Chapter II

Experimental

Materials

The following materials obtained from commercial sources were used as received

1 Ascorbic acid (Distributed by Pharmaceutical Traders Co.,Ltd, Thailand, Lot no.812732)
2. Hydrochlorothiazide (Marsing, Denmark, Batch no.M0802)
3. Isoniazid (Korea, Batch no.725055)
4. Rice flour (Cho-heng, Thailand)
5. Spray dried rice starch (Era-Tab^{R}, Erawan Pharmaceutical, Thailand)
6. Pregelatinized corn starch (Starch 1500^{R}, Colorcon, England, Batch no.611035)
7. Microcrystalline cellulose (Avicel PH 102^{R}, FMC, USA., Lot no. 2611)
8. Dicalcium phosphate, dihydrate (Emcompress^{R}, Mendell, USA., Lot no.K27A)
9. Trisodium trimetaphosphate (Sigma Chemical, USA., Lot no.103 H0282)
10. Sodium carbonate, anhydrous (Carlo Erba, Italy, Code no.479307)
11. Sodium hydroxide (Eka Nobel, Sweden, Lot no.080992)
12. Hydrochloric acid (Carlo Erba, Italy, Code no.403872)

13. Potassium dihydrogen phosphate (Merck, Germany, Lot no. A739373)
14. Magnesium stearate (Peter Greven Fett-Chemic, Germany)
15. Talc (Haicheng, Thailand, Code 21943-010323)

Apparatus

1. Analytical balance (Sartorious, Model A200s, Germany)
2. Top load balance (Sartorious, Model 1264MP, Germany)
3. Magnetic stirrir (Thermolyne, Model no.SP46920-261
4. Hot air oven (Memmert, Type UL 80, Germany)
5. Spectrophotometer (Bausch&Lomb, Spectronic-2000, USA.)
6. Carver laboratory press (Perkin Elmer, Model C, Fred&Corver Inc., USA.)
7. Hardness tester (Schleuniger, Model 2E/205, Dr.K. Schleuniger&Co., Switzerland)
8. Erweka friabilator (Erweka, Type TAP, Germany)
9. Disintegration apparatus (Hanson Research, USA.)
10. Dissolution apparatus (Hanson Research, Model SR2, USA)
11. Single punch tabletting machine (Viuhang Engineering, Model A3, Thailand)
12. Scanning electron microscope (Jeol, T220A, Japan)
13. Brabender viscometer (Brabender, Type 801260, Germany)
14. Spray drying apparatus (NIRO ATOMZER Mobile Minor unit, Denmark)
15. Differential scanning calorimetry (Netzsch, Model DSC 200, Germany)

Methods

Preparation of Modified Rice Starch

1. Deproteinization of Rice Starch

500 g of rice starch flour was dispersed in 500 ml water and mixed with 380 ml of 0.87 % NaOH for 5 min by high speed mixer (Janke & Kunkel, Germany) The starch slurry was treated at 50 ^{0}C and held for 4 hrs with agitation at this temperature. After complete these processes, treated starch slurry was centrifuged at 3,000 rpm by supercentrifuge (Hitachi, Japan) for 20 min. The starch sediment was collected, washed with distilled water and repeated this step until the least protein remained in rice starch.

2. Crosslinking of Deprotienized Rice Starch

350 g of deproteinized rice starch dispersed in water was mixed with 210 ml of solution in which 11 g of sodium trimetaphosphate, 10 g of sodium carbonate anhydrous and 3.3 g of sodium hydroxide had already been dissolved. The pH of starch was adjusted to 11.0 by sodium hydroxide and then heated to 50 ^{0}C with continuous stirring and kept at this temperature for 6 hrs . Then it was neutralized with 1 N HCl and washed with distilled water, centrifuged and dried.

3. Spray Drying Process

Rice starch that modified by chemical reaction from 1 and 2 was milled and dispersed in water until homogeneous slurry was obtained. Then 50 % w/w of slurry was sprayed into the spray drying chamber, using proper condition described as follows.

