CHAPTER 3

Materials and Methods

3.1 Materials

- Tranexamic acid (Daiichi Pharmaceutical Co., Ltd., Japan)
- Hydroxymethylpropylcellulose (Methocel ®) E 4M (Colorcon Co., Ltd, USA)
- Hydroxymethylpropylcellulose (Methocel ®) E 50 (Colorcon Co., Ltd, USA)
- Carbopol ® 980 NF (BF Goodrich Co., Ltd., USA)
- Naphthalene-2,3-dicarboxaldehyde (NDA) (Fluka Chemical Corp., WI, USA)
- DC 200/350 (Dow Corning Co., Ltd., USA)
- DC 7-9245 (Dow Corning Co., Ltd., USA)
- Polyvinylpyrrolidone (PVP) K 90 (Serva Feinbiochemica GmbH&Co., Germany)
- Acrylax ® 1061 (Neoplast Co., Ltd., Thailand)
- Disodium tetraborate (BHD Chemical Ltd., England)
- Potassium cyanide (KCN) (Merck Co., Ltd., Germany)
- Methanol, HPLC grade (Merck Co., Ltd., Germany)
- Sodium chloride (Merck Co., Ltd., Germany)
- Potassium chloride (Merck Co., Ltd., Germany)
- Propylene glycol (S. Tong Chemical Co., Ltd., Thailand)
- Phenoxy ethanol (Nipa Hardwicke Inc., USA)
- Disodium-hydrogenphosphate anhydrous (Na_2HPO_4) (Merck Co., Ltd., Germany)
- Potassium dihydrogen orthophophate (KH_2PO_4) (BHD Chemical Ltd., England)
- Sodium hydroxide (NaOH) (BHD Chemical Ltd., England)
- Lactic acid (Mallinckrodt Inc., USA)
- N-methylpyrrolidone (Fluka Chemical Corp., WI, USA)
- Backing membrane and release liner for patch (Neoplast Co., Ltd., Thailand)

- Cellophane membrane
- Laminated aluminum foil

3.2 Apparatus

- Spectrofluorometer Model FP-777, 150 W Xenon lamp (Jasco Japan Co., Ltd., Japan)
- pH meter :Inolab electrode senTix81 (Wissenschaftlich Technische Werkstätten, Germany)
- Horizontal diffusion cell (Japan)
- Sonicator Vibra cell (Sonic&Material Inc., USA)
- Analytical balance: Precisa XT220A (Precisa Instruments, Ltd., Switzerland)
- Micrometer (NSK Co., Ltd., Japan)
- Hot air oven: Memmert® 854 Schwabach (Edelstahl Rost Frei Co., Ltd., Germany)
- Glass plates for preparing the patches
- Adhesive test Model

3.3 Methods

3.3.1 Tranexamic acid patch formulation

3.3.1.1 Preparation of tranexamic acid patches

The hydrogel formulas M35 and M38 were prepared with the composition shown in table 3.1. In each formula, the liquid medium of HPMC E4M dispersion was prepared by weighting and adding agents to a beaker in steps as followed;

- Methylparaben was melted in propylene glycol by heating. Distilled water was added followed by an exact amount of tranexamic acid powder. The powder was stirred or sonicated until dissolved.

- Other agents were added in the order of; phenoxy ethanol, 10%w/w PVP K90 and 8%w/w DC7-9245, respectively, (for preparing formula M38 , other two agents, DC 200/350 and 30%w/w acrylax ®1061, were added).
- This liquid mixture was directly heated to approximately 55-65°C. The HPMC E4M was dispersed and blended into the hot mixture. After the viscosity of dispersion rises, the dispersion was frozen for a while until a gel-liked compound can be noticed. Then the patches were prepared by pressing the gel mixture between the two glass plates as shown in figure 3.1.

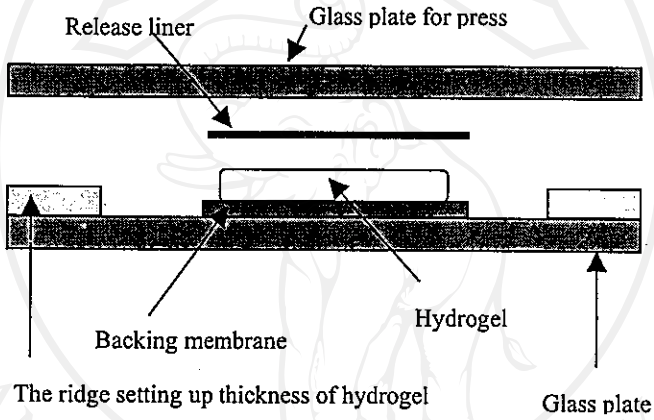


Figure 3.1 Diagram of patch making.

