CHAPTER III

EXPERIMENTAL

Materials

All chemicals were analytical or HPLC grade and were used as received.

1. Ranitidine HCl (a gift from Glaxo Wellcome, Thailand, Lot No. XR04R 9701).
2. β - cyclodextrin (supplied by Rama Production Co., Ltd., Thailand, Lot No. 23111).
3. 2-Hydroxypropyl-β-cyclodextrin with degree of substitution = 0.2 (Fluka, Switzerland, Lot No. 375315/1 24597).
4. Procaine HCl (Fluka, Switzerland, Lot No. 376890/1 45097).
5. Sodium dihydrogen phosphate (Fluka, Switzerland, Lot no. 348694/1 795).
6. Methanol HPLC grade (Mallinckrodt Baker, Inc., USA, Lot No 3041 KVBT).
7. Acetonitrile HPLC grade (Mallinckrodt Specialty Chemicals Co., USA, Lot No 2856 KLTK).
8. Triethanolamine (Fluka, Switzerland, Lot No. 30911881).
9. Sodium chloride (Farmitalia Carlo Erba, Italy, Lot No. 479687).
10. Sodium hydroxide (Merck KGaA, Germany, Lot No. B870498 625).
11. Phosphoric acid (Farmitalia Carlo Erba, Italy, Lot No. 4D050294G).
12. Disodium hydrogen phosphate (Merck KGaA, Germany, Lot No. F127286 628).
13. Glacial acetic acid (J.T. Baker Inc., USA, Lot No. E45830).
14. Sodium acetate trihydrate (E. Merck, Germany, Lot No. 306 TA 404665).
15. Glycine (Fluka, Switzerland, Lot No. 322642/1 493).
16. Trisodium phosphate dodecahydrate (E. Merck, Germany, Lot No. 305 A 687278).

17. Magnesium nitrate hexahydrate (Fluka, Switzerland, Lot No. 360693/1 30498).
18. Potassium nitrate (Fluka, Switzerland, Lot No. 371010/1 22697).
19. Potassium carbonate (E. Merck, Germany, Lot No. 606 A 99628).
20. Sodium nitrite (supplied by Srichand United Dispensary Co., Ltd., Thailand, Lot No. SER 49/894).

Equipment

1. Analytical balance (Satorius GMPH, Scientific Promotion Co. Ltd., Germany).
2. Ultra sonic bath (T900/H, Elma, Germany).
3. Hot air oven (UL 50, Memmert, Germany).
4. pH meter (Beckman, USA).
5. Micropipet (Pipet man, Gilson Medical Electronics, France).
6. Digital hygrometer, thermometer, dew point (Fisher Scientific, USA).
7. FTIR Spectrometer (Spectrum 2000, Perkin Elmer, USA).
8. Ball mill (The Pascall Engineering co., Ltd., England).
9. Shell-freezer (SF-4, Just-A-Tilt shell freezer, USA).
10. Freeze-dryer (FD-6-85 DMPO, Dura-Dry, Japan).
11. Differential scanning calorimeter (DSC 7, Perkin Elmer, USA).
12. Scanning electron microscope (JSM-6400, Joel, Japan).
13. X-ray diffractometer (JDX-8030, Joel, Japan).
14. NMR (DPX 300, Bruker, Switzerland).
15. High-Performance Liquid Chromatography (HPLC) equipped with:
    a solvent delivery module (LC10AD, Shimadzu Corp., Japan),
    a variable wavelength UV detector (SPD10A, Shimadzu Corp., Japan),
    a data integrating software (LC10, Shimadzu Corp., Japan),
    an automatic sample injector (SIL-10A, Shimadzu Corp., Japan),
    a communications bus module (CBM-10A, Shimadzu Corp., Japan),

a C_{18} reverse phase chromatographic column (μ - Bondapak C18 125Å, 300x3.9 mm, 10 μm , Lot No. T71043, Water Corp., USA).