Concentration of dispersion : 50 % W/W

Inlet air temperature : 130 ^{0}C

Atomizing air pressure : 3.0 bar

Feed rate : 11.5 ml/min

Detection of Phosphate and Crosslinking Reaction in Modified Rice Starch

Phosphate content in modified rice starch was determined by spectrophotometry (Maurice, 1975 ; Smith, 1967). The crosslinking reaction was confirmed by differential scanning calorimetry, X-ray diffraction and the evaluation of viscosity.

1. Percent Phosphate

Ten grams of modified rice starch was accurately weighed into porcelain dish. Ten millitres of 2 % calcium acetate solution was added, distributing the solution uniformly through the sample by adding distilled water. The sample was evaporated to dryness on a steam bath, heated on a hot plate until thoroughly charred and then ignited 2 hr in a muffle furnace (Muffle furnace, England) at 600 $\pm$ 25^{0}C. The dish was cooled to room temperature, and the ash was washed by 15 ml of distilled water followed by 5 ml of 29 % nitric acid. The solution was quantitatively transferred to a 250 ml volumetric flask. The transfer was completed by washing the dish with distilled water, and the solution was diluted to volume and mixed thoroughly. An aliquot selected to contain not more than 2.5 mg of phosphorus was pipetted into a 100 ml volumetric flask and 25 ml of distilled water was added into another 100 ml volumetric

flask serving for the reagent blank. The following reagent were added to both flasks, in the order stated, mixing after each addition : 7.5 ml of ammonium molybdate and 2.5 ml of hydroquinone are added, and then, after 5 min, 12.5 ml of sulfite-carbonate buffer. The solution can be made up to 100 ml with distilled water and the absorption measured after a few minutes at 753.9 nm in a 1-cm matching cuvette. Potassium dihydrogen phosphate concentration (μg/ml) of the sample solution was read from the standard curve in Figure 61 (in Appendex I) that was prepared follows.

Potassium dihydrogen phosphate 4.394 g was accurately weighed and dissolved in distilled water. The solution was adjusted to 1,000 ml with distilled water and used as stock solution. The stock solution was individually pipetted 0.075, 0.09, 0.10, 0.13, 0.15, 0.20 and 0.25 ml into 50 ml volumetric flask. The following reagent were added to both flasks, in the order stated, mixing after each addition : 7.5 ml of ammonium molybdate and 2.5 ml of hydroquinone are added, and then, after 5 min, 12.5 ml of sulfite-carbonate buffer. The solution can be made up to volume with distilled water. The final concentration of each solution was 0.15, 0.18, 0.20, 0.26, 0.30, 0.40 and 0.50 μg/ml, respectively.

The absorbance of known drug concentration was determined using the reagent blank in UV absorption spectrophotometer in a 1-cm cell at 753.9 nm. Each concentration was determined in duplicated.

The degree of crosslinking was measured directly by analysis of phosphate content in modified rice starch (see Appendix VIII).

2. Differential Scanning Calorimetry

Thermal properties of modified starch were examined using differential scanning calorimetry (Netzsch, Germany). Each sample was milled and mixed with distilled water in the ratio of 1:3. Samples (15-20 mg) were transferred into previously weighed aluminium pans. The pans were immediately sealed in a sample encapsulating press. The reference pan contained 15 µl of distilled water. DSC was carried out at a heating rate of 10 ^{0}C/min.

3. Powder X-ray Diffraction

X-ray diffractograms of samples were determined by the reflexion method with nickle-filtered Cukα radiation of Jeol diffractometer (model JDX8030, Japan) operated in the ϖ-2θ scanning mode between 5^0 and 85^0.

4. Viscosity

Ten grams of dry starch was dispersed in 100 ml water and the slurry was transferred to Brabender viscometer. The measurements of viscosity were performed at the three cycles, respectively, as follows.

