CHAPTER II

MATERIALS AND METHODS

METERIALS

Raw materials were used as received without further purification.

1. Drug substances

   Indomethacin (Lot no. T98-034, China)

   Mefenamic acid (Lot no. 860932, Supatpasat Co.,Ltd., Thailand)

2. Carriers

   Beta cyclodextrin (Ringdex®, supplied by Rama Production Co., Ltd. Thailand)

3. Excipeints

   Microcrystalline cellulose (Avicel PH 102, AMC Corporation Co.,Ltd., Thailand)

   Sodium lauryl sulfate (Lot & Ctrl. no. SEPO5, Srichansahaosod, Thailand)

4. Reagents

   Disodium hydrogen phosphate anhydrous (Carlo Erba Reagent, Italy)

   Phosphoric acid (AJAX Chemical, Australia)

   Methanol (HPLC grade; BDH Laboratory Supplies, England)

   Methanol (Analytical grade; Labguard®, Mallinckrodt Baker, Inc., Paris)

   Capsule No. 0

EQUIPMENT

1. Analytical balance (Model A200S, Sartorious GmbH, Germany and model PB 3002 Mettler, Switzerland)
2. Dissolution apparatus (Model SR-2, Erweka, Germany)
3. Differential scanning calorimeter (Model DSC7, Perkin Elmer, USA)
4. Fourier transform infrared spectrophotometer (Model SP 2000, Perkin Elmer Ltd., England)
5. High Performance Liquid Chromatography (HPLC) instrument equipped with the following:
   - a tunable absorbance detector (Model 484, Waters, USA)
   - a constant flow pump (Model 600E, Waters, USA)
   - an integrator (Model 746, Waters, USA)
   - an autoinjector (Model 712 WISP, Waters, USA)
   - a C_{18} reverse phase chromatography column (Spherisorb ODS2, 250 x 4.6 mm, 10 micron, Phase Separations, USA)
5. Light scattering particle analyzer (Master sizer, Malvern®, UK)
6. Magnetic stirrer (Model SP 46920-26, Limare CZ, Thermolyne, USA)
7. Powder flow meter equipped with the following:
   - an analog-to-digital convector (MacLab™)
   - a personnel computer (Macintosh®)
   - a MacLab™ front-ends
   - an isotronic transducer
   - a printer
8. pH Meter (Model 292, Pye Unicam Ltd., England)
9. Powder X-ray diffractometer (Model JDX-3530, Jeol Ltd., Japan)
10. Pneumatic pump (Model 505S, Watson-Marlow Ltd., England)
11. Scanning electron microscope (Model JSM-6400 LV, Jeol Ltd., Japan)
12. Shaking water bath (Model TBVS01, Hetomix® and DT Hetotherm®, Heto, Denmark)
13. Spray dryer (Mobile Minor Unit, Niro Atomizer, Denmark)
14. Thermal gravimeter-differential thermal analyzer (Model TGA7, Perkin Elmer, USA)
15. Ultrasound transonic digital sonicator (Model T900, Elma, Germany)
16. Ultraviolet-visible recording spectrophotometer (Model UV-160A, Shimadzu Corp., Japan)

METHODS

1. Methods of quantitative analysis of indomethacin

1.1 HPLC assay for indomethacin analysis

1.1.1 HPLC conditions

The high pressure liquid chromatography technique was used for analysis of indomethacin (IMC). The system consisted of constant flow pump, a variable wavelength UV detector, an integrator and an autoinjector. The conditions used for analysis IMC method are adapted from Analytical Profiles of Drugs (Mathew, James and Edward, 1984) and presented as follows:

Column	: Spherisorb C18 ODS2 (250 x 4.6 mm), 10 micron
Mobile phase	: Methanol:0.1% Phosphoric acid (65:35)
Detector wavelength	: 240 nm
Flow rate	: 2 ml/min
Attenuation	: 512
Chart speed	: 0.25 cm/min
Injection volume	: 20 microlitres
Internal standard	: mefenamic acid 1000 mcg/ml

1.1.2 Preparation of standard solutions

A stock solution of internal standard was prepared by completely dissolving 0.2 g of mefenamic acid in methanol in a 200-ml volumetric flask. The solution was adjusted to volume, giving the final concentration of 1000 mcg/ml.

A stock solution of IMC was prepared by dissolving 0.05 g of IMC in 8 ml-methanol. The solution volume was adjusted to volume with phosphate buffer pH 7.4 in a 100 ml-volumetric flask.

