CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Chemicals and Reagents

1. Beeswax (Miki Chemical, Japan)
2. Benzophenone - 3 (Escalol 567) (American Cyanamide Co., USA)
3. Borax (Miki Chemical, Japan)
4. Carbomer (Carbopol 934) (B.F. Goodrich Co., Ltd., UK)
5. Cetearyl Glucoside (Seppic, USA)
6. Cetearyl Octanoate (Seppic, USA)
7. Disodium EDTA (Azko, Netherland)
8. Ethanol 95% (J.T.Baker, USA)
9. Glacial acetic acid (E. merck, Germany)
10. Glyceryl stearate (Glyceryl monostearate SE) (Inolex Chemical Co., Ltd., USA)
11. Homosalate (Eusolex HMX) (E. merck, Germany)
12. Hydrochloric acid (J.T. Baker, USA)
13. Imidazolidinyl urea (ISP, USA)
14. Isopropyl palmitate (Seppic, USA)
15. Methanol HPLC grade (J.T. Baker, USA)
16. Mineral oil 65 - 70 cps (Hanwha, korea)
17. Methyl paraben (Ueno, Japan)

18. Octyl dimethyl PABA (Escalol 507) (Vandyk, USA)
19. Octyl methoxycinnamate (Escalol 2292) (Givaudan SA, Switzerland)
20. Propylene glycol (Dow Chemical, USA)
21. Propyl paraben (Ueno, Japan)
22. Serum bovine albumin (Sigma Chemical Co., Ltd., USA)
23. Silcone oil 350 cps (Dow Corning Co., USA)
24. Sodium Chloride (Farmitalia Carlo Erba, Italy)
25. Sodium hydroxide (E. Merck, Germany)
26. Sodium lauryl sulfate (Reidel - Dettaen, Germany)
27. Sorbitan Sesquioleate (ICI, USA)
28. Stearic acid (Henkel, Germany)
29. Stearyl alcohol (Henkel, Germany)
30. Sulfamerazine (Sigma Chemical Co., Ltd., USA)
31. Titanium dioxide (Micronized) (Sun Smart, USA)
32. Toluene (J.T. Baker, USA)
33. Triethanolamine (Farmitalia Carlo Erba, Italy)
34. Transpore tape (3M Company, USA)
35. White petrolatum (Witco, USA)

2. Instruments

1. Centrifugation (Sigma-302k, Germany)
2. High Performance Liquid Chromatography, Model LC-10A (Shimadzu, Japan)
3. Modified franz diffusion cell (Crown Glass, USA)
4. Multiport solar UV simulator, Model 601 (Solar Light Co., USA)
5. pH meter, SA 520 pH meter (Orion, USA)
6. SPF 290s analyzer (Optomrics Ltd., USA)
7. Spherisorb 5 ODS 2 (250x4.6 mm) (Waters Associates, USA)
8. UV meter (Solar Light Co., USA)
9. UV-visible spectrophotometer, Model 7800 (Jasco, Japan)
10. Vortex mixer (Scientific Industries, Thailand)

METHODS

Preparations of sunscreen formulations

1.1 Formulations of sunscreen preparations

Two groups of emulsion bases were formulated for this study ; oil in water emulsion base and water in oil emulsion base. The formula were shown in Table 9 and Table 10. Sunscreen emulsions were prepared using modified emulsion bases which consisting of sunscreen agents and water resistant ingredients. The sunscreen agents used were micronized titanium dioxide, octyl dimethyl PABA, octyl methoxycinnamate and oxybenzone. The water resistant was silicone oil viscosity 350 cps. The sunscreen emulsions were prepared by method of a two-phase heat system. The formula were shown in Table 11. Procedure of the preparation was done as following: the ingredients of part A and part B were separately heated to 77 ^{0}C to 82 ^{0}C with constant stirring until the contents of each part were dissolved completely. Part B was slowly added to part A with continuous stirring until the emulsion was formed and allowed to cool down to 50 ^{0}C. Sufficient purfied water was added to the emulsion to obtained 100g of the formulation. If sunscreen agent was octyl dimethyl PABA, octyl methoxycinnamate or oxybenzone were added in Part A, micronized titanium dioxide (in aqueous) was added in Part B.

