CHAPTER III

MATERIALS AND METHODS

Materials

1. Calcium chloride dihydrate (Merck Co., Ltd., Germany), Lot No. 614 TA176581.
2. Calcium D-gluconate monohydrate (Fluka Chemi GmbH, Switzerland), Lot No. 381225/1.
3. Citric acid (Carlo Erba Co., Ltd., Italy), Lot No. 403727.
4. Disodium hydrogen orthophosphate anhydrous (Asia Pacific Specialty Chemicals Ltd., Australia), Lot No. F2F136.
5. Ethyl alcohol absolute anhydrous, AR grade (Mallinckrodt Co., Ltd., Mexico), Lot No. V569 Y50 D54.
6. Ethyl cellulose, 45 cps (Sigma Co., Ltd.), Lot No. 100K0137.
7. Eudragit® NE 30 D (Rohm GmbH & Co. KG, Germany) (donated by JJ-Degussa (T) Ltd., Thailand), Lot No. 1240312040.
8. Eudragit® RL 100 (Rohm GmbH & Co. KG, Germany) (donated by JJ-Degussa (T) Ltd., Thailand), Lot No. 8320106011.
9. Eudragit® RS 100 (Rohm GmbH & Co. KG, Germany) (donated by JJ-Degussa (T) Ltd., Thailand), Lot No. 8370408031.
10. Fresh porcine buccal mucosa (approximately 2 X 2 cm^2) (donated by Somchai Farm, Nakornpathom province, Thailand).
11. Glycerin USP (supplied by S. Tong Chemicals Co., Ltd., Thailand), Lot No. 12821203.
12. Kenalog® in orabase (Bristrol-Myers Squibb).
13. Kieselguhr® (Merck Co., Ltd., Germany), Lot No. 28458819.
14. Kollicoat® SR 30 D (BASF, Germany) (donated by BASF (Thai) Ltd.), Lot No. 92-1632.
15. Magnesium chloride (Ajax Finechem, Australia), Lot No. AF309081.

16. Methanol, HPLC grade (Lab Scan Co., Ltd., Thailand).
17. Mucin from porcine stomach, Type III (Sigma Co., Ltd., Singapore), Lot No. 014K7002.
18. Potassium chloride (Ajax Finechem, Australia), Lot No. AF307085.
19. Polyethyleneglycol 6000 (East Asiatic Public Company Ltd, Thailand).
20. Prednisolone base micronized BP (Wyeth Lederle Limited, India) (donated by V & S Chemi Group Co., Ltd.), Lot No. 223.
21. Sodium bicarbonate (Fisher Scientific UK Ltd.), Lot No. 0247414.
22. Sodium chloride (Merck Co., Ltd., Germany), Lot No. K28555404049.
23. Sodium hexametaphosphate (Carlo Erba Co., Ltd.), Lot No. 368357.
24. Sodium hydroxide (Mallinckrodt Co., Ltd., Sweden), Lot No. 7708.
25. Sodium sulphide X-hydrate QP (Panreac Quimica SA., E.U.), Lot No. 805867KP.
26. Sorbitol (70 % solution) (supplied by Srichan United Dispensary Co., Ltd., Thailand), Lot No. 180/9.
27. Tetra-Natriumdiphosphat-Decahydrate (Merck Co., Ltd.), Lot No. 636 A174391.
28. Triamcinolone acetonide micronized USP (Pharmacia & Upjohn Co., Ltd., USA) (donated by V & S Chemi group Co., Ltd.), Lot No. 01HKT.
29. Urea (supplied by S. Tong Chemicals Co., Ltd., Thailand).

Equipment

1. Analytical balance (Model AX105, Mettler toledo).
2. Analytical balance (Model PG 403-S, Mettler toledo).
3. Brookfield® Digital Viscometer (Model RVT DCP S/N A03969, Brookfield Engineering Laboratories, Inc., USA).
4. Disintegrator (Model ZT31, Erweka).
5. Hot air oven (Model B40, Memmert, Germany).
6. High performance liquid chromatography (HPLC) equipped with
    - System controller (SCL-10AVP) (Shimadzu Corporation, Japan).
    - Liquid chromatography (LC-10ADVP) (Shimadzu Corporation, Japan).
    - Degasser (DGU-12A) (Shimadzu Corporation, Japan).

- UV-visible detector (SPD-10AV) (Shimadzu Corporation, Japan).
- Auto injector (SIL-10ADVP) (Shimadzu Corporation, Japan).
- Column oven (CTO-10ASVP) (Shimadzu Corporation, Japan).
- C 18 column, Inertsil ODS 5 micron 4.6 X 150 mm (GL Science, Japan) with a guard column, Inertsil ODS 5 micron 4.6 X 10 mm (GL Science, Japan).

7. Modified Franz diffusion cell.
8. pH meter (Sartorius, Germany).
9. Rotavapor (Model R-220, Buchi, Switzerland).
10. Rotavapor (Model R-114, Buchi, Switzerland).
11. Stability chamber (Eurotherm Axyos).
12. Universal tensometer (Model H5KS 1509, Tinius olsen Ltd., United Kingdom).
13. UV-Visible spectrophotometer (Model UV-1601, Shimadzu, Japan).
14. Water bath (Gilson, England).

