CHAPTER II

EXPERIMENTAL

Materials

The following materials were obtained from commercial sources, except nifedipine crystal powder and nitrendipine which kindly supported by Bayer (Germany), and Pluronic F-127 was donated by BASF Corporation (USA).

Nifedipine crystal powder : Batch no. PT.254335D ; Bayer AG. Leverkusen, Germany.

Nitrendipine reference standard : Batch no. R-147-1 ; Bayer AG. Leverkusen, Germany.

Pluronic F-127 : Batch no. 620505 ; BASF Corporation, Chemical Division, New Jersy, USA.

Aerosil A-200 : Wacker Chemie GMBH Munchen, Germany

Glycerin : Lot & Control no. GB 10/4 ; Srichand United Dispensary Co., Ltd., Thailand

Sodium lauryl sulfate : Lot & Control no. SEP 05 ; Srichand United Dispensary Co., Ltd., Thailand

4-Dimethylaminobenzaldehyde AR grade: Lot no. G461458; E.Merck , Germany

N-Cetyl-N,N,N-trimethyl-ammonium bromide : Lot no. K14887542; E.Merck, Germany

Cremophor A25 : BASF Corporation , Chemical Division,New Jersy, USA.

Sodium hydroxide : Batch no. 040991 ; Eka Noble AB, Surte, Sweden

Potassium phosphate monobasic AR grade : Lot no. 1890T200 ; Farmitalia Carlo Erba

Cremophor RH40 : Lot no. 1860130 ; BASF Corporation , Chemical Division, USA.

Potassium hydroxide : E. Merck , Darmstadt, Germany

Urea : Lot no. 296834391 ; Fluka Chemie AG, Switzerland ;

Ammonium acetate : E.Merck, Damstadt, Germany

Glacial acetic acid : E. Merck, Damstadt, Germany

Cetylpyridinium chloride : Product no. 27921 ; BDH Chemical Ltd, England

Adalat ® 10 mg : Bayer, Germany

Methanol, HPLC grade : J.T. Baker Chemical Incorporation , USA

Ethyl acetate, AR grade : E. Merck, Darmstadt, Germany

Heparin : Upjohn, USA

Water : Reversed osmosis treated water was used throughout the experiment. In addition, it was filtered through 0.45 μm membrane filter (FH-type Millipore), and sonicated before the chromatographic testing was performed

Apparatus

Analytical balance : Sartorius , model A200s, Sartorius Ltd., Co., Germany

Vortex mixer : Vortex Genie-2, Model G-500E, Scientific Industries Inc., USA.

Stir-hot plate : Nuova 7 stir plates, model no.SP-18420, Thermodyne Sybron Corporation, USA

Micropipet : Pipetman®, Gilson, U.K.

High Performance Liquid Chromatography, HPLC:

Pump : Multiple solvent delivery system, Milton Roy model CM 4000, Milton Roy, USA

UV absorption detector : Programmatic wavelength detector, Milton Roy model SM 4000, Milton Roy, USA

Integrator : Computer integrator, Milton Roy model 4100, Milton Roy, USA

Disposable needle no.21 : Terumo, USA

Disposable syringe 5 ml : Terumo, USA

Screw-capped tube : Pyrex, USA

Yellow lamp : Tungsram ,40w,Hungary.

Chromatographic column : Spherisorb, octadecylsilica particle size 5 μm, 25 cm length, 4.6 mm I.D., Phenomenex, Florida, USA

Guard Column : Bondapack$^{®}$C$_{18}$/Coracil size 37-50 μm, 5 cm length, 2.0 mm I.D., Phenomenex, Florida USA

Methods

Due to photo-sensitivity properties of NFP, the whole experiments had to be performed in the room illuminated with yellow lamp. All glasswares used had to be wrapped with aluminium foil for light protection throughout the experiment.

