CHAPTER II

MATERIALS AND METHODS

Materials

1. Chemicals

Acetonitrile was HPLC grade obtained from Mallinkrodt., USA.

Polyethylene glycol 6000 (PEG 6000) was analytical reagent grade obtained from Fluka., Switzerland.

Hydroxypropyl methylcellulose 15 cps. (Methocel®E15). Distributed by Rama Production Co., Ltd., Bangkok, Thailand.

Sorbitol. Distributed by Namsiang Company Limited, Thailand.

Triamcinolone acetonide. Distributed by Siam Chemi-Pharm L.P., Thailand.

Miconazole nitrate. Distributed by S. Tong Chemicals Co.,Ltd., Thailand.

Ethyl alcohol absolute, Hydrochloric Acid were analytical grade obtained from E. Merck, Germany.

Sodiumhydrogen carbonate, Sodium chloride, Potassium chloride, Potassium dihydrogen phosphate, disodium hydrogen phosphate, were analytical grade obtained from E. Merck, Germany.

polyoxyethylene 20 cetyl ether, polyethylene glycol 400 (PEG 400) obtained from sigma chemical, Germany.

2. Equipments

- Analytical balance (Sartorious Model A200S, Germany)
- Micrometer (Thickness Gauge 0-10 mm., Code No. 17389 Inspector 3)
- pH meter (MP 230, Mettler Toledo, LE413, ME 51340 251, Switzerland)
- Viscometer (Brookfield, Model LVDV-I+, Brookfield Engineering Laboratories INC., USA)
- Magnetic stirrer (Model SP 46920-26, Barnstead/Hermodyne, USA)
- Tensile tester (Instron 5565, Instron Corp., England)
- Oven (Mammert, Becthai Co.,Ltd., Thailand)
- Diffusion cells (Modified from Franz's diffusion cell)
- High-performance liquid chromatography (HPLC) instrument equipped with the following
    - Liquid chromatograph pump : LC-10AD, Shimadzu, Japan.
    - UV-VIS detector : SPD-10A, Shimadzu, Japan.
    - Recorder : C-R6A chromatopac, Shimadzu, Japan.
    - Microsyringe 100 μl : SGE, Australia.
    - C-18 column, 150 x 3.9 mm, 5 μm : Water, Ireland.
- UV-VIS spectrometer (Spectronic® GENESYS 5, Becthai Co.,Ltd., Thailand)
- IR spectrometer (Perkin Elmer, FTIR spectrometer spectrum 2000)
- Suction apparatus (Buchner Funnel, Aspirator, SIBATA circulating aspirator WJ-20, Japan)
- Rotary evaporator (Buchi R-200, Switzerland)
- Water bath (Julabo D-7633, Germany)
- Stirrer (KMO2, Janke & Kunkel GMBA & Co. KG)

Methods

1. Preparation of Polysaccharide Gel From Fruit-Hulls of Durian

Fresh durian fruit-hulls were collected, cleaned, ground and kept at -20 °C. One kilogram of ground fresh durian fruit-hull was dried by hot air oven at 70 °C for 48 hrs, about 200 grams of dried durian fruit-hulls were obtained. Dried fruit-hulls of durian were kept in cold room (4 °C) until use. Hot acidic water extraction and acid alcohol precipitation of polysaccharide gel from fruit-hulls was performed by the procedure modified by Pongsamart and Panmaung (1998).

2. Physical Properties of PG Films

2.1 pH

Acidic PG solutions (2% w/w) were adjusted to the different pH ranges 2-7 with 0.1M $NaHCO_3$. The pH and viscosity were individually measured by using pH meter and viscometer, respectively.

2.2 The Viscosity

PG solutions were prepared at 2% concentration, the pH of different values range 2-7 were adjusted with 0.1M $NaHCO_3$, the viscosity was determined using Brookfield Viscometer (model LVDV-I+) at each pH of PG solution. Viscosity of PG and pH were then plotted against $NaHCO_3$ concentration to determine the pH-viscosity relationship.

