CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Chitosan (squid type) (Ta Ming Enterprises Co., Ltd., Thailand)

L(+)-lactic acid (min. assay 88%) (Carlo Erba, Italy)

Vancomycin hydrochloride for injection, Edicin (Lex Pharmaceutical, Slovenia)

Tears Naturalle II (Alcon Laboratories, Belgium)

Reference standard of vancomycin hydrochloride (Sigma Chemical Co., USA)

Sodium Chloride (Dominion salt Ltd, New Zealand)

Boric acid (BL-Hua, Thailand)

Sodium Tetraborate (Borax) (Ajax, New Zealand)

Standard buffer solution pH 4 and 7 (Merck, Germany)

Mueller-Hinton broth (Merck, Germany)

TDx vancomycin reagent (Abbott, USA)

TDx vancomycin calibration (Abbott, USA)

TDx vancomycin control (Abbott, USA)

TDx buffer diluent (Abbott, USA)

3.1.2 Animals

New Zealand white rabbit, male, weight 2,500-3,500 gm (The National Laboratory Animal Centre, Nakornpathom, Thailand)

3.1.3 Microorganism

The bacterial strain used in this study was *Staphylococcus aureus* American Type Culture Collection (ATCC) 29213.

3.1.4 Equipments

Abbott TDX analyzer (Abbott, USA)

UV- visible spectrophotometer, 160A (Shimadzu, USA)

pH meter, EA 920 (Orion, USA)

Osmometer, Osmomat 030 (Gonotec, Germany)

Incubator, UE 400 (Memmert, Germany)

Automatic Viscosity Measuring System, AVS 310 (Schott-Geräte, Germany)

Heating vacuum desicator (Gallenkamp, England)

3.1.5 Other Materials

TDx cartridge sample (Abbott, USA)

TDx cuvettes (Abbott, USA)

Microcap 2.0 µl (Alttech, USA)

3.2 Methods

3.2.1 Determination of loss on drying [84]

The loss on drying (% by weight) of chitosan was determined by heating at 60°C for 24 hours to a constant weight in a vacuum oven (Figure 3.1) and calculating the weight loss. The loss on drying was then obtained from:

$$Loss\ on\ drying \quad = \quad \frac{weight\ before\ drying - weight\ after\ drying}{weight\ before\ drying} \times 100\%$$

Due to its nature as a hydrogel, chitosan absorbs a significant amount of moisture when exposed to air. This absorption continues until the chitosan reaches its equilibrium water content (EWC) according to ambient conditions (temperature, pressure, relative humidity).

Figure 3.1 Heating vacuum desiccator.

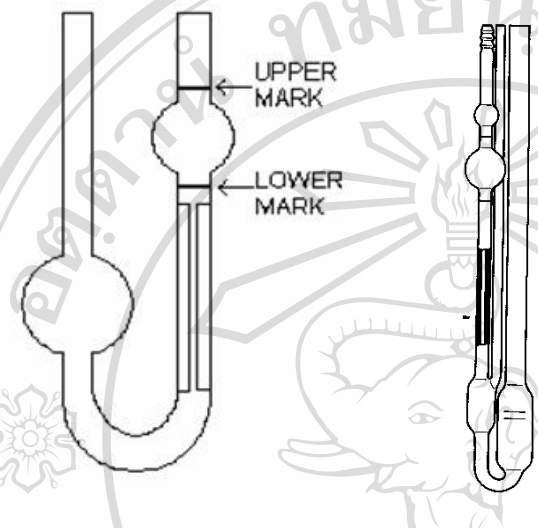
3.2.2 Determination of viscosity-average molecular weight ($\overline{M}_v$)

Principles and Theory of Dilute-Solution Viscometry [85]

Dilute-solution viscosity is one of the simplest and most widely used techniques for routinely determining polymer molecular weights. It is known as a secondary method for molecular weight determination, that is to say, it is not an absolute method like primary ones (e.g., light scattering, ultracentrifugation, osmometry). However, this method has the important advantages of being easy to perform and requiring relatively inexpensive equipment.

