Chapter II

Experiment

1. Materials

All materials obtained from commercial sources were used as received.

- Indomethacin (Batch no. T93-068, Vertex Chemical Co., Hong Kong)

- Chitosan (MW. 30000-70000 Dalton, Kyowa Technos Co., Ltd., Japan , Distributed by G.T. Chemical Co., Ltd., Bangkok)

- Carboxymethylcellulose sodium (High viscosity, Lot no. 7532C6, Distributed by Pharmaceutical Traders Co.,Ltd., Bangkok)

- Pectin (Pectin citrus type B rapid set, Lot no. P008, Distributed by Srichand United Dispensary Ltd. Part., Bangkok)

- Glutaraldehyde (Ucacide-250, IPD.8342, Union Carbide, Thailand)

- Calcium chloride dihydrate (E. Merck, Germany)

- Acetic acid glacial AR grade (E. Merck, Germany)

- Sodium hydroxide AR grade (E. Merck, Germany)

- Isopropanol commercial grade (Distributed by Chaimongkol Trading, Bangkok)

- Nitrogen gas (Distributed by Num Heng Co.,Ltd.,Bangkok)

- Methanol HPLC grade (J.T. Baker, USA)

- Acetonitrile HPLC grade (J.T. Baker, USA)

- Potassium dihydrogen phosphate AR grade (E. Merck, Germany)

- Sodium acetate (Carlo Erba, Milano)

- Ibuprofen (Batch no. L9012197, Shasun Drugs, India)

2. Equipment

- Spray nozzle (UniGlatt Laboratory unit, Germany)
- Peristaltic pump (Verder VRX88, Germany)
- Air pump (UniGlatt Laboratory unit, Germany)
- Suction pump (serial no. 357552, USA)
- Plastic bath (11 cm diameter)
- Magnetic stirrer (Heidolph, MR 3001, Germany)
- Magnetic bar (10.5 cm long)
- Analytical balance (Sartorius, model A200S, Germany)
- pH meter (model 292, Pye Unicam, England)
- Spectrophotometer (Spectronic 3000 Array, Milton Roy, USA)
- Dissolution apparatus (model SR2, Hanson Research, USA)
- High Performance Liquid Chromatography (System controller :Waters 600E, Turnable Absorbance Detector : Walters 484 , Intelligent Sample Processor Walters: Walters 712 WISP , Data module : Walters 746, Millipore, USA)
- Column chromatography (Spherisorb ODS2, 5 μm, Milton Roy, USA)
- Photomicrograph microscope (BHB, Olympus, Japan)
- Scanning Electron Microscope (model S2360N, Hitachi, Ltd., Japan)
- Viscometer (Brookfield viscometer, model RVTDCP, Brookfield Engineering Laboratories, Inc. USA)
- Sonicator (Transonic Digital S, Elma, Gemany)
- Infrared spectrophotometer (model Perkin-Elmer 16 PC FT-IR, Perkin Elmer Ltd., USA)
- Differential Scanning Calorimeter (Netzsch DSC 200, Germany)
- Capsule filling machine

3. Microencapsulation process

3.1 Preliminary study

Preparation of polymer solutions :

Chitosan solutions of 0.25-1.0 %w/v were prepared by dispersing chitosan in 970 ml of acetic acid solution (1.0%w/v) and allowing it to hydrate overnight. The hydrate chitosan solution was filtered to remove insoluble fraction and adjusted to pH 4 with 1.0 N sodium hydroxide. Finally, chitosan solution was adjusted to 1000 ml by acetic acid solution.

Carboxymethylcellulose sodium (CMC) solutions of 0.5-1.5 %w/v and pectin solutions of 2.5-10.0 %w/v were prepared by dispersing CMC or pectin in deionized water and allowing them to hydrate overnight. The viscosities of CMC solution or pectin solution were determined by a Brookfield viscometer.

Process of microencapsulation :

CMC solution or pectin solution of 150 ml was passed through rubber tube (inner diameter 0.6 cm) to a nozzle by peristaltic pump at 2±0.25 rpm. The polymer solution was sprayed through the nozzle with 0.2 bar air pressure as small droplets into a bath of 1000 ml chitosan solution which had been kept at a temperature of 10± 2 °c. During the spraying, the chitosan solution was stirred at a speed of 200±50 rpm in order to produce a vortex with less or without air bubbles. After spraying, glutaraldehyde 0.25 gm /gm polymer was added into the reaction bath and the mixture was continuously stirred for 2 hours to harden the

microcapsule wall. The processes for chitosan-CMC and chitosan-pectin are shown in Figures 3 and 4 , respectively.

