APPENDICES

APPENDIX A

The raw data of viscosity-average molecular weight ($\overline{M}_v$) of chitosan were determined by dilute-solution no 2 and 3.

Table 1 Dilute-solution viscosity data and calculated parameters for chitosan solution at 25.0 $\pm$ 0.1°C (Run No.2)

Concentration (g/dl)	**Flow-time (s)**	**η_{rel}**	**η_{sp}**	**η_{red} (dl/g)**	**η_{inh} (dl/g)**
0.000	373.76	-	-	-	-
0.015	441.19	1.1804	0.1804	12.0273	11.0574
0.030	518.72	1.3878	0.3878	12.9281	10.9250
0.045	614.57	1.6443	0.6443	14.3176	11.0513
0.060	703.56	1.8824	0.8824	14.7064	10.5423
0.075	812.50	2.1739	1.1739	15.6514	10.3534

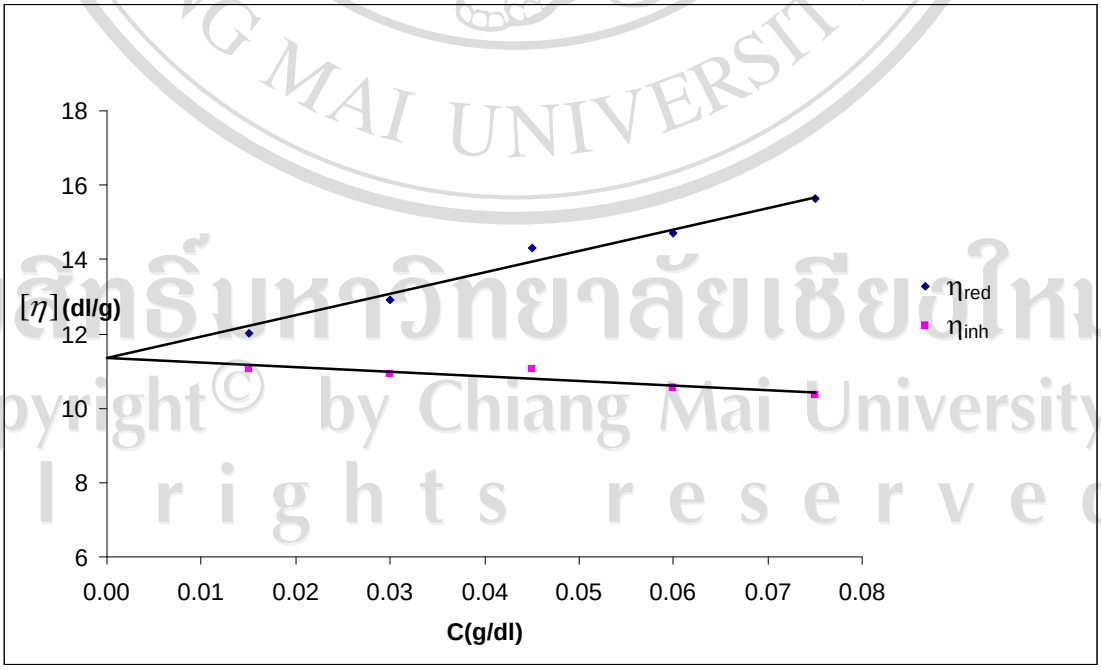

Figure 1 The reduced viscosity, η_{red}, and inherent viscosity, η_{inh}, against concentration. (Run No.2).

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

From Table 1 and Figure 1, the value of [η] is estimated as 11.45 dl/g, Therefore,

$$[\eta] = K\overline{M}_v^a$$

$$K = 8.93 \times 10^{-4}\ dl/g$$

$$a = 0.71$$

$$[\eta] = 11.45\ dl/g$$

$$11.45 = 8.93 \times 10^{-4}\ \overline{M}_v^{0.71}$$

$$\boxed{\overline{M}_v = 6.03 \times 10^5}$$

Table 2 Dilute-solution viscosity data and calculated parameters for chitosan solution at 25.0 ± 0.1°C (Run No.3)

Concentration (g/dl)	**Flow-time (s)**	**η_{rel}**	**η_{sp}**	**η_{red} (dl/g)**	**η_{inh} (dl/g)**
0.000	375.20	-	-	-	-
0.015	445.29	1.1868	0.1868	12.4538	11.4178
0.030	527.48	1.4059	0.4059	13.5288	11.3551
0.045	619.73	1.6517	0.6517	14.4829	11.1517
0.060	720.14	1.9193	0.9193	15.3225	10.8664
0.075	834.22	2.2234	1.2234	16.3120	10.6538

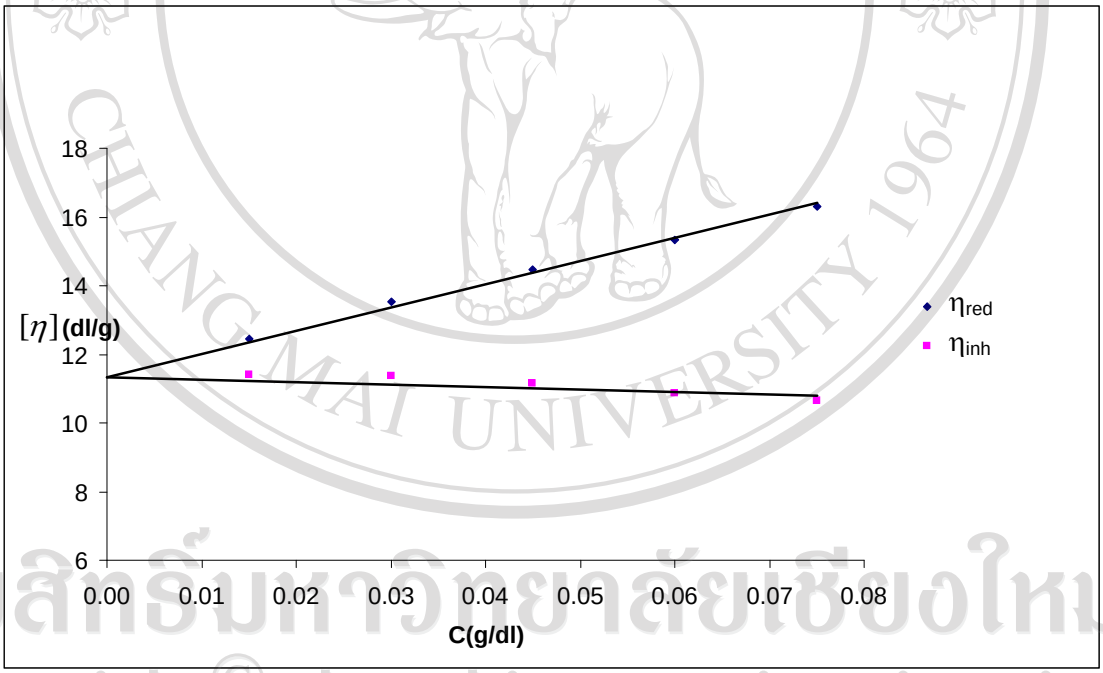

Figure 2 Graphs of reduced viscosity, η_{red}, and inherent viscosity, η_{inh}, against concentration. (Run No. 3).

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

From Table 2 and Figure 2 the value of [η] is estimated as 11.45 dl/g.

Therefore:

$$[\eta] = K\overline{M}_v^a$$

$$K = 8.93 \times 10^{-4}\ dl/g$$

$$a = 0.71$$

$$[\eta] = 11.45\ dl/g$$

$$11.45 = 8.93 \times 10^{-4}\ \overline{M}_v^{0.71}$$

$$\boxed{\overline{M}_v = 6.03 \times 10^5}$$

APPENDIX B

The raw data of assay validation reports.

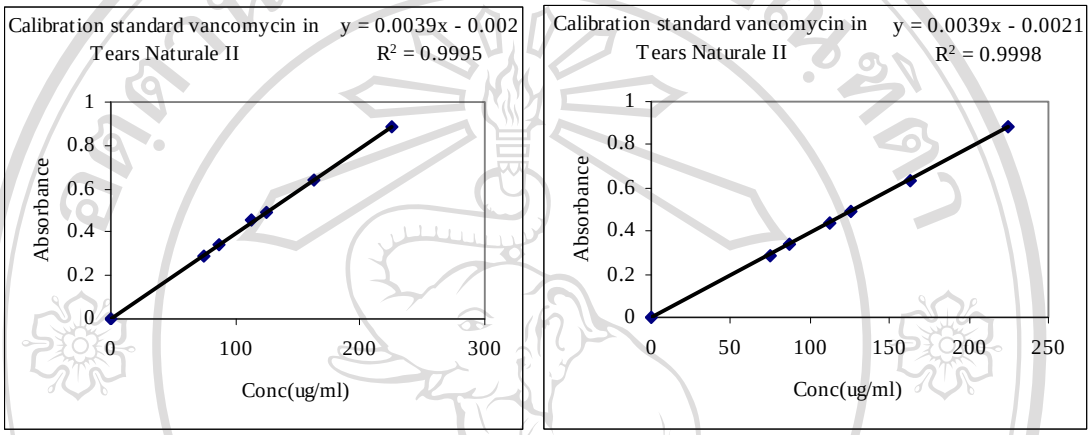

Figure 1 Calibration curve of standard vancomycin in Tears Naturale II™ no. 2 and 3 (From left to right).

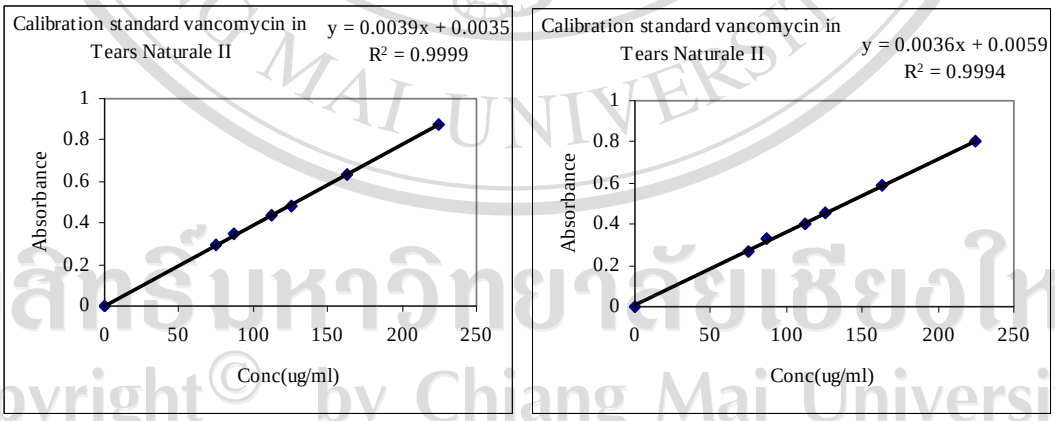

Figure 2 Calibration curve of standard vancomycin in Tears Naturale II™ no. 4 and 5 (From left to right).

