CHAPTER II

EXPERIMENT

2.1 Instruments

For in vitro experiments, specific instruments were used; Franz diffusion cells, 13 ml, 2.27 cm^2, a pH Meter (Model 744 pH Meter, Metrohm Ltd., Switzerland), a vacuum suction (Model Sam 12, Aerosal Medical, USA) and an UV/VIS Spectrophotometer (Model UV-2550, Shimadzu, Japan)

2.2 Chemicals

Octyl methoxycinnamate (Eusolex®2292), butyl methoxy dibenzoylmethane (Eusolex®9020), 4-methyl benzylidene camphor (Eusolex®6300) and Eusolex UV pearl OMC were kindely given by Merck Co. Ltd. (Thailand). The newly developed UV filters including di(2-ethylhexyl)-2,4,5-trimethoxybenzalmalonate, 2-ethylhexyl-2,4,5-trimethoxycinnamate, dihexyl-2,4,5-trimethoxybenzalmalonate, diethyl-2,4,5-trimethoxy benzalmalonate, poly-(3-hydroxy-propoxy) cinnamic acid, poly-(6-hydroxy-hexyloxy) cinnamic acid, poly-(11-hydroxy-undecyloxy) cinnamic acid, poly penta-ethylene glycol cinnamate, poly (*p*-propoxycinnamate)-co-(*p*-undecyloxycinnamate), 4-methoxycinamoylphthaloyl chitosan, 4-methoxycinamoylphthaloyl irradiated chitosan, poly [vinyl-2,4,5-trimethoxycinnamate(vinyl alcohol)]copolymer. Solvents were obtained other student in the lab.

Skin specimens

The skin specimens of 2 weekolds baby mice (*Mus Musculus* Linn.) were purchased from National Laboratory Animal Centre (Mahidol University, Buddhamonthon 4 Rd., Salaya, Nakhonpathom, Thailand). The abdominal skin was removed by surgical procedure. The skin composed of epidermis and dermis layers. They were kept at room temperature,- 20 $^{\circ}C$ and -80 $^{\circ}C$, and cut into approximately 2.0x2.5 cm^2 pieces.

2.3 Determination of percutaneous absorption of sunscreen.

2.3.1 Study of transdermal penetration of various sunscreen through baby mice skin by Franz diffusion cell method.

Preparation of sunscreen solutions.

Table 2.1 Protocol showing the preparation of sunscreen solutions for Franz diffusion cell experiments. Two hundread µl of the prepared solution were dropped onto 2.27 cm^2 baby mouse skin at the top compartment of the diffusion cell.

UV filters	solvent	concentration		Coverage on the skin mg/cm^2
		Molar(M)	w/v	
OMC	MeOH	0.172	0.01/200	4.4
BMDBM	MeOH	0.161	0.01/200	4.4
4-methyl benzylidene camphor	MeOH	0.197	0.01/200	4.4
Eusolex UV pearl OMC	MeOH	0.173	0.01/200	4.4
di(2-ethylhexyl)-2,4,5-trimethoxybenzalmalonate	MeOH	0.099	0.01/200	4.4
2-ethylhexyl-2,4,5-trimethoxycinnamate	MeOH	0.143	0.01/200	4.4
dihexyl-2,4,5-trimethoxybenzalmalonate	MeOH	0.112	0.01/200	4.4
diethyl-2,4,5-trimethoxybenzalmalonate	MeOH	0.112	0.01/200	4.4

Table 2.1 Protocol showing the preparation of sunscreen solutions for Franz diffusion cell experiments. Two hundread µl of the prepared solution were dropped onto 2.27 cm^2 baby mouse skin at the top compartment of the diffusion cell (continued).

UV filters	solvent	concentration		Coverage on the skin mg/cm^2
		Molar (M)	w/v	
poly-(3-hydroxy-propoxy)cinnamic acid	CH_2Cl_2	-	0.01/200	4.4
poly-(6-hydroxy-hexyloxy) cinnamic acid	CH_2Cl_2	-	0.01/200	4.4
poly-(11-hydroxy-undecyloxy)cinnamic acid	CH_2Cl_2	-	0.01/200	4.4
poly penta ethylene glycol cinnamate	CH_2Cl_2	-	0.01/200	4.4
poly-(*p*-propoxycinnamate)-co-(*p*-undecyloxy cinnamate)	CH_2Cl_2	-	0.01/200	4.4
4-methoxycinamoylphthaloylchitosan	DMSO	-	0.01/200	4.4
4-methoxycinamoylphthaloyl irradiated chitosan	DMSO	-	0.01/200	4.4
poly[vinyl2,4,5trimethoxycinnamate(vinyl alcohol)]copolymer	MeOH	-	0.01/200	4.4

