CHAPTER III
MATERIALS AND METHODS

Materials

Nifedipine was generously supplied by MOEHS, S.A. Barcelona Spain, Eudragit RS100 by Rohm Pharma GmbH (Darmstadt, Germany) and Polyvinylpyrrolidone K30 by BASF, Germany. The other materials were purchased from commercial sources. Deionized and HPLC water were used throughout this study.

4-Dimethylaminobenzaldehyde, HPLC grade, lot no. 362184/1 40897, Fluka chemika, Switzerland

Absolute ethanol, analytical grade, lot no. L868107, E. Merck, Germany

Absolute methanol, HPLC grade, lot no. L912502, E. Merck, Germany

Curcumin crude extract, Vejchapong Osoth, Thailand

Curcumin, analytical grade, lot no. 327874/1 295, Fluka chemika

Dichloromethane, analytical grade, lot no. K25290117825, E. Merck, Germany

Eudragit RS100, lot no. 8381008148, Rohm Pharma, Germany

FD&C Yellow No.5 (tartrazine), Supported by Department of Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand

FD&C Yellow No.6 (sunset Yellow), Supported by Department of Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand

Hydrochloric acid 37%, analytical grade, lot no. K25290117 825, E. Merck, Germany

Light Liquid Paraffin, lot no. 143605, S. Tong Co. Ltd., Thailand

Nifedipine, batch no. 71/2, MOEHS, S.A., Barcelona Spain

Polyvinylpyrrolidone K30, lot no. 51-4960, BASF, Germany

Sodium bisulfite, analytical grade, lot no. 7448KCLZ, Mallinckrodt Inc.

Sodium chloride, lot no. 47/874, E. Merck, Germany

Sodium hydroxide, lot no. 305C242198, E. Merck, Germany

Apparatus

Analytical balance, Sartorius 1615 MP, Germany

Digital illuminance meter, Model Tes-1332, Tes Electrical Electronic Corp., Taiwan

Dissolution apparatus, Model AT7, Sotax, Switzerland

Fluorescent tube, daylight, 15 watt, Asia Lighting, Thailand

High performance liquid chromatography (HPLC), Waters 745 Data module, USA

Hot air oven, Mammert

Image analyzer, Model KS400 rel. 2.0, Kontron electronik, Germany

Light cabinet, locally constructed, Thailand

Low pressure sodium lamp, SOX-EXWC 121 K, Phillips, UK

pH Meter, Model SA 520, Orion Research Inc., USA

Spray dryer, B-190, Buchi, Switzerland

UV spectrophotometer, Model 160A, Shimadzu, Japan

Methods

In order to prevent any influences to photodegradation of nifedipine which is very sensitive to light, experiments were conducted under yellow sodium light which wavelength region radiated is nonabsorbed by nifedipine (Florey, 1989; Soons et al., 1991). In addition, when needed, nifedipine containers were wrapped with aluminium foil.

1. Preparation of nifedipine spray dried microspheres

Nifedipine and combined polymers, Eudragit RS 100 and PVP K30, were weighed and dissolved in 1:1 mixture of ethanol-dichloromethane. Then the solution was spray dried using a Mini Spray Dryer (Buchi, B-190). The experimental parameters were set as follows (Sinsuebpol, 1999)

Inlet air temperature	55, 65 and 75 $^{\circ}$C
Aspirator setting	10
Pump setting	5 ml/min
Spray flow	600 NL/h
Spray concentration	5 % w/v
Nozzle opening	0.5 mm.

The spray-dried microspheres were collected from cyclone and collector and they were kept in a light protected glass desiccator at room temperature in order to protect them from any chemical and physical instabilities.