In dilute-solution viscometry, the polymer is dissolved in an appropriate solvent and the viscosity is measured. When a material of high molecular weight is dissolved in a suitable solvent, the viscosity of the resulting solution (polymer + solvent) is greater than that of the solvent alone. The magnitude of the viscosity increase depends upon the concentration, length, geometry and flexibility of the polymer molecules, as well as the polymer solvent interactions.

Dilute-solution viscosities are usually measured at concentrations of up to 1 g per 100 ml of solvent (1% w/v) by determining the flow-time of a certain volume of solution through a fixed length capillary. Two typical viscometers are shown in Figure 3.2



(a) (b)

Figure 3.2 Viscometers commonly used in dilute-solution viscometry.
(a) Ostwald viscometer
(b) Ubbelohde viscometer

The device is placed in a bath at constant temperature. In dilute-solution viscometry, the following viscosity-related quantities are defined:

i) the relative viscosity or viscosity ratio (η_{rel}) is expressed as the ratio of the solution viscosity, η, to the solvent viscosity, η_0, which, to a good approximation, is equal to the ratio of the flow-time solution (t) to the flow- time of the solvent (t_0).

$$\eta_{rel} = \frac{\eta}{\eta_0} = \frac{t}{t_0} \qquad (3.1)$$

ii) the specific viscosity (η_{sp}) is the relative increment in viscosity of the solution over the viscosity of the solvent.

$$\eta_{sp} = \frac{\eta - \eta_0}{\eta_0} = \frac{t - t_0}{t_0} = \eta_{rel} - 1 \qquad (3.2)$$

iii) the reduced viscosity or reduced specific viscosity (η_{red}) is the specific viscosity per unit of concentration (c), where the units of c are usually g/dl (g/100 ml).

$$\eta_{red} = \frac{\eta_{sp}}{c} \qquad (3.3)$$

For polymer solutions, η_{sp}/c is generally found to increase with increasing c.

iv) the inherent viscosity (η_{inh}) is defined as:

$$\eta_{inh} = \frac{In\,\eta_{rel}}{c} \qquad (3.4)$$

v) the intrinsic viscosity $[\eta]$ is the reduced viscosity or the inherent viscosity extrapolated to infinite dilution (c=0).

$$[\eta] = \lim_{c=0} \eta_{sp} / c \qquad (3.5)$$

$$[\eta] = \lim_{c=0} (In\,\eta_{rel}) / c \qquad (3.6)$$

The intrinsic viscosity, $[\eta]$, is a function of the size of polymer molecules in the solution, polymer-solvent system, and temperature. All of these viscosity terms are collected together in Table 3.1. The concentration dependence on polymer solution viscosity is conveniently expressed by two empirical equations, known as the Huggins Equation, given by equation (3.7), and the Kraemer Equation, given by equation (3.8).

$$\eta_{red} = \frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c \qquad \text{(Huggins Equation)} \qquad (3.7)$$

$$\eta_{inh} = \frac{In\,h_{rel}}{c} = [\eta] + k''[\eta]^2 c \qquad \text{(Kraemer Equation)} \qquad (3.8)$$

where k' is the Huggins Constant

k'' is the Kraemer Constant

Table 3.1 Definitions and nomenclature of dilute-solution viscosity quantities [85]

Common Name	Official Name *	Quantity
Viscosity	Viscosity coefficient	η
Relative viscosity	Viscosity ratio	$\eta_{rel} = \eta / \eta_0 = t/t_0$
Specific viscosity	Specific viscosity	$\eta_{sp} = \eta_{rel} - 1$
Reduced viscosity	Viscosity number	$\eta_{red} = \eta_{sp} / c$
Inherent viscosity	Logarithmic viscosity number	$\eta_{inh} = (\ln \eta_{rel}) / c$
Intrinsic viscosity	Limiting viscosity number	$[\eta] = \lim_{c=0} \eta_{sp} / c$
Intrinsic viscosity	Limiting viscosity number	$[\eta] = \lim_{c=0} (\ln \eta_{rel}) / c$