The hydrogel formula J33 was prepared with the composition shown in table 3.1. The liquid compound was prepared as M35 preparation except 50%w/w DC7-9245 was used instead of 8%w/w DC7-9245, 10%w/w PVP K90 and HPMC E4M. The HPMC E50 was dispersed in hot mixture, then 6%w/w carbopol® 980 NF was immediately added. The freezing and preparation steps were performed in the same procedure as M35 and M38 preparations.

3.3.1.2 Preparation of tranexamic acid hydrogel with releasing accelerants

Only lactic acid was used as a releasing accelerant in the formula M35 hydrogel, whereas N-methylpyrrolidone (NMP) was used in both formulae M35 and J33. Either lactic acid or NMP was added in a step following the addition of all liquid ingredients. This was done

before the liquid mixture was heated. Another procedure of lactic acid addition was the same as that of M35 preparation and another procedure of NMP addition was the same as M35 and/or J33 preparation. The compositions of these new preparations (P1, P4, P7, P8, P9, P10 and P11) are shown in tables 3.2.

Table 3.1 There main formulae of tranexamic acid hydrogel.

Component	Amount (g in 5g)		
	M35	M38	J33
HPMC E4M	0.75	0.70	-
HPMC E50	-	-	0.59
Distilled water	3.10	2.63	2.16
Tranexamic acid powder*	0.21	0.21	0.21
Propylene glycol	0.25	0.21	0.21
Methyl paraben	0.01	0.02	0.02
Phenoxy ethanol	0.04	0.04	0.04
8%w/w DC7-9245	0.17	0.17	-
50%w/w DC7-9245	-	-	0.34
DC 200/350	-	0.17	0.17
10%w/w PVP K90	0.47	0.17	-
30%w/w Acrylax®	-	0.68	-
6% w/w Carbopol® 980 NF	-	-	1.26

*0.21 g of tranexamic acid in 5 g hydrogel equals to 4.20 % of drug content

3.3.2 The pH measurement

The pH of tranexamic acid matrix was directly measured using pH meter three times at room temperature and the average pH was recorded.

Table 3.2 The component of tranexamic acid hydrogels with various amount of releasing accelerants

Component	Amount (g in 5g)						
	P1	P4	P7	P8	P9	P10	P11
HPMC E4M	0.75	0.75	0.75	0.75	-	-	-
HPMC E50	-	-	-	-	0.59	0.59	0.59
Distilled water	3.10	3.10	3.10	3.10	2.16	2.16	2.16
Tranexamic acid	0.21	0.21	0.21	0.21	0.21	0.21	0.21
Propylene glycol	0.25	0.25	0.25	0.25	0.21	0.21	0.21
Methyl paraben	0.01	0.01	0.01	0.01	0.02	0.02	0.02
Phenoxy ethanol	0.04	0.04	0.04	0.04	0.04	0.04	0.04
8%w/w DC7-9245	0.17	0.17	0.17	0.17	-	-	-
50%w/w DC7-9245	-	-	-	-	0.34	0.34	0.34
DC 200/350	-	-	-	-	0.17	0.17	0.17
10%w/w PVP K90	0.47	0.47	0.47	0.47	-	-	-
30%w/w Acrylax®	-	-	-	-	-	-	-
6% w/w Carbopol® 980 NF	-	-	-	-	1.26	1.26	1.26
Lactic acid	0.25	0.50	-	-	-	-	-
N-methylpyrrolidone	-	-	0.21	0.42	0.42	0.63	0.21

3.3.3 Spectrofluorimetric determination of tranexamic acid

3.3.3.1 Reagent and dilution medium preparation

Solution of NDA (5.36 mM) was prepared weekly in methanol and stored at 4°C protected from light. Aqueous solutions of KCN (5.36 mM) and disodium tetraborate buffer (pH 9.5, 50 mM) were prepared as required. The dilution medium for tranexamic acid was a phosphase-buffer saline solution (PBS, pH 7.4, 0.15 M), which was prepared by mixing 0.80, 0.02, 0.115, 0.02 %w/v of NaCl, KCl, Na_2HPO_4 and KH_2PO_4 respectively, in deionized water.

