CHAPTER III

EXPERIMENTAL

Materials

The following materials were obtained from commercial sources and deionized water was used throughout the study.

1. Model drug

Acyclovir (Lot No.2002/20, Zhejiang wuyi Pharmaceutical Factory, China)

2. Additives

- Hydroxypropyl methylcellulose (4000 cps)
  (Methocel® K4M PremiumCR EP, Lot No. PL15012N01, Colorcon Asia Pacific Pte Ltd, Singapore)
- Xanthan gum (200 mesh)
  (Rheogel® 200 mesh, Lot No. OH0977A, CNI Colloid natural international, France)
- Sodium alginate NF 19
  (Lot No. 105035/2001BL669, Germany)
- Carbopol 934 P
  (Lot No. CC24HBB151, Noveon, U.S.A.)
- Lactose
  (Lot No. R1 45/00164, Wyndale, New Zealand)
- Dibasic calcium phosphate dihydrate
  (Emcompress®, Lot No. 7031X, Peawest Pharmaceuticals Co.,UK)
- Talcum
  (Lot No. 21023 010701,China)

- Magnesium stearate
  (Lot No. F1G253, Asia Pacific Pte Ltd., Australia)

3. Other chemicals

- Hydrochloric acid solution 37%, AR grade
  (Lot No. 03 02 0186, Lab-scan Analytical Sciences , Ireland)
- Potassium dihydrogen orthophosphate, AR grade
  (Lot No. F1F125, Asia Pacific Specialty Chemicals Ltd.,Australia)
- Sodium chloride crystals, AR grade
  (Lot No. F1K256, Asia Pacific Specialty Chemicals Ltd.,Australia)
- Sodium hydroxide pellets, AR grade
  (Lot No. B 131198 241, Merck, Germany)
- Methanol, HPLC grade
  (Lot No. 02 09 0153, Lab-scan Analytical Sciences, Ireland)
- Acetonitrile, HPLC grade
  (Lot No. 01 02 0099, Lab-scan Analytical Sciences, Ireland)
- Ammonium acetate, AR grade
  (Lot No. 238 C220716, Merck, Germany)
- Glacial acetic acid
  (Lot No. 227 K18049863, Merck, Germany)

4. Equipment

- Analytical balance
  (Model A200s, Sartorius GmbH, Germany)
- Dissolution apparatus
  (Model DT-6R, Erweka, Germany)
- Digital camera
  (Model C-720 Ultra zoom, Olympus)

- Hot air oven
  (Memmert, Germany)
- Image analyzer
  (Image-Pro®plus version 4.5 For Window™)
- Magnetic stirrer
  (HS-101, Harikul Ltd. Partnership, Thailand)
- pH meter
  (Model 210A$^+$, Thermo Orion, Germany)
- Single punch tabletting machine
  (EKO, Viuhang Engineering, Thailand)
- Tablet hardness tester
  (Model 2E/205, Schleuniger, Switzerland)
- Tablet thickness tester
  (Type SM-112, Teclock, Japan)
- Ultrasound transonic digital sonicator
  (Model D-78224, Elma, Germany)
- Ultraviolet / visible recording spectrophotometer
  (Model V-530, Jasco, Japan)
- Viscometer
  (Model DV-II$^+$, Brookfield, U.S.A.)
- Shaker bath
  (Polyscience®, U.S.A.)
- High performance liquid chromatography system
  (Model SCL-10A VP, Shimadzu, Japan)
- Friabilator
  (Erweka TAR20, Germany)
- Scanning electron microscope
  (Model JSM-5410 LV, Joel Ltd., Japan)

Methods

1. Preparation of acyclovir matrices

1.1 Formulation of acyclovir matrices

The formulation for matrices preparation is presented in Table 2.