*Official Names are those recommended by the International Union on Pure and Applied Chemistry (IUPAC)

In most cases, the value of k' is in the range of $0.3 < k' < 0.5$ and increases as the solvent power decreases. Furthermore, it can be shown that the values of k' and k'' are related through the simple equation:

$$k' - k'' = 0.5 \quad \ldots(3.9)$$

Thus, the two equations, (3.7) and (3.8), should yield linear plots against concentration, c, with their common intercept equal to $[\eta]$ at c=0, as shown in Figure 3.3, The double extrapolation facilitates an accurate estimation of $[\eta]$.

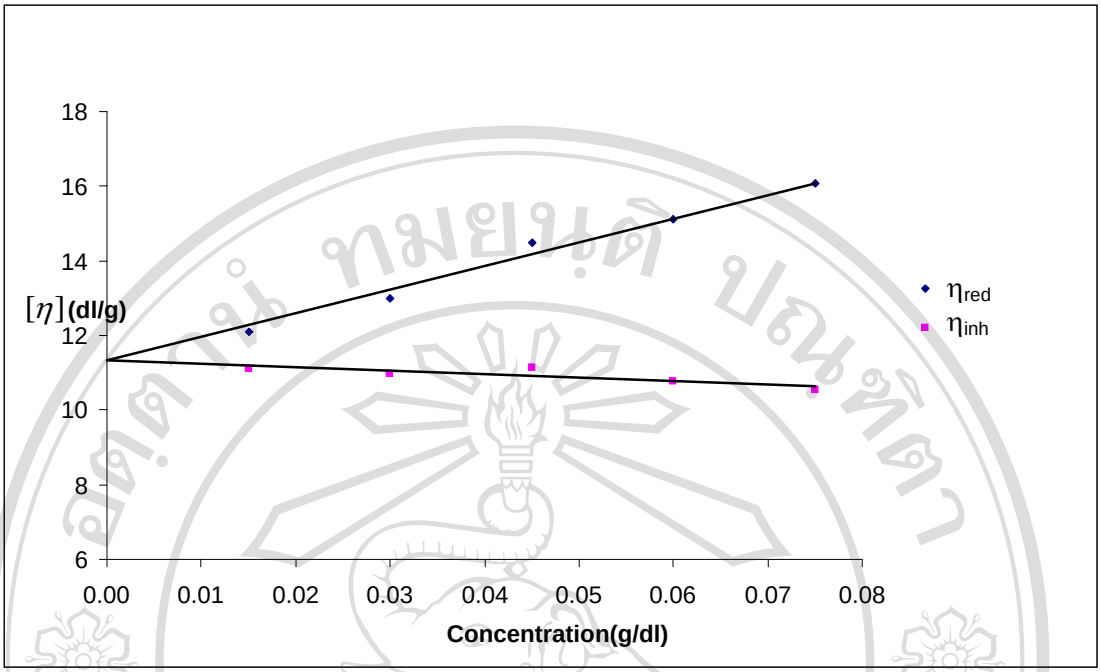


Figure 3.3 Reduced and inherent viscosity-concentration plots (data from this research).

The intrinsic viscosity, $[\eta]$, is related to the polymer molecular weight by the empirical Mark-Houwink-Sakurada Equation (3.10) :

$$[\eta] = K\overline{M}_v^a \quad \ldots(3.10)$$

where K and a are polymer-solvent interaction constants at a given temperature and usually obtained from the "Polymer-Handbook" [85], while $\overline{M}_v$ is the viscosity-average molecular weight.

The exponent 'a' in the equation (3.10) has a value within the range of $0.5 \leq a \leq 1.0$ and is used as a molecular conformation parameter. When a is > 0.8, the polymer molecule has an extended rod shape in the solution; for a = 0.6 – 0.8 (the most common range), and the molecule is shaped like a random coil, while for a < 0.6, the molecule is a very compact globular sphere.

