CHAPTER 4
RESULTS AND DISCUSSION

4.1 Loss on drying of chitosan

The average loss on drying of chitosan in this study was 13.50 % (Table 4.1).

Table 4.1 Weight data and calculated loss on drying of chitosan

Analysis No	Chitosan weight before heating (gm)	Chitosan weight after heating (gm)	Weight loss (gm)	Loss on drying (%)
1	1.2938	1.1353	0.1585	12.25
2	1.5662	1.3425	0.2237	14.28
3	1.3780	1.1885	0.1895	13.75
			Average	**13.50**

Chitosan is hygroscopic in nature [96], hence it is affected by moisture absorption during storage. Therefore, chitosan should be kept in a closed container in a cool and dry location. Chitosan solution is prepared from the calculated weight of chitosan raw material minus the weight of chitosan loss on drying.

4.2 Viscosity-average molecular weight ($\overline{M}_v$) of chitosan

The viscosity-average molecular weight ($\overline{M}_v$) of chitosan was 6.03 x 10^5 (Run No.1).

$$[\eta] = (\eta_{red})_{c=0} = (\eta_{inh})_{c=0} \quad 11.45\ dl/g$$

In Table 4.2 and Figure 4.1, the value of [η] is estimated at 11.45 dl/g (Run No.1).

- Dilute-solution viscosity data and calculated parameters for chitosan solution at 25.0 $\pm$ 0.1°C (Run No.2 and 3) are shown in appendix A.

- Graph of reduced viscosity, η_{red}, and inherent viscosity, η_{inh}, against concentration (Run No. 2 and 3) are shown in appendix A.

The average value of [η] and $\overline{M}_v$ for the chitosan raw material used in this research project was calculated as:

$$[\eta] = 11.45\, dl/g$$
$$\overline{M}_v = 6.03\, x\, 10^5$$

Table 4.2 Dilute-solution viscosity data and calculated parameters for chitosan solution at 25.0 $\pm$ 0.1°C (Run No.1)

Concentration (g/dl)	**Flow-time (s)**	**η_{rel}**	**η_{sp}**	**η_{red} (dl/g)**	**η_{inh} (dl/g)**
0.000	375.18	-	-	-	-
0.015	443.27	1.1815	0.1815	12.0991	11.1182
0.030	521.47	1.3899	0.3899	12.9973	10.9749
0.045	619.73	1.6518	0.6518	14.4849	11.1528
0.060	715.43	1.9069	0.9069	15.1150	10.7580
0.075	827.56	2.2058	1.2058	16.0769	10.5477

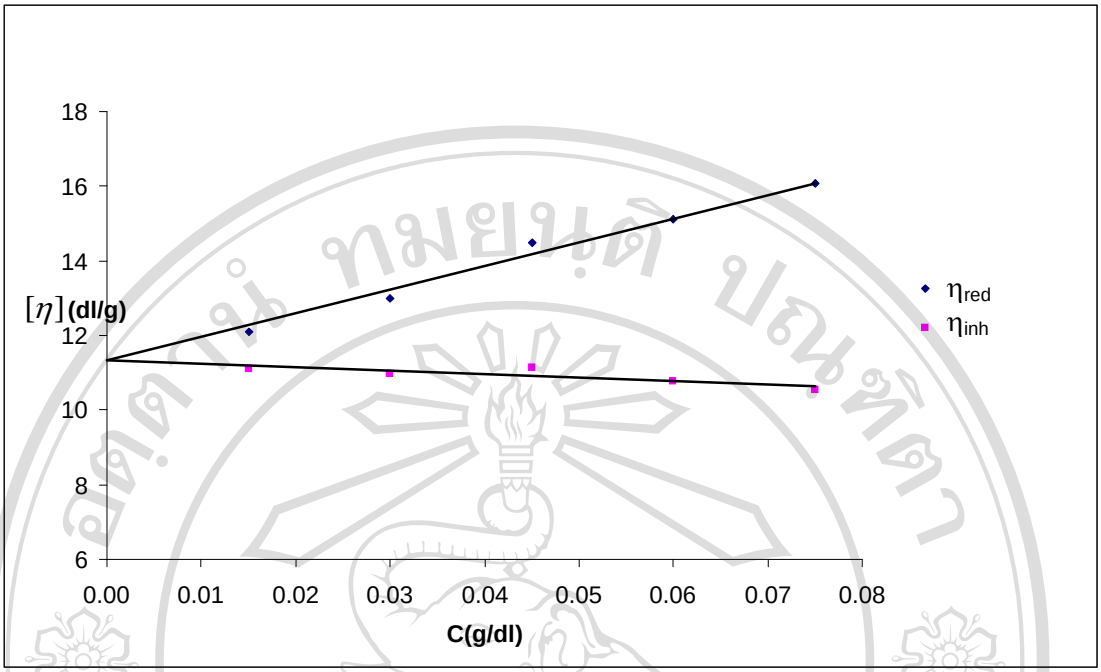


Figure 4.1 Graph of reduced viscosity, η_{red}, and inherent viscosity, η_{inh}, against concentration (Run No. 1).

4.3 Degree of deacetylation of chitosan

The average degree of deacetylation (DD) of chitosan was 94.0% (Table 4.3).

Table 4.3 Experimental results and calculated degrees of deacetylation (DD)

Analysis No.	Chitosan Weight (g)	Chitosan Solution Vol. (ml)	Concentration of NaOH (M)	Vol. Of NaOH (ml)	DD Value (%)
1	0.56	10.00	0.1	2.6	91.70
2	0.55	10.00	0.1	2.6	93.36
3	0.55	10.00	0.1	2.7	96.95
			Average DD =		94.00

It has been reported that the DD and MW are important chemical characteristics, which could influence the performance of chitosan in many of its applications [97, 98]. Application of chitosan in ocular drug delivery is proposed in this study. Mucoadhesion is the important mechanism to sustain drugs in the eye, when chitosan is used as a vehicle [17]. Mucus in the eye is composed a of glycoprotein called mucin, which is rich in negative charges, since it has sialic acid residues. When diluted in diluted acid, chitosan protonates the positive charge and can interact with mucin by electrostatic forces. The extent of this union depends on the amount of sialic acid present in the mucin and the MW and DD of chitosan. It has been found that when the MW of chitosan increases, the penetration in the mucin layer also increases and hence the mucoadhesion is stronger [18]. A higher DD leads to an increase in the charge density of the molecule, and adhesive properties become more relevant [98].

4.4 Preparation of chitosan solution

Chitosan 1% w/v was dissolved in 1% aqueous L(+)- lactic acid (Carlo Erba, 88%) at room temperature by magnetic stirring until completely soluble in 7 days. The solution was then diluted to 0.1% and 0.3% w/v using Feldman buffer for ophthalmic preparations (Table 4.4) and sterilized by autoclaving at 121 ^{o}C and 15 psi for 15 mins. The preparation and stability of 0.1% and 0.3% chitosan solutions were described by Khangtragool et al. [99]. The preparation of 0.1% chitosan solution was a modification of that of artificial tears from 0.1% chitosan solution [88]. The preparation of 0.3% chitosan solution was based on the concentration of artificial tears using hydroxypropyl methylcellulose at 0.3% w/v [100].

In general, ophthalmic solutions are formulated in the range of pH 4 to 8.0 [101]. If the pH of the dosage form is outside the physiological range (pH of 7.4 [102]), eye irritation or ocular discomfort may become an issue. Clear eye solution drops are more advantageous than aqueous suspensions or ointments that produce blurred vision. Chitosan solution of 0.1% and 0.3% were clear when diluted with Feldman buffer for ophthalmic preparations at pH 7.3 and 7.7, respectively. The pH of 0.1% and 0.3% chitosan, after dilution with Feldman buffer for ophthalmic preparations, was 5.95 and 4.56, respectively. After that, 0.1% and 0.3% chitosan

were sterilized by autoclaving. The pH values after autoclaving were 5.17 and 3.91. Increased pH was required to precipitate the chitosan, which had a pKa of 6.5 [50]. The pH of 0.1% and 0.3% chitosan after being autoclaved and stabilized are shown in Table 4.5.

Feldman buffer for ophthalmic preparations

The compositions of Feldman buffer for ophthalmic preparations were acid stock solution and alkaline stock solution in pH 5.0-8.2.

Table 4.4 Preparation of 0.1% and 0.3% chitosan solutions by using Feldman buffer for ophthalmic preparations

pH	Boric Acid Solution, ml	Sodium Borate Solution, ml	0.1 % chitosan solution	0.3 % chitosan solution
5.0	100	0	Clear	Clear
6.0	100	0.4	Clear	Clear
7.0	95	5	Clear	Clear
7.1	94	6	Clear	Clear
7.2	93	7	Clear	Clear
7.3	91	9	Clear	Clear
7.4	89	11	Not clear	Clear
7.5	87	13	Not clear	Clear
7.6	85	15	Not clear	Clear
7.7	82	18	Not clear	Clear
7.8	80	20	Not clear	Not clear
7.9	76	24	Not clear	Not clear
8.0	73	27	-	Not clear
8.1	69	31	-	Not clear
8.2	65	35	-	Not clear

Table 4.5 The pH stability of 0.1% and 0.3% chitosan solutions

Days	pH 0.1% chitosan (n=3)*		pH 0.3% chitosan (n=3)*	
	2-8 ºC**	30 ºC	2-8 ºC**	30 ºC
0	5.17	5.17	3.91	3.91
3	5.18	5.12	4.12	4.16
7	5.20	5.10	4.06	4.02
10	5.27	5.23	4.11	4.08
14	5.30	5.20	4.13	4.11
21	5.26	5.21	4.19	4.13
28	5.27	5.21	4.12	4.08
60	5.40	5.13	4.11	3.98
90	5.02	4.82	4.17	3.99
120	5.29	5.15	4.10	3.97
150	5.25	5.16	4.12	4.07
180	5.04	4.80	4.11	3.99
210	5.27	5.11	4.15	4.10
240	5.35	5.20	4.08	4.02
270	5.93	5.46	4.17	4.10
300	5.06	4.82	4.12	3.99
330	5.08	4.99	4.18	3.98
360	5.10	5.05	4.12	4.08

* pH readings accurate to ± 0.01

** according to standard method (see Appendix C)
actual experimental temperature = 4 ºC

4.4.1 Viscosity stability of 0.1% chitosan solution

The viscosity stability of 0.1 % chitosan solutions was studied at 2-8 ºC and 30 ºC (Table 4.6) and plotted on the graph in Figure 4.2 and 4.3.

Viscosity stability of 0.1% chitosan solution, determined by flow time and intrinsic viscosity [η] before autoclaving, was 235.0 seconds and 14.1 dl/g. Flow time of solvent (Feldman buffer for ophthalmic preparations) was 74.9 seconds.

Table 4.6 Viscosity stability of 0.1% chitosan solution

Days	Flow time 2-8 ºC (s) n=3	Flow time 30 ºC (s) n=3	[η] (dl/g) 2-8 ºC	[η] (dl/g) 30 ºC
0	175.4	175.4	9.90	9.90
3	173.3	173.1	9.75	9.73
7	171.6	172.0	9.61	9.64
10	171.6	171.9	9.61	9.64
14	171.2	170.4	9.58	9.52
21	170.9	169.2	9.56	9.42
28	171.2	168.6	9.58	9.38
60	136.9	133.1	6.70	6.36
90	137.5	132.5	6.76	6.30
120	140.1	135.2	6.99	6.55
150	139.5	133.3	6.93	6.39
180	137.7	131.5	6.78	6.21
210	145.8	138.4	7.49	6.84
240	150.5	141.1	7.89	7.08
270	169.9	161.7	9.48	8.82
300	169.4	162.1	9.44	8.86
330	169.4	161.8	9.44	8.83
360	170.1	161.2	9.50	8.78

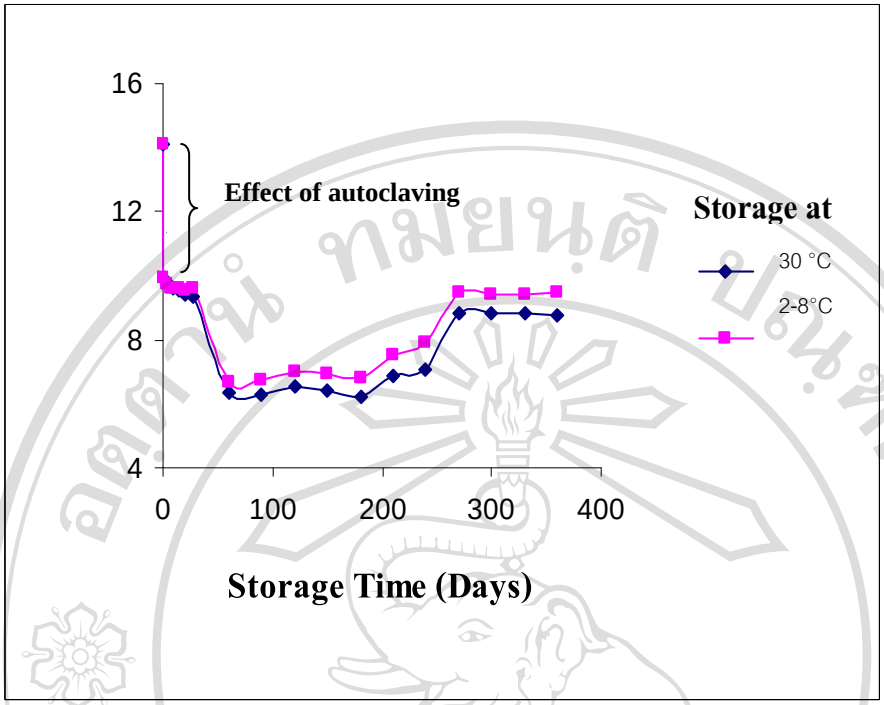


Figure 4.2 Variations in intrinsic viscosity,[η], of the 0.1% w/v chitosan solution with autoclaving and storage time at different temperatures.