Table 2 Formulation of acyclovir matrix tablets

Ingredient	% W/W		
Acyclovir	68.97		
Polymer [a]	10	15	20
Diluent [b]	17.03	12.03	7.03
Talcum	3		
Magnesium stearate	1		

[a] Hydroxylpropyl methylcellulose (4000 cps; HPMC), xanthan gum (200 mesh; XG), sodium alginate (SA) or carbopol 934P (CP)

[b] Lactose or dibasic calcium phosphate

The amount of acyclovir in each matrix tablet was 400 mg which used for administration twice a day. This was equivalent to administrate conventional acyclovir tablet 200 mg five times per day. The dose of acyclovir matrices was calculated from pharmacokinetic parameters which were previously mentioned in chapter II (section 5.5). Moreover, the dose and dosage regimen of acyclovir matrices prepared in this study was equivalent to those of patented acyclovir controlled-release capsule (Autant et al., 2000). The amount and the type of polymer and diluent used in each formulation of acyclovir matrices are shown in Table 3.

Table 3 The amount and the type of polymer and diluent used in each formulation of acyclovir matrices

Formulation	% Polymer (W/W)				% Diluent (W/W)	
	HPMC	XG	SA	CP	Lactose	Dibasic calcium phosphase
Blank A	-	-	-	-	27.03	-
Blank B	-	-	-	-	-	27.03
F1	10	-	-	-	17.03	-
F2	15	-	-	-	12.03	-
F3	20	-	-	-	7.03	-
F4	-	10	-	-	17.03	-
F5	-	15	-	-	12.03	-
F6	-	20	-	-	7.03	-
F7	-	-	10	-	17.03	-
F8	-	-	15	-	12.03	-
F9	-	-	20	-	7.03	-
F10	-	-	-	10	17.03	-
F11	-	-	-	15	12.03	-
F12	-	-	-	20	7.03	-
F13	10	-	-	-	-	17.03
F14	15	-	-	-	-	12.03
F15	20	-	-	-	-	7.03
F16	-	10	-	-	-	17.03
F17	-	15	-	-	-	12.03
F18	-	20	-	-	-	7.03
F19	-	-	10	-	-	17.03
F20	-	-	15	-	-	12.03
F21	-	-	20	-	-	7.03
F22	-	-	-	10	-	17.03
F23	-	-	-	15	-	12.03
F24	-	-	-	20	-	7.03

1.2 Preparation of acyclovir matrices

Acyclovir matrices were prepared by wet granulation method. Acyclovir, polymer and diluent in each formulation were weighed and mixed in a tumbling mixer for 20 minutes. Wet granulation was performed by spraying 96% isopropanol to the powder mixture in the mortar and then kneading by the pestle until the damp mass was formed. The damp mass was granulated through the oscillating granulator equipped with a 16-mesh screen and dried in hot air oven at 50 °C for 30 minutes. The dried granules were screened through a 18–mesh screen. Talcum and magnesium stearate were added and the mixture was blended in tumbling mixer for 3 minutes. The tablets were compressed using a single-punch tabletting machine with 1/2-inch diameter round concave punch and die. The tablet weight and hardness were adjusted to 580 mg and 7-9 kp, respectively. Two hundred tablets of acyclovir matrices were prepared for each formulation.

2. Preparation of placebo tablets

2.1 Formulation of placebo tablets

Under the condition selected for drug release studies, the peaks of non-active ingredients in the matrices must not interfere with the peak of acyclovir. The placebo tablets of similar composition without acyclovir were also prepared. The compositions of placebo tablet formulations are shown in Table 4.

Table 4 Formulation of placebo tablets

Ingredient	Amount per tablet (mg)		
Polymer	58 [b]	87 [d]	116 [f]
Diluent [a]	95.29 [c]	66.29 [e]	37.29 [g]
Talcum	5.8		
Magnesium stearate	17.4		

[a] Lactose or dibasic calcium phosphate

[b,c] Polymer (HPMC (4000 cps), xanthan gum (200 mesh, XG), sodium alginate (SA) or carbopol 934P (CP)) and diluent in placebo tablets were in the same amount of acyclovir matrices containing 10 % polymer, respectively.

[d,e] Polymer (HPMC (4000 cps), xanthan gum (200 mesh;XG), sodium alginate (SA))and diluent in placebo tablets were in the same amount of acyclovir matrices containing 15 % polymer, respectively.