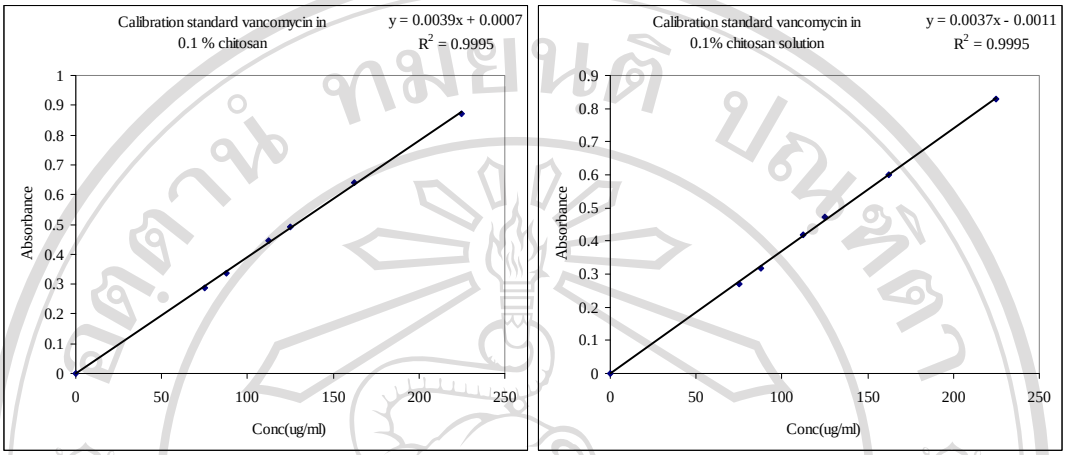

Figure 3 Calibration curve of standard vancomycin in 0.1% chitosan no. 2 and 3 (from left to right).

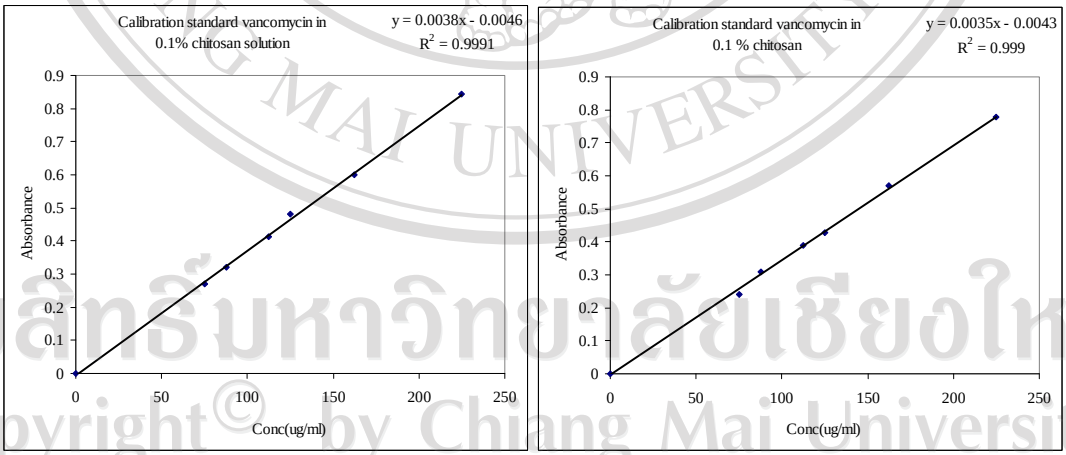

Figure 4 Calibration curve of standard vancomycin in 0.1% chitosan no. 4 and 5 (from left to right).

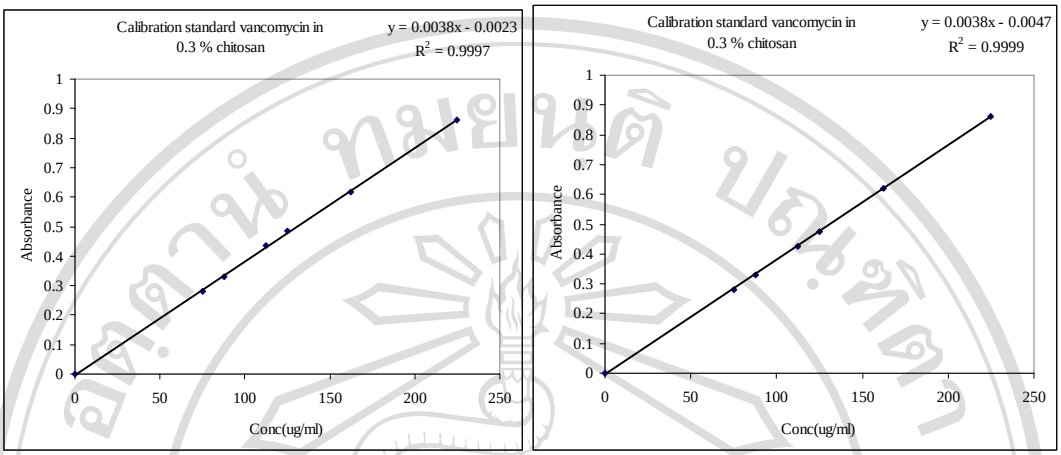

Figure 5 Calibration curve of standard vancomycin in 0.3% chitosan no. 2 and 3 (from left to right).

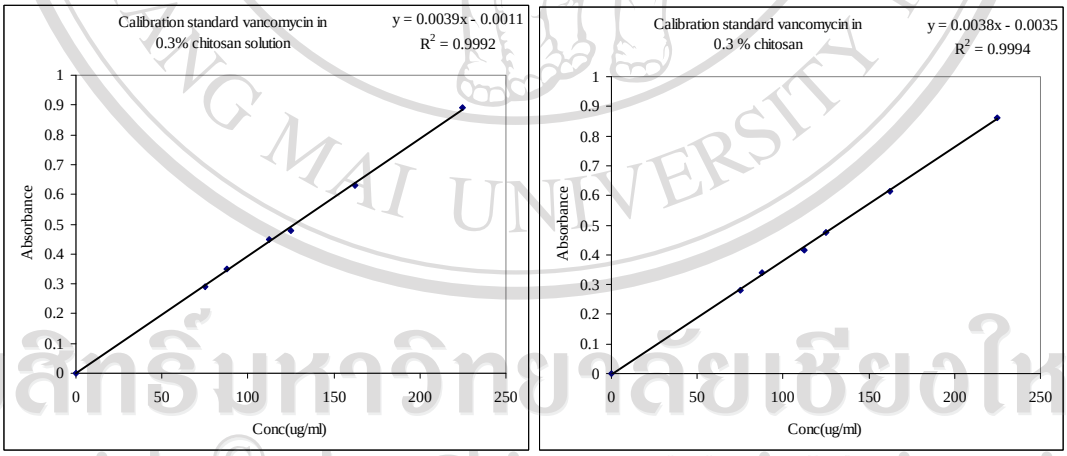

Figure 6 Calibration curve of standard vancomycin in 0.3% chitosan no. 4 and 5 (from left to right).

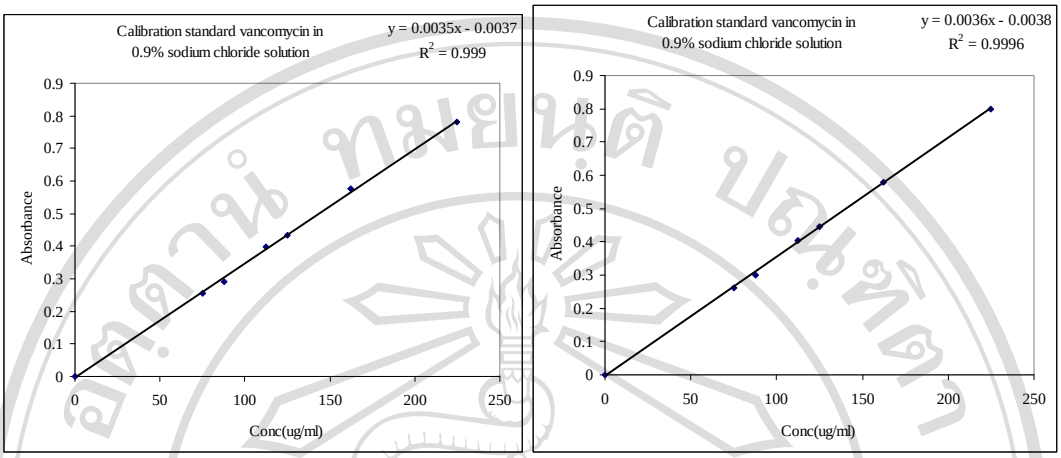

Figure 7 Calibration curve of standard vancomycin in 0.9% sodium chloride no. 2 and 3 (from left to right).

