CHAPTER III

EXPERMENTAL

Materials

1. Lidocaine hydrochloride: Batch No. R3 44/00784. Supplement from The Government Pharmaceutical Organization, Bangkok, Thailand.
2. Sodium carboxymethylcellulose 1500 cps.: Batch No. E3103/294. Distributed by Srichan United Dispensary, Thailand.
3. Hydroxypropyl methylcellulose 4000 cps. (Methocel®E4M premium): Batch No. MM94040112E. Distributed by Rama Production Co., Ltd., Bangkok, Thailand.
4. Hydroxypropyl methylcellulose 15 cps. (Methocel®E15): Batch No. MM110921E. Distributed by Rama Production Co., Ltd., Bangkok, Thailand.
5. Hydroxypropyl cellulose type H, Batch No. CE-211. Nippon Soda, Japan.
6. Chitosan (85% deacetylation MW 227,000): Distributed by Seafresh Chitosan Co., Ltd., Bangkok, Thailand.
7. Polyethyleneglycol 400: Distributed by Srichand United Dispensary, Thailand.
8. Citric acid, Batch No. 0086978, Fisher Scientific, England.
9. Menthol: Batch No. 1010715. Distributed by Srichand United Dispensary, Thailand.
10. Ethanol 95%: Distributed by The Government Pharmaceutical Organization, Bangkok, Thailand.
11. Potassium dihydrogen orthophosphate: Batch No. F1F125. APS Finechem, Australia.
12. Di-sodium hydrogen phosphate anhydrous: Batch No. 1E708110F. Carlo Erba Reagenti, Italy.
13. Sodium hydroxide pellets: Batch No. 7708MVKK. Mallinckrodt Baker, Maxico.
14. Glacial acetic acid: Batch No. 428580717. BDH Laboratory Supplies, England.
15. Methylparaben: Batch No. 406565/1 21600. Fluka, Switzerland.

16. Potassium chloride: Batch no. TA915536 124. Merck, Germany.
17. Potassium nitrate: Batch no. A264163 117. Merck, Germany.
18. Magnesium nitrate: Batch no. A325253 132. Merck, Geramany.
19. Sodium chloride: Batch no. K2367963 705. BDH Laboratory Supplied, England.
20. Acetonitrile HPLC grade: Batch No. 01070042. Labscan Asia, Co. Ltd., Thailand.
21. Methanol HPLC grade: Batch No. 01070114. Labscan Asia, Co. Ltd., Thailand.
22. Ultrapure Water equipped with filter system (Balson®, Balson Inc., USA),
23. Standard buffer solutions: Beckman, USA.

All chemicals were analytical or pharmaceutical grades and were used as received.

Equipments

1. Analytical balance: Satorius, model A200s, Satorius Co., Ltd., Germany.
2. Magnetic stirrer: Heidolph, model MR3001, Germany.
3. pH meter: Beckman, USA.
4. Micropipet: Pipetman®, Gilson, UK.
5. Sonicator: Ultrasound ELMA, model T900, Elma, Germany.
6. Diffusion cells: Modified from Franz's diffusion cell.
7. Water Bath: Heto, model TBVS01, Denmark.
8. 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.
    - Microsyring 100 µl: SGE, Australia.
    - C-18 column, 250 x 4.6 mm, 5 µm: Hypersil®, BDS, England.
9. Tensile tester, Instron 5565, Instron Corp., England.
10. Ball mill

11. Hot air oven: Memmert type BM600, GmbH, Germany.
12. Micrometer: Teclock Co., Japan.
13. Differential scanning calorimeter: NETZCH DSC 200, NETZSCH-Geratebau, GmbH, Germany.
14. Scanning-electron microscope: JSM-5410LV, Jeol, Japan.
15. X-ray diffractometer: Model JDX-8030, Jeol, Japan.
16. Fourier transform infrared spectrometer: Model 1760X, Perkin Elmer, USA.
17. Surface area determination equipment: Model Flowsorb 230FC, Micromeritics Instrument Corporation, USA.

