CHAPTER III
MATERIALS AND METHODS

Materials

1. Ascorbic acid (Lot no. B/NO. F2E201, Asia Pacific Specially Chemicals Limited, Australia)
2. Carbon dioxide (Thai Industrial Gases Public Company Limited)
3. Chlorofrom (A.R.Grade, Batch no. 01031125, Labscan Asia Co., Ltd.)
4. Dimethyl formamide (HPLC Grade, Batch no. 01090008, Labscan Asia Co., Ltd.)
5. Dimethyl sulfoxide (DMSO) (Analytical Grade, Labscan Asia Co., Ltd.)
6. Di-Potassium hydrogen orthophosphate (Lot no. B/NO. F2A246, Asia Pacific Specially Chemicals Limited, Australia)
7. Di-Sodium hydrogen orthophosphate (Lot no. B/NO. F3C152, Ajax Finechem, Australia)
8. Lactose (Lactose super-tab anhydrous, lot no. CN020070, Maxmay Co., Ltd. New Zealand)
9. Methanol (HPLC Grade, Batch no. 03120083, Labscan Asia Co., Ltd.)
10. Methylene chloride (HPLC Grade, Lot no. 0309696, Fisher Scientific)
11. Middlebrook 7H11 (Lot 3034793 Exp. 2006-12-31)
12. Orthophosphoric acid 85% (Lot no.B/NO. A3B017, Ajax Finechem, Australia)
13. Poly (DL-lactide) (DL-PLA) (mol wt 90,000-120,000 Da, Lot no. 073K1159 Sigma, Germany)
14. 50:50 Poly (DL-lactide-co-glycolide) (PLGA) (Lactel, mol wt 40,000-65,000 Da, Lot no. D01070, Birmingham Polymer, USA)
15. Poly (L-lactide) (L-PLA) (Lactel BP-0600, mol wt 85,000-160,000 Da, Lot no.112K0661, Sigma, Germany)
16. Poly (L-lactide) (L-PLA) (mol wt ~67,400 Da, Lot no.427973/1 35102139,Fluka, Switzerland)

17. Poly(L-lactide) (L-PLA) (mol wt ~101,700 Da, Lot no.427974/1 35102142 Fluka, Switzerland)
18. Poly(L-lactide) (L-PLA) (mol wt ~152,000 Da, Lot no.439303/1 52503188 Fluka, Switzerland)
19. Rifampicin (Lot no. RMP-C_050/01, supplied by Siam Bheasach, Thailand)
20. Sodium Chloride (Lot no. B/NO. AF309070, Ajax Finechem,Australia)
21. Sodium dihydrogen orthophosphate (Lot no. B/NO. F3E048, Ajax Finechem, Australia)
22. Tween 80 (Lot no. 807870, B.L. Hua & Co., Ltd., Thailand)

Equipment

1. Analytical balance (Model A2005, Sartorius, Germany)
2. Anderson Cascade Impactor (Andersen Sampler Inc., Atlanta, USA)
3. Centrifuge (Eppendrof Centrifuge 5810, Germany)
4. Dissolution apparatus (Model VK7000, Vankel, USA)
5. DSC (Mettler Toledo, DSC 821e, Switzerland)
6. Gas Chromatograph (Series II 5890, Hewlett Packard, USA)
7. High performance liquid chromatography (HPLC) (Model SCL-10AVP, Shimadzu, Japan):
   - Auto injector (Model SIL-10A, Shimadzu, Japan)
   - Column oven (Model CTD-10AS, Shimadzu, Japan)
   - Degasser (Model DGU-14A, Shimadzu, Japan)
   - Pump A, B liquid chromatography (Model LC-10AD, Shimadzu, Japan)
   - System controller (Model SCL-10A, Shimadzu, Japan)
   - UV-VIS detector (Model SPD-10A, Shimadzu, Japan)
8. HPLC column: a Apollo C18 5μ (250mm x 4.6mm) column Serial No. 02100422.1 (Altech, Deerfield, IL)
9. HPLC Pump (MILTONROY® CM 4000 multiple solvent delivery system, USA)
10. Laser particle size analyzer (Mastersizer 2000Ver 5.1 (Malvern Instruments Ltd, Malvern UK)
11. pH meter (Model 210 A+, Thermo Orion, Germany)
12. Scanning electron microscope (Model JSM-6400, Jeol Co., Ltd., Japan)
13. Sonicator (Transsoni Digitals, Elma)
14. Supercritical Fluid Extractor (SFE400, Superlco, USA)
15. UV spectrophotometer (V530 Jasco, USA)