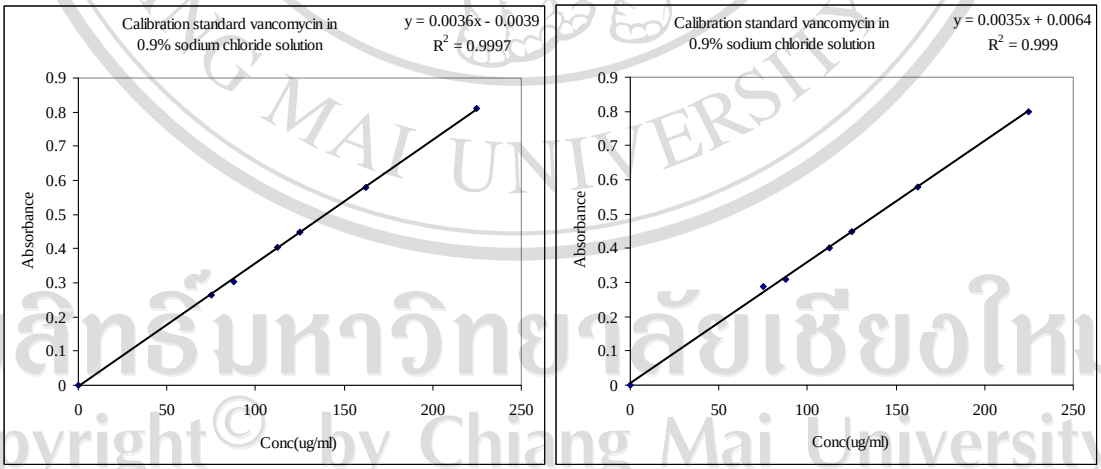

Figure 8 Calibration curve of standard vancomycin in 0.9% sodium chloride no. 4 and 5 (from left to right).

Table 1 Interday assay validation of standard vancomycin in Tears Naturale II™

Standard vancomycin concentration (µg/ml)	Calculated concentration (µg/ml)							
	day 1	day 2	day 3	day 4	day 5	Mean	S.D.	%CV
100	101.2	102.91	103.38	99.40	101.03	101.58	1.60	1.60
175	180.73	171.71	178.80	175.00	180.00	177.38	3.94	2.21
212.5	219.10	218.68	220.43	219.10	219.32	219.32	0.66	0.30
					Average %CV = 1.37			

Table 2 Interday assay validation of standard vancomycin in 0.1% chitosan

Standard vancomycin concentration (µg/ml)	Calculated concentration (µg/ml)							
	day 1	day 2	day 3	day 4	day 5	Mean	S.D.	%CV
100	103.28	103.92	99.48	103.41	103.71	102.76	1.85	1.80
175	175.08	174.0	174.65	173.71	172.34	173.96	1.05	0.60
212.5	207.77	208.97	206.49	208.88	211.83	208.79	1.98	0.95
					Average %CV = 1.12			

Table 3 Interday assay validation of standard vancomycin in 0.3% chitosan

Standard vancomycin concentration (µg/ml)	Calculated concentration (µg/ml)							
	day 1	day 2	day 3	day 4	day 5	Mean	S.D.	%CV
100	101.91	104.69	100.24	104.18	104.5	103.10	1.95	1.89
175	175.85	174.77	175.41	174.48	173.11	174.72	1.05	0.60
212.5	207.81	211.05	208.17	210.54	213.53	212.20	2.33	1.11
					Average %CV = 1.20			

Table 4 Interday assay validation of standard vancomycin in 0.9% sodium chloride

Standard vancomycin concentration (µg/ml)	Calculated concentration (µg/ml)							
	day 1	day 2	day 3	day 4	day 5	Mean	S.D.	%CV
100	101.55	102.65	104.55	100.03	104.55	102.67	1.96	1.90
175	175.06	172.35	176.02	175.66	178.55	175.53	2.22	1.26
212.5	213.50	216.50	212.00	213.00	213.00	213.60	1.71	0.80
					Average %CV = 1.32			

Table 5 Intraday assay validation of standard vancomycin in Tears Naturale II™

No.	Conc (µg/ml)	Vancomycin (absorbance)	Conc (µg/ml)	Accuracy (%)
1	100	0.389	98.85	98.85
2	100	0.389	98.85	98.85
3	100	0.393	99.87	99.87
4	100	0.381	96.79	96.79
5	100	0.387	98.33	98.33
6	100	0.384	97.56	97.56
7	100	0.375	95.26	95.26
8	100	0.379	96.28	96.28
9	100	0.380	96.54	96.54
10	100	0.386	98.08	98.08
	Mean		97.64	
	SD		1.45	
	%CV		1.49	
1	175	0.690	176.03	100.59
2	175	0.689	175.77	100.44
3	175	0.695	177.31	101.32
4	175	0.697	177.82	101.61
5	175	0.692	176.54	100.88
6	175	0.696	177.56	101.46
7	175	0.689	175.77	100.44
8	175	0.698	178.08	101.76
9	175	0.697	177.82	101.61
10	175	0.688	175.51	100.29
	Mean		176.82	
	SD		0.57	
	%CV		0.32	
1	212.5	0.840	214.49	100.94
2	212.5	0.845	215.77	101.54
3	212.5	0.838	213.97	100.69
4	212.5	0.839	214.23	100.81
5	212.5	0.841	214.74	101.05
6	212.5	0.847	216.28	101.78
7	212.5	0.845	215.77	101.54
8	212.5	0.846	216.03	101.66
9	212.5	0.847	216.28	101.78
10	212.5	0.839	214.23	100.81
	Mean		215.18	
	SD		0.43	
	%CV		0.20	

Average %CV = 0.67

Table 6 Intraday assay validation of standard vancomycin in 0.1% chitosan

No.	Conc (µg/ml)	Vancomycin (absorbance)	Conc (µg/ml)	Accuracy (%)
1	100	0.385	98.54	98.54
2	100	0.386	98.79	98.79
3	100	0.377	96.49	96.49
4	100	0.387	99.05	99.05
5	100	0.388	99.31	99.31
6	100	0.390	99.82	99.82
7	100	0.388	99.31	99.31
8	100	0.390	99.82	99.82
9	100	0.384	98.28	98.28
10	100	0.386	98.79	98.79
	Mean		98.82	
	SD		0.97	
	%CV		0.98	
1	175	0.684	175.21	100.12
2	175	0.685	175.46	100.26
3	175	0.670	171.62	98.07
4	175	0.675	172.90	98.80
5	175	0.686	175.72	100.41
6	175	0.687	175.97	100.55
7	175	0.679	173.92	99.38
8	175	0.681	174.44	99.68
9	175	0.684	175.21	100.12
10	175	0.686	175.72	100.41
	Mean		174.62	
	SD		0.81	
	%CV		0.46	
1	212.5	0.811	207.77	97.77
2	212.5	0.809	207.26	97.53
3	212.5	0.820	210.08	98.86
4	212.5	0.797	204.18	96.08
5	212.5	0.795	203.67	95.84
6	212.5	0.790	202.38	95.24
7	212.5	0.789	202.13	95.12
8	212.5	0.805	206.23	97.05
9	212.5	0.820	210.08	98.86
10	212.5	0.816	209.05	98.38
	Mean		206.28	
	SD		1.48	
	%CV		0.72	

Average %CV = 0.72

Table 7 Intraday assay validation of standard vancomycin in 0.3% chitosan

No.	Conc (µg/ml)	Vancomycin (absorbance)	Conc (µg/ml)	Accuracy (%)
1	100	0.375	99.92	99.92
2	100	0.377	100.45	100.45
3	100	0.369	98.34	98.34
4	100	0.375	99.92	99.92
5	100	0.374	99.66	99.66
6	100	0.374	99.66	99.66
7	100	0.376	100.18	100.18
8	100	0.380	101.24	101.24
9	100	0.379	100.97	100.97
10	100	0.377	100.45	100.45
	Mean		100.08	
	SD		0.81	
	%CV		0.81	
1	175	0.664	175.97	100.55
2	175	0.667	176.76	101.01
3	175	0.665	176.24	100.71
4	175	0.660	174.92	99.95
5	175	0.670	177.55	101.46
6	175	0.660	174.92	99.95
7	175	0.670	177.55	101.46
8	175	0.670	177.55	101.46
9	175	0.660	174.92	99.95
10	175	0.660	174.92	99.95
	Mean		176.13	
	SD		1.17	
	%CV		0.66	
1	212.5	0.799	211.50	99.53
2	212.5	0.802	212.29	99.90
3	212.5	0.801	212.03	99.78
4	212.5	0.798	211.24	99.41
5	212.5	0.801	212.03	99.78
6	212.5	0.799	211.50	99.53
7	212.5	0.802	212.29	99.90
8	212.5	0.799	211.50	99.53
9	212.5	0.798	211.24	99.41
10	212.5	0.797	210.97	99.28
	Mean		211.66	
	SD		0.47	
	%CV		0.22	

Average %CV = 0.56

Table 8 Intraday assay validation of standard vancomycin in 0.9% sodium chloride solution

No.	Conc (μg/ml)	Vancomycin (absorbance)	Conc (μg/ml)	Accuracy (%)
1	100	0.359	100.81	100.81
2	100	0.357	100.25	100.25
3	100	0.358	100.53	100.53
4	100	0.357	100.25	100.25
5	100	0.357	100.25	100.25
6	100	0.358	100.53	100.53
7	100	0.357	100.25	100.25
8	100	0.357	100.25	100.25
9	100	0.358	100.53	100.53
10	100	0.358	100.53	100.53
	Mean		100.42	
	SD		0.19	
	%CV		0.19	
1	175	0.629	175.81	100.46
2	175	0.628	175.53	100.30
3	175	0.627	175.25	100.14
4	175	0.628	175.53	100.30
5	175	0.627	175.25	100.14
6	175	0.627	175.25	100.14
7	175	0.628	175.53	100.30
8	175	0.627	175.25	100.14
9	175	0.628	175.53	100.30
10	175	0.627	175.25	100.14
	Mean		175.42	
	SD		0.19	
	%CV		0.11	
1	212.5	0.761	212.47	99.99
2	212.5	0.762	212.75	100.12
3	212.5	0.761	212.47	99.99
4	212.5	0.762	212.75	100.12
5	212.5	0.760	212.19	99.85
6	212.5	0.760	212.19	99.85
7	212.5	0.760	212.19	99.85
8	212.5	0.761	212.47	99.99
9	212.5	0.762	212.75	100.12
10	212.5	0.761	212.47	99.99
	Mean		211.47	
	SD		0.23	
	%CV		0.11	

