รายงานการวิจัยฉบับสมบูรณ์

การประเมินการใช้ไคโตซานเป็นระบบนำส่งยาหยอดตาแวนโคมัยซิน

Evaluation of the use of chitosan in ocular drug delivery for vancomycin

ผศ.ดร.ภก. ภูริวัฒน์ ลี้สวัสดิ์	สังกัดคณะเภสัชศาสตร์ มหาวิทยาลัยเชียงใหม่
ภญ. อนุตรา มังตระกูล	สังกัดงานผลิตยา ฝ่ายเภสัชกรรม คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่
รศ.พญ. สมสงวน อัษญดุณ	สังกัดภาควิชาจักษุวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่
Dr. Robert Molloy	สังกัดคณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่

เสนอต่อ

ศูนย์วิศวกรรมชีวการแพทย์ มหาวิทยาลัยเชียงใหม่

กันยายน 50

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Dr. Phuriwat Leesawat

Evaluation of the use of chitosan in ocular drug delivery for vancomycin

Anutra Khangtragool*[1], Somsanguan Ausayakhun[2], Phuriwat Leesawat[3], Robert Molloy[4] and Chutiporn Laokul[4]

[1]Division of Pharmacy, [2]Department of Ophthalmology, Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, [3]Department of Pharmaceutical Science, Faculty of Pharmacy and Biomedical Engineering Center, [4]Biomedical Polymers Technology Unit, Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

ABSTRACT

In this study, the physicochemical properties of chitosan and its use in the ocular drug delivery of vancomycin were studied. 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 1.45×10^6 respectively. The chitosan 0.1% solution was found to be more stable if stored at 2-8°C rather than 30°C. Similary the compatibility and stability of vancomycin 50 mg/ml eye drops in Tears Naturale II™ and chitosan solution were enhanced if stored at 2-8°C.

In pharmacokinetic study, 0.3% chitosan solution is able to increase precorneal residence time of vancomycin when compared with simple aqueous solutions.

The conclusion to be drawn from this study is that a 0.3% chitosan solution may be of value for the delivery of vancomycin because of its favourable compatibility and stability.

การประเมินการใช้ไคโตซานเป็นระบบนำส่งยาหยอดตาแวนโคมัยซิน

อนุตรา ฆังตระกูล*[1], สมสงวน อัษญคุณ[2], ภูริวัฒน์ ลี้สวัสดิ์[3], โรเบอร์ต มอลลอย[4] และ ชุติพร เล้ากุล[4]

[1]ฝ่ายเภสัชกรรม [2]ภาควิชาจักษุวิทยา โรงพยาบาลมหาราชนครเชียงใหม่ คณะแพทยศาสตร์ [3]สายวิชาวิทยาศาสตร์เภสัชกรรม คณะเภสัชศาสตร์และศูนย์วิศวกรรมชีวการแพทย์ [4] ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่

บทคัดย่อ

การศึกษาลักษณะทางเคมีกายภาพของไคโตซานและการใช้ไคโตซานเป็นระบบนำส่งยาหยอดตาแวนโคมัยซินพบว่าความชื้นของไคโตซาน, ค่าของการอะเซทิเลชั่น, น้ำหนักโมเลกุลที่คำนวณจากความหนืดมีค่าเท่ากับ 13.5%, 94.0% และ 1.45×10^6 ตามลำดับ จากการศึกษาความคงตัวของสารละลายไคโตซาน 0.1% เมื่อเก็บไว้ที่อุณหภูมิ 2-8°C พบว่ามีความคงตัวดีกว่าที่อุณหภูมิ 30°C เช่นเดียวกับความคงตัวของยาหยอดตาแวนโคมัยซินใน Tears Naturale II™ และสารละลายไคโตซาน เมื่อเก็บไว้ที่อุณหภูมิ 2-8°C จะคงตัวมากกว่าที่ 30°C

การศึกษาเภสัชจลนศาสตร์ของยาหยอดตาแวนโคมัยซินเมื่อใช้สารละลายไคโตซาน 0.3% เป็นระบบนำส่งยาหยอดตาแวนโคมัยซินพบว่า มีชีวประสิทธิผลที่มากกว่าเมื่อใช้สารละลายอื่นๆ เป็นระบบนำส่งยาหยอดตาแวนโคมัยซิน