Standard solutions were prepared by pipetting 1, 2, 3, 4 and 7 ml of IMC stock solution transferring each one to 10-ml volumetric flasks. Three millilitres of

mefenamic acid stock solution was added into each of these volumetric flasks. The solutions were adjusted to volume with mobile phase so that the concentrations of IMC in standard solutions were 50, 100, 150 and 350 mcg/ml, respectively.

1.1.3 Validation of HPLC for the quantitative determination of indomethacin

The parameters essential to ensure the acceptability of the performance of analytical method are accuracy, precision, limit of quantitation, specificity and linearity (USPXXIII).

1.1.3.1 Accuracy

A) Analysis of indomethacin in phosphate buffer pH 7.4

Indomethacin solutions were prepared by dissolving IMC exactly 0.05 g in 8-ml methanol and adjusted to volume 100 ml by phosphate buffer pH 7.4. Three sets of solution were prepared at IMC concentrations of 50, 150 and 350 mcg/ml. Percent analytical recovery of each sample was detected and calculated.

B) Analysis of indomethacin with beta cyclodextrin in phosphate buffer pH 7.4

Three lots of IMC were prepared at each of the following IMC concentrations: 50, 150 and 350 mcg/ml. The solutions were prepared by dissolving 0.05 g of IMC in 8-ml methanol. When the solution was clear, the volume was adjusted to 100 ml by a solution of 0.1585 g of beta cyclodextrin (BCD) in phosphate buffer
pH 7.4. The final solutions were prepared by pipetting 1, 3 and 7 ml of IMC/BCD in phosphate buffer to a-10 ml volumetric flask. Then the solutions were adjusted to volume with mobile phase. Percent analytical recovery of each sample was detected and calculated.

C) Analysis of indomethacin with sodium lauryl sulfate in phosphate buffer pH 7.4

The solutions for HPLC analysis were prepared by dissolving 0.05 g

of IMC in 8-ml methanol and adjusted to 100 ml by solution of sodium lauryl sulfate in phosphate buffer pH 7.4. The analytical solutions were pipetted 1, 3 and 7 ml to 10-ml volumetric flask and adjusted to volume by mobile phase. Three concentrations were 50, 150 and 350 mcg/ml, respectively. Percent analytical recovery of each sample was detected and calculated.

1.1.3.2 Precision

a) Within run precision

The within run precision was determined by analyzing three sets of the calibration curves in the same day. Peak ratios of indomethacin to mefenamic acid were compared and the percent coefficient of variation (%CV) for each concentration was determined.

b) Between run precision

The between run precision was determined by comparing each concentration of three sets of the calibration curves prepared on different days. Peak area ratios for the three standard curves injected on different days were determined and %CV of each concentration was calculated.

1.1.3.3 Specificity

Under the chromatographic conditions selected, the peaks of other components in the sample must not interfere with the peak of indomethacin. The samples containing degradation products were prepared by dissolving 1 g of IMC in phosphate buffer pH 9.0 and heating this solution for 5 hours. The sample solutions of indomethacin with degradation products were pipetted 1 ml to 10-ml volumetric flask and adjusted to volume by mobile phase. Chromatograms were evaluated by comparing with those of the standard solutions of indomethacin.

1.1.3.4 Linearity

Seven IMC standard solutions in the concentration range of 50 - 350 mcg/ml were prepared and analyzed. Linear regression analysis of the peak area ratios versus their concentrations were performed.

2. Determination of the effect of pH on stability of indomethacin

Indomethacin 14 mg was dissolved into each 10-ml phosphate buffer (pH 6.0, 7.4 and 8.0). Three sets of each buffer solution were shaken in water bath at 37 °C for 12 hours. The amount of IMC was assayed by high performance liquid chromatography at wavelength 240 nm.

3. Solubility study

3.1 Solubility study of indomethacin in various pH solutions

An excess quantity of IMC (100 mg) was weighed into each pH solution (which adjusted by 1.0 N sodium hydroxide pH 6.0, 7.4 and 8.0) about 100 ml. The solutions were shaken in a water bath at ambient temperature (23 °C) for 12 hours. The clear solution of the substance was assayed by UV spectrophotometrically at the wavelength 319 nm after filtration and suitable dilution.

