CHAPTER II

MATERIALS AND METHODS

Materials

A. Chemical for preformulation studies

All materials obtained from commercial sources were used as received.

1. Diclofenac diethylamine : Distributed by Siam Chemical Product Co., Ltd., Bangkok, Thailand.
2. Propylparaben : Lot no. VB19/1 : Distributed by Pharmaceutical Traders Co., Ltd., Bangkok, Thailand.
3. Pluronic F-127 : Distributed by BASF Corporation Chemical Division, Bangkok, Thailand.
4. Hydroxypropyl methylcellulose, 50 cps : Distributed by Pharmaceutical Traders Co., Ltd., Bangkok, Thailand.
5. Sodium carboxymethylcellulose, high viscosity : Distributed by Pharmaceutical Traders Co., Ltd., Bangkok, Thailand.
6. Sodium alginate : Distributed by Pharmaceutical Traders Co., Ltd., Bangkok, Thailand.
7. Propylene glycol : Distributed by Srichand United Dispensary Co., Ltd., Bangkok, Thailand.
8. Polyethylene glycol 400 : Distributed by Srichand United Dispensary Co., Ltd., Bangkok, Thailand.
9. Ethyl alcohol (95%) : BDH Laboratory Supplies Poole, England.

10. Isopropyl alcohol : BDH Laboratory Supplies Poole, England.
11. Benzyl alcohol : BDH Laboratory Supplies Poole, England.
12. N,N-dimethyl acetamide : Fluka Chemika, Germany.
13. Potassium dihydrogen phosphate : E. Merck, Germany.
14. Potassium phosphate dibasic : E. Merck, Germany.
15. di-Sodium hydrogen phosphate anhydrous : E. Merck, Germany.
16. Sodium dihydrogen phosphate monohydrate : E. Merck, Germany.
17. Sodium hydroxide : E. Merck, Germany.
18. Sodium chloride : E. Merck, Germany.
19. Voltaren® emulgel : Ciba Geigy, Thailand.

B. Chemical for HPLC

1. Sodium acetate : Carlo Erba, Milano, Italy.
2. Glacial acetic acid, AR grade : E. Merck, Germany.
3. Methanol, HPLC grade : J.T. Baker, USA.
4. Acetonitrile, HPLC grade : J.T. Baker, USA.
5. Water : Reversed osmosis treated water was used throughout the experiment.

C. Shed snake skin

Shed snake skin of *Elaphe obsoleta* (Black rat snake) donated by Pata departmentstore's zoo was used throughout the experiment.

D. Chemical for *in-vivo* evaluation

1. Carrageenan Lambda, (Gelatin, vegetable; Irish Moss) Type IV : Sigma Chemical Co., Ltd., USA.

Apparatus

1. Analytical balance : Sartorius, model A200s, Sartorius Co., Ltd., Germany.
2. Vortex mixer : Vortex Genie-2, model G-560E, Scientific Industries Inc., Bohemia, New York, USA.
3. Magnetic stirrer : Heilidolph, MR 3001, Germany.
4. pH meter : Hanna Instruments 8417, USA.
5. Micropipet : Pipetman®, Gilson, UK.
6. Sonicator : Transsonic Digital S, Elma, Germany.
7. Diffusion cell : Modified from Keshary-Chien diffusion cell.
8. Spectrophotometer : Spectronic 300 Array, Milton Roy, USA.
9. High Performance Liquid Chromatography, HPLC :
   System controller : Waters 600E
   Tunable absorbance detector : Waters 484
   Intelligent sample processor Waters : Waters 712 WISP
   Data module : Waters 746, Millipore, USA.
   Column chromatography : ODS Hypersil C_{18} (4.6 mmx15 cm), Phenomenex, CA, USA.
10. Plethysmometer : Cat. no. 7150, Ugo Basile, Italy.

