CHAPTER II

MATERIALS AND METHODS

1. Materials

Nifedipine crystal powder : Bayer AG. Leverkusen, Germany., Batch No. 224437C

Pluronic F-127 : BASF corporation chemical division, New Jersy, USA. Batch No. WPTK 500B

Aerosil A-200 : Wacker chemie GMBH Munchen, Germany., Batch No. N20

Dimethylaminobenzaldehyde AR grade : E. Merck, Darmstatdt, Germany., Lot No. 8053

Methanol HPLC grade : JT Baker Incorporation, Phillipsberg, New Jersy, USA

Water : Reversed osmosis treated water was used throughout the experiment. In addition, filtering through a 0.45 um membrane filter, FH-type Millipore, prior to the chromatographic testing was performed.

2. Apparatus

Analytical balance : Sartorius model A2005, Sartorius Ltd., Co. Germany.

pH meter : Henna model HI8417 microprocessor, Henna Instrument, USA.

Shaking water-bath : Hotech shaker Bath mode 905, Hotech Instruments Corporation, Taipei, Taiwan.

Spectrophotometer : Shimadzu model UV 180, Shimadzu Corporation, Kyoto, Japan.

Recorder : Shimudzu model U-135, Shimudzu Corporation, Kyoto, Japan.

Stir-Plate with temperature thermostat : Nuova 7 stir plate model No. SP-18420, Thermolyne Sybron Corporation, Dubuque, Iowa, USA.

High performance liquid chromatography (HPLC) :

Pump : Multiple solvent delivery system, Milton Roy model CM 4000, Milton Roy, LDC division, Florida, USA.

UV absorption detector : Programmatic wavelength detector, Milton Roy model SM 4000, Milton Roy, LDC division, Florida, USA.

Integrator : Computor integrator, Milton Roy model 4100, Milton Roy, LDC division, Florida, USA.

HPLC-Reverse phase column : Spherisorb, octadecylsilica particle size 5 um, 25 cm lenght, 4.6 mm. I.D., Phenomenex, Milton Roy LDC, Florida, USA.

Guard column : Upchurch model C-130B, octadecylsilica particle size 10 um, Phenomenex, Milton Roy LDC, Florida, USA.

Vortex mixer : Vortex Genie-2, model G-560E, Scientific Industries Inc., Bohermia, New Yorl, USA.

Microsyringe : 100 ul Unimetrics, Unimetrics, Storewood, Illinois, USA.

Golden fluorescence lamp : Tungsram, Hungary.

Since NFP is very sensitive to light, it undergoes ready photochemical conversion to the corresponding pyridine derivatives when exposed to normal laboratory and ultraviolet light. Thus, the whole experiment had to be done in a dark room and under golden fluorescence which had low actinic light. All glasswares used had to be carefully protected from light by wrapping with aluminum foil.

3. Solubility of Nifedipine

Each 50 mg of NFP was weighed accurately and put into a 50 ml aluminium-foil wrapped glass vial with 50 ml of various solvents including water. These preparations were equilibrated with constant shaking in a shaking water-bath which was kept at a constant temperature of 37^{o}C for 24 hours. Withtime, the preparations were withdrawn and the saturated solution was then quickly filtered through a 0.45 um membrane, Millipore FH type, and the filtrate was assayed for NFP concentration after proper dilution by high performance liquid chromatographic method which was described for this reserch study in section 6.2. After 48 hours, the fitrate was repeated assayed for NFP to confirm its solubility.

Each preparation was carried out in triplicated run.

4. Preparation of Nifedipine Transdemal delivery system

4.1 Formulation

In order to fabricate NFP transdermal drug delivery system by using gelling agents as drug carriers, optimal ranges of concentrations of PF-127, a hydrophilic agent, and Ae-200, a hydrophobic agent, were selected from preliminary studies to be 30 - 50% w/w and 20 - 30% w/w,

respectively. Other additives were then incorporated to improve physical appearance of the preparation and release mechanism of the drug.