3. Preparation of PG Films

PG film (without plasticizer) and PG film base (with plasticizer) were prepared by a casting/solvent evaporation method (Remunanlopez and Bodmeier, 1996). The concentration of 2% PG (w/v) was used as a casting solution of PG film (without plasticizer). The composition of casting solution with plasticizer composed of 2 % PG solution and plasticizers/co-film forming agent at concentrations based on PG weight

using 10-50 % sorbitol, 30 % PEG 400, 1-2 % PEG 6000, 1-20 % HPMC E15, respectively. Casting solution of PG film without plasticizer or with plasticizer/co-film forming agent (PG film base) were prepared by dissolving PG in deionized water and stirred at room temperature, PG film base was prepared by adding each plasticizer to PG solution, respectively. The casting solutions were adjusted to pH 3.7 with 0.1M $NaHCO_3$ and poured on the glass plate (8.8 cm in diameter) and left to stand until the air bubbles were removed. The films were dried for 6-8 hours or until constant weight in a hot air oven at 50 °C. The dried films were stored in desiccator until use.

The 3 layers of PG film base was prepared by the same method of PG film on the ground glass (area of casting = 9.7 x 10.5 cm). The bottom layer was 1% (w/v) HPMC, the middle layer was 1% (w/v) PG and the top layer was 2% (w/v) PG with 30% (w/w) sorbitol the pH was adjusted to 3.7. The 3 layers PG films with drugs were prepared by adding 0.1% (w/w) of triamcinolone acetonide or 2% (w/w) of miconazole nitrate based on PG into the casting solution of top layer (2% w/v of PG with 30% w/w sorbitol), and adjusted pH to 3.7.

3.1 The film preparations of PG were prepared using the following formulas :

3.1.1) PG film without plasticizer (PG film), the formula composed of 2% (w/v) of PG in water.

3.1.2) PG film base, the formulations of PG film with different types of plasticizer were indicated in the following formula :

3.1.2.1) The formulations of PG film base using sorbitol (S) as a plasticizer

Formula	Ingredients in % based on PG (g/100 ml of casting solution)	
	Polysaccharide gel (PG)	Sorbitol (S)
S10PG	100 (2)	10 (0.2)
S20PG	100 (2)	20 (0.4)
S30PG	100 (2)	30 (0.6)
S40PG	100 (2)	40 (0.8)
S50PG	100 (2)	50 (1.0)

3.1.2.2) The formulations of PG film base using PEG 400 and PEG 6000 as plasticizer

	Ingredients in % based on PG (g/100 ml of casting solution)		
Formula	Polysaccharide gel (PG)	PEG 400 (P4)	PEG 6000 (P6)
P4/30PG	100 (2)	30 (0.6)	-
P6/1PG	100 (2)		1 (0.02)
P6/1.5PG	100 (2)		1.5 (0.03)
P6/2PG	100 (2)		2 (0.04)

3.1.2.3) The formulations of PG film base using PG with HPMC E15

	Ingredients in % based on PG (g/100 ml of casting solution)	
Formula	Polysaccharide gel (PG)	HPMC E15 (H)
H1PG	100 (2)	1 (0.02)
H3PG	100 (2)	3 (0.06)
H5PG	100 (2)	5 (0.1)
H10PG	100 (2)	10 (0.2)
H20PG	100 (2)	20 (0.4)

3.2 Preparation of Triamcinolone Acetonide Mucoadhesive Films (TPG)

Triamcinolone-PG films were prepared by following the method. The casting solution composed of 2% w/v PG. PG powder was dissolved in deionized water and stirred at room temperature overnight in order to completely hydrate and swell the polymer. Sorbitol (30% w/w of PG) was added as a plasticizer and a previously dissolved triamcinolone acetonide (0.1% w/w of PG) in 95% ethanol solution was added into the casting solutions. Adjust pH to 3.7 with 0.1M $NaHCO_3$ and adjusted to 100 g weight with distilled water and poured on glass plate (8.8 cm in diameter) and left to stand until the trapped bubbles were removed. The films were dried for 6-8 hours or until constant weight in a hot air oven at 50 °C. The dried films were stored in a desiccator.

3.3 Preparation of Miconazole Nitrate Mucoadhesive Films (MPG)

Miconazole-PG films were prepared by the same method as triamcinolone-PG films except that miconazole nitrate was dissolved in melted PEG 6000 (1:1) and frozen immediately. The solid mixture was pulverized in a mortar and passed through 80-mesh sieve. The powder mixture of miconazole nitrate (2% w/w of PG and PEG 6000 2% w/w based on weight of PG) was added into the casting solutions of 2% w/v PG with sorbitol (30% w/w based on PG) as a plasticizer then adjust pH to 3.7 with 0.1M $NaHCO_3$. The casting solutions were poured on glass plate following the method as described in the preparation of triamcinolone acetonide mucoadhesive films.