Methods

1. Extraction of PG from Durian-Fruit Hulls

Polysaccharide gel (PG) was extracted by the method modified by Pongsamart and Panmaung (1998), and Hokputsa et al. (2004). Fresh durian-fruit hulls were collected, cleaned and ground. One kg of the ground fresh durian-fruit hull was dried in a hot air oven at 60 °C for 48 hr, and approximately 200 g of dried durian-fruit hulls were obtained. The dried fruit-hulls of durian were kept in a refrigerator (4 °C) until use. PG in dried durian-fruit hulls was extracted with water at 100 °C, added hexametaphosphate and adjusted to pH 4 with citric acid. The water extract was concentrated and precipitated using acid ethanol (5% v/v HCl in 75% v/v ethanol). The precipitate of PG was dried at 50 °C for 6 hr, crude PG was obtained. The crude PG was further purified by redissolved in water and re-precipitation in 75% v/v ethanol dried the PG precipitate at 50 °C and ground. A creamy-white powder was obtained.

2. Determination of Physico-chemical Properties of PG

Physical properties of PG extract were determined as the following:

2.1 pH Measurement

The pH of PG in distilled water was measured using the Sartorius® pH meter. An electrode was immersed into the sample. The pH value was recorded. The pH measurements were performed in triplicate.

2.2 Viscosity Measurement

The viscosity of PG in distilled water was determined using the Brookfield® cone and plate viscometer (cone #41). The determination of the viscosity of samples was performed by applying about 2 ml of the samples in the lower plate of the viscometer.

The cone #41 was moved down to touch the sample and rotated at 2.5-50 rpm. The measurements of samples were performed in triplicate.

3. Selection of Types and Concentrations of Calcium Salts

Calcium salts, calcium chloride and calcium gluconate were used as suitable crosslinking agents in PG gel. A dispersion of 2.00 %w/w PG in distilled water was prepared. Each of calcium chloride and calcium gluconate solution which equivalent to calcium ion at 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 %w/w based on the PG weight was slowly added to the PG gel dispersion and stirred gently using a magnetic stirrer.

The viscosity of PG gel was determined using the Brookfield® cone and plate viscometer (cone #40). The determination of the viscosity was performed by applying about 0.5 ml of samples in the lower plate of the viscometer. The cone #40 was lowered to touch the sample and rotated at 5-50 rpm. The measurements of samples were performed in triplicate.

The calcium salt which provided the concentration with optimum viscosity of PG gel (~ 400-600 cps) was chosen for using in a casting mixture in preparation of buccal mucoadhesive films in **4**.

4. Preparation of Buccal Mucoadhesive Film Base

4.1 Preparation of Buccal Mucoadhesive Layers

4.1.1 Selection of Plasticizers

Effects of plasticizers was studied by using the following ingredients; glycerin (20 and 30 %w/w based on the PG weight), polyethylene glycol 6000 (PEG 6000) (1 and 2 %w/w based on the PG weight), and sorbitol (20 and 30 %w/w based on the PG weight). A full factorial design was performed (Table 3). The formulas are shown in Table 4. The procedures for preparing buccal mucoadhesive films are shown in Figure 14 and described as follows. The 4.00 %w/w PG was dispersed in distilled water and left at room temperature for 12 hr in order to remove entrapped air bubbles and allow the PG

gel to completely hydrate and swell. The calcium gluconate solution which contained calcium ion at 1.0 %w/w based on the PG weight (selected from **3.**) was added gently into the PG gel dispersion to avoid air entrapment stirred gently until a homogeneous viscous gel was obtained. The mixture pH was adjusted with 0.5 N NaOH to 5.5. The plasticizers were added to the mixture. Then, the mixture was stirred gently for 2 hr and left until air bubbles were removed. A film was cast by pouring the mixture into a hollow space of a stainless steel box placed on top of a silicone paper strip. The gage at left side of the stainless steel box was adjusted up to allow 2.18 mm of casting mixture thickness after the stainless steel box had been dragged towards the right side of the silicone paper. The thin casting mixture was allowed to dry and followed by drying in a hot air-oven at 50 °C for 4 hr.

The plasticizers in concentrations that provided a film with satisfactory mechanical properties (as determined in **6.2**), maximum detachment force and work of adhesion (as determined in **7.**) were selected.

Table 3 Plasticizers included using full factorial design (2^3)

Film No.	Plasticizers		
	glycerin[a]	PEG 6000[b]	70 % sorbitol[c]
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+

Note: [a] – and + are 20 and 30 %w/w glycerin based on the PG weight, respectively.

[b] – and + are 1 and 2 %w/w PEG 6000 based on the PG weight, respectively.

[c] – and + are 20 and 30 %w/w sorbitol (70 %) based on the PG weight, respectively.

Table 4 Formulation of the mucoadhesive layers containing plasticizers

Ingredients	Film No.							
	1	2	3	4	5	**6**	7	8
PG (g)	4.00	4.00	4.00	4.00	4.00	**4.00**	4.00	4.00
Calcium gluconate (g)	0.45	0.45	0.45	0.45	0.45	**0.45**	0.45	0.45
Glycerin (g)	0.80	1.20	0.80	1.20	0.80	**1.20**	0.80	1.20
PEG 6000 (g)	0.04	0.04	0.08	0.08	0.04	**0.04**	0.08	0.08
70 % sorbitol (g)	0.80	0.80	0.80	0.80	1.20	**1.20**	1.20	1.20
0.5 N NaOH (ml)	20	20	20	20	20	**20**	20	20
Distilled water qs to (g)	100	100	100	100	100	**100**	100	100