Average %CV = 0.14

Table 9 Standard vancomycin in Tears Naturale II™ recovery

Concentration	Conc (μg/ml)	Absorbance	Conc (μg/ml)	Recovery(%)
Low	100	0.390	99.10	
	100	0.400	101.67	
	100	0.390	99.10	
	100	0.390	99.10	
	100	0.390	99.10	
	Mean	0.393	99.96	99.96
	SD	1.31	1.32	
Medium	175	0.681	173.72	
	175	0.682	173.97	
	175	0.680	173.46	
	175	0.681	173.72	
	175	0.682	173.97	
	Mean	0.682	173.85	99.34
	SD	0.15	0.15	
High	212.5	0.830	211.92	
	212.5	0.830	211.92	
	212.5	0.836	213.46	
	212.5	0.835	213.21	
	212.5	0.840	214.49	
	Mean	0.835	213.25	100.35
	SD	0.54	0.54	

Average recovery = 99.88 %

Table 10 Standard vancomycin in 0.1 % chitosan recovery

Concentration	Conc (μg/ml)	Absorbance	Conc (μg/ml)	Recovery(%)
Low	100	0.380	97.26	
	100	0.385	98.54	
	100	0.388	99.31	
	100	0.391	100.08	
	100	0.375	95.97	
	Mean	0.384	98.24	98.24
	SD	1.49	1.49	
Medium	175	0.710	181.87	
	175	0.699	179.05	
	175	0.689	176.49	
	175	0.679	173.92	
	175	0.710	181.87	
	Mean	0.696	178.24	101.85
	SD	1.82	1.82	
High	212.5	0.810	207.51	
	212.5	0.821	210.33	
	212.5	0.832	213.15	
	212.5	0.799	204.69	
	212.5	0.840	215.21	
	Mean	0.825	211.27	99.42
	SD	2.19	2.19	

Average recovery = 99.84 %

Table 11 Standard vancomycin in 0.3% chitosan recovery

Concentration	Conc (µg/ml)	Absorbance	Conc (µg/ml)	Recovery(%)
Low	100	0.375	99.92	
	100	0.380	101.24	
	100	0.375	99.92	
	100	0.381	101.50	
	100	0.382	101.76	
	Mean	0.379	100.87	100.87
	SD	0.003	0.88	
Medium	175	0.680	175.56	
	175	0.675	174.28	
	175	0.680	175.56	
	175	0.690	178.13	
	175	0.680	175.56	
	Mean	0.681	175.82	100.47
	SD	0.804	1.4	
High	212.5	0.825	212.74	
	212.5	0.824	212.49	
	212.5	0.823	212.23	
	212.5	0.824	212.49	
	212.5	0.825	212.74	
	Mean	0.824	212.54	100.02
	SD	0.001	0.21	

Average recovery =100.45%

Table 12 Standard vancomycin in 0.9% sodium chloride recovery

Concentration	Conc (μg/ml)	Absorbance	Conc (μg/ml)	Recovery (%)
Low	100	0.358	100.53	
	100	0.359	100.81	
	100	0.358	100.53	
	100	0.357	100.25	
	100	0.359	100.81	
	Mean	0.358	100.58	100.58
	SD	0.001	0.23	
Medium	175	0.680	175.36	
	175	0.679	175.10	
	175	0.679	175.10	
	175	0.680	175.36	
	175	0.679	175.10	
	Mean	0.679	175.21	100.12
	SD	0.001	0.14	
High	212.5	0.825	212.54	
	212.5	0.824	212.28	
	212.5	0.824	212.28	
	212.5	0.825	212.54	
	212.5	0.824	212.28	
	Mean	0.824	212.38	99.94
	SD	0.001	0.140	

Average recovery=100.21 %

Table 13 Lower limit of quantitation of standard vancomycin in Tears Naturale II™

No.	Conc (µg/ml)	Vancomycin (absorbance)	Conc (µg/ml)	Recovery(%)
1	75	0.295	74.74	99.66
2	75	0.308	78.08	104.10
3	75	0.289	73.21	97.61
4	75	0.301	76.28	101.71
5	75	0.297	75.26	100.34
6	75	0.297	75.26	100.34
	Mean	0.298	75.47	100.63
	% CV	2.13	2.15	2.15

Table 14 Lower limit of quantitation of standard vancomycin in 0.1% chitosan

No.	Conc (µg/ml)	Vancomycin (absorbance)	Conc (µg/ml)	Recovery(%)
1	75	0.288	73.67	98.22
2	75	0.300	76.74	102.32
3	75	0.299	76.49	101.98
4	75	0.290	74.18	98.91
5	75	0.292	74.69	99.59
6	75	0.283	72.38	96.51
	Mean	0.292	74.69	99.59
	%CV	2.24	2.25	2.25

Table 15 Lower limit of quantitation of standard vancomycin in 0.3% chitosan

No.	Conc (μg/ml)	Vancomycin (absorbance)	Conc (μg/ml)	Recovery(%)
1	75	0.283	75.71	100.95
2	75	0.281	75.18	100.25
3	75	0.282	75.45	100.60
4	75	0.282	75.45	100.60
5	75	0.281	75.18	100.25
6	75	0.282	75.45	100.60
	Mean	0.282	75.40	100.54
	%CV	0.267	0.26	0.26

Table 16 Lower limit of quantitation of standard vancomycin in 0.9% sodium chloride

No.	Conc (μg/ml)	Vancomycin (absorbance)	Conc (μg/ml)	Recovery(%)
1	75	0.269	75.81	101.07
2	75	0.267	75.25	100.33
3	75	0.268	75.53	100.70
4	75	0.268	75.53	100.70
5	75	0.267	75.25	100.33
6	75	0.268	75.53	100.70
	Mean	0.268	75.48	100.64
	% CV	0.281	0.277	0.277

APPENDIX C

Storage Temperature (United States Pharmacopeia)

Freezer

A place in which the temperature is maintained thermostatically between -25 ^{o}C and -10 ^{o}C (-13 ^{o}F and 14 ^{o}F).

Cold

Any temperature not exceeding 8 ^{o}C (46 ^{o}F). A refrigerator is a cold place in which the temperature is maintained thermostatically between 2 ^{o}C and 8 ^{o}C (36 ^{o}F and 46 ^{o}F).

Cool

Any temperature between 8 ^{o}C and 15 ^{o}C (46 ^{o}F and 59 ^{o}F). An article directed for storage in a cool place may be stored alternatively and distributed in a refrigerator, unless otherwise specified by the individual monograph.

Controlled Cold Temperature

This temperature is defined as that maintained thermostatically between 2 ^{o}C and 8 ^{o}C (36 ^{o}F and 46 ^{o}F), which allows for excursions in temperature between 0 ^{o}C and 15 ^{o}C (32 ^{o}F and 59 ^{o}F) and may be experienced during storage, shipping, and distribution when the allowable calculated mean kinetic temperature (MKT) is not more than 8 ^{o}C (46 ^{o}F). Transient spikes of up to 25 ^{o}C (77 ^{o}F) may be permitted if the manufacturer so instructs, and providing such spikes do not exceed 24 hours, unless supported by stability data, or the manufacturer instructs otherwise.

Room Temperature

The temperature that prevails in a working area.

Controlled Room Temperature

A temperature maintained thermostatically that encompasses the usual and customary working environment of 20 ^{o}C to 25 ^{o}C (68 ^{o}F to 77 ^{o}F); resulting in a mean kinetic temperature calculated to be not more than 25 ^{o}C; and thus allowing for excursions between 15 ^{o}C and 30 ^{o}C (59 ^{o}F and 86 ^{o}F), as experienced in pharmacies,

hospitals, and warehouses. Provided the mean kinetic temperature remains in the allowed range, transient spikes of up to 40 °C are permitted as long as they do not exceed 24 hours. Spikes above 40 °C may be permitted if the manufacturer so instructs. Articles may be labeled for storage at "controlled room temperature" or at "up to 25 °C", or other wording based on the same mean kinetic temperature. The mean kinetic temperature is a calculated value which may be used as an isothermal storage temperature that simulates the nonisothermal effects of storage temperature variations. An article for which storage at Controlled Room Temperature is directed may be stored alternatively and distributed in a cool place, unless otherwise specified in the individual monograph or on the label.

Warm

Any temperature between 30 °C and 40 °C (86 °F and 104 °F).

Excessive Heat

Any temperature above 40 °C (104 °F).

Protection from Freezing

Where, in addition to the risk of breakage of the container, freezing subjects an article to loss of strength or potency, or to destructive alteration of its characteristics, the container label bears an appropriate instruction to protect the article from freezing.

Dry Place

The term "dry place" denotes a place that does not exceed 40% average relative humidity at Controlled Room Temperature or the equivalent water vapor pressure at other temperatures. The determination may be made by direct measurement at the place, or may be based on reported climatic conditions. Determination is based on not less than 12 equally spaced measurements that encompass either a season, a year, or, where recorded data demonstrate, the storage period of the article. There may be values of up to 45% relative humidity provided that the average value is 40% relative humidity.

APPENDIX D

Terms describe of the pharmacokinetics

Pharmacokinetics

Pharmacokinetics is one of the two basic areas of pharmacology, in addition to pharmacodynamics. It deals with the quantitation of the process drug absorption, distribution, biotransformation, and excretion. These factors, coupled with prescribed drug dose, determine the time course of drug concentrations in vivo. Pharmacokinetic studies of drugs are clinically useful in predicting the intensity of drug effects if a relationship exists between drug concentrations and the pharmacologic or toxic effects of drugs.

Area under the curve (AUC):

The area under the plot of drug concentration (not logarithm of the concentration) against time after drug administration is conveniently determined by the “trapezoidal rule”: the data points are connected by straight line segments, perpendiculars are erected from the abscissa to each data point, and the sum of the triangular and trapezoidal areas so constructed is computed.

The trapezoidal rule

The trapezoidal rule is a numerical method used to approximate the integral or area under a curve. Using the trapezoidal rule to approximate the area under a curve first involves dividing the area into a number of equally wide strips. Then, the area of each strip is approximated by the area of the trapezium formed, when the upper end is replaced by a chord. The sum of these approximations gives the final numerical result of the area under the curve.