The formulations of PG film with triamcinolone acetonide and miconazole nitrate are indicated in the following formula :

3.3.1) The formulations of triamcinolone and miconazole PG film

Formula	Ingredients in % based on PG (g/100 ml of casting solution)				
	PG	Sorbitol	PEG 6000	Triamcinolone acetonide	Miconazole nitrate
1 layer film					
Triamcinolone acetonide film (1TPG)	100 (2)	30 (0.6)		0.1 (0.002)	
Miconazole nitrate film (1MPG)	100 (2)	30 (0.6)	2 (0.04)		2 (0.04)
Top layer of the 3 layer film					
PG film base (3S30PG)	100 (2)	30 (0.6)			
Triamcinolone acetonide film (TPG)	100 (2)	30 (0.6)		0.1 (0.002)	
Miconazole nitrate film (MPG)	100 (2)	30 (0.6)	2 (0.04)		2 (0.04)

4. In vitro Evaluation of PG Films

4.1 Physical Characteristics of PG Films

An appearances of PG film preparations were visually observed including color, transparency, flexibility and ease of detachment from glass plates.

4.2 Film Thickness Measurement

The thickness of PG films were measured by micrometer. Each sample was measured at five locations. The mean values of thickness were calculated.

4.3 Mechanical Properties Analysis of PG Films

The mechanical properties of PG films were analyzed by an universal tensile testing machine (Instron 5565, Instron Crop.) equipped with 10N tensile load cell. The relative humidity of the laboratory for testing was about 55% and temperature was 25±2 °C, the data of tensile properties obtained by the following procedure.

The film samples were cut into small strips (2x20 mm). The films were left to expose to room humidity for 1 hour before tested. The film strip was carefully clamped by an upper and lower pneumatic flat-faced grips and extended by the test machine at speed of 1.5 mm/min until it was ruptured. The breaking force displayed in digital was recorded, the values of Young's modulus, stress at break, %strain at break and toughness were evaluated. The mean values and standard deviation of five measurements in each group were compared.

4.4 Infrared Spectra Patterns

Fourier transform infrared spectrophotometry (FTIR) was used to study the change in the functional groups of the PG, drug and the mucoadhesive films. The PG films were examined by using KBr disc. Approximately 1 part of PG was triturated with 100 parts of dried, finely powdered KBr. The mixture was thoroughly ground with an agate mortar and pestle to obtain a uniform mixture, spread in die of 7 mm diameter

and compressed using the Qwik Handi-Press. The scanning range used was 400-4000 cm^{-1}. These spectra were compared to the spectra of drug and mucoadhesive films using KBr disc.

4.5 Moisture Sorption

The determination of the moisture sorption of the film strips was operated by following procedure. Films of 2% PG with 30% sorbitol (S30PG), 2% HPMC and 3 layer film (3S30PG) composed of bottom layer of 1% HPMC, middle layer of 1% PG and top layer of 2% PG with 30% sorbitol were observed. The films were cut into size 5x5 cm. The films were determined the initial dry weight (W_o) at room temperature (about 25 °C) by keeping them in the desiccator filled with silica gel for 24 hours or until constant weight. Then they were placed inside a desiccator containing saturated sodium chloride solution (75% RH) and stored at 25 °C. At appropriate time intervals, the films were taken out and weigh immediately (W_t). The percentages of moisture sorption of the films were calculated by the following equation.

$$\% \text{ moisture sorption} = \frac{W_t - W_o}{W_o} \times 100$$

Where W_o is the initial weight of the strip, W_t is the weight of the strip at time t. The measurement was made in triplicate.

4.6 In Vitro Drug Release Profile of PG Films

The release and penetration of drug from PG films were determined using modified Franz diffusion cells (Figure 9). The internal diameter of each cell was 1.7 cm. The receptor compartment contained 13.4 ml of 8 mM phosphate buffer pH 7.4 as receiving solution.