ผลการวิจัยนี้จึงสรุปได้ว่า สารละลายไคโตซานน่าจะเป็นระบบนำส่งยาหยอดตาแวนโคมัยซินที่ดี เนื่องจากเข้ากันได้กับตัวยาและดวงตา, มีความคงตัว, มีชีวประสิทธิผลที่ดีกว่าสารละลายอื่น และ มีราคาถูก

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LIST OF ABBREVIATIONS

AUC_{0-t}	=	area under concentration-time curve from administration and time
%CV	=	percent coefficient of variation
^{o}C	=	degree Celsius
Min	=	minute
µg	=	microgram
µl	=	microliter
ml	=	milliliter
PK	=	pharmacokinetics
SD	=	standard deviation
$T_{1/2}$	=	half-life

INTRODUCTION

The eye is a unique organ that is virtually impermeable to most environmental agents. Continuous tear flow, aided by the blink reflex, mechanically washes substances from the ocular surface and prevents the accumulation of microorganisms. In addition, lysozyme, lactoferrin, secretory immunoglobulins, and defensins are present at high levels in tears and can specifically reduce bacterial colonization of the ocular surface[1].

Since most pathogens cannot penetrate the intact corneal layer, corneal infections derive essentially from a failure of the protective mechanisms that maintain ocular surface integrity. Defects in the tear film, chemical or foreign body trauma, allergic hypersensitivity reactions, and overuse of contact lenses, as well as complications after laser *in situ* keratomileusis, can result injury to the ocular surface and predispose the cornea to infection[2,3,4]. Because of its high incidence and potential complications, bacterial keratitis is one of the most threatening ocular infection. *Pseudomonas aeruginosa* and *Staphylococccus aureus* frequently cause severe keratitis that may lead to progressive destruction of the corneal epithelium and stroma. Infection keratitis due to these organisms often causes corneal scarring, corneal perforation, and blindness if aggressive and appropriate is not promptly initiated[3,4].

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 antibiotic and the corneal epithelium acts as a barrier against drug penetration. For this reason, standard treatment of severe bacterial keratitis requires administration of frequent intervals (every 15 to 60 minute for 48 to 72 hours) of eye drops containing fortified (more concentrated than commercially available solutions) solutions of fluoroquinolones or multiple antibiotics, usually a cephalosporin, an aminoglycoside and glycopeptides[2-6]. However, this regimen not only is disruptive to the patient and usually necessitates hospitalization, but it has also been associated with *in vitro* toxicity to the corneal epithelium. Efforts are now directed to testing new antimicrobials that better permeate the cornea and to develop systems capable of prolonging the

contact time between antibiotics and the corneal tissue, thereby potentially enhancing intracorneal delivery of ophthalmic medication.

Chitosan, cationic polymer, is biodegradable, biocompatible and non-toxic. Chitosan is in this 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[10]. Chitosan is a very promising biomaterial in ophthalmology not only because of the favourable biological properties indicated above but also because of its inherent biological activity, which may also have an impact in ocular therapeutics. The various forms in which chitosan has been investigated in ophthalmology are indicated in Table 1.

Table 1 Forms of chitosan investigated in ophthalmology.

Chitosan form	**Application**	**Drug incorporate**	**Reference**
Solution	Prolonged retention	Tobramycin	7
Microspheres	Improved corneal penetration	Ofloxacin	8
Nanoparticles	Improved corneal penetration	Cyclosporin	9

In this study, chitosan used for ocular delivery for vancomycin, a glycopeptides that has never been tested for its delivery. The rationale for choosing chitosan for ocular delivery for vancomycin was based on its excellent tolerance after topical application, bioadhesive properties, prolong retention and good spreading over the entire cornea[7,10].