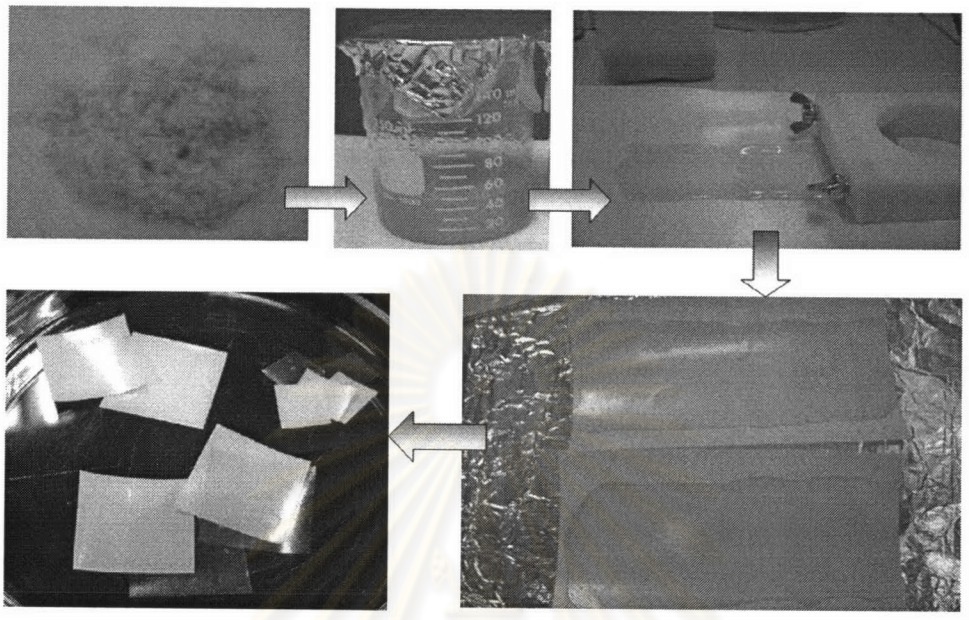

Figure 14 Preparation scheme of buccal mucoadhesive films.

4.1.2 Determination of a Suitable Concentration of PG

Dispersions of PG in distilled water at concentrations of 4.00, 5.00, and 6.00 %w/w were prepared (Table 5) and left at room temperature for 12 hr in order to remove entrapped air bubbles and allow the PG gel to completely hydrate and swell. The calcium gluconate solution which contained calcium ion of 1.0 %w/w based on the PG weight was added gradually into the PG gel dispersion with gentle stirring to prevent air entrapment until a homogeneous viscous gel was obtained. The mixture pH was adjusted with 0.5 N NaOH to 5.5. The types and concentrations of plasticizers chosen from **4.1.1** were added with stirring gently to prevent air entrapment. The stirring had been continued for 2 hr and left until air bubbles were removed. A film was cast by pouring the mixture into a hollow space of a stainless steel box placed on top of silicone paper strip. The gage at left side of the stainless steel box was adjusted up to allow 2.18 mm of casting mixture thickness after the stainless steel box had been dragged towards the right side of the silicone paper. The thin casting mixture was allowed to dry and followed by drying in a hot air-oven at 50 °C for 4 hr.

The concentration of PG that provided a film with appropriate mechanical properties (as determined in **6.2**), maximum detachment force, and work of adhesion (as determined in **7.**) was selected.

Table 5 Compositions of mucoadhesive layers with varied concentrations of PG

Ingredients	Film No.		
	6	9	10
PG (g)	4.00	5.00	6.00
Calcium gluconate (g)	0.45	0.56	0.67
Glycerin (g)	1.20	1.50	1.80
PEG 6000 (g)	0.04	0.05	0.06
70 % sorbitol (g)	1.20	1.50	1.80
0.5 N NaOH (ml)	20	25	30
Distilled water qs to (g)	100.00	100.00	100.00

4.1.3 Selection of Water-insoluble Polymers

The dispersion of 5.00 %w/w PG in distilled water was prepared and left at room temperature for 12 hr in order to remove entrapped air bubbles and allow the PG gel to completely hydrate and swell. The calcium gluconate solution which contained calcium ion at 1.0 %w/w based on the PG weight was added gently into the PG gel dispersion with gentle stirring to prevent air entrapment until a homogeneous viscous gel was obtained. The mixture pH was adjusted with 0.5 N NaOH to 5.5. The plasticizers chosen from **4.1.1** were added with gentle stirring to prevent air entrapment. The stirring had been continued for 2 hr then left until air bubbles were removed.

Water-insoluble polymers were added to retard the dissolution of mucoadhesive layer by using Eudragit® RL 100 (12.5-45.5 %w/w based on the PG weight) or Eudragit® RS 100 (0.1-0.3 %w/w based on the PG weight) or Eudragit® NE 30D (0.2-1.0 %w/w based on the PG weight) or Kollicoat® SR 30 D (0.1-1.0 % w/w based on the PG weight) (Table 6). Eudragit® RL 100 and Eudragit® RS 100 were dispersed separately in absolute alcohol at concentration 15.00 %w/w with continuous

stirring for 4 hr and then used as a stock solution to add to the PG mixture. Eudragit® NE 30D or Kollicoat® SR 30 D dispersions were used as received. The water-insoluble polymer was added to the PG mixture with gentle stirring for 12 hr until a homogeneous viscous gel was obtained. A film was cast by pouring the mixture into a hollow space of a stainless steel box placed on top of silicone paper strip. The gage at left side of the stainless steel box was adjusted up to allow 2.18 mm of casting mixture thickness after the stainless steel box had been dragged towards the right side of the silicone paper. The thin casting mixture was allowed to dry and followed by drying in a hot air-oven at 50 °C for 4 hr.

The water-insoluble polymers that provided a homogeneous mixture and a film with mechanical properties (as determined in **6.2**), maximum detachment force (as determined in **7.**), work of adhesion, (as determined in **7.**), and dissolution time (as determined in **8.**) were selected for further studies.