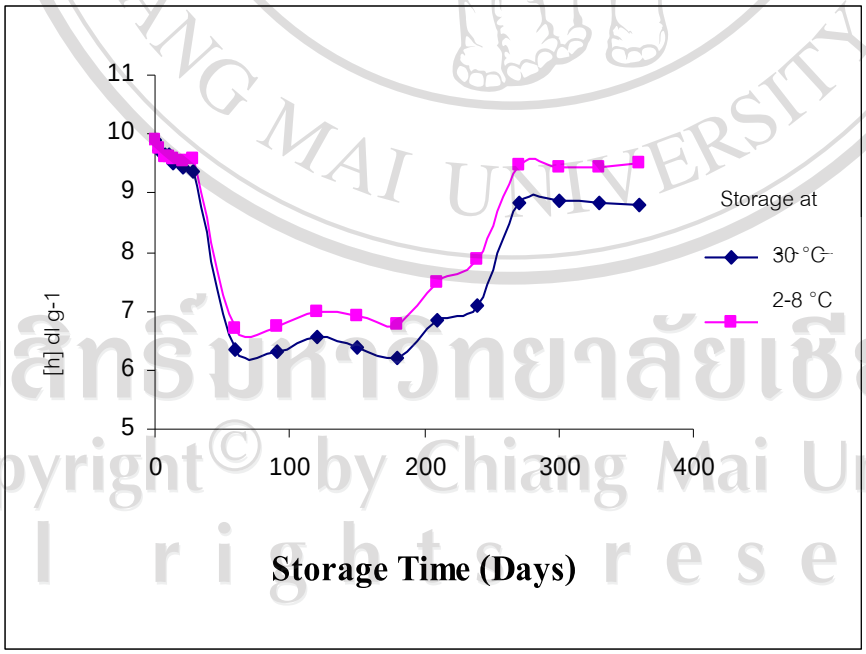


Figure 4.3 Variations in intrinsic viscosity,[η], of the 0.1% w/v chitosan solution with storage time only at different temperatures.

4.4.2 Viscosity stability of 0.3% chitosan solution

The viscosity stability of 0.3% chitosan solutions was studied at 2-8 ºC and 30 ºC (Table 4.7) and plotted on the graph in Figure 4.4 and 4.5.

Viscosity stability of 0.3% chitosan solution, determined by flow time and intrinsic viscosity [η] before autoclaving, was 2885.8 seconds and 25.11 dl/g. Flow time of solvent (Feldman buffer for ophthalmic preparations) was 87.8 seconds.

Table 4.7 Viscosity stability of 0.3% chitosan solution

Days	Flow time 2-8 ºC (s) n=3	Flow time 30 ºC (s) n=3	[η] dl/g 2-8 ºC	[η] dl/g 30 ºC
0	473.2	473.2	7.75	7.75
3	535.2	463.3	7.62	8.55
7	537.3	459.6	7.57	8.57
10	480.6	467.9	7.68	7.85
14	478.2	464.0	7.63	7.82
21	467.0	523.5	8.40	7.67
28	467.7	519.9	8.36	7.68
60	495.6	447.1	7.40	8.05
90	494.4	442.8	7.34	8.03
120	473.1	439.5	7.30	7.75
150	472.2	435.4	7.20	7.74
180	470.1	426.8	7.12	7.71
210	457.5	411.4	6.90	7.54
240	445.2	403.3	6.78	7.37
270	435.4	382.8	6.48	7.24
300	440.3	377.8	6.40	7.31
330	450.3	409.1	6.87	7.44
360	441.1	375.2	6.36	7.32

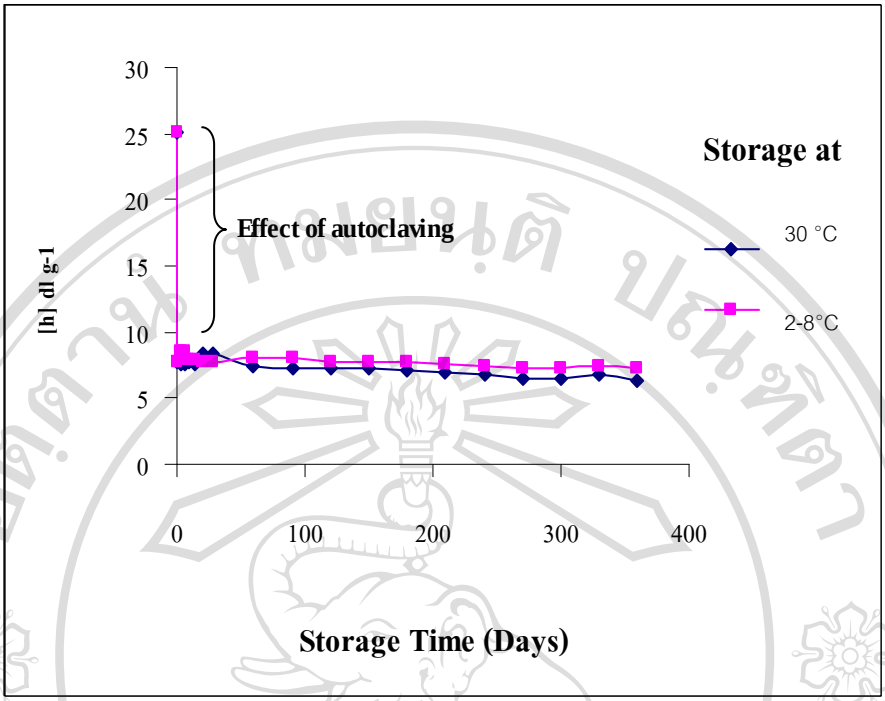


Figure 4.4 Variations in intrinsic viscosity,[η], of the 0.3% w/v chitosan solution with autoclaving and subsequent storage time at different temperatures.

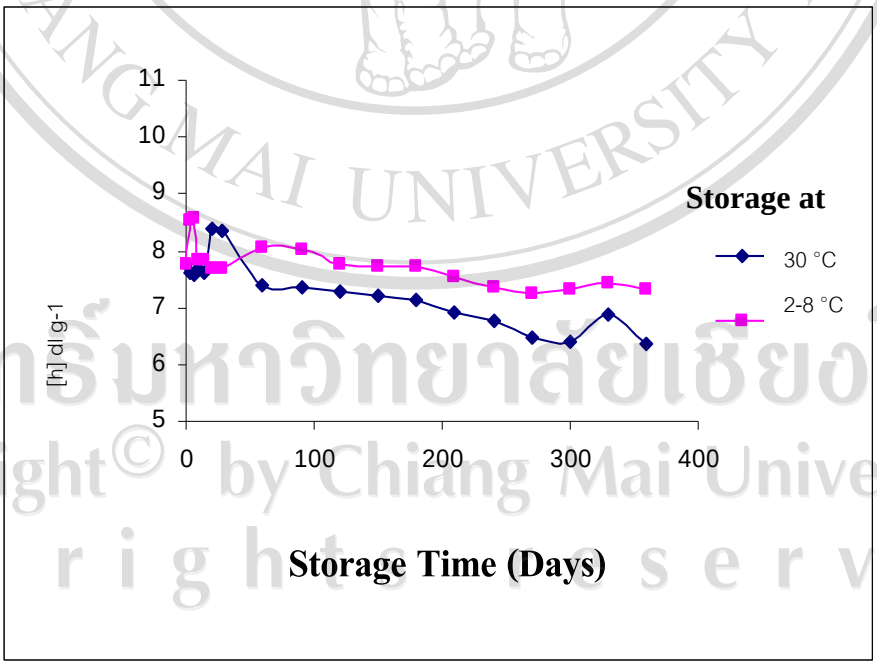


Figure 4.5 Variations in intrinsic viscosity,[η], of the 0.3% w/v chitosan solution with storage time only at different temperatures.

The storage stability of the chitosan solution was studied at two different temperatures: ambient (Asian) temperature (30 ºC) and refrigerated temperature (2-8 ºC) [103]. Stability was monitored in terms of intrinsic viscosity which, in turn, reflected changes in the chitosan molecular weight.

The decreases in intrinsic viscosity, [η], of the 0.1% and 0.3% w/v chitosan solutions, with sterilization and storage time at two different temperatures, are shown in Figure 4.2 and 4.4. The decreases are seen as biphasic, with an initially rapid sterilization phase followed by a much slower storage phase. As the results show, the effect of storage is relatively small compared with that of sterilization. The effect of storage time only is seen more clearly on expanded scales in Figure 4.3 and 4.5. The intrinsic viscosity decreases further on storage, but only relatively slowly and with little difference between the two temperature regimens of 2-8 °C and 30 °C. The effect of increasing the solution concentration from 0.1% to 0.3% w/v is for mainly increasing solution viscosity for practical purposes (e.g., prolonged ocular retention time) rather than influencing storage stability. Finally, the pH values of the 0.1% and 0.3% w/v solutions fluctuated between 4.80-5.93 and 3.91-4.19, respectively (Table 4.5), with no obvious trend with storage time.

Chitosan is well known for undergoing acid-catalyzed hydrolytic chain scission of its glucosidic linkages in diluted acid solution [104]. This chain scission, which occurs at random points along the chain, results in a rapid molecular weight decrease with a correspondingly rapid decrease in solution viscosity. For a random chain scission process, in which there is an equal probability of chain scission occurring at any glucosidic linkage along the chain, most cellulose derivatives in diluted acid solution show a decrease in average molecular weight $\overline{M}$,with time t able to fit approximately to the second-order rate equation (4.1) [105], i.e.,

$$\frac{1}{\overline{M}_t} - \frac{1}{\overline{M}_o} = kt \quad \ldots(4.1)$$

where $\overline{M}_t$ = average molecular weight at time t

$\overline{M}_o$ = initial average molecular weight at t=0

k = rate constant for chain, scission which is a function of temperature and acid catalyst concentration

i.e., k = f (T, [Acid])

For good approximation, the viscosity-average molecular weight, $\overline{M}_v$, from viscometry of chitosan in diluted aqueous acid solution can be considered directly proportional to the intrinsic viscosity, $[\eta]$, of the solution (i.e., $\overline{M}_v \propto [\eta]$). This is because the value of exponent 'a' in the Mark-Houwink Equation (4.2) is approximately equal to 1 for chitosan in diluted acid solution. Therefore, since K is a contant, and the following equation applies:

$$[\eta] = K\overline{M}_v^{\,a} \qquad \ldots(4.2)$$

approximates to $$[\eta] = K\overline{M}_v \qquad \ldots(4.3)$$

hence $$[\eta] \propto \overline{M}_v \qquad \ldots(4.4)$$

Therefore, combining equations (4.1) and (4.3) gives

$$\frac{1}{[\eta]_t} - \frac{1}{[\eta]_o} = k't \qquad \ldots(4.5)$$

which implies that a plot of $(1/[\eta]_t - 1/[\eta]_o)$ against time t should yield a linear graph of slope k'.

In this work, the values of $[\eta]_o$ and $[\eta]_t$ were estimated, again to good approximation, from a single solution concentration via the Solomon-Ciuta One-Point Equation [106] (4.6):

$$[\eta] = [2(\eta_{sp} - \ln \eta_{rel})]^{1/2} / C \qquad \ldots(4.6)$$

where η_{sp} is the specific viscosity, η_{rel} is the relative viscosity, and C is the concentration of the solution; in this case, C= 0.1 % or 0.3 % w/v (g dl^{-1}). The variations in $[\eta]$, with sterilization by autoclaving and storage time (days) at 30^0C and 2-8 °C, are compared in Figure 4.2 and 4.4. As the results clearly show, the main decrease in $[\eta]$ is brought about by autoclaving, during which the solutions are subjected to high temperature (121 °C at 15 psi for 15 mins). The combination of high temperature and the presence of acid causes rapid hydrolytic degradation of the chitosan in solution, even during only a short period of time (15 mins). Further degradation then occurs during storage, although much more slowly and to a much

lesser extent. The lower storage temperature of 2-8 °C marginally increases storage stability (Figure 4.3 and 4.5), although this effect is overshadowed in Figure 4.2 and 4.4 by the much greater effect of autoclaving.