The hardened microcapsules were filtered and then washed 4 times and 6 times with water 200 ml for chitosan-CMC microcapsule and chitosan-pectin microcapsule, respectively, in order to remove excess glutaraldehyde and followed by 4 times washing with 50 ml of isopropanol (IPA) to remove the remaining water. The microcapsules were dried with nitrogen gas. Finally, the dried microcapsules were passed through a no. 40 sieve and collected.

For the chitosan-CMC microencapsulation process, the step of hardening the microcapsule wall was varied in hardening time between 2 and 3 hours by using of drug-CMC dispersion instead of CMC solution.

3.2 Microencapsulation of indomethacin

The process of drug encapsulation followed the preliminary microencapsulation process described in section 3.1, but used drug-CMC or drug-pectin dispersion instead of polymer solution. The drug-polymer dispersions were prepared by dispersing indomethacin, which was passed through no. 80 sieve, in CMC or pectin solution.

For the chitosan-pectin microencapsulation process, calcium chloride had to be dissolved into chitosan solution before adjusting the pH to 4 in order to prevent agglomeration of pectin droplets.

Figure 8 : Schematic illustration of microencapsulation method using chitosan and CMC as wall materials.

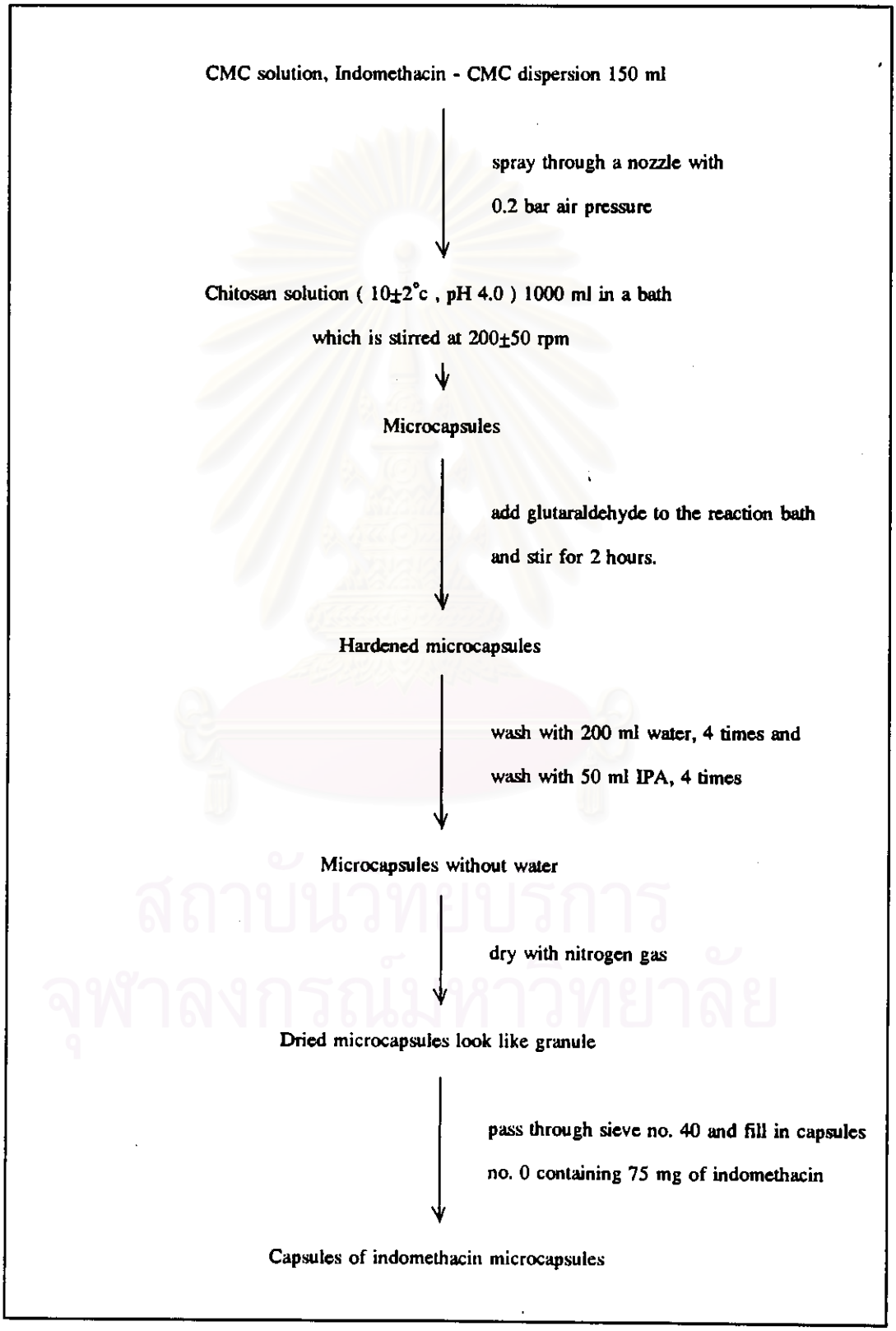
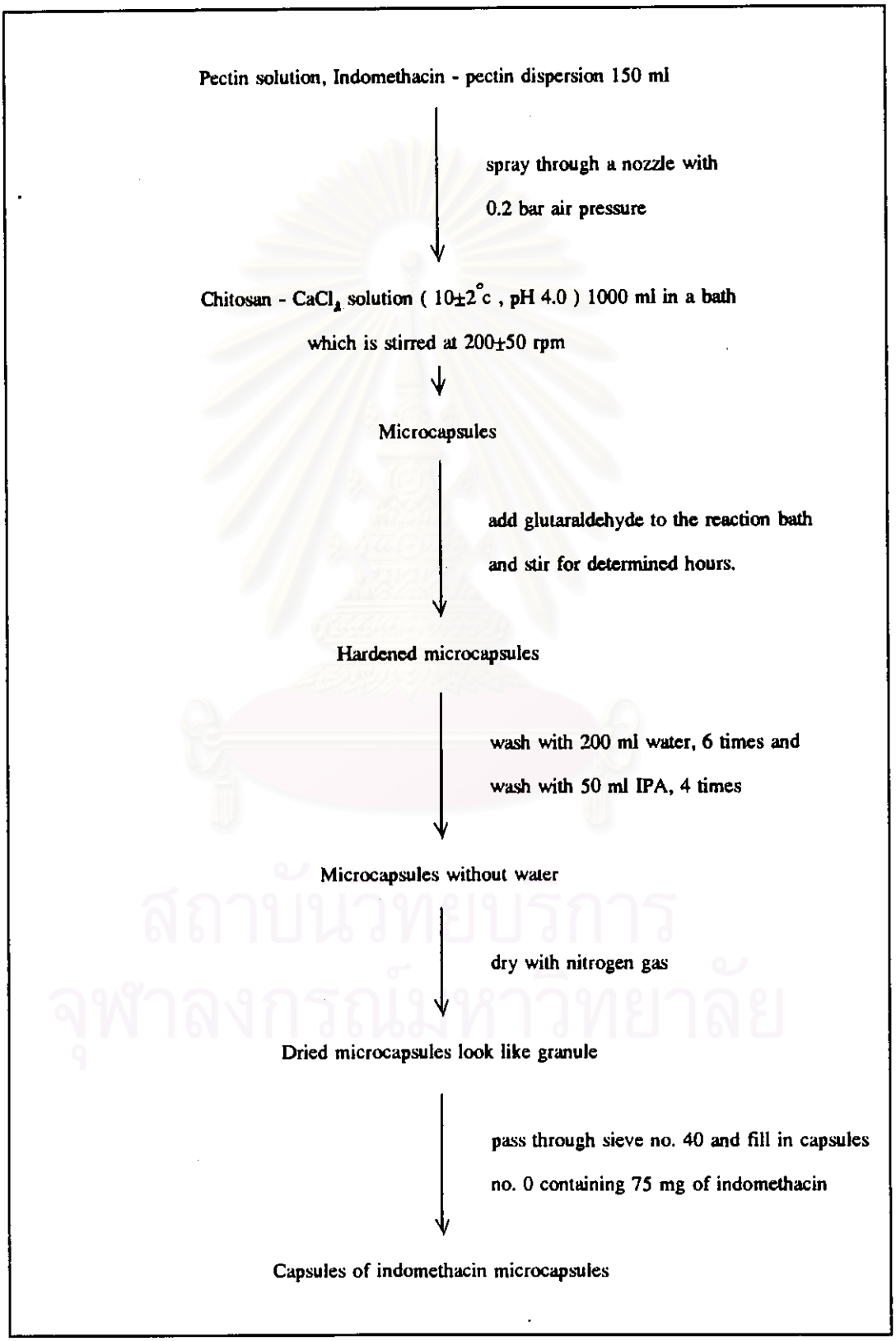


Figure 4 : Schematic illustration of microencapsulation method using chitosan and pectin as wall materials.



