CHAPTER II

MATERIALS AND METHOD

Materials.

- Nifedipine (Batch No.74404/87,Nobel Chemical, Sweden)
- Methocel® A 4M (Lot. No. 83101602A, Premium grade,The Dow Chemical Company, USA)
- Methocel® K 4M (Lot. No. 5022101K, Premium grade,The Dow Chemical Company, USA)
- Methocel® K 100M (Lot. No. 85100501K, Premium grade, The Dow Chemical Company, USA)
- Methanol AR grade (E.Merck, Darmstadt, Germany)
- Methanol HPLC grade (E.Merck, Darmstadt, Germany)
- Ethanol AR grade (E.Merck, Darmstadt, Germany)
- Disodium hydrogen phosphate (Srichand-United Dispensary, Thailand)
- Polyvinyl alcohol 72000 (Lot. No. 81384, Fluka A G, CH-9470 Buchs, Switzerland)
- Polyvinyl pyrrolidone K 30 (Batch. No. 65-2955,BASF, Germany)
- Polyethylene glycol 4000 (Pharmaceutical Science Ltd., Part.,Thailand)
- Polyethylene glycol 400 (Pharmaceutical Science Ltd., Part.,Thailand)
- Glycerin AR grade (E.Merck, Darmstadt, Germany)
- Pluronic® F 127 (WPAH-613B,BASF Corporation Chemical Division, USA)

- Dimethyaminobenzaldehyde AR grade (E. Merck, Darmstadt, Germany)
- Dichlormethane AR grade (E. Merck, Darmstadt, Germany)
- Pentane AR geade (BDH, Limited Pool, England)

Apparatus :

- Analytical Balance (Sartorius A200s, Germany)
- Hot Air Oven (Memmert, Type UL 80, Germany)
- pH meter (Pye Model 232, Pye Unichem Ltd.,England)
- Spectrophotometer (Spectronic 2000, BAUSCH & LOMB, USA)
- Centrifuge (Hettich Zentrifugen, Type 2000, Germany)
- Stir-Plate (Model No. SP-18420, Thermplyne Sybron Corporation, Iowa, USA)
- Terumo disposable needle and syringe (23 G * 1 1/2 and 2.5 ml.)
- Diffusion cell, diameter 4.0 cm. (Modified from Keshary-Chien diffusion cell)
- Durapore 0.45 μm. (Lot. No. J5B98705A, Millipore Corp. Bedford, Mass.,USA)
- High performance liquid chromatography
  - : pump (Shimadzu, model C-R3A)
  - : UV Absorption detector (Shimadzu, model SPO-6AV)
  - : Intregator (Shimadzu, model C-R3A)
  - : Reverse-Phase Column : Zorbax-ODS 7 um. 4.6 mm. * 25 cm.

Since nifedipine is sensitive to light, it is necessary that the whole experiment has to be done in the dark room and all of the glasswares used have to wrapped aluminium foil to avoid directly contact with the white light.

1. Preparation of 1% w/w Nifedipine Transdermal Delivery System.

1.1 Formulation :

Nifedipine TDDS preparations were formulated by single or combined hydrophilic polymers. Every formulation consists of the fixed concentration of nifedipine (1% w/w). The formulations containing various amount of the polymers were prepared as following;

- Pluronic(R)F127 in water in the range of 30-50% w/w
- PEG 4000 and 400 copolymer in the ratio of 1:4, 1:2,and 1:1
- Copolymers PVA:PVP (1:1) in glycerine and water in the range of 30-50% w/w
- Methocel(R)A 4M in the range of 5-20% w/w
- Methocel(R)K 4M in the range of 5-20% w/w
- Methocel(R)K 100M in the range of 5-20% w/w

1.2 Procedure for Preparing Drug-Polymer Matrix Preparations.

A weight of 20.0 g. was obtained for each preparation. If necessary, another 20.0 g. was prepared.

Pluronic(R)F 127

The TDDS preparations of nifedipine with Pluronic(R)F 127 were prepared by cold process (51). The required amount of Pluronic(R)F 127 was weighed in the beaker and an accurate weight of 200 mg. of nifedipine was poured. Then cold water (5-10°C) was slowly added under constant agitation until the total weight of the preparation was as required. This dispersion was stored overnight in a refrigerator in order to form a clear and viscous solution.

Transformation to gel state occurred when the solution was later warmed at room temperature.

PEG 4000 and PEG 400 copolymers

Two hundred milligrams of nifedipine was dispersed in a required amount of PEG 400 and warmed to 65°C. The calculated amount of PEG 4000 was melt on a water bath to 65°C and was mixed with the previously dispersed solution. The mixture was allowed to cool, and stirred until congealed.

PVA - PVP copolymers

Equal amount of PVA and PVP as required and 5 g. of glycerine were dissolved in 60 ml. of water by heating on a water bath at 90°C. until the solution was remained to a total weight as required. Two hundred milligrams of nifedipine was dispersed in this solution under constant agitation until a uniform mixture was occured. This mixture solution was poured into the donor of diffusion cell and warmed in hot air oven at 37°C for 5-6 hours to form gel.