Each preparation consisted of a fixed concentration of NFP as 1% w/w with PF-127 gel matrices or Ae-200 gel matrices. Glycerol and propyleneglycol (PG) were used as plasticizers or organic modifier for polymer to reduce stiffness of the polymer backbone and increase polymer chain flexibility therefore increasing drug diffusion rate from devices. In addition, both glycerol and PG including PEG 400 were used as co-solvents of NFP. Methocel K 4M (HPMC) was used as thickening agent to increase the consistency of gel matrices to improve physical appearance of the preparations. The prepared formulas of NFP-polymer matrices were shown in Table 4 - 9.

4.2 Method of Matrices Preparation

4.2.1 Pluronic F-127 gel matrix

The NFP transdermal delivery systems which used PF-127 gel matrices as drug carrier were prepared by "cold method" by Schmolka (1972). The required amount of PF-127 was slowly added to cold reversed-osmosis treated water, 5 - 10^{o}C, under constant agitation. The obtained dispersion was stored overnight in a refrigerator to deaerate and formed a cleared viscous dispersion. With

Table 4 Formulas of 30% w/w Pulronic F-127 Gel Matrices.

Ingredients	Percentage concentration (% w/w)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Pluronic F-127	30	30	30	30	30	30	30	30	30	30	30	30	30	30
Glycerol	-	5	10	15	-	-	-	-	-	-	10	10	-	-
Propylene glycol	-	-	-	-	5	10	15	-	-	-	-	-	10	10
PEG 400	-	-	-	-	-	-	-	5	10	15	5	10	5	10
water	70	65	60	55	65	60	55	65	60	55	55	50	55	50

Table 5 Formulas of 40% w/w Pulronic F-127 Gel Matrices.

Ingredients	Percentage concentration (% w/w)													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Pluronic F-127	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Glycerol	-	5	10	15	-	-	-	-	-	-	10	10	-	-
Propylene glycol	-	-	-	-	5	10	15	-	-	-	-	-	10	10
PEG 400	-	-	-	-	-	-	-	5	10	15	5	10	5	10
water	60	55	50	45	55	50	45	55	50	45	45	40	45	40

Table 6 Formulas of 50% w/w Pulronic F-127 Gel Matrices.

Ingredients	Percentage concentration (% w/w)													
	29	30	31	32	33	34	35	36	37	38	39	40	41	42
Pluronic F-127	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Glycerol	-	5	10	15	-	-	-	-	-	-	10	10	-	-
Propylene glycol	-	-	-	-	5	10	15	-	-	-	-	-	10	10
PEG 400	-	-	-	-	-	-	-	5	10	15	5	10	5	10
water	50	45	40	35	45	40	35	45	40	35	35	30	35	30

Table 7 Formulas of 20% w/w Aerosil A-200 Gel Matrices.

Ingredients	Percentage concentration (% w/w)																	
	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Aerosil A-200	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
Glycerol	25	35	45	-	-	-	25	25	35	35	-	-	-	-	25	25	-	-
Propylene glycol	-	-	-	25	35	45	-	-	-	-	25	25	35	35	-	-	25	25
PEG 400	-	-	-	-	-	-	20	30	20	30	20	30	20	30	20	20	20	20
HPMC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	3	1	3
water	55	45	35	55	45	35	35	25	25	15	35	25	25	15	34	32	34	32

Table 8 Formulas of 30% w/w Aerosil A-200 Gel Matrices.

Ingredients	Percentage concentration (% w/w)																	
	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78
Aerosil A-200	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30
Glycerol	25	35	15	-	-	-	25	25	35	35	-	-	-	-	25	25	-	-
Propylene glycol	-	-	-	25	35	45	-	-	-	-	25	25	35	35	-	-	25	25
PEG 400	-	-	-	-	-	-	20	30	20	30	20	30	20	30	20	20	20	20
HPMC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	3	1	3
water	45	35	25	15	35	25	25	15	15	5	25	15	15	5	24	22	24	22

Table 9 Formulas of Combination of Pluronic F-127 and Aerosil A-200 Gel Matrices.