Methods

1. Preparation of Rifampicin and Rifampicin-Biodegradable Microparticles by Supercritical Fluid Technique

The microparticles of rifampicin and rifampicin-biodegradable polymers were prepared by supercritical fluid technique. The polyhydroxy acids [50:50 poly(DL-lactide-co-glycolide) copolymer (PLGA), poly(DL-lactide)(DL-PLA), and poly(L-lactide)(L-PLA)] were used for preparation of drug-loaded microparticles. A schematic diagram of the supercritical anti-solvent apparatus (modified SFE 400, Supelco) is shown Figure 3-1. Carbon dioxide was preliminary introduced by thermal pump into the stainless steel precipitation vessel (250 ml) to set the operating pressure. The liquid solution composed of drug and biodegradable polymer dissolved in methylene chloride. The liquid solution was sprayed by HPLC pump through a 50 μm internal diameter capillary. The flow rate of CO_2 was kept constant at 1.5 g/min. The organic solvent was extracted into the supercritical fluid, resulting in the formation of solid microparticles in the vessel. When liquid solution flow was stopped, the precipitation vessel was depressurized. The microparticles were collected from both vessel wall and the vessel bottom and stored in a desiccator at room temperature, pending analysis.

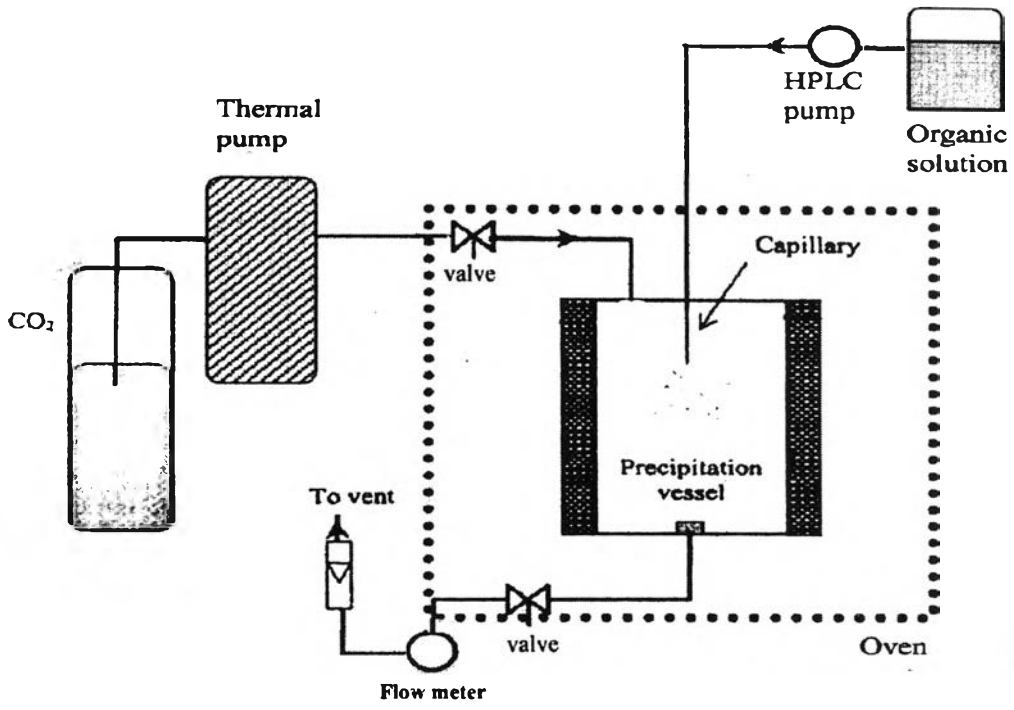

Figure 3-1 Schematic diagram of supercritical antisolvent apparatus.