1.2 Preparation of the standard US-FDA sunscreen formulation

The SPF of a sunscreen product is to be determined in reference to a standard sunscreen preparation, which is an 8% homosalate preparation in an oil in water emulsion having an SPF value of 4.1 ± 0.8 (11). Procedure of the preparation was done as following: the ingredients of part A and part B were

separately heated to 77 ^{0}C to 82 ^{0}C with constant stirring until the contents of each part were dissolved completely. Part B was slowly added to part A with continuous stirring until the emulsion was formed and allowed to cool down to 50 ^{0}C. Sufficient purified water was added to the emulsion to obtained 100g of the standard US-FDA formulation.

Table 9 Compositions of oil in water emulsion base.

Ingredients	Percent by weight
Part A	
Glyceryl stearate	2.00
Cetearyl Gluscoside	4.00
Cetearyl Octanoate	10.00
Octyl dimethyl PABA/octyl - methoxycinnamate	qs
Oxybenzone	qs
Part B	
Carbomer (Carbopol 934)	0.50
Triethanolamine	0.55
Disodium EDTA	0.02
Imidazolidinyl urea	0.30
Propyl paraben	0.015
Methyl paraben	0.025
Propylene glycol	1.00
Micronized titanium dioxide	qs
Deionized water	qs to 100.00

Table 10 Compositions of water in oil emulsion base.

Ingredients	Percent by weight
Part A	
Mineral oil	25.00
Isopropyl palmitate	20.00
Beeswax	8.00
Octyl dimethyl PABA/octyl - methoxycinnamate	qs
Oxybenzone	qs
Part B	
Sorbitan Sesquioleate	2.00
Borax	0.40
Propylene glycol	5.00
Propyl paraben	0.015
Methyl paraben	0.025
Micronized titanium dioxide	qs
Deionized water	qs to 100.00

Table 11 Compositions of sunscreen emulsions.

Formula	Compositions	Percent by weight
1.	Micronized Titanium dioxide	5.0
	Oil in water emulsion base	95.0
2.	Octyl dimethyl PABA	7.0
	Oil in water emulsion base	93.0
3.	Octyl methoxycinnamate	8.0
	Oil in water emulsion base	92.0
4.	Oxybenzone	3.0
	Oil in water emulsion base	97.0
5.	Micronized Titanium dioxide	5.0
	Octyl dimethyl PABA	7.0
	Oil in water emulsion base	88.0
6.	Micronized Titanium dioxide	5.0
	Octyl methoxycinnamate	8.0
	Oil in water emulsion base	87.0
7.	Micronized Titanium dioxide	5.0
	Oxybenzone	3.0
	Oil in water emulsion base	92.0
8.	Micronized Titanium dioxide	5.0
	Octyl dimethyl PABA	7.0
	Oxybenzone	3.0
	Oil in water emulsion base	85.0
9.	Micronized Titanium dioxide	5.0
	Octyl methoxycinnamate	8.0
	Oxybenzone	3.0
	Oil in water emulsion base	84.0
10.	Micronized Titanium dioxide	5.0
	Water in oil emulsion base	95.0
11.	Octyl dimethyl PABA	7.0
	Water in oil emulsion base	93.0
12.	Octyl methoxycinnamate	8.0
	Water in oil emulsion base	92.0
13.	Oxybenzone	3.0
	Water in oil emulsion base	97.0

Table 11 Compositions of sunscreen emulsions (continued).

Formula	Compositions	Percent by weight
14.	Micronized Titanium dioxide	5.0
	Octyl dimethyl PABA	7.0
	Water in oil emulsion base	88.0
15.	Micronized Titanium dioxide	5.0
	Octyl methoxycinnamate	8.0
	Water in oil emulsion base	87.0
16.	Micronized Titanium dioxide	5.0
	Oxybenzone	3.0
	Water in oil emulsion base	92.0
17.	Micronized Titanium dioxide	5.0
	Octyl dimethyl PABA	7.0
	Oxybenzone	3.0
	Water in oil emulsion base	85.0
18.	Micronized Titanium dioxede	5.0
	Octyl methoxycinnamate	8.0
	Oxybenzone	3.0
	Water in oil emulsion base	84.0
19.	Micronized Titanium dioxide	5.0
	Silicone 350 cps	3.0
	Oil in water emulsion base	92.0
20.	Octyl dimethyl PABA	7.0
	Silicone 350 cps	3.0
	Oil in water emulsion base	90.0
21.	Octyl methoxycinnamate	8.0
	Silicone 350 cps	3.0
	Oil in water emulsion base	89.0
22.	Oxybenzone	3.0
	Silicone 350 cps	3.0
	Oil in water emulsion base	94.0
23.	Micronized Titanium dioxide	5.0
	Octyl dimethyl PABA	7.0
	Silicone 350 cps	3.0
	Oil in water emulsion base	85.0

Table 11 Compositions of sunscreen emulsions (continued).