The method studied was divided into three parts as follow ;

1. Preparation of NFP transdermal patch by using various surfactants

2. *In vitro* evaluation of NFP transdermal patch using diffusion cells

3. *In vivo* evaluation of NFP transdermal patch in rabbits

Preparation of NFP Transdermal Patch

Formulations

According to the master formula of NFP transdermal patch already studied (Tattawasart, 1992) that consists of fixed concentrations of 1 % w/w NFP, 50 % w/w Pluronic F-127 gel matrices, 10 % w/w glycerin, 6 % w/w Aerosil A-200, only the types and concentration of surfactant as enhancer were formulated in this study. Ten surfactants (0.5-2.0 % w/w) as named in Table 1 were individually incorperated to the aforementioned NFP preparation. The net weight of each formulation prepared was 10 gram.

Method of NFP patch preparation

Pluronic F-127 gel preparation

Pluronic F-127 gel was prepared by cold method developed by Schmolka (1972). The required amount of Pluronic F-127 was weighed in the beaker with stirring rod, then cold water (5-10°C) was slowly added under constant agitation until the required total weight was met. The dispersion was stored overnight in the refrigerator in order to form a clear and viscous dispersion. Pluronic F-127 dispersion was transformed to gel state at room temperature before used.

Formulation procedures

The required amount of NFP and surfactant were mixed with glycerin on slab. Upon completely incorporated, an appropriate amount of Aerosil A-200 and Pluronic F-127 gel were added by geometric dilution to obtain uniform matrix.

Table 1   Various types and concentrations of surfactants in each preparation

Surfactant	Percentage Concentration (w/w)																			
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16	F17	F18	F19	F20
Sodium lauryl sulfate	-	0.5	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cetyltrimethyl ammonium bromide	-	-	-	-	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cetylpyridinium chlor	-	-	-	-	-	-	1	2	-	-	-	-	-	-	-	-	-	-	-	-
Cremophor A25	-	-	-	-	-	-	-	-	1	2	-	-	-	-	-	-	-	-	-	-
Cremophor RH40	-	-	-	-	-	-	-	-	-	-	1	2	-	-	-	-	-	-	-	-
Stearic acid	-	-	-	-	-	-	-	-	-	-	-	-	1	2	-	-	-	-	-	-
Oleic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	2	-	-	-	-
Stearyl alcohol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	2	-	-
Cetyl alcohol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	2

The matrix was stored in a beaker at room temperature for 24 hours, in which it would be transformed to gel state before evaluating their physical properties.

Aluminium mold for transdermal patch

Aluminium mold was fabricated by patch-pressed machine in which the shape was designed by using a single punch hand-tableting machine as a reference model. The diagrammatic illustration of the machine was shown in Figure 9 . It consisted of two major parts; a moving upper punch with 3.45 cm in diameter; and a fixed lower die with 3.5 cm internal diameter and 1.55 mm in depth circular hold at the center. For fabrication of an aluminium mold, a circular piece of aluminium foil with 5.5 cm in diameter was placed over the lower die. Then the upper punch was slowly pressed over the aluminium foil into the hold forming an aluminium mold with a fixed circular diameter at 3.44 cm and a fixed depth at 1.5 mm. This mold, with 9.2941 cm^2 surface area, was used to fill NFP gel matrix to be transdermal patch used in this study.