Methods

1. Analytical Methods and Conditions

1.1 Spectrophotometric Analysis of Diclofenac Diethylamine

A spectrophotometer was employed to determine the maximum spectrum of diclofenac diethylamine (DD). It was performed by scanning the UV absorption within the wavelength range of 350-200 nm. A concentration of 10 μg/ml DD in various solvent systems such as ethyl alcohol (EtOH), isopropyl alcohol (IPA), benzyl alcohol (BZA), propylene glycol (PG), polyethylene glycol 400 (PEG 400), and N,N-dimethyl acetamide (DMA), were prepared to obtain the maximum absorption wavelength. The procedure was done at an ambient condition. The characteristic peak was observed for DD at a maximum wavelength of 282 nm for ethyl alcohol, isopropyl alcohol, propylene glycol, and polyethylene glycol 400, except for benzyl alcohol and N,N-dimethyl acetamide, maximum wavelength were observed at 306 and 264 nm, respectively.

The absorbance value at the maximum wavelength was read and the corresponding DD concentration was calculated from the calibration curve. The calibration curves of DD in various solvents were plotted between the concentration of drug as a function of the absorbance. Table 2 shows a concentration of DD in various solvents versus its absorbance. A typical calibration plot, showed a linear relationship between the absorbance and DD concentration. The calibration curves of diclofenac diethylamine after regression analysis are illustrated in Figures 39-44.

Table 2 Absorbance of diclofenac diethylamine (DD) in different solvents at 282 nm (for EtOH, IPA, PG, and PEG 400), at 306 nm (for BZA), and at 264 nm (for DMA) by UV spectrophotometry.

Conc of DD (μg/ml)	Absorbance					
	282 nm				306 nm	264 nm
	EtOH	IPA	PG	PEG 400	BZA	DMA
10	0.360	0.343	0.393	0.499	0.259	0.613
15	0.545	0.552	0.591	0.604	0.317	0.686
20	0.730	0.743	0.765	0.704	0.371	0.761
25	0.901	0.931	0.958	0.820	0.433	0.837
30	1.076	1.116	1.143	0.925	0.489	0.915
r^2	0.999	0.999	0.999	0.999	0.999	0.999
Y-intercept	0.035	0.036	0.036	0.021	0.011	0.015
Slope	0.007	-0.033	0.023	0.283	0.143	0.460

Remark : BZA = benzyl alcohol, DMA = N,N-dimethyl acetamide,
EtOH = ethyl alcohol, IPA = isopropyl alcohol,
PG = propylene glycol, PEG 400 = polyethylene glycol 400

1.2 HPLC Analysis of Diclofenac Diethylamine

DD was quantitated by reversed-phase high performance liquid chromatography (HPLC). Paramerters for the HPLC system was set as follows :

column : HPLC-reverse phase column : ODS Hypersil C_{18} (4.6 mmx15 cm) settled at an ambient temperature

mobile phase : pH 4.2, 0.01 M sodium acetate : acetonitrile (50:50 v/v)

injected volume : 20 µl
flow rate : 1.5 ml/min
pressure : 1500 psi
detector : UV detector was set at 282 nm
chart speed : 0.25 cm/min

Concentration of DD in the samples, taken from the receptor solution at predetermined intervals were obtained by a reversed phase HPLC as outlined above. The mobile phase was filtered through a 0.45 µm membrane filter and then degassed by sonication for 30 min prior to use. Under these conditions, excellent linearity and reproducibility were obtained between 0.2-100 µg/ml. DD produced a very sharp, clear absorption peak at the retention time of 7.5 min and propylparaben (PP, internal standard) at 4.6 min. The run time per sample was within 11 min. Chromatogram of HPLC, shown in Figure 9, represents a good resolution between the drug and the internal standard.

Standard solution containing 2, 4, 6, 8, and 10 µg/ml of DD and 5 µg/ml of PP and 10, 20, 30, 40, and 50 µg/ml of DD and 30 µg/ml of PP in each dilution were prepared. The calibration curves (Figures 10-11) were constructed by plotting the ratio of the peak area of DD and PP versus the concentration of DD. The calibration plot showed a linear relationship with a good correlation coefficient. For calculation of the concentration of DD in the *in-vitro* evaluation, the calibration was repeated prior to every course of the analysis.