Glassware and Miscellaneous

1. Dialysis membrane: cut off size 12,000-14,000. Lot No. 28H0141, Sigma, Germany.
2. 0.45 μm membrane filter: Waters, USA.
3. Beaker: Pyrex, USA.
4. Cylinder: Pyrex, USA.
5. Test tube: Pyrex, USA.
6. Transfer pipette: HBG, Germany.
7. Volumetric flask: HBG, Germany.
8. Disposable syringe: Terumo, Thailand.
9. Filter device: Swinnex, Millipore, USA.
10. Aluminium foil: MMP packing, Thailand.
11. Parafilm: American National Can., USA.
12. Desiccator

Methods

1. Preparation of Lidocaine Hydrochloride Mucoadhesive Films

Mucoadhesive films containing 20 mg of lidocaine hydrochloride per 2.5 cm^2 were prepared. The compositions of the formulas are indicated in Tables 1-6. The procedures for preparing mucoadhesive films were as follows:

Chitosan was pulverized in a ball mill and passed through a 80-mesh screen. Required quantity of chitosan (1% w/w) was gradually dispersed in half of required volume of water. The amount of citric acid giving 3% w/w acid in final solution was dissolved in another part of water and then added into the previous dispersion. The plasticizer was added and the polymer solution was adjusted to weight with water. HPMCs 15 and 4000 cps, HPC-H, and CMC 1.5%w/w solution were prepared by dissolving the polymer in distilled water. All polymeric solutions were stirred at room temperature overnight in order that the polymer would completely hydrate and swell. The resulting solution was filtered through a gauze cloth in order to remove the extraneous matter before used. Then lidocaine hydrochloride, citric acid and the 50 %w/w alcoholic solution of menthol were added into the polymeric solutions. The solutions were poured on glass plate (9.7 cm in diameter) and left to stand until the trapped bubbles were removed. The films were dried for 12 hours or until constant weight in a hot air oven at 55°C. The dried films were stored in desiccator until the time of analysis.

The codes of the lidocaine HCl mucoadhesive films are presented in Tables 2-7. HPMC E15, HPMC E4M, HPC-H, chitosan films were coded as E15, E4M, HPC and CS, respectively. And the numbers after these code, 1:1, 1:0.67 and 1:0.5 or 2:1, described the drug content in the term of weight ratio of drug and polymer. Combinations of HPMC E15 and HPC were coded as E15HPC and the numbers following this code were the ratio of HPMC and HPC. For the combination films, drug to polymer ratio was fixed of 1:1.

Table 2 The formulations of lidocaine hydrochloride mucoadhesive films using CMC as mucoadhesive polymer

Substance	Formulation code			
	CMC 1:1 (g/plate)	CMC 1:0.67 (g/plate)	CMC 1:0.5 (g/plate)	CMC 0.5:1 (g/plate)
Lidocaine HCl	0.6	0.6	0.6	0.3
NaCMC (1.5% w/w in water)	40	26.67	20	40

Table 3 The formulations of lidocaine hydrochloride mucoadhesive films using HPMC E15 as mucoadhesive polymer

Substance	Formulation code		
	E15 1:1 (g/plate)	E15 1:0.67 (g/plate)	E15 1:0.5 (g/plate)
Lidocaine HCl	0.6	0.6	0.6
HPMC E15 (1.5% w/w in water)	40	26.67	20
Citric acid	0.18	0.18	0.18
Menthol (50% w/w in ethanol)	0.36	0.36	0.36

Table 4 The formulations of lidocaine hydrochloride mucoadhesive films using HPMC E4M as mucoadhesive polymer

Substance	Formulation code			
	E4M 1:1 (g/plate)	E4M 1:0.67 (g/plate)	E4M 1:0.5 (g/plate)	E4M 2:1 (g/plate)
Lidocaine HCl	0.6	0.6	0.6	0.12
HPMC E4M (1.5% w/w in water)	40	26.67	20	40
Citric acid	0.18	0.18	0.18	0.36
Menthol (50% w/w in ethanol)	0.36	0.36	0.36	0.72

Table 5 The formulations of lidocaine hydrochloride mucoadhesive films using chitosan as mucoadhesive polymer