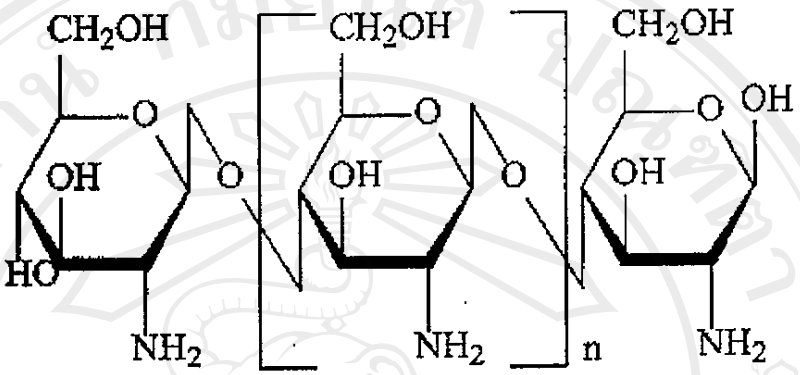
LITERATURE REVIEW

Topical application of drugs to the eye is the most popular and well-accepted route of administration for the treatment of various eye disorders. The bioavailability for ophthalmic drug is, however, very poor due to efficient protective mechanisms of the eye. Blinking, baseline and reflex lachrymation, and drainage remove rapidly foreign substances, including drugs, from the surface of the eye. Moreover, the anatomy, physiology and barrier function of the cornea compromise the rapid absorption of drugs[11]. In addition, the relative impermeability of the cornea to both hydrophilic and hydrophobic molecules accounts for the poor ocular bioavailability and systemic adverse effects as well.

Ophthalmic drug delivery is one of the most interesting and challenging endeavors facing the pharmaceutical scientist. The anatomy, physiology, and biochemistry of the eye render this organ highly impervious to foreign substances. A significant challenge to the formulator is to circumvent the protective barriers of the eye without causing permanent tissue damage. The goal of pharmacotherapeutics is to treat a disease in a consistent and predictable fashion. Whenever an ophthalmic drug is applied topically to the eye, only a small amount (<5%) actually penetrates the cornea and reaches the internal anterior tissues of the eyes. The amount of the drug that ultimately penetrates the cornea is often determined during the first 4-6 min after topical dosing. Frequent instillation of eye drops are necessary to maintain a therapeutic drug level in the tear film or at the site of action. But the frequent use of highly concentrated solutions may induce toxic side effects and cellular damage at the ocular surface. As a results, optimal absorption depends on achieving a satisfactory and rapid penetration rate across the cornea to minimize the competing, but non-absorptive factor[12].

Basic research concerning the physiochemical properties of the tears and cornea and their potential impact on ocular drug delivery was performed [13-15], and this knowledge is still exploited now in the development of new ophthalmic delivery systems. The various approaches that have been attempted to increase the bioavailability and the duration of the therapeutic action of ocular drugs can be divided into two categories[12]. The first one is based on the use of sustained drug delivery systems, which provide the controlled and continuous delivery of ophthalmic drugs. The second involves maximizing corneal drug absorption and minimizing precorneal drug loss (viscosity and penetration enhancers, prodrugs, colloids). Cationic dispersions can provide

simultaneously both advantages, by interacting with the negatively charged corneal surface components and the epithelium cellular membrane. In addition, their administration via conventional liquid dosage form is an attractive feature for patient acceptability and compliance.



Chitosan

Fig 1.Chemical structure of chitosan

As Lehr et al. [16]suggested, cationic polymers were probably superior mucoadhesive due to an ability to develop molecular attraction forces by electrostatic interactions with the negative charges of the mucus, the polycationic chitosan (see fig 1) was investigated as an ophthalmic vehicle. The polymer is biodegradable, biocompatible and non toxic. It possesses antimicrobial and wound-healing properties. Moreover, chitosan exhibits a pseudoplastic and viscoelastic behavior. [10,17] The mucoadhesive properties of chitosan are determined by the formation of either secondary chemical bonds such as hydrogen bonds or ionic interactions between the positive charged amino groups of chitosan and the negative charged sialic acid residued of mucins, depending on environmental pH. The mucoadhesive performance of chitosan is significantly highly at neutral or slightly alkaline pH as in the tear film[16]. The rationale for choosing chitosan as a viscosifying agent in artificial tear formulations was based on its excellent tolerances after topical application, bioadhesive properties, hydrophilicity, and good spreading over the entire cornea[18].The antibacterial activity of chitosan is an advantage, because *in keratoconjuctivitis sicca*, secondary infections due to the diminished tear secretion, which contains antibacterial lysozyme and lactoferrin, are frequently observed. In rabbits, a radiolabeled chitosan formulation remained at the ocular surface as long as a 5 fold more viscous PVP solutions[18]. A 3-folds