The variable factors in the process were concentrations of chitosan solution, CMC solution, and pectin solution, the amount of calcium chloride (for chitosan-pectin microencapsulation), the amount of glutaraldehyde, and content of indomethacin in polymer solution.

The yield dried microcapsules were filled into capsules no. 0 by capsule filling machine without additional diluent. Each capsule contained 75 mg of indomethacin. The various conditions for prepared pharmaceutical microcapsules are shown in Tables 5 and 6 for chitosan-CMC and chitosan-pectin microcapsules, respectively.

Table 5 : Variable factors in indomethacin microcapsules (chitosan - CMC) preparations.

Preparation	Chitosan soln (%w/v)	CMC soln (%w/v)	Glutaraldehyde (gm) /polymer 1 gm	Indomethacin %(W/V) /CMC soln
1	0.25	0.75	0.25	1.00
2	0.25	1.00	0.25	1.00
3	0.25	1.50	0.25	1.00
4	0.35	1.00	0.25	1.50
5	0.50	0.75	0.10	1.00
6	0.50	0.75	0.25	1.00
7	0.50	1.00	0.10	1.00
8a,b	0.50	1.00	0.25	1.00
9	0.50	1.00	0.25	1.50
10	0.50	1.50	0.25	1.00

a = hardening time 2 hours

b = hardening time 3 hours

other prep. = hardening time 2 hours

Table 6 : Variable factors in indomethacin microcapsules (chitosan - pectin) preparations.

Prep.	Chitosan soln (%w/v)	Pectin soln (%w/v)	$CaCl_2$ (gm) /chitosan 1 gm	Glutaraldehyde (gm) /polymer 1 gm	Indomethacin (%w/v) /pectin soln
11	0.25	5.00	1.00	0.10	1.00
12	0.25	5.00	1.00	0.10	2.00
13	0.25	5.00	1.00	0.25	1.00
14	0.25	5.00	3.00	0.00	3.00
15	0.25	5.00	3.00	0.10	1.00
16	0.25	5.00	3.00	0.25	1.00
17	0.25	5.00	3.00	0.25	2.00
18	0.25	7.50	1.00	0.10	1.00
19	0.25	7.50	1.00	0.10	2.00
20	0.25	7.50	1.00	0.25	1.00
21	0.25	7.50	3.00	0.10	1.00
22	0.25	7.50	3.00	0.25	1.00
23	0.25	7.50	3.00	0.25	2.00
24c,d	0.25	7.50	3.00	0.25	3.00
25d	0.25	10.00	3.00	0.25	3.00
26	0.50	5.00	1.00	0.10	1.00
27	0.50	5.00	1.00	0.25	1.00
28	0.50	5.00	3.00	0.10	1.00
29	0.50	5.00	3.00	0.25	1.00
30	0.50	7.50	1.00	0.10	1.00
31	0.50	7.50	1.00	0.25	1.00
32	0.50	7.50	3.00	0.00	1.00
33	0.50	7.50	3.00	0.00	5.00
34	0.50	7.50	3.00	0.10	1.00
35	0.50	7.50	3.00	0.10	2.00
36	0.50	7.50	3.00	0.10	3.00
37	0.50	7.50	3.00	0.25	1.00
38c,d	0.50	7.50	3.00	0.25	3.00

Table 6 : Variable factors in indomethacin microcapsules (chitosan - pectin) preparations. (continue)