In addition to the experiment performed by using UV filter in solution form by the Franz diffusion method, experiments performed with lotion containing the interested UV filters were also done. In this latter case, appropriated amouts of liquid UV filters were added directly into a fix amout of lotion. For the solid UV filters, 500 mg of the compounds were dissolved in alcohol before being mixed into 5 mL of lotion. For liquid UV filter (OMC), 500 mg of the compound were directly added to 5 ml lotion. The UV filter tested by this method include: OMC, BMDBM, di(2-ethylhexyl-2,4,5-trimethoxybenzalmalonate and 2-ethylhexyl-2,4,5-trimethoxycinnamate. The lotion used contains aqua, petrolatum, isopropyl palmitate, paraffinum liquidum, glyceryl stearate, ceteth-20. hypoallergenic anhydrous lanolin, phenoxyethanol, methylparaben, hydroxyethylcellulose, carbomer, propyl-paraben, sodium hydroxide, BH. Five experimental repititions were carried out for each UV filter.

Diffusion cell study.

In vitro permeation studies were conducted with vertical Franz diffusion cells (Figure 2.1) with a 13 ml capacity receptor compartment and 2.27 cm^2 diffusion area. The abdominal skin of 2 weekolds baby mice (*Mus Musculus* Linn.) was removed by surgical procedure. The skin samples were stored at room temperature, -20°C (freezer) and -80 $^{\circ}$C. Skin was cut into two suitable small pieces and carefully mounted onto the receiver compartment of the diffusion cells with the stratum corneum facing in the direction of the donor compartment. When the donor compartment was fastened to the receptor compartment with a clamp, the skin acted as a seal between the two half-cells. The receptor medium consisted of isotonic phosphate buffered saline pH 7.4 (PBS-buffer) with 1% w/v Tween 20. The buffer was prepared by dissolving 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.44 g Na_2HPO_4 and 10 mL Tween 20 in 1L distilled water. This medium was maintained at 37°C and constantly stirring with magnetic bar. The sunscreen solution (200 µl) was added into the donor compartmant of the cell. At five time intervals; 0 min, 1, 2, 4 and 24 hours, the 3.4 ml of receptor were withdrawn and replaced with fresh receptor medium. Care was given to avoid any air bubble in the receptor fluid. The concentration of the UV absorbers in each withdrawn receptor volume was then determined by UV/VIS spectrophotometer. The test was done at least in duplicate. Since skins from different mice gave different penetration rates, each sample was compared to the penetration rate of OMC using skin from the same mouse.