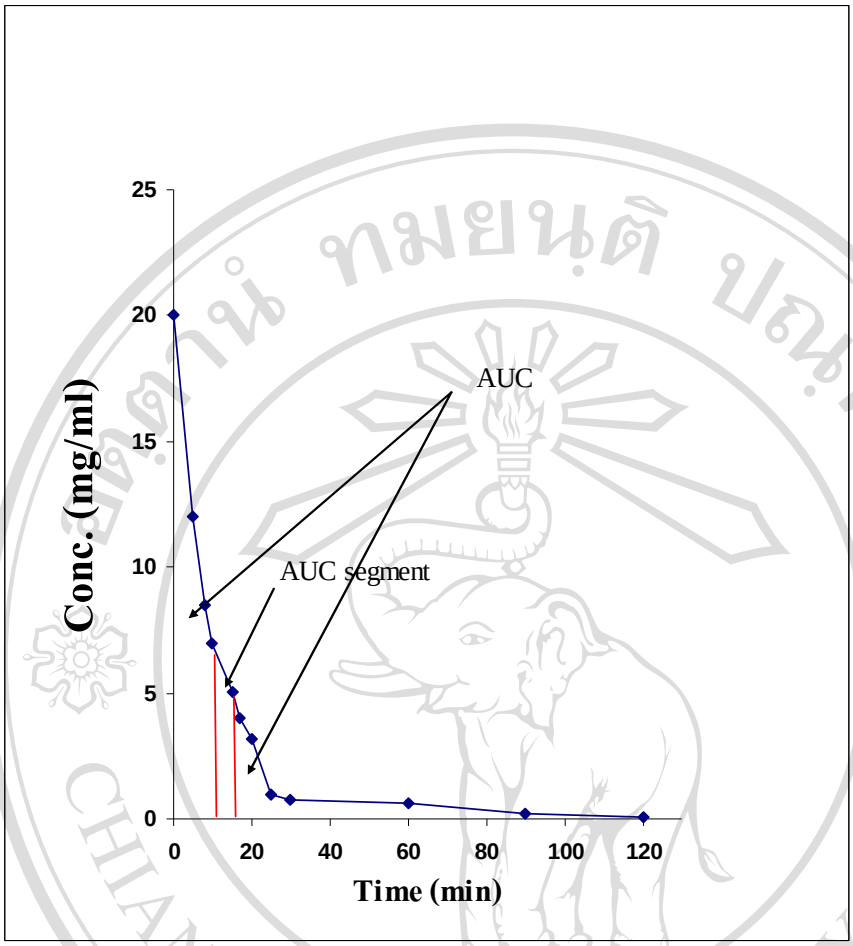

Figure 1 Linear Plot of Concentration versus Time showing the AUC and AUC segment.

Area under a curve from time = 0 to time t [AUC0_t]

Area under the curve is calculated for concentration vs. time data using the linear trapezoidal rule, which is used as a widely accepted method for approximating the area under a concentration-time curve. The accuracy of the approximation for true area under a curve depends on the number of concentration-time points within the time interval under consideration. The formula to calculate the area under the curve is as follows:

$$AUC0\_t = \sum_{i=0}^{n-1} (t_{i+1} - t_i) * (C_i + C_{i+1}) / 2$$

Where, AUC0_t = Area under a curve for time 0 to t

t = time values

C = Concentration values, e.g. C_i at time t_i

n = Total numbers of time concentration points

i = Reference index for i^{th} concentration-time value.

APPENDIX E

Certificate of approval for use of animals

Faculty of Medicine, Chiang Mai University

Certificate of Approval
For Use of Animals
Faculty of Medicine, Chiang Mai University

Protocol Number:	**4/2550**
Title of project	**Evaluation of the use of chitosan in ocular drug delivery for vancomycin**
Principal investigator:	**Ms. Anutra Khangtragool**
Affiliation:	**Division of Pharmacy**

The Faculty of Medicine, Chiang Mai University, supported by the results of Animal Ethics committee review in the meeting **1/2550** dated **30 January 2007** that the use of animals in the project conforms with international and national guidelines for ethical conduct on the care and use of animals,

Hereby approves the research proposal to be conducted under its proposed scheme. The approval is effective from **30 January 2007**

Niwat Maneekarn
Niwat Maneekarn,D.V.M.,Ph.D.
Associate Professor
Chair
Date 15th March 2007

Niwes Nantachit
Niwes Nantachit , M.D.
Associate Professor
Dean
Date 21st March 2007

Animal Ethics Committees (AECs) (26 Oct 2006 – 25 Oct. 2008)

Name/Surname	Qualification	Field of Study	Present/Absent
Assoc.Professor Dr.Niwat Maneekarn, Chair	D.V.M.,Ph.D.	Microbiology	Present
Assoc.Professor Dr. Nirush Lertprasertsuke	M.D., Ph.D.	Pathology	Absent
Asist.Professor Dr. Tawat Taesotikul	Ph.D.	Pharmacology	Present
Asist.Professor Dr. Chatchawann Apichartpiyakul	Ph.D.	Microbiology	Present
Asist.Professor Dr. Chucheep Praputhpitaya	Ph.D.	Physiology	Present
Asist. Professor Dr. Raweewan Puatanachokchai	Ph.D.	Medical Science	Absent
Dr. Chaisuree Suphavilai	Ph.D.	Tropical Health	Present
Ms. Tassanee Laima, Secretary	D.V.M	Veterinary Medicine	Present
Ms. Sanhajuta Suwannachat	B.Ag.Tech	Animal Science	Present

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Birth place Uttaradit

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High school Uttaradit Darunee School

1982 – 1987 Uttaradit

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Scientific presentation

Khangtragool, A., N. Phattana, and W. Wanaratwichit. *Hypersensitivity induced by etoposide-a case report*. 2009. The Empress Chiang Mai.

Khangtragool, A., et al. *Evaluation of the use of chitosan in ocular drug delivery for vancomycin*. in *Research path: Innovation for life*. 2008. Chiang Mai University Convention Hall: Chiang Mai University

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Publication

Khangtragool, A., et al., *Evaluation of the use of chitosan in ocular drug delivery of vancomycin.* CMU. J. Nat. Sci, 2009. **8**(1): p. 1-9.

Khangtragool, A., et al., *Stability of chitosan solutions for potential use in ocular drug delivery.* CMU. J. Nat. Sci, 2008. **7**(2): p. 209-17.

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Khangtragool, A., *Stability of cefazolin sodium eye drops.* CMU. J. Nat. Sci, 2008. **7**(1): p. 89-94.

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Khangtragool, A., B. Kumsorn, and N. Rojanasthien, *Bioequivalence study of generic finasteride in healthy male volunteers.* Chiang Mai Med Bull, 2003. **42**(4): p. 131-7.

Training Course

- The 2/2551 North- Pharmacist: The Empress Chiang Mai; Chiang Mai 13-14 January 2009
- The 8th National Conference on Thailand towards Center of Excellence in Clinical Trials “Healthy and powerful infrastructures for clinical researches in Thailand” : Faculty of Medicine, Chiang Mai University; Chiang Mai 14-15 August 2008
- Living Organization: The Empress Chiang Mai; Chiang Mai 23-24 June 2008
- Association of hospital pharmacy Thailand: Integration of pharmacy service for patient safety: The Medical Association of Thailand; Bangkok 21-23 May 2008
- Scientific program of the first Thailand forum on Renin-Angiotensin-Aldosterone System: from basic science to clinical practice: Shangri-La Chiang Mai ; 7-9 March 2008
- The 8th HA regional forum Chiang Mai Thailand: Humanized healthcare: Kad Suan Kaew ; Chiang Mai 28-29 June 2007
- Dabur Oncology meeting: Controversies in oncology: CMU experiences: The Imperial Phukaew Hill Resort; Petchaboon 24-16 November 2006
- GCP-Principles for the conduct of clinical research: Data quality: Amity Green Hill; Chiang Mai 28-29 October 2003

การประเมินการใช้ไคโตซานเป็นระบบนำส่งยาหยอดตาแวนโคมัยซิน

อนุตรา ผังตระกูล[1], สมสงวน อัษญคุณ[2], ภูริวัฒน์ ลี้สวัสดิ์[3], โรเบิร์ต มอลลอย[4] และ ชุติพร เล้ากุล[4]
[1]ห้ายนาสัชกรรม [2]ภาควิชาจักษุวิทยา โรงพยาบาลมหาราชนครเชียงใหม่ คณะแพทยศาสตร์
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วัตถุประสงค์

เพื่อศึกษาลักษณะทางเคมีกายภาพของไคโตซานและการใช้ไคโตซานเป็นระบบนำส่งยาหยอดตาแวนโคมัยซิน

วัสดุและวิธีการ

ความชื้นของไคโตซาน,ค่าของการอะเซทิเลชั่น,น้ำหนักโมเลกุลที่คำนวณจากความหนืดมีค่าเท่ากับ13.5%,94.0% และ 6.03×10^5 เตรียมยาหยอดตาแวนโคมัยซิน 50 mg/ml ใน Tears Naturale II™, 0.9% โซเดียมคลอไรด์, และ สารละลายไคโตซาน 0.1% และ 0.3% ศึกษาความคงตัวโดยการวัด UV absorbance ,pH และความเข้มข้นต่ำสุดที่ใช้ยับยั้งเชื้อ *Staphylococcus Aureus* ศึกษาในสัตว์ทดลองศาสตร์ในตาของกระต่ายหลังจากหยอดยาตา 25µl แล้วเก็บตัวอย่างที่เวลา 0, 30, 60, 90 และ 120 นาที

Tears samples were obtained by using 2.0 µl calibrated glass capillaries (microcaps Drummond)

ผลการศึกษา

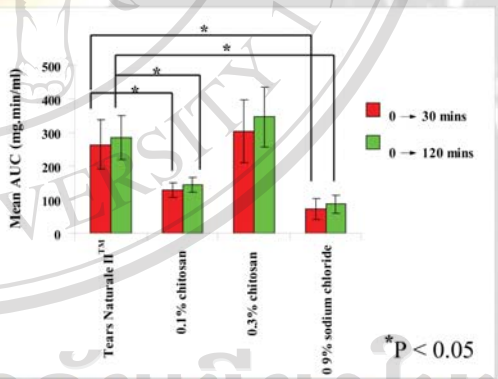
ยาหยอดตาแวนโคมัยซิน 50 mg/ml ในสารละลายไคโตซาน 0.1% และ 0.3% คงตัว 28 วันเมื่อเก็บไว้ที่อุณหภูมิ 2-8 °C ยาหยอดตาแวนโคมัยซิน 50 mg/ml ในสารละลายไคโตซาน 0.3% มีพื้นที่ใต้กราฟที่มากกว่าใน Tears Naturale II™

Concentrations of vancomycin hydrochloride in tears samples were determined by fluorescent polarization immunoassay (TDx-FLx system Abbott, USA)

สรุป

สารละลายไคโตซาน 0.3 % น่าจะเป็นระบบนำส่งยาหยอดตาแวนโคมัยซินที่ดี เนื่องจากเข้ากันได้กับตัวยาและดวงตา, มีความคงตัว, มีชีวประสิทธิผลที่ดีกว่าสารละลายอื่นและมีราคาถูก

Eye drops were instilled into the rabbit eyes



Area under the curve of vancomycin hydrochloride in the various diluents used.