Methods

1. **Preparations of Ranitidine HCl (R) : Cyclodextrin (β-CD, 2HP-β-CD) Inclusion Complexes**

Three methods were used for preparation of the inclusion complexes. These included co-grinding, freeze-drying, and kneading. Physical mixtures were also prepared for comparison. Three molar ratios of R : CD (1 : 1, 1 : 2, and 2 : 1) were used in this study.

1.1 Co-Grinding Method

Ranitidine HCl and cyclodextrins were accurately weighed according to certain ratios showed in Table 4. Ranitidine HCl and each cyclodextrin were co-ground in a ball mill (The Pascall Engineering co., Ltd., England) using 200 balls, at a speed of 75 rpm. Samples were collected at 30 min, 1 hr, and 3 hr, respectively, and then screened through a 40-mesh sieve. The products were kept in amber glass bottles and stored in a desiccator for further study.

1.2 Freeze-Drying Method

Ranitidine HCl and cyclodextrins were accurately weighed according to certain ratios showed in Table 4. The cyclodextrin was dissolved in distilled water to obtain a clear solution at ambient temperature. Then ranitidine HCl was added to the solution that was agitating vigorously by a magnetic stirrer, and the agitation was prolonged for an hour. Approximately 100-150 mL of this solution was transferred to

Table 4. Composition of ranitidine HCl (R) : CD (β-CD, 2HP-β-CD) inclusion complexes.

System	Preparation methods	Molar ratios of R : CD	Amount (g)		
			R	β-CD	2HP-β-CD
R:β-CD	Co-grinding	1 : 1	2.81	9.08	-
		1 : 2	1.40	9.08	-
		2 : 1	3.51	5.68	-
	Freeze-drying	1 : 1	0.35	1.14	-
		1 : 2	0.35	2.27	-
		2 : 1	0.70	1.14	-
	Kneading	1 : 1	0.70	2.27	-
		1 : 2	0.70	4.54	-
		2 : 1	1.40	2.27	-
	Physical mixture	1 : 1	0.35	1.14	-
R:2HP-β-CD	Co-grinding	1 : 1	2.10	-	8.28
		1 : 2	1.05	-	8.28
		2 : 1	3.51	-	6.90
	Freeze-drying	1 : 1	0.35	-	1.38
		1 : 2	0.35	-	2.76
		2 : 1	0.70	-	1.38
	Kneading	1 : 1	0.70	-	2.76
		1 : 2	0.70	-	5.52
		2 : 1	1.40	-	2.76
	Physical mixture	1 : 1	0.35	-	1.38

500-mL erlenmeyer flasks, and then frozen at a temperature of about - 40°C in a shell - freezer (Just - A - Tilt, Model SF-4, USA) containing ethanol. The 500-mL erlenmeyer flasks were used to yield a large surface area producing a thin layer of substance that would dry rapidly by Dura -Dry (Model FD-6-85 DMPO, Japan). During the whole procedure, the substance was protected from light. Then the products were kept in amber glass bottles and stored in a desiccator for further study.

1.3 Kneading Method

Ranitidine HCl and cyclodextrins were accurately weighed according to certain ratios showed in Table 4. The kneaded mixture was prepared by gently triturating the drug and CD in a glass mortar with a pestle and was mixed for 5 minutes. Then 50% ethanol in water was added drop-wise and the mixture was kneaded for 10 minutes to obtain homogeneous paste. The paste was dried in a hot air oven (UL 50, Memmert, Germany) at 40°C overnight. Then, the kneaded mixture was grounded and screened through a 40-mesh sieve. The powder was collected in amber glass bottles and stored in a desiccator for further study.

1.4 Physical Mixture

Ranitidine HCl and cyclodextrins were accurately weighed according to certain ratios showed in Table 4. The physical mixture was prepared by simply blending of ranitidine HCl and cyclodextrin in a glass mortar with a pestle for 5 minutes. Then the physical mixture was kept in amber glass bottles and stored in a desiccator for further study.