The hydrolytic degradation of chitosan in aqueous acid solution is well documented [107]. The fact that it results in random chain scission means that the molecular weight of the chitosan decreases very rapidly. Furthermore, the rate of hydrolysis increases with both acid concentration and temperature [108].

In this work, the chitosan solutions were prepared in a diluted (1% v/v) aqueous solution of weak acid (L-lactic acid) and stored at moderate (30 °C) or low (2-8 °C) temperatures. Under these conditions, the hydrolysis rate of the chitosan in solution was expected to be relatively slow over a period of days, if not weeks. However, chitosan solutions used in ocular drug delivery need to be sterilized before use, with the recommended method, i.e., autoclaving at 121 °C at 15 psi pressure for 15 mins. By subjecting the solution to this high temperature, even for such a short time, it is sufficient to cause the chitosan to hydrolyse rapidly with a resultant, drastic reduction in molecular weight. This effect is observed in Figure 4.2 and 4.4 as the large reductions in intrinsic viscosity, $[\eta]$. Consequently, because the chitosan had already been degraded to such a large extent by autoclaving, the subsequent effects of storage, at 2-8 °C and 30 °C, were both small and slow in comparison. Autoclaving, necessary as it is in order to sterilize the solution, is a highly-degradative process in terms of the chitosan molecular weight. While this does not adversely affect the sterility of the solution, it drastically reduces its viscosity.

The pH values of the 0.1% and 0.3% chitosan solutions stored at 2-8 °C and 30 °C were in the range of 4.80-5.93 and 3.91-4.19, respectively (Table 4.5). The pH range of 3.5-10.5 is usually tolerated by the human eye [109]. The pH values of the 0.3% chitosan solution were slightly lower than those of the 0.1% chitosan solution. At the same time, the osmolalities of the 0.1% and 0.3% solutions were 267 and 193 mOsmol/kg, respectively. Osmolality that can be tolerated by the eye is 160-670 mOsmol/kg [80].

4.5 Preparation of ophthalmic formulations

4.5.1 Compatibility and stability studies

The compatibility and stability studies of vancomycin at 50 mg/ml in formulation A, B, C and D showed that solutions of vancomycin eye drops at 50 mg/ml in Tears Naturale II™ remained clear until day 7, when stored at 2-8 °C and 30 °C. In 0.9% sodium chloride, the solutions remained clear until day 14, when stored at 2-8 °C and 30 °C. In the 0.1% chitosan solution, the eye drops remained clear throughout the 28-day study period, when stored at 2-8 °C, but only until day 21 when stored at 30 °C. In the 0.3% chitosan solution, the eye drops remained clear throughout the 28-day study period when stored at 2-8 °C, but only until day 14 when stored at 30 °C (Table 4.8).

Table 4.8 Compatibility and stability studies of vancomycin eye drops at 50 mg/ml in Tears Naturale II™, 0.9% sodium chloride and the 0.1% and 0.3% chitosan solutions

Days	Clarity of vancomycin at 50 mg/ml							
	Tears Naturale II™ (A)		0.1% chitosan solution (B)		0.3% chitosan solution (C)		0.9% sodium chloride (D)	
	2-8 °C	30 °C	2-8 °C	30 °C	2-8 °C	30 °C	2-8 °C	30 °C
0	clear	clear	clear	clear	clear	clear	clear	clear
3	clear	clear	clear	clear	clear	clear	clear	clear
7	clear	clear	clear	clear	clear	clear	clear	clear
10	not clear	not clear	clear	clear	clear	clear	clear	clear
14	not clear	not clear	clear	clear	clear	clear	not clear	not clear
21	not clear	not clear	clear	clear	clear	not clear	not clear	not clear
28	not clear	not clear	clear	not clear	clear	not clear	not clear	not clear

4.5.2 Validation of the UV spectrophotometer

The validation of method is an important issue in pharmaceutical analysis. The process confirms that the analytical procedure employed for the analysis is suitable for its intended use, and shows reliability of the method. Therefore, the validation of method is necessary in pharmaceutical analysis. In this study, all validation parameters for quantitative analysis of vancomycin in vehicles were tested, and validation data were evaluated according to their acceptance criteria.

Assay Validation Report

Specificity

UV spectrophotometric scan of blank and standard vancomycin in vehicles is shown in Figures 4.6-4.8 and Tables 4.9-4.11. The absorbance in this research used 282 nm [91, 110]. Figures 4.6-4.9 and Tables 4.9-4.11 show that standard vancomycin in Tears Naturale II^{TM}, 0.1%, 0.3% chitosan and 0.9% sodium chloride solution has maximum absorption in the range 270-290 nm, with no interference from blank absorption.

Linearity

The calibration curve of standard vancomycin was linear over the range of 75.0-225 µg/ml (Tables 4.12-4.15, Figures 4.9-4.12). The calibration curve of absorbance versus concentration for standard vancomycin at six different concentrations over the afore-mentioned range gave a linear plot ($r^2 > 0.999$).

Precision and accuracy

The results of the intraday and interday assay were found to have good accuracy, indicating an agreement between the true value and the value found (Table 4.16 and 4.17). All of the intraday and interday assays were determined as coefficients of variation and were less than 5%.

The overall mean recovery of vancomycin in Tears Naturale II^{TM}, 0.1%, 0.3% chitosan and 0.9% sodium chloride solution were 99.84%, 99.84%, 100.45% and 100.21%, respectively (Table 4.18).

The lower limit of detection (LLQ) was 75 µg/ml (Table 4.19) and coefficient variations of standard vancomycin in Tears Naturale II^{TM}, 0.1%, 0.3% chitosan and 0.9% sodium chloride solution were 2.15%, 2.25%, 0.26% and 0.28%, respectively.

The raw data for assay validation reports are shown in Appendix B.

Table 4.9 UV spectrophotometric scan of standard vancomycin in Tears Naturalle II™ and Tears Naturalle II™

Lambda (nm)	Absorbance std. vancomycin in Tears Naturalle II™	Absorbance of Tears Naturalle II™
200	2.505	0.018
210	2.505	0.015
220	2.505	0.008
230	2.505	0.005
240	1.968	0.008
250	0.606	0.008
260	0.234	0.007
270	0.343	0.005
280	0.484	0.006
290	0.307	0.005
300	0.030	0.006
310	0.003	0.005
320	0.001	0.005
330	0.001	0.005
340	0.002	0.006
350	-0.001	0.003

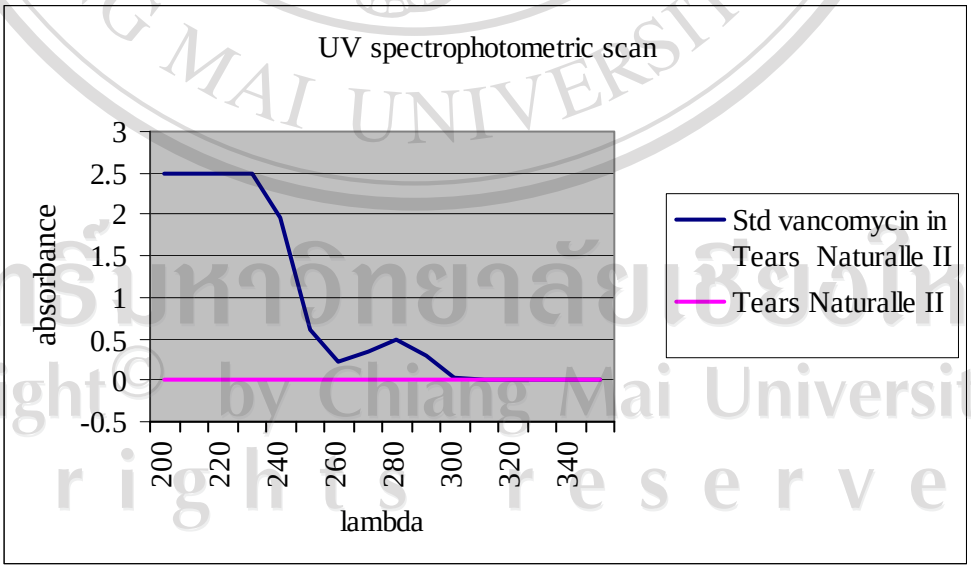


Figure 4.6 UV spectrophotometric scan (in graph) of standard vancomycin in Tears Naturalle II™ and Tears Naturalle II™.

Table 4.10 UV spectrophotometric scan of standard vancomycin in 0.1% chitosan and 0.1% chitosan

Lambda (nm)	Absorbance std. vancomycin in 0.1% chitosan	Absorbance of 0.1% chitosan
200	2.505	0.027
210	2.505	0.022
220	2.505	0.011
230	2.505	0.008
240	2.290	0.009
250	0.726	0.010
260	0.282	0.008
270	0.414	0.007
280	0.583	0.007
290	0.369	0.005
300	0.038	0.006
310	0.006	0.006
320	0.003	0.006
330	0.003	0.005
340	0.003	0.006
350	0.002	0.003

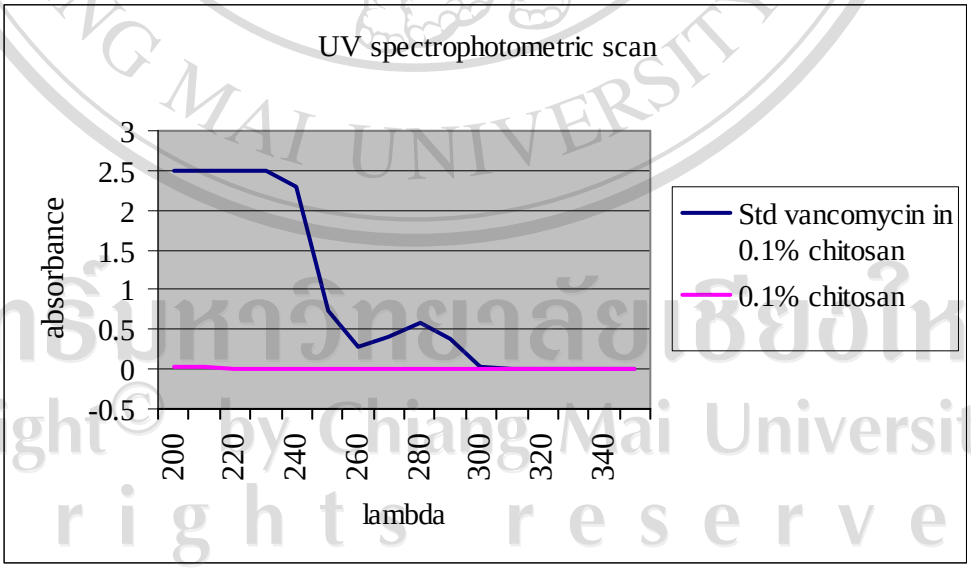


Figure 4.7 UV spectrophotometric scan (in graph) of standard vancomycin in 0.1% chitosan and 0.1% chitosan.

Table 4.11 UV spectrophotometric scan of standard vancomycin in 0.9% sodium chloride and 0.9% sodium chloride

Lambda (nm)	Absorbance std. vancomycin in 0.9% sodium chloride	Absorbance of 0.9% sodium chloride
200	2.505	1.883
210	2.505	0.114
220	2.505	0.020
230	2.505	0.011
240	1.939	0.011
250	0.599	0.011
260	0.238	0.009
270	0.346	0.009
280	0.484	0.010
290	0.308	0.011
300	0.035	0.011
310	0.008	0.009
320	0.008	0.007
330	0.007	0.006
340	0.007	0.006
350	0.005	0.003

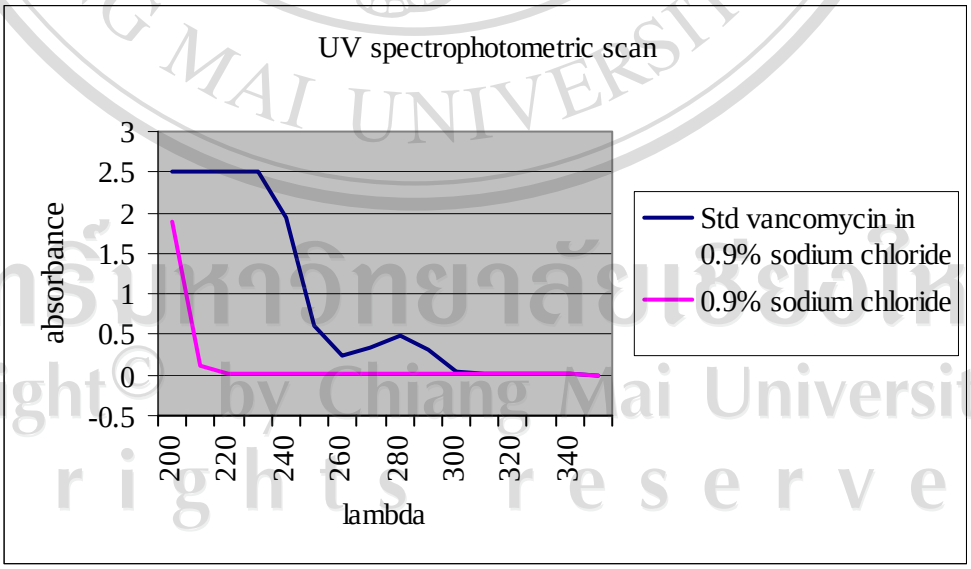


Figure 4.8 UV spectrophotometric scan (in graph) of standard vancomycin in 0.9% sodium chloride and 0.9% sodium chloride.

