CHAPTER III

MATERIALS AND METHODS

Materials.

The following materials were purchased from commercial sources except nifedipine was kindly donated by MOEHS,S.A. Barcelona Spain , polyethylene glycols and poloxamers were donated by BASF. Deionized water was used throughout this study.

Model drug.

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

Carriers.

1. Polyethylene glycol 4000 (lot no.49-4429, BASF, Germany)
2. Polyethylene glycol 6000 (lot no.32-3729, BASF, Germany)
3. Poloxamer 188 (lot no.87-0807, BASF, Germany)
4. Poloxamer288 (lot no. WPWT-566B, BASF, Germany)
5. Poloxamer 407 (lot no.12-0226, BASF, Germany)
6. 2-Hydroxypropyl-β-cyclodextrin (lot no.369003/1 21697, Fluka, Switzerland)
7. β-cyclodextrin (Ringdex-B® lot no. 23723, Merician Corporation).

Other substances.

1. Absolute ethyl alcohol, analytical grade (lot no. K25846283 844, E.Merck, Germany)
2. Hydrochloric acid 37% (lot no. K25290117 825, E.Merck, Germany)
3. Methyl alcohol (lot no. 980060049, Lab- Scan)
4. Acetone (lot no. 98081038, Lab-Scan)
5. Sodium chloride (lot no. 47/874, E.Merck, Germany)
6. Potassium bromide (lot no. 378170/1 50398, Fluka, Switzerland)

Apparatus.

1. Analytical balance (Satorius, GMPH,Germany)
2. Hot air oven (UL 50, Memmert, Germany)
3. Ultrasonic bath (3210, Branson, Swithkline Co.,U.S.A.)
4. Vertical rotator apparatus (EWPC 902/T/R/P,Eliwell)
5. Rotary evaporator (RE120, Buchi, Switzerland)
6. UV Spectrophotometer (Model 7800, Jasco Corporation, Thailand)
7. Dissolution apparatus (Model AT7, Sotax, Switzerland)
8. Fourier transform infrared spectrometer (Perkin Elmer Spectrum 2000, U.S.A.)
9. X-ray diffractrometer (Rigaku Denki 2027, Japan)
10. Differential scanning calorimetry (Model TA9900,Du Pont)
11. Scanning electron microscope (JSM-6400, Jeol, Japan)
12. Low pressure sodium lamp (SOX-E XWC121K, Phillips)
13. Fluorescent lamp (TFC FL-15D,15 watt, 43 cm., daylight, Taiwan)
14. Full flow™ filters (10μm,VanKel Industries, Inc.)
15. Membrane filters (lot. No.-7295-17, 0.8μm, Domnick hunter, Asypor)

Methods.

As nifedipine is sensitive to light, all experiments were conducted under yellow sodium light which has nonabsorbed wavelength by nifedipine to prevent any influences from photodegradation (Abrahamsson et al., 1998). In addition, containers used for nifedipine were wrapped with aluminium foil, when needed, throughout the experiment.

A. Preparation of powder dispersion

Nifedipine was solid-dispersed in PEG family (PEG4000 and PEG6000), poloxamer family (poloxamer188, poloxamer288 and poloxamer407) and cyclodextrin family (β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin). The ratios between drug and all the carriers were standardized at 1:1, 1:3, 1:5 and 1:10 on the weight per weight basis.

Each combinations were prepared by 3 methods which are melting method, solvent method and kneading method compared to physical mixing. Each method is detailed in 1-4. Then the solidified products were stored in a desiccator overnight. The dried mass was ground with mortar and pestle before passed through 60 mesh sieve. The final products were store in a desiccator ready for further experiments.