1.1 Effect of Polymer Type and Polymer Content

The microparticles were prepared using above procedure to investigate the effect of polymer type and polymer content on properties of rifampicin-polymer microparticles. The operation parameters are shown in Table 3-1. During the particle formation, the pressure was fixed at 2500 psi and the temperature at 40 °C for L-PLA except DL-PLA and DL-PLGA. Because of the low glass transition temperature (*Tg*) of DL-PLA and DL-PLGA., these polymers were processed at 33 °C (Bodmeier et al. 1995). Microparticles of rifampicin were prepared with various types of polymer and polymer content, using poly (L-lactide) (L-PLA), poly(DL-Lactide) (DL-PLA) and 50:50 poly(DL-Lactide-co-glycolide) (PLGA) and 20, 30, 40, 50, 60, 70, 80, 90, and 100 %w/w biodegradable polymer in microparticles. These formulations are shown in Table 3-2.

Tables 3-1 Operating conditions used for microparticle preparation.

Operating condition	Level
Supercritical carbon dioxide pressure	2500 psi
Temperature	40 °C for L-PLA 33 °C for DL-PLA and PLGA
Concentration of solution	20 mg/ml
Feed rate	0.5 ml/min

1.2 Effect of the Solvent Type

In this experiment, methylene chloride or/and dimethyl sulfoxide (DMSO) were used to dissolve drug and polymer. Mixtures of DMSO and methylene chloride at ratio of 1:9, 1:4, 3:7, 2:3, 1:1, 3:2, 7:3, 4:1 and 9:1 were used. The prepared solution was sprayed into the pressurized vessel as described in section 1.

1.3 Operating Parameters: Pressure, Temperature, Concentration of Solution and Feed Rate

The Formulation containing 70% Poly(L-lactide) and 30% rifampicin was chosen for further experiments. Poly(L-lactic acid) has excellent biodegradable properties. Poly(L-lactide) has also been shown to consistently precipitate from the supercritical process as discrete microparticles which are desirable for pharmaceutical applications.

Table 3-2 The parameters used in the preparation of rifampicin microparticles with various types of polymer and polymer content.

Polymer type	Polymer content (%w/w)	Rifampicin (%w/w)	Drug : polymer ratio	Concentration of Drug-polymer solution (mg/ml)
PLGA 50:50	100%	0%	-	20
	90%	10%	1:9	20
	80%	20%	1:4	20
	70%	30%	3:7	20
	60%	40%	2:3	20
	50%	50%	1:1	20
	40%	60%	3:2	20
	30%	70%	7:3	20
	20%	80%	4:1	20
DL-PLA	100%	0%	-	20
	90%	10%	1:9	20
	80%	20%	1:4	20
	70%	30%	3:7	20
	60%	40%	2:3	20
	50%	50%	1:1	20
	40%	60%	3:2	20
	30%	70%	7:3	20
	20%	80%	4:1	20
L-PLA	100%	0%	-	20
	90%	10%	1:9	20
	80%	20%	1:4	20
	70%	30%	3:7	20
	60%	40%	2:3	20
	50%	50%	1:1	20
	40%	60%	3:2	20
	30%	70%	7:3	20
	20%	80%	4:1	20

Table 3-3 Formulation of microparticles: Formula I.

Material	%
Rifampicin	70
L-PLA	30
Dissolve in methylene chloride to make concentration of 20 mg/ml	

The formula I containing L-PLA and rifampicin was prepared to have drug: polymer ratio at 7:3 and sprayed into precipitation vessel at different operating conditions. Operating parameters, such as antisolvent pressure, temperature, solution concentration and solution feed rate were varied to determine their effects on precipitate characteristics. The same procedure as described in section 1 was conducted with different supercritical carbon dioxide pressures (Table 3-4). When pressure was varied other operation parameters were fixed as presented in Table 3-1. Various temperatures were used ranging from 33, 40 and 50 °C. Solution concentrations were varied at three levels as follows: 10, 20 and 30 mg/ml. Feed rates were varied ranging from 0.4, 0.5 and 0.6 ml/min. The effects of operating conditions on the particle size and particle size distribution were investigated.

Tables 3-4 Various operating conditions used for microparticle preparation.

Operating conditions	Level
Supercritical carbon dioxide pressure	2000, 2500, 3000 psi
Temperature	33, 40 , 50 °C
Concentration of solution	10, 20, 30 mg/ml
Feed rate	0.4, 0.5, 0.6 ml/min

1.4 Reproducibility of Supercritical Fluid Technique in Preparation Polymer-Drug Loaded Microparticles.

This experiment was performed to investigate the consistent of supercritical antisolvent process. Formula I was prepared as described in section 1. The operation parameters were fixed as in Table 3-1. Three consecutive batches of microparticles were prepared. Those microparticles from three batches were evaluated.