Prep.	Chitosan soln (%w/v)	Pectin soln (%w/v)	$CaCl_2$ (gm) /chitosan 1 gm	Glutaraldehyde (gm) /polymer 1 gm	Indomethacin (%w/v) /pectin soln
39	0.50	10.00	3.00	0.00	3.00
40c,d	0.50	10.00	3.00	0.25	3.00
41	0.75	10.00	3.00	0.00	3.00
42c,d	0.75	10.00	3.00	0.10	3.00
43c,d	0.75	10.00	3.00	0.25	3.00

c = hardening time 6 hours

d = hardening time 18 hours

other prep. = hardening time 2 hours

4. Evaluation of pharmaceutical microcapsules

4.1 Morphology

Morphology of microcapsules was determined by using optical photomicroscope and scanning electron microscope (SEM). Optical photomicroscope at x40 and x200 magnifications was used for showing microencapsulation of indomethacin of chitosan-pectin microcapsules. For determining the morphology and drug entrapment in the microcapsules, samples were selected and mounted with water prior to the microscopic examination at x200 magnification. Shape and surface topography of microcapsules, before drug release, were observed by SEM at x60, x80 and x800 magnifications by coating the samples with gold prior to the microscopic examination using ion sputtering.

4.2 Size and size distribution

Size and size distribution of microcapsules were determined by optical photomicroscope at x40 magnification. Sample size of 350 microcapsules was used for size distribution analysis. The particle size was determined by measuring the Feret's diameter, which was the distant between two tangents on opposite sides of the particle parallel to some fixed direction (Martin, 1993). The number distribution was analysed. The percentage frequency and cumulative percentage frequency undersize were calculated. In order to calculate the geometric mean diameter at D_{50} value of microcapsules, particle diameter was transformed into logarithm value. The D_{50} value of microcapsules was computed from the logarithm of particle diameter and cumulative percentage frequency undersize.

4.3 Determination of indomethacin content in indomethacin microcapsules

The method for determining indomethacin content employed in this study was modified from the USP XXIII, under the monograph of Indomethacin Extended-Release Capsules.

4.3.1 Calibration curve of indomethacin content

Indomethacin 25 mg and ibuprofen 260 mg were individually and accurately weighed into each 100-ml volumetric flask and dissolved in a solvent mixture of acetonitrile and pH 4.2 acetate buffer (55:45). Each solution was diluted to volume. The solution was used as standard stock solution and internal standard stock solution, respectively.

The standard stock solutions of 1.0, 2.0, 3.0, 4.0, and 5.0 ml were individually pipetted into 50-ml volumetric flasks. The internal standard stock solution of 2.0 ml was pipetted into each flask and then diluted to volume with the solvent mixture. The final concentrations of each standard solution were 5.0, 10.0, 15.0, 20.0, and 25.0 μg/ml, respectively. The final concentration of internal standard solution was 104 μg/ml.

The standard solution mixtures were sonicated for 15 minutes and injected equal volumes (about 20 μl) into the column chromatograph. The system using acetonitrile : pH 4.2 acetate buffer (55:45) (Jarukumjorn, 1993) was used as mobile phase. The flow rate was 1.0 ml per min. The chromatogram was detected at 230 nm. The maximum absorbance was chosen from ultraviolet absorbance spectra of indomethacin and ibuprofen in Figures 89 and 90 in the Appendix. The chromatograms of ibuprofen, phenylbutazone, mefenamic acid for choosing as internal standard and indomethacin are shown in Figures 87 and 88 in the Appendix. The calibration curve of indomethacin is illustrated in Figure 91 in the Appendix. Each concentration was determined in triplicate.

4.3.2. Assay of indomethacin content in indomethacin microcapsules

Indomethacin microcapsules of 400 mg were approximately weighed and ground in motar to destroy the microcapsule wall. Sample 200.0 mg was accurately weighed into a 100-ml volumetric flask. The sample was added with 60 ml of solvent mixture, made up of acetonitrile and pH 4.2 acetate buffer (55:45). The sample suspension was left to stand for 4 hours with occasional shaking. After standing, the sample suspension was sonicated for 15 minutes. Then, the sample solution was diluted with the solvent mixture to volume, mixed

and centrifuged a portion of the solution at 3000 rpm. The supernatant liquid was filtrated through a filter having a pore size of 0.45 μm. The filtrate was used as the assay preparation.

The assay preparation of 1.0 ml and the internal standard solution of 2.0 ml were pipetted into 50-ml volumetric flask and diluted to volume with the solvent mixture. The sample solution mixtures were sonicated for 15 minutes and injected equal volumes (about 20 μl) into the column chromatograph. The indomethacin content was calculated from a calibration curve of peak area ratio of indomethacin and ibuprofen. Each sample was determined in triplicate.