Substance	Formulation code		
	CS 1:1 (g/plate)	CS 1:0.67 (g/plate)	CS 1:0.5 (g/plate)
Lidocaine HCl	0.6	0.6	0.6
Chitosan (1% w/w in water)	60	40	30
PEG400	0.12	0.08	0.06
Citric acid	1.8	1.2	0.9
Menthol (50% w/w in ethanol)	0.36	0.36	0.36

Table 6 The formulations of lidocaine hydrochloride mucoadhesive films using HPC as mucoadhesive polymer

Substance	Formulation code		
	HPC 1:1 (g/plate)	HPC 1:0.67 (g/plate)	HPC 1:0.5 (g/plate)
Lidocaine HCl	0.6	0.6	0.6
HPC-H (1.5% w/w in water)	40	26.67	20
Citric acid	0.18	0.18	0.18
Menthol (50% w/w in ethanol)	0.36	0.36	0.36

Table 7 The formulations of lidocaine hydrochloride mucoadhesive films using mixture of HPMC E15 and HPC-H as mucoadhesive polymers with drug to polymer ratio of 1:1

Substance	Formulation code				
	E15HPC 1:3 (g/plate)	E15HPC 2:3 (g/plate)	E15HPC 3:3 (g/plate)	E15HPC 3:2 (g/plate)	E15HPC 3:1 (g/plate)
Lidocaine HCl	0.6	0.6	0.6	0.6	0.6
HPMC E15 (1.5% w/w in water)	10	16	20	24	30
HPC-H (1.5% w/w in water)	30	24	20	16	10
Citric acid	0.18	0.18	0.18	0.18	0.18
Menthol (50% w/w in ethanol)	0.36	0.36	0.36	0.36	0.36

2. In vitro Evaluation of the Mucoadhesive Films

2.1 Physical characteristics of the mucoadhesive films

Transparency of the films was visually observed. Ease to peel off detachment from glass plates, glossiness, flexibility, and stickiness were also observed.

2.2 Film thickness measurement

The thickness of the mucoadhesive films was measured by micrometer. The samples were measured in triplicate. Each sample was measured at five locations. The mean and the standard deviation of thickness were calculated.

2.3 Content uniformity determination

The content uniformity of lidocaine HCl in the bioadhesive films was quantitatively determined by mean of absorption peak area ratio by HPLC method. The standard USP method was followed:

For analysis purpose, the film was cut into ten small rectangular pieces (1 x 2.5 cm) with an accurate weight. Each piece was determined to contain 20 mg of lidocaine HCl. They are individually tested for their content uniformity. Each piece was analyzed by dissolving in 100 ml of water in 100-ml volumetric flask and shaken at room temperature until the film was completely dissolved in water. The obtained solution was taken to measure the absorbance by HPLC. The percentage of active drug within the test films was calculated from the calibration curve.

2.4 Analysis of lidocaine hydrochloride

2.4.1 HPLC chromatographic condition

The chromatographic condition used was adapted from USP24. Its condition was presented as follows:

Column	: Hypersil® C18 column (250x4.6 mm), 5 μm (UK)
Detector	: UV detector was set at 254 nm
Flow rate	: 1.2 ml/min
Attenuation	: 2
Chart speed	: 2 cm/min
Injection volume	: 20 μl
Internal standard	: methyl paraben 10 μg/ml
Mobile phase	: 50 ml of glacial acetic acid was mixed with 930 ml of water, and then it was adjusted to pH 3.4 with 1N sodium hydroxide. 8 volume of this solution was mixed with 3 volume of acetonitrile. The mobile phase was prepared freshly and filtered through a 0.45 μm membrane filter then degassed by sonication for 30 min prior to use.