Formula	Compositions	Percent by weight
24.	Micronized Titanium dioxide	5.0
	Octyl methoxycinnamate	8.0
	Silcone 350 cps	3.0
	Oil in water emulsion base	84.0
25.	Micronized Titanium dioxide	5.0
	Oxybenzone	3.0
	Silicone 350 cps	3.0
	Oil in water emulsion base	89.0
26.	Micronized Titanium dioxide	5.0
	Octyl dimethyl PABA	7.0
	Oxybenzone	3.0
	Silicone 350 cps	3.0
	Oil in water emulsion base	82.0
27.	Micronized Titanium dioxide	5.0
	Octyl methoxycinnamate	8.0
	Oxybenzone	3.0
	Silicone 350 cps	3.0
	Oil in water emulsion base	81.0
28.	Micronized Titanium dioxide	5.0
	Silicone 350 cps	3.0
	Water in oil emulsion base	92.0
29.	Octyl dimethyl PABA	7.0
	Silicone 350 cps	3.0
	Water in oil emulsion base	90.0
30.	Octyl methoxycinnamate	8.0
	Silicone 350 cps	3.0
	Water in oil emulsion base	89.0
31.	Oxybenzone	3.0
	Silicone 350 cps	3.0
	Water in oil emulsion base	94.0

Table 11 Compositions of sunscreen emulsions (continued).

Formula	Compositions	Percent by weight
32.	Micronized Titanium dioxide	5.0
	Octyl dimethyl PABA	7.0
	Silicone 350 cps	3.0
	Water in oil emulsion base	85.0
33.	Micronized Titanium dioxide	5.0
	Octyl methoxycinnamate	8.0
	Silicone 350 cps	3.0
	Water in oil emulsion base	84.0
34.	Micronized Titanium dioxide	5.0
	Oxybenzone	3.0
	Silicone 350 cps	3.0
	Water in oil emulsion base	89.0
35.	Micronized Titamium dioxide	5.0
	Octyl dimethyl PABA	7.0
	Oxybenzone	3.0
	Silicone 350 cps	3.0
	Water in oil emulsion base	82.0
36.	Micronized Titanium diowide	5.0
	Octyl methoxycinnamate	8.0
	Oxybenzone	3.0
	Silicone 350 cps	3.0
	Water in oil emulsion base	81.0

Table 12 Compositions of the standard US-FDA sunscreen formulation.

Ingredients	Percent by weight
Part A	
Homosalate	8.00
White petrolatum	2.00
Stearic acid	3.00
Stearyl alcohol	2.00
Part B	
Propyl paraben	0.015
Methyl paraben	0.025
Disodium EDTA	0.05
Sodium lauryl sulfate	0.50
Propylene glycol	12.00
Deionized water	qs to 100.00

2. Evaluation of the sunscreen formulations

2.1 Determination of physical properties of the sunscreen formulations

The physical properties of all of the prepared sunscreen emulsions were determined as the following :

2.1.1 Physical appearance

The physical appearance of the prepared sunscreen emulsions such as color, phase seperation recorded at room temperature and stored in the hot air oven at 40 ^{0}C, 50 ^{0}C and 60 ^{0}C for 3 months.

Freeze-thaw cycle : prepared sunscreen emulsions were stored in the hot air oven at 40 ^{0}C for 48 hours and in the refrigerator at - 4 ^{0}C for 48 hours; it was one cycle, six cycles were repeat.

2.1.2 pH

The pH of the prepared sunscreen emulsions was measured by using the digital pH-meter with accurate flow combination pH electrode.

2.2 Analysis of sunscreen agents

2.2.1 Analysis of homosalate in the standard US-FDA sunscreen formulation

The standard US-FDA sunscreen emulsion was determined by the following method to ensure that it had proper concentration of homosalate.