3.3.3.2 Derivatization procedure for tranexamic acid standard and sample solutions

Derivatization of tranexamic acid was performed in test tubes by adding 50 µl of tranexamic acid standard or sample solution in a 1.85 ml of pH 9.5 disodium tetraborate buffer, then a 50 µl of NDA and a 50 µl of KCN were sequentially added. The final concentrations of both NDA and KCN were 0.134 mM. The solution was reacted for 5 minutes at room temperature that was 29°C (35) before transferring to a normal size cuvette for fluorimetric detection

3.3.3.3 Preparation of solutions for calibration curve

The highest concentration of tranexamic acid solution was 0.53 mM (84.0 µg/ml). Six solutions were prepared at various concentrations as followed; 8.4, 16.8, 33.6, 50.4, 67.2 and 84.0 µg/ml, respectively. The procedure was started by preparing a stock solution of tranexamic acid at the concentration of 26.72 mM (4.20 mg/ml). The exact amount of tranexamic acid powder (0.42 g) was dissolved in 0.15 M PBS, pH 7.4. The solution was adjusted to the volume of 100-ml in a volumetric flask with PBS buffer. Then a 0.50 ml of a stock solution was pipetted into a 25 ml volumetric flask to attain 84.0 µg/ml solution (referred as a standard solution A). The standard solution was diluted into five other concentrations by pipetting 0.5, 1.0, 2.0, 3.0 and 4.0 ml of the standard solution A in each of 5-ml volumetric flask. The series of final concentration were 8.4, 16.8, 33.6, 50.4 and 67.2 µg/ml respectively. These working standard solutions were included in an assay validation. The assay was carried out in triplicates.

3.3.3.4 Fluorescence detection

The measurement of tranexamic acid samples and solutions for calibration curve examination was performed by spectrofluorometer with 150W Xenon lamp. The measurement conditions, excitation and emission spectral bandwidths, were set up to 5 nm. The excitation wavelength was 420 nm and emission wavelength was 480 nm. PMG gain was assigned to the "very low" level.

3.3.3.5 Assay validation

Accuracy and precision examination

The intensity of each standard solution of tranexamic acid for each calibration curve was measured in triplicates. The detection was carried out in 5 times of its triple measurements to examine the inter-day accuracy and precision. Only one of the measurements was carried out 5 times in the same day to examine for the intra-day accuracy and precision.

Method selectivity examination

The selectivity of determination method was investigated by the examination of derivative stability. Stability of the derivative was monitored for 30 minutes. After reacting a 50.4 μg/ml tranexamic acid with the reagents, the fluorescence intensities of the derivative were measured at predetermined time intervals compared with the response obtained from the reagent blank.

Limit of detection of tranexamic acid

The series of tranexamic acid samples diluted to various concentrations, blank and reagent blank were detected by spectrofluorometer. The blank was only 50 mM pH 9.5 disodium tetraborate buffer solution and the reagent blank consisted the three components of 0.15 M pH7.4 PBS, NDA and KCN solutions. The measurement was terminated when the minimum concentration of sample showing its intensity had been found. However, the signal of acceptable concentration shown as intensity value needs to be 3 times more than the signal of reagent blank.

3.3.4 Quantitative analysis of tranexamic acid in hydrogels

3.3.4.1 Examination of gel base influence

Since hydrogel matrix consists of tranexamic acid, an active ingredient, and its gel base that probably interferes with the detection of tranexamic acid by spectrofluorimetry. Therefore, it is necessary to examine the interference of the gel base.

To achieve the examination, the gel solutions were prepared by two methods; 1) spiking method and 2) the direct-dissolved gel from a patch. The gel solutions prepared from both methods were compared in terms of percent of drug recovery and drug content.

For spiking method, an exact amount of tranexamic acid was placed in each solution of gel base. Three solutions of different gel base consisted in M35, M38 and J33 with added tranexamic acid were defined as M35s, M38s and J33s respectively. In each sample preparation, the 0.0084 g of tranexamic acid powder and 0.1916 g of gel base were placed in 100 ml volumetric flask. The PBS was added to dissolve the compounds and adjusted to the volume.

The direct examination of gel from a patch was carried out by dissolving each cut piece of gel of M35, M38 and J33 patches. Each solution was prepared from 0.20 g of a gel formulation dissolved in PBS. The volume of solvent was adjusted to 100 ml in volumetric flask.

Standard solution of tranexamic acid in concentration of 84.0 μg/ml was assumed to be 100% of drug recovery. Therefore, its average intensity detected by spectrofluorometer was compared with the average intensities of those solutions that were prepared by two methods.