ACKNOWLEDGEMENTS: This study has been accomplished with the support of BioMedical Engineering Center and Faculty of Pharmacy, Chiang Mai University

Evaluation of the Use of Chitosan in Ocular Drug Delivery of Vancomycin

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Phuriwat Leesawat[3], Robert Molloy[4] and Chutiporn Laokul[4]

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Evaluation of the Use of Chitosan in Ocular Drug Delivery of Vancomycin

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ABSTRACT

In this study, the physicochemical properties of chitosan and its use in the ocular drug delivery of vancomycin were evaluated. The physicochemical properties of the chitosan used were characterized in terms of moisture content, degree of deacetylation (DD) and viscosity-average molecular weight ($\overline{M}_v$) and were found to be 13.5%, 94.0% and 6.03 x 10^5, respectively. The vancomycin 50 mg/ml was prepared by reconstituting with Tears Naturale II™, 0.9% sodium chloride and 0.1% and 0.3% chitosan solutions. The antimicrobial potency was measured by the minimum inhibitory concentration against Staphylococcus aureus. The stabilities of the solutions were evaluated by measuring their UV absorption and pH.

The results of this study showed that vancomycin 50 mg/ml eye drops in 0.1% and 0.3% chitosan solutions were stable for 28 days when stored at 2-8°C.

The main conclusion to be drawn from this study is that the 0.1% and 0.3% chitosan solutions may be useful for the ocular drug delivery of vancomycin due to their biocompatibility, storage stability and cost effectiveness.

Key words: Vancomycin hydrochloride, Chitosan, Eye drops, Storage stability

INTRODUCTION

Bacterial keratitis is one of the most threatening ocular infections (Schaefer et al., 2001; Keay et al., 2006). Successful therapy of bacterial keratitis must be able to rapidly attain drug concentrations at the site of infection. Since the cornea is not vascularized, it is not readily permeated by systemically-administered drugs,

which are therefore generally not used for the treatment of keratitis. On the other hand, topical treatment may fail to achieve therapeutically-active drug levels in the cornea, as continuous tear flow reduces the bioavailability of topically- applied antibiotics and the corneal epithelium acts as a barrier against drug penetration. For this reason, the standard treatment of severe bacterial keratitis requires administration at frequent intervals (every 15 to 60 minutes for 48 to 72 hours) of eye drops containing fortified solutions (more concentrated than commercially-available solutions) of fluoroquinolones or multiple antibiotics, usually a cephalosporin, an aminoglycoside and glycopeptides (Fleischer et al., 1986; Gangopadhyay et al., 2000; Schaefer et al., 2001; Ghelardi et al., 2004). However, this regimen is not convenient to the patient and usually necessitates hospitalization. Efforts are now being directed at testing vehicles that better deliver antibiotics that can permeate through the cornea and at developing systems capable of prolonging the contact time between antibiotics and the corneal tissue, thereby potentially enhancing intracorneal delivery of ophthalmic medication (Ghelardi et al., 2004).

Chitosan, a cationic polymer, is biodegradable, biocompatible and non-toxic and falls into the category of mucoadhesive polymers. When using a mucoadhesive material, the clearance of the drug is controlled by the mucus turnover rate, which is much slower than the tear turnover rate. This prolonged retention of the drug formulation implies, for a drug with good permeability properties, an enhanced ocular drug bioavailability (Alonso and Sanchez, 2003). Chitosan is a very promising biomaterial in ophthalmology, not only because of its favourable biological properties, but also because of its inherent biological activity which together may have an impact on ocular therapeutics (Felt et al., 1999; Alonso and Sanchez, 2003).

In this study, chitosan has been used in the ocular delivery of vancomycin, an application which, to the best of our knowledge, has not yet been reported. The rationale for choosing chitosan for the ocular delivery of vancomycin was based on its excellent tolerance after topical application, bioadhesive properties, prolonged retention and good spreading over the entire cornea (Felt and Gurny 2001; Alonso and Sanchez, 2003). The method of preparation of the chitosan solution was adopted from the literature and modified accordingly (Leesawat et al., 2005).

MATERIALS AND METHODS

Materials

Chitosan prepared from squid chitin was purchased from Ta Ming Enterprises Co., Ltd., Thailand. Vancomycin hydrochloride for injection and Tears Naturalle II™ were purchased from Lex Pharmaceutical and Alcon Laboratories, respectively. A pure reference standard of vancomycin hydrochloride was purchased from Sigma Chemical Co., USA.

Chitosan was characterized by determining its viscosity-average molecular weight, M_v, by dilute-solution viscometry using a Schott-Gerate AVS 300 Automatic Viscosity Measuring System and its degree of deacetylation, DD, by a

chemical titration method following the procedure described by Hayes and Davies (1978). An $\overline{M}_v$ of 6.03 x 10^5 and a DD of 94.0% resulted from the analyses. The moisture content of the chitosan of 13.5% was determined by heating at 60°C to constant weight in a vacuum oven and noting the weight loss.

Microorganism

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

Preparation and characterization of chitosan solution

The method of preparation of the chitosan solution was adopted from the literature, as described by Leesawat et al. (2005). Chitosan 1% w/v was dissolved in 1% aqueous L(+)- lactic acid (Carlo Erba, 88%) at room temperature with magnetic stirrer. The solution was then diluted to 0.1% and 0.3% w/v, using Feldman's ophthalmic buffer pH 7.3 and 7.7, respectively, and sterilized by autoclaving at 121°C and 15 psi for 15 mins.

Preparation of ophthalmic formulations

Ophthalmic solutions were prepared extemporaneously by dissolving vancomycin (as the hydrochloride salt) sterile powder 500 mg in 10 ml of Tears Naturale II™ (i.e., to a concentration of 50 mg/ml) and placed into Tears Naturale II™ containers. Similarly, the vancomycin sterile powder 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. The osmolalities of these vancomycin 50 mg/ml in Tears Naturale II™, 0.9% sodium chloride and the 0.1% and 0.3% chitosan solutions were determined by an Osmomat 030.

Design of compatibility and stability studies

The compatibilities and stabilities of the vancomycin 50 mg/ml eye drops in Tears Naturale II™, the 0.9% sodium chloride solution and the chitosan solutions 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°C in a refrigerator and Group II (n=10) was stored at 30°C in an incubator.

Validation of UV spectrophotometer

A standard stock solution of vancomycin 50 mg/ml was prepared for validating the vancomycin in the Tears Naturale II™ and chitosan solutions. Further 6 solutions were prepared by dilution of 6, 7, 9, 10, 13 and 18 µl of the vancomycin 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 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

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17 µl of their vancomycin 50 mg/ml stock solutions with distilled water and adjusting the volumes 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.

Minimum inhibition concentration analysis

Minimum inhibitory concentration (MIC) was determined by a broth dilution method according to CLSI guidelines (CLSI, 2005). Ophthalmic solutions were prepared extemporaneously in a Class 100 clean-room environment by dissolving 500 mg of vancomycin hydrochloride sterile powder 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 of 50 mg/ml. A control vancomycin hydrochloride 50 mg/ml solution was prepared by dissolving 500 mg of vancomycin hydrochloride in 10 ml of 0.9% sodium chloride solution.

Each stock solution was divided into two halves for storage at room temperature (30°C) and under refrigeration (2-8°C) with testing on days 0 (day of preparation), 3, 7, 10, 14, 21 and 28. The vancomycin hydrochloride 50 mg/ml control 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 (1.0×10^8 CFU/ml) was prepared in a Mueller-Hinton broth and diluted 100-fold (to 1.0×10^6 CFU/ml). The vancomycin solutions were further diluted with water for injection to a concentration of 250 µg/ml before serial dilutions with the Mueller-Hinton broth were carried out for the tests to be performed in sterile test tubes closed with cotton plugs. Two-fold dilutions of vancomycin were prepared in Mueller-Hinton broth, as in Table 1. For each dilution tube, 0.5 ml of each bacterial suspension and the antimicrobial agent were incubated together at 35°C in an aerobic environment for 24 hours. Standard quality control reference strain of *Staphylococcus aureus* ATCC 29213 (CLSI, 2005) with sensitivity to vancomycin hydrochloride was 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) is defined as the lowest concentration of antibiotic that yields no growth in the Mueller-Hinton broth.

Statistical analysis

Different significant percentages of the labeled amounts between day 0 and days 3, 7, 10, 14, 21 and 28 at 30°C and 2-8°C were determined by using an SPSS 12.0 for Windows One-Way ANOVA and Multiple-Comparison Post Hoc Test. Results with $p<0.05$ were considered to be statistically significant.

RESULTS

The physicochemical properties of the chitosan used were characterized in terms of its moisture content, degree of deacetylation (DD) and viscosity-average molecular weight ($\overline{M}_v$) and were found to be 13.50%, 94.0% and 6.03×10^5,

Table 1. Scheme for preparing dilutions of vancomycin to be used in Mueller-Hinton (MHB) broth dilution susceptibility tests.