1. Preparation of nifedipine physical mixtures.

1.1 The required amount of nifedipine and carrier were accurately weighed in weight ratio as shown in Table 5.

1.2 Both components were thoroughly mixed in glass a mortar with pestle for five minutes as illustrated in Figure11.

1.3 The mixture was then screened through a 60 mesh sieve and stored in a desiccator.

2. Preparation of nifedipine solid dispersion by melting method

2.1 The required amounts of nifedipine and carrier were accurately weighed and physically mixed.

2.2 The mixture was melted on the sand bath. It was continuously stirred until both components were completely melted.

2.3 Rapid cooling was then conducted in an icebath .

2.4 The solid dispersion was placed in a desiccator overnight.

2.5 Solid dispersion was scrapped, grounded, passed through 60 mesh sieve and stored in a desiccator.

β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin can not be prepared by melting method because of too high melting points (about 280°C)of both carriers even though extremely high temperature was applied (about 200°C) which higher than melting point of nifedipine, may be the degradation products of nifedipine will occur.

The procedure was shown in Figure 12.

3. Preparation of nifedipine solid dispersion by solvent method.

3.1 The accurately weighed amount of nifedipine was dissolved in 20 ml of acetone, except for 2-hydroxypropyl-β-cyclodextrin solid dipersion, nifedipine was dissolved in methanol (0.1g: 50 ml).

3.2 Carriers were dissolved in 30 ml of absolute ethanol and sonicated until solution obtained, except for 2-Hydroxypropyl-β-cyclodextrin was dissolved in methanol 40 ml.

3.3 After that the dissolved drug was thoroughly mixed with the dissolved carrier in a round bottom flask.

3.4 The mixture was then evaporated by the rotary evaporator under vacuum condition until solvent completely evaporated. (about 10 hours) and placed in a desiccator overnight.

3.5 The solid dispersion was grounded in a mortar and pestle and screened through 60 mesh and kept in a desiccator

The procedure was shown in Figure 13 and the types and amount of solvent used in all treatments were summarized in Table 6.

The solid dispersion of β-cyclodextrin cannot prepared by solvent method, since an appropriate solvent systems at appropriate volume to dissolve both nifedipine and β-cyclodextrin cannot be obtained.

4. Preparation of nifedipine solid dispersion by kneading method.

4.1 The physical mixture of accurately weighed nifedipine and carrier as shown in Table 5 was made in mortar for 5 minutes.

4.2 The mixture was kneaded with deionized water in the amount of 0.1 times of total weight for PEGs and poloxamers but 0.4 times of total weight for cyclodextrins. Water was gradually added while continuously kneading. Kneading time was controlled at 30 minutes. This should have given the mixture homogeneous texture.

4.3 Water can be added during kneading to maintain moist homogeneous texture. The procedures were illustrated in Figure 14.

Table 5 The weight of nifedipine : carrier in each ratio used in the preparation.

Method	Carrier	Drug : Carrier (g)			
		Ratio 1: 1	Ratio 1: 3	Ratio 1: 5	Ratio 1: 10
Physical mixing	PEG4000	2.5 : 2.5	2.5 : 7.5	1.67 : 8.33	0.9091 : 9.099
	PEG6000				
	Poloxamer188				
	Poloxamer288				
	Poloxamer407				
	β-cyclodextrin				
	2-hydropropyl-β-cyclodextrin				
Melting	PEG4000	2.5 : 2.5	2.5 : 7.5	1.67 : 8.33	0.9091 : 9.099
	PEG6000				
	Poloxamer188				
	Poloxamer288				
	Poloxamer407				
	β-cyclodextrin	-	-	-	-
	2-hydropropyl-β-cyclodextrin	-	-	-	-
Solvent	PEG4000	2.5 : 2.5	2.5 : 7.5	1.67 : 8.33	0.9091 : 9.099
	PEG6000				
	Poloxamer188				
	Poloxamer288				
	Poloxamer407				
	β-cyclodextrin	-	-	-	-
	2-hydropropyl-β-cyclodextrin	0.5 : 0.5	0.5 : 1.5	0.5 : 2.5	0.5 : 5
Kneading	PEG4000	2.5 : 2.5	2.5 : 7.5	1.67 : 8.33	0.9091 : 9.099
	PEG6000				
	Poloxamer188				
	Poloxamer288				
	Poloxamer407				
	β-cyclodextrin				
	2-hydropropyl-β-cyclodextrin				