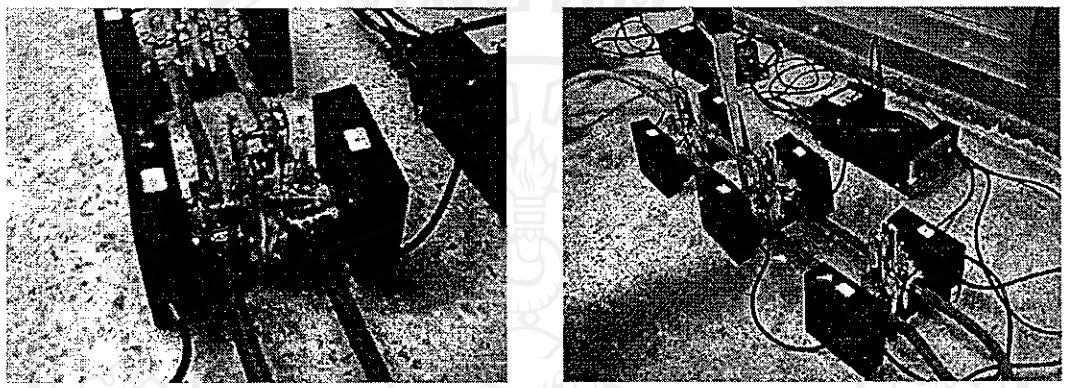
3.3.4.2 Tranexamic acid content in the patches

The gel part of the patch was cut by spatula and weighed accurately to 0.20 g. PBS was used for dissolving the patch sample in 100 ml volumetric flask. During solubilized process, the mixed compound had to be kept at least 12 hours for the complete dissolve before the volume of solvent was adjusted. Every sample was diluted 50 times in order to fit within a range of calibration curve. The diluted sample was derivatized with NDA for the next process. The intensity measurement of samples was carried out in triplicate.

3.3.4.3 Release study of tranexamic acid from the hydrogels

A horizontal diffusion cell was utilized for the release experiment. One diffusion system

consisted of a hydrogel patch and a diffusion cell. A pair of diffusion cells were arranged side by side and independently functioned as a receptor compartment for each patch sample. Tranexamic acid patch samples were carefully mounted back to back between the two cells. Figure 3.2 show the diffusion systems performed by this equipment.



a b

figure 3.2 a) Arrangement of horizontal diffusion cells with the patches, b) the diffusion systems and fluid sampling during the diffusion process.

The capacity of the receiver compartment is 2.5 ml. A soak cellophane membrane was used as a barrier between matrix on the patch and receiver medium. The receiver compartment was filled with 0.15 M pH7.4 PBS at 32 °C. Diffusion surface area of horizontal cell is 1.54 cm^2. 1.00 ml of sample solution was withdrawn periodically (10, 20, 30, 60, 90 and 180 minutes) from the receptor compartment and the same volume of 0.15-M pH7.4 PBS was added after sampling. The tranexamic acid concentration in each sample was derivatized and determined by spectrofluorometer. All release experiments were carried out in triplicate and the results are expressed as percentage of release amount.

3.3.5 Stability evaluations

The stability of tranexamic acid patches had been evaluated from several factors which were tranexamic acid content, release profiles appearance, thickness and adhesive property of the patches.

3.3.5.1 Tranexamic acid content examination

The formulae M35, M38 and J33 were sealed in laminated aluminum foils and stored for 4 months in 3 conditions; 4°C, room temperature (RT = 29°C) and 45°C. The content of tranexamic acid in these 3 formulae had been determined since the first day of the storage. The intervals of an examination were divided into 1, 30, 60, 90 and 120 days. The drug content was carried out by the protocol mentioned in 3.3.4.2.

3.3.5.2 Release profile examinations

The release profiles of M35, M38 and J33 formulas were studied on the first day of preparation and again on the last day of storage (day 120th). Each tranexamic acid patch prepared for release study was stored in the same condition as the patch for drug content examination.

3.3.5.3 Thickness of tranexamic acid patch

The thickness of tranexamic acid patch was measured by micrometer on the first and the last days of the storage period. To ease of the measurement, a patch was sandwiched between two small thin glass plates. The net thickness of a patch can be calculated from the different results of a whole patch with two glasses and a pair of glasses alone.

3.3.5.4 The appearance

The colour and texture of M35, M38 and J33 patches were observed on the first day of preparation and compared with their 120 days-stored patches.