Methocel(R) A 4M, K 4M and K 100M

Methocel(R) powder was dispersed with 1/3 of the required total weight of water as hot water (75-80° C). The dispersion was then cooled for a few minutes, then mixed throughly with accurately weighed nifedipine. The remainder of the water was added as cold water to a total weight as required. Agitation was continued until clear solution appeared. This mixture was stored in a refrigerator for completely solubilized of a clear, viscous gel.

1.3 Selection of Nifedipine TDDS Formulations

Three formulations of each hydrophilic polymers were selected for further experiment. These formulations were selected by depending on the difficulty in preparing and the physical appearance of the obtained gel matrix. The physical appearances of nifedipine preparations were visually observed including ; color, clarity, and air bubbles. The rigidity, the residue on the skin when spreading the gel matrix were also observed.

2. Analytical Quantitation of Nifedipine

2.1. Analysis of Nifedipine in Preparations

Various analytical techniques for quantitative determination of nifedipine in preparation were reported such as thin layer chromatography (TLC) (52), UV Spectrophotometry (52-56), and high performance liquid chromatography (HPLC) (57). In USP XXI, UV spectrophotometric technique was officially used for determining the content uniformity of nifedipine preparation and thin layer chromatographic technique was recommanded for the assay of drug in preparation. At present, HPLC method is the official assay in USP XXII for drug preparation.

In this study, UV Spectrophotometric technique was used for determination of the concentration of nifedipine released from the preparation according to USP XXI.

Analytical Procedure.

2.1.1. Determination of the maximum absorption wavelength of nifedipine :

Since the medium solution was not methanol, the absorption wavelength used was not the same as that in monograph. It is necessary to find out the maximum absorption wavelength of nifedipine in the medium of PEG 400 and ethanol co-solvent.

A concentration of 0.01 mg/ml nifedipine in the medium was prepared for scanning to determine the maximum absorption wavelength of nifedipine by UV Spectrophotometry. The same concentration of nifedipine was prepared again to confirm the obtained wavelength.

In order to ensure no interference of the absorbance of polymeric base in the observed drug absorbance, the polymeric bases were test whether they were absorbed in this obtained wavelength or not. The medium was added into the polymers base and shaken by vortex mixer for 15 min. The supernatant was taken and filtered. The absorbance of this filtered solution was observed.

2.1.2. Calibration Curve

The calibration curve of nifedipine was constructed spectrophotometrically before drug determination of nifedipine for *in-vitro* study. It was plotted between the concentration of drug against the absorbance. The series of drug in medium solution is in the concentration ranged of 0.01-0.08 mg/ml. This calibration curve was used in the whole *in-vitro* study.

2.1.3. Determination of the concentration of nifedipine released :

The nifedipine sample solution was assayed immediatly. The absorbance of the sample was read vs. the medium at the maximum absorption wavelength. The concentration of drug in the sample could be calculated according to the linear regression equation obtained from the calibration curve.

Only two polymers which gave the maximum percentages of nifedipine released and could sustained for 12 hours or release the drug with a constant controlled rate were selected as the bases of choice for further the *in-vivo* diffusion evaluation.

2.2. Analysis of Nifedipine in Biological Fluids

The analysis of nifedipine in biological fluids was developed by various techniques. High performance liquid chromatographic method was widely used to determine nifedipine or its metabolites in plasma (58, 59, 60, 61-67) and in serum (68). The other techniques are gas-liquid chromatography (69-73). An isocratic reverse-phase high performance liquid chromatographic technique was used to determine nifedipine concentration in serum. The analytical procedure was modified from Pietta et al (58), Kleinbloesem and Vanharten (64) and Miyasaki et al (65).

2.2.1. Analytical Procedure

In every assay run, a series of spiked nifedipine standard solution in blank serum was analysed concurrently along with the serum sample. For a serum sample, a 0.5 ml of the treated rabbit serum was accurately pipetted into 10-ml aluminum foil

wrapped test tube. A 10 µl of methanol and 10 µl of 0.1 mg/ml 4-dimethylaminobenzaldehyde as the internal standard solution were added. The mixture was vortexed for 30 seconds. A 10 µl of 0.5004 N sodium hydroxide solution was then added and vortexed to mix. A 5 ml of the mixture of dichloromethane:pentane in the ratio of 3:7 was added and then the mixture was rotated for 30 minutes with vertically rotary instrument. After that the mixture was centrifuged at 2000 rpm for 15 minutes. A 2 ml of organic layer was seperated into an aluminum foil wrapped test tube and the organic solution was evaporated on the warm 35°C water bath. The evaporated residue was dissolved into a 100 µl of the mobile phase which consisted of 0.01 M.disodium hydrogen phosphate, pH 6.1 : methanol in the ratio of 40 : 60. A 10 µl aliquot of the dissolved solution was injected into the liquid chromatograph. The peak areas ratio of nifedipine and 4-dimethylaminobenzaldehyde (IS) calculated were used to determined the concentration of nifedipine in the sample through the linear regression equation obtained from the calibration curve in that day.