- Heating cycle: heat the starch dispersion from room temperature (25 ^{0}C) to 95 ^{0}C.
- Holding cycle: hold the temperature at 95 ^{0}C for 30 min.
- Cooling cycle: let the dispersion cool down from 95 ^{0}C to 50 ^{0}C.

The heating rate was 3.0 ^{0}C/min. The stirring rate was 75 rpm.

Properties of Modified Rice Starch

The following properties of modified rice starch powder according to USP XXIII, were investigated.

1. Identification

- A water slurry of modified rice starch was colored reddished violet to deep blue by iodine TS

- Ten grams of modified rice starch was dispersed in 20 ml of cold water. The sample suspension was transferred into 150 ml of boiling water, boiled gently for 2 minutes, and cooled. The product was a translucent, whitish jelly.

2. pH

About 10 g of modified rice starch was shaked with 80 ml of water for 10 min and centrifuged. The pH of the supernatant liquid was between 4.5 and 7.0.

3. Loss on Drying

Modified rice stach was dried at 120 ^{0}C for 4 hours. It lost not more than 14.0 % of its weight.

4. Residue on Ignition

Two grams of modified rice starch was weighed accurately in a tared crucible dish, ignited gently until the substance was thoroughly chared. Then, it was cooled, moisted with 1 ml of 1 N H_2SO_4 and heated again until white fume no longer evolved. The residue was ignited in the

furnace (Muffle furnace, England) at 800±25 ^{0} C until the carbon was consumed. The crucible was cooled in a dessicator, weighed and calculated based on the initial dry weight. The percentage of ash was an average of three determinations.

5. Iron

The determination was made by concomitant visual comparision with a control prepared from a standard iron solution.

Standard iron solution : 863.4 mg of ferric ammonium sulfate was dissolved in water before adding 10 ml of 2 N H_2SO_4 and diluted with water to 100 ml. 10 ml of this solution was pipetted into a 1000 ml volumetric flask, 10 ml of 2 N H_2SO_4 was added and diluted with water to volume mix. This solution contains the equivalent of 0.01 mg of iron per ml.

Ammonium thiocyanate solution : 30 g Ammonium thiocyanate was dissolved in water to make 100 ml.

Standard preparation : 1 ml of standard iron solution was transferred into a 50 ml color comparision tube.The specimen was diluted with water to 50 ml, added with 2 ml of 1 N HCl and mixed.

Test preparation : The 0.5 g of residue obtained in the test for residue on ignition in 8 ml of 1 N HCl with the aid of gentle heating was diluted with water to 100 ml, and mixed. 25 ml of this solution was transferred into with water to 50 ml volumetric flask, and diluted to volume with water.

Procedure : 50 mg of ammonium persulfate crystals and 3 ml of ammonium thiocyanate solution were added to each of the tubes containing the standard preparation and the test preparation, and mixed. The color of the solution from the test preparation was not darker than that of the solution from the standard solution.

6. Oxidizing Substances

4.0 g of modified rice starch was transferred into a glass-stoppered, 125 ml conical flask containing 50 ml of water. The sample was swirled for 5 minutes, decanted into a glass-stoppered, 50 ml centrifuge tube and centrifuged to clarify, respectively. 30 ml of clear supernatant liquid was transferred into a glass-stoppered 125 ml conical flask containing 1 ml of glacial acetic acid and 0.5 g to 1.0 g of potassium iodide. The sample was swirled and allowed to stand for 25 to 30 minutes in the dark. 1 ml of starch TS was added. The sample was titrated with 0.002 N sodium thiosulfate until the starch-iodine color was disappeared. Not more than 1.4 ml of 0.002 N sodium thiosulfate was required (0.002 %).