In this research project, the chitosan was characterized further by determining its viscosity-average molecular weight ($\overline{M}_v$) by dilute-solution viscometry using a Schott-Gerate AVS 300 Automatic Viscosity Measuring System (Figure 3.4). The solvent used was an aqueous solution containing 0.2 M acetic acid, 0.1 M sodium chloride and 4 M urea. A series of chitosan solutions were prepared with concentrations of 0.015, 0.030, 0.045, 0.060 and 0.075 g/dl. For flow-time measurements, 15 ml of each solution were accurately pipetted into a Ubbelohde-type viscometer (Type and Capillary No. 531 03/0C) and clamped vertically in a water bath at the constant temperature of 25.0 ± 0.1° C. At least 15 minutes were allowed for temperature equilibration before flow-time measurements were made. The value of $\overline{M}_v$ was calculated from the following equation (3.10) [86]:

$$[\eta] = 8.93 \times 10^{-4} \overline{M}_v^{0.71} \; dl/g \qquad \ldots(3.11)$$

where $[\eta]$ is the intrinsic viscosity of the chitosan in units of dl/g.

Figure 3.4 The Schott-Gerate AVS Automatic Viscosity Measuring System used in dilute-solution viscometry showing, from left to right, the Ubbelohde viscometer, main control unit, and water bath at constant temperature.

3.2.3 Determination of the degree of deacetylation

Although, both chitin and chitosan are copolymers of N-acetylglucosamine and glucosamine, the chemical and physical properties of chitin and highly deacetylated chitosan are very different. Therefore, accurate determination of the degree of deacetylation (% DD) is an essential part of chitosan characterisation. Many methods have been developed to determine analyticial accuracy. Also, advanced analyticial methods such as IR and ^{13}C-NMR spectrometry can be used to measure the % DD [85]. The degree of deacetylation, DD, of the chitosan was determined by a chemical titration method following the procedure described by Hayes and Davies (1978) [87]. Pre-dried chitosan was dissolved in 10% aqueous acetic acid and chitosan hydrochloride precipitated by dropwise addition of hydrochloric acid. From the titration of 10 ml of a known weight solution comprising hydrochloride dissolved in 100 ml of distilled water with 0.1 M sodium hydroxide solution, the DD of the original chitosan was calculated from :

$$DD = \frac{(C \times V_1 \times V_2 \times MW \times 100)}{(1000 \times V_3 \times W)} \ \%$$

where C is the exact molar concentration of the sodium hydroxide (≈0.1),

V_1 is the volume of sodium hydroxide (ml), V_2 is the made-up volume of the chitosan hydrochloride solution in ml (=100),

MW is the molecular weight of chitosan hydrochloride (=197.5),

V_3 is the volume of the chitosan hydrochloride solution in ml (=10), and

W is the weight of chitosan hydrochloride dissolved in V_2 (g).

3.2.4 Preparation and characterization of chitosan solution

The preparation method for the chitosan solution was taken from the literature, as described by Leesawat et al. [88]. Chitosan 1% w/v was dissolved in 1% aqueous L(+)- lactic acid (Carlo Erba, 88%) at room temperature with magnetic stirring (Figure 3.5). The solution was then diluted to 0.1% and 0.3% w/v using Feldman buffer for ophthalmic preparations (Table 3.2) and sterilized by autoclaving at 121 ^{o}C and 15 psi for 15 mins. The osmolalities of these 0.1 % and 0.3% chitosan solutions

were determined by an Osmomat 030 (Figure 3.6). The stabilities of the solutions were evaluated in terms of their intrinsic viscosity ($[\eta]$) changes during storage at 2-8 ^{o}C and 30 ^{o}C.

These storage temperatures were chosen according to the standard procedure described in the United States Pharmacopeia (see Appendix C). For the lower storage temperatures of 2-8 ^{o}C, the actual temperature used in these experiments was 4 ^{o}C.