Table 4.12 Calibration of standard vancomycin in Tears Naturale II™

Conc standard vancomycin(ug/ml)	In Tears Naturale II absorbance				
	No 1	No 2	No 3	No 4	No 5
75	0.270	0.288	0.288	0.295	0.270
87.5	0.328	0.338	0.338	0.349	0.328
112.5	0.403	0.454	0.441	0.439	0.403
125	0.455	0.487	0.495	0.486	0.455
162.5	0.588	0.639	0.631	0.631	0.588
225	0.800	0.884	0.882	0.874	0.800
r^2	0.9994	0.9995	0.9998	0.9999	0.9994

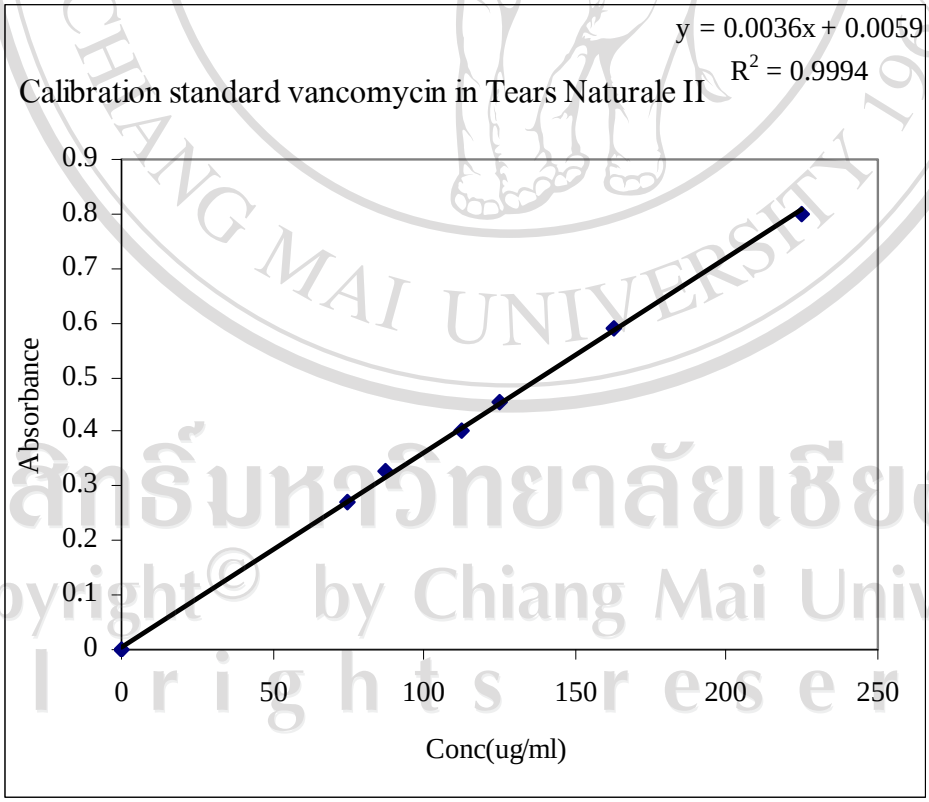


Figure 4.9 Calibration curve of standard vancomycin in Tears Naturale II™ no. 1.

Table 4.13 Calibration of standard vancomycin in 0.1 % chitosan

Conc standard vancomycin(ug/ml)	In 0.1 % chitosan absorbance				
	No 1	No 2	No 3	No 4	No 5
75.0	0.250	0.286	0.269	0.27	0.242
87.5	0.308	0.338	0.318	0.320	0.308
112.5	0.399	0.446	0.420	0.413	0.388
125.0	0.428	0.492	0.471	0.480	0.428
162.5	0.570	0.639	0.600	0.599	0.570
225.0	0.777	0.870	0.828	0.843	0.777
r^2	0.9992	0.9995	0.9995	0.9991	0.9990

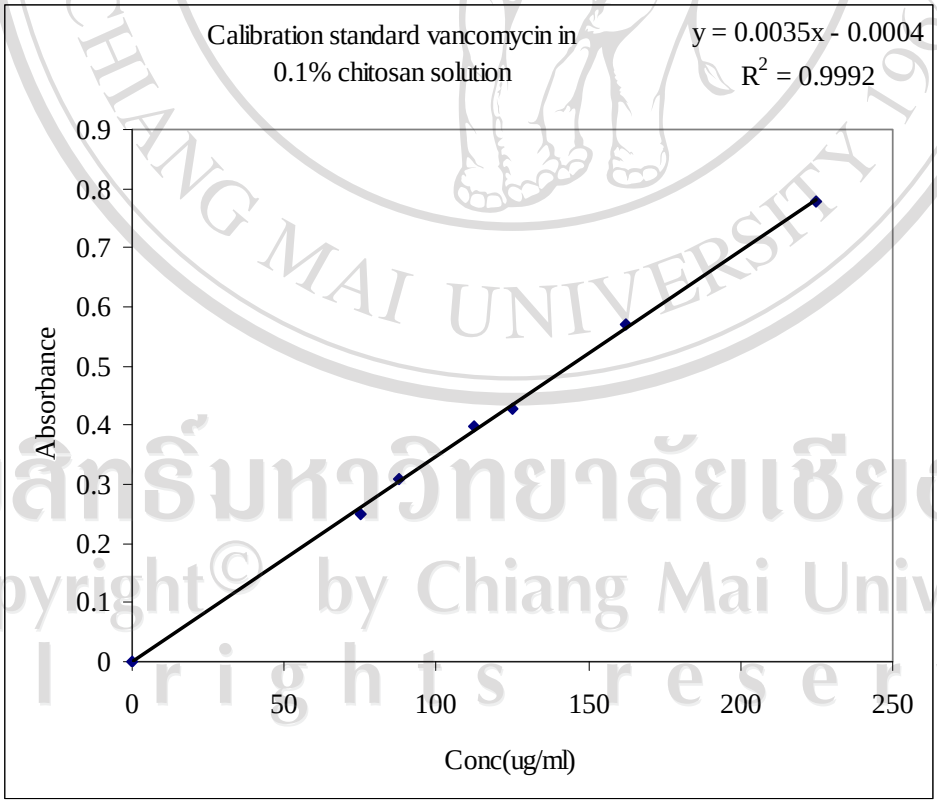


Figure 4.10 Calibration curve of standard vancomycin in 0.1% chitosan no. 1.

Table 4.14 Calibration of standard vancomycin in 0.3 % chitosan

Conc standard vancomycin(ug/ml)	In 0.3 % chitosan absorbance				
	No 1	No 2	No 3	No 4	No 5
75.0	0.280	0.279	0.280	0.290	0.280
87.5	0.340	0.331	0.330	0.350	0.340
112.5	0.450	0.435	0.425	0.450	0.415
125.0	0.480	0.484	0.475	0.480	0.475
162.5	0.635	0.617	0.620	0.630	0.615
225.0	0.890	0.863	0.863	0.890	0.863
r^2	0.9992	0.9997	0.9999	0.9992	0.9994

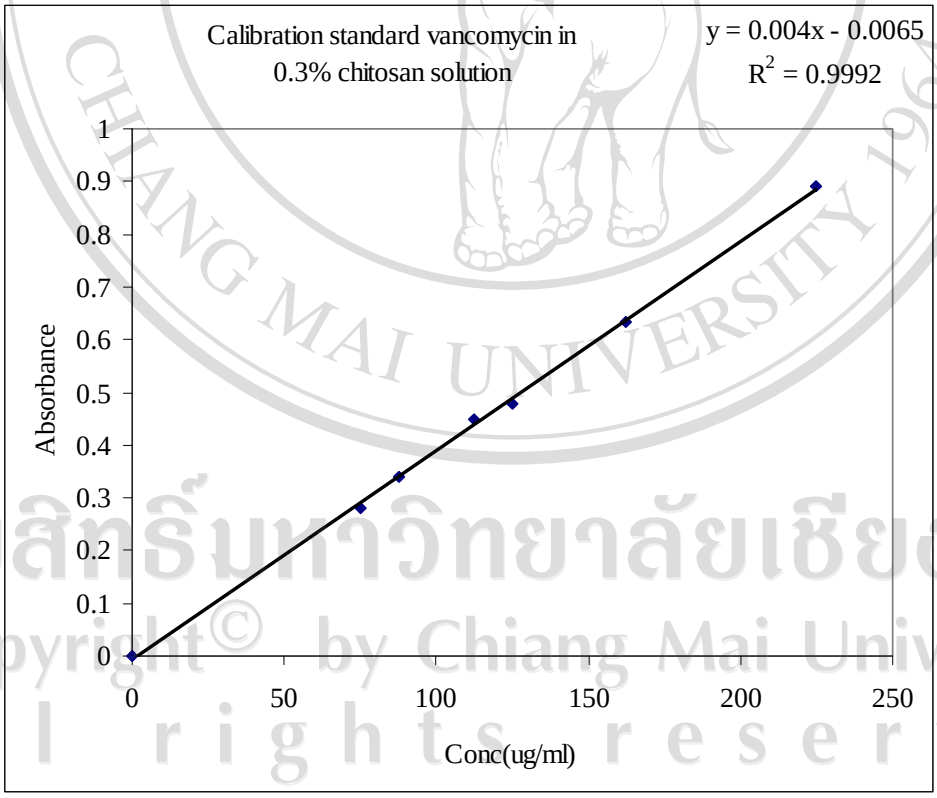


Figure 4.11 Calibration curve of standard vancomycin in 0.3% chitosan no.1.

Table 4.15 Calibration of standard vancomycin in 0.9 % sodium chloride

Conc standard vancomycin(μg/ml)	**In 0.9 % sodium chloride absorbance**				
	No 1	**No 2**	**No 3**	**No 4**	**No 5**
75.0	0.256	0.256	0.260	0.265	0.288
87.5	0.299	0.290	0.300	0.304	0.310
112.5	0.396	0.397	0.404	0.404	0.401
125.0	0.434	0.435	0.447	0.450	0.450
162.5	0.550	0.577	0.580	0.580	0.580
225.0	0.772	0.780	0.800	0.810	0.800
r^2	0.9995	0.999	0.9996	0.9997	0.999

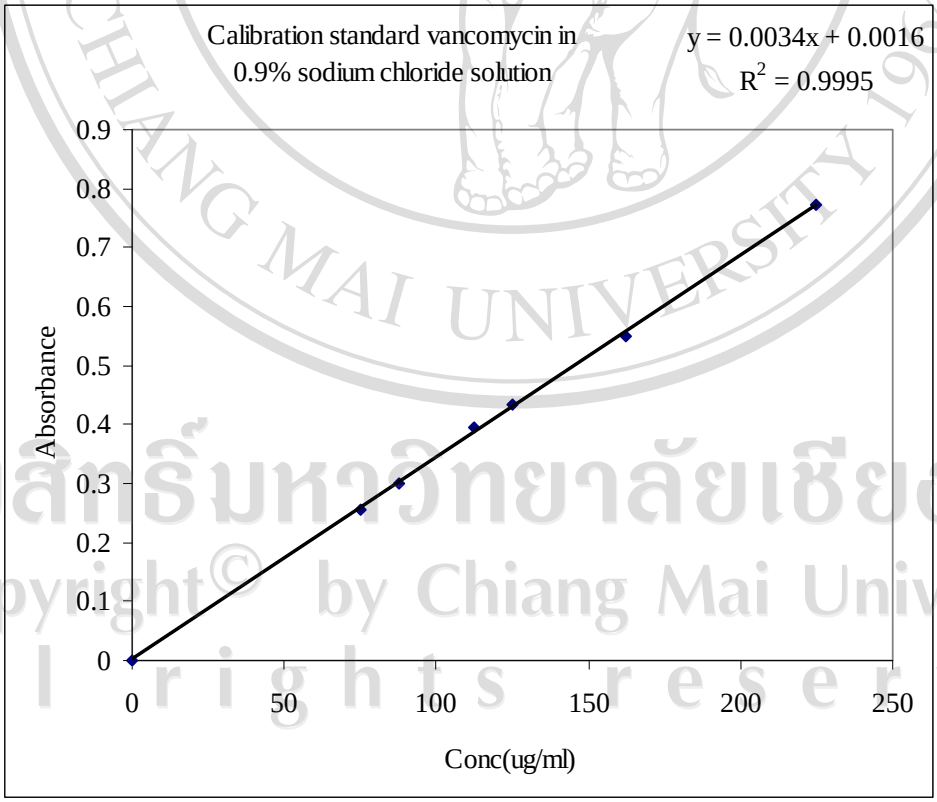


Figure 4.12 Calibration curve of standard vancomycin in 0.9% sodium chloride no. 1.