7. Sulfur Dioxide

20 g of modified rice starch was mixed with 200 ml of water until a smooth suspension was obtained and filtered. 3 ml of starch TS was added to 100 ml of the clear filtrate. The sample was titrated with 0.01 N iodine to the first permanent blue color: Not more than 2.7 ml was consumed (0.008 %).

8. Microbial Limits: Test for *Salmonella Species* and *Escherichia coli*

Fluid Lactose Medium was added and made to 100 ml in a 500 ml Erlenmeyer flask containing 10 g of modified rice starch. It was incubated and examined for growth. 1 ml portions was pipetted into 100 ml Erlenmeyer flask containing, respectively, 10 ml of fluid Selenite-Cystine Medium and Fluid Tetrathionate Medium. They were mixed and incubated for 24 hours.

Test for *Salmonella Species*

By means of an inoculating loop, both the selenite-cystine and tetrathionate media was streaked portions from on the surface of Brilliant Green Agar Medium, Xylose-Lysine-Desoxycholate Agar Medium, and Bismuth sulfite Agar Medium contained in petri dishes. The dishes were covered, inverted and incubated for 24 hrs. Upon examination, if none of the colonies conforms to the description given in Table 2 , the specimen meets requirements of the test for absence of the genus *Salmonella*.

Table 2 Morphologic Characteristics of *Salmonella Species* on Selective Agar Medium.

Selective Medium	Characteristic Colonial Morphology
Brilliant Green Agar Medium	Small, translucent, colorless or pink to white opaque (frequently surrounded by pink to red zone)
Xylose-Lysine-Desoxycholate Agar Medium	Red, with or without black centers
Bismuth Sulfite Agar Medium	Black or Green

Test for *Escherichia coli*

By means of an oculating loop, The remaining Fluid Lactose Medium was streaked on the surface of MacConkey Agar Medium. The dishes was covered, inverted and incubated. Upon examination, if none of the colonies conforms to the description given in Table 3 for this medium, the specimen meets the requirements of the test for absence of *Escherichia coli.*

Table 3 Morphologic Characteristics of *Escherichia coli* on MacConkey Agar Medium.

Gram Stain	Characteristic Colonial Morphology
Negative rods (cocci-bacilli)	Brick-red; may have surrounding zone of precipitate bile

Evaluation of Physical Properties of Modified Rice Starch Compared with Commercial Diluents

1. Powder Morphology

Morphology of powder samples were determined with scanning electron microscopy. The sample were coated with gold prior to the microscopic examination using ion sputting. Size, shape and surface topography of powders were observed, and then photographed at appropriate magnification.

2. Particle Size Distribution

Particle size distribution was determined using sieve analysis. Twenty grams of powders were put on the top of a sieve series (Endecotts Ltd, England) ranging from 125, 106, 90, 75 to 45 μm, respectively. The nest of sieve was placed on the sieve shaker (Josef Deckelman, Germany) for 20 minutes. The results averaged from two determinations were reported as percentage of weight retained on each sieve size. The geometric mean diameter was taken from graph.

3. Bulk and Tapped Density

Bulk density was performed by pouring an accurate weight of each sample (about 20 g) into a 100 ml graduated cylinder and measuring the bulk volume. Tapped density was performed by dropping the graduated cylinder containing the sample onto a hard wood surface from a height of 5 cm. until the powder attained a constant tapped volume.

The bulk and tapped densities were calculated by dividing the weight of sample by its bulk volume and tapped volume. The results were presented as an average of three trials. The compressibility was calculated from the following equation.

$$\% \text{ Compressibility} = \frac{(T-B)}{T} \times 100$$

T = Tapped density

B = Bulk density

4. Angle of Repose Determination

Angle of repose was determined by cylinder method. Appropriate amount of powder was carefully filled in the cylinder (height = 8.15 cm., radius = 4.3 cm.) placed on the graph paper. When powder was filled at the top of cylinder, slowly lifted the cylinder in the vertical direction, producing round heap. The results averaged from three determinations were reported. Angle of repose was calculated from the following equation.

$$\propto = \tan^{-1}\frac{H}{R}$$

$\propto$ = Angle of repose

H = Height of heap

R = Radius of heap

5. Flow Rate

Accurate weight of about 30 g of powder was filled in a glass funnel 6.5 mm internal stem diameter fixed on a clamp. The time was recorded when the powder started to flow until finished. The flow rate was expressed in g/sec.