PHYSICAL MIXTURE

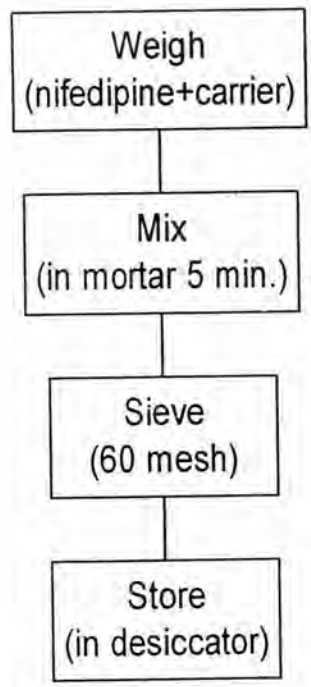

Figure 11 A schematic diagram for preparing nifedipine solid dispersion as physical mixture.

MELTING METHOD

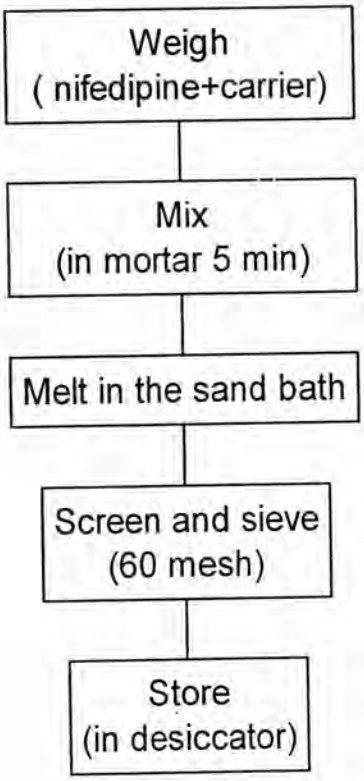

Figure 12 A schematic diagram for preparing nifedipine solid dispersion by melting method.