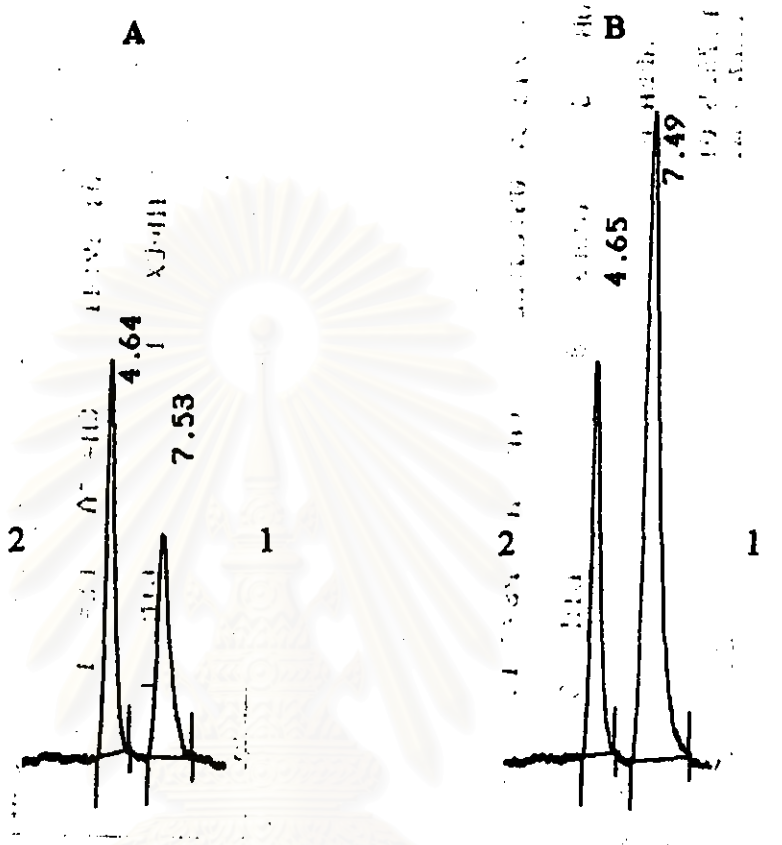

Figure 9 High performance liquid chromatogram of diclofenac diethylamine and propylparaben (internal standard) at 282 nm [1; diclofenac diethylamine (A) 10 μg/ml (B) 30 μg/ml 2; propylparaben (A) and (B) 30 μg/ml].

STANDARD CURVE OF DICLOFENAC DIETHYLAMINE

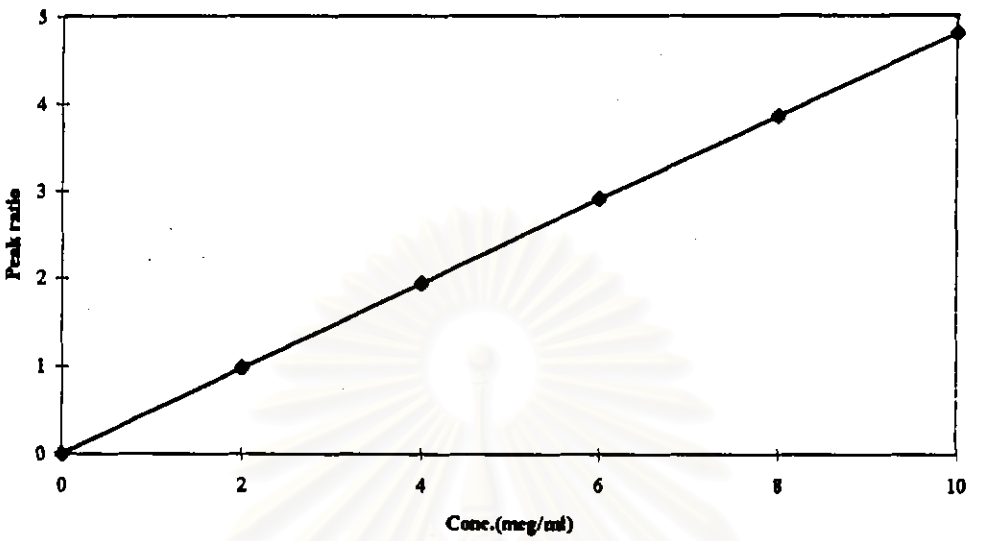

Figure 10 Calibration curve of diclofenac diethylamine-internal standard peak area ratio as a function of diclofenac diethylamine concentration range of 2-10 mcg/ml (r^2=0.999, intercept= - 0.021, slope=2.065).