Tube No.	Working solution (ml)	MHB (ml)	MHB from previous tube (ml)	Inoculum (ml)	Final concentration of vacomycin (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

respectively. The compatibility and stability studies of vancomycin 50 mg/ml eye drops in Tears Naturale II™, 0.9% sodium chloride and the 0.1% and 0.3% chitosan solutions showed that the solutions of vancomycin 50 mg/ml eye drops 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. The calibration curve of absorbance versus concentration for standard vancomycin at six different concentrations over the afore-mentioned range gave a linear plot ($r^2 > 0.99$). All of the within-day and between-day precision and accuracy levels were determined as coefficients of variation and were less than 5%. The concentration of the vancomycin eye drops was calculated from the standard curves. The percentage of the labeled amount of vancomycin 50 mg/ml eye drops 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 28-day storage. However, at 30°C, there was a statistically significant decrease in the percentage of the labeled amount from days 28 and 21 onwards for vancomycin 50 mg/ml eye drops in Tears Naturale II™ and the 0.1% chitosan solution, respectively ($p<0.05$). The percentage of the labeled amount of vancomycin 50 mg/ml eye drops in the 0.3% chitosan and the 0.9% sodium chloride solutions showed statistically significant decreases in the percentages of the labeled amounts from day 21 (Table 2).

Table 2. Percentage of the labeled amounts of vancomycin hydrochloride 50 mg/ml eye drops.

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

Table 3. pH of vancomycin hydrochloride 50 mg/ml eye drops.

Day	pH (n=10)							
	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	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

The pH values of the vancomycin 50 mg/ml eye drops in Tears Naturale II™, 0.9% sodium chloride and 0.1% and 0.3% chitosan solutions stored at 2-8°C and 30°C were in the ranges of 3.23-3.73, 3.19-3.63, 3.52-4.03 and 3.55-3.79, respectively (Table 3).

This study has also been concerned with the antimicrobial potency and the stability of extemporaneous preparations of vancomycin 50 mg/ml eye drops in the

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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 the vancomysin 50 mg/ml eye drops in Tears Naturale II™, 0.9 % sodium chloride and the 0.1% and 0.3% chitosan solutions were between 0.5-2.0 µg/ml (Table 4). According to the Clinical and Laboratory Standards Institute (CLSI, 2005), the standard MIC value of vancomycin hydrochloride is 0.5-2.0 µg/ml. All positive controls without added vancomycin hydrochloride showed positive results. Negative controls not inoculated with *Staphylococcus aureus* ATCC 29213 showed negative results. Thus, this study has demonstrated that vancomycin 50 mg/ml eye drops in the various solutions stored at 2-8°C and 30°C resulted in no loss of MIC during 28 days.

The osmolalities of vancomycin 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.

Table 4. Minimum inhibitory concentrations of vancomycin 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

DISCUSSION

In this study, attention has been focused on chitosan, a polysaccharide that has not previously been tested for its ocular delivery of vancomycin. Vancomycin hydrochloride is currently available for the treatment of external ocular diseases, such as blepharitis, conjunctivitis and bacterial keratitis (Keay et al., 2006). However, vancomycin hydrochloride eye drops (50 mg/ml) are not commercially available, instead they are made up by reconstitution in artificial tears (Ahmed and Day, 1987). Chitosan, a well-known polycationic biopolymer of natural origin, has shown excellent ocular compatibility, prolonged retention and also the ability to interact with the negatively charged conjunctiva and cornea (Felt et al., 1999; Felt and Gurny, 2001). Thus, chitosan was chosen in this study for the ocular delivery of vancomycin.

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

Table 4 compares the antibacterial activities of vancomycin 50 mg/ml eye drops 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 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., (1998) studied the stability of vancomycin 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 pH range 3.5-10.5 is usually tolerable by the eyes (Lund, 1994). The pH of vancomycin 50 mg/ml eye drops in Tears Naturale II™ and 0.9 % sodium chloride in this study are slightly lower than this pH range and are therefore not well tolerated by the eyes. In contrast, the pH values of the vancomycin 50 mg/ml eye drops in the 0.1% and 0.3% chitosan solutions stored at 2-8°C and 30°C are within this range and should be well tolerated by the eyes.

Finally, the osmolality which can be tolerated by the human eye is 160-670 mOsmol/kg (Charlton et al., 1998). The osmolalities of the vancomycin 50 mg/ml in Tears Naturale II™, 0.9% sodium chloride and the 0.1% and 0.3% chitosan solutions in the present study are all within a well-tolerated range.

CONCLUSION

The results of this study show that the 0.1% and 0.3% w/v chitosan solutions may be of value for the delivery of vancomycin since vancomycin 50 mg/ml eye drops in the chitosan solutions have a stability comparable with or even better than Tears Naturale II™. Furthermore, chitosan offers other potential benefits as regards to its antimicrobial properties, particularly in the treatment of bacterial keratitis.

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Stability of Chitosan Solutions for Potential Use in Ocular Drug Delivery

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ABSTRACT

In this study, the physicochemical properties of chitosan and its stability in solution for potential use as an ocular drug delivery vehicle were studied. The physicochemical properties of the chitosan used were characterized in terms of its moisture content, degree of deacetylation (DD) and viscosity-average molecular weight ($\overline{M}_v$) and were found to be 13.5%, 94.0% and 6.03×10^5, respectively. Chitosan solutions of 0.1% and 0.3% w/v concentrations in 1% aqueous L(+)-lactic acid were prepared. Sterilization of the solutions by autoclaving at 121°C at 15 psi pressure for 15 mins resulted in rapid acid-catalysed hydrolytic chain scission of the chitosan which, in turn, resulted in a drastic reduction in solution viscosity. Thereafter, the solutions remained stable during storage at 30°C, slightly more so at 2-8°C, with only slow and relatively small further decreases in viscosity over a period of 60 days.

The main conclusion to be drawn from this study is that 0.1% and 0.3% w/v chitosan solutions may be of value as ocular drug delivery vehicles because of their low toxicity, good ocular tolerance and storage stability.

Key words: Chitosan solution, Storage stability, Ocular drug delivery vehicle

INTRODUCTION

Chitosan has found widespread application in conventional pharmaceutical devices as a potential formulation excipient due to its suitable binding, disintegrating and tablet-coating properties (Singla and Chawla, 2001). The polymer has also been investigated as a potential adjuvant for swellable-controlled drug delivery systems. The use of chitosan in novel drug delivery as a mucoadhesive, in gene and peptide drug administration via the oral route, as well as its absorption-enhancing effect

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has been explored by a number of researchers (Singla and Chawla, 2001). Chitosan is soluble in dilute acidic solutions wherein it becomes protonated. The positive charges on the protonated chitosan molecule enable it to interact with polyanions, a process that has been used to obtain complexes as well as micro and nanoparticulate drug delivery systems. Chitosan is a very promising biomaterial in ophthalmology because of its mucoadhesive and antimicrobial activity, as recently corroborated by the findings of Felt et al., (2000).

Chitosan is biodegradable, biocompatible and non-toxic. The chemical structure of chitosan is shown in Fig. 1. When used as a mucoadhesive material in drug delivery, the clearance of the drug is controlled by the mucus turnover rate which is much slower than the tear turnover rate. This prolonged retention of the drug formulation implies, for a drug with good permeability properties, an enhanced ocular drug bioavailability (Alonso and Sanchez, 2003). Consequently, chitosan is a very promising biomaterial in ophthalmology, not only because of the favourable biological properties mentioned above, but also because of its inherent biological activity which may also have an impact on ocular therapeutics.

The objectives of this present research were to evaluate the effects of sterilization by autoclaving and the subsequent storage stability (via intrinsic viscosity, [η]) of chitosan solutions stored at 2-8°C and 30°C for 60 days. The method of preparation of the chitosan solutions was taken from the literature and modified accordingly (Leesawat et al., 2005).

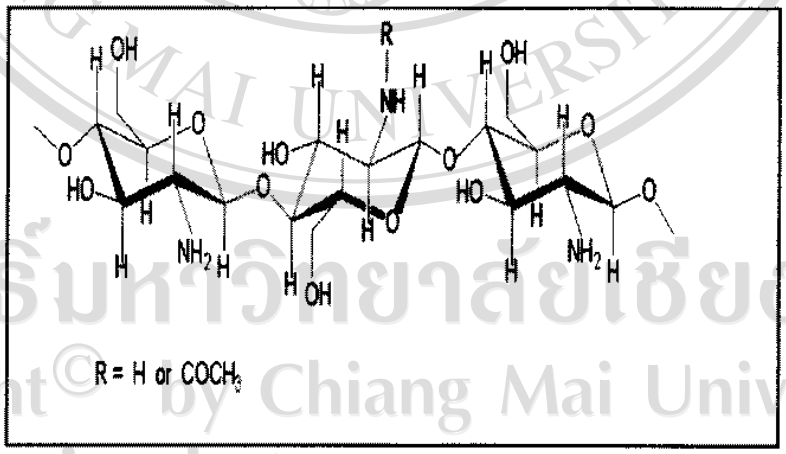

Figure 1. Chemical structure of chitosan.

MATERIALS AND METHODS

Materials

Chitosan prepared from chitin (squid type) was purchased from Ta Ming Enterprises Co., Ltd., Thailand. L(+)-lactic acid (min. assay 88%) was purchased from Carlo Erba.

Determination of moisture content

The moisture content (% by weight) of the chitosan was determined by heating at 60°C to constant weight in a vacuum oven and calculating the weight loss. The moisture content was then obtained from:

$$\text{Moisture Content} = \frac{\textit{initial weight–dry weight}}{\textit{initial weight}} \times 100\% \qquad (1)$$

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 the ambient conditions (temperature, pressure, relative humidity).

Determination of degree of deacetylation

The degree of deacetylation, DD, of the chitosan was determined by a chemical titration method following the procedure described by Hayes and Davies (1978). 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 solution of a known weight of the hydrochloride dissolved in 100 ml of distilled water with 0.1 M sodium hydroxide solution, the DD of the original chitosan was calculated from :

$$\text{DD} = \frac{(C \times V_1 \times V_2 \times MW \times 100)}{(1000 \times V_3 \times W)} \quad \% \qquad (2)$$

where C is the exact molar concentration of the sodium hydroxide (≈0.1), V1 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 weight of chitosan hydrochloride dissolved in V_2 (g).