3.2 Solubility study of indomethacin in beta cyclodextrin solutions

Solubility studies was carried out according to the method of Higuchi and Connor (1965). Excess amounts of IMC (100 mg) was added to phosphate buffer pH 7.4. Ten millitres of beta cyclodextrin solutions in phosphate buffer pH 7.4 containing various concentrations of 0, 2×10^{-3}, 4×10^{-3}, 6×10^{-3}, 8×10^{-3}, 10×10^{-3}, 12×10^{-3} and 14×10^{-3} M were added to each erenmayer flask. The erenmayer flasks were sealed and shaken in a constant temperature water bath controlled at 37 ± 1 °C and equilibrated for 12, 24, 48, 72 and 144 hours. The suspensions were filtered through Whatman No. 1 filter after equilibrium. The filtered solutions were diluted and the amount of dissolved IMC was determined UV spectrophotometrically at 319 nm. The absorbance of BCD did not interfere with the assay.

Phase solubility diagram between concentration of BCD and concentration of the dissolved drugs were constructed. Apparent solubility constants, K_c was calculated from the straight line of the solubility profile according to the equation.

$$Kc = \frac{Slope}{S_0(1\text{-}slope)}$$

4. Preparation of indomethacin sample

4.1 Spray dried indomethacin

Indomethacin 27 g. was suspended in 10 litres of phosphate buffer pH 7.4 and devided to 5 litres. These solutions were heated at 80 ±5 °C to obtain a clear solution. The solution was fed into the drying chamber with a rotating centrifugal wheel atomizer.

The spray drying conditions were as follows:

Inlet air temperature	140	°C
Feed rate	15	ml/min
Outlet air temperature	> 75	°C
Atomizing air pressure	1	bar

4.2 Preparation of indomethacin with beta cyclodextrin

Indomethacin and beta cyclodextrin samples were prepared by physical mixture and spray drying method in the molar ratios of 2:1, 1:1 and 1:2 of the drug and BCD, respectively.

4.2.1 Physical mixture

Indomethacin and beta cyclodextrin mixed together in a plastic bag for 5 minutes and kept in closed containers for further investigation.

4.2.2 Spray drying

Dissolved exact amount of BCD (as shown in Table 7) in about 2000 ml phosphate buffer pH 7.4, then warmed the solution to about 80±5 °C until the solution was clear. Add IMC in the amount in Table 7 and mixed thoughroughly. The resulting solutions were fed into the spray dryer under the condition shown in Table 8.

Table 7 The amount of IMC and BCD used throughout this study

Molar ratio of IMC:BCD	amount of substances in each molar ratio (g)	
	IMC	BCD
2:1	30.93	49.07
1:1	19.17	60.83
1:2	10.89	69.11

4.3 Preparation of indomethacin with sodium lauryl sulfate

Although there was SLS in phosphate buffer pH 7.4, the solubility of IMC in this solution was minute. The volume of phosphate buffer pH 7.4 was about 4000 ml. Dissolved sodium lauryl sulfate in concentration of 13% or 20% or 27% or 33% of amount of indomethacin (60 g) in phosphate buffer pH 7.4, then warmed the solution to 80± 5 °C. Add IMC and mixed until the solutions were clear. The solution was sprayed into the spray-drying chamber under suitable conditions as shown below:

Parameter	Condition
Inlet air temperature (°C)	140
Oultet air temperature (°C)	>75
Feed rate (ml/min)	15
Atomizing air pressure (bar)	1
Dilution medium	1.782% disodium phosphate solution

Table 8 The spray drying conditions used throughout the study (For indomethacin and beta cyclodextrin preparation)

Parameter	Condition
Inlet air temperature (°C)	130-150
Outlet air temperature (°C)	>75
Feed rate (ml/min)	10-20
Atomizing air pressure (bar)	1
Dilution medium	1.782 % disodium phosphate solution

5. Evaluations of Physiochemical Properties of Spray Dried Powders

5.1 Powder Morphology

The microscopic aspect of the raw materials as compared with that of the spray dried powders by examination under the scanning electron microscopy (SEM). The samples were coated with gold prior to the microscopic examination with ion sputtering. Size, shape and surface topography of the spray dried powders were observed.