STANDARD CURVE OF DICLOFENAC DIETHYLAMINE

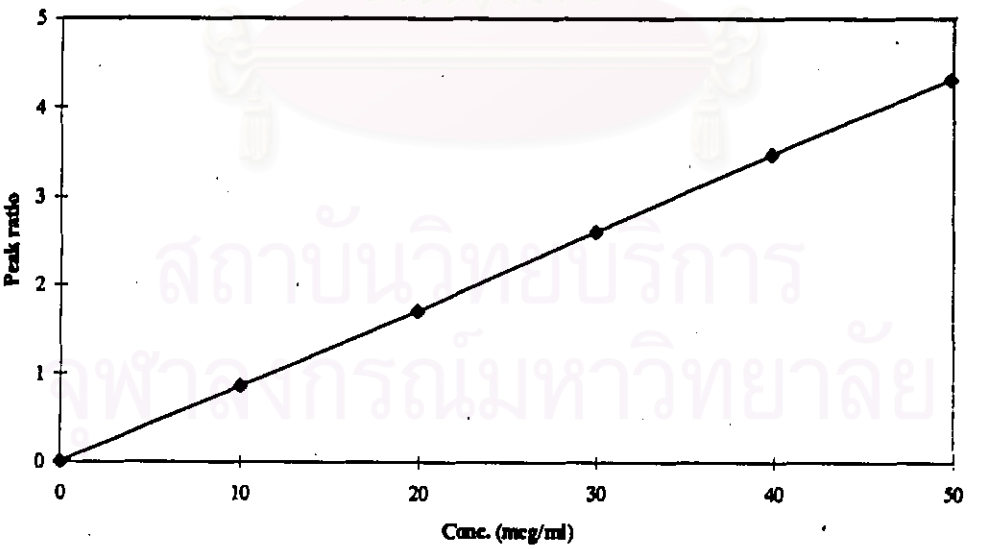

Figure 11 Calibration curve of diclofenac diethylamine-internal standard peak area ratio as a function of diclofenac diethylamine concentration range of 10-50 mcg/ml (r^2=0.999, intercept=0.011, slope=11.53).

2. Design of Improved Diffusion Cell

An *in-vitro* release and permeation study was carried out by using a diffusion cell (Figure 12) modified from Franz diffusion cell (Franz, 1975), Keshary-Chien diffusion cell (Keshary and Chien, 1984), and Patch cell (Mueller, Roberts, and Scott, 1990). This diffusion cell consists of two compartments, namely the donor and the receiver compartments vertically attached to each other via a metal clamp. The capacity of the receptor compartment was 60 ml and the cross-sectional area of the donor compartment corresponded to the effective permeation area of 12.5 cm^2. In the meantime, a water-jacket was extended to envelope a greater area of the receptor compartment than the Franz diffusion cell to provide a better temperature control and equilibrium release of drug.

3. Preformulations of Drug Reservoir for Diclofenac Diethylamine-TDS

This section of the experiment will include the solubility and stability studies, which will determine the suitable solvent, pH value of buffer system, and the gelling agent. These parameters will be employed for further formulation development.

3.1 Determination of Drug Solubility

Drug solubility study was done in various solvents (such as ethyl alcohol, isopropyl alcohol, benzyl alcohol, propylene glycol, polyethylene glycol 400 and N,N-dimethyl acetamide). Approximately 0.5 g of drug was weighed into a test tube. Ten milliliters of solvent was added. The test tube was then covered with parafilm and aluminium foil before enclosing with a cap. The