2.4.2 Validation of HPLC method

The analytical parameter used for the assay validation were specificity, accuracy, precision and linearity (USP24/NF19, 1999).

a) Specificity

Under the chromatographic condition used, the peak of lidocaine HCl had to be completely separated from and not be interfered by the peak of other components in the sample. Phosphate buffer and non-active ingredients, including HPMC E15, HPMC E4M, HPC-H, chitosan, menthol and citric acid were injected. Chromatograms were evaluated by comparing with the standard solution of lidocaine HCl.

b) Accuracy

Three sets of standard solutions of lidocaine HCl having concentrations of 5-25 μg/ml were prepared and injected. The percentage of the analytical recovery of each standard solution was calculated.

c) Precision

Within Run Precision

The within run precision was determined by analyzing three sets of five standard solutions of lidocaine HCl in the same day. Peak area ratios of lidocaine HCl to methyl paraben were compared and percentage coefficient of variation (%CV) of each concentration were determined.

Between Run Precision

The between run precision was determined by comparing each concentration of lidocaine HCl standard solutions that were prepared and injected on different days. The percentage coefficient of variation (%CV) of lidocaine HCl to its internal standard peak area ratios from the three sets of standard solutions having the same concentration were determined.

d) Linearity

Lidocaine HCl standard solutions ranging from 5 to 25 μ g/ml were prepared and analyzed. Linear regression analysis of peak area ratios versus their concentrations was performed.

2.4.3 System suitability

System suitability tests were used to verify that the resolution and reproducibility of the chromatographic system were adequate for analysis to be done.

a) Resolution

The resolution was a function of column efficiency and was specified to ensure that lidocaine HCl was resolved from methyl paraben. The resolution, R, was determined by the equation

$$R = \frac{2(t_2-t_1)}{W_2+W_1} \quad \ldots\ldots\ldots\ldots (1)$$

In which t_1 and t_2 were the retention times of lidocaine HCl and methyl paraben, respectively. W_2 and W_1 were the corresponding widths at the base of the peaks obtained by extrapolating the relatively straight sides of the peak to the baseline.

b) Tailing factor

Tailing factor was performed by collecting data from injected standard solutions. This test was determined by equation

$$T = \frac{W_x}{2f} \quad \ldots\ldots\ldots\ldots (2)$$

in which W_x was the width of peak of lidocaine HCl and methyl paraben at 5% height, f was the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

2.4.4 Calibration curve of lidocaine hydrochloride

a) Stock solutions

A stock solution of internal standard was prepared by transferring about 20 mg of methyl paraben, accurately weighed, to a 100-ml volumetric flask. Deionized water was used to adjust to volume. Then 1 ml of

this solution was pipetted and transferred into 100-ml volumetric flask then adjusted to volume with deionized water.

A stock solution of lidocaine HCl was prepared by transferring about 50 mg of lidocaine HCl, accurately weighed, to a 100-ml volumetric flask. Deionized water was used to adjust to volume. Then 10 ml of this solution was pipetted and transferred into 100-ml volumetric flask. Deionized water was used to adjust to volume.

b) Standard solutions for calibration curve

Standard solutions of lidocaine HCl (5, 10, 15, 20 and 25 μg/ml) containing 0.2 μg/ml of methyl paraben were prepared from stock solution of lidocaine HCl and methyl paraben by diluted and adjusted to volume with ultrapure water.

2.5 Surface topography

Surface topography of mucoadhesive films was observed under a scanning electron microscope. The samples were attached to the slab surfaces with double-sided adhesive tapes and then coated with gold to a thickness of approximately 30 nm under vacuum to produce the samples conductive. Scanning electron photomicrographs were taken at 100 and 500 x magnifications.

2.6 Specific surface area determination

The specific surface area and the total pore volume of the films were determined by BET adsorption method using surface area analytical equipment. The prepared films were cut into very small pieces (about 0.2 x 0.7 mm). Then they were kept in desiccator for 1 weak in order to remove the residual moisture prior determined. The specific surface area was calculated automatically.

2.7 The physicochemical characterization

Infrared spectrometry, X-ray diffractometry and differential thermal analysis were used to characterize the substances in the films.

2.7.1 Infrared spectrometry

Fourier transform infrared spectrophotometry (FT-IR) was to study the change in the functional groups of the polymers, drug and the mucoadhesive films.

Infrared spectra were examined by using a Fourier transform infrared spectrometer. The obtained films were examined by using KBr disc. The sample disc was determined by FT-IR spectrometer in the wavenumbers 400-4000 cm^{-1}. These spectra were compared to the spectra which were taken from initial powder of lidocaine HCl, polymers, menthol and citric acid using KBr disc.