Table 4.16 Interday assay validation of standard vancomycin in each vehicle

	Vehicles	Standard vancomycin concentration (µg/ml) (n=5)		
		100 (µg/ml)	175 (µg/ml)	212.5 (µg/ml)
%Accuracy	Tears Naturale II™	101.58	101.36	103.21
	0.1% Chitosan	102.76	99.41	98.25
	0.3% Chitosan	103.10	99.84	99.86
	0.9% sodium chloride	102.67	100.3	100.52
Mean	Tears Naturale II™	101.58	177.38	219.32
	0.1% Chitosan	102.76	173.96	208.79
	0.3% Chitosan	103.10	174.72	212.20
	0.9% sodium chloride	102.67	175.53	213.60
S.D.	Tears Naturale II™	1.60	3.94	0.66
	0.1% Chitosan	1.85	1.05	1.98
	0.3% Chitosan	1.95	1.05	2.33
	0.9% sodium chloride	1.96	2.22	1.71
%CV	Tears Naturale II™	1.60	2.21	0.30
	0.1% Chitosan	1.80	0.60	0.95
	0.3% Chitosan	1.89	0.60	1.11
	0.9% sodium chloride	1.90	1.26	0.80

Table 4.17 Intraday assay validation of standard vancomycin in each vehicle

	Vehicles	Standard vancomycin concentration (µg/ml) (n=10)		
		100 (µg/ml)	175 (µg/ml)	212.5 (µg/ml)
%Accuracy	Tears Naturale II™	97.64	101.04	101.26
	0.1% Chitosan	98.82	99.78	97.07
	0.3% Chitosan	100.08	100.65	99.60
	0.9% sodium chloride	100.42	100.24	99.99
Mean	Tears Naturale II™	97.64	176.82	215.18
	0.1% Chitosan	98.82	174.62	206.28
	0.3% Chitosan	100.08	176.13	211.66
	0.9% sodium chloride	100.42	175.42	212.47
S.D.	Tears Naturale II™	1.45	0.57	0.43
	0.1% Chitosan	0.97	0.81	1.48
	0.3% Chitosan	0.81	0.66	0.22
	0.9% sodium chloride	0.19	0.11	0.11
%CV	Tears Naturale II™	1.42	1.00	0.93
	0.1% Chitosan	0.96	1.42	3.04
	0.3% Chitosan	0.81	1.17	0.47
	0.9% sodium chloride	0.20	0.20	0.23

Table 4.18 Recovery validation of standard vancomycin in each vehicle

Vehicles	% Recovery			
	Std vancomycin added 100 (µg/ml)	Std vancomycin added 175 (µg/ml)	Std vancomycin added 212.5 (µg/ml)	Average
Tears Naturale II™	99.96	99.34	100.35	99.88
0.1% Chitosan	98.24	101.85	99.42	99.84
0.3% Chitosan	100.87	100.47	100.02	100.45
0.9% sodium chloride	100.58	100.12	99.94	100.21

Table 4.19 Lower limit of standard vancomycin in each vehicle

Vehicles	Conc standard add (µg/ml)	Found conc (µg/ml)	% CV	% Mean recovery
Tears Naturale II™	75	75.47	2.15	100.63
0.1% Chitosan	75	74.69	2.25	99.59
0.3% Chitosan	75	75.40	0.26	100.54
0.9% sodium chloride	75	75.48	0.28	100.64

The validation of method in this study showed that the UV method for the determination of vancomycin in Tears Naturale II™, 0.1%, 0.3% chitosan and 0.9% sodium chloride solution was simple, rapid, precise, accurate and sensitive. The UV method is suitable for investigation of the chemical stability of vancomycin in Tears Naturale II™, 0.1%, 0.3% chitosan and 0.9% sodium chloride solution in extemporaneous eye drops.

4.5.3 Stability of vancomycin eye drops at 50 mg/ml in each vehicle

The pH values of vancomycin eye drops at 50 mg/ml in formulation A, B, C and D, stored at 2-8 °C and 30 °C, were in the ranges of 3.23-3.73, 3.52-4.03, 3.55-3.79 and 3.19-3.63, respectively (Table 4.20). The effect of temperature slightly influenced the above pH values for 28 days (Figure 4.13). Vancomycin eye drops at 50 mg/ml, which used 0.1% and 0.3% chitosan solutions as a vehicle, had a higher pH than that in Tears Naturale II™ and 0.9% sodium chloride. The 0.1% and 0.3% chitosan solutions used Feldman buffer for ophthalmic preparations, therefore, it had a buffer effect that could control the pH of eye drops better than that of Tears Naturale II™ and 0.9% sodium chloride.

The pH range of 3.5 to 10.5 is usually tolerated by the eye [109]. However, the pH of vancomycin eye drops at 50 mg/ml in Tears Naturale II™ and 0.9 % sodium chloride in this studied, was slightly lower than this pH range and, therefore, not well tolerated by the eye. In contrast, the pH values of vancomycin eye drops at 50 mg/ml in the 0.1 % and 0.3 % chitosan solutions, stored at 2-8 °C and 30 °C, were within this range and should be well tolerated by the eye (Table 4.20 and Figure 4.13).

Vancomycin has been reported most stable at pH 3 to 5 and degradation is principally deamidation [111]. Vancomycin in distilled water or 0.9% sodium chloride has a pH of about 3.9 [111]. The pH of vancomycin at 50 mg/ml in formulation A, B, C and D was between 3 to 5. The precipitate of vancomycin at 50 mg/ml in any solution may have a higher concentration of vancomycin and temperature. Vancomycin at 20 and 40 mg/ml in 5% dextrose exhibited little or no loss after 96 hours and 30 days of storage at 25 °C and 5 °C, respectively [111]. For ophthalmic solutions, which have been prepared extemporaneously by diluting vancomycin sterile powder with artificial tears to a final concentration of 50 mg/ml, recommendations concerning stability and storage were not possible, since pH of the resulting ophthalmic solution decreased rapidly to less than 3.5 by the seventh day [112]. In general, the rate of a chemical reaction increases exponentially for each 10° increase in temperature [92]. In this study, vancomycin at 50 mg/ml in formulation A, B, C and D was more stable when stored in 2-8 ^{o}C than at 30 ^{o}C.

Table 4.20 The pH of vancomycin eye drops at 50 mg/ml in each vehicle

Day	pH (n=10)*							
	Vancomycin 50 mg/ml in Tears Naturale II^{TM} (A)		Vancomycin 50 mg/ml in 0.1% chitosan solution (B)		Vancomycin 50 mg/ml in 0.3% chitosan solution (C)		Vancomycin 50 mg/ml in 0.9% sodium chloride (D)	
	2-8 ^{o}C	30 ^{o}C	2-8 ^{o}C	30 ^{o}C	2-8 ^{o}C	30 ^{o}C	2-8 ^{o}C	30 ^{o}C
0	3.23	3.23	3.52	3.52	3.72	3.69	3.35	3.40
3	3.57	3.50	3.80	3.90	3.56	3.63	3.19	3.32
7	3.45	3.58	3.71	3.86	3.71	3.79	3.49	3.51
10	3.40	3.40	3.78	3.89	3.63	3.79	3.63	3.45
14	3.51	3.66	3.84	4.03	3.55	3.65	3.36	3.55
21	3.45	3.73	3.75	4.02	3.70	3.65	3.35	3.63
28	3.41	3.55	3.60	3.86	3.65	3.66	3.45	3.54

*pH readings accurate to ± 0.01

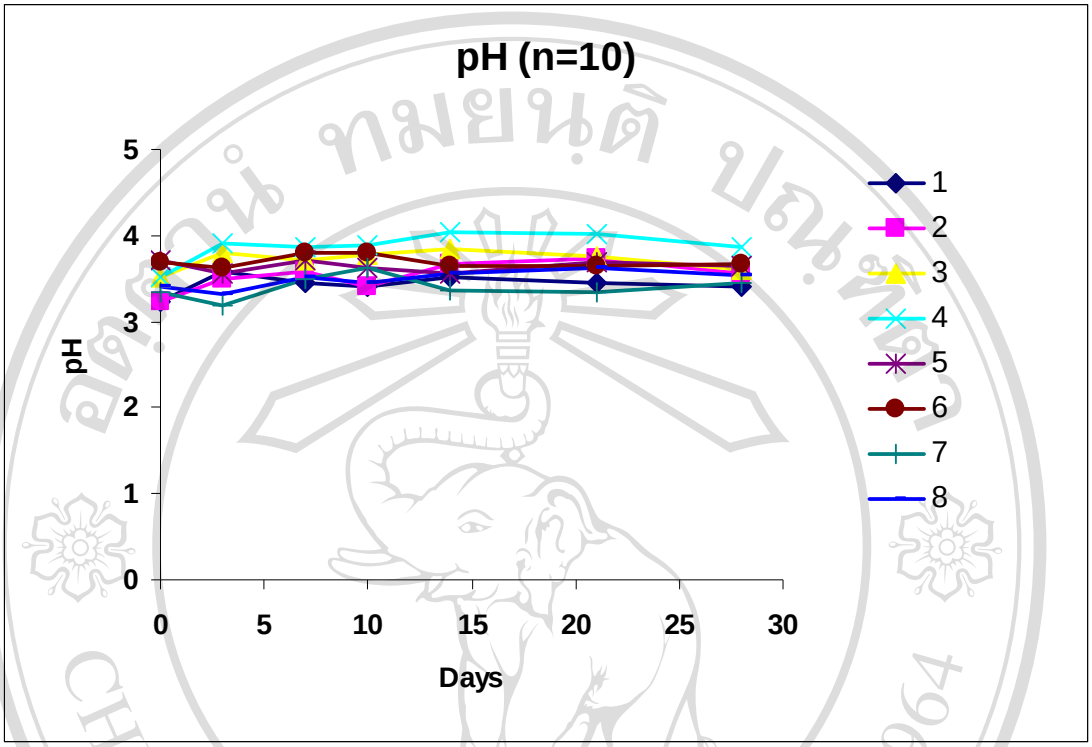


Figure 4.13 The pH graph of vancomycin eye drops at 50 mg/ml in each vehicle.

1 = Vancomycin 50 mg/ml in Tears Naturale II™ 2-8° C

2 = Vancomycin 50 mg/ml in Tears Naturale II™ 30° C

3 = Vancomycin 50 mg/ml in 0.1% chitosan 2-8° C

4 = Vancomycin 50 mg/ml in 0.1% chitosan 30° C

5 = Vancomycin 50 mg/ml in 0.3% chitosan 2-8° C

6 = Vancomycin 50 mg/ml in 0.3% chitosan 30° C

7 = Vancomycin 50 mg/ml in 0.9% sodium chloride 2-8° C

8 = Vancomycin 50 mg/ml in 0.9% sodium chloride 30° C

The percentage of the labeled amount of vancomycin eye drops at 50 mg/ml in Tears Naturale II™, 0.9% sodium chloride and the 0.1% and 0.3% chitosan solutions, stored at 2-8 °C, showed no loss of stability during a 28-day storage period. However, at 30 °C, there was a statistically significant decrease in the percentage of the labeled amount from day 28 and 21 onwards for vancomycin eye drops at 50 mg/ml in Tears Naturale II™ and the 0.1 % chitosan solution, respectively ($p<0.05$). The percentage of the labeled amount of vancomycin eye drops at 50 mg/ml in the 0.3% chitosan and 0.9% sodium chloride solutions showed statistically significant decreases in the percentages of the labeled amounts from day 21 (Table 4.21 and Figure 4.14).

In this work, it was found that the change in percentage labeled amounts of vancomycin at 50 mg/ml was affected by both storage temperature and vehicle. Vancomycin at 50 mg/ml in 0.1 % and 0.3 % chitosan solutions remained clear and stable at 2-8 °C throughout the study period (28 days).