2.2.1.1 Calibration curve of homosalate in 1% glacial acetic acid in 95% ethanol

The calibration curve of homosalate in 1% glacial acetic acid in 95% ethanol was prepared by dissolving 100 mg of accurately weighed homosalate into a 100 ml volumetric flask and adjusted to volume with 1% glacial acetic acid in 95% ethanol. Appropriate dilutions were made with 1% glacial acetic acid in 95% ethanol to obtain standard solutions of known concentrations. The absorbances of these solutions were determined in a 1-cm cuvette cell at the maximum absorption

wavelength of 306 nm with spectrophotometer, using 1% glacial acetic acid in 95% ethanol as a blank in the reference cell. The obtained absorbances were plotted against known concentrations and linear regression analysis was used to obtain the best straight line.

2.2.1.2 Determination of homosalate content

The analytical procedure for homosalate content in the sunscreen emulsions was done as following : one gram of the standard homosalate sunscreen emulsions was accurately weighed in a 100-ml volumetric flask and mixed with 50 ml of 1% glacial acetic acid in 95% ethanol. The mixture was heated on a steam bath and mixed throughly. The solution was cooled to room temperature (25 ^{0}C to 32 ^{0}C) and then diluted with 1% glacial acetic acid in 95% ethanol to make 1% solution. The 1% solution was filtered through number 1 filter paper. The filtrate was collected and then 1 ml of this filtrate was pipeted into a 50-ml volumetric flask and adjusted to volume with 1% glacial acetic acid in 95% ethanol. This was the test solution for measuring the absorbance at 306 nm. The percentage of homosalate in the standard sunscreen was calculated by means of a calibration curve.

2.2.2 Analysis of sunscreen agent in sunscreen emulsions

2.2.2.1 Preparation of internal standard

Thirty milligrams of sulfamerazine was dissolved in 50 ml methanol and diluted with methanol to give final concentration of 6 mcg/ml. The internal standard solution was freshly prepared on each day of analysis.

2.2.2.2 Preparation of standard solution

Stock standard solution : 10, 20, 30, 40, 50 and 60 milligrams of each sunscreen agent were dissolved in 50 ml methanol and diluted with methanol to give final concentration of 2, 4, 6, 8, 10 and 12 mcg/ml. The internal standard solution (sulfamerazine) 1 ml was added into each standard solution.

2.2.2.3 Assay procedure

Twenty microliters of solution was injected onto the column under the following chromatographic conditions.

Column	:	Spherisorb ODS2, 5 micron, 4.6x250 mm.
mobile phase	:	100% Methanol
flow rate	:	1.0 ml/min
attenuation	:	32
AUFS	:	0.01
detector	:	UV detector, 254 nm
inject volume	:	20 µl

Quantitative analysis was achieved by calculating peak area ratio of each sunscreen agent and sulfamerazine from calibration curve using least-square linear regression method.

2.2.2.4 Calibration curve

The standard solutions 2, 4, 6, 8, 10 and 12 mcg/ml were assayed using the same procedure as in 2.2.2.2 and 2.2.2.3. The calibration curve was obtained by plotting the peak area ratio of each sunscreen agent to sulfamerazine versus the concentration of each sunscreen agent using linear regression. Standard curve was obtained from the average of the three determinations.

2.2.2.5 Content analysis of the prepared sunscreen emulsions

Eight mg of sunscreens was accurately weighed and mixed with 10 ml of methanol in a test tube and centrifuge at 3,000 rpm for 60 min. The solution was filtered with cellulose acetate membrane with pore size of 0.45 micron. The internal standard solution (sulfamerazine) 1 ml was added into each sunscreen preparation and adjust to final volume 50 ml with methanol into a volumetric flask.

2.3 Determination of SPF of sunscreen products by SPF-290 analyzer

The SPF of prepared sunscreen emulsions and standard sunscreen preparations were evaluated by SPF-290 analyzer as shown in Figure 16. The procedures were as follows. A reference was made by measuring the transmission of UVB and UVA wavelengths (290-400 nm) through the transpore tape which was placed in the incident beam. The reference preparation data will be use to compensate for wavelength-dependent variables in the source, substrate, monochromator, and detector. The sunscreen to be tested was applied using a 1-cc syringe onto the transpore tape in rows of small "dabs" or "spots" and rubbed gently. An area approximately 2.75 x 2.75 inch (7.0x7.0 cm) should be covered with the tested sample. This technique will distribute a layer of sample about 2 $\mu l/cm^2$ over the specific area, therefore, a sample thickness will be equivalent to that used in standard *in vivo* SPF tests. The measurement of transmission of UVB and UVA wavelengths was similar to a reference run. After all runs were completed, the mean (average) MPF and their standard deviation were calculated for each wavelength. The SPF was calculated from the MPF as described by Diffey and Robson (9).