In vitro evaluation of NFP transdermal patch

Physical properties

Difficulty in preparing

Every formulation was prepared within one day.The difficulty in preparing each sample was evaluated according to the time consumes to obtain the complete preparation.
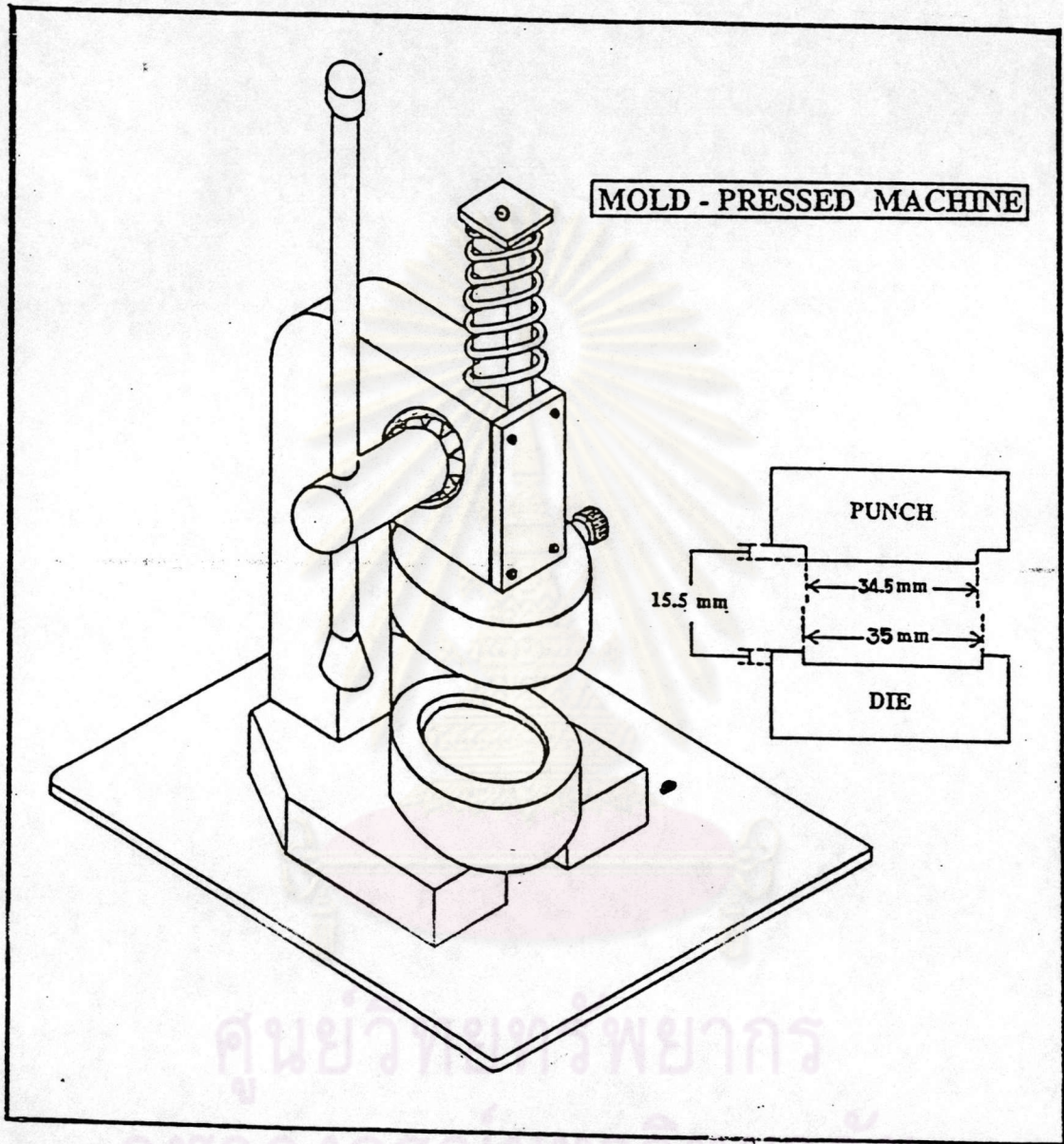
Figure 9   Schematic illustration of mold-pressed machine

Clarity

Overall clarity was observed visually. Insoluble materials in preparations was also searching via microscope.

Air bubbles

Air bubbles of each products were detected by microscopic investigation.

Residue

The residue on skin after applying each preparation of NFP transdermal patch was observed and compared.

Suitable formulations were selected for further study from their acceptable physical properties described above.