2. Detection of Ranitidine HCl (R) : Cyclodextrin (β-CD, 2HP-β-CD) Inclusion Complex Formation

FTIR spectroscopy, differential scanning calorimetry (DSC), and powder X-ray diffractometry (XRD) were used to characterize the solid state complexes, while proton nuclear magnetic resonance (^1H NMR) spectrometry was performed to detect the interactions between the drug and CDs in solution. In addition, the appearance and morphology of the pure components and the obtained products were examined using a scanning electron microscope.

2.1 FTIR Spectroscopy

Fourier-transform infrared spectra of the pure components, all the inclusion complexes and the physical mixture were obtained using potassium bromide (KBr) disks detected by an FTIR spectrometer (Spectrum 2000, Perkin Elmer, USA). The data of each sample were averaged from 32 scans.

2.2 Differential Scanning Calorimetry (DSC)

DSC thermograms were obtained using a Perkin Elmer DSC-7 apparatus (Perkin Elmer, USA). The temperature axis was calibrated using indium and zinc standard. Powder samples of 2-5 mg in weight were crimped in perforated 30 μL aluminum pans. All DSC runs of the pure components, all the inclusion complexes, and the physical mixture were performed at a heating rate of 10°C/min from 80 or 100°C to 200°C, using nitrogen as a purging gas. An empty pan, which was sealed in the same way as the sample, was used as a reference.

2.3 Powder X-ray Diffractometry (XRD)

Powder X-ray diffractograms of the pure components, the inclusion complexes having molar ratios (R:CD) of 1:1, and the physical mixture were obtained using a Joel Model JDX-8030 diffractometer (Joel, Japan). Samples were irradiated using nickel-filtered CuK_{α} at a wavelength of 1.542 Å operated using a voltage of 45 kV, a current of 30 mA, a time constant of 1.50 s, and a scanning speed of 1°/min. The samples were investigated in the 2 θ range of 5 - 60°.

2.4 Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR)

Proton NMR experiments were performed at 300 MHz using a Bruker DPX 300 spectrometer (Bruker, Switzerland) at a temperature of 300 K. Samples of the pure components and the 1:1 freeze-dried inclusion complexes were solubilized in D_2O and water signal (4.70 ppm) was used as a reference.

2.5 Scanning Electron Microscopy

The appearances and morphological features of the pure components were compared with those of the inclusion complexes having molar ratio of 1:1 using the scanning electron microscopy. The scanning electron micrographs of the particulate samples covered with gold were made using a Joel Model JSM-6400 electron microscope (Joel, Japan) with an accelerating voltage of 20 kV. The scanning electron micrographs were taken at magnifications of 200 and 700, respectively.

A preparation method providing homogeneous inclusion complexes with the least amount of CDs used were chosen for preparing inclusion complexes for further study of the effects of pH and relative humidity.

3. **Effect of pH on the Chemical Stability of Inclusion Complexes of Ranitidine HCl : Cyclodextrin (β-CD, 2HP-β-CD)**

Buffer solutions having pH values of 1, 3, 5, 7, 9, 11, and 13 were prepared and their details are presented in Table 5. The ionic strength of all buffer solutions were kept constant at 0.5 using sodium chloride (NaCl). Ranitidine HCl or selected R:β-CD or selected R:2HP-β-CD inclusion complex was dissolved in the buffer solutions to possess the final ranitidine HCl concentration of 10 mg/mL. One - mL of the solutions was pipetted by using a micropipet and transferred to 2-mL transparent borosilicate glass vials. The vials were closed with rubber closures and covered with aluminum caps using a hand crimper. These vials were stored in a hot air oven at 70°C. Four vials were sampling for analysis of ranitidine HCl remaining at appropriate time intervals.

Appropriate rate constants were calculated using regression analysis. General and/or specific acid-base catalyses were determined.