Ingredients	Percentage concentration (% w/w)																				
	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99
Pulronic F-127	30	30	30	30	30	30	30	40	40	40	40	40	40	40	50	50	50	50	50	50	50
Glycerol	-	-	-	10	10	-	-	-	-	-	10	10	-	-	-	-	-	10	10	-	-
Propylene glycol	-	-	-	-	-	10	10	-	-	-	-	-	10	10	-	-	-	-	-	10	10
PEG 400	-	-	-	10	10	10	10	-	-	-	10	10	10	10	-	-	-	10	10	10	10
Aerosil A-200	2	5	10	5	10	5	10	2	5	10	5	10	5	10	2	5	10	5	10	5	10
water	68	65	60	45	40	45	40	58	55	50	30	35	30	35	48	45	40	20	25	20	25

time, the accurate weight of NFP (1% w/w) was dispersed in required amount of co-solvent and other required amount of additive(s) were then added, well dispersed with constant agitation to obtain a uniform dispersion.

For the selection of PF-127 concentration, the dispersion was stored in a 25 ml beaker at room temperature for 24 hours, to incubate into the gel state for observing their physical appearance.

For the evaluation of NFP transdermal drug delivery preparation, the uniform dispersions was then poured into tared weighing aluminium molds. The molds were filled to desired weight, and allowed to gel under vacuum at room temperature prior to evaluation.

4.2.2 Aerosil A-200 gel matrix

The desired amount of Ae-200 and organic modifier were weighed into a 50 ml beaker. The mixture was, then, agitated to thoroughly mix. Then, the accurate weight of NFP was dispersed in required weight of co-solvent and other additives were subsequently added. The mixture was mixed well for complete homogeniety.

The filling and gelling of preparation for both selection of Ae-200 concentration and for evaluation the NFP transdermal delivery system were carried out as previously described.

4.2.3 Pluronic - Aerosil gel matrix

The required amount of PF-127 gel was prepared by the method as previously described. Then, the mixture of Ae-200 and organic modifier was added to the gel with thoroughly mixing. The accurate weight of NFP which was dispersed in required weight of co-solvent and other additive(s) were subsequently added into the mixture with thoroughly mixing for complete homogeniety. Each finished product was used for selection and evaluation as in 4.2.1.

4.3 Aluminium mold for TDDs

Aluminium mold was frabricated by mold-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 plate or die with 3.5 cm in internal diameter and 1.55 mm in depth circular hole at the centre. For fabrication of an aluminium mold, a circular piece of aluminium foil with

