CHAPTER II

EXPERIMENTAL

Materials.

Chemicals:

Diclofenac Sodium, supplied by Atlantic pharmaceutical Co., Ltd.

Phenylbutazone, supplied by S. Co. Pharma Ltd.

Isopropyl alcohol AR, Batch No. K 17894034, E. Merck.

Methanol HPLC grade, Batch No. 9093-03, J.T. Baker.

Ethanol AR, Batch No. K 91078583, E. Merck.

Orange oil, HC-4029, Kieth Harris & Co. (Far East) P.T.E. Ltd.

Polysorbate 20, Lot No. TGA02, Mark.

Propylene glycol BP 1973, Batch No. QC 000136, Viddhyasom Co.

Tetraglycol, Batch No. 77187, Lehman & Voss Co. Ltd.

Glacial acetic acid AR, Batch No. K 11297763, E. Merck.

Polyoxyethylenelauryl ether (Brij 35), Batch No. 801962, Merck-Schuchardt.

Monobasic potassium phosphate AR, Batch No. A 646173, E. Merck.

Disodium hydrogen phosphate AR, Batch No. A 713186, E Merck.

Sodium chloride AR, Batch No. 13445704, E. Merck.

Sodium acetate AR, Batch No. TA 461186, E. Merck.

Membranes:

- Newborn pig skin, Local farm in Sri-sakes.

- Human amnion and placental membrane, supplied by Chulalongkorn Hospital.

Equipments:

Diffusion apparatus, Atlantic Pharmaceutical Co., Ltd.

Modified Franz diffusion cells, FDC-400, Crown Glass Company, Inc., USA.

UV spectrophotometer, Model U2000, Serial No. 0402-040, Hitashi, Japan.

pH meter, Model SA 520, Orion, USA.

Analytical balance, Sartorius GMPH, Range 80/0.1 mg, Germany.

Thermostatted shaker bath, Julabo VSW 1, Jucheim Labortechnik HG, West Germany.

Mitutoyo micrometer, 0-25 mm, 0.01, Mitutoyo Mfg. Co., Ltd., Japan.

Vortex mixer, Scientific Industries Inc.

Ultrasonic bath, Branson 3210, Smithkline Company, USA.

High Performance Liquid Chromatography (HPLC) equipped with

a tunable absorbance detector, Model M 484, Serial No. 484-PRA 902, Waters, USA.

a 20 μl injector, Rheodyne.

a constant flow pump, Waters 510 HPLC pump, Millipore, USA.

an integrator, Waters 745 B Data Modules, Serial No. 7 BE / 400678, USA.

an autoinjector, Waters 712 WISP, Serial No. 712-007617, Millipore, USA.

a Spherisorb HPLC cartridge column phase separations S5 ODS 2 (250x4.6 mm) 5 μ, Phase Separations Inc, USA.

Methods

1. Solubility Determination of Diclofenac Sodium in Donor Solutions at 33 ± 1°C.

An excess amount of diclofenac sodium was added into a screw-capped test tube containing 5 ml of a donor vehicle. The donor vehicles studied included water, 0.01 mg/ml tween 20 in water, 0.05 mg/ml tween 20 in water, 0.4% w/v Brij 35 in water, 1% w/v Brij 35 in water, 10% w/v propylene glycol in water, 1% w/v tetraglycol in water, ethanol, isopropanol and 1% w/v orange oil in ethanol. The solubility of drug in each donor solution was determined in six replicates. The test tubes were vigorously shaken and swirled by a vortex mixer which placed in a thermostatted shaker bath at 33 ± 1°C. The mixtures were allowed to equilibrate for at least 48 hours.

After an equilibrium had been reached, the test tubes were placed upright for about twelve hours and then an aliquot portion of the supernatant was diluted with water in an appropriate amount. It was then determined for drug content using a UV spectrophotometer referring to a calibration curve performed as follows.

An accurate amount of diclofenac sodium (about 40 mg) was weighed in a 100-ml volumetric flask and dissolved in one of the donor vehicles. The solution was adjusted to the final volume to make a concentration of 400 μg/ml. The stock solution was pipetted and diluted to make final concentrations of 4, 8, 12, 24, 32, and 40 μg/ml, respectively. The standard solutions with the donor vehicles being water, 0.01 mg/ml tween 20 in water, 0.05 mg/ml tween 20 in water, 0.4% w/v brij 35 in

water and 1% w/v brij 35 in water were measured at a wavelength of 275.5 nm. The standard solutions with donor vehicles being ethanol and 1% w/v orange oil in ethanol were measured at a wavelength of 284 nm. And the solutions having isopropanol vehicle was measured at a wavelength of 283 nm.