Figure 16 The SPF - 290 analyzer used to appraised SPF of tested products in the *in vitro* method.

2.4 Determination of *in vitro* skin penetration.

The highest SPF Sunscreen emulsions were selected from for each cream base of Table 11 to determine the *in vitro* skin penetration as follows :

2.4.1 Diffusion studies

A modified franz diffusion cell (Figure 17) were used for the *in vitro* diffusion studies. The diffusion cell was made up from glass. It consists of two compartments with the donor compartment in the upper and the receptor compartment in the lower which has double jacket around it. A small magnetic bar (d = 1.0 cm) was inserted inside and filled with about 10 - 11 ml of physiological saline with albumin (1.5% w/v). The test sample was applied over the skin of diffusion area of 1.76 cm^2 which placed between the donor and receptor compartment. The temperature was controlled by passing water through double jacketed from a water bath controlled at 37 $\pm$ 1^{0}C. The content of the receptor cell was stirred continuously during the experiment. The entire receiving sample was taken periodically and replaced with the equivalent volume of fresh receptor fluid.

2.4.2 Skin samples

Human skin was obtained from the abdominal region after surgery. Samples were stored at freezer. The excess fatty tissue was removed from the flaps of the skin. Skin was cut into suitable small pieces and then set in diffusion cells.

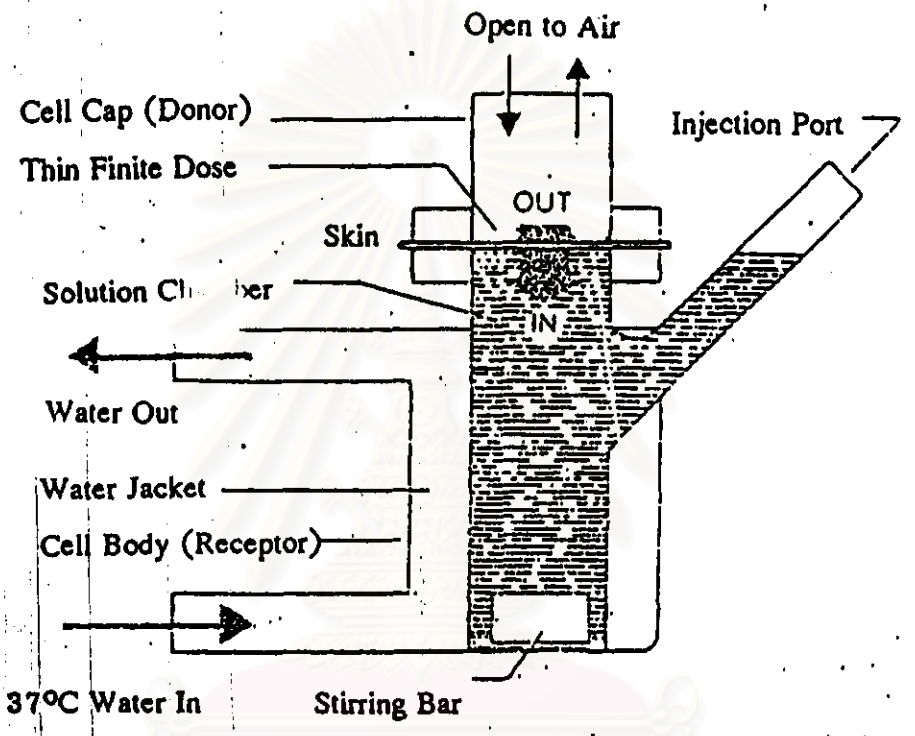

Figure 17 A modified franz diffusion cell (34).

2.4.3 Skin penetration

Skin penetration was measured using modified franz diffusion cell with a 1.76 cm^2 surface area of exposed skin (4). The preparations were applied at a dose of 5 mg/cm^2. The receptor fluid was physiological saline with albumin (1.5% w/v) maintained at 37 ± 1^0C . Six appropriate time interval were investigated at 2 min, 0.5, 2, 4, 6 and 8 hours. All drug concentration in the receiving solution and in the donor solution after 8 hours were assayed by HPLC.

Three experiments (n=3) were conducted for each sunscreen emulsions and two control cells were studied (without product application on the skin surface). After completion of the preset time (T=8 hours), skin samples were taken out of the diffusion cells.