Table 6 Compositions of mucoadhesive layers containing water-insoluble polymers

Ingredients in 100 g of PG casting mixture	Film No.																	
	11	12	13	14	15	16	17	18	19	20	21	22	**23**	**24**	**25**	**26**	**27**	**28**
PG (g)	**5.00**	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	**5.00**	**5.00**	**5.00**	**5.00**	**5.00**	**5.00**
Calcium gluconate (g)	**0.56**	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	**0.56**	**0.56**	**0.56**	**0.56**	**0.56**	**0.56**
Glycerin (g)	**1.50**	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	**1.50**	**1.50**	**1.50**	**1.50**	**1.50**	**1.50**
PEG 6000 (g)	**0.05**	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	**0.05**	**0.05**	**0.05**	**0.05**	**0.05**	**0.05**
70% sorbitol (g)	**1.50**	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	**1.50**	**1.50**	**1.50**	**1.50**	**1.50**	**1.50**
0.5 N NaOH (ml)	**25**	25	25	25	25	25	25	25	25	25	25	25	**25**	**25**	**25**	**25**	**25**	**25**
Eudragit® RL 100[a] (g)	**0.625**	1.250	1.500	1.750	2.000	2.275	-	-	-	-	-	-	-	-	-	-	-	-
Eudragit® RS 100[b] (g)	-	-	-	-	-	-	0.005	0.010	0.015	-	-	-	-	-	-	-	-	-
Eudragit® NE 30D[c] (g)	-	-	-	-	-	-	-	-	-	0.010	0.025	0.050	-	-	-	-	-	-
Kollicoat® SR 30 D[d] (g)	-	-	-	-	-	-	-	-	-	-	-	-	**0.005**	**0.010**	**0.020**	**0.030**	**0.040**	**0.050**
Distilled water qs to (g)	**100**	100	100	100	100	100	100	100	100	100	100	100	**100**	**100**	**100**	**100**	**100**	**100**

Note: [a] Eudragit® RL 100 is a copolymer of acrylic and methacrylic acid esters with 10 % of quaternary ammonium groups.

[b] Eudragit® RS 100 is a copolymer of acrylic and methacrylic acid esters with 5 % quaternary ammonium groups.

[c] Eudragit® NE 30D is an aqueous dispersion of a neutral copolymer consisting of 30 %w/v polymethacrylic acid esters; (ethyl acrylate, methyl methacrylate) 2:1.

[d] Kollicoat® SR 30 D is an aqueous dispersion of 30 %w/v polyvinyl acetate stabilized with polyvinylpyrrolidone and sodium lauryl sulfate.

4.2 Preparation of Bilayered Buccal Mucoadhesive Films

Bilayered buccal mucoadhesive films consisted of the buccal mucoadhesive layer prepared in **4.1** and a backing layer. Ethylcellulose film was used as the backing layer. Ethylcellulose (45 cps) was accurately weighed, gradually dispersed in absolute ethanol, and adjusted to give a final concentration of 5.00 and 10.00 %w/v. This dispersion was stirred until a clear solution was obtained. The film of backing layer was produced by casting the 5.00 or 10.00 %w/v of ethylcellulose solution on top of the dried mucoadhesive layer laying on a silicone paper strip. The gage at left side of the stainless steel box was adjusted up to allow 0.05 mm of the ethylcellulose solution thickness after the stainless steel box had been dragged towards the right side of the silicone paper. It was then allowed to dry on a leveled surface in a hot air-oven at 50 °C for 15 min. The completely dried bilayered film was kept in a tightly-closed container. The concentration of ethylcellulose solution that provided appropriate thickness was chosen for further studies.

5. Preparation of Bilayered Buccal Mucoadhesive Film Containing Triamcinolone Acetonide

Bilayered buccal mucoadhesive films containing 0.1 %w/w triamcinolone acetonide was prepared. Triamcinolone acetonide (9.3 mg) was dissolved in 3 ml absolute ethanol. The solution was added to the mixture of mucoadhesive layer in **4.1.3** with gentle stirring for 12 hr until a homogeneous viscous gel was obtained. The mixture was cast by the same procedure described previously with the mixture thickness of 2.18 mm and the mixture was hardened and allowed to dry completely in a hot air-oven at 50 °C for 4 hr. The ethylcellulose film was also cast on top of the dry mucoadhesive layer as described previously with the thickness of 0.05 mm and was allowed to dry on a leveled surface in a hot air-oven at 50 °C for 15 min. Then the completely dry bilayered film was kept in a tightly-closed container.

6. Physical Evaluation of the Buccal Mucoadhesive Films

6.1 Appearance of Buccal Mucoadhesive Film Products

Color, transparency and integrity of all film products were visually observed. The film flexibility was roughly determined by hand stretching and bending. The thickness of 4×30 cm^2 films was measured using a micrometer having a sensitivity of 0.01 mm. The mean results of thickness of 30 randomly assigned positions were calculated.

6.2 Mechanical Properties of Buccal Mucoadhesive Films

Mechanical properties of the buccal mucoadhesive film products were evaluated using a tensiometer (Tinius olsen®, Model H5KS 1509). The mechanical properties studied included the tensile strength, percent elongation at break, work of failure, and Young's modulus; five replications were performed. The procedure employed was based on the guideline of the American Society for Testing and Material (1995). A film specimen was cut into small strips (2×20 mm) using a standard template. Only the strips that were free from air bubbles and physical imperfections were used. The mean thickness of each strip was the average value of five measurements taken along the length 2-cm distance using a micrometer. Both ends of the test specimen were carefully clamped using flat-faced grips and extended by the test machine according to conditions as follows:

Rate of grip separation	=	6 mm/min
Gauge length	=	5 mm
Loading weight	=	10 N
Temperature	=	25 ± 2 °C
Relative humidity	=	45 ± 5 %