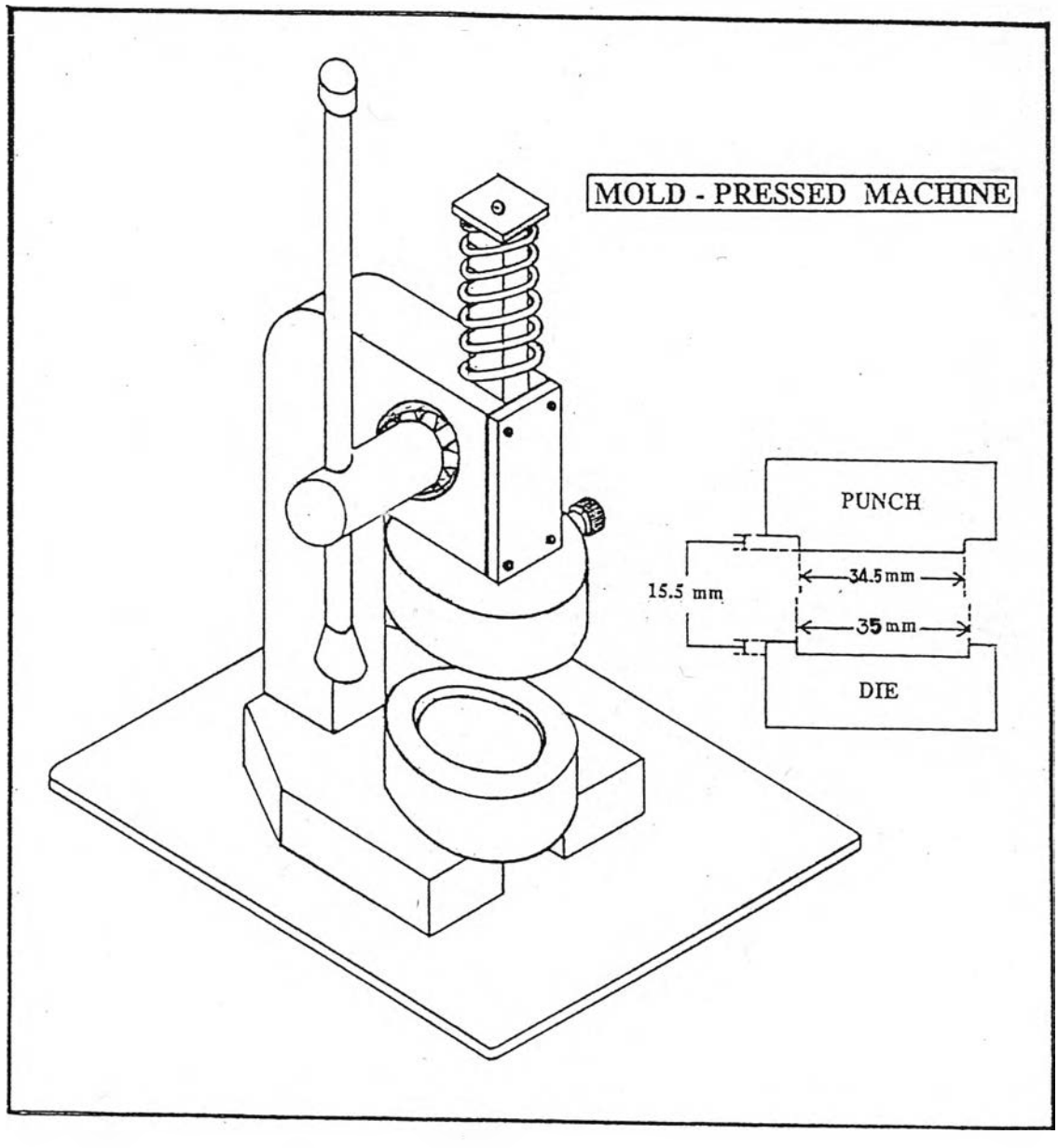

Figure 9 Schematic Illustration of Mold-Pressed Machine.

5.5 cm in diameter was placed over the lower die, then the upper plunger was pressed down the aluminium foil slowly into the hole to form an aluminium mold with fixed circular dimeter at 3.44 cm and a fixed depth at 1.5 mm. A circular mold, which provided 9.2941 square centimetres in surface area, was used to fabricate NFP transdermal delivery systems in this research study.

5. In-vitro evaluation of NFP transdermal delivery system

5.1 Physical Characteristics

In order to evaluate NFP matrix preparations the suitable formulas was selected. The formulations were selected according to the difficulty in preparing and the physical appearance of the obtained gel matrix. The physical appearance including clarity and air bubble were visually observed, the residue was observed by applying gel matrix on the skin and the consistency of gel matrix was observed by hand pressed (Viegas, Hikal and Cleary, 1988).

5.2 Permeation Study

5.2.1 Skin permeation cell

An *in-vitro* skin permeation cell which was selected to be used for the investigation of long term skin permeation kinetic of NFP transdermal delivery system was a modified Keshary-Chien finite-dose diffusion cell. All components were frabricated from Pyrex glass. Diagrammatic illustration and comparison with Keshary-Chien diffusion cell were shown in Figure 10. The modification was aimed to overcome the defection of Keshary-Chien diffusion cell. First, the receptor compartment was enlarged because the volume of sample needed was much greater than the one-ml per sampling that could be provided from the Keshary-Chien diffusion cell and to reduce interference in sink condition of system and intact intimate between skin and receptor solution that might due to a large volume of sampling. Second, dued to the bending of the sampling port, the way to pipette the sample was inconvieience and air bubbles often entered into the receptor compartment while pipetting the sample and to keep the temperature of the sampling port at a constant and desired control temperature. Thus, the inner diameter of the receptor compartment was enlarged from the former Keshary-Chien diffusion cell of 20 mm to 35.8mm. In additon, to change in the dimension, the sampling port was modified to be straight at the end of the receptor