Figure 3.5 Preparation of chitosan solution.

Figure 3.6 Osmomat 030.

Borate buffer (Feldman buffer for ophthalmic preparations)

This study used Feldman buffer for ophthalmic preparations, which had the composition of acid stock solution and alkaline stock solution pH 5.0-8.2 (Table 3.2).

ACID STOCK SOLUTION

Boric acid	12.368	gm
Sodium chloride	2.925	gm
Purified Water, q.s	1,000	ml

ALKALINE STOCK SOLUTION

Sodium borate decahydrate	19.07	gm
Purified Water, q.s	1,000	ml

Table 3.2 Feldman buffer for ophthalmic preparations

pH	Boric Acid Solution, ml	Sodium Borate Solution, ml
5.0	100	0
6.0	100	0.4
7.0	95	5
7.1	94	6
7.2	93	7
7.3	91	9
7.4	89	11
7.5	87	13
7.6	85	15
7.7	82	18
7.8	80	20
7.9	76	24
8.0	73	27
8.1	69	31
8.2	65	35

3.2.5 Preparation of ophthalmic formulations

Ophthalmic solutions were prepared extemporaneously by dissolving vancomycin (as the hydrochloride salt) sterile powder at 500 mg in 10 ml of Tears Naturale II™ (i.e., to a concentration of 50 mg/ml) and then placing into Tears Naturale II™ containers. Similarly, the vancomycin sterile powder at 500 mg was dissolved in 10 ml of 0.9% w/v aqueous sodium chloride, and the 0.1% and 0.3% w/v chitosan solutions to a final concentration of 50 mg/ml, and placed into sterile eye drop containers (Table 3.3). All processing was performed using the aseptic technique in a clean room with a laminar air flow hood.

Table 3.3 Composition of each formulation

Formulation	Composition			
	Vancomycin (mg/ml)	Tears Naturale II™ (ml)	Chitosan Solution (ml)	Sodium Chloride solution (ml)
A	50	10	-	-
B	50	-	10 (0.1%)	-
C	50	-	10 (0.3%)	-
D	50	-	-	10 (0.9%)

3.2.5.1 Design of compatibility and stability studies

The compatibilities and stabilities of the 50 mg/ml of vancomycin eye drops in formulation A, B, C and D were examined by absorbance (UV Spectrophotometer, Shimadzu), and pH at days 0, 3, 7, 10, 14, 21 and 28 (day 0 = immediately following preparation). The samples were divided into 2 groups: Group I (n=10) was stored at 2-8 ^{o}C in a refrigerator and Group II (n=10) at 30 ^{o}C in an incubator.

3.2.5.2 Validation of the UV spectrophotometer

A standard stock solution of vancomycin at 50 mg/ml was prepared for validating the vancomycin in the Tears Naturale II™, 0.9% sodium chloride and the 0.1% and 0.3% w/v chitosan solutions. A further 6 solutions were prepared by dilution of 6, 7, 9, 10, 13 and 18 µl of the vancomycin at 50 mg/ml stock solution, with distilled water and the volumes adjusted to 4 ml. Thus, solution concentrations of 75, 87.5, 112.5, 125, 162.5 and 225 µg/ml were obtained for the construction of a calibration curve. The precision and accuracy of standard vancomycin determination in Tears Naturale II™**,** 0.9% sodium chloride, and the 0.1% and 0.3% w/v chitosan solutions were tested by diluting 8, 14 and 17 µl of their vancomycin at 50 mg/ml stock solutions, with distilled water and the volumes adjusted to 4 ml to obtain concentrations of 100, 175 and 212.5 µg/ml. Each solution was then analysed by UV spectrophotometry by measuring the absorbance at 282 nm.

3.2.5.3 Determination of vancomycin concentration and pH

The method to determine the vancomycin concentration used was modified from the UV spectrophotometer in ceramic implants, heparin lock and extemporaneous eye drops [89-91]. This method was validated by following the United States Pharmacopeia (USP) [92]. The pH of vancomycin was determined by a pH meter.