increase of the precorneal residence time of tobramycin was achieved when adding chitosan to the formulations, compared to the commercial solution of the drug. Only a minimum influence was observed from the concentration and molecular weight of chitosan employed, indicating a saturable bioadhesive mechanism based on ionic interactions of the cationic polymer with the negative charges of the ocular mucus[17]. Various chitosan derivatives were synthesized not only to improve the mucoadhesion, but also to enhance the penetration of drugs and peptides through the mucosa by opening the tight juctions between epithelial cells or by intracellular routes[10]. Felt et al. [17] reported that co-administration of ofloxacin and chitosan in eyedrops resulted in increased antibiotic bioavialbility and time of efficacy in tear fluid compared to commercial eye drops and this effect to the high viscosity of the chitosan solution. These results are of relevance to the treatment of external ocular infections. The purpose of the present study will be evaluation of chitosan in ocular drug delivery for vancomycin.

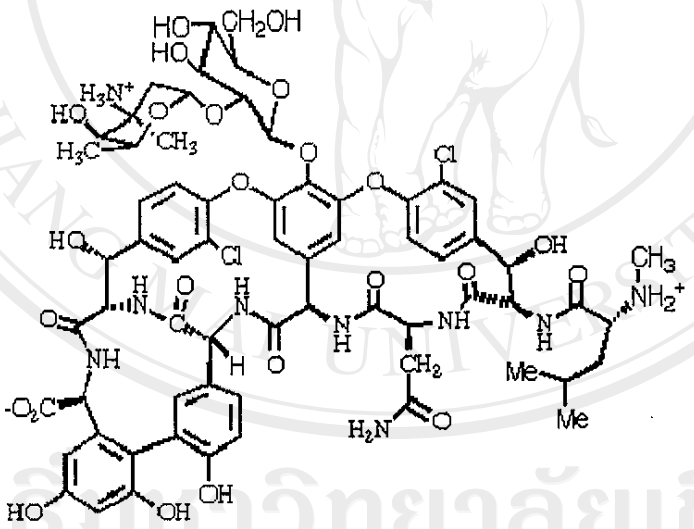


Fig 2. Chemical structure of vancomycin

Vancomycin, as shown in Figure 2, is an antibiotic produced by *Streptococcus orientalis*. Vancomycin is a glycopeptides of molecular weight 1500. Vancomycin inhibits cell wall synthesis by binding firmly to the D-Ala-D-Ala terminus of nascent peptidoglycan pentapeptide. This inhibits the transglycosylase, preventing futher elongation of peptidoglycan and cross-linking. The peptidoglycan is thus weakened and the cell becomes susceptible to lysis. The cell

membrane is also damaged, which contributes to antibacterial effect. Vancomycin eye drops (25-50 mg/ml) is use for the treatment of certain eye infections[19-20]. Ophthalmic infection by *Staphylococcus aureus* and *Staphylococcus epidermidis* are two causes of conjunctivitis and blepharoconjuctivitis. Over time, these pathogens have become resistant to cephalosporin therapy. Furthermore, strains of methicillin-resistant *S. aureus* (MRSA) have become prevalent infectious pathogens on many hospitals and long term facilities. Conventional therapy for MRSA and methicillin-resistant *S. epidermidis* includes the use of vancomycin[21]. Depending on the serious of the condition and the bacterial combinations implicated need strengthened eye drops containing a high concentration of antibiotics[22]. As these are not commercially available, they are made up for the treatment of eye infections due to sensitive bacterial combinations after isolation. The vancomycin 50 mg/ml eye drops will be prepared by adding 10 ml of Tears Naturale II™ to vancomycin 500 mg inj.[21,23] In this study chitosan solution will be used as a vehicle for vancomycin and evaluation of chitosan in ocular drug delivery for vancomycin eye drops.

OBJECTIVES

The aim of this study is to evaluate chitosan in ocular drug delivery for vancomycin. This study determined:

- Physicochemical characteristic of chitosan and chitosan solution stabilities
- Compatibility and stabilities of vancomycin 50 mg/ml eye drops in Tears Naturale II™ and chitosan solution
- Minimum inhibition concentration (MIC) of vancomycin 50 mg/ml in Tears Naturale II™ and chitosan solution
- *In vivo* pharmacokinetics studies on rabbits

MATERIAL AND METHOD

Materials

Chitosan prepared from squid chitin (polymer type) 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, ($\overline{M}_v$) by dilute-solution viscometry capillary viscometry (Schott-Gerate AVS 300 Automatic Viscosity Measuring System) and its deacetylation degree, DD, by a chemical titration method following the procedure described by Hayes et al. (1978). Viscosity-average molecular weight of 1.45×10^6 and a DD of 94.0% resulted from the analyses. The water content of the chitosan, 13.5%, was determined by heating at 100°C to constant weight in a vacuum desiccator.