[f,g] Polymer (HPMC (4000 cps), xanthan gum (200 mesh;XG), sodium alginate (SA) or carbopol 934P (CP)) and diluent in placebo tablets were in the same amount of acyclovir matrices containing 20 % polymer, respectively.

The amount and the type of polymer and diluent used in each formulation of placebo tablet are displayed in Table 5.

Table 5 The amount and the type of polymer and diluent used in each formulation of placebo tablets

Formulation	Polymer (per tablet, mg)				Diluent (per tablet, mg)	
	HPMC	XG	SA	CP	Lactose	Dibasic calcium phosphate
FP1	58	-	-	-	95.29	-
FP2	87	-	-	-	66.29	-
FP3	116	-	-	-	37.29	-
FP4	58	-	-	-	-	95.29
FP5	87	-	-	-	-	66.29
FP6	116	-	-	-	-	37.29
FP7	-	58	-	-	95.29	-
FP8	-	87	-	-	66.29	-
FP9	-	116	-	-	37.29	-
FP10	-	58	-	-	-	95.29
FP11	-	87	-	-	-	66.29
FP12	-	116	-	-	-	37.29
FP13	-	-	58	-	95.29	-
FP14	-	-	87	-	66.29	-
FP15	-	-	116	-	37.29	-
FP16	-	-	58	-	-	95.29
FP17	-	-	87	-	-	66.29
FP18	-	-	116	-	-	37.29
FP19	-	-	-	58	95.29	-
FP20	-	-	-	116	37.29	-
FP21	-	-	-	58	-	95.29
FP22	-	-	-	116	-	37.29

2.2 Preparation of placebo tablets

The procedure for preparation of placebo tablets was the same as that for preparation of acyclovir matrices. But the punch and die of smaller diameter were used because the weight of placebo tablets was lower than that of acyclovir matrices. The 10–cm. diameter round flat faced punch and die were used to compress the placebo tablet. The tablet weight and hardness were adjusted to 176.5 mg and 7-9 kp, respectively.

3. Evaluation of acyclovir matrices and placebo tablets

The following evaluation procedures were used for acyclovir matrices. The placebo tablets were evaluated for drug release only (see section 3.5.2.1).

3.1 Determination of weight variation

Weight variation test was carried out by weighing 20 tablets individually. The mean and standard deviation were calculated.

3.2 Determination of thickness and hardness

Tablets thickness and hardness were evaluated by using tablet thickness tester and tablet hardness tester, respectively. Twenty tablets were measured individually. The mean and standard deviation were calculated. The thickness and hardness of tablets were reported in terms of millimeters and kilopounds, respectively.

3.3 Determination of tablet friability

The friability was performed on 20 tablets using the Roche type friabilator. The drum was rotated at 25 rpm for 4 minutes. Loss of tablet weight with respect to the initial value was calculated as percent friability.

3.4 Determination of acyclovir content in matrices

The drug content of acyclovir in matrices was quantitatively determined by means of absorption peak area from HPLC method. The validation of analysis of acyclovir by HPLC is in Appendix A.

3.4.1 HPLC conditions

The chromatographic conditions were developed as follows.

Column	:	Alltech®, 4.6 x 250 mm (C18, 5μm)
Detector	:	UV detector at 250 nm
Flow rate	:	1 ml/min
Injection volume	:	20 μl
Mobile phase	:	A mixture of 0.02 M ammonium acetate buffer pH 4.5 and acetonitrile in a volume ratio of 88 : 12
Diluent medium	:	A mixture of acetonitrile and ultrapure water in volume ratio of 60 : 40

Preparation of 0.02 M ammonium acetate buffer pH 4.5

Ammonium acetate 1.54 g. was dissolved in 980 ml of ultrapure water. The pH of solution was adjusted to 4.5 with dropwise addition of glacial acetic acid. The final volume was subsequently adjusted to 1000 ml with ultrapure water.