4. **Effect of % Relative Humidity (RH) on the Chemical Stability of Inclusion Complexes of Ranitidine HCl : Cyclodextrin (β-CD, 2HP-β-CD)**

One hundred milligrams of ranitidine HCl or selected R:β-CD or selected R:2HP-β-CD inclusion complex was accurately weighed and transferred to a 5-mL amber glass vial. Four vials of the drug and each complex were prepared and placed in a desiccator containing a saturated aqueous solution of a salt. Table 6 displays the salts used to obtain relative humidities of 41, 51, 62, 75, and 91%, respectively, at 37°C. Actual relative humidities were measured by Digital Hygrometer, Thermometer, Dew Point (Fisher Scientific, USA). After two weeks of storage, the final weight of vials were accurately weighed by an analytical balance (Satorius GMPH, Scientific Promotion, Germany) and the amount of ranitidine HCl remaining was determined by HPLC.

Table 5. Details of buffer solutions.

pH*	Conc.	Buffer composition (M)					Added
	(M)	H_3PO_4	NaH_2PO_4	Na_2HPO_4	CH_3COOH	CH_3COONa	NaCl
1	0.10	0.0890	0.0110	-	-	-	0.4000
phosphate	0.20	0.1780	0.0220	-	-	-	0.3000
	0.30	0.2671	0.0329	-	-	-	0.2000
3	0.05	0.0038	0.0462	-	-	-	0.4500
phosphate	0.10	0.0075	0.0925	-	-	-	0.4000
	0.20	0.0150	0.1850	-	-	-	0.3000
5	0.10	-	-	-	0.0261	0.0739	0.4000
acetate	0.20	-	-	-	0.0523	0.1477	0.3000
	0.30	-	-	-	0.0784	0.2216	0.2000
7	0.05	-	0.0137	0.0363	-	-	0.3774
phosphate	0.10	-	0.0274	0.0726	-	-	0.2548
	0.20	-	0.0547	0.1453	-	-	0.0094

Table 5. Details of buffer solutions (continued).

pH*	Conc.	Buffer composition (M)				Added
	(M)	Glycine	NaOH	Na_2HPO_4	Na_3PO_4	NaCl
9	0.10	0.1000	0.0157	-	-	0.4843
glycine-NaOH	0.20	0.2000	0.0314	-	-	0.4686
	0.30	0.3000	0.0471	-	-	0.4529
11	0.10	0.1000	0.0950	-	-	0.4050
glycine-NaOH	0.20	0.2000	0.1900	-	-	0.3100
	0.30	0.3000	0.2850	-	-	0.2150
13	0.03	-	-	0.0120	0.0288	0.3236
phosphate	0.05	-	-	0.0020	0.0480	0.2060
	0.08	-	-	0.0032	0.0768	0.0296

* pH's were calculated by:

$$pH = pK_a + \log \frac{[salt]}{[acid]_j} - \frac{0.51\,(2n-1)\,\mu^{1/2}}{1+\mu^{1/2}} \qquad \text{at } 25^\circ C$$

$$\mu = \frac{1}{2} \sum_1 C_i Z_i^2$$

where μ is the ionic strength

C_i is the concentration in moles per liter of the i^{th} ion

Z_i is the valence of the i^{th} ion

Table 6. Saturated salt solutions for maintaining constant relative humidities in desiccators (from Wade and Weller, 1994).

Saturated salt solutions*	% Relative humidity at 37°C
Potassium carbonate	41
Magnesium nitrate	51
Sodium nitrite	62
Sodium chloride	75
Potassium nitrate	91

* Prepared from reagent grade salts dissolved in purified water.

5. Assay of Ranitidine HCl

The high-pressure liquid chromatographic (HPLC) method of Gupta (Gupta, 1988) was modified and used for analysis of remaining ranitidine HCl in the presence of its degradation products.

5.1 Chromatographic Conditions

The properly modified high-performance liquid chromatographic conditions for analysis of ranitidine HCl remaining are as follows:

Column : a μ - bondapak C_{18} column (300 x 3.9 mm),10 μm.

Mobile phase : a mixture of 35% v/v acetonitrile and 65%v/v 0.02 M pH 6.2 sodium dihydrogen phosphate buffer.