5.2 Bulk, Tapped Density and Percent Compressibility

Thirty grams of the spray dried powder was accurately weighed and carefully transferred into a 100-ml cylinder, three times at 2-second intervals. The volume of the powder is then read and used to calculate the bulk density.

$$\text{Bulk density (g/ml)} = \frac{\text{weight of the powder}}{\text{bulk volume}} \quad (3)$$

Tapped density is an extension of bulk density measurements. It was performed by dropping graduate cylinder filled with powder on a hard surface from 5 cm height, until the volume was stable. Division of the weight by this constant volume presented the tapped density.

$$\text{Tapped density (g/ml)} = \frac{\text{weight of the powder}}{\text{volume after tapped}} \quad (4)$$

The percent compressibility of the powder can be estimated from the tapped and bulk density measurements:

$$\text{Percent compressibility} = \frac{(T - B)}{T} \times 100 \quad (5)$$

B and T were bulk and tapped density, respectively. Both densities and percent compressibility were averaged from three determinations.

5.3 Determination of flowability of spray dried powder

The rate of flow of spray dried powders was adapted from MacLab™ program. Adequate amount of powder was weighed and filled into the funnel which was placed on the holder. There was a plastic bottle on the sensor. The isotronic transducer was placed on the other holder and below the funnel. Then the powder was allowed to pass through the funnel into the plastic bottle. The graph showed the changing weight of the sample when time passed. Slope of this graph showed the rate of flow of the powder.

5.4 Particle size determination

The Master sizer light-scattering instrument equipped with a computer was used to determine the mean particle size of the samples. Each sample was dispersed in mineral oil. It should be a dilute suspension. The sample cell filled with the diluted sample was inserted into the instrument. The particle size analysis outputs used for this study include a display of a relative size distribution (volume-based) histogram,a cumulative undersize distribution curve, a mean particle size and the standard deviation of the distribution.

5.5 Moisture content determination (Thermogravimetry)

About 5-6 mg of spray dried powders was placed in a platinum sample pan and transferred to the heating compartment of the TGA instrument (Model TGA7, Perkin Elmer) under a dry nitrogen atmospheres. The nitrogen flow was given instead of air throughout the process. Thermograms were recorded during heating at a rate of 10 °C/min. Percent weight loss was determined.

5.6 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy was used to study the interactions of the functional groups of the products induced by spray drying process by observing the positions and intensities of IR peaks.

The FTIR spectra of pure IMC, pure BCD, pure SLS and spray dried powders was examined using the potassium bromide (KBr) disc method in the range of 4000-400 cm^{-1}.

5.7 The powder X-ray diffraction analysis

The powder X-ray diffractometry was used to determined the diffraction angles of substances which exhibit crystallinity and interplanar spacing of the crystal planes of the products after spray drying process.

The analysis of pure IMC, pure BCD, pure SLS and spray dried powders was carried out using powder X-ray diffractometer.

Each sample powder was packed into a thin rectangular quartz slide by the other cover slide. After firmly packed, the cover slide was taken off and the sample-packed slide was exposed to the X-ray beam in the X-ray diffraction chamber. The X-ray diffraction pattern was recorded at the speed of 1° 2θ per minute from 5° to 90° 2θ angle.

5.8 Differential Scanning Calorimetry (DSC)

DSC analyses for IMC original substances, pure BCD, pure SLS and spray dried products, were carried out using differential scanning calorimeter.

About 3-5 mg of sample was accurately weighed onto the aluminium sample pan. The pan filled with spray dried powder was placed in the equipment beside the reference pan made by the same method except without powder. Nitrogen was used as a purge gas at the rate of 40 ml/min, and the scanning speed of 10 °C/min. All thermal runs are in the range from 30 to 240 °C.

5.9 Determination of Indomethacin Content of Spray Dried Powders

Standard Curve of indomethacin

The stock solution of indomethacin was compared as follows. Indomethacin of 100 mg was accurately weighed in to 200 ml volumetric flask through the aid of a glass funnel. The powder was rinsed off the funnel by absolute methanol, then dissolved and adjusted to volume with absolute methanol.

Different amount of the standard stock solution, 2, 3, 4, 5 and 6 ml, was individually pipetted into the 100 ml volumetric flask and diluted to volume with phosphate buffer pH 7.4. The final concentrations of the obtained standard solutions were 10, 15, 20, 25 and 30 mcg/ml, respectively.

The absorbances of the standard solutions were determined by a UV/visible spectrophotometer at 319 nm with absolute phosphate buffer pH 7.4 as a blank reference. The absorbance and the calibration curves of IMC were presented in Table 1B and Figure 1B , in Appendix B , respectively. The result of each concentration of the standard solution was averaged from three determinations.