Preparation of mobile phase

The 0.02 M ammonium acetate buffer pH 4.5 (880 ml) was mixed with 120 ml acetonitrile. The mobile phase was freshly prepared and filtered through a 0.45 μm membrane filter. It was degassed by sonication for 30 minutes prior to use.

3.4.2 Calibration curve of acyclovir

About sixty milligrams of acyclovir was accurately weighed into a 100-ml volumetric flask. The drug was dissolved and adjusted to volume with diluting medium. This solution was used as standard stock solution. Then 0.2, 0.4, 0.6, 0.8 and 1 ml of standard stock solution were individually pipetted and transferred into 10 ml-volumetric flasks, diluted and adjusted to volume with diluting medium. The final concentrations of the standard solutions were 12, 24, 36, 48 and 60 μg/ml, respectively.

3.4.3 Assay of acyclovir content in matrices

Twenty tablets of each formulation were taken by random sampling. They were pulverized by mortar and pestle. The powder (44 mg) was accurately weighed into a 50-ml volumetric flask which was equivalent to about 30 mg of acyclovir. The powder was dissolved in diluting medium and sonicated for 60 minutes. Then, the solution was adjusted to volume with the same medium and mixed thoroughly. This solution was filtered through 0.45 μm membrane. The filtrate (0.6 ml) was pipetted into a 10-ml volumetric flask, diluted and adjusted to volume with the same medium. The final concentration of the sample solution was about 36 μg/ml.

3.5 Determination of drug release from matrices

3.5.1 Calibration curves of acyclovir

Calibration curves of acyclovir in deionized water, 0.1 N HCl solution, phosphate buffer pH 6.8 solution, phosphate buffer pH 6.8 solution with ionic strength adjusted to 0.1 with sodium chloride, 0.05, 0.1 and 0.2 M NaCl solutions were constructed.

Thirty five milligrams of acyclovir was accurately weighed into a 200-ml volumetric flask. The drug was dissolved and adjusted to volume with various media as mentioned above. This solution was used as standard stock solution. The standard stock solutions of 2, 3, 4, 5, 6, 7 and 8 ml were individually pipetted into the 100 ml volumetric flasks, diluted and adjusted to volume with the same medium. The final concentrations of each standard solution were 3.5, 5.25, 7.0, 8.75, 10.5, 12.25 and 14.0 μg/ml, respectively.

The standard solutions were assayed spectrophotometrically at a wavelength of 252 nm, which was the λ_{max} of acyclovir in each medium, except in 0.1 N HCl solution. The λ_{max} of acyclovir in 0.1 N HCl solution was at 255 nm. The calibration curve of each medium was determined in triplicate. The relationship between absorbances and concentrations of acyclovir was established using linear regression analysis.

3.5.2 Dissolution study

The dissolution study was performed by USP 24 standard apparatus II (paddle) at 37 °C ± 0.5 °C with a rotation speed of 50 rpm. In addition, perforated stainless steel plates were placed on the bottom of vessels in order to keep the matrices from sticking to the wall. The distal paddles were calibrated at 2.5 cm above the perforated stainless steel plates. In order to investigate the influences of pH and ionic strength of dissolution media on drug release from matrices, 900 ml of deionized water; 0.1 N HCl solution; phosphate buffer pH 6.8 solution (PBS pH 6.8); phosphate buffer pH 6.8 solution with ionic strength adjusted to 0.1 with sodium chloride (PBS pH 6.8 + NaCl); 0.05; 0.1; 0.2 M NaCl solutions were employed as dissolution media. The dissolution test of each formulation was done in triplicate.

The sample of 10 ml were withdrawn at the time intervals of 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7, 8,10 and 12 hrs. The same volume of the medium was immediately added after sampling to keep the constant volume of the medium in the vessel throughout the experiment.

Each sample was filtered through filter paper. The filtrate was diluted to suitable concentration in order to be determined by UV/visible spectrophotometry at the maximum wavelength of the drug in each dissolution medium.

The amounts of acyclovir release at any times were calculated from the calibration curve of each dissolution medium. A cumulative correction was performed to account for the previously removed sample to determine the total amount of drug release. The drug release values reported were based on the average of three determinations of each formulation.