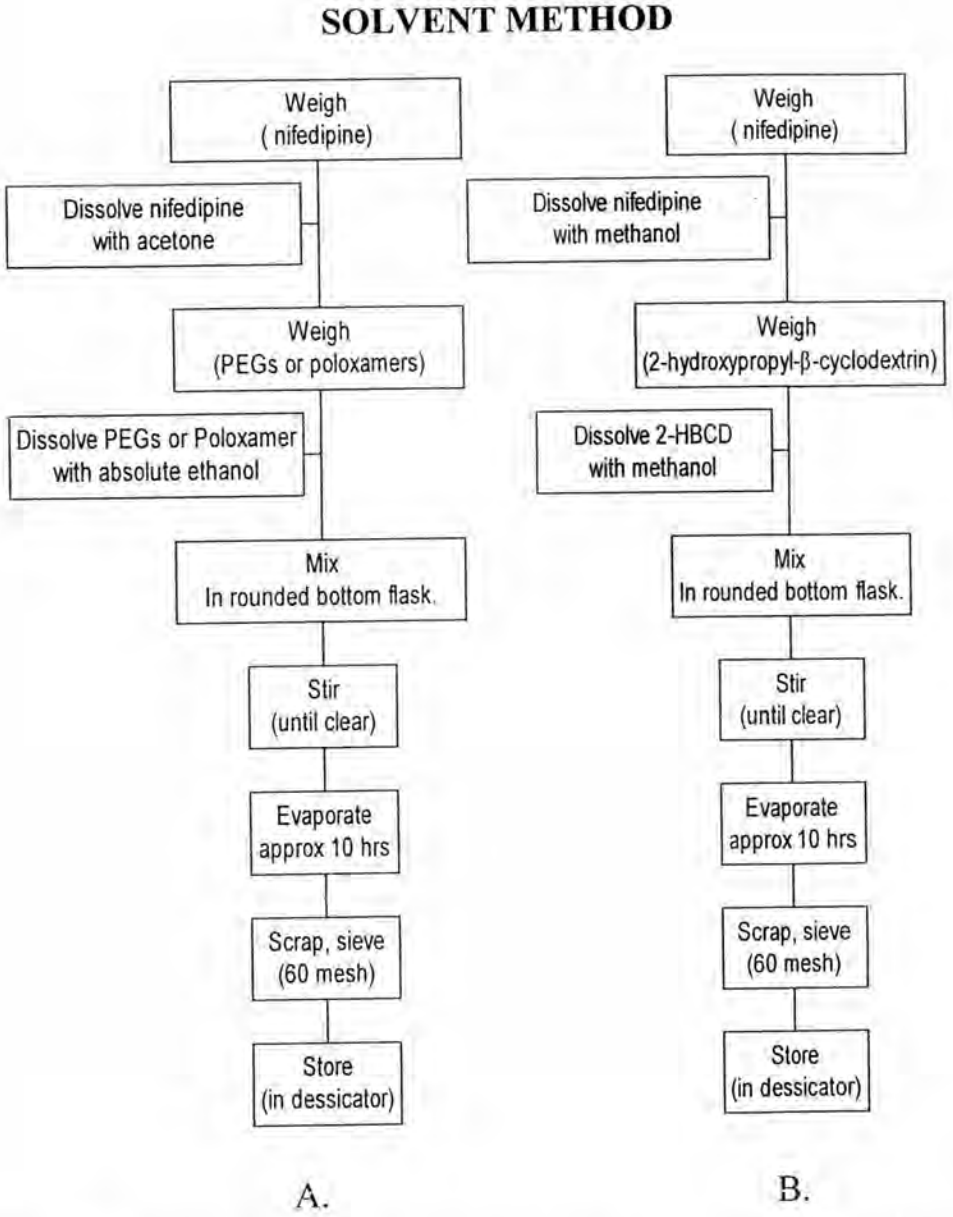

Figure 13 Schematic diagrams for preparing nifedipine solid dispersion by solvent method for PEGs and poloxamers (A) and 2-hydroxypropyl-β-cyclodextrin (B).

Table 6 Types of solvent and volume used in preparation of solid dispersion.

Carrier	Solvent for carrier	Vol. (ml)	Solvent for nifedipine	Vol. (ml)	Drug: Carrier			
					1:1	1:3	1:5	1:10
PEG4000	Absolute ethanol	30	acetone	20	2.5 : 2.5	2.5 : 7.5	1.67 : 8.33	0.9091 : 9.091
PEG6000								
polox188								
polox288								
polox407								
BCD	-	-	-	-	-	-	-	-
2-HBCD	methanol	40	methanol	0.1g/ 50ml	0.5:0.5	0.5:1.5	0.5:2.5	0.5:5