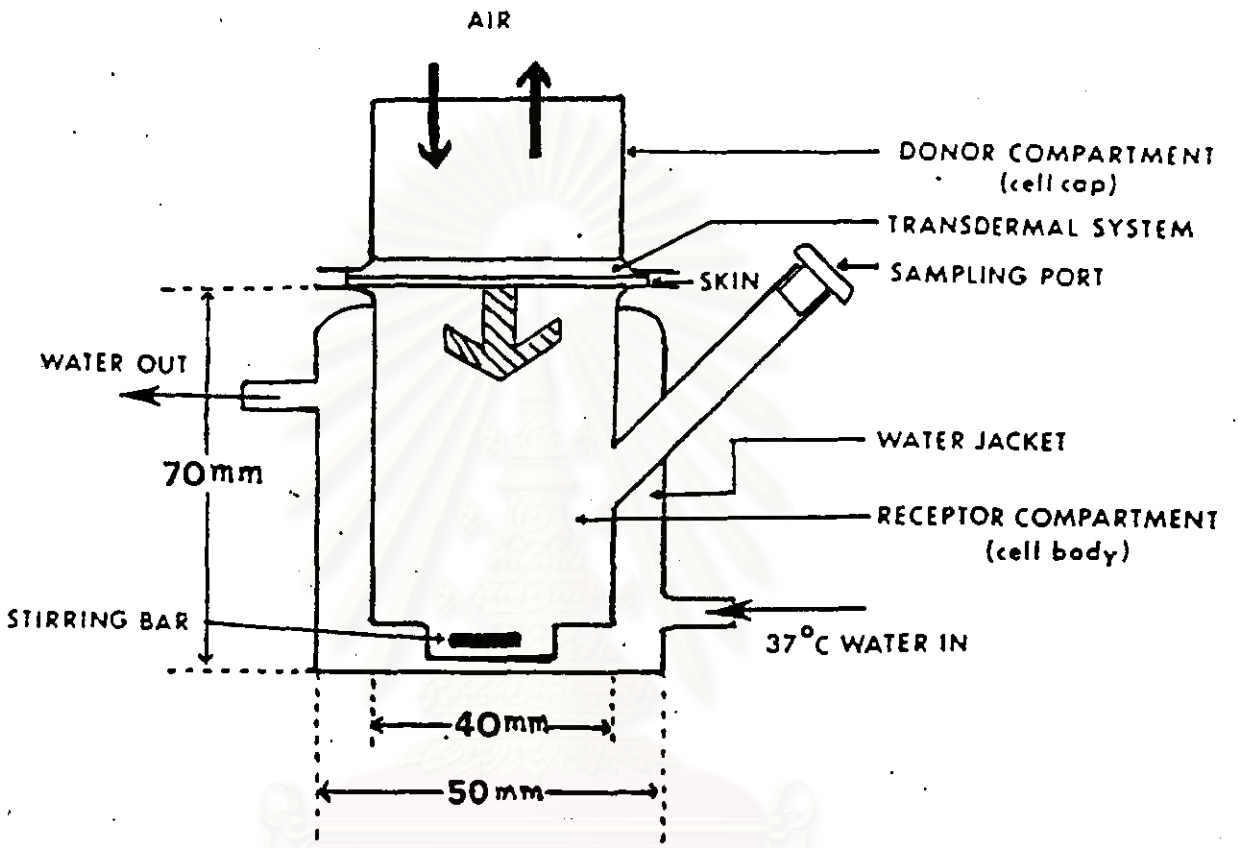

Figure 12 Schematic illustration of the modified diffusion cell used in this study.

mixture was agitated using the Vortex apparatus and was observed for excess drug powder. If the clear mixture was obtained, another 0.5 g of drug was then added until excess drug powder was achieved. The mixture was allowed to equilibrate using the shaker at 37±1°C for 24 hours. The supernatant was diluted to optimum concentration. The amount of drug was determined by spectrophotometric method.

3.2 Effect of Various Solvents on the Physical Change at Accelerated Condition

Drug was added to various solvents (as described in 3.1), at 1.16 %w/w concentration. The solutions were filled into 6 ml vials and closed with the rubber stopper and aluminium cap. The vials were kept at 45±2°C incubator for 1 month. From previous studies, it was found that after degradation of DD the color intensity increased noticeably. This event was probably due to the degradation product present in the sample. For this reason, the vials were observed after 1 month and compared for any color changes to select a suitable solvent for the active drug.

3.3 Effect of pH Values of Buffer Solution on the Physical Change at Accelerated Condition

Sorensen phosphate buffer was prepared with two separate stock solutions, di-sodium hydrogen phosphate solution and sodium dihydrogen phosphate solution. The pH 6, 7, 8, 9, and 10 buffer solutions were prepared by mixing di-sodium hydrogen phosphate stock solution and sodium dihydrogen phosphate stock solution using different ratios to obtain the desired pH value, then was detected using a pH meter. DD was dissolved in the buffer phase to

achieve a concentration of 1.16 %w/w. Short-term stability studies were conducted by keeping in the incubator at 45±2°C for 1 month and then observed for color changes to select a suitable pH value for the active drug.