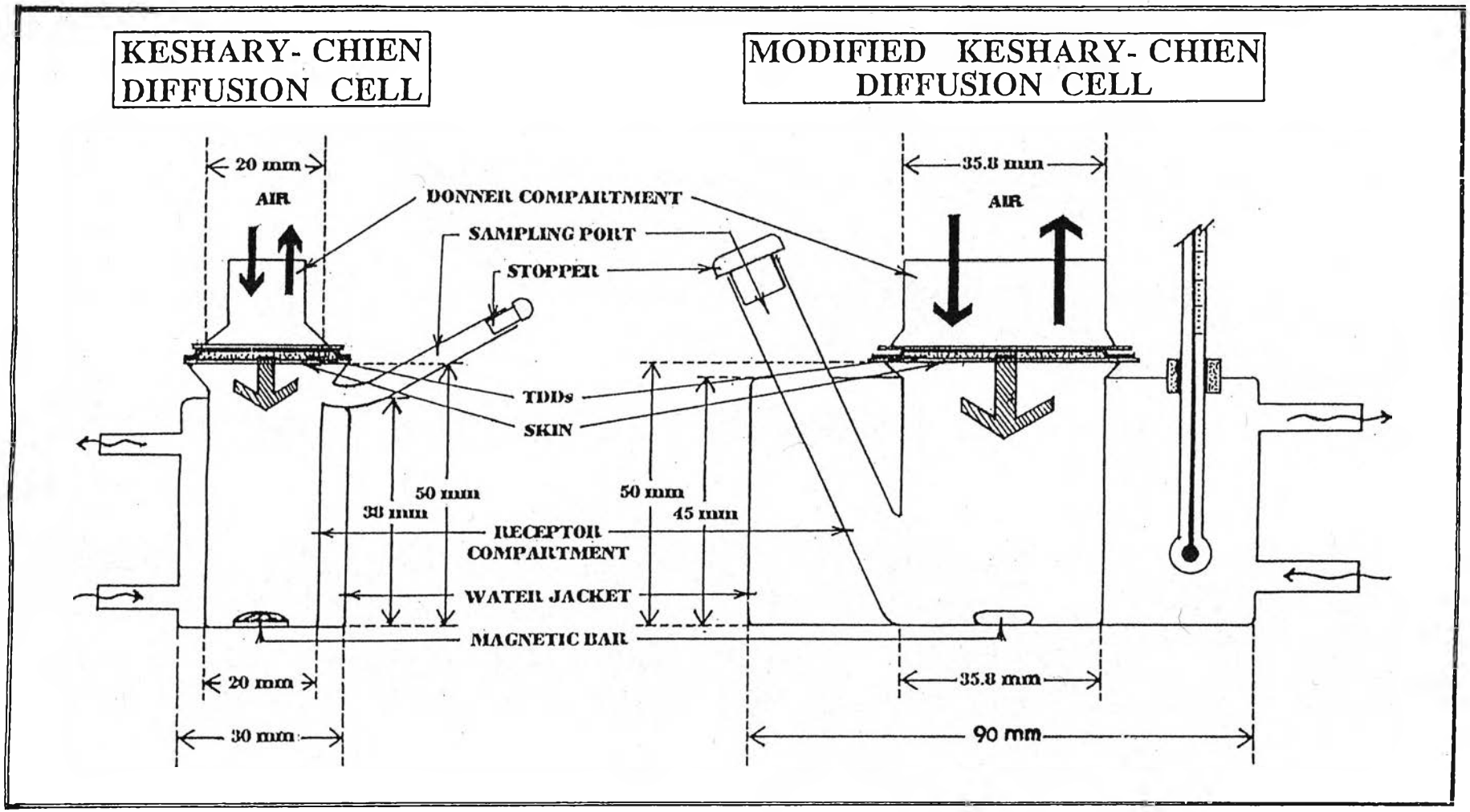

Figure 10 Schemetic Illustration of The Modified Diffusion Cell Used in this Study Compared with Keshary-Chien Diffusion Cells.

compartment. In the mean time, the water-jacket compartment was extended to envelop a greater surface area of the receptor compartment with enlargement its total volume to provide a constant temperature control and equilibium while maintaining the diffusion cell at 37°C by individual stir-plate with temperature thermostat.