KNEADING METHOD

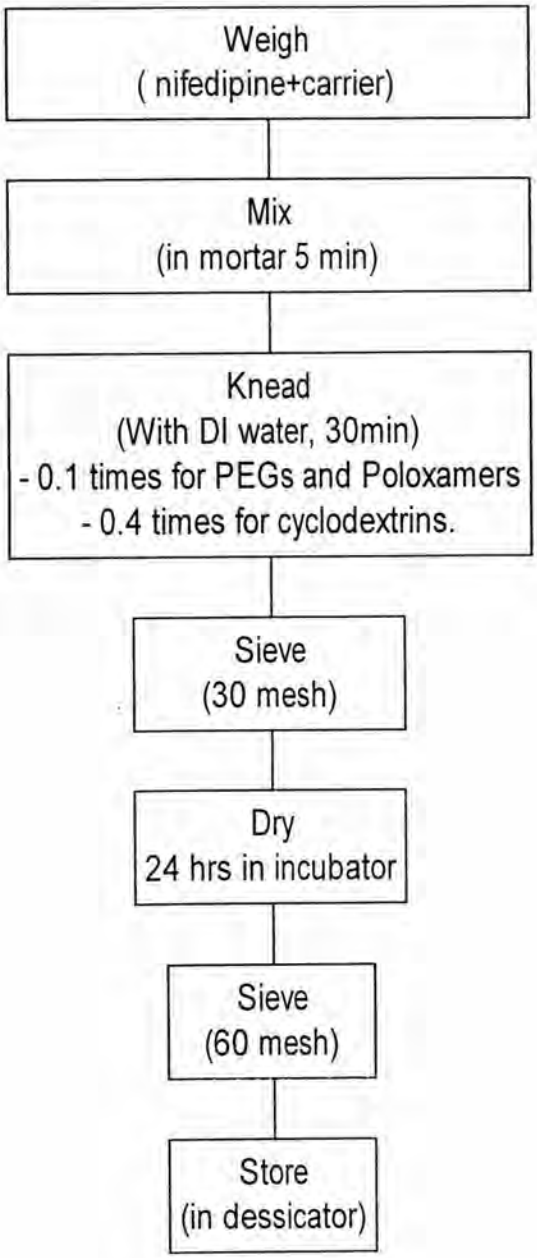

Figure 14 A schematic diagram for preparing nifedipine solid dispersion by kneading method.

B. In-Vitro Evaluation.

1. Analysis and calibration curve of nifedipine

1.1 Nifedipine was weighed accurately 0.031g into a 100 ml volumetric flask.

1.2 Nifedipine was diluted to 100 ml using absolute ethanol.

1.3 Transferred 10 ml nifedipine solution was diluted to 100 ml. in a volumetric flask and keep it as a stock solution.

1.4 Appropriate dilution of nifedipine standard solutions were made by diluting nifedipine stock solution as shown in Figure 15 using simulated gastric fluid without pepsin as solvent (USPXXIII).

1.5 Absorbances of nifedipine solutions were measured by spectrophotometric method at 238 and 280 nm (maximum wavelength for reduced form and oxidized form respectively). These two wavelengths were previously investigated before and after the solution was irradiated to a 15 watt fluorescent lamp for 4 hours using a double beams spectrophotometer in a 1-cm cell (Al-Turk et al., 1989).

1.6 Measured nifedipine solution was then transferred to a light cabinet which had a 40 cm fluorescent lamp hanging 30 cm above the sample solutions. The intensity of light is about 1000-1300 lux.

NIFEDIPINE DILUTION FOR CALIBRATION CURVE

Nifedipine = 0.031g.

↓

100 ml with absolute ethanol

↓

10 ml of nifedipine solution

↓

100 ml with SMG* (stock solution)

↓

1 ml. ⇓ 10 ml.	1.5 ml. ⇓ 10 ml.	2 ml. ⇓ 10 ml.	2.5 ml. ⇓ 10 ml.	3 ml. ⇓ 10 ml.	4 ml. ⇓ 10 ml.	4.5 ml. ⇓ 10 ml.	5 ml. ⇓ 10 ml.
3.1 μg/ml	4.65 μg/ml	6.2 μg/ml	7.75 μg/ml	9.3 μg/ml	12.4 μg/ml	13.95 μg/ml	15.5 μg/ml

- * simulated gastric fluid without pepsin.
- Dilution in the table, diluted by simulated gastric fluid without pepsin.

Figure 15 A schematic diagram of nifedipine dilution for calibration curve.

1.7 After irradiating for 4 hours, nifedipine was oxidized to be nitrosopyridine (Al-turk et al., 1989) at 238 nm and 280 nm. Absorbance of each solution was measured again at 238 and 280 nm.

1.8 A linear regression between concentration and absorbance was made to obtain 4 slope values and Y-interceptions (two values before and after irradication at 238 nm and also two values at 280 nm)

An equation was derived as shown in Appendix A by using 4 slopes and Y-interception values for calculating the reduced form of nifedipine in further study of dissolution and determination of percentage drug content. The validation of calibration curve is in the Appendix A.

2. Dissolution study

2.1 The dissolution studies were performed in triplicate with Sotax dissolution test apparatus (USPXXIII, apparatus 2), in simulated gastric fluid without pepsin at 37°C using the paddle method at a rotation speed of 150 rpm.