3.4 Preparation of Drug Reservoir for Transdermal Delivery System (TDS)

The suitable solvent and buffer pH value obtained from previous studies, were selected and were utilized in the following experiments. The quantities used for preparing the drug solutions in propylene glycol and the gel formulations are listed in Table 3.

3.4.1 Preparation of Diclofenac Diethylamine-Propylene Glycol

Since propylene glycol was selected to be the solvent in every formulations, the formulations consisted of various concentrations of DD (1.16, 1.74, and 2.32 %w/w) in propylene glycol were prepared.

3.4.2 Preparation of Diclofenac Diethylamine-Poloxamer F-127

Poloxamer F-127 was used as a gelling agent in the formulation. This investigation was aimed at the effect of poloxamer F-127 on the *in-vitro* release and skin permeation. The poloxamer F-127 was slowly added to cold buffer pH 7 and stored in a refrigerator to form a clear viscous solution. DD was then dissolved in an appropriate quantity of propylene glycol and mixed with poloxamer F-127 gel.

Table 3 Formulas of diclofenac diethylamine-TDS in propylene glycol and various gelling agents.

Formula #	Concentration of				
	DD	PLX	HPMC	CMC	SALG
1	1.16	-	-	-	-
2	1.74	-	-	-	-
3	2.32	-	-	-	-
4	1.16	20	-	-	-
5	1.74	20	-	-	-
6	2.32	10	-	-	-
7	2.32	20	-	-	-
8	2.32	30	-	-	-
9	1.16	-	2	-	-
10	1.74	-	2	-	-
11	2.32	-	1	-	-
12	2.32	-	2	-	-
13	2.32	-	3	-	-
14	1.16	-	-	2	-
15	1.74	-	-	2	-
16	2.32	-	-	1	-
17	2.32	-	-	2	-
18	2.32	-	-	3	-
19	1.16	-	-	-	1
20	1.74	-	-	-	1
21	2.32	-	-	-	1
22	2.32	-	-	-	2
23	2.32	-	-	-	3

Remark : DD = diclofenac diethylamine, PLX = poloxamer F-127,
HPMC = hydroxypropyl methylcellulose,
CMC = carboxymethylcellulose, SALG = sodium alginate,
Propylene glycol was used as solvent in every formulations,14 %w/w
Phosphate buffer pH 7 was used as stabilizer in Formulas #4-23.

3.4.3 Preparation of Diclofenac Diethylamine-Hydroxypropyl Methylcellulose, Sodium Carboxymethylcellulose, or Sodium alginate

Hydroxypropyl methylcellulose, sodium carboxymethylcellulose, and sodium alginate were separately weighed and dispersed in phosphate buffer pH 7. DD was dissolved in an appropriate quantity of propylene glycol and mixed with each gel.

3.5 Stability Study of Drug in Gelling Agents at Accelerated Condition

Drug was incorporated into various gelling agents such as poloxamer F-127, hydroxypropyl methylcellulose, sodium carboxymethylcellulose, and sodium alginate, at 1.16 %w/w which was equal to Voltaren® emulgel concentration. The solutions were filled into 6 ml vials and closed with the rubber stopper and aluminium cap. The vials were stored in 45±2°C incubator for 4 months. After 1 month interval, the vials were observed for color changes and analyzed for drug content using HPLC method.

4. Evaluation of Diclofenac Diethylamine-TDS Formulations

4.1 Preparation of Transdermal Patch

The transdermal patch used in this study was prepared using impermeable backing membrane of heat sealable tan polyester film laminate. The membrane was obtained from microporous polyethylene film. The adhesive layer of the system was hypoallergic acrylate pressure sensitive adhesive. The delivery device was protected using a release liner. Backing

membrane and microporous polyethylene film were cut into pieces (5x5 cm^2 each) and sealed four rims with heat sealing machine. The system is a square, multilaminate rate-controlling membrane, which the drug can be delivered across the membrane to the skin. A system with a contact surface area of 9 cm^2 was used throughout this study (Figure 13).