The surface horny layers of stratum corneum were removed using an adhesive tape, and stripping were introduced in vials. Vials were filled with 5 ml of toluene, then shaking. Strong shaking was necessary to loosen the adhesive layer in toluene, 3 ml of methanol was added, and thus enable determinations by HPLC.

Epidermis and dermis were separated on a hot plate at 60^0C for 2 hours. Epidermis was treated by 3 ml of methanol and placed in an ultrasound bath for 20 minutes. After filtration with filtered membrane (pore size 0.45 micron), solutions were assayed by HPLC.

Dermis was treated by 1 ml of 1 N NaOH and kept in a steamer at $37\ ^0C$ for 48 hours. Samples were then neutralized with 1 N hydrochloric acid prior to the analysis by HPLC.

2.5 Evaluation of SPF of sunscreen products by *in vivo* method

The SPF of prepared sunscreen emulsions and standard sunscreen preparations were evaluated by US-FDA procedures under indoor condition. In this study a 150-watt xenon arc solar simulator with six light guides was used as the light source as shown in Figure 18.

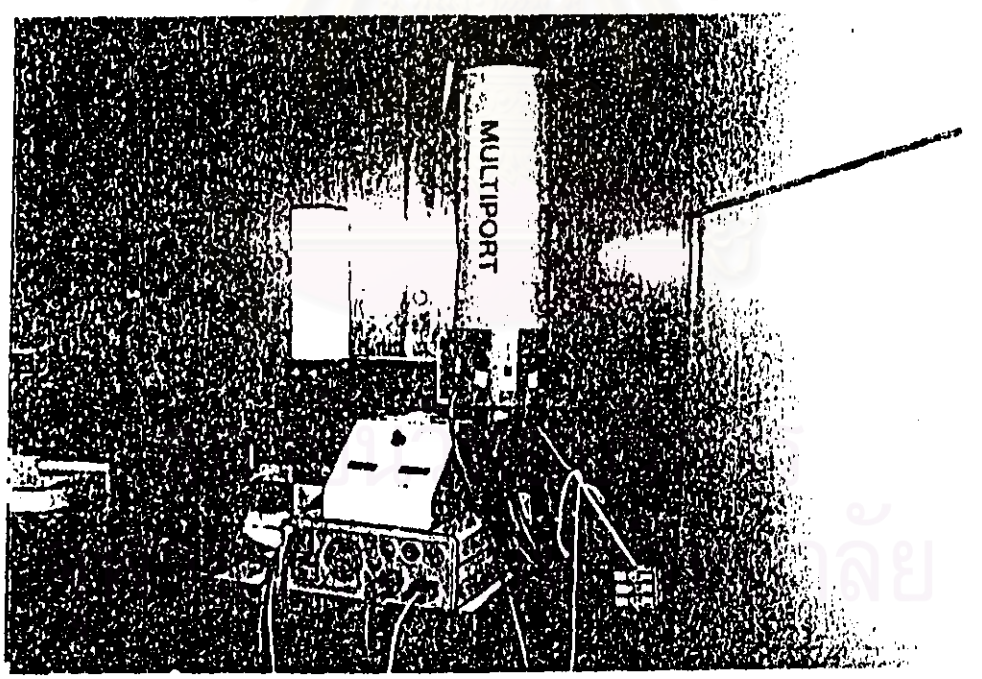

Figure 18 A 150-watt xenon arc solar simulator.

This light source has a 1 mm thick WG 320 filter which produces UV spectrum wavelength in the 290-400 nm range and a UG5 filter which reduces visible and infrared wavelengths. The irradiated energy of light source was measured in MED/minute unit by erythema ultraviolet intensity meter in order to estimate UV doses for the test. The volunteers were healthy male and were between 18 and 40 years of age with the following criteria :

1) not pregnant and breast-feeding,
2) no history of skin diseases such as eczema or psoriasis,
3) no any abnormal responses to sunlight (i.e., phototoxic or photoallergic rections),
4) no previous use of either topical or systemic medicines known to produce photosensitivity,
5) each volunteer should receive similar tests not more than 5 times per year,
6) each test must be done at least on month apart in the same volunteer,
7) only volunteers with skin types III and IV were allowed to enroll in this study.