PG film was cut into size 1x1 cm and clamped between the donor and the receptor compartments on cellulose acetate membrane for penetration study. The cellulose acetate membranes were previously soaked in phosphate buffer pH 7.4 at least 30 min before used. A small magnetic bar (4x7 mm) was placed in the receptor

compartment and rotated at 750 rpm. The diffusion cell was filled with phosphate buffer solution pH 7.4 until it reached to the top level of the receiver chamber on which the membrane or film was placed, leaving no air bubbles in the chamber. The temperature of the assembled diffusion cells were maintained at 37±1 °C by the means of a circulating water jacket connected to a constant temperature water bath. A portion of the receiver medium (10 ml each) was withdrawn at predetermined time interval. The same volume of the solution withdrawn was returned to the chamber at each withdrawal. The triplicate determinations of each of sample were evaluated.

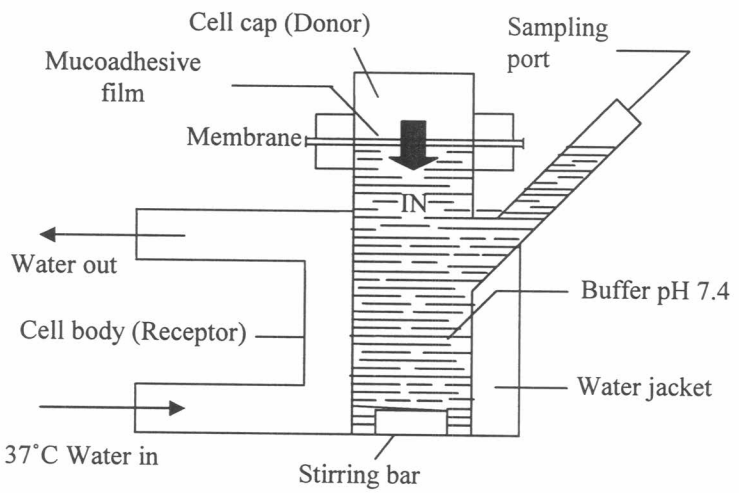

Figure 9 Schematic diagram of the diffusion cell apparatus for the in vitro release and penetration studies

4.6.1 Analysis of Triamcinolone Acetonide

The amount of drug release was assayed by HPLC method (Sveinsson et al., 1993). Its condition was operated as follows :

Column	:	Symmetry® C18 column (150x3.9 mm), 5 μm (Ireland)
Detector	:	UV detector was set at 254 nm
Flow rate	:	1 ml/min
Attenuation	:	3
Chart speed	:	1 cm/min
Injection volume	:	20 μl
Mobile phase	:	420 ml of acetonitrile was mixed with 580 ml of water. The mobile phase was prepared freshly and filtered through 0.45 μm membrane filter then degassed by sonication for 30 min prior to use

The 1 ml of sample solutions volume was to separate from the receiving medium (10 ml) and filtered through 0.45 μm membrane filter before injected into HPLC column. The standard solutions were freshly prepared and injected regularly during the analysis of each release experiment. The drug concentration was then determined using peak area and the standard curve.

4.6.2 Analysis of Miconazole Nitrate

The amount of drug release was assayed by Spectrophotometry method at 232 nm (Minghetti et al., 1999). About 1 ml of sample solutions from the receiving medium (10 ml) was used to measured an absorbance at 232 nm in quartz cuvette using spectrophotometer (Spectronic® GENESYS 5). The standard solutions were freshly prepared. The value of standard curve was used to determined the concentration of drug.

4.7 Sensory Analysis of PG Film Base

The PG film base formula 3S30PG was cut into pieces of 1x2 cm and used for sensory analysis using questionnaires to evaluate ; taste, ease of application, adhesiveness, non-irritation, no-residue, non-annoyance, product appearance satisfaction and product satisfaction after use. The opinions were analyzed by interpreting the percentage of frequency scores. The scores ranged from 1 = poor, 2 = fair, 3 = good, 4 = excellent. The film was prepared in 3 layers; the bottom layer (rough) was 1% (w/v) HPMC, the middle layer was 1% (w/v) PG and the top layer (smooth) was 2% (w/v) PG with 30% (w/w) sorbitol as a plasticizer. The thickness of films were 0.08 mm. The 32 healthy male and female volunteers participated in the study. They were 21 to 38 years old and they had no pathological or physiological disorder in oral cavity. Volunteers were applied PG film base using the top layer attach to the any area of the mucous membrane in oral cavity. No food or drink was allowed until the film dissolved completely (about 5 to 10 minutes).