3.2.6 Minimum inhibitory concentration analysis

Minimum inhibitory concentration (MIC) was determined by a broth dilution method according to CLSI 2005 guidelines [93] . Ophthalmic solutions were prepared extemporaneously in a Class 100 clean-room environment by dissolving vancomycin sterile powder at 500 mg in 10 ml of Tears Naturale II™, 0.9% sodium chloride, and the 0.1% and 0.3% chitosan solutions to give a final concentration in each solution of 50 mg/ml. A control vancomycin at 50 mg/ml solution was prepared by dissolving vancomycin at 500 mg in 10 ml of 0.9% sodium chloride solution.

Each stock solution was divided into two halves for storage at room temperature (30 ^{o}C) and under refrigeration (2-8 ^{o}C), with testing on days 0 (day of preparation), 3, 7, 10, 14, 21 and 28. The control of vancomycin at 50 mg/ml was stored in a freezer and also tested on days 0, 3, 7, 10, 14, 21 and 28. On each test day, a bacterial suspension equal to a 0.5 McFarland turbidity standard (1.0 x 10^{8} CFU/ml)

was prepared in a Mueller-Hinton broth and diluted 100-fold (to 1.0 x 10^6 CFU/ml). The vancomycin solutions were diluted further with water for injection to a concentration of 250 µg/ml before serial dilutions with the Mueller-Hinton broth were carried out for tests in sterile test tubes closed with cotton plugs. Two-fold dilutions of vancomycin were prepared in Mueller-Hinton broth, as shown in Table 3.4. For each dilution tube, 0.5 ml of each bacterial suspension, and the antimicrobial agent, were incubated together at 35 ^oC in an aerobic environment for 24 hours. Standard quality control reference strains of *Staphylococcus aureus* ATCC 29213 (CLSI 2005), with sensitivity to vancomycin, were chosen for this study. The bacteria were transferred daily to ensure purity and good growth. On each test day, a bacterial suspension equal to the 0.5 McFarland turbidity standard was prepared in Mueller-Hinton broth. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic that yielded no growth in the Mueller-Hinton broth (Figure 3.7).

Table 3.4 Scheme for preparing dilutions of vancomycin for use in Mueller-Hinton broth (MHB) dilution susceptibility tests

Tube No.	Working solution (ml)	MHB (ml)	MHB from previous tube (ml)	Inoculum (ml)	Final concentration of vancomycin (µg/ml)
1	0.5	0.0	0.0	0.5	125.00
2	0.5	0.5	0.0	0.5	62.50
3	0.0	0.5	0.5	0.5	31.25
4	0.0	0.5	0.5	0.5	15.63
5	0.0	0.5	0.5	0.5	7.81
6	0.0	0.5	0.5	0.5	3.91
7	0.0	0.5	0.5	0.5	1.95
8	0.0	0.5	0.5	0.5	0.98
9	0.0	0.5	0.5	0.5	0.50
10	0.0	0.5	0.5	0.5	0.24
11	0.0	0.5	0.0	0.5	Positive control
12	0.5	0.5	0.0	0.0	Negative control