2. Partition Coefficient Determination (Moeckly, 1986).

All human placental membrane, human amnion and newborn pig skin were included in this study. The membrane was cut into small squares (~ 4 cm^2) and allowed to equilibrate with the donor vehicle for at least 24 hours. The donor vehicles studied were water, 0.05 mg/ml tween 20 in water, 1.0% w/v brij 35 in water, 10% w/v propylene glycol in water and 10% w/v tetraglycol in water. The thickness of the membrane which had already absorbed the donor vehicle was measured to approximate the volume (Vo) of the membrane plus the sorbed donor vehicle. The membrane were stored in the donor vehicle for further use.

The solvent absorbing membranes were cut into small squares (~ 1 cm^2) and placed into a screw-capped test tubes containing 5 ml (Va) of the donor solution containing Ci mg/ml of drug. The tubes were lined with a teflon tape, close with a closure sealed with an electrical tape and shaked in a water-bath for at least 48 hours at 33±1°C.

A high pressure liquid chromatographic (HPLC) technique was used to assay the donor concentration (C_E) after the equilibrium had been reached. The HPLC assay was used both to increase sensitivity and to avoid potential problems with contaminates leaching out of the membrane material.

Calculation of the experimental partition coefficient was accomplished using a method derived by Takamatsu and Eisenberg (1979) and later extended by Dahl (1983):

$$\text{Partition Coefficient} = \frac{V_a (C_i - C_E)}{V_o C_E} \qquad (9)$$

where Va is the volume of the donor solution, Ci is the initial concentration of donor solution which is the saturated concentration in this case, C_E is the concentration of donor solution after it was absorbed by the membrane, and Vo is the volume of the membrane which had already absorbed the donor vehicle.

3. *In Vitro* Permeation Studies.

3.1 Experimental conditions.

Steady state fluxes of diclofenac sodium through newborn pig skin, human amnion and human placental membrane were determined using the following conditions.

Donor Solutions: Two sets of donor solutions, saturated concentration and 25 mg/ml of diclofenac sodium, were studied. Saturated donor solutions had been used in part of this study to obtain a constant activity so that the enhancing effect was resulted only from enhancers used. For 25 mg/ml of diclofenac sodium solutions, the effect of drug solubility was the main purpose to study.

In the case of saturated solutions of diclofenac sodium, the following vehicles were studied:

water.

0.01 mg/ml tween 20 in water (below CMC).

0.05 mg/ml tween 20 in water (above CMC).

0.4% w/v brij 35 in water (below CMC).

1% w/v brij 35 in water (above CMC).

10% w/v propylene glycol in water.

10% w/v tetraglycol in water.

ethanol.

isopropanol.

1% w/v orange oil in ethanol.

In the case of 25 mg/ml diclofenac sodium, the following vehicles were studied:

water.

0.05 mg/ml tween 20 in water.

1% w/v brij 35 in water.

10% w/v propylene glycol in water

10% w/v tetraglycol in water.

Receiving Solutions:

Monobasic potassium phosphate	0.190	gm
Disodium hydrogen phosphate	0.810	gm
Sodium chloride	0.411	gm
Distilled water to	1000	ml

Membranes: Newborn pig skin.

Human amnion.

Human placental membrane.

Diffusion cells: Modified Franz diffusion cells with an inside diameter of 1.57-1.64 cm which corresponds to an effective diffusional area of 1.93-2.11 cm^2. Receptor volumes are varied between 12.2-12.7 ml.

3.2 Preparation and Treatment of Membranes.

Newborn Pig Skin:

An excised full thickness skin was used for the penetration study. An abdominal skin of a newborn pig was carefully excised and inspected for any defects; for example, scratches, bruises and bites. Subcutaneous fat and other extraneous tissues adhering to the dermis were completely removed and trimmed if necessary using forceps and scissors. The skin was cleaned and bathed in the receiving solution. Then it was wrapped in an aluminium foil and stored in a freezer. The skin membranes might be used immediately. The frozen skin was thawed and allowed to dehydrate by immersing in the receiving solution at room temperature for about one hour. Then the skin was cut to a circle shape with a diameter of about 3 cm. Its thickness was measured at twelve different points using a Mitutoyo micrometer. An average of the twelve values were used for subsequent calculations.

Human Placental Membrane:

Human placental membrane delivered from a healthy mother was used. Extraneous tissues facing the mother side were carefully peeled off using forceps and scissors. The placental membrane facing the fetus side which was smoother than the mother side was used. All microvilli adhering to the membrane were also carefully removed. The placental membrane was then cleaned using tapped water, immersed in the receiving solution, wrapped with aluminium foil, kept in a plastic bag and placed in a freezer. It was thawed prior to use by the same procedure as the frozen pig skin mentioned previously.

Human amnion:

Human amnion delivered from a healthy mother was used. The amnion could be peeled off very easily using forceps. The membrane was then washed using tapped water, immersed in the receiving solution and kept well in a plastic bag placed in a freezer. It was thawed prior to use by the same procedure as the frozen pig skin mentioned previously.