3.5.2.1 Dissolution studies of placebo tablets
(Formulations FP1-FP22)

The objective of the dissolution studies of placebo tablets was to verify specificity of the method for quantitative analysis of drug release. Dissolution test of low polymer containing (10% polymer; corresponding to high level of diluents) and high polymer containing (20% polymer; corresponding to low level of diluents) formulation was performed in 0.1 N HCl solution and PBS pH 6.8. In case of 15% polymer containing formulation, dissolution test was performed in deionized water, 0.2 M NaCl solution and PBS pH 6.8+NaCl. The samples were withdrawn at the time intervals of 1, 6 and 12 hours. The concentration of sample was determined without further dilution. The other procedures were conducted as specified above. The results obtained from the dissolution studies of placebo tablets are shown in Appendix B.

3.5.2.2 Dissolution studies of matrices blank A and B

Dissolution tests of blank A and B were performed in 0.1 N HCl solution, phosphate buffer pH 6.8 solution, deionized water and 0.2 M NaCl. The procedure was conducted as specified above.

3.5.2.3 Dissolution studies of matrices containing 15% polymer (Formulation F2, F5, F8, F11, F14, F17, F20, F23)

Dissolution test of each formulation was performed in all dissolution media. The procedure was conducted as specified above.

3.5.2.4 Dissolution studies of matrices containing10% polymer (Formulation F1, F4, F7, F10,F13, F16, F19,F22) and 20%polymer (Formulation F3, F6, F9, F12, F15, F18, F21, F24)

Each formulation was studied for drug release by using 0.1 N HCl solution and phosphate buffer pH 6.8 solution as dissolution media. The procedure was conducted as specified above.

3.5.2.5 pH change dissolution studies

The pH change dissolution method (changing the pH of the dissolution medium while running the dissolution test) was also used in order to simulate the environment in the gastro-intestinal tract. The selective formulations were tested by transferring the perforated plates after 2 hours from 0.1 N HCl solution to phosphate buffer pH 6.8 solution. The samples were withdrawn at the time intervals of 0.25, 0.5, 0.75, 1, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 10 and 12 hours. The procedure was conducted as specified above.

3.5.3 Data analysis

The dissolution data were fitted according to the following well-known exponential equation given below, which is often used to describe the drug release behavior from polymeric systems:

$$\frac{M_t}{M_\infty} = kt^n$$

where M_t/M_∞ is the fraction of drug released, t is the release time, k is a kinetic constant (with units of t^{-n}) incorporating structural and geometric characteristics of the release device and n is the release exponent indicative of the mechanism of release. This equation can be used to analyze the first 60% of a release profile where the release profile is linearly related to t^{-n}, regardless of geometric shape (Ritger and Peppas, 1987). Values for n and k for each matrix formulation were obtained by plotting the logarithm of the fractional release against the logarithm of time. The slope of the line is n while log k is the intercept. The drug release data were plotted using values of M_t/M_∞ within the range of 0-0.60.

Although the constant k is one of the measures of the drug release rate, it should not be used for comparison because different kinetics are usually involved in different test conditions (Talukdar et al., 1996). Therefore, to characterize the drug release rate in different experimental conditions, relative dissolution time (RDT) was calculated from dissolution data by using following equation.

$$RDT = \frac{ABC}{M_\infty}$$

This equation was calculated based on that for determining mean dissolution time (MDT) (Brockmeier and Hattingberg, 1982). The diagrammatic of dissolution profile for explaining RDT calculation is illustrated in Figure 7.