Five specimens were used for the study of each film formulation. After the specimen was ruptured, the breaking force and the change in length at the moment of rupture were recorded by the QMAT 4.10 S-Series-5K program. Only the data obtained from the strips

ruptured at the bilateral section were accepted. The following equations were used to calculate the mechanical properties of the films:

$$Tensile\ strength(MPa) = \frac{maximum\ load}{original\ minimum\ cross\text{-}sectional\ area\ of\ the\ specimen} \quad (5)$$

$$\%\ elongation = \frac{extension\ at\ the\ moment\ of\ rupture\ of\ the\ sample \times 100}{initial\ gage\ length\ of\ the\ specimen} \quad (6)$$

$$Young's\ modulus = \frac{tensile\ stress}{elastic\ strain\ in\ tension} \quad (7)$$

$$Work\ of\ failure(mJ) = area\ of\ a\ curve\ \ plotting\ \ between\ \ force\ \ and\ \ extension \quad (8)$$

7. *In vitro* Mucoadhesion Study

The experiment was modified from the *in vitro* method of assessment of the maximum detachment force and work of adhesion described by Eouani et al. (2001); Kok, and Choy (1999); and Repka, Prodduturi, and Stodghill (2003). The apparatus employed in this study is depicted in Figure 15.

7.1 Preparation of Buccal Tissue Sample

Fresh porcine buccal tissue, cut into pieces (approximately 2 × 2 cm^2), of 6-7 months old pigs weighing between 70 and 100 kg was obtained from a slaughterhouse. The tissue was stored in normal saline at 4 °C upon collection and was rapidly frozen in a freezer until use. The buccal tissue sample was thawed by immersing in a bath of artificial saliva at ambient temperature (Shojaei, Paulson, and Honary, 2000). Its epithelium with a thickness of 1000-1200 μm was separated from the underlying connective tissues using surgical scissors and clamped on top of a support platform. During the force of adhesion measurements, the tissue surface was damped periodically using artificial saliva to maintain moist tissue throughout the experiments.

The formula of the artificial saliva was modified from Fusayama, Katayori, and Nomoto (1963) and composed of the following ingredients:

Artificial Saliva with Mucin

$Na_4P_2O_7$	1.91 mg
Na_2HPO_4	600.00 mg
NaCl	398.32 mg
KCl	400.00 mg
$MgCl_2$	1.37 mg
$CO(NH_2)_2$	1000.00 mg
Na_2S	1.60 mg
$CaCl_2$	600.00 mg
Mucin	4000.00 mg
85 % o-phosphoric acid	qs to pH 6.2
Distilled water qs	1000 ml

7.2 Measurement of Adhesive Force

A tensiometer (Tinius olsen®, Model H5KS 1509) with a 10 N load cell and a software-controlled program, QMAT 4.10 S-Series-5K, was used in tensile mucoadhesive experiments. The withdrawal and return speeds were set at 30 and 100 mm/min, respectively. A probe used was an aluminium cylinder having a diameter of 2.5 cm. The experiments were carried out at 37 °C by using circulating bath.

A film product (1×1 cm) was taped to the base of aluminium probe fixed to the mobile arm of the tensometer. A piece of porcine buccal mucosa (2 × 2 cm) was then mounted securely in place on a platform within a jacketed water bath containing 50 ml artificial saliva with or without mucin at 37 °C. The porcine buccal mucosa was spread with 0.1 ml of artificial saliva started at the center. The film was brought into contact with the mucous membrane with a constant force of 0.5 N for 10 s. The maximum detachment force (N) and work of adhesion (mJ) were obtained by measuring the resistance to withdrawal of the probe indicating the mucoadhesive character of the film to the mucous membrane. The backing film was used as a negative control in this study.

Five replications were performed. A new porcine buccal mucosa was replaced for each run.

The reproducibility of the measurement was determined by using the new porcine buccal mucosa from three different pigs. Five replications were performed for each mucosa and the coefficients of variation were calculated.