2.2 A certain amount of each sample, containing equivalent amount to 10 mg nifedipine was put into a vessel with 900 ml of simulated gastric fluid without pepsin.

2.3 After 5, 10, 15, 20 min and so on until the dissolution was in steady state, 5 ml of solution were withdrawn through 10 μm filters. The initial volume of the vessel was maintained by adding 5 ml of the same medium after each sampling.

2.4 The withdrawn solution was assayed spectrophotometrically with Jasco UV-spctrophotometer at 238 and 280 nm .

2.5 The concentration of reduced form of nifedipine present in solution was calculateed from the derived equation as previously described in 1.8.

The investigated samples were those prepared from 1:1, 1:3, 1:5 and 1:10 nifedipine–carrier mixing ratios with 7 carriers (PEG4000, PEG6000, poloxamer188, poloxamer288, poloxamer407, β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin) with various methods. The untreated and treated nifedipine were also investigated.

2.6 The dissolution profile of % dissolution of nifedipine was plotted against time and dissolution rate constant was analyzed at 30 minute. Then the statistical significance of dissolution rate constants of each carrier were determined by the two way analysis of variance at 95% confidence interval (Appendix D). In addition, the time 80% of nifedipine dissolved was also discussed.

3. Solubility Study

Solubility study of nifedipine-carrier were carried out according to the method of Higuchi and Connors (1965). Each concentration of carriers were investigated in triplicate . All steps have to be protected from light .

3.1 Excess amount of nifedipine was added to 5 ml solutions containing different concentrations of carriers and rotated for 24 hours (The preliminary

test showed that the equilibrium was obtained at about 24 hours.) by vertical rotator, previously adjusted to 30 ± 2°C.

3.2 Then, the solution was filtered passed through 0.8 μm membrane filter and suitably diluted with deionized water.

3.3 The solution was analyzed spectrophotometrically at 238 and 280 nm to define the solubility characteristics. Each concentration of carriers were performed in tripicate. All steps of the study have been protected from light.

4. Scanning electron microscope study.

Electron photomicrographs of samples were taken with the scanning electron microscopy. The samples were coated with gold before examination, using ion sputtering. Then they were photographed at appropriate magnification scales. The samples were all ratios of 7 carriers by 4 methods in this experiment except for melting and solvent method β-cyclodextrin and melting method 2-hydroxypropyl-β-cyclodextrin.The samples including nontreated pure nifedipine, treated pure nifedipine by four methods and seven pure carriers.

5. Powder X-ray diffraction study

The powder X-ray diffraction (XRD) pattern was investigated on Rigaku Denki 2027 Diffractometer with target Cu and filter Ni. The measurement condition was as follows:

Voltage	30 KV
Current	5 mA

Scanning speed	4°C/min
Scanning range (2θ)	5-40°

6. Differential scanning calorimetry study.

Differential scanning calorimetry (DSC) was investigated on a differential scanning calorimeter (DuPont, Model TA9900). The 2-3 mg samples was accurately weighed and placed in a closed aluminium pan. The measurement condition was as follows:

Scanning speed	5°C/min.
Temperature range	35-250 °C
Atmosphere	Nitrogen gas, flow rate 60ml./min.

7. Infrared spectrophotometric study.

Infrared (IR) spectra were measured by the KBr disc method using Perkin-Elmer Spectrum 2000 infrared spectrophotometer in the range of 4000-400 cm^{-1}, the characteristic bands were observed.

8. Wettability Study

The wettability of powder samples was investigated by the modified method of Imai et al (1989).

8.1 The sample powder of 200 mg weight was compressed into a cylindrical tablet (11 mm diameter) using a single punch compressing machine at a pressure of 400 psi for 1 min.

8.2 A 20 µl drop of deionized water was placed on the flatted tablet surface using a micropipette.

8.3 After 2 seconds, the drop was photographed, and the contact angle was measured directly from the photographs.