2.7.2 Powder X-ray diffractometry

Powder X-ray diffractometry was used to determine the diffraction angles of substances that showed crystallinity and interplannar spacing of the crystal planes. This mode was used to study the change of crystallinity of polymers and drug after preparation which could explain some physiochemical properties of mucoadhesive films.

The X-ray diffractograms from the films and the initial powder of lidocaine HCl, polymers, menthol and citric acid were examined by the diffraction method with nickle-filtered CuKα radiation of Jeol diffractometer operated in the ω-2θ scanning mode between 5° and 60° 2θ at 1.50° per second.

2.7.3 Differential scanning calorimetry

Differential scanning calorimetry was used to determine the thermograms of polymers and drug. The differences in thermal energy patterns between the original substances and their products were evaluated. This method was used to study interaction between components after preparation.

About 2 mg of original powder or 5 mg of products of each sample were accurately weighed into the DSC pan. Then it was crimped with the sealed pan and was placed in the equipment beside the reference pan made by the same method except without powder. The thermal runs were controlled at a

heating rate of 10°C per minute and in the range of 30°C to 200°C, sensitivity ± 50 μv and chart speed 10 mm/min in static air atmosphere.

2.8 Tensile properties

The ultimate tensile strength, modulus of elasticity (Young's modulus) and percentage of strain at point of break were examined by using an universal tensile testing machine (Instron 5565, Instron Crop.) equipped with 10N tension load cell under ASTM standard D882-88. 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 specimens were cut into small strips (2 x 80 mm) by using a standard knife. The specimens were left to expose to room humidity for 1 hour before tested. The thickness of each strip was the mean value of five separate measurements taken along the length of the sample by using micrometer. Then the strips were carefully clamped by an upper and lower pneumatic flat-faced grip and were extended by the test machine at speed of 5.0 mm/min until it was ruptured.

The tensile stress was plotted against the percent strain to give stress-strain curve, and ultimate tensile strength as well as elongation at break was reported. The mean and standard deviation of the values were obtained from six-determinations. Statistical analyses were performed using two-way ANOVA and differences were considered at a level of $p<0.05$.

2.9 Moisture sorption and swelling property

2.9.1 Moisture sorption study

The mucoadhaesive films were cut into 1 x 2.5 cm small strip. The strips were determined the original dry weight (W_0) at room temperature (about 25°C) by keeping them in the desiccator which filled with silica gel for 24 hours. Then they were placed inside a desiccator containing saturated solutions of magnesium nitrate (53%RH), sodium chloride (75%RH), potassium chloride (84%RH), and potassium nitrate (94%RH) and stored at 25°C (Umprayn and Mendes, 1987). At appropriate time intervals (1, 3, 5, and 7 days),

the films were taken out, and weigh immediately (W_t). The percentages of moisture sorption of the films were calculated by the following equation.

$$\% \text{ moisture sorption} = \frac{W_t - W_0}{W_0} \times 100\% \quad \ldots\ldots\ldots (3)$$

where W_0 is the original dry weight of the strip films, W_t is the weight of the strip films at time t. The measurement was made in triplicate.

2.9.2 Swelling measurement

The mucoadhaesive films were cut into 1 x 2.5 cm small strips. The strips were determined the initial thickness at room temperature (about 25°C) by keeping them in the desiccator which filled with silica gel for 24 hours and calculated the initial volume (V_0). Then they were placed inside a desiccator containing saturated solutions of sodium chloride (75%RH), potassium chloride (84%RH), and potassium nitrate (94%RH) and stored at 25°C. At an appropriate time intervals (1, 3, 5, and 7 day), the films were taken out, and the size (wide and length) and thickness of the films was measured immediately and then calculated the volume (V_t). The percentage of swelling was calculated by the following equation.

$$\% \text{ swelling} = \frac{V_0 - V_t}{V_0} \times 100 \quad \ldots\ldots\ldots (4)$$

where V_0 is the original volume of the strip films, V_t is the volume of the strip films at time t. The measurement was made in triplicate.