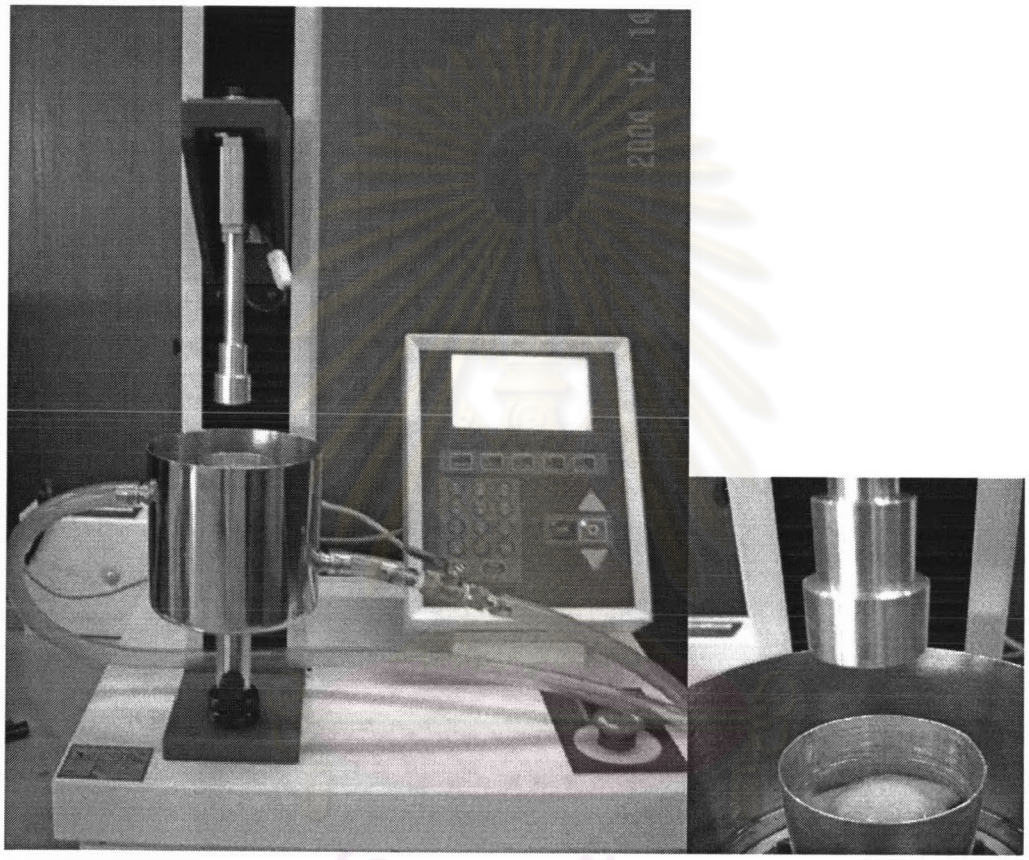

Figure 15 A tensiometer used in the *in vitro* mucoadhesive study (Tinius olsen®, Model H5KS 1509).

8. Determination of Dissolution Time of Buccal Mucoadhesive Films

The dissolution time of the buccal mucoadhesive films was determined using a disintegrator (Model ZT31, Erweka). The apparatus consisted of a basket-rack assembly, 1000-ml beaker containing immersion fluid, a heating unit equipped with a thermostat controlling the temperature at 37 ± 2 °C, and a device for raising and lowering the basket in the immersion fluid at a constant frequency rate between 29 and 32 cycles per min (USP 27, 2004). The immersion fluid in the vessel was artificial saliva without mucin. A buccal mucoadhesive film was secured in a disk, placed in the basket, and immersed in the fluid. The raising-lowering device was then turned on and the dissolution time was recorded.

9. Quantitative Analysis of Triamcinolone Acetonide in Buccal Mucoadhesive Films

9.1 HPLC Conditions

Figure 16 shows a UV spectrum of triamcinolone acetonide in methanol. The maximum absorbance of triamcinolone acetonide was found at the wavelength of 239 nm. Therefore, the analysis of triamcinolone acetonide was performed at 239 nm.

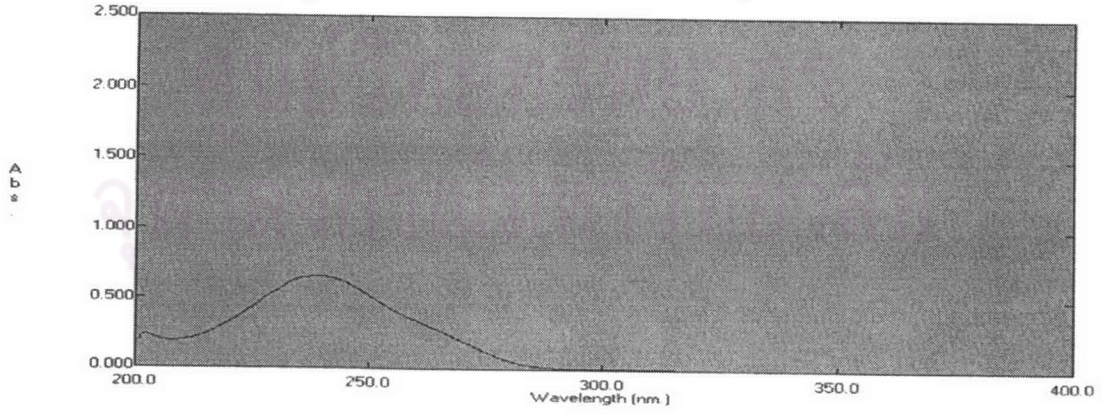

Figure 16 A UV spectrum of triamcinolone acetonide in methanol.

Prednisolone base (10.00 µg/ml) was used as an internal standard due to its appropriate retention time and optimal resolution from the triamcinolone acetonide peak. The HPLC conditions for the analysis of triamcinolone acetonide were as follows:

Column	:	Inertsil ODS C 18, 5 micron, 4.6 × 150 mm
Mobile phase	:	60:40 methanol : water
Flow rate	:	1 ml/min
Run time	:	14 min
Detector	:	UV detector
Wavelength	:	239 nm
Temperature	:	ambient
Internal standard	:	prednisolone base

9.2 Preparation of Internal Standard Solutions

A stock solution of prednisolone base was prepared by accurately weighing of 10.00 mg prednisolone base in a 10-ml volumetric flask. The drug was dissolved and adjusted to volume with methanol. Then 1.0 ml of this solution was transferred to a 100-ml volumetric flask and adjusted to volume with 60 %v/v methanol. The final concentration of prednisolone base was 10.00 µg/ml.

9.3 Preparation of Standard Solutions

An accurate weight of 10.00 mg triamcinolone acetonide was placed in a 10-ml volumetric flask. The drug was dissolved and adjusted to volume with methanol. Then, 1.0 ml of the solution was transferred to a 10-ml volumetric flask and adjusted to volume with 60 %v/v methanol. This stock solution had a final concentration of 100.00 µg/ml. Certain volumes of 60.0, 100.0, 140.0, 180.0, 220.0 and 260.0 µl of the drug stock solution and 1.0 ml of the stock internal standard solution were transferred into 10-ml volumetric flasks. They were adjusted to volume with 60 %v/v methanol and final concentrations of 0.60, 1.00, 1.40, 1.80, 2.20, and 2.60 µg/ml triamcinolone acetonide, respectively, and 1.00 µg/ml prednisolone base were obtained. A calibration curve of triamcinolone acetonide was plotted between its concentrations and peak area ratios.