5.2.2 Skin preparation

The pig skin sample used in this research study was a full-thickness skin which exised from a 2 - 3 weeks old miniature pig. The miniature pig was sacrificed and the whole skin was surgically removed and cleaned with sterile normal saline several times. The subcutaneous tissue and blood vessel were carefully removed until the epidermis was achieved. The full thickness skin was cleaned with normal saline and finally with sterile water. After evaporation of the excess water at room temperature, the excised pig skin was wrapped with aluminium foil and stored at freezing temperature, -20°C, which the freez-thaw process had little effect on *in-vitro* permeation kinetics (Gummer, 1989). To perform *in-vitro* skin permeation experiment of NFP transdermal delivery system, excised pig skin was thawed at room temperature and a circular section, 4 cm in diameter, was cut off for skin permeation study.

5.2.3 Determination of optimal stirring rate and temperature

To carry out the optimal stirring rate of skin permeation studies, the method of Gummer and et al., (1987) was introduced for this experiment. The three diffusion cells were equipped as in permeation study of NFP preparations, excepted the synthetic membrane was used to represent the skin sample. Each receptor compartment along with a 3 mm x 10 mm stirring bar and a crystal of potassium permanganate. The rate of stirring was equipped at 60, 70, 80, 90, 100 rpm. The time of each rate which dispersal of mauve coloration from the permaganate crystal was used to quantitatively assess the degree of stirring. The stirring rate which completed and even coloration within 30 seconds was selected for evaluation of NFP permeation study.

For the determination of the temperature control of skin permeation cell, the water jacket envelop receptor compartment was regulated by the individual external thermostat plate. The degree for setting the temperature-adjustable knob of each individual thermostat plate was calibrated for each diffusion cell which was equipped as in permeation study of NFP transdermal delivery preparation as previously described and stirred at the desired rate. The temperatures in receptor compartment and in the water jacket were measured to

calibrate for the desired scale of temperature-adjustable knob. The desired scale was the scale that could create the temperature of water in water-jacket, which provided and maintained constant internal temperature of the receptor compartment at 37 ± 1°C for at least 24 hours of experiment.

5.2.4 Permeation procedure

An aluminium mold of 1% w/w NFP-polymer preparation with constant surface of 9.2941 square centimetre was used as the subject for *in-vitro* permeation study using the aforementioned modified Keshary-Chien diffusion cell. The full-thickness pig skin was mounted onto the receptor compartment with the stratum corneum side facing upwards into the donor compartment and the dermal side facing downwards into the receptor compartment, then a unit of NFP-patch was placed with the drug-releasing surface intimate contract the stratum corneum surface of pig's skin. The donor compartment was placed on the top and the whole assembly was securely clamped together following the elution solution, which preheated at 37°C, was introduced into the receptor compartment as the corrected volume that predetermined. The water jacket was at predetermined temperature and the hydrodynamic in the receptor compartment was controlled with a predetermined optimal stirring rate by external stir-plate with thermostat,

while the doner compartment was maintained at the room temperature of 25 ± 1°C.

After completed assembly, one ml of elution solution was pipetted out as a sample for assaying the drug content at a predetermined time intervals (0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 18, 21 and 24 hours) and immediatly replaced with the same volume of 37°C preheated free drug elution solution to maintain the same total volume and to ensure a good intimate to the dermal side of the skin. After appropriate dilution, the sample was assayed for NFP concentration by a sensitive method which described later. The skin permeation study of each NFP preparation was performed at least 24 hours and carried out in triplicate runs.

The quantitation of NFP permeation (Q), and permeation per unit surface area (Qs) at interval time from each preparation were plotted against time (Q vs t, Qs vs t), against square root of time (Q vs $t^{1/2}$, Qs vs $t^{1/2}$) and logarithm of remained drug (RQ) and remained drug per area (RQs) were plotted against time (log RQ vs t, log RQs vs t), so the correlation coefficient was calculated for determination the relationship of the drug premeation against time to evaluate NFP transdermal delivery preparations.