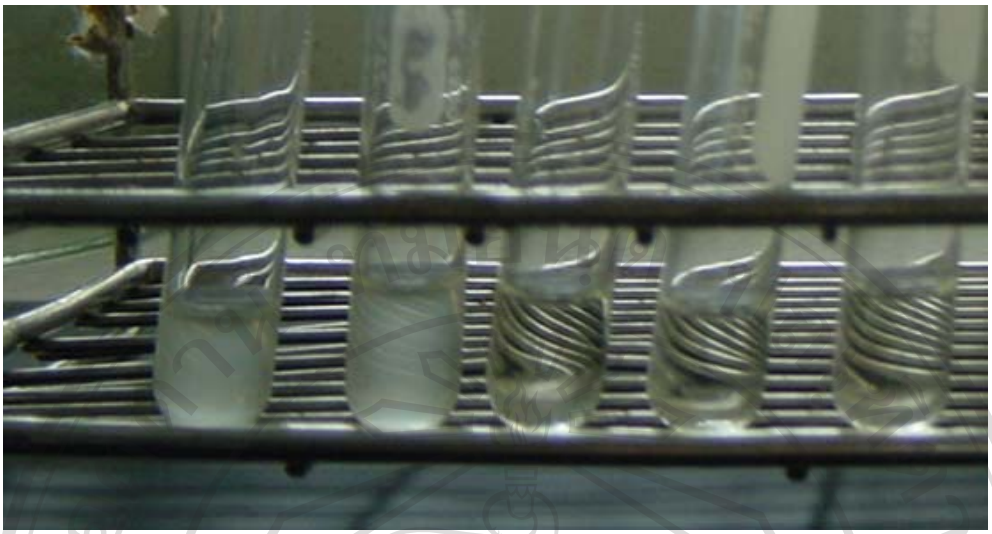


Figure 3.7 Growth (not clear) and no growth (clear) of bacteria.

3.2.7 Kinematic viscosity measurements

The kinematic viscosities of Tears Naturale II™, the 0.1% and 0.3% w/v chitosan solutions, 0.9% sodium chloride solution and their solutions containing vancomycin at 50 mg/ml were determined with a Schott-Gerate AVS 300 Automatic Viscosity Measuring System according to the German Standard Test Method DIN 51562 Part 1. For flow-time measurements, 15 ml of each solution were accurately pipetted into a calibrated Ubbelohde-type viscometer (Type and Capillary No. 532 10/ I) and clamped vertically in a water bath at a constant temperature of 25.0 ± 0.1 °C. At least 15 minutes were allowed for temperature equilibration before flow-time measurements were made. The kinematic viscosity, v, was calculated from the equation:

$$v = kt \qquad \text{(for Newtonian liquids)}$$

where v is the kinematic viscosity (mm^2/s), k is the viscometer constant (0.009679) (mm^2/s^2) and t is the flow-time (s). Flow-times were determined as the average of at least 3 readings, which all agreed to within ± 0.2% of the average value.

3.2.8 Pharmacokinetics of topically applied vancomycin in rabbit eyes

Animals

Male albino New Zealand rabbits weighing approximately 2.5-3.5 kg and free of any ocular damage were used throughout the study, as approved by the Ethics Committee for Animal Experimentation (Faculty of Medicine, Chiang Mai University, Thailand). The animals were maintained under conventional, standardized conditions in a single cage with free access to pelleted food and drinking water (Figure 3.8).

Figure 3.8 The animals were maintained under conventional, standardized conditions in a single cage with free access to pelleted food and drinking water.

3.2.8.1 Topical administration and sampling

Vancomycin at 50 mg/ml in formulation A, B, C and D were dropped into the rabbit's eyes (Figure 3.9-3.11). After applying one drop containing 25 µl of vancomycin at 50 mg/ml into the lower conjuctival eye sac, with care to avoid spillage, tear samples were obtained using 2.0 µl of calibrated glass capillaries (microcaps Drummond^{TM}). Tear samples were collected after 0, 30, 60, 90 and 120 minutes. Each formulation was tested on 6 rabbits.

Figure 3.9 Preparation of rabbits for applying eye drops.

Figure 3.10 Eye drops instilled into the rabbit's eye.