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

The chitosan was further characterized by determining its viscosity-average molecular weight (M_v) by dilute-solution viscometry, using a Schott-Gerate AVS 300 Automatic Viscosity Measuring System. 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 0.025, 0.050, 0.075 and 0.100 g/dl. For flow-time measurements, 15 ml of each solution were accurately pipetted into a Ubbelohde-type viscometer, clamped vertically in a constant temperature water bath at 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 equation (3) (Rathke and Hudson, 1994):

$$[\eta] = 8.93 \times 10^{-4} \overline{M}_v^{\,0.71} \; dl/g \qquad (3)$$

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

Preparation of chitosan solutions for storage stability studies

The method of preparation of the chitosan solutions was taken from the literature and modified accordingly (Leesawat et al., 2005). Chitosan 1% w/v was dissolved in 1% aqueous L(+)-lactic acid at room temperature with magnetic stirring. It was then diluted to 0.1% and 0.3% w/v using Feldman,s ophthalmic buffer pH 7.3 and 7.7. respectively, and sterilized by autoclaving at 121°C and 15 psi for 15 mins. The osmolalities of these 0.1% and 0.3% chitosan solutions were determined by an Osmomat 030. The stability of the chitosan solutions was investigated in terms of their pH and intrinsic viscosity ([η]) changes during storage at 2-8°C and 30°C .

RESULTS

The physicochemical properties of the chitosan which were characterized, namely: moisture content, degree of deacetylation (DD) and viscosity-average molecular weight ($\overline{M}_v$) were found to be 13.50%, 94.0% and 6.03 x 10^5, respectively. The storage stability of the chitosan solution was studied at two different temperatures: ambient (Asia) temperature (30°C) and refrigeration temperature (2-8°C) (Prakongpan, 2540). 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 Figs. 2 and 3. The decreases are seen to be biphasic with an initial rapid sterilization phase, followed by a much slower storage phase. As the results show, the effect of storage is relatively small compared with the effect of sterilization. The effect of storage time only is seen more clearly in Figs. 4 and 5 on expanded scales. The intrinsic viscosity does decrease 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 mainly to increase the solution viscosity for practical purposes (e.g., prolonged ocular retention time) rather than to influence storage stability. Finally, the pH values of the 0.1% and 0.3% w/v solutions fluctuated between 5.10-5.40 and 3.91-4.19, respectively (Table 1), with no obvious trend with storage time.

DISCUSSION

Chitosan is well known to undergo acid-catalysed hydrolytic chain scission of its glucosidic linkages in dilute acid solution. This chain scission, which occurs at random points along the chain, results in a rapid molecular weight decrease with a corresponding 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, it has been shown that, for most cellulose derivatives in dilute acid solution, the decrease in average molecular weight $\overline{M}$ with time t can be fitted approximately to the second-order rate equation (4) (Haward, 1950), i.e.,

$$\frac{1}{\overline{M}_t} - \frac{1}{\overline{M}_o} = kt \quad (4)$$

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., $k = f(T, [Acid])$

To a good approximation, the viscosity-average molecular weight, $\overline{M}_v$, from viscometry of chitosan in dilute aqueous acid solution can be considered to be directly proportional to the intrinsic viscosity, $[\eta]$, of the solution (i.e., $\overline{M}_v \propto [\eta]$). This is because the value of the exponent 'a' in the Mark-Houwink Equation (5) is approximately equal to 1 for chitosan in dilute acid solution. Therefore, since K is a contant, the equation below

$$[\eta] = K\overline{M}_v^{\,a} \quad (5)$$

approximates to $[\eta] = K\overline{M}_v$ (6)

hence $[\eta] \propto \overline{M}_v$ (7)

Therefore, combining equations (4) and (6) gives

$$\frac{1}{[\eta]_t} - \frac{1}{[\eta]_o} = k't \quad (8)$$

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 a good approximation, from a single solution concentration via the Solomon-Ciuta One-Point Equation (9):

$$[\eta] = [2(\eta_{sp} - \ln \eta_{rel})]^{1/2} / C \quad (9)$$

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°C and 2-8°C are compared in Figs. 2 and 3. 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). 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 (Figs. 4 and 5), although this effect is overshadowed in Figs. 2 and 3 by the much greater effect of autoclaving.

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 5.10-5.40 and 3.91-4.19, respectively (Table 1). The pH range 3.5-10.5 is usually tolerable by the human eye (Lund, 1994). 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. The osmolality which can be tolerated by the eye is 160-670 mOsmol/kg (Charlton and Dalla, 1998).

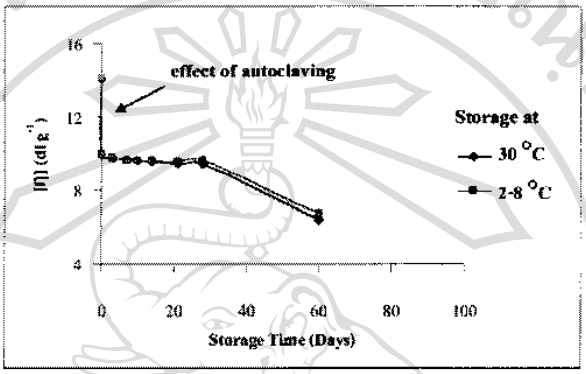

Figure 2. Variations in intrinsic viscosity, [η], of the 0.1% w/v chitosan solutions with autoclaving and storage time at different temperatures.

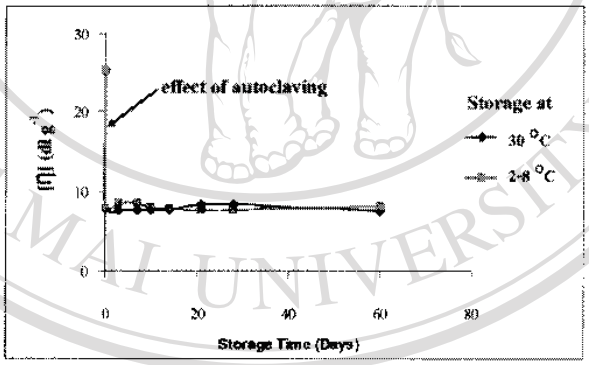

Figure 3. Variations in intrinsic viscosity, [η], of the 0.3% w/v chitosan solutions with autoclaving and subsequent storage time at different temperatures.

CONCLUSION

The hydrolytic degradation of chitosan in aqueous acid solution is well documented (Biskup et al., 2007). The fact that this hydrolytic degradation 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 (Varum et al., 2001).

In this work, the chitosan solutions were prepared in a dilute (1% v/v) aqueous solution of a weak acid (L-lactic acid) and stored at moderate (30°C) or low (2-8°C) temperature. Under these conditions, the rate of hydrolysis of the chitosan in solution would be expected to be relatively slow over a period of days, if not weeks. However, chitosan solutions for use in ocular drug delivery need to be sterilized

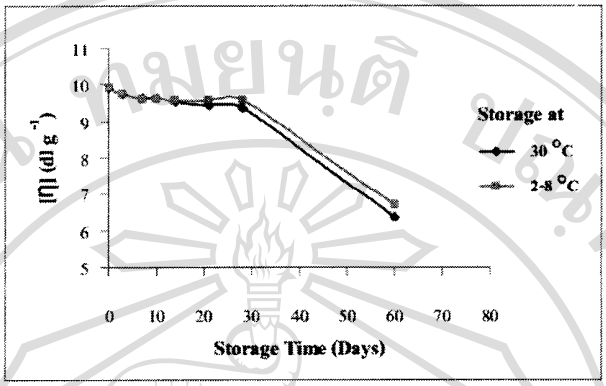

Figure 4. Variations in intrinsic viscosity, [η], of the 0.1% w/v chitosan solutions with storage time only at different temperatures.

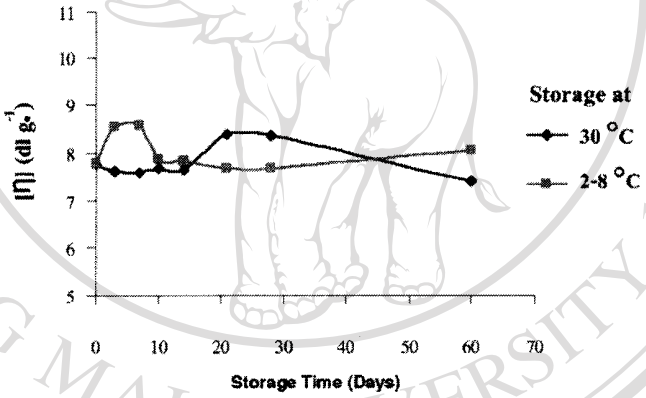

Figure 5. Variations in intrinsic viscosity, [η], of the 0.3% w/v chitosan solutions with storage time only at different temperatures.

Table 1. Variations in pH of the 0.1% and 0.3% w/v chitosan solutions with storage time at different temperatures.

Day	pH (±0.01)			
	Chitosan solution 0.1%		Chitosan solution 0.3%	
	2-8°C	30°C	2-8°C	30°C
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

before use with the recommended method, i.e., being autoclaved 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 has been observed here as the large reductions in intrinsic viscosity, $[\eta]$, in Figs. 2 and 3. 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 though 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 does drastically reduce its viscosity.

In conclusion, these results need to be viewed within the wider context of this work which is to examine the potential use of these chitosan solutions as vehicles for ocular drug delivery. Bearing in mind the unavoidable effect of autoclaving, the main considerations are (a) whether or not the residual solution viscosity after autoclaving is still sufficient for ocular retention and (b) how stable the solutions are on storage. In answer to these questions, the results of this work have shown that (a) if the initial molecular weight and solution concentration of the chitosan are high enough, the residual solution viscosity after autoclaving is both sufficient and adjustable for practical use and (b) the autoclaved chitosan solutions can be stored safely for extended periods of up to 60 days at 30°C; storage at 2-8°C improves storage stability still further, as would be expected, but only marginally so. On the basis of these results, it is concluded that chitosan solutions in dilute aqueous L-lactic acid have considerable potential for use as ocular drug delivery vehicles. Further work is continuing in order to develop this potential.

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