2.2.2. Calibration curve :

Standard solutions containing 0.2-2.4 µg/ml nifedipine in methanol were prepared. Instead of 10 µl of methanol, 10 µl of each standard solution was added to 0.5 ml of drug free serum. This sample was processed as described above. The calibration curve was constructed by plotting the ratio of the peak area of nifedipine and 4-dimethylaminobenzaldehyde (internal standard) against the concentration of nifedipine. Stock solution of both nifedipine (1 mg/ml) and internal standard (0.1 mg/ml) in methanol were stored in the aluminium foil wrapped volumemetric flask. These solutions were prepared every week.

2.2.3 Chomatographic conditions :

The modified isocratic reversed-phase HPLC technique was used for this study. Various parameters for setting the analysis are shown as following:

column :	Zorbax-ODS, 7 μm., 25 cm * 4.6 mm.I.D.
mobile phase :	0.01 M disodium hydrogen phosphate pH 6.1 : methanol (40 : 60)
flow rate :	1.5 ml/min
pressure :	3200 psi.
detector :	UV 236 nm at 0.002 a.u.f.s
chart speed :	3 mm/min
column temperature :	ambient

3. Evaluation of the Nifedipine TDDS Preparations

Nifedipine TDDS preparations were evaluated via the *in-vitro* and the *in-vivo* diffusion study. *In the in-vitro* diffusion study using the diffusion cell and synthetic membrane, the quantitation of drug release from the preparations were determined according to the analysis of drug in preparations as described above. The obtained data were plotted between drug release vs time, drug release vs the square root of time and logarithm of drug release vs time, also the correlation coefficient was calculated for determination whether the relationship of the drug release against time was as zero-order, Higuchi's model or first-order, respectively.

For the *in-vivo* diffusion study, rabbits were selected as an animal model. Since it has been reported that several compounds such as; drug, pesticides, paraquat etc., could penetrate into the

rabbit's skin more than the skin of common laboratory animals (74-76). The profile of serum concentration of nifedipine against time was determined for evaluating the preparation prepared.

3.1. In-Vitro Diffusion Stdies.

The drug-polymer preparation was accurately weighed into the diffusion cell which was modified from Keshary-Chien diffusion cell with a surface area of 12.256 cm^2. Durapore, with the pore size of 0.45 μm was clamped between the donor and the recipient compartments. The drug-polymer preparation was placed in the donor compartment over the Durapore whereas the recipient compartment which contained a medium solution of 63 ml of 1:1 ethanol:polyethylene glycol 400 co-solvent at 37°C was stirred at 100 rpm. by a magnetic stirrer. A 5 ml aliquots of the medium solution in the receptor compartment was pipetted out as a sample for assaying of the drug content at 0.5 hours and hourly intervals up to 12 hours. The volume of the medium solution withdrawn at each time interval was replaced immediately with fresh warmed medium solution of the same quantity for maintaining the sink condition of the system.

3.2. In-Vivo Diffusion Studies.

Male New Zealand white rabbits weight range 2-4 kg. were used in this study. Three rabbits were designated for each formulation. The rabbits were fasted overnight before the day of the experiment.

3.2.1 Application of nifedipine TDDS preparations and blood sampling

Six rabbits were used for the application. On the day of the experiment, the rabbit's back skin was carefully shaved. The blood had been drawn before application of nifedipine TDDS preparation and kept as blank blood serum for the whole analysis. The 1% w/w nifedipine preparation was filled into the plastic lid with a surface area of 12.571 cm^2 and the thickness of 0.5 cm. This lid was put on the left hand side of body between the neck and foreleg of rabbit and then the whole lid was covered with the gauze. The rabbit was then placed in the restrainer. Blood samples were obtained by directly drawing from the ear veins at time interval of 1,2,4,6,8,10,12,and 24 hours after the drug administration. The blood samples were allowed to clot at room temperature for approximately 15 minutes and centrifuged at 2000 rpm. for 30 mimutes. The serum was immediately seperated and transferd into the aluminum foil wrapped test tube and kept at -20° C for subsequent analysis.

3.2.2 Intravenous administration (38)

The total of three rabbits were used for this part of the study. The blood sample were drawn before drug administration. A concentration of 0.625 mg/ml of nifedipine in 30% v/v polyethylene glycol 400 was bolusly injected into the rabbit's ear veins in the volume of 1 ml. The series of the blood samples were taken from the other ear veins at the same time interval as those for nifedipine tdds administration. The serum was collected as already described and kept at -20°c for subsequent analysis .

Note : The frozen serum samples of the rabbits were thawed to the room temperature before drug analysis.