In vitro permeation study

Skin permeation cell

An selected *in vitro* skin permeation used for investiging of long term skin permeation of NFP transdermal patch in this study was a modified Keshary-Chien finite-dose diffusion cell. All components were fabricated from pyrex glass. Diagrammatic illustration and comparison with Keshary-Chien diffusion cell were shown in Figure 10. It consisted of two main compartments; a donor compartment, which had a wide opening at the top exposing to the air without any temperature control and an enlarged receptor compartment, in which the solution in the inner chamber was thermostated at 37 $^\circ$c by water in the outer The sampling port was modified to be straight
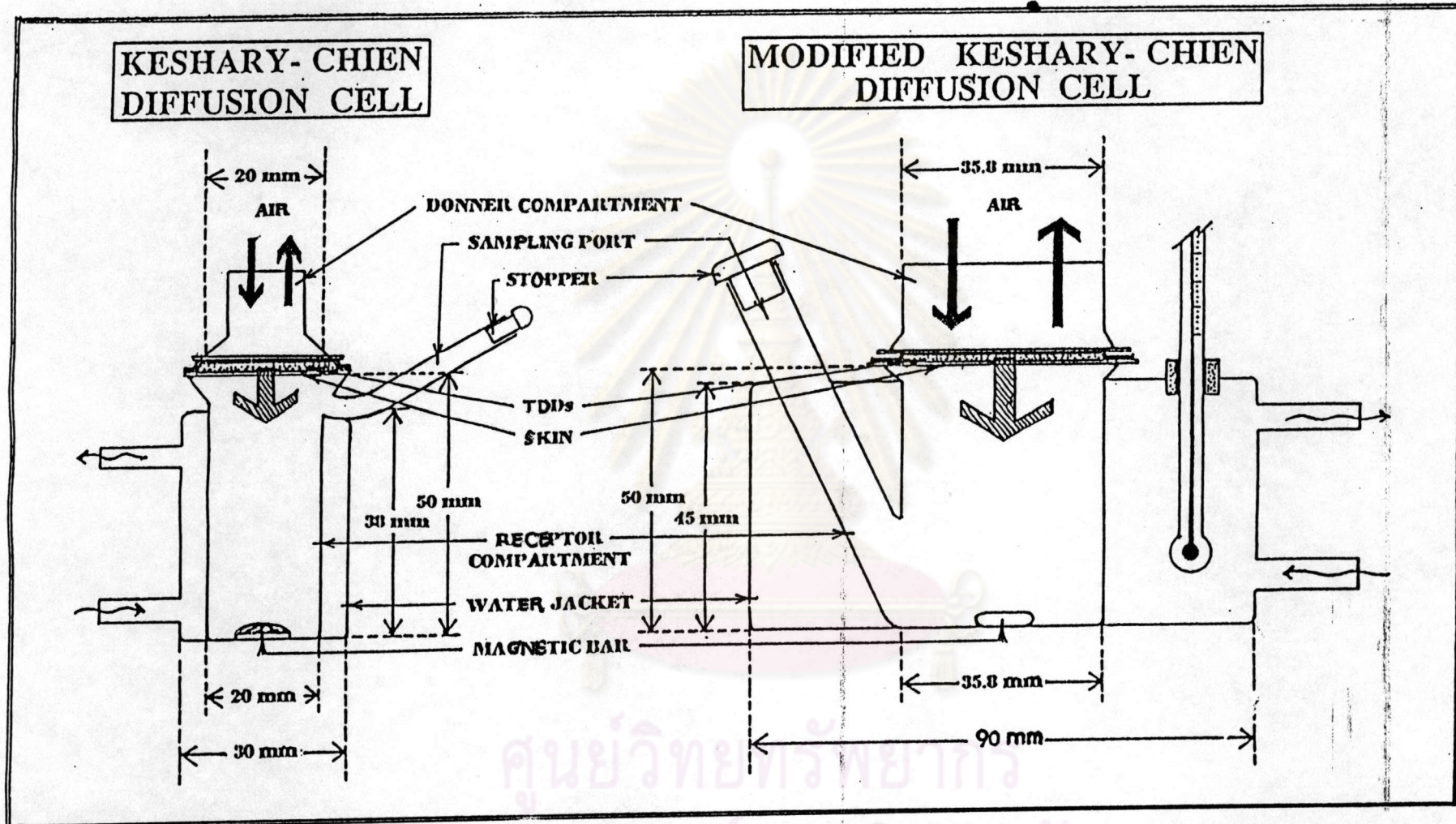
Figure 10   Schematic illustration of the modified diffusion cell used

in this study compared with Keshary-Chien diffusion cell

and enlarged for conveniency to pipette. A star-head magnet was replaced by amagnetic stirring bar. In the experiment, modified Keshary-Chien diffusion cell was maintained temperature and controlled stirring rate at 90 rpm by individual stir-hot plate.