2. Characterization of Microparticles

2.1 Yield of Microparticles

The prepared microparticle was accurately weighed. The percent yield of microparticles was determined from equation (1).

$$\% \text{ Yield} = \frac{\text{Weight of microparticles (g)} \times 100}{\text{Theoretical weight of microparticles (g)}} \quad (1)$$

2.2 Surface Morphology

The powders were sputter-coated with gold prior to microscopic examination. Shape and surface morphology of microparticles were observed using scanning electron microscope (SEM, JSM-5410LV, Jeol, Ltd., Japan) and photographed at appropriate magnification.

2.3 Particle Size and Size Distribution

The volume particle size distribution of microparticles was determined by laser diffraction (Mastersizer 2000 Ver 5.1, Malvern Instruments Ltd.). The microparticles were suspended in an aqueous medium with small amount of Tween 80 (0.01%w/v). The particle size distribution was measured after 90s of sonication. (Muller and Thies 1998) The polydispersity of the microparticles was expressed by the span.

$$\text{Span} = \frac{[D_{90\%}-D_{10\%}]}{D_{50\%}} \quad (2)$$

where $D_{90\%}$, $D_{10\%}$ and $D_{50\%}$ were the equivalent volume diameters at 90, 10 and 50% cumulative volume, respectively. The particle size of the primary microparticles was described by the volume median diameter (VMD).

2.4 Drug Content and % Entrapment Efficiency

Drug content was determined for microparticles using a modified reverse phase high performance liquid chromatography (HPLC) method. A calibration curve was prepared for rifampicin in a concentration range of 10- 50 μg/ml using a solvent medium containing chloroform (1 ml), methanol (9 ml), and dissolved polymer (10 mg). The mobile phase consisted of 70% methanol and 30 % phosphate buffer (pH 5.2) (Panchagnula et al., 1999). Analysis was performed on a 20 μl injection at a flow rate of 1 ml through a Apollo C18 (5μ 250mm x 4.6mm) column (Altech, Deerfield, IL) and the absorbance measured at a wavelength of 238 nm.

A known mass of microparticles was dissolved in 1 ml of chloroform followed by the addition of 9 ml of methanol to precipitate the polymer. The sample was then centrifuged for 30 min at 4,000 rpm and 1 ml aliquot taken from supernatant and analyzed by HPLC.

Drug incorporation efficiency was expressed both as drug content (%w/w) and entrapment efficiency (%); represented by equations (3) and (4), respectively. The individual values for three replicate determinations and their mean values are reported. Mean values of three determinations were reported.

$$\% \text{ Drug content} = \frac{\text{Actual drug in microparticles} \times 100}{\text{Mass of microparticles}} \quad (3)$$

$$\% \text{ Entrapment efficiency} = \frac{\text{Actual drug in microparticles} \times 100}{\text{Theoretical drug in microparticles}} \quad (4)$$

2.5 *In vitro* Release Study

Release of rifampicin from rifampicin-loaded L-PLA microparticles with different polymer content (60%, 70%, 80%), rifampicin before and after supercritical process were investigated. The dissolution apparatus II was operated using constant temperature at 37 ± 0.5 °C with constant stirring rate of 50

rpm. The dissolution medium was two hundred milliliters of 0.05 M phosphate buffer in saline (PBS) with 200 μg/ml ascorbic acid added as an antioxidant to prevent oxidative degradation. The powder containing 10 mg of drug was placed in dissolution medium (n= 3). Three milliliters of each sample was withdrawn after the dissolution apparatus was operated at 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24 hours, respectively. Those sampled solutions were centrifuged at 4,000 rpm for 15 min. The equivalent amount of dissolution medium was added immediately after sampling to maintain a constant volume of dissolution medium. Samples were measured by UV spectrometer at 475 nm.