4.6 Minimum inhibitory concentrations of vancomycin at 50 mg/ml in Tears Naturale II™, sodium chloride and chitosan solutions stored at different temperatures over time

This study was also concerned with the antimicrobial potency and stability of extemporaneous preparations of vancomycin eye drops at 50 mg/ml in various solutions. On examining the minimum inhibitory concentrations (MIC), it was found that the MIC values at 2-8 °C and 30 °C on days 0, 3, 7, 10, 14, 21 and 28 for vancomycin eye drops at 50 mg/ml in Tears Naturale II™, 0.9 % sodium chloride and the 0.1 % and 0.3 % chitosan solutions were between 0.5 and 2.0 µg/ml (Table 4.22). According to the Clinical and Laboratory Standards Institute (CLSI, 2005 [93]), the standard MIC value of vancomycin is 0.5-2.0 µg/ml. All positive controls without added vancomycin showed positive results. Negative controls, which were not inoculated with *Staphylococcus aureus* ATCC 29213, showed negative results. Thus, this study demonstrated that vancomycin eye drops, at 50 mg/ml stored in those varied solutions at 2-8 °C and 30 °C, resulted in no loss of MIC during the 28 day period.

Table 4.21 Percentage of the labeled amounts of vancomycin eye drops at 50 mg/ml in each vehicle

Day	Percentage of the labeled amounts[a]							
	Vancomycin 50 mg/ml in Tears Naturale II™		Vancomycin 50 mg/ml in 0.1% chitosan solution		Vancomycin 50 mg/ml in 0.3% chitosan solution		Vancomycin 50 mg/ml in 0.9% sodium chloride	
	2-8 °C	30 °C	2-8 °C	30 °C	2-8 °C	30 °C	2-8 °C	30 °C
0	108.63 ± 1.13	108.48 ±0.95	108.22 ± 0.63	108.22 ± 0.63	108.70 ± 0.98	108.95 ±0.23	109.73 ± 0.48	109.14 ± 0.66
3	107.88 ± 0.87	109.45 ± 1.40	107.78 ± 1.89	108.87 ± 0.46	108.93 ± 0.27	108.96 ± 0.17	109.56 ± 0.76	109.85 ± 1.01
7	107.74 ± 1.22	108.75 ± 0.93	108.94 ± 0.46	108.80 ± 1.12	108.95 ± 0.17	108.98 ± 0.24	109.14 ± 1.08	109.24 ± 0.84
10	108.07 ± 0.53	108.82 ± 1.09	108.75 ± 0.38	108.74 ±0.90	109.00 ± 0.16	108.98 ± 0.19	109.58 ± 0.58	109.62 ±0.65
14	107.73 ± 0.76	108.74 ± 0.56	108.43 ± 0.49	108.94 ± 0.68	108.75 ± 0.44	108.95 ± 0.46	108.88 ± 0.91	109.87 ± 0.37
21	108.29 ± 0.32	108.53 ± 0.48	108.15 ± 0.67	100.77 ± 1.00*	108.96 ± 0.2	104.26 ± 1.23*	109.35 ± 0.78	108.27 ± 0.97*
28	107.71 ± 0.81	102.07 ± 0.33*	107.88 ± 1.00	99.52 ± 0.80*	108.86 ± 0.22	99.25 ± 1.76*	109.92 ± 1.05	96.69 ± 0.54*

[a] mean ± SD of 10 samples * P< 0.05

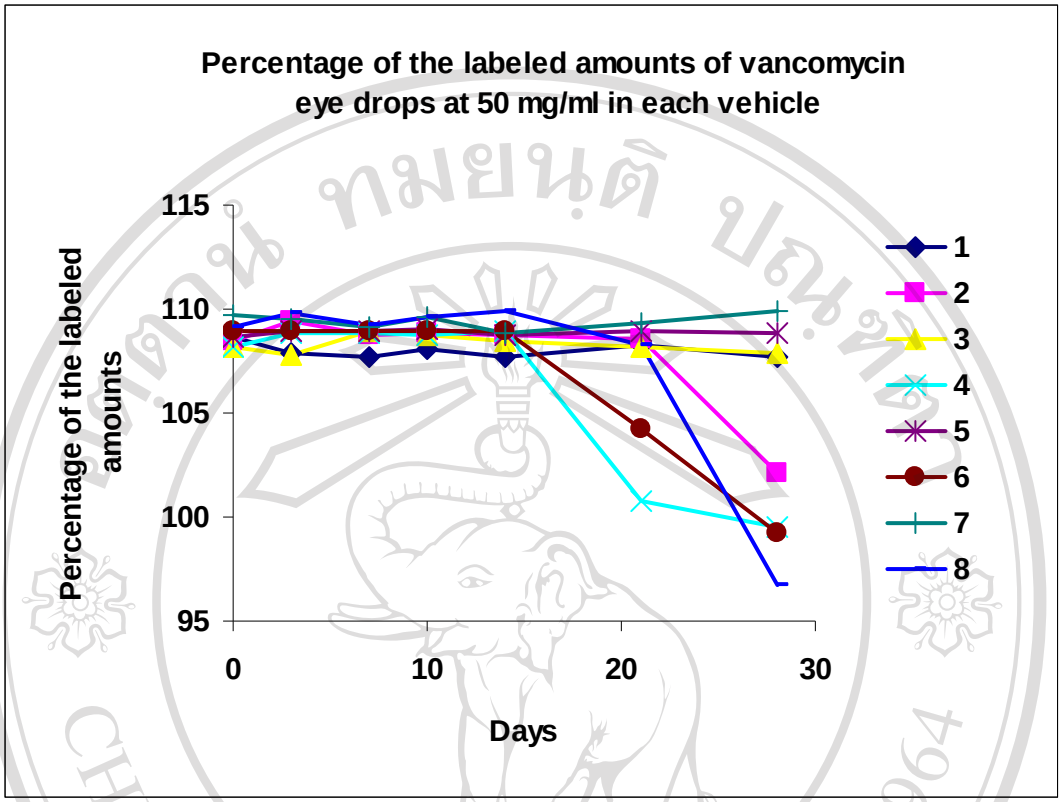


Figure 4.14 Percentage graph of the labeled amounts of vancomycin eye drops at 50 mg/ml in each vehicle.

1 = Vancomycin 50 mg/ml in Tears Naturale II™ 2-8° C

2 = Vancomycin 50 mg/ml in Tears Naturale II™ 30° C

3 = Vancomycin 50 mg/ml in 0.1% chitosan 2-8° C

4 = Vancomycin 50 mg/ml in 0.1% chitosan 30° C

5 = Vancomycin 50 mg/ml in 0.3% chitosan 2-8° C

6 = Vancomycin 50 mg/ml in 0.3% chitosan 30° C

7 = Vancomycin 50 mg/ml in 0.9% sodium chloride 2-8° C

8 = Vancomycin 50 mg/ml in 0.9% sodium chloride 30° C

Table 4.22 Minimum inhibitory concentrations of vancomycin at 50 mg/ml in Tears Naturale II™, sodium chloride and chitosan solutions stored at different temperatures over time

Day	Minimum inhibitory concentration (µg/ml) (n=2)								
	Vancomycin 50 mg/ml in sodium chloride	Vancomycin 50 mg/ml in Tears Naturale II™		Vancomycin 50 mg/ml in 0.1% chitosan solution		Vancomycin 50 mg/ml in 0.3% chitosan solution		Vancomycin 50 mg/ml in 0.9% sodium chloride	
	Freezer	2-8°C	30°C	2-8°C	30°C	2-8°C	30°C	2-8°C	30°C
0	0.98	0.98	0.98	0.98	0.98	0.50	0.98	0.50	0.50
3	0.98	0.98	0.98	0.98	0.98	0.50	0.98	0.50	0.50
7	0.50	0.74	0.50	0.74	0.50	0.98	0.74	0.74	1.49
10	0.50	0.50	0.74	0.50	0.50	2.00	1.49	2.00	0.50
14	0.74	0.98	2.00	1.49	2.00	1.49	1.49	1.49	2.00
21	0.98	0.98	0.98	0.98	2.00	0.98	0.74	0.74	1.49
28	0.98	0.98	1.49	0.98	0.98	0.74	1.49	0.50	1.49

Table 4.22 compares the antibacterial activities of vancomycin eye drops at 50 mg/ml in Tears Naturale II™, 0.9% sodium chloride and the 0.1% and 0.3% chitosan solutions during storage at 2-8 °C and 30 °C for 28 days. The growth of *Staphylococcus aureus* ATCC 29213 was suppressed by vancomycin throughout the study period. The potency of vancomycin at 50 mg/ml in Tears Naturale II™ and the 0.9% sodium chloride solution is comparable with that in the 0.1% and 0.3% chitosan solutions. Charlton et al. [80] studied the stability of vancomycin at 50 mg/ml in artificial tears and found that the drug activity did not vary with temperature (4 °C, 25 °C) or storage time (28 days).

The osmolalities of vancomycin at 50 mg/ml in Tears Naturale II™, 0.9% sodium chloride and the 0.1% and 0.3% chitosan solutions were determined as 334, 315, 310 and 235 mOsmol/kg, respectively.

The inflammation of the cornea made tear film to hyperosmolar [113]. Therefore, the preparation of vancomycin at 50 mg/ml in 0.3% chitosan solution has an advantage over any other vehicles because it is hypoosmolar.

Finally, an osmolality of 160-670 mOsmol/kg can be tolerated by the human eye [80]. The osmolalities of vancomycin at 50 mg/ml in Tears Naturale II™, 0.9% sodium chloride and the 0.1% and 0.3% chitosan solutions in this study were all within a well-tolerated range.

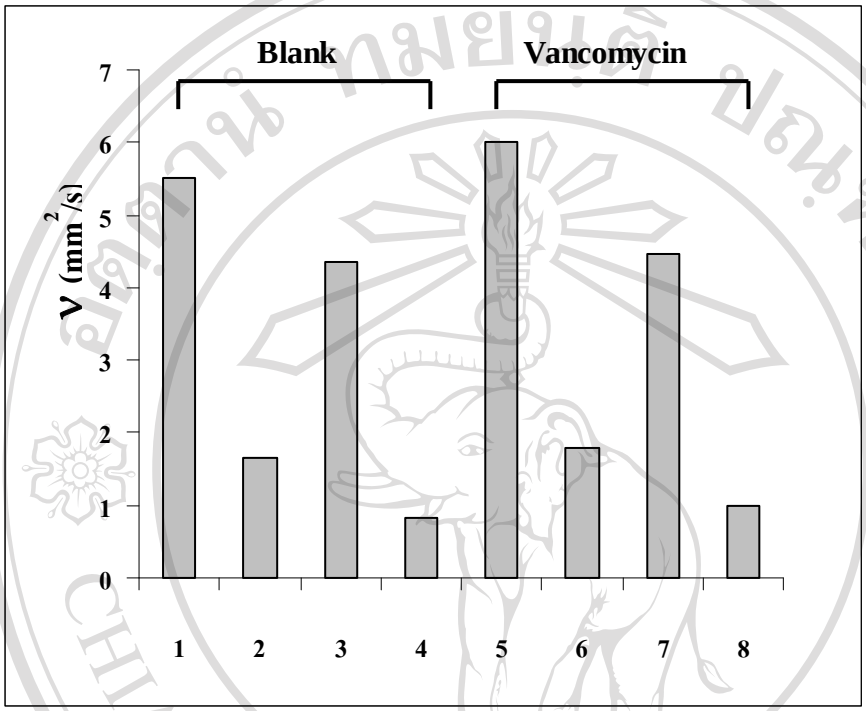
4.7 Kinematic viscosity measurements

In this study, the kinematic viscosities of blank samples of artificial tears, 0.1% and 0.3% w/v chitosan solutions and 0.9% sodium chloride solution were 5.50, 1.65, 4.36 and 0.84 mm^2/s, respectively. When loaded with vancomycin at 50 mg/ml, these kinematic viscosities increased to 6.00, 1.78, 4.47 and 0.98 mm^2/s, respectively (Table 4.23 and Figure 4.15). Thus, it could be seen that the kinematic viscosities of vancomycin at 50 mg/ml in the 0.1% and 0.3% w/v chitosan solutions and 0.9% sodium chloride solution were significantly lower than in artificial tears.

