 2, 4-9, and 13), and 3:2 (Formulation no. 3 and 14), respectively, were accurately weighed and transferred into 900 ml of dissolution medium which was maintained at 37±0.1°C and stirred at a constant stirring rate of 100 rpm. Five-milliliter samples were withdrawn at appropriate time intervals and immediately replaced with fresh dissolution medium over a period of 4 h and 8 h for microcapsules prepared by temperature induced coacervation technique and solvent evaporation technique, respectively. The samples were then filtered through a 0.22 μm membrane filter unit and diluted with the dissolution medium to an appropriate concentration. The concentrations of ascorbic acid were then determined using a UV spectrophotometer at a wavelength of 265 nm. The mean percentage of ascorbic acid released, which was based on the total drug in the sample determined after each dissolution test as 100%, and standard deviation were computed. The mean percentage of ascorbic acid released was plotted against time (min) to obtain release profiles. Finally, the drug release profiles were fitted to Higuchi square root of time model according to equation (6). The correlation coefficient (r) and release rate constant (K) were calculated from an appropriate portion of the plots. Each release determination was performed in triplicate.

3. Chemical Stability of Ascorbic Acid Microcapsules

The formulations of ascorbic acid microcapsules that provided slow release rates were selected for chemical stability study. The formulations were stored in opened clear-glass bottles at 40°C and 75% R.H. for a period of 5 months.

Quadruplicate samples of each formulation were drawn and drug contents were assayed occasionally using the HPLC method according to 2.1.2. The ascorbic acid contents remaining in microcapsules were calculated using equation (12) and compared with those in the dry powder form stored under the same conditions.

$$\% \text{ Drug remaining} = \frac{\% \text{ Content at time t X 100}}{\% \text{ Content at initial time}} \qquad (12)$$