Detector wavelength	: 290 nm.
Flow rate	: 1.0 mL/min.
Attenuation	: 16.
Injection volume	: 5 μL.
Internal standard	: 10 μg/mL procaine HCl.
Retention times	: ranitidine HCl, 5.5 - 7.0 min.
	: procaine HCl, 7.5 - 9.0 min.

The buffer solution used for preparing the mobile phase was prepared by weighing of 2.76 grams of anhydrous sodium dihydrogen phosphate in a 1000-mL volumetric flask. It was dissolved and the final volume was adjusted using distilled water. The pH was adjusted to 6.2 using triethanolamine.

5.2 Standard Solutions

A stock solution of internal standard was prepared by accurately weighing of 50 milligrams of procaine HCl in a 25-mL volumetric flask. Purified water was added to dissolve the internal standard and adjust the final volume.

A stock solution of ranitidine HCl was prepared by accurately weighing of 20 milligrams of ranitidine HCl in a 25-mL volumetric flask. Purified water was used to dissolve the drug and adjust the final volume. Then, 5 mL of this solution was pipetted and transferred to a 100-mL volumetric flask. Purified water was used to adjust the final volume.

Standard solutions of ranitidine HCl were prepared by pipetting 0.5, 1, 2, 3, 4, 5, and 6 mL of the ranitidine HCl stock solution and transferring each aliquot to each one of seven 10-mL volumetric flasks, respectively. Then 50 μL of the procaine HCl stock solution was added into each of these volumetric flasks. The solutions were

adjusted to volume with purified water so that the concentrations of the standard solutions were 2, 4, 8, 12, 16, 20, and 24 μg/mL, respectively, and that of procaine HCl was 10 μg/mL. Three sets of standard solutions were prepared for each HPLC run.

5.3 Validation of the High-Pressure Liquid Chromatographic Method

The analytical parameters used for the assay validation were specificity, precision, accuracy, and linearity (USPXXIII).

5.3.1 Specificity

Under the chromatographic conditions used, the peak of ranitidine HCl must be completely separated from and not be interfered by the peaks of other components in the sample.

5.3.1.1 In the Presence of CDs and Buffer Compositions

Blank buffer solutions having the highest concentration at each pH in Table 5, sample solutions of 10 mg/mL ranitidine HCl, and selected R:CD inclusion complexes having ranitidine HCl concentration of 10 mg/mL in those buffer solutions were injected. Chromatograms were evaluated by comparing them with the standard solutions of ranitidine HCl.

5.3.1.2 In the Presence of Degradation Products of Ranitidine HCl

Sample solutions of 10 mg/mL ranitidine HCl and R:CD inclusion complexes selected having ranitidine HCl concentration of 10 mg/mL in 0.30 M pH 1 and 0.08 M pH 13 phosphate buffer solutions were forced to decompose by storing them in a hot air oven at 70°C for 1 month. The decomposed solutions and the blank

buffer solutions used in 5.3.1.1 were injected. Their chromatograms were compared with the standard solutions of ranitidine HCl.

5.3.2 Precision

5.3.2.1 Within Run Precision

The within run precision was determined by analyzing three sets of the seven standard solutions of ranitidine HCl in the same day. Peak area ratios of ranitidine HCl to procaine HCl were compared and the percent coefficient of variation (%CV) for each concentration was determined.

5.3.2.2 Between Run Precision

The between run precision was determined by comparing each concentration of three sets of ranitidine HCl standard solutions prepared and injected on different days. The coefficients of variation (%CV) of ranitidine HCl to its internal standard peak area ratios from the three sets of standard solutions having the same concentration were calculated.

5.3.3 Accuracy

Three sets of the seven standard solutions of ranitidine HCl were prepared and injected. The percentage of analytical recovery of each standard solution was calculated.

5.3.4 Linearity

Seven ranitidine HCl standard solutions were prepared and analyzed. Linear regression analysis of the peak area ratios versus their concentrations was performed by the method of least squares.