4.4 Percentage of drug entrapment, drug recovery, and yield

From the determination of indomethacin content in microcapsules, the percentage of drug entrapment, drug recovery, and yield were computed from the following formulas.

$$\% \text{ Drug Entrapment} = \frac{DM \times 100}{MC} \quad \text{..... Eq. 2}$$

$$\% \text{ Drug Recovery} = \frac{MT \times DM \times 100}{DI \times MC} \quad \text{..... Eq. 3}$$

$$\% \text{ Yield} = \frac{MT}{CS + (CMC \text{ or } PT) + DI} \quad \text{.....Eq. 4}$$

DM = Drug content in the pharmaceutical microcapsule which was determined from MC (mg)

MT = Total pharmaceutical microcapsule yield (gm) of each preparation

DI = The initial amount of drug used in the microencapsulation process (gm)

MC = The weight of microcapsule which was used in the determination of drug content in the pharmaceutical microcapsule (mg)

CS = The weight of chitosan used in the each preparation (gm)

CMC or PT = The weight of CMC or pectin used in the each preparation (gm)

4.5 Drug release study of indomethacin microcapsules

The method of drug release of indomethacin employed in this study was determined according to the USP XXIII, using apparatus I (the basket method).

4.5.1 Calibration curve of indomethacin release

Indomethacin 50 mg was accurately weighed into 100-ml volumetric flask and dissolved in 8.0 ml methanol. The solution was diluted to volume with pH 6.2 phosphate buffer and used as standard stock solution.

The standard stock solutions of 3.0, 4.0, 5.0, 6.0, and 8.0 ml were individually pipetted into 100-ml volumetric flasks and diluted to volume with pH 6.2 phosphate buffer. The final concentrations of each solution were 15.0, 20.0, 25.0, 30.0, and 40.0 μg/ml, respectively.

The absorbance of standard solution was detected by a double beam spectrophotometer in a 1-cm cell at 323 nm and pH 6.2 phosphate buffer was used as a blank solution. The absorbance maximum spectrum is shown in Figure 92 in the Appendix. Each concentration was determined in triplicate. The calibration curve of indomethacin is illustrated in Figure 93 in the Appendix.

4.5.2 Release of indomethacin from indomethacin microcapsules

Seven hundred and fifty millilitres of pH 6.2 phosphate buffer were filled into a glass vessel specified in the USP drug release test, and equilibrated at 37 ± 0.5 °c. One capsule was placed in a dry basket, specified in the compendium, and immersed in the medium at the centre of the vessel and at 20 mm above the bottom of the vessel. The apparatus was operated at speed of 75 rpm. Three capsules of each formulation were evaluated.

At the time interval of 1, 2, 3, 4, 6, 9, 12, 18, and 24 hours, ten millilitres of the specimen was withdrawn and the medium was added immediately in the same quantity after each sampling to keep the volume constant during the experiment. The absorbance of each sample was detected by using a spectrophotometer at 323 nm. The sample was diluted to a suitable concentration, if necessary.

The amount of indomethacin release at any time interval was calculated from the calibration curve. A cumulative correction was made for the previously removed sample to determine the total amount of drug release.

4.6 Determination of infrared spectra

Infrared spectra of samples were measured by using a model Perkin-Elmer 16 PC FT-IR spectrophotometer which used KBr disk method and run at 4400-450 cm^{-1}. Samples tested were chitosan, CMC, pectin, indomethacin, $CaCl_2$, microcapsules, and indomethacin microcapsules.

4.7 Determination of differential scanning calorimetric thermogram

Differential Scanning Calorimeter (DSC) is used as a source of information in the study of solid state interaction of drug with polymer.

DSC thermograms were obtained from Netzsch Thermal Analyzer model Netzsch DSC 200. The instrument was calibrated with indium. All measurements were carried out using 5-8 mg of samples placed in aluminium pan and sealed. Scanning was performed at the temperature range of 40-240 $^{\circ}c$ with the heating rate 10 $^{\circ}K/min$. The samples tested were chitosan, CMC, pectin ,chitosan-CMC microcapsules, chitosan-pectin microcapsules, and chitosan-pectin with $CaCl_2$ microcapsules.

4.8 Reproducibility study

Preparations which conformed to the USP drug release specification were selected for this reproducibility study and were prepared according to the conditions described in 3.2. Study on reproducibility of size, size distribution, drug entrapment, drug recovery, yield, and drug release pattern of indomethacin microcapsules were investigated for three consecutive batches.