2. Quantitative analysis of nifedipine by HPLC

2.1. HPLC conditions

Stationary phase, μ-Bondapak C18 column 3.9 x 300 mm, particle size 10 μm, internal standard, 4-Dimethylaminobenzaldehyde, and detection wavelength, 254 nm, were chosen from previous reports (Pietta, Rava, and Biondi, 1981; Connors, 1982; Suzuki et al., 1985; Teraoka, Matsuda and Sugimoto, 1988; Al-Turk et al., 1989; Matsuda, Teraoka, and Sugimoto, 1989; Matsuura, Imaizumi and Sugiyama, 1990; Soons et al., 1991; Bechard et al., 1992; Ohkubo, Noro, and Sugawara, 1992; Swarbrick and Boylan, 1993; Grundy, Kherani, and Foster, 1994; Walily, 1997). Mobile phase and flow rate were optimized regarding to reported experiments to obtain a sharp peak which had an appropriate retention time, gave good resolution between nifedipine, nifedipine degradation products and the internal standard and had no interference by other substances, i.e., polymers, UV absorbers and antioxidants.

2.2. Calibration curve of nifedipine

1) Nifedipine of 31.25 mg was accurately weighed into 50 ml volumetric flask and then, dissolved in 1:1 mixture of methanol and water to 50 ml.

2) 10 ml of nifedipine solution was transferred and diluted to 50 ml. This solution was kept and used as nifedipine stock solution.

3) An amount of 93.75 mg of internal standard was accurately weighed into 50 ml volumetric flask and then, dissolved in 1:1 mixture of methanol and water to 50 ml.

4) 10 ml of internal standard solution was transferred and diluted to 50 ml. This solution was kept and used as internal standard stock solution.

5) Appropriate amounts of both nifedipine and internal standard stock solution were individually pipetted into separated volumetric flasks and adjusted to volume, the final concentration of nifedipine were between 2.5 and 35.0 ng/ml, and of internal standard was 15.0 ng/ml.

6) Peak area ratio between nifedipine and internal standard were measured using HPLC.

7) The curve plotted from the concentration against the peak area ratio was equated using the linear regression. All studies were run in triplicate.

3. Effects of processing and formulation factors on nifedipine degradation

3.1. Effects of PVP K30 content and inlet air temperature

1) Fifteen formulas of spray dried nifedipine microspheres were prepared. The ratio between nifedipine-combined polymers in all formulas were fixed at 1:10. The amount of PVP K30 in the combined polymers was varied as 0, 20, 50, 80, and 100% to make 5 ratios of nifedipine:Eudragit RS100:PVP K30 as 1:0:10, 1:2:8, 1:5:5, 1:8:2, and 1:10:0. The solution of each specified mixing ratio was spray dried by varying inlet air temperature as 55, 65 and 75 $^{\circ}$C.

2) The particle size analysis of nifedipine microspheres was performed with the image analyzer (Kontron electronik, KS400 rel. 2.0). At least 600 particles were measured for their longest diameters. The frequency distribution histogram and the percentage cumulative frequency curve were determined, afterthat the median diameter was obtained from 50% cumulative frequency size. The statistical difference of the median diameter among formulas were compared by Kruskal-Wallis test.

3) The fifteen formulas of nifedipine microspheres were individually and accurately weighed with equivalent amount of nifedipine 2.25 mg into fifteen sets of clear, 3 ml glass vials. The thickness of powdered samples in the vials was controlled to be uniformly less than 3 mm. All vials were tightly closed with rubber stoppers and sealed with aluminum seals in order to prevent from extraneous humidity.

4) Each set of vials was then divided into two groups. One was experimental group and the other one was control group of which vials were wrapped with aluminum foil.

5) All vials were stored in the light cabinet equipped with daylight fluorescent tubes and gave 1,200 lux light intensity. The temperature inside the cabinet were determined occasionally as $30 \pm 1\,^{\circ}C$

6) Three vials from the experimental group and one vial from the control group were drawn at appropriate periods and were analyzed for nifedipine content by HPLC method.

7) The order of degradation reaction was determined from the plots of remaining nifedipine content versus time according to zero-order, first-order and second-order plots.

8) The statistical difference among groups were compared by three-way analysis of variance (factorial design) at the significant level (α) of 0.05 using PVP K30 content, inlet air temperature, and sampling time as independent variables. If there was a significant difference, multiple comparisons were performed by Scheffe test.

3.2. Effect of microsphere particle size

1) Four formulas of 1:2:8 nifedipine:Eudragit RS100:PVP K30 spray dried microspheres were prepared by varying inlet air temperature, pump

setting, spray-flow, and solid concentration of spray solution in order to obtain 4 different particle sizes.