The permeation of NFP from saturated solution in water was also evaluated in the same maner.

6. **Quantitative Analysis of Nifedipine preparation**

6.1 Determination of Maximum Absorption Wavelength of Nifedipine

Preliminary study to determine the maxima spectra of NFP and 4-dimethylaminobenzaldehyde, an internal standard (IS), were performed by scanning the UV absorption in a wavelength range of 400 - 200 nm. It is necessary to find out the suitable absorption of NFP and the IS to perform a suitable wavelength for HPLC method in this research study. A concentration of 39.68 ug/ml NFP in methanol and 4.03 ug/ml IS in methanol were prepared for this purpose. The procedure was performed at ambient condition with a scan speed of 50 mm/min and a chart speed of 50 mm/min. The absorption spectra of NFP and IS were evaluated for suitable spectum for quantitate the amount of NFP permeation.

6.2 Chromatographic conditions

The isocratic reverse-phase technique was used for quantitative analysis of NFP in this research study. HPLC system was setted to various parameter for analysis as follow :

column : Spherisorp-ODS, particle size 5 um, 25 long and 4.6 mm ID.

guard column : Upchurch model C-130B, ODS, 10 um

mobile phase : Degassed mixture of 0.01 M acetate buffer pH 6.1 and methanol as 35 : 65.

injected volume : 20 ul

flow rate : 1 ml/min

pressure : 3200 psi

detector : UV detector set at predetermined spectum, peak area was calculated by using an integrator, Milton Roy-computer integrator model CI 4100.

chart speed : 2 mm/min

column temperature : room temperature 25 $\pm$ 1^{o}C

The mobile phase was fleshly prepared, consisted of a mixture of 65% methanol and 35% 0.01 M acetate buffer prepared by mixing 0.7708 g ammonium acetate with 1 litre of degassed reversed osmosis treated water then adjusting the pH of solution to 6.1 with 50% glacial acetic acid.

The mixture solution was filtered through a 0.45 um membrane filter, type FH Millipore, and then was degassed by sonication for 30 min prior to use.

The high performance liquid chromatography was carried out at room temperature, 25°C, and in a dark room under golden light.

6.3 Preparation of Calibration curves

The calibration curves of NFP was constructed within the concentration range of 0.02 - 0.24 ug/ml. Standard solutions containing 0.02, 0.04, 0.08, 0.1, 0.12, 0.16, 0.18, 0.2, 0.24 ug/ml of NFP and 0.12 ug/ml of IS in each dilution, in mobile phase were prepared under very subdued light. A 20 ul aliquout standard solution was injected into HPLC and the calibration curve was constructed by plotting the ratio of peak areas under curve of NFP and IS against the concentration of NFP and a least square fitted of linear regression equation was used to calculate the concentration of NFP in each elution sample.

Stock solution of both NFP (1 mg/ml) and IS (1.5 mg/ml) in methanol were stored in aluminium foil wrapped volumetric flasks at refrigerator temperature (2 - 10°C), these solutions were freshly prepared every month.

6.4 Sample preparation

Each sample of elution solution had to be appropriate dilution for assaying the NFP concentration. The 1 ml of sample was accurately pipetted into a 10 ml volumetric flask, then 1 ml of 1.2 ug/ml of IS in methanol was added, the mixture solution was then diluted with freshy mobile phase to achieve the corrected volume of volumetric flask. The final solution was agitated with a Vortex mixer for 1 minute, then filtered through a 0.22 um pore size membrane filter, type FH Millipore. The filtrate solution was transfered to an aluminium foil-wrapped screw-capped test tube and a 20 ul aliquot of sample solution was injected into the HPLC. The ratio of peak areas under curve of NFP and IS was calculated to determine the concentration of NFP in each sample from the calibration curve. The amount of NFP permeating through the skin was then calculated from the NFP concentration in the receptor solution at each sampling time after correcting for the total volume of the solution and the amount of NFP withdrawn for assay.