2.6 *In vitro* Drug Deposition

Dry powder inhaler formulations were prepared according to the formula II and III in Table 3-5 and 3-6, respectively. The microparticles were mixed with carrier lactose. The ratio of inhalation lactose type and polymer-drug loaded microparticles was 2:1. A cascade impactor comprising a preseparator, eight stages, glass throat and device (Andersen Sampler Inc., Atlanta, USA) was used (Figure 3-2). All parts were cleaned and rinsed with deionised water and were then sonicated for 15 minutes to ensure that there was no clogging in any of the orifices. The stages and plates were dried in a hot air oven before being employed in deposition studies which conducted at 60 l/min for 10 seconds (Srichana et al. 1998). Two formulations with different lactose types were employed for this study. The formulation comprised of the microparticles containing 5 mg rifampicin mixed with micronised lactose as presented in Table 3-5 and 3-6. The formulation was introduced to the cascade impactor using the glass device. The determination was carried out for five times. After actuating the dose into the Andersen cascade impactor at 60 l/min, glass device, the glass throat, preseparator and each stage were rinsed with methanol containing 0.02 % ascorbic acid solution into a volumetric flask and diluted to a known volume to give appropriate drug concentrations for subsequent HPLC analysis and calculate % deposition on each stage. Mass median aerodynamic diameter (MMAD) of each formulation was calculated.

Table 3-5 Dry powder inhaler formulations of Formula II

Materials	Lactose : microparticle ratio
Inhalation Lactose Type < 45 μm	2:1
Drug-polymer loaded Microparticles	

Table 3-6 Dry powder inhaler formulations of Formula III

Materials	Lactose : microparticle ratio
Inhalation Lactose Type 45-90 μm	2:1
Drug-polymer loaded Microparticles	

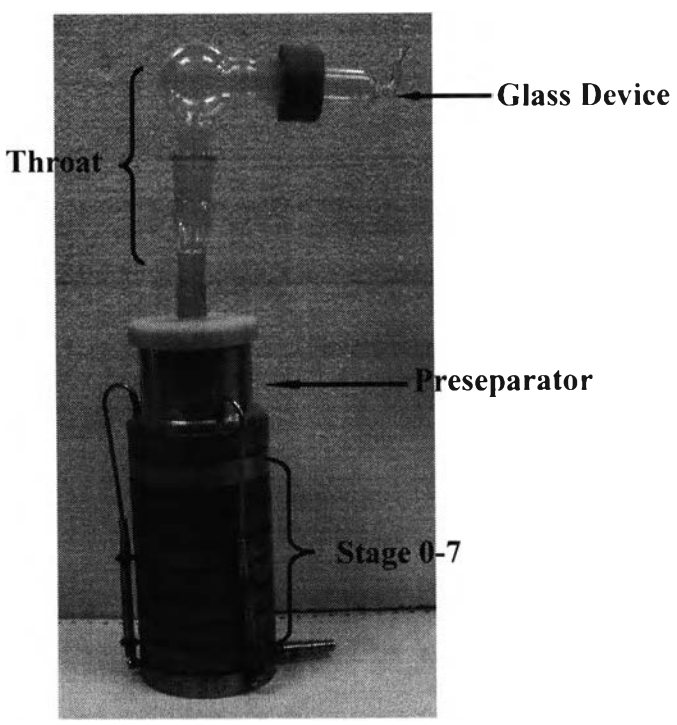

Figure 3-2 Andersen 1 CFM ambient samplers with preseparator, throat and glass device.

3. Physicochemical Characterization of Rifampicin and Microparticles

3.1 Differential Scanning Calorimetry (DSC)

Thermograms of microparticles and rifampicin were recorded on a DSC (Mettler Toledo, DSC 821e, Switzerland) using Mettler Stare system. The temperature axis and cell constant of DSC were previously calibrated with indium. A heating rate of 10 °C/min was employed over a temperature range of 25–300 °C with nitrogen purging (100 ml/min). Powder sample (2–8 mg) was weighed into an aluminum pan and analyzed as sealed with pinhole and an empty aluminum pan was used as a reference.

3.2 X-ray Diffraction (XRD)

XRD patterns of microparticles and rifampicin were acquired at room temperature on X-ray diffractometer (Model JDX-8030, JEOL, Japan) using Ni-filtered Cu Kα radiation (tube operated at 45 kV, 30 mA). Data were collected over an angular range from 5 to 60 ° 2θ in continuous scan mode using a step size of 0.04 °.

3.3 Fourier Transformed Infrared Spectroscopy (FTIR)

Spectra were recorded on a FTIR spectrophotometer (Model 1760X, Perkin Elmer, USA). Potassium bromide disk technique was employed. Each spectrum was collected in the region 400–4000 cm^{-1}.