5.10 Solubility of The Mixtures

Excess amount of sample was added to phosphate buffer pH 7.4 solution. The mixture was shaken in the erlenmeyer flask in a constant temperature water bath controlled at 37±1 °C and equilibrated for 72 hours. Sampling the sample at 2, 4, 6, 12, 24, 30, 48 and 72 hours intervals. After sampling, the suspensions were filtered through Whatman no. 1 membrane filter. The filtrates were analyzed UV spectrophotometrically at 319 nm.

5.11 Quantitation of Indomethacin Spray Dried Powders

Indomethacin spray dried powders were kept in amber glass bottles placed in desiccator at room temperature. The amount of spray dried powders was analyzed using high performance liquid chromatography. The sample of spray dried powders were prepared by dissolving the powder in the mobile phase to obtain concentration of 25 mcg/ml and the process continued according to that described in 1.1.1.

6. Preparation of Indomethacin Capsules

The pure IMC, spray dried powders of IMC/BCD, the physical mixtures of IMC/BCD, the spray dried IMC/SLS, the physical mixtures of IMC/SLS were formulated into capsules with compositions as shown in Tables 8, 9, 10 and 11.

Table 9 The compositions of indomethacin spray dried powder capsules

Ingredients	2:1 molar ratio	1:1 molar ratio	1:2 molar ratio
Indomethacin	25.00 mg	25.00 mg	25.00 mg
Beta cyclodextrin	39.66 mg	79.33 mg	158.64 mg
Phosphate buffer pH 7.4	28.81 mg	46.48 mg	81.82 mg
Avicel pH 102	86.53 mg	29.19 mg	94.54 mg

Table 10 The compositions of indomethacin physical mixtures capsules

Ingredients	2:1 molar ratio	1:1 molar ratio	1:2 molar ratio
Indomethacin	25.00 mg	25.00 mg	25.00 mg
Beta cyclodextrin	40.13 mg	80.26 mg	185.53 mg
Avicel pH 102	114.87 mg	74.74 mg	149.47 mg

Table 11 The compositions of indomethacin capsules for indomethacin and sodium lauryl sulfate (spray dried powders)

Ingredients	13% SLS	20% SLS	27% SLS	33% SLS
Indomethacin	25.00 mg	25.00 mg	25.00 mg	25.00 mg
SLS	3.25 mg	5.00 mg	6.75 mg	8.25 mg
Phosphate buffer pH 7.4	29.70 mg	29.70 mg	29.70 mg	29.70 mg
Avicel pH 102	122.05 mg	120.30 mg	118.55 mg	117.05 mg

Table 12 The compositions of indomethacin capsules for indomethacin and sodium lauryl sulfate (physical mixtures)

Ingredients	13% SLS	20% SLS	27% SLS	33% SLS
Indomethacin	25.00 mg	25.00 mg	25.00 mg	25.00 mg
SLS	3.25 mg	5.00 mg	6.75 mg	8.25 mg
Avicel pH 102	151.75 mg	150.00 mg	148.25 mg	146.75 mg

Method for preparation of indomethacin capsules

The required quantities of the spray dried powders or the physical mixtures were mixed with Avicel pH 102 in closed container for 5 minutes. The final mixtures were then filled in capsule No. 0. The dissolution of capsules were evaluated.

7. Dissolution of Indomethacin Capsules

The dissolution of IMC capsules were determined using rotating basket (USP XXIII Apparatus I). The dissolution medium was 750 ml of phosphate buffer pH 7.4 warmed at 37±0.5 °C. The baskets were rotated at 100 rpm. At time intervals 6,

10, 12, 15, 30,40 and 60 minutes, successive portions of 10 ml of sample solution were withdrawn and analyzed for IMC content by UV spectrophotometer at maximum absorbance at 319 nm and 10 ml of fresh buffer was replaced. The amount of dissolved IMC was calculated from standard curve prepared with the same medium. The dissolution profile was obtained by plotting the percentage of drug dissolved versus time.

(Phosphate buffer pH 7.4: Dissolved 17.82 g of dibasic sodium phosphate in water. 85% v/v of phosphoric acid was added to adjust the solution to pH 7.4 and adjusted to 1000 ml with deionized of water.)

8. Statistical analysis

Statistical analysis of the difference in moisture content, particle size, solubility values and initial dissolution rate of drug among spray dried products compared with pure drug were performed using one way analysis of variance and multiple comparison test. The P-value of 0.05 was to judge the significance of the difference.