2) Particle size and size distribution of samples were determined by using an image analyzer. A small amount of samples was dispersed with light liquid paraffin and mounted on slide. The average size and size distribution were determined from 600 particles.

3) Photostability study of these four formulas of microspheres were followed the procedure in 3.1.

4) The statistical difference among groups were compared by two-way analysis of variance (randomized blocked design) at the significant level (α) of 0.05 using particle size of the microspheres, and sampling time as independent variables. If there was a significant difference, multiple comparisons were performed by Scheffe test.

3.3. Effect of drug-polymer ratio

1) Eight formulas of spray dried nifedipine microspheres were prepared using single polymer, Eudragit RS100 or PVP K30, instead of combined polymer. The ratio of drug-polymer was varied as 1:1, 1:3, 1:5 and 1:10. The inlet air temperature was fixed at 65 ^{o}C.

2) Photostability study of these eight formulas of microspheres were followed the procedure in 3.1.

3) The statistical difference among groups were compared by two-way analysis of variance (randomized blocked design) at the significant level (α) of 0.05 using the drug-polymer ratio, and sampling time as independent variables. If there was a significant difference, multiple comparisons were performed by Scheffe test.

3.4. Effect of light intensity

1) Spray dried microspheres of 1:2:8 nifedipine:Eudragit RS100:PVP K30 were prepared using inlet air temperature of 65 $^{\circ}$C.

2) Sample was stored separately in 4 groups in the light cabinet, which gave varied light intensities of 400, 800, 1200, and 2000 lux. The temperature inside the cabinet was determined occasionally as 30 ± 1 $^{\circ}$C.

3) Photostability study of these 4 groups of microspheres were followed the procedure in 3.1

4) The statistical difference among groups were compared by two-way analysis of variance (randomized blocked design) at the significant level (α) of 0.05 using the light intensity, and sampling time as independent variables. If there was a significant difference, multiple comparisons were performed by Scheffe test.

3.5. Effect of UV absorbers and antioxidants

3.5.1. Photostabilization of nifedipine in solution state

1) Nifedipine of 31.25 mg was accurately weighed into 50 ml volumetric flask and then, dissolved in 1:1 mixture of methanol-water to 50 ml. This solution was kept and used as stock solution.

2) Twenty formulas of 2 mg% (20 ng/ml) nifedipine solution with the UV absorbers or antioxidant were prepared by transferring 10 ml of nifedipine stock solution into a 250 ml volumetric flask. Appropriate amounts of the UV absorbers or antioxidant were added to make four final concentrations as 2, 4, 8 and 16 mg% for the UV absorbers, tartrazine,

sunset yellow, curcumin and curcumin crude extract, and as 0.05, 0.1, 0.5, and 1% for the antioxidant, sodium bisulfite.

3) Ten ml of the solution were transferred into a number of clear glass screwed-cap test tubes.

4) Photostability study of these solutions were followed the procedure in 3.1 using 2 mg% nifedipine solution with neither UV absorber nor antioxidant as the control.

5) The statistical difference among groups were compared by two-way analysis of variance (randomized blocked design) at the significant level (α) of 0.05. If there was a significant difference, multiple comparisons were performed by Scheffe test.

6) The type and concentration of UV absorber or antioxidant that gave the most stabilization was chosen for further study.

3.5.2. *Photostabilization of nifedipine in solid state*

1) Spray dried microspheres of 1:2:8 nifedipine:Eudragit RS100:PVP K30 with the UV absorber chosen from 3.5.1. were prepared using inlet air temperature of 65 ^{o}C.

2) Photostability study of these microspheres were followed the procedure in 3.1 using microspheres of 1:2:8 nifedipine:Eudragit RS100:PVP K30 without UV absorber as the control.

3) The statistical difference among groups were compared by two-way analysis of variance (randomized blocked design) at the significant level (α) of 0.05. If there was a significant

difference, multiple comparisons were performed by Scheffe test.