Table 4.23 Comparison of the kinematic viscosity, ν , of various solvents and vancomycin at 50 mg/ml in solutions at 25°C

Active ingredient	Vehicle	Flow-time (n=3), t (s)	ν (mm^2/s)[a]
Blank	Tears Naturale II™	568.3	5.50
	0.1% chitosan solution	170.1	1.65
	0.3% chitosan solution	450.3	4.36
	0.9% sodium chloride solution	86.3	0.84
Vancomycin	Tears Naturale II™	619.7	6.00
	0.1% chitosan solution	183.6	1.78
	0.3% chitosan solution	462.2	4.47
	0.9% sodium chloride solution	101.2	0.98

[a] Kinematic viscosity = ν = 0.009679t mm^2/s [where 1 mm^2/s = 1 cSt (centistoke)]



1 = Tears Naturale II™

2 = 0.1% chitosan

3 = 0.3% chitosan

4 = 0.9% sodium chloride

5 = Vancomycin 50 mg/ml in Tears Naturale II™

6 = Vancomycin 50 mg/ml in 0.1% chitosan

7 = Vancomycin 50 mg/ml in 0.3% chitosan

8 = Vancomycin 50 mg/ml in 0.9% sodium chloride

Figure 4.15 Comparison of the kinematic viscosity, ν , of various solvents and vancomycin at 50 mg/ml in solutions at 25°C by bar graph.

4.8 Pharmacokinetics of topically applied vancomycin in rabbit eyes

The concentrations of vancomycin in tear films were determined at 0, 30, 60, 90 and 120 minutes after addition of the vancomycin eye drops. The effects of the solvent on the delivery of vancomycin over time were compared by studying their respective pharmacokinetics. The precision of vancomycin in solutions (control test) is shown in Table 4.24.These concentration-time profiles for vancomycin in the tear films are shown in Tables 4.25-4.28 and Figures 4.16-4.19. The areas under the curves (AUC 0→30, 0→120 minutes) are shown in Figure 4.20 and Table 4.29 and 4.30.

There were statistically significant differences in the areas under the curves from 0 to 30 and 0 to 120 minutes for vancomycin in Tears Naturale II™, the 0.1% w/v chitosan solution, and 0.9% sodium chloride solution (Table 4.29 and 4.30). The AUC from 0 to 30 and 0 to 120 minutes for vancomycin at 50 mg/ml in 0.3% chitosan was not significantly different from that in Tears Naturale II™ (Table 4.29 and 4.30) and showed a 1.15- and 1.22-fold improvement, respectively.

Table 4.24 Control test of vancomycin

Control test (μg)	Acceptable range (μg)	Analysis No 1 (μg)	Analysis No 2 (μg)	Analysis No 3 (μg)	Analysis No 4 (μg)
7.0	5.5-8.5	7.07	7.64	7.68	7.51
35.0	30.0-40.0	34.90	35.30	35.65	35.35
75.5	63.0-87.0	72.5	72.37	73.54	73.93

Table 4.25 Concentration of vancomycin at 50 mg/ml in Tears Naturale II™ in tear film

Time(Min)	Conc vancomycin in Tears Naturale II™ (µg/ml)					
	No 1	No 2	No 3	No 4	No 5	No 6
0	13499	10052	16656	17250	23084	22008
30	750	329	762	605.5	459.5	240
60	599	209.5	141	160	344.5	204
90	70	98.5	103	82.5	77.5	124
120	61.35	95.5	66	46.5	46	69.5

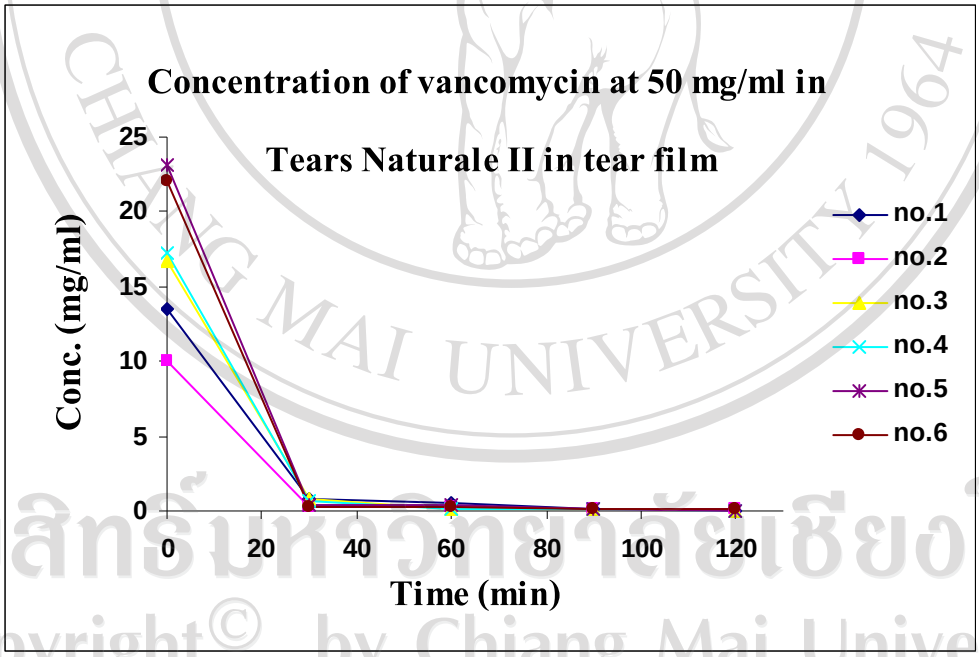


Figure 4.16 Concentration-time profiles of vancomycin at 50 mg/ml in Tears Naturale II in tear film (6 samples).

Table 4.26 Concentration of vancomycin at 50 mg/ml in 0.1% chitosan in tear film

Time(Min)	Conc vancomycin in 0.1% chitosan (µg/ml)					
	No 1	No 2	No 3	No 4	No 5	No 6
0	7284	9412	5972	9332	7814	8036
30	331	691	114.5	232	971.5	600
60	149.5	157.5	120	120.5	102.5	238
90	145	118.5	106.5	145.5	83.5	134.5
120	126.5	64.5	98	45	90	82.5

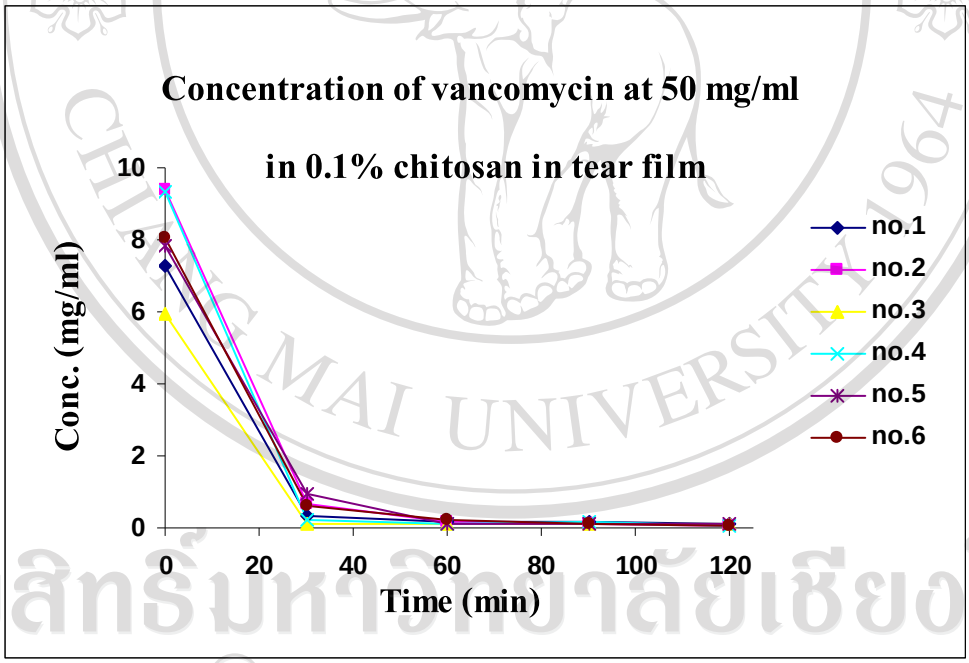


Figure 4.17 Concentration-time profiles of vancomycin at 50 mg/ml in 0.1% chitosan solution in tear film (6 samples).

Table 4.27 Concentration of vancomycin at 50 mg/ml in 0.3% chitosan in tear film

Time(Min)	Conc vancomycin in 0.3% chitosan (µg/ml)					
	No 1	No 2	No 3	No 4	No 5	No 6
0	15416	10808	19000	29104	20529	22928
30	882.5	473	625.5	619.5	533.5	713.5
60	1148	299	951	410	356.5	471.5
90	349.5	175.5	677	467.5	332	247
120	225	145	125.5	459.5	123.5	142.5

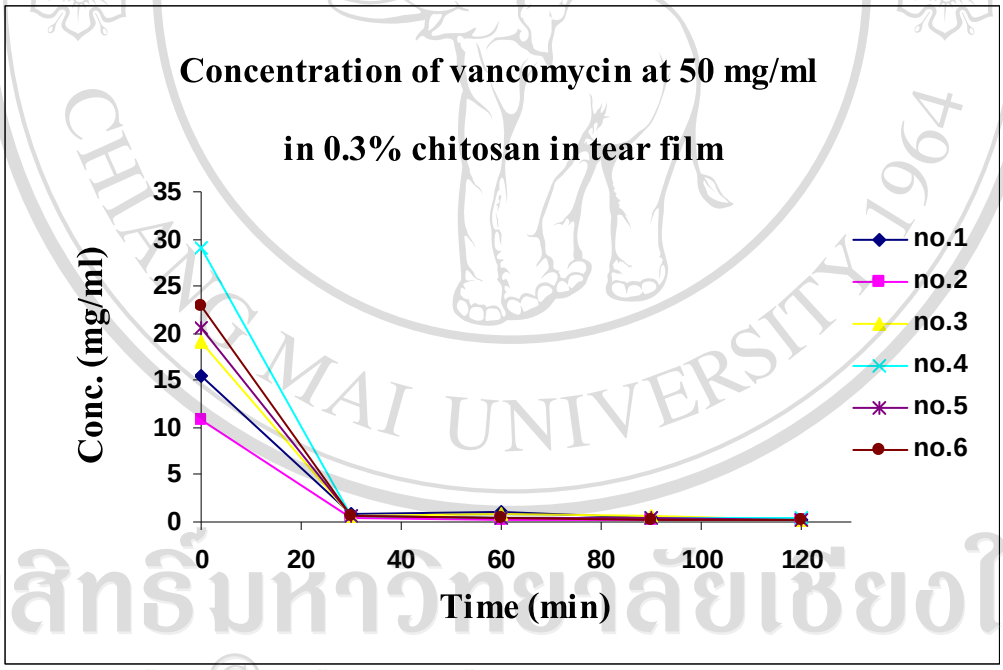


Figure 4.18 Concentration-time profiles of vancomycin at 50 mg/ml in 0.3% chitosan solution in tear film (6 samples).

Table 4.28 Concentration of vancomycin at 50 mg/ml in normal saline solution (NSS) in tear film

Time(Min)	Conc vancomycin in NSS (µg/ml)					
	No 1	No 2	No 3	No 4	No 5	No 6
0	4078	7724	3056	3942	2290	5540
30	418.5	302	284	298.5	265	450.4
60	186.5	150	118	185	154	165
90	166	105	107	149	126	135
120	153.5	90	77	105	120	86

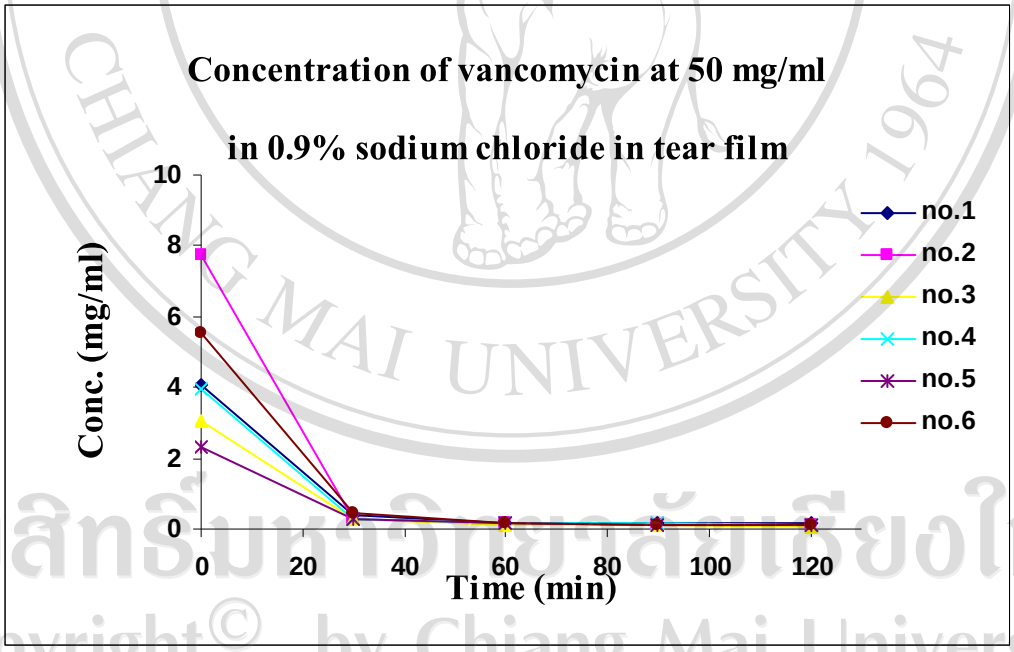


Figure 4.19 Concentration-time profiles of vancomycin at 50 mg/ml in 0.9% sodium chloride solution in tear film (6 samples).