4. Effect of Processing on Stability of Rifampicin

Rifampicin was prepared using above procedure described in section 1 and 1.1. Then unprocessed rifampicin and processed rifampicin by supercritical fluid technique were dissolved in methanol. These solutions were evaluated for percentage recovery of drug by HPLC method which modified from Panchagnula et al., 1999. The mobile phase consisted of 70% methanol and 30 % phosphate buffer (pH 5.2). Analysis was performed on a 20 µl injection at a flow rate of 1 ml through

a Apollo C18 (5μ 250mm x 4.6mm) column (Altech, Deerfield, IL) and the absorbance measured at a wavelength of 238 nm.

5. Determination of Residual Methylene Chloride Content in the Microparticles

Determination of residual methylene chloride content in the microparticles was modified from a method described by Spenlehauer, Veillard and Benoit (1986). A weighed quantity of microparticles (~10 mg) was dissolved in 2 ml of dimethylformamide. The solution was injected into a gas chromatography. Gas chromatography conditions for determined of methylene chloride were as follows:

Column: HP OV-101(100/120 mesh), 6Ft
Carrier Gas: Nitrogen, flow rate of 15 ml/min
Detector: FID
Hydrogen: flow rate of 35 ml/min
Air: 350 ml/min
Injector Temperature: 150 °C
Detector Temperature: 250 °C
Oven temperature program: Initial temperature of 40 °C, initial time 7 min,
rate of 45 °C/min
final temperature of 200 °C, final time 5 min

The amount of residual methylene chloride was determined from standard curve. The standard curve was plotted between peak area and concentration in ppm. of methylene chloride which was prepared to contain 1, 5, 10 and 20 ppm. ın dimethylformamide.

6. Effect of Molecular Weight of Polymer (L-PLA)

The formulation containing of L-PLA and rifampicin was prepared at drug: polymer ratio of 7:3. The microparticles were prepared using a procedure described in section 1 and 1.1 to investigate the effect of molecular weight of polymer on rifampicin-polymer microparticles properties. The operation parameters are shown in Table 3-1. During the particle formation, the pressure was fixed at 2500 psi and the temperature at 40 °C. Various molecular weights of L-PLA (Fluka) were used in this experiment as follows.

Table 3-7 Various molecular weights of L-PLA used for microparticle preparation.

polymer	Molecular weight	Drug: polymer ratio
L-PLA M1	~67,400 Da	7:3
L-PLA M2	~101,700 Da	7:3
L-PLA M3	~152,000 Da	7:3

7. Bactericidal Efficacy of the Drug-Loaded Microparticles Against *Mycobacterium Tuberculosis.*

The Middlebrook 7H11 Agar base (19 g) was weighed and dissolved in 900 ml distilled water and 5 ml glycerol. An aliquot of (180 ml) medium was transferred to each flask and autoclaved at 121 °C and 15 min. Then, the flask was incubated in water bath at 50 °C and 20 ml Middlebrook OADC was added. Stock solutions were prepared from powders of rifampicin, rifampicin-L-PLA microparticle and L-PLA in dimethyl formamide or 95 % methanol. When the medium was mixed homogenously, rifampicin, rifampicin-loaded microparticles or polymer stock solution was added to give final concentration of 0.5, 1.0 and 2.0 μg/ml and filled into Mac Cartney bottle. The slanting agar in Mac Cartney bottle was obtained from 7 ml of those medium.

The experiments were done with *Mycobacterium fortuitum* and *Mycobacterium avium complex* and fresh-isolated, drug susceptible and resistant obtained from patients, as well as with standard strain H37Rv as follows:

Mycobacterium fortuitum

Mycobacterium avium complex

Mycobacterium tuberculosis Rifampicin resistant strains

Mycobacterium tuberculosis Rifampicin sensitive strains

Mycobacterium tuberculosis standard strains (H37Rv)

The Mac Farland No.1 suspension of mycobacterium (0.1 ml) was taken into 4.9 ml distilled water. Then, it was inoculate (10 µl) on agar using standard loop. The organisms were grown on 7 ml slanting agar and were incubated for 28 days at 37 °C. The colonies were counted after of incubation.