9.4 Validation of the HPLC Method

Analytical parameters validated were specificity, precision, accuracy, and linearity.

9.4.1 Specificity

Triamcinolone acetonide standard solutions were prepared as previously described in **9.3**. They were injected into the HPLC column using the chromatographic conditions inferred in **9.1**. The resolution of peak was then calculated.

9.4.2 Precision

9.4.2.1 Within run precision

The within run precision was determined by analyzing five concentrations of standard solutions containing 0.70, 1.10, 1.50, 1.90, and 2.30 μg/ml triamcinolone acetonide in the same day. Three determinations per concentration were performed. The percent coefficient of variation (%CV) of the estimated concentration of triamcinolone acetonide at each concentration was then calculated. The within run precision was concluded if the %CV were within 2 % (USP 27, 2004).

9.4.2.2 Between run precision

The between run precision was determined by comparing the same estimated concentrations of standard solutions containing 0.70, 1.10, 1.50, 1.90, and 2.30 μg/ml of triamcinolone acetonide, respectively, prepared and injected in different three days. Three determinations per concentration were performed. The percent coefficients of variation (%CV) of each triamcinolone acetonide concentration analyzed in different three days were calculated. The between run precision could be concluded if the %CV was less than 2 % (USP 27, 2004).

9.4.3 Accuracy

Five sets of 0.70, 1.10, 1.50, 1.90, and 2.30 μg/ml of triamcinolone acetonide solutions were prepared and injected into the HPLC column for analysis of triamcinolone acetonide in terms of percent recovery. The percent recovery of each concentration was calculated from the ratio of inversely estimated concentration to

known concentration of triamcinolone acetonide multiplied by 100. Three determinations per concentration were performed. The percent recovery should be within 2 % of each nominal concentration (USP 27, 2004).

9.4.4 Linearity

Five standard solutions (0.70, 1.10, 1.50, 1.90, and 2.30 μg/ml, respectively) were prepared and analyzed. The linear regression analysis of the peak area ratios versus their concentrations was performed by the method of least square.

9.5 Content Uniformity of Triamcinolone Acetonide in Mucoadhesive Films

The film sample (n=10) was cut into small pieces (1 × 1 cm^2). A small piece of the film was placed in a 25-ml volumetric flask. Then 9.0 ml of methanol was pipetted into the flask, and allowed the film to dissolve completely on a magnetic stirrer for 6 hr. The stock internal standard (1.0 ml) was added, and the stirring was continued for 2 hr until the 10-ml homogeneous solution was obtained. The solution was filtered through a 0.45 μm nylon membrane and was analyzed using the HPLC method described previously. Ten replications were performed.

In a preliminary study, an isocratic system (60:40 methanol:water, 0-12 min) could be used to analyze triamcinolone acetonide in the films. However, due to large differences in polarity of ingredients in the film, a gradient system was employed to elute the other components in formulation in order to shorten the length of study time. The gradient system is shown in Table 7.

Table 7 Time program and ratios of mobile phase for analyzing triamcinolone acetonide in films

Time (min)	Methanol : Water
12.0	60:40
12.1	80:20
12.5	100:0
35.0	100:0
36.0	80:20
37.0	60:40
47.0	60:40

10. Stability Study of Triamcinolone Acetonide Buccal Mucoadhesive Films

The stability testing was determined from the film preparations that have been stored according to two conditions. Firstly, the test preparations (1 × 1 cm^2) were stored in glass vials, which tightly sealed with rubber closures and aluminium caps, and stored in a chamber controlling the temperature at 40 ± 2 °C and the humidity at 75 ± 5 % RH for three months (Carstensen, 1990). Secondly, the stability of the same preparations was also studied at ambient condition for three months. The temperature and relative humidity were examined using a digital hygrometer. Three samples of each preparation were randomly taken at time intervals of 0, 1, 2, and 3 months. The % amount of triamcinolone acetonide was analyzed by the procedure described in **9.5**. The % labeled amount of test preparations was also determined at the initial time.

11. *In Vitro* Release Study of Triamcinolone Acetonide from Buccal Mucoadhesive Films and Kenalog® in Orabase

A buccal mucoadhesive film or Kenalog® in orabase was adhered on a cellophane membrane, which were placed on a 14-ml receiving chamber of a modified Franz diffusion cell. A donor chamber was then placed on top of the membrane and securely fixed to the receiving chamber using a metal clamp. The receiving compartment contained pH 7.4 isotonic phosphate buffer which was maintained at 37 °C by a circulating water jacket, which was connected to a constant temperature water bath. Uniform mixing of the receiving solution was provided by a magnetic stirrer at 500 rpm throughout the time of release study. Any air bubbles formed under the preparation had necessarily been removed before the experiment was started. Each of film preparation and Kenalog® in orabase was tested in five replications.

Samples of 4 ml were taken from the receiving medium at certain time intervals (0, 10, 20, 30, 45, 60, 120, 180, 240, 300, 360, 420, and 480 min) via the side arm sampling port of diffusion cell. The entire receiving solution was removed using a syringe fitted with a piece of flexible tubing and the receiving compartment was immediately replaced with the same amount of medium and the run was continued.

All receiving solutions taken were analyzed by using the HPLC method as previously described in **9.1** and the drug concentration was then calculated from the calibration curve. The amount of drug released was calculated by multiplying the drug concentration with the receiving volume.