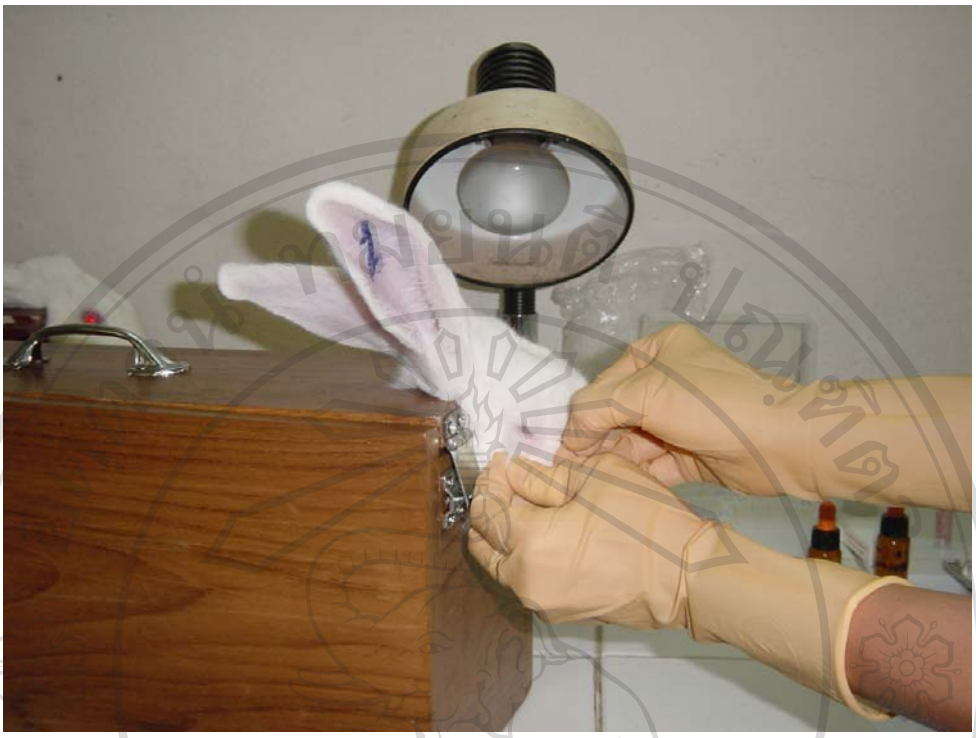


Figure 3.11 Tear samples were obtained using 2.0 µl of calibrated glass capillaries (microcaps Drummond™).

3.2.8.2 Determination of vancomycin concentration in tears

The concentrations of vancomycin in the tear samples were determined by fluorescent polarization immunoassay (TDx-FLx System Abbott, USA) (Figure 3.12) after tests were finished each day. Concentration of vancomycin in tear film was determined by a modified method of that by Alster et al. [94]. Volume of vancomycin from 2.0 µl of calibrated glass capillaries was diluted with TDx buffer diluent at 98 µl. Further dilution with TDx buffer was performed if the concentration of vancomycin was higher than the calibration curve.

Concentration of vancomycin with known concentrations of vancomycin, as provided by the manufacturer, was included in each run for calibration and quality control purposes. Concentration of vancomycin for controls contained 7, 35 and 75 µg/ml. Concentration of vancomycin for calibration was 0, 5, 10, 25, 50 and 100 µg/ml. Sensitivity of fluorescent polarization immunoassay for vancomycin was 2.0 µg/ml.

Figure 3.12 Fluorescent polarization immunoassay (TDx-FLx System Abbott, USA).

Statistical analysis

Different significant percentages of the labeled amounts between day 0 and days 3, 7, 10, 14, 21 and 28 at 30 ^{o}C and 2-8 ^{o}C were determined by using the SPSS 12.0 program for Windows, One-Way ANOVA, and Multiple-Comparison Post Hoc Test (n=10). Results with $p<0.05$ were considered as statistically significant.

Pharmacokinetic analysis

Pharmacokinetic calculations were performed on a personal computer using Microsoft Excel (Version 2003) with relevant add-ins (PK Functions for Microsoft Excel, JI Usansky, A Desai, and D Tang-Liu, Department of Pharmacokinetics and Drug Metabolism, Allergan, Irvine, Calif) [95]. Pharmacokinetic parameters were calculated by a non-compartment model.

Different significant areas under the curves between Tears Naturale II^{TM} and the other solvents were determined using the SPSS 12.0 program for Windows, One-Way ANOVA, and Tukey Test (n=6). Results with $p<0.05$ were considered as statistically significant.