4. Effect of relative humidity on nifedipine microspheres stability

4.1. Moisture uptake study

1) Fifteen formulas of spray dried nifedipine microspheres were prepared. The ratio between nifedipine-combined polymers in all formulas were fixed at 1:10. The amount of PVP K30 in the combined polymer was varied as 0, 20, 50, 80, and 100% to make 5 ratios of nifedipine:Eudragit RS100:PVP K30 as 1:0:10, 1:2:8, 1:5:5, 1:8:2, and 1:10:0. Every individual ratio was spray dried by varying inlet air temperature as 55, 65 and 75 $^{\circ}$C.

2) The amount of 100 mg of the fifteen formulas was accurately weighed into open amber glass vials.

3) The vials were kept in 4 varied relative humidities (RH) which prepared and controlled by using different saturated salt solutions in glass desiccators incubated at 40 $^{\circ}$C. The saturated solutions of following salts, i.e. magnesium chloride hexahydrate, ammonium nitrate, sodium chloride and potassium sulfate, in water were prepared and equilibrated in the desiccators for 24 h before the study and gave 31, 53, 75 and 96% RH, respectively (Lide, 1995).

4) Each vial was accurately weighed periodically until the moisture sorption was in equilibrium, that was the weight was constant.

5) Critical relative humidity (CRH) of each formula was obtained from the plot of moisture uptaken against percentage relative humidity.

6) The statistical difference among groups were compared by Kruskal-Wallis test at the significant level (α) of 0.05. If there is a significant difference, multiple comparisons were performed.

4.2. Chemical stability study

1) The final microspheres samples of the fifteen formulas from 4.1 were analyzed for remaining nifedipine content by HPLC using samples which were protected from moisture as the control.

2) The statistical difference among groups were compared by Friedman test at the significant level (α) of 0.05. If there was a significant difference, multiple comparisons were performed.

5. Effect of light, relative humidity and temperature in ambient atmosphere on stabilized nifedipine microspheres.

5.1. Chemical stability study

1) Two formulas of spray dried microspheres of 1:2:8 nifedipine:Eudragit RS100:PVP K30 with and without the UV absorber chosen from 3.5.1. were prepared using inlet air temperature of 65 $^{\circ}$C.

2) The two formulas were then accurately weighed equivalent to 2.25 mg of nifedipine into clear glass vials.

3) The vials of the two formulas were stored separately into two groups. One was experimental group which was kept open, and the other one was the control group which was tightly closed with rubber stopper, sealed with aluminum cap and wrapped with aluminium foil.

4) All vials were kept in the light cabinet which gave 1,000 lux of light intensity and the temperature was $30 \pm 1\ ^{\circ}C$

5) Samples of 3 vials from the experimental group and 1 vial from the control group were drawn periodically and analyzed by HPLC method for remaining nifedipine content.

6) Nifedipine degradation rate constant in each group was determined and compared. The statistical difference among groups were compared by two-way analysis of variance (randomized blocked design) at the significant level (α) of 0.05. If there was a significant difference, multiple comparisons were performed by Scheffe test.

5.2. Dissolution study

1) The two formulas of spray dried microspheres of 1:2:8 nifedipine:Eudragit RS100:PVP K30 with and without the UV absorber were studied for their dissolution characteristics of the control and the experimental group. Microspheres of equivalent amount to 10 mg of nifedipine were weighed accurately for each dissolution study.

2) The dissolution of each formula was performed in triplicate with the dissolution apparatus II (USP 24) as follows:

   a) Each vessel contained 900 ml of simulated intestinal fluid without enzyme, pH 7.5 as the dissolution medium which was allowed to equilibrate to a temperature of $37 \pm 0.5\ ^{\circ}C$ using a rotation speed of 150 rpm.

   b) Five ml of solution was withdrawn through a 10 μm filter at appropriate time intervals and replaced with five ml of fresh dissolution medium after each sampling to maintain a constant volume. The dissolution study was operated for 24 hours.

c) The withdrawn solution was determined spectrophotometrically at 238 nm.

d) The dissolution profile was obtained from the plot of the percentage dissolved of nifedipine against time.

3) The statistical difference among dissolution profiles of each group were compared using the two-way analysis of variance (randomized blocked design) at the significant level (α) of 0.05. If there was a significant difference, multiple comparisons were performed by Scheffe test.