2.10 Mucoadhsive property

The adhesive forces of the mucoadhesive films in contact between aluminium flat surfaces were determined using Instron tensile tester equipped with computer integrated data acquisition system. The relative humidity of the laboratory for testing was about 55% and temperature was 25±2°C. The films were cut into small pieces (1 x 2.5 cm). Each piece of sample was placed on the surface of aluminium stationary platform then moistened with 200 µl of phosphate

buffer pH 6.8 and left its swell for 2 min. The moving part, which was aluminium, was then brought in contact with the film. Adhesion of films to substrate was brought on after the application of constant force of 2 g. After a pre-set time (2 mim) of contact, the crosshead was raised at a constant speed (20 mm/min) and the force required for detachment between the sample and the aluminium was recorded. The data were analyzed using Series IX software (Instron corp.) and were reported as the maximum force required for detachment per cross sectional area. Experiments were run in ten times. Statistical analysis were performed using single factor ANOVA and differences were considered at a level of $p<0.05$.

2.11 In vitro drug release and penetration from mucoadhesive films

The release and penetration of drug from mucoadhesive films were determined using modified Franz diffusion cells (Figure 25). The internal diameter of each cell was 1.7 cm, corresponding to an effective permeable surface area of 2.27 cm^2. The receptor compartment contained about 14 ml of pH 6.8 phosphate buffer as penetration medium.

The mucoadhesive film was clamped between the donor and the receptor compartments with dialysis membrane for penetration study (Senel et al., 2000) or without dialysis membrane for release study. In case of drug penetration through dialysis membrane, the mucoadhesive film was placed directly onto the dialysis membrane. The dialysis membranes were pretreated by immersing in deionized water at room temperature for overnight and then rinsing with 80°C of deionized water for 2 min in order to wash off any water soluble contaminates. After that, they were soaked in phosphate buffer pH 6.8 until used. A small magnetic stirring bar (4 x 7 mm) was placed in the receptor compartment and rotated at 750 rpm. The diffusion cell (capacity 14 ml) was filled with phosphate buffer solution pH 6.8 until it reached to the top level of the receiver chamber on which the dialysis membrane or mucoadhesive film was placed, leaving no air bubbles in the chamber. The temperature of the assembled diffusion cell was 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 through the 0.8 μm membrane filter at predetermined time interval.

The same volume of the solution withdrawn was returned to the chamber at each withdrawal. The amount of drug released or penetrated was assayed by the HPLC method. The triplicate determinations of each of sample were measured.

Saturated solution of lidocaine HCl was determined for drug penetration through dialysis membrane in order to compare drug penetration profiles of solution and mucoadhesive films.

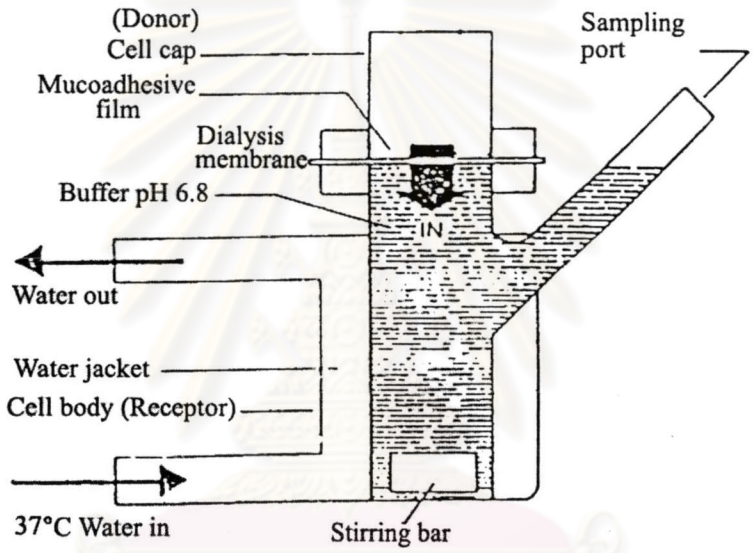

Figure 25 Schematic diagram of the apparatus for the in vitro release and penetration studies