3.3.5.5 Adhesive property

M35, M38 and J33 patches were wrapped in laminated aluminum foils and kept in zip-locked plastic bags for 4 months at room temperature. The relative adhesion of the hydrogel was

determined as the distance of the metal ball traveling on the gel surface of a patch that was experimented by a manual model (figure 3.3). The distances were finally converted to the friction coefficient (μ). The model has 10 degrees slope. Small metal ball, 0.62 mm diameter and 1.05 g weight, was rolled down to a 6 cm slope from its starting point to the edge of attached gel. The ball was continually allowed to roll on horizontal direction until it stopped. The distance between the edge of gel attached to the slope and roll-ended point of a ball was reported. Each patch was measured by the intervals of 1, 3, 7, 14, 28, 60, 90 and 120 days. The 120 days-stored patches of M35, M38 and J33 formulae stored in both 4°C and 45°C condition were also measured the adhesive property by this model.

The friction efficiency was calculated from an equation below;

$$\mu = h/S \qquad [3.1]$$

where μ is the friction efficiency, h is the height of starting point and S is measuring length or the distance between an attached gel to a point where the ball stopped on a patch.

The approximate adhesion time was evaluated by the investigator. Each patch was applied on a forearm and allowed to attach the skin until the slip was observed.

3.3.6 Irritation test

Nine volunteers (3 males and 6 females), age 20 to 54 years old, had been applied the patches for 24 hours. The formulas J33, P9, P10, P11 and a gel base of J33, used as a control formula, were applied on inner side of the upper arm of each volunteer. The patches were secured in place with the plaster bandages. The irritation was evaluated from redness and/or scaling with the visual scoring by the investigator on the occluded positions after the patches were applied and when the patches were removed within 2 hours (day1). Then those positions were observed again after removal of the patches in 24 hours (day2).

The visual scores are demonstrated as follow (36);

0	=	No reaction
?	=	Very weak erythema or scaling
1	=	Mild erythema or scaling across most of the patch test area

2 = Distinct erythema and/or oedema

3 = Erythema and oedema spreading outside the test area

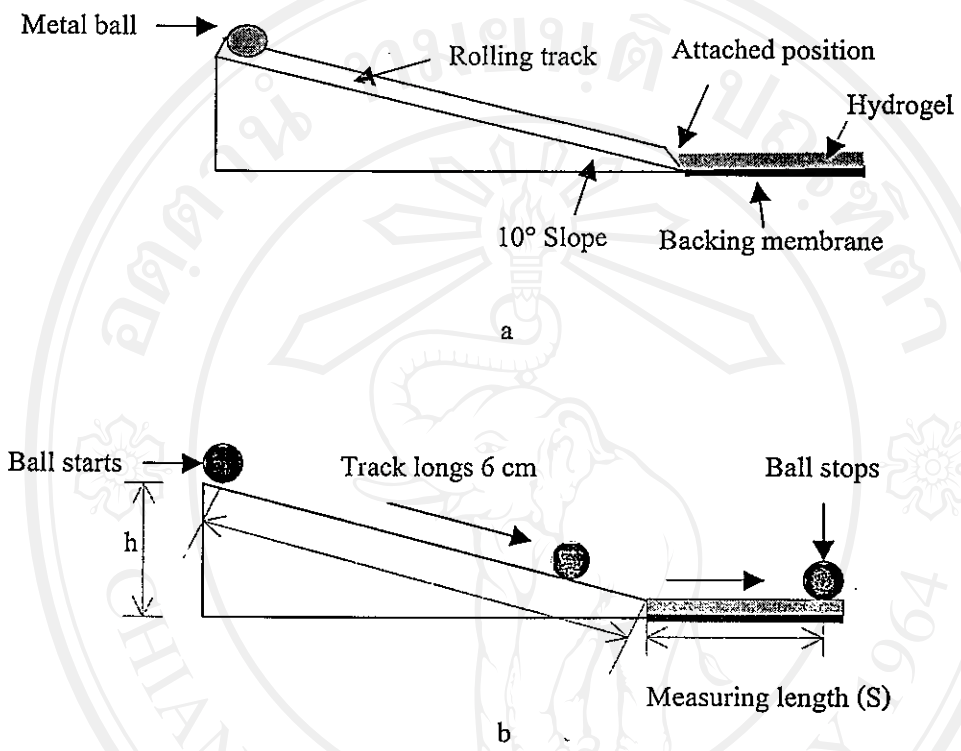


Figure 3.3 Diagrams of a) adhesive test ınodel b) The distance measurement of a ball on hydrogel surface.