Evaluation of each volunteer's skin types was performed by experts in this field. The brief history of sunburning and tanning questionaires that agree with the Fitzpatrick phototype definitions was also used to evaluate the skin types. The written consent provided for all of the volunteers before they encounter the test. After they were qualified for the study, each of them was irradiated to determine his/her minimal erythema dose (MED). The back position of volunteer during the test is shown in Figure 19. The MED of each volunteer was determined by administering a series of six doses of ultraviolet exposures to the unprotected skin of the back. The dose intervals form a arithmetic series of 25% increments and was based on the volunteer's

skin type. Approximately 24 hours after exposing, the site showing minimal perceptible erythema was selected as the MED. The sunscreen formulation to be tested was applied onto 50 cm^2 test site area at the concentration of 2 $\mu l/cm^2$. The test site was allowed to air dry for at least 15 minutes before it was exposed to the series of six doses of ultraviolet radiation. The series of ultraviolet doses at the test site was based on the volunteer's inherent MED and the estimated or claimed SPF of the tested sunscreen formulation. The MED for protected skin was evaluated after about 16 to 24 hours. A minimal erythema of skin protected with a tested product is shown in Figure 20. An individual SPF of each volunteer was calculated using the following equation :

SPF = MED of protected skin/MED of unprotected skin

However, the SPF of each product is determined by calculating the arithmetic mean of SPF values obtained from the panel of volunteers tested (at least twenty volunteers for US-FDA procedure). All of the volunteers encountered a testing of the US-FDA procedure. The standard error should not exceed $\pm$ 5% of the mean for US-FDA procedure.

In order to compare the results from different methods for determining the SPF of sunscreen products, ranging from SPF-290 analyzer and US-FDA procedures. The t test and Pearson 's test statistic were used for this purpose. The results of this analysis were used to justify the differences from each methods.

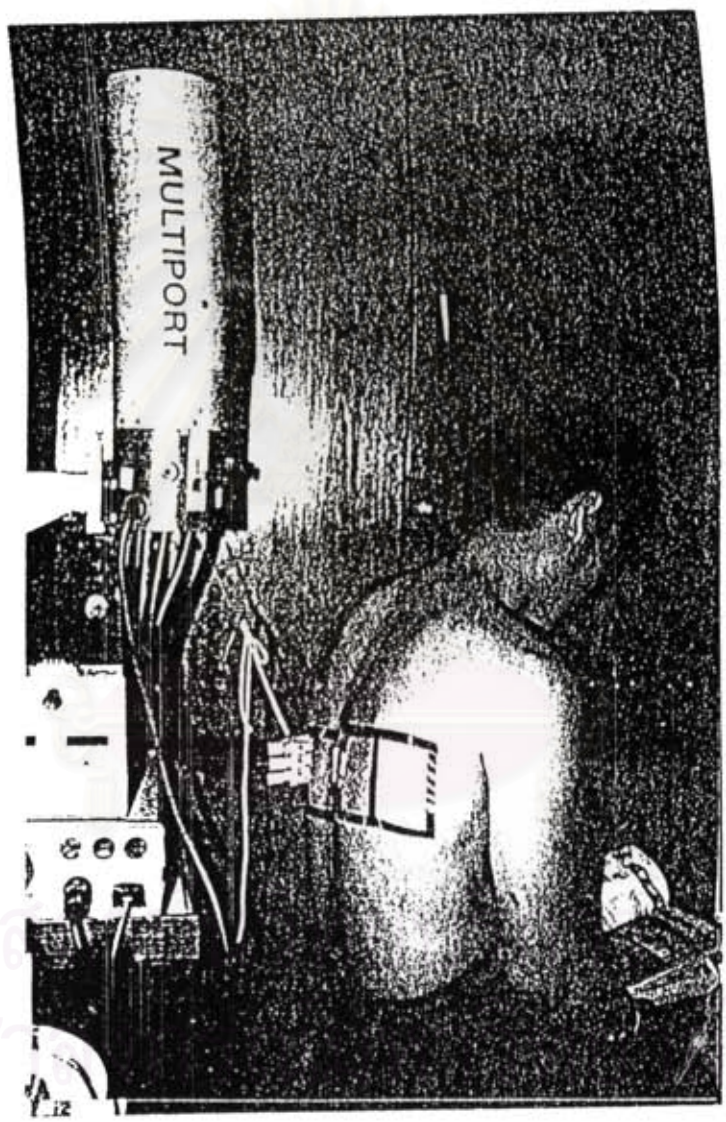

Figure 19 Volunteer's position during the test.

Figure 20 A minimal erythema of skin protected with a tested sunscreen product.