6. Moisture Determination

Ten grams of sample was accurately weighed on a pan of the moisture determination balance (OHAUS, USA.), dried until constant weight was obtained. The result was shown as percent moisture content.

Tabletting Characteristics of Modified Rice Starch Compared with Commercial Diluents

Four diluents: Era-Tab^{R}, Starch 1500^{R}, Avicel PH102^{R} and modified rice starch were determined for their tabletting characteristics. Each diluent was compressed into tablets by Carver laboratory press. The 300 mg of each sample without additives was weighed and compressed at compression forces of 500 , 1,000 , 1,500 and 2,000 pounds using 9.5 mm in diameter round, flat-faced punch. The tablets were tested as follows.

Tablet Evaluation

1. Thickness

The thickness of tablets was determined by using a micrometer (Teclock, Japan) and expressed in mm. The thickness value was an average of ten determinations.

2. Hardness

The hardness of compressed tablets was measured by hardness tester (Schleuniger-2E, Switzerland). The mean and standard deviation of ten determinations were calculated and expressed in kilopound (kp).

3. Disintegration

Disintegration time of tablets was determined , using the USP XXIII apparatus (Hanson Research, USA) with purified water at $37\pm2\ ^{0}C$ as a disintegration fluid. The test was performed without disk

and the average disintegration time was calculated from six determinations.

4. Friability

Twenty tablets were weighed accurately and transferred into the friabilitor (Erweka, Germany) rotating at 25 rpm for 4 min, then they were dedusted and reweighed. Friability was calculated as percent weight loss.

Comparative Dilution Potential Ability of Modified Rice Starch and Commercial Diluents

1. Preparation of Tablets

The poorly compressible drug: Ascorbic acid was added to each diluent material as concentration i.e. 10% , 20% , 30% , 40% and 50% W/W. Ascorbic acid was mixed with each diluent in the plastic box for 5 min. and mixed with 0.75% magnesium stearate for 2 min. The tablets were compressed by Carver laboratory press using 9.5 mm in diameter, round, flat-faced punch. The tablet weight was adjusted to 350 mg and the compression forces applied varied from 500 to 2,000 pounds.

2. Tablet Evaluation

Tablets were evaluated in thickness and hardness as previously described.

Effect of Lubricant on Tabletting Characteristics

Magnesium stearate was chosen as representative lubricant in this study because of its widespread use in tabletting. Magnesium stearate was screened through a 80-mesh sieve immediately prior to blending. The use of diluents uniquely allow tabletting without lubricant, there by providing an unlubricated control for compression. Each diluent was lubricated with magnesium stearate at 0.25 %, 0.5 %, 0.75 % and 1.0 % levels, except dicalcium phosphate dihydrate. Dicalcium phosphate dihydrate was lubricated with 0.75 %, 1.0 % and 1.25 %. Each mixture was blended in a plastic box for 3 minutes. The tablet were compressed by Carver laboratory press using 9.5 mm in diameter, round, flat-faced punch. The tablet weight was adjusted to 350 mg and the compression forces applied were from 500 to 2,000 pounds.

Tablet Evaluation

Tablets were evaluated in thickness, hardness and disintegration time as previously presented.

Application in Manufacture of Tablet Products

1. Preparation of Tablets

Hydrochlorothiazide and isoniazid were chosen to represent water insoluble and water soluble drugs, respectively. Two drugs were mixed with other excipients, according to the formula in Table 4 and 5. Each formula was compressed into 350 mg tablet using 3/8 inch round, flat-faced punch to the target hardness of 4-5 kps.