4.2 Skin Preparation

4.2.1 Preparation of Receiver Medium

A 1.9 g of potassium dihydrogen phosphate was dissolved in 400 ml of water. Slowly adding 4.11 g of sodium chloride to this solution. Dibasic sodium phosphate, 8.1 g, was separately dissolved in another 400 ml of water. The two solutions were then mixed together and the final volume was adjusted to 1,000 ml with water. The pH of solution was adjusted to 7.4$\pm$0.02 with 1 N sodium hydroxide. Finally, 400 ml of polyethylene glycol 400 was added to this solution.

4.2.2 Pretreatment of Shed Snake Skin

Shed snake skin specimens from *Elaphe obsoleta* were selected as the representative of the stratum corneum, the major barrier to percutaneous drug absorption. They were kept in the refrigerator at 4°C. Prior to the experiment, they were thawed at room temperature and the dorsal part of the specimens were cut into pieces (5x5 cm^2 each) and then immersed in the receiver medium for 12 hours before use.

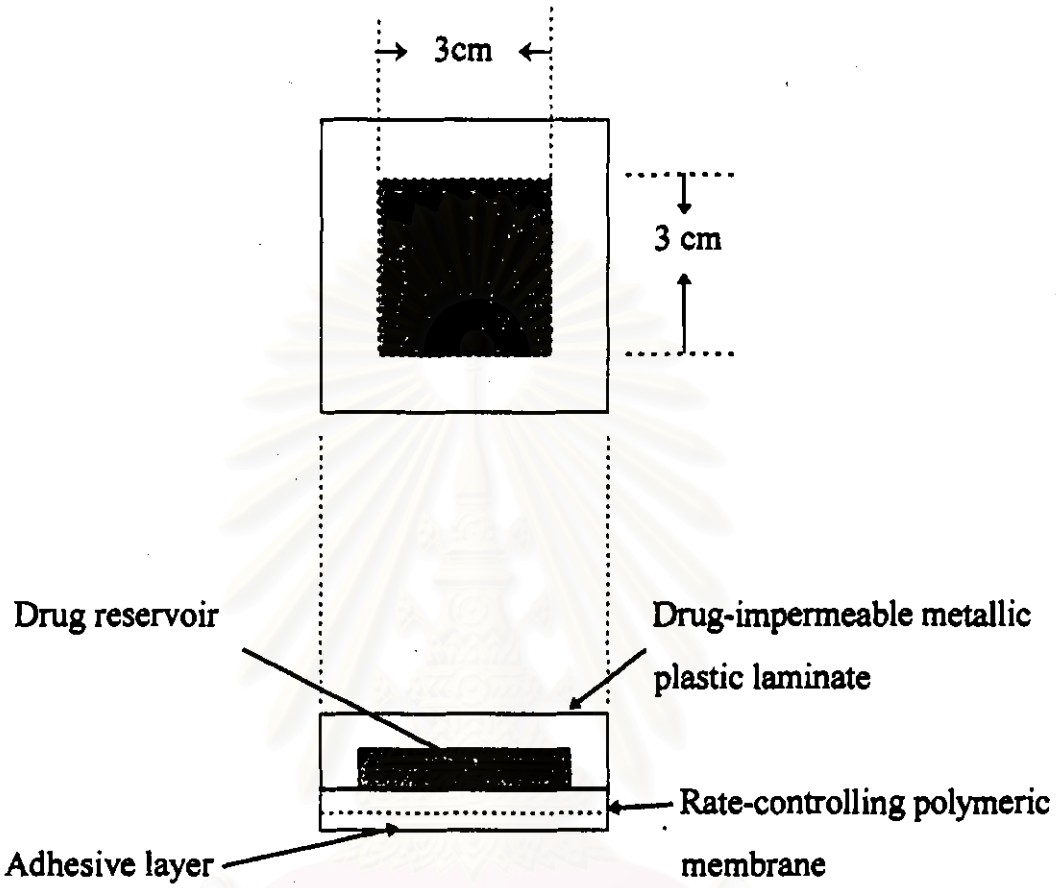

Figure 13 Cross sectional view of a membrane-moderated transdermal drug delivery system, used in this study.