3.3 Diffusion Experiments.

A sheet of membrane was mounted and clamped in place between the donor and receptor compartments of diffusion cell. The excised newborn pig skin was set in place with the stratum corneum facing the donor compartment. The placing side of the other membranes did not affect the steady state flux values. The receptor fluid was filtered under vacuum prior to use . The receptor fluid in the receptor compartment had been continually stirred with a magnetic stirring bar

rotating at 300 ± 5 rpm with syncronous motors and maintained at 37 ± 1° C by circulated water which was pumped through a jacket surrounding the cell body throughout the time of diffusion studies.

The membrane and the receptor solution were equilibrated and warmed for fifteen minutes and air bubbles under the membranes were carefully removed by tipping or gently rocking the cell . After the equilibration, an excess amount of the donor solution was applied on top of the membrane and the donor compartment was covered with a parafilm to prevent the evaporation of donor vehicle. The ambient temperature of donor solution was about 33 ± 1° C.

At appropriate time intervals, the entire receiving solution was drawn using a syringe flitted with a piece of flexible tubing through the sampling side arm of receptor cell. The volume of the receptor compartment was immediately replaced with an equal volume of fresh receiving solution and the run was continued. All drug concentrations in the receiving solution withdrawn were determined using the HPLC assay. The appropriate dilution of samples with the receiving solution was made prior to the analysis.

A perfect sink condition must be maintained throughout the experiment. A time period of the run must be long enough to ensure that the steady state permeation was achieved. Each donor solution was run in triplicate.

At the completion of each experiment, the drug concentrations in the receptor phase were determined by the HPLC method. The permeating amount was calculated by multiplying the drug concentration by the receptor volume. For each

membrane specimen, the cumulative permeating amount was plotted versus time and the slope and intercept of the steady state portion were derived by the regression analysis. The observed steady state flux (Jss) was calculated by dividing the slope by the diffusional area. The steady state flux was also expressed as a normalized flux which was Jss x $h/\bar{h}$, where h is the thickness of membrane and $\bar{h}$ is the average thickness of membranes.

4. HPLC Assay.

The high pressure liquid chromatographic technique was used for analysis of drug concentrations from the partition and permeability experiments. The system consisted of a constant flow pump, an autoinjecting sample processor, a variable wavelength UV detector, an integrator, and a fixed volume sample injector with a 20 μl loop. An internal standard was used to determine the drug concentration. The appropriate conditions for analyzing of the drug content by the HPLC techniques are as follows.

4.1 HPLC Conditions (Sane, Samant, and Nayak, 1987).

Column	:	Spherisorb ODS 2.
Mobile phase	:	a mixture of 70 % methanol and 30 % pH 4.0, 0.01 M sodium acetate buffer by volume.
Detector wavelength	:	280 nm.
Flow rate	:	1 ml/min.
Attenuation	:	4.

Chart speed	:	0.25 cm/min.
Injected Volume	:	20 μl.
Internal standard	:	0.25 mcg/ml phenylbutazone.
Retention time of diclofenac sodium	:	7.48 - 8.10 min.
Retention time of phenylbutazone	:	4.58 - 4.70 min.
Standard Solutions	:	0.125, 0.25, 0.05, 0.75, 1.00, 1.25 mcg/ml.

Preparation of pH 4.0 , 0.01 M sodium acetate buffer:

0.82 grams of anhydrous sodium acetate was dissolved and adjusted to a volume of 1000 ml with distilled water. The solution pH was adjusted to 4.0 with glacial acetic acid.

4.2 Preparation of Standard Solutions.

Stock Solution of Phenylbutazone:

Twenty-five milligrams of phenylbutazone was accurately weighed in a 50-ml volumetric flask. Methanol was added and swirled until phenylbutazone was completely dissolved and then adjusted to volume. An aliquot of 0.5 ml of this solution was pipetted to a 100-ml volumetric flask and was further adjusted to volume with methanol so that the final concentration of phenylbutazone was 2.5 mcg/ml.

Stock Solution of Diclofenac Sodium:

Twenty-five milligrams of diclofenac sodium was accurately weighed in a 100-ml volumetric flask. The mobile phase was used to dissolve the drug and adjust the volume. One ml of the solution was pipetted into a 100-ml volumetric flask and was adjusted to the volume with the mobile phase, so the final concentration of 2.5 mcg/ ml was obtained.

Standard Solutions:

One ml of the phenylbutazone stock solution was pipetted to six 10-ml volumetric flasks. The stock solution of diclofenac sodium was pipetted (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 ml) and transferred to the six 10-ml volumetric flasks, respectively. The solutions were then adjusted to the volume with receiving solution so that the final concentrations of the standard solutions were 0.125 0.25, 0.5, 0.75, 1.0 and 1.25 mcg/ml, respectively.