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

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

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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