Skin preparation

Full-thickness skin from pig's infant with perinatal death was surgically removed and cleaned with sterile normal saline solution. Blood vessels, subcutaneous tissues, and fat were separated and discarded. Then the skin was cleaned again with sterile normal saline solution and finally with sterile water. After that,it was wrapped with aluminium foil and stored frozen at -20°c. The frozen skin was thawed at room temperature and cut at the similar regions for each formulation in circular section, 4 cm in diameter before mounting on the diffusion cell for the skin permeation study.

Permeation procedure

Phosphate buffer, pH 7.4, as diffusion medium was filled in the receptor compartment with a magnetic bar. Water was filled in the outer jacket, which is preheated at 37°C. Skin sample (4 cm in diameter) was mounted onto the modified Keshary-Chien diffusion cell. Its stratum corneum was faced up into the donor compartment, which was maintained at 37°C by a stir-hot plate. A unit of NFP patch with drug-releasing surface of 9.2941 cm^2 was placed over the skin in intimate contact with the stratum corneum and the whole assembly was clamped together with the donor cap on the top.

At predetermined time intervals (1, 2, 4, 7, 10, 13, 16, 20, 24, and 28 hours), one ml of diffusion medium in the receptor compartment was withdrawn and replaced with preheated diffusion medium (drug free). After

appropriate dilution, concentration of NFP in the sample was determined by the HPLC method. The skin permeation study of each NFP formula was carried out in triplicate runs.

The cumulative amount of NFP permeating through a unit surface area of the skin (Q_S) at interval time, were determined using HPLC technique.

Chromatographic conditions

The isocratic reversed - phase high performance liquid chromatographic (HPLC) technique was used for NFP analysis in this study. The chromatographic system consisted of :

Chromatographic column :- Spherisorb-ODS, particle size 5 μm with dimension of 250 * 4.6 mm.

Guard column :- A stainless steel 50 * 2 mm column packing with coracil (37-50 μm)

Mobile phase :- 0.01M Acetate buffer pH 6.1 : Methanol = 35:65

Flow rate :- 1 ml/min

Injection volume :- 20 μl

Detector :- UV 238 nm

Chart speed :- 2 mm/min

Attention :- 2

The 0.01 M Acetate buffer was prepared by dissolving 0.7708 g ammonium acetate in 990 ml reversed osmosis treated water. Then the pH of solution was adjusted to 6.1 with 50 % glacial acetic acid. The volume was adjusted up to 1000 ml with water.

The mobile phase was freshly prepared by mixing 65 % methanol and 35 % 0.01 M acetate buffer pH 6.1. The mixture solution was filtered through a 0.45 μm membrane filter, and was degassed by sonication for 15 min before used.

Calibration curve for *in vitro* permeation study

The concentration of NFP in the range of 0.02-1.0 μg/ml was used for constructing the calibration curve. Standard solution containing 0.02, 0.04, 0.08, 0.16, 0.20, 0.24, 0.48, 0.60, 0.80, and 1.00 μg/ml of NFP and 0.12 μg/ml of 4-dimethylaminobenzaldehyde (internal standard, IS) in each dilution was prepared in mobile phase under yellow light.

Sample preparation

The 1.0 ml of sample solution eluted from NFP permeation study was accurately pipetted into a 10 ml volumetric flask, then 1 ml of 1.2 μg/ml of IS in methanol was added and adjusted to 10 ml with mobile phase. The mixture was vortex-mixed for 15 second, and filtered through a 0.45 μm membrane filter and transferred into an aluminium foil-wrapped screw-capped test tube. The 20 μl aliquot was injected into HPLC system. The ratio of peak area of NFP and IS was used to determine the concentration of NFP in each sample through the calibration curve. The amount of NFP permeating through the skin was then calculated from adjusted NFP concentration which has been corrected for the total volume of the solution and amount of drug withdrawn for assay.

The formula that NFP can be maximum permeated through the skin was selected for further *in vivo* study.

In Vivo Evaluation of NFP Transdermal Patch

Analysis of NFP in biological fluids

The analysis of NFP in biological fluids was developed by various techniques (Suzuki et al., 1985; Miyazaki, Kohri, and Arita, 1984; Kleinbloesem, and Harten, 1984; Sadanaga et al., 1982; Pietta,Rava, and Biondi, 1981). In this study, an isocratic reverse-phase high performance liquid chromatographic technique was used to determine NFP concentration in rabbit plasma. The analytical procedure was modified from Thongnopnua and Wiwattanawongsa's (1992).