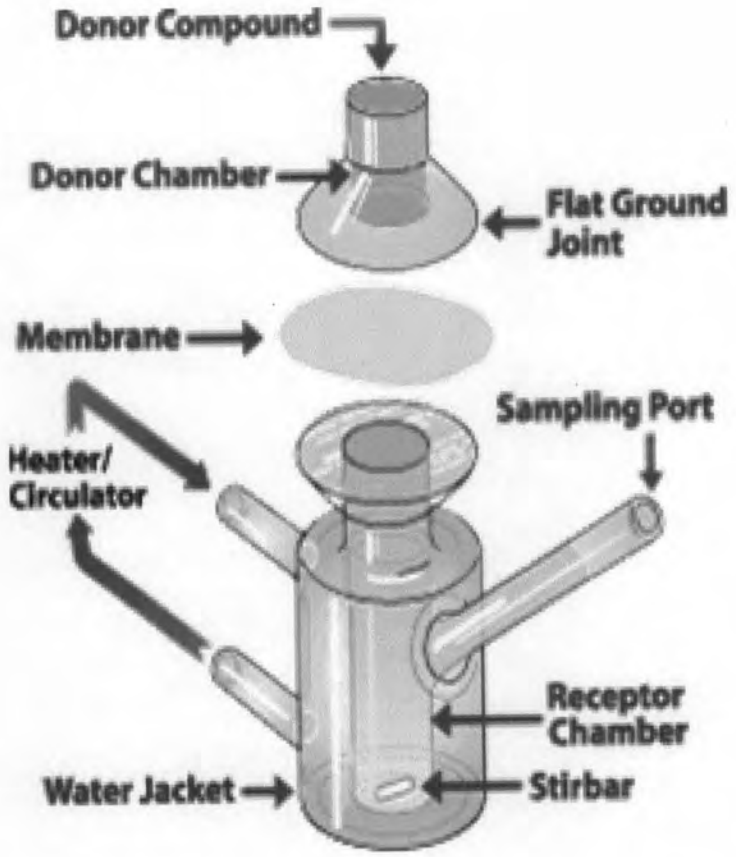


Figure 2.1 A Franz diffusion cell

Quantification of UV filter in receptor fluid

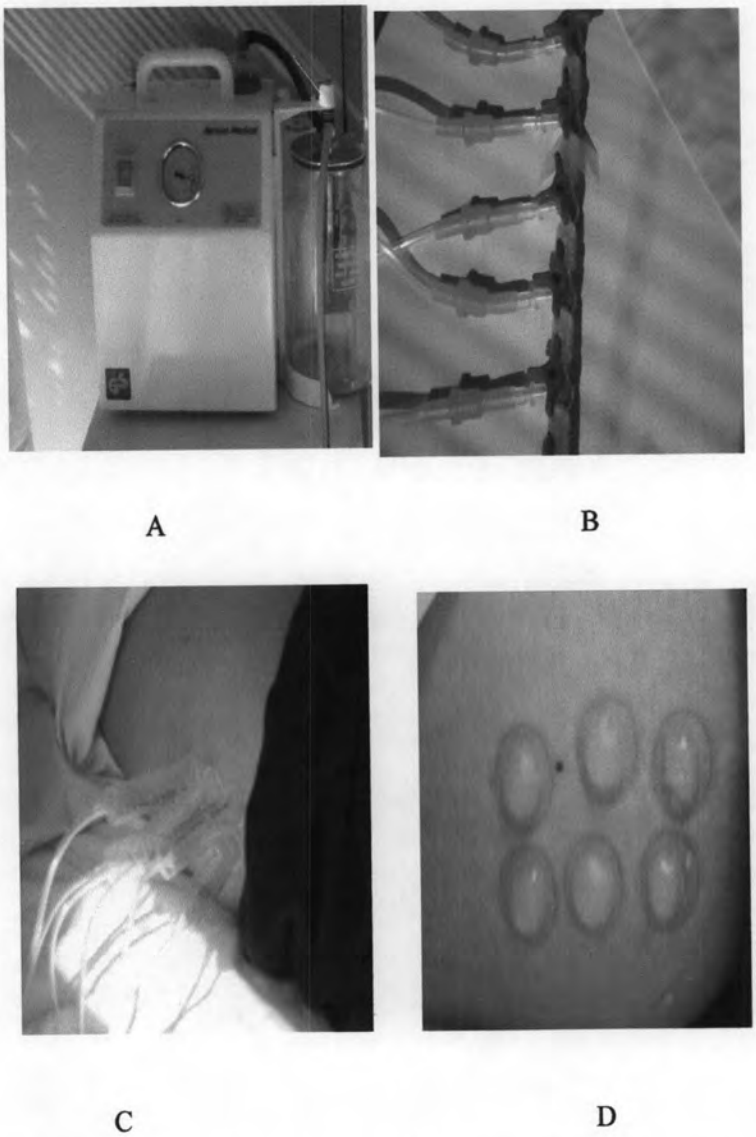
Concentration of UV filter in the receptor fluid was determined by UV/Vis spectrophotometer. The calibration curve of each UV filter in receptor fluid was constructed by measuring the absorbance of the UV filter solution (in receptor fluid) at its λ max. Receptor fluid was used as a blank in the reference cell. The graph between absorptions and concentrations of the UV filters was then constructed and used for determination of UV filter in the receptor fluid obtained during the Franz Diffusion Cell experiments.

2.3.2 Study of transepidermal penetration of various sunscreen through volunteer human skin by suction blister method.

Preparation of sunscreen formulations for suction blister

The same UV filters spiked lotions used in the Franz Diffusion cell experiment were used in this experiment.

Suction Blister study.



A B C D

Figure 2.2 A) The vacuum suction used in this technique B) The IV tube connected to the suction C) The syringe pressed against volunteer skin D) Six simultaneous suction blisters on thigh of volunteer skin.

The suction was connected to the syringe through IV tube (see Fig.2.2B). The syringe was pressed against volunteer skin and negative pressure (200 mmHg) was then applied. Six spots of suction blister (15 mm diameter) (Fig. 2.2D) were induced by keep applying the pressure for 2-3 h on thigh skin. After the roof of the blisters, epidermis was separated from the basement membrane and the resulting space (blisters) was filled with interstitial fluid with the diameter at the base of the blister of ≅ 15 mm, twenty μL of sample (covered on skin at 2.27 mg/cm^2) was dropped over the surface of the swelling skins using micro pipete. Spatula was used to spread sample over the blister roof. The suction blister fluid (SBF) was then collected after an appropriate time using a fine needle. The collected SBF was then subjected to further analysis.

Quantification of UV filter in suction blister fluid

The calibration curve between absorbances at the λ max of each UV filter and concentrations of the standard UV filter in receptor fluid was constructed. Standard UV filters were prepared using receptor fluid as solvent. The concentration used were 0.1 mg/ml, 0.3 mg/ml and 0.5 mg/ml. The absorbance of the standard solutions were determined with UV/VIS spectrophotometer, using suction blister fuid as a blank in the reference cell. The obtained absorbances were plotted against concentrations and linear regression analysis was used to obtain the best straight line. The calibration curve of each compound was constructed for each individual using the individual's receptor fluid.

The t test and Pearson's test statistic were used for the comparison of results obtained from the Franz diffusion cell method and the suction blister method.