8. High Performance Liquid Chromatographic Technique for Drug Analysis

The HPLC method was used to determinate percentage of drug loading from processed microparticles and drug deposition each stage in cascade impactor. The reverse phase HPLC assay with UV detection was modified from Panchagnula et al. 1999 and validated as the following:

HPLC Analysis

HPLC chromatographic conditions:

Column	: a Apollo C18 5µ (250mm x 4.6mm) (Altech, Deerfield, IL)
Mobile phase	: Methanol and phosphate buffer pH 5.2 (70:30). Mobile phase was prepared freshly and filtered through a 0.45 µm membrane filter. It was then degassed by sonication for a 30 min prior to use.
Flow rate	: 1.0 ml/min
Detector	: UV detector at 238 nm

Injection volume : 20 µl
Temperature : ambient
Retention time : 7.7 min

The typical analytical parameters considering in the validation of the quantitative determination of rifampicin by HPLC method were modified from USP (United States Pharmacopoeial Convention, 2002).

8.1 Specificity

The specificity of the HPLC method used to determine rifampicin quantity was evaluated by comparing the chromatograms of rifampicin and the peaks of other components in the microparticle systems. The blank preparation that had the same component as rifampicin-loaded microparticles was prepared and the peak of other component in the sample must not interfere with the peak of rifampicin.

8. 2 Precision

(a) Within Run Precision

The within run precision was evaluated by analyzing peak area of drug of three repetitions of each concentration of standard solution on the same day. The mean, the standard deviation (SD) and the percentage coefficient of variation (% CV) of each concentration were determined.

(b) Between Run Precision

The between run precision was evaluated by analyzing peak area of drug of three repetitions of each concentration of standard solution on 3 different days. The mean, the standard deviation and the percentage coefficient of variation of each concentration were determined.

8.3 Accuracy

The accuracy of rifampicin assay was evaluated by analyzing percent recoveries of each concentration of known rifampicin solutions. The known rifampicin samples were prepared from physical mixture of rifampicin and polymer. Percent recovery of each injection was calculated by comparing the concentration

fitted from a calibration curve with the known concentration. The mean, the standard deviation and the percentage coefficient of variation of each concentration were determined.

8.4 Linearity

The linearity was evaluated by plotting the standard curve between the peak area of rifampicin and the concentrations of rifampicin. Linear regression analysis was performed. The equation and the coefficient of determination (R-square) were calculated.

9. UV Spectrophotometric Method for Drug Analysis

The UV spectrophotometric method used to determine amount of rifampicin in dissolution test. The UV method was validated under the following condition.

9.1 Specificity

The specificity of UV spectrophotometric method was evaluated by comparing the spectrum of vehicle used to prepare standard solutions, rifampicin and the other components of a combined formulation. The maximum absorbance peak of drug must not be interfered by those of other compounds.

9.2 Precision

(a) Within Run Precision

The determination of within run precision of rifampicin assayed by UV spectrophotometric method was done by comparing the absorbance of drug of three repetitions of each concentration of standard solution in the same day. The mean, the standard deviation (SD) and the percentage coefficient of variation (% CV) of the absorbance of rifampicin of each concentration were determined.

(b) Between Run Precision

The determination of between run precision of rifampicin assayed by UV spectrophotometric method was done by comparing the absorbance

of drug of three repetitions of each concentration of standard solution on three different days. The mean, the standard deviation and the percentage coefficient of variation of each concentration were determined.

9.3 Accuracy

The determination of accuracy of rifampicin assayed by UV spectrophotometric was done by analyzing percent recoveries of three sets of 2, 4, 8 μg/ml (absorbance <0.2) and 12, 24, 32 μg/ml (absorbance >0.2) of known rifampicin solutions. The known rifampicin samples were prepared from physical mixture of rifampicin and polymer. Percent recovery of each concentration was calculated by comparing the concentration fitted from a calibration curve with the known concentration. The mean, the standard deviation and the percentage coefficient of variation of each concentration were determined.

9.4 Linearity

The linearity was determined by plotting the standard curve between the absorbance of rifampicin prepared to contain 0.5, 1, 2, 4, 6, 8, 10 μg/ml (absorbance <0.2) and 12, 16, 20, 24, 32, 40 μg/ml (absorbance >0.2) and the concentrations of rifampicin. Linear regression analysis was performed. The equation and the coefficient of determination (R-square) were calculated.