Table 4 Formulation of isoniazid tablets and hydrochlorothiazide tablets using Era-tab® and modified rice starch as directly compressible diluents

Ingredients	Weight of Ingredients per Tablet (mg)	
	Isoniazid Tablets	Hydrochlorothiazide
Isoniazid	100	-
Hydrochlorothiazide	-	50
Diluents*	248.25	298.25
Magnesium stearate	1.75	1.75
Tablet weight (mg)	350	350

* Diluents are Era-Tab® or Modified Rice Starch

Table 5 Formulation of isoniazid tablets and hydrochlorothiazide tablets using Starch 1500® and Avicel PH 102® as directly compressible diluents

Ingredients	Weight of Ingredients per Tablet(mg)	
	Isoniazid Tablets	Hydrochlorothiazide Tablets
Isoniazid	100	-
Hydrochlorothiazide	-	50
Diluents*	244.75	294.75
Magnesium stearate	1.75	1.75
Talc	3.5	3.5
Tablet weight (mg)	350	350

* Diluents are Starch 1500® or Avicel PH 102®

2. Tablet Evaluation

2.1 Physical Properties of Tablets:

2.1.1 Weight Variation

Twenty tablets from each batch were individually weighed, using an analytical balance (Sartorius, Germany) and determined for average weight and standard deviation.

2.1.2 Thickness, Hardness and Disintegration Time of tablets were evaluated as described earlier.

2.2 Percent Label Amount of Tablets

2.2.1 Isoniazid Tablets

Twenty tablets of each diluent were weighed and grounded to fine powder. A quantity of the powder containing 100 mg of isoniazid was transferred to a 100 ml volumetric flask containing 80 ml of water and shaked for 20 min. The final volume was made with water, filtered and discarded the first 10 ml of filtrate. 10 ml of this filtrate was pipetted into 100 ml volumetric flask and adjusted to volume with water. Then, 10 ml of this solution was transferred to another 100 ml volumetric flask. 0.1 N HCl was added to volume. The absorbance of sample was measured spectrophotometrically in a 1 cm cell at 266.8 nm The amount of isoniazid was calculated from the calibration absorbance-concentration curve in Figure 58 (Appendix I).

2.2.2 Hydrochlorothiazide Tablets

Twenty tablets of each diluent were weighed and grounded to fine powder. A quantity of powder containing 50 mg of hydrochlorothiazide was transferred to a 100 ml volumetric flask containing 50 ml of 0.1 N NaOH, shaked for 30 min, and diluted to volume with 0.1 N NaOH. The sample was filtered and discarded the first 10 ml of filtrate. Then, 10 ml of this filtrate was pipetted into 100 ml volumetric flask and adjusted to volume with 0.1 N NaOH. Finally, 10 ml of this solution was transferred to another 100 ml volumetric flask. 0.1 N NaOH was added to volume. The absorbance of sample was measured spectrophotometrically in a 1 cm cell at 276.0 nm The amount of hydrochlorothiazide was calculated from the calibration absorbance-concentration curve in Figure 60 (Appendix I).

2.3 Dissolution Time

Dissolution test of tablets was carried out using dissolution apparatus (Hanson Research, USA). Dissolution profile was obtained from the average of six determinations.

2.3.1 Isoniazid Tablets

A volume of 900 ml. of 0.1 N HCl was placed in the vessel that permitted holding the temperature at 37 ± 0.5 ^{0}C. Each dried basket containing one tablet was placed in the vessel. The rotation speed of the basket was 100 rpm.