12. Clinical Efficacy of Buccal Mucoadhesive Films with and without Triamcinolone Acetonide

The protocol of this study was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

12.1 Criteria for Selection of Subjects

Seventy-two subjects showing a sign of aphthous stomatitis were recruited according to the following criteria:

Inclusion criteria:

1. Men or women showing a sign of aphthous stomatitis for not more than 2 days; the ulcer must be located either on the buccal mucosa or on the upper or lower labial mucosa where the preparations could be easily applied and the ulcer size could be easily measured.
2. Age of over 18 years old.
3. No evidence of chronic dental neglect.
4. Not being pregnant or breast-feeding.
5. Not being under any medications.
6. Must be willing to read and sign an informational letter of consent.
7. Having no factors in their medical history that could pose a potential risk to themselves (such as allergy to a test agent) or to the study personnel.
8. Having potential for full participation through the study.

Exclusion criteria:

1. Not being able to perform the inclusion criteria.
2. Determine to quit the study.

A questionnaire used to inquire the information is shown in Appendix VI. The subjects were allowed to quit the study at any time.

12.2 Comparisons of the Clinical Efficacy of Buccal Mucoadhesive Films with and without Triamcinolone Acetonide and Kenalog® in Orabase

To investigate the efficacy of prepared buccal mucoadhesive films with and without triamcinolone acetonide (TAA), a well-known commercial triamcinolone acetonide in oral paste (Kenalog® in orabase) was used as a positive control. The seventy-two recruited subjects were randomized into 4 groups (Cohen, 1977): group No. 1, 2, 3, and 4, respectively. The test preparations as shown in Table 8.

Table 8 Preparations given to subjects of four groups

Group No.	Preparations
1	Control
2	Kenalog® in orabase
3	Buccal mucoadhesive film from PG
4	Triamcinolone acetonide buccal mucoadhesive film from PG

The procedures for applying the preparations were as follows:

12.2.1 Group No. 1

Eighteen subjects were randomized into group No. 1, which was assigned to be a control group. The subjects were not permitted to use any medications. The subjects had to observe their own symptoms such as pain and irritation, and measure the size of the lesions (mm) with a calibrated paper strip once every morning until the lesions disappeared or up to seven days. How long the lesion was cured, which was indicated by the disappearance of lesion, was recorded. The curing rate (mm/day) was calculated from the difference between the lesion sizes under initial and final observations divided by number of days as shown in Equation (9).

$$curing\ rate = \frac{l_0 - l_c}{d} \qquad (9)$$

where l_o was the longest side (mm) of the lesion initially, l_c was the longest side (mm) of the lesion at the 7^{th} day or 0 if the lesion disappeared, and d was the time period needed to cure the lesion, respectively.

12.2.2 Group No. 2

Eighteen subjects were randomized into group No. 2, a positive control group. The subjects were not allowed to eat, drink, and talk during the test. Prior to application of this preparation, the subjects had to rinse their mouths and use cotton buds to absorb excess saliva on the lesions. They were instructed to apply a small dab of Kenalog® in orabase (about ¼ inches) with a cotton bud to the lesions before bedtime once daily until the lesions disappeared. They were asked to observe symptoms such as

pain and irritation, and measure the size of the lesions (mm) with a calibrated paper strip once every morning until the lesions disappeared or up to seven days. The resident time of mucosal adhesion of the preparation was the time span between time at initial adhesion and when the paste completely lost its adhesive contact with the mucosa. How long the lesion was cured, which was indicated by the disappearance of lesion, was also recorded. The curing rate (mm/day) was calculated from the difference between the lesion sizes under initial and final observations divided by number of days as shown in Equation (9).

12.2.3 Group No. 3

Eighteen subjects were randomized into group No. 3, a treatment 1 group. The subjects were not allowed to eat, drink, and talk during the test. Prior to application of buccal mucoadhesive films without triamcinolone acetonide, the subjects had to rinse their mouths, and use cotton buds to absorb excess saliva. Then the 1×1 cm^2 film was peeled off from the silicone paper and applied the silicone contacted side to the lesion before bedtime once daily until the lesion disappeared. The subjects had to observe symptoms such as pain and irritation, and measure the size of the lesions (mm) with a calibrated paper strip once every morning until the lesions disappeared or up to seven days. The resident time of mucosal adhesion of the preparation was the time span between the time at the initial adhesion and the time when the film completely lost its adhesive contact with the mucosa. How long the lesion was cured, which was indicated by the disappearance of lesion, was also recorded. The curing rate (mm/day) was calculated from the difference between the lesion sizes under initial and final observations divided by number of days as shown in Equation (9). At the end of the study, the subjects were asked to fill in a questionnaire informing their perception of the preparation.

12.2.4 Group No. 4

Eighteen subjects were randomized into group No. 4, a treatment 2 group. The subjects were not allowed to eat, drink, and talk during the test. Prior to application of buccal mucoadhesive films containing triamcinolone acetonide, the subjects had to rinse their mouths, and use cotton buds to absorb excess saliva. Then the 1×1 cm^2 film was peeled off from the silicone paper and applied the silicone contacted side to the lesion before bedtime once daily until the lesion disappeared. The subjects had

to observe symptoms such as pain and irritation, and measure the size of the lesions (mm) with a calibrated paper strip once every morning until the lesions disappeared or up to seven days. The resident time of mucosal adhesion of the preparation was the time span between the time at the initial adhesion and the time when the film completely lost its adhesive contact with the mucosa. How long the lesion was cured, which was indicated by the disappearance of lesion, was also recorded. The curing rate (mm/day) was calculated from the difference between the lesion sizes under initial and final observations divided by number of days as shown in Equation (9). At the end of the study, the subjects were asked to fill in a questionnaire informing their perception of the preparation.

12.3 Statistical Analysis

The curing rate and the time period indicating the lesion disappearance among group No.1-4 were compared. The analysis of variance (ANOVA) with equal subsamples was used to determine the difference between groups. Significant difference between groups was assumed if p-value was less than 0.05. The Least significance difference (LSD) was used to identify which preparation gave different clinical efficacy.