4.3 *In-vitro* Evaluation of Voltaren® emulgel

4.3.1 *In-vitro* Drug Release Evaluation of Voltaren® emulgel in patch

Voltaren® emulgel was transferred into transdermal patch in the drug reservoir layer. Then, the transdermal delivery system was clamped between

the donor and the receptor compartments, with the drug releasing surface facing the receptor compartment. Receiver medium which was sonicated for 1 hour and preheated at 37±1°C, was filled into the receiver compartment. A small magnetic stirring bar was placed in the receptor compartment and rotated at 130 rpm. The temperature of the assembled diffusion cell was maintained at 37±1° C by means of a circulating water jacket connected to a constant temperature water bath. A portion of the receiver medium (1.0 ml each) was withdrawn at predetermine time interval (i.e. at 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12 hours) and was filled with an equal volume of freshly prepared (drug-free) receiver medium. The amount of drug released was analyzed by the HPLC method.

4.3.2 *In-vitro* Skin Permeation Evaluation of Voltaren® emulgel in patch

Voltaren® emulgel was carefully transferred into transdermal patch in the drug reservoir layer. A skin which had been previously treated (as described in 4.2.2), was mounted between the donor and the receiver compartments by means of a clamp. The skin was placed in such a way that the upper part was in contact with the preparation in the donor chamber and the other side with the receiver medium. The method of *in-vitro* skin permeation was done in same way as previously described in 4.3.1.

4.3.3 *In-vitro* Evaluation of a Direct Skin Permeation Study of Voltaren® emulgel

Voltaren® emulgel was subjected to the *in-vitro* tests using a modified Franz diffusion cell. Excess amount of Voltaren® emulgel (5 g) was applied

over the skin surface in the donor chamber. The method of *in-vitro* skin permeation was done in same way as previously described in 4.3.1.

4.4 *In-vitro* Evaluation of Diclofenac Diethylamine-TDS Formulations

After obtained suitable pH value, solvent system, and polymer, these formulation factors will provided useful information to develop diclofenac diethylamine-TDS formulations. In this section, effect of drug concentrations will be evaluated to select optimal gradient force and release rate compared to that of Voltaren® emulgel in patch.

4.4.1 *In-vitro* Drug Release Evaluation of Diclofenac Diethylamine-TDS Formulations

All formulations, consisted of various concentrations of diclofenac diethylamine (1.16, 1.74, and 2.32 %w/w) and gelling agents (Table 3), were placed into the transdermal patch in the drug reservoir layer. The method of *in-vitro* drug release was done as previously described in 4.3.1. The results were compared to that of Voltaren® emulgel in patch.

4.4.2 *In-vitro* Skin Permeation Evaluation of Diclofenac Diethylamine-TDS Formulations

From section 3.5, the suitable drug reservoirs were chosen. All formulations consisted of various concentrations of gelling agents were placed into the transdermal patch in the drug reservoir layer. The method of *in-vitro* skin permeation was done as previously described in 4.3.1. The results were compared to that of Voltaren® emulgel in patch.

4.5 *In-vivo* Evaluation of Diclofenac Diethylamine-TDS Formulations

From section 4.4.1, the suitable formulation was chosen. The formulation was placed into the transdermal patch in the drug reservoir layer. Anti-inflammatory studies were performed to evaluate the *in-vivo* skin permeation of DD-TDS formulation, using a plethysmometer to measure carrageenan-induced paw volume following the mothod of Winter et. al. (1963).

Male wistar strain albino rats, 100-120 g, were grouped into 3. Each group consisted of 6 animals. The animals were fasted over night but had free access to water. First group served as a control, second and third groups were used to evaluate transdermal applications of DD patch and Voltaren® emulgel respectively. Gel on a suitably cut paper was placed in contact with the lightly shaved dorsal skin of the rat, secured in place using adhesive tape in an area of application of approximately 4 cm^2. Transdermal patch with the same area was place in the same way. In all the cases, drug was administered 3 and 12 hours prior to the subplantar injection of 0.1 ml of 1% carrageenan in normal saline into the right hind paw. The paw volume upto the ankle joints was measured using plethysmometer immediately before and 3 hours after the carrageenan injection. Percentage anti-inflammatory activity was calculated using the formula

$$(1-V_t/V_c)x100,$$

where V_t and V_c stand for mean oedema volume in the treated and control group respectively (Jayanthi and Udupa, 1993; Medhavan and Hwang, 1992).