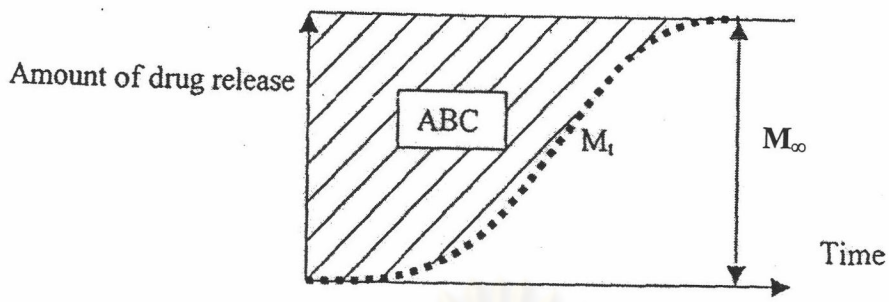

Figure 7 Diagrammatic of dissolution profile for explaining RDT calculation. ABC is area between upper line (M_∞) and the dissolution curve; M_∞ is maximum drug release at infinite time and M_t is amount of drug release at any time t.

ABC was calculated indirectly by subtracting total area (M_∞ multiplied with time function) with area under dissolution curve (AUC). The trapezoidal method was used to calculate AUC. In this study, relative dissolution time (RDT) was calculated from dissolution data up to 12 hours and was used for comparing the release profile under different test conditions.

3.6 Determination of drug solubility

Solubility of acyclovir was investigated in all dissolution media. Excess amounts of acyclovir were mixed with each dissolution medium in erlenmeyer flask. The samples were shaken in a controlled temperature shaker bath at 37 °C ± 0.5 °C. An aliquot was withdrawn, filtered and assayed in triplicate for acyclovir concentration periodically, until constant concentration was obtained. The concentration of acyclovir was analyzed by UV/visible spectrophotometry at λ_{max} of the drug in each dissolution medium.

3.7 Determination of polymer solution viscosity

The 2% w/w solutions of HPMC, XG, SA and CP were prepared with all dissolution media. In order to complete the hydration of the polymer, the gel was kept at room temperature overnight. The measurement of viscosity was performed with viscometer (model DV-II$^+$, Brookfield, U.S.A.). The viscosity was determined in triplicate with a shear rate of 50 rpm at 37 °C ± 0.5 °C.

3.8 Determination of matrix swelling and matrix erosion

Only formulations F2, F5 and F8 were investigated for matrix swelling and matrix erosion. The following dissolution media were used for investigating matrix swelling and matrix erosion: 0.1 N HCl solution, phosphate buffer solution pH 6.8, deionized water and 0.2 M NaCl solution.

The dissolution apparatus was set as described in the method of dissolution study. The perforated plate with individual tablet was removed at the time intervals of 0.5, 1, 2, 4, 6, 8 and 12 hours. In order to examine the radial swelling of the tablet, the area of swollen tablet was determined by photographing the tablet with the calibrated scale at the top view by a digital camera. The areas of swollen tablets were determined at all time intervals by using image analyzer and then the percent swellings were calculated. After that, the swollen tablets were dried in hot air oven at 60 °C until constant weight was achieved. The total weight loss of swollen tablets after drying were determined and estimated as the percent erosion. The test was done in triplicate for each time interval. The percent swelling and erosion of swollen tablets could be calculated according to the equations (1) and (2), respectively.

$$\% \text{ Swelling} = (At-Ao)/Ao*100 \qquad (1)$$

where At is the area of swollen tablet at a time interval, Ao is the area of tablet at the initial time.

$$\% \text{ Erosion} = (Wo-Wt)/Wo*100 \quad (2)$$

where Wt is the weight of dried tablet at a time interval, Wo is the weight of tablet at the initial time

3.9 Surface morphology of acyclovir matrices

Only formulations F2, F5 and F8 were investigated for their surface morphology by scanning electron microscopy. The matrices were investigated both before and after the dissolution test. In preparation of samples for scanning electron microscopy, tablet of each formulation was hydrated in either 0.1 N HCl solution , phosphate buffer pH 6.8 solution, deionized water or 0.2 M sodium chloride solution using the same condition of the dissolution test. Afterwards, these swollen tablets at various time intervals (2, 6 and 12 hours for formulation F2 and F5 and 2, 4 and 6 hours for formulation F8) were dried in hot air oven under the same procedure for matrix erosion test. The dried matrices with constant weight were used to evaluate their surface morphology. The sample was positioned on the sample holder and then observed after being coated with gold.