Table 4.29 Areas under the curves of vancomycin at 50 mg/ml 0→30 minutes for the range of vehicles used

No.	Area under the curve (AUC) $_{0\rightarrow30}$ (mg.min/ml)			
	Vancomycin 50 mg/ml in Tears Naturale II™	Vancomycin 50 mg/ml in 0.1% chitosan	Vancomycin 50 mg/ml in 0.3% chitosan	Vancomycin 50 mg/ml in 0.9% sodium chloride
1	213.74	114.23	244.48	67.45
2	155.72	151.55	169.22	120.39
3	261.27	91.30	294.38	50.10
4	267.83	143.46	445.85	63.61
5	353.15	131.78	315.94	38.33
6	333.72	129.54	354.62	89.86
Mean	264.24	126.98*	304.08	71.62*
SD	73.59	21.64	94.47	29.54
AUC ratio[a]	1	0.48	1.15	0.27

[a] Mean AUC of vancomycin at 50 mg/ml in each vehicle / Mean AUC of vancomycin at 50 mg/ml in Tears Naturale II™

* $P < 0.05$

Table 4.30 Areas under the curves of vancomycin at 50 mg/ml 0→120 minutes for the range of vehicles used

No.	Area under the curve (AUC) $_{0\rightarrow120}$ (mg.min/ml)			
	Vancomycin 50 mg/ml in Tears Naturale II™	Vancomycin 50 mg/ml in 0.1% chitosan	Vancomycin 50 mg/ml in 0.3% chitosan	Vancomycin 50 mg/ml in 0.9% sodium chloride
1	245.98	129.92	306.02	86.60
2	171.32	171.16	192.72	133.92
3	281.01	101.28	354.49	62.27
4	284.89	155.60	488.36	79.68
5	373.40	153.29	346.45	52.50
6	348.20	150.95	389.02	106.90
Mean	284.13	143.70*	346.18	86.98*
SD	66.16	22.47	88.77	27.24
AUC ratio[a]	1	0.51	1.22	0.31

[a] Mean AUC of vancomycin at 50 mg/ml in each vehicle / Mean AUC of vancomycin at 50 mg/ml in Tears Naturale II™

* $P < 0.05$

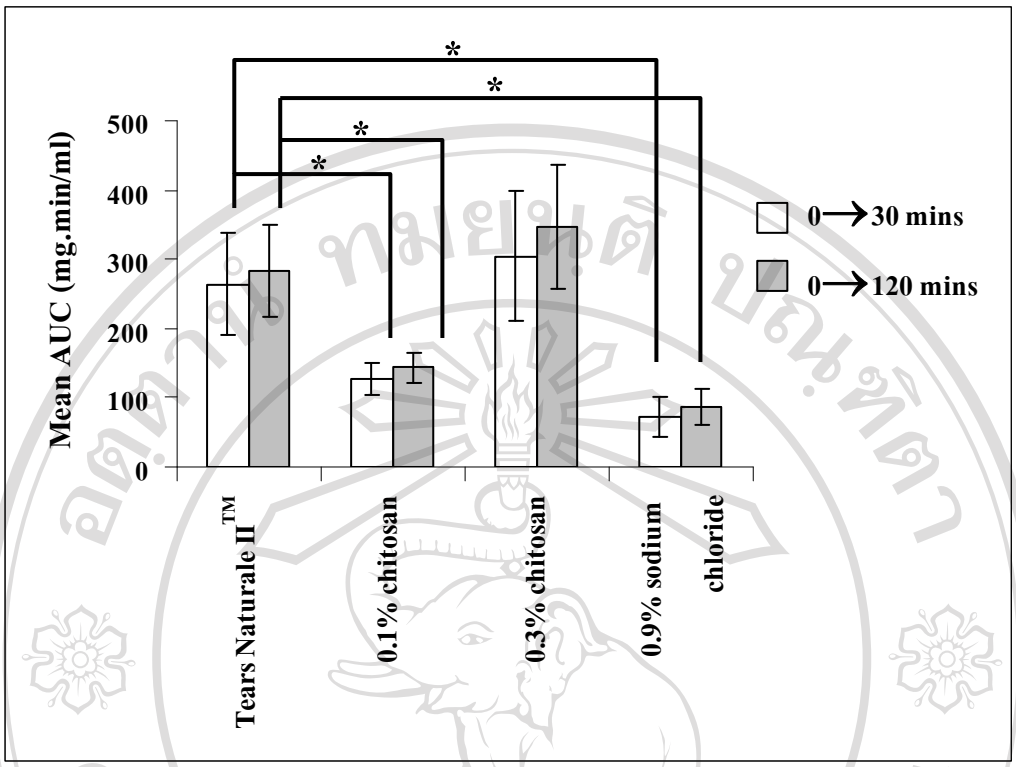


Figure 4.20 Area under the curve of vancomycin at 50 mg/ml in the various vehicles used. * $P < 0.05$

In this work, the collection of tear samples after 0, 30, 60, 90 and 120 minutes was based on the minimum inhibition concentration (MIC) and sensitivity of fluorescent polarization immunoassay (TDx-FLx System Abbott test kit) of vancomycin. Murphy et al. [114] reported that the MIC of vancomycin for gram positive endophthalmitis causing pathogens is 4 mg/l, and the majority of cases is in the range of 0.5-1.0 mg/l [115, 116]. The sensitivity of the fluorescent polarization immunoassay (TDx-FLx System Abbott, USA) was 2 µg/ml. The method for determining the concentration of vancomycin assays by fluorescent polarization immunoassay (TDx-FLx System Abbott, USA) was a modified version of that described by Alster et al. and Huerva et al. [94, 117]. The limit of detection of the vancomycin concentration in rabbit eyes came from the small volume of samples taken in the glass capillaries (2 µl). Since the volume of samples determined by the TDx-FLx System Abbott test kit was 100 µl, each tear fluid sample was diluted by

Abbott buffer to 100 µl before determination by fluorescent polarization immunoassay. The concentration of vancomycin at the last sampling time (120 minute) was about 50 µg/ml, above the MIC of vancomycin. The area under the concentration-time curve is measured the bioavailability of the drug.

Delivery mechanism of chitosan solution

After applying the eye drops, the drug mixes with the lacrimal fluid. The contact time of drug with ocular tissue is very short, at about 1-2 minutes, because the production of lachrymal fluid is permanent [118]. Chitosan has been reported to enhance drug penetration through the mucosa, thereby increasing the transcorneal permeation of the drug [17, 56]. In addition, other properties of chitosan such as its ability to change into a hydrogel at ocular pH (pH 7.4), and its viscous nature and bioadhesiveness make it a promising candidate for ocular drug delivery [18, 20-22, 56].

A. Sol-gel transition of chitosan

The phase change (sol to gel) of chitosan solution can be triggered by a change in pH. The transition can occur at ocular pH [56]. After application of the eye drops, the transition from sol to gel of vancomycin at 50 mg/ml in 0.1% chitosan solution requires an increase of about 3 pH units to ocular pH. This is different from the study of Leesawat et al. [88], in which 0.1 % w/v chitosan solution was used as an artificial tear fluid that had a pH of 5.97, close to the pKa of chitosan (pKa 6.5). A chitosan solution of pH 5.97 can change more rapidly to a gel than vancomycin at 50 mg/ml in 0.1% chitosan solution. From Table 4.29 and 4.30, it is seen that the $AUC_{0\rightarrow30}$ and $AUC_{0\rightarrow120}$ for vancomycin at 50 mg/ml in Tears Naturale II™ is 2 times more than for vancomycin at 50 mg/ml in 0.1% chitosan. The discomfort caused by the low pH [119] of vancomycin at 50 mg/ml, prepared extemporaneously using a 0.1% chitosan solution, may induce lacrimation and lead to rapid drainage before the sol-gel transition.

B. Viscous nature of chitosan

The increase in bioavailability and duration of the therapeutic action of ocular drugs can be achieved in two ways [1, 120]. The first is to use a sustained drug delivery system, while the second involves maximizing corneal drug absorption and minimizing precorneal drug loss (viscosity effect). In this study, the kinematic

viscosities of vancomycin at 50 mg/ml in Tears Naturale II™, the 0.1% and 0.3% w/v chitosan solutions and 0.9% sodium chloride were determined by flow-time measurement and comparison. Vancomycin at 50 mg/ml in the 0.3% chitosan solution had about 3 time higher kinematic viscosity and about 2.5 time higher bioavailability than in the 0.1% chitosan solution (Table 4.26, Figure 4.28).

Commercially available artificial tears are often acceptable vehicles for topical extemporaneous eye drops [33]. Artificial tears contain emollients and buffer systems for eye comfort, and viscosity agents for prolonged contact time [33].

C. Mucoadhesive properties of chitosan

The amino group in chitosan has a pKa value of 6.5 [50]; thus, chitosan is positively charged and soluble in acidic to neutral solutions, with a charge density dependent on pH and DD. In other words, chitosan is bioadhesive and readily binds to negatively charged surfaces such as mucosal membranes. The mucoadhesive character of chitosan derives from the attraction between its positively charged amino groups and the negatively charged residues of sialic acid in the mucus of the eye along with other forces such as hydrogen bonds [6, 17].

Although vancomycin at 50 mg/ml in Tears Naturale II™ had a higher kinematic viscosity than in 0.3% chitosan (Table 4.23), its bioavailability was equivalent (Table 4.29 and 4.30). The mucoadhesive properties of chitosan can influence the bioavailability of vancomycin in eye drops.

Eye drops drain rapidly from the ocular surface, so the time for drug absorption is in the order of only a few minutes, and bioavailability is very low, typically less than 5% [8]. The AUC from 0 to 30 and 0 to 120 minutes in 0.3% chitosan showed a 1.15 and 1.22-fold improvement, respectively. From these results, it appears that the combination of its polycationic nature and viscosity makes the 0.3% chitosan solution equivalent to artificial tears.

These improvements may be explained by the viscosity and mucoadhesive nature of chitosan prolonging the drug's residence time [17]. The mucoadhesive characteristics of chitosan result from the attraction between its positively charged amino groups and negatively charged residues of sialic acid in the mucus, along with other forces such as hydrogen bonds [6, 17]. Topical eye drops have a short time of contact with the eye surface. The contact time, and therefore the

duration of drug action, can be prolonged by using a mucoadhesive material [19]. With a mucoadhesive, the clearance of the drug is controlled by the mucus turnover rate, which is much slower than the tear turnover rate [17]. Hence, a correspondingly prolonged drug residence time in tears could be expected, based on the general hypothesis that prolonging the precorneal residence time enables therapeutic drug levels to be maintained over longer periods of time [19]. Thus, the mucoadhesive nature of chitosan can control the clearance of vancomycin by slowing down the elimination of the drug by the lachrymal flow.

This study has demonstrated that the viscosity and mucoadhesive nature of chitosan are more important than the sol-gel transition because the 0.3% w/v chitosan solution is equivalent in terms of bioavailability to artificial tears for the delivery of vancomycin at 50 mg/ml. Furthermore, the kinematic viscosity of the 0.3% chitosan solution is lower than that of artificial tears, providing greater ease of application for the patient.

Chitosan is also proposed for use in artificial tear formulations, since it is endowed with good hydrating properties as well as an antibacterial effect that is desirable in cases of dry eye, which are often complicated by secondary infections [21]. These findings are consistent with those of Singla and Chawla [121] and Ludwig [6], who reported that chitosan has an antimicrobial effect. The antibacterial activity of chitosan is an advantage for treating bacterial keratitis when using it for the delivery of eye drops.

A previous report claimed that an artificial tear formulation containing chitosan caused no irritation in rabbit eyes during testing [88]. In economic terms, the cost of chitosan solution is much less than that of artificial tears (about 200 times cheaper), due to the natural abundance of chitosan in Thailand and the small amount required for a 0.3% chitosan solution.

This study shows that 0.3% chitosan solution has two mechanisms of sustained release: viscosity and mucoadhesive property. However sol-gel transition is not a significant mechanism. In addition, this study also suggests that 0.3% chitosan solution offers several advantages as a vehicle for the ophthalmic delivery of vancomycin, for example: biocompatibility, storage stability, and cost effectiveness. The physical properties of the formulation do not change when

prepared extemporaneously in eye drops. Its use in topical ocular administration provides an attractive alternative to the use of extemporaneous solutions of vancomycin by allowing a reduced frequency of topical eye drop application and is therefore more convenient for health care teams.