The 5 ml of sample solution was withdrawn and filtered at 2, 4, 6, 8, 10, 15, 20, 25, 30 and 45 minutes interval. Then, the equal volume of 0.1 N HCl was substituted immediately. The withdrawn sample was

diluted with the same medium to a suitable volume and measured at the absorbance wavelenght of 266.8 nm with spectrophotometer (Bausch&Lomb, USA). The amount of drug dissolved in each sample at various times was calculated by comparing with the standard curve presented in Figure 58 (Appendix I). The time when eighty percent of drug dissolved ($T_{80\%}$) was determined from dissolution profiles. The amount of isoniazid dissolved in 45 min was not less than 80 % (Q).

2.3.2 Hydrochlorothiazide Tablets

The dissolution profile of each tablet was measured in a vessel containing 900 ml of 0.1 N HCl at 37±0.5 ^{0}C . Each tablet was placed in the vessel and the basket was rotated at 100 rpm. The 5 ml of sample was withdrawn and filtered at 2, 5, 10, 15, 20, 25, 30, 40, 50 and 60 minutes interval. The volume taken was substituted immediately by an equal volume of 0.1 N HCl. The withdrawn sample was suitably diluted with 0.1 N HCl and assayed at the absorbance wavelength of 273.8 nm with spectrophotometer. The amount of drug in each sample at various times was calculated from the standard curve presented in Figure 59 (Appendix I). The time when sixty percent of drug dissolved ($T_{60\%}$) was determined from dissolution profiles. The amount of hydrochlorothiazide dissolved in 60 min was not less than 60 % (Q).

2.4 Standard Curve of Isoniazid

Isoniazid 100 mg was accurately weighed and dissolved in 0.1 N HCl. The solution was adjust to 100 ml with 0.1 N HCl. 5 ml of this solution was transferred into 100 ml volumetric flask and diluted to volume with 0.1 N HCl and used as stock solution. The stock solution

was individually pipetted 5.0, 5.5, 7.5, 10.0, 15.0 and 20.0 ml into 50 ml volumetric flask and diluted to volume with 0.1 N HCl. The final concentration of each solution was 5.0, 5.5, 7.5, 10.0, 15.0 and 20.0 μg/ml, respectively.

The absorbance of known drug concentration was determined using UV absorption spectrophotometer in a 1-cm cell at 266.8 nm. The 0.1 N HCl was used as blank solution. Each concentration was determined in duplicated. The standard curve of isoniazid was illustrated in Figure 58 (Appendix I).

2.5 Standard Curve of Hydrochlorothiazide

Hydrochlorothiazide 50 and 100 mg was accurately weighed and dissolved in 0.1 N HCl and 0.1 N NaOH, respectively. The solution was individually adjusted to 100 ml with 0.1 N HCl or 0.1 N NaOH.

For hydrochlorothiazide in 0.1 N HCl, 10 ml of this solution was transferred into 100 ml volumetric flask and diluted to volume with 0.1 N HCl and used as stock solution. The stock solution was individually pipetted 3.0, 3.5, 5.0, 7.5 and 10.0 ml into 50 ml volumetric flask and diluted to volume with 0.1 N HCl. The final concentration of each solution was 3.0, 3.5, 5.0, 7.5 and 10.0 μg/ml, respectively.

For hydrochlorothiazide in 0.1 N NaOH, 5 ml of this solution was transferred into 100 ml volumetric flask and diluted to volume with 0.1 N NaOH and used as stock solution. The stock solution was individually pipetted 3.5, 5.0, 7.5, 10.0, 15.0 and 17.0 ml into 50 ml volumetric flask and diluted to volume with 0.1 N NaOH. The final

concentration of each solution was pipetted 3.5, 5.0, 7.5, 10.0, 15.0 and 17.0 μg/ml, respectively.

The absorbance of known drug concentration was determined using UV absorption spectrophotometer in a 1-cm cell at 273.8 nm for 0.1 N HCl and 276.0 nm for 0.1 N NaOH. The 0.1 N HCl and 0.1 N NaOH were used as blank solution. Each concentration was determined in duplicated. The standard curves of hydrochlorothiazide were illustrated in Figure 59 and 60 (Appendix I), respectively.