Analytical procedure

A 0.5 ml of rabbit plasma was accurately pipetted into 10 ml aluminium foil-wrapped screw-capped test tube, then added 0.6 ml of 1.0 M KOH solution and vortex-mixed for 15 second. After that, a 1.0 ml of 8.0 M urea solution and 20 μl of 6.0 μg/ml of nitrendipine in methanol (IS) were added and vortex-mixed for 20 second. The mixture was extracted once with 4.0 ml of ethyl acetate by vortex mixer and centrifugation at 1500 rpm for 10 min at room temperature. A 3.5 ml of supernatant was transferred into a new aluminium foil-wrapped test tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 200 μl of methanol and then 50 μl of solution was injected into the liquid chromatograph.

Chromatographic conditions

The modified HPLC technique was used in this study. The chromatographic system consisted of :

Chromatographic column and guard column (the same as *in vitro* study)

Mobile phase :- 0.05M Acetate buffer pH 6.1: Methanol = 38:62

Flow rate	:-	1 ml/min
Injection volume	:-	50 μl
Detector	:-	UV 247 nm
Chart speed	:-	0.25 cm/min
Attention	:-	2

Calibration curve for *in vivo* evaluation

Standard solutions containing 40, 80, 120, 160, 240, 400, and 600 ng/ml NFP in methanol were prepared for constructing the calibration curve for each day of drug analysis. A 20 μl of each dilution was spiked into 0.5 ml of drug free plasma and processed along with sample analysis under the same analytical procedure. The calibration curve was then constructed by plotting the ratio of peak area of NFP and IS against NFP concentration. Stock solution of both NFP (0.5 mg/ml) and IS (0.5 ml/ml) in methanol were stored in the aluminium foil-wrapped volumetric flask in the freezer. These solutions were prepared every week.

***In vivo* permeation study**

The total five male Mixed New Zealand white rabbits weighing range 2.5-4.0 kg were used in this study. They were all designated to administration NFP as both oral and transdermal dosage forms with the washed out period of one week. All rabbits were fasted overnight for preparing for each administration..

Single-oral dose application

Before starting the experiment, approximatelt a 1 ml of whole blood was drawn from each rabbit and the separated plasma was kept as blank. A single oral dose of NFP (Adalat$^{®}$ 10 mg) was administered to each rabbit by put on its tonge and forced to swallow with 10 ml of water. Blood samples were drawn from ear vein at time intervals of 0.5, 1, 2, 4, 8, 12, and 24 hours after drug administration. Each blood sample was immediately centrifuged at 1500 rpm for 20 minutes. Plasma was separated into aluminium foil wrapped vial and kept at -20 $^{°}$C for subsequent analysis.

Transdermal patch application

Before the day of experiment, the back area (between neck and foreleg) of all five rabbits were shaven and designated NFP patching sites ($\sim$ 4 cm I.D.) .Then on the experimental day, a 1 % w/w NFP patch was put on the designated area and covered with gauze. Approximately 1.0 ml of blood sample was directly withdrawn from the ear vein using heparinized needle no.21 at consecutive time intervals of 1, 2, 4, 8, 12, 18, and 24 hours following drug administration. The blood samples were immediately centrifuged and the plasma separated was kept frozen for subsequent analysis using HPLC technique as already described.

In vivo data interpretation

The data of NFP plasma concentration at various sampling times for both oral and transdermal patch administrations were used for evaluating dosage forms. Parameters of the maximum NFP concentration (C_{max}) and the time to reach maximum plasma drug concentration (t_{max}) were determined directly from NFP plasma concentration-time profile plots. The

area under NFP plasma concentration-time curves were determined from time up zero to 24 hrs after drug administration (AUC $\mid_0^{24}$) using trapezoidal rule.

Statistically significant difference between the administration of oral and transdermal patch dosage forms to rabbits were also figured out in term of C_{max} value and AUC $\mid_0^{24}$ parameters at significant level 0.05. Additionally, the AUC $\mid_0^{24}$ value from *in vitro* study was also compared with *in vivo* study to determine the release pattern of NFP from transdermal patch.