Preparation and characterization of chitosan solution

The method of preparation of the chitosan solution was taken from the literature and modified accordingly [24]. Chitosan 1% w/v was dissolved in 1% aqueous L(+)- lactic acid (Carlo Erba, 88%), at room temperature with magnetic stirring. It was then diluted to 0.1% w/v using Feldman' s ophthalmic buffer pH 7.3 and sterilized by autoclaving at 121 °C for 15 mins. The osmolality of this 0.1 % chitosan solution was determined by an Osmomat 030. The stability of the chitosan solution was investigated in terms of its color, pH and viscosity (molecular weight) during storage at 2-8°C and 30 °C .

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™ to a final concentration of 50 mg/ml and placed into Tears Naturale II™ containers. Similarly, vancomycin (as the hydrochloride salt) sterile powder 500 mg was dissolved in 10 ml of the 0.1% w/v chitosan solution to a final concentration of 50 mg/ml and placed into sterile eye drop containers.

Design of compatibility and stability studies

The compatibilities and stabilities of the vancomycin 50 mg/ml eye drops in Tears Naturale II™ and chitosan solution were examined by absorbance (UV spectrophotometer, Shimadzu), color, pH and viscosity at day 0, 3, 7, 10, 14, 21, 28. (Day 0 = immediately following preparation). The samples were divided into 2 groups: Group I (n=10) stored at 2-8 °C and Group II (n=10) stored at 30 °C.

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 solution. A 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 vancomycin determination in Tears Naturale II™ and chitosan solution were tested by diluting 8, 14 and 17 µl of the vancomycin 50 mg/ml stock solution with distilled water and adjusting the volumes adjusted to 4 ml to obtain concentrations of 100, 175 and 212.5 µg/ml. Each solution was then analysed using UV spectrophotomety by measuring the absorbance at 282 nm.

Minimum inhibition concentration analysis

Minimum inhibition concentration (MIC) was determined by a broth dilution method according to NCCLS guidelines[27]. Ophthalmic solutions was prepared extemporaneously in a Class 100 clean-room environment by dissolving vancomycin (as the hydrochloride salt) sterile powder 500 mg in 10 ml of Tears Naturale II™ and the 0.1% chitosan solution to give a final concentration of 50 mg/ml. A control vancomycin hydrochloride 50 mg/ml solution was prepared by dissolving vancomycin hydrochloride 500 mg in 10 ml of aqueous with 0.9% sodium chloride solution.

The 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 was prepared in a Mueller-Hinton broth. The vancomycin solutions were further diluted to a concentration of 250 μg/ml by water for injection before serial dilutions with the Mueller-Hinton broth were carried out for the tests to be performed the tests in sterile test tubes closed with cotton plugs. Two-fold dilutions of vancomycin were prepared in Mueller-Hinton broth (Table 2). For each dilution tube, 0.5 ml of each bacterial suspension and the antimicrobial agent were incubated together at 35^{0}C in an aerobic environment for 24 hours. Standard quality control reference strains of *Staphylococcus aureus* ATCC 29213(3) with sensitivity to vancomycin hydrochloride were chosen for this study. The bacteria were transferred daily to ensure purity and good growth. On each test day, a bacterial suspension equal to the 0.5 McFarland turbidity standard was prepared in Mueller-Hinton broth. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of antibiotic that yields no growth in the Mueller-Hinton broth.

***In vivo* pharmacokinetic studies on rabbits**

***In vivo* pharmacokinetic experiments**

Drug formulation

Ophthalmic solutions prepared extemporaneously by diluting vancomycin sterile powder 500 mg with 10 ml of Tears Naturale II™ and chitosan solution to final concentration of 50 mg/ml. Vancomycin 50 mg/ml in Tears Naturale II™ and chitosan divided into 2 groups. Group I stored at 2-8 °C and group II stored at 30 °C.

Animals

Male albinos New Zealand rabbits weighing approximately 2.0-3.0 kg and free of any ocular damage were used throughout the whole study as approved by ethics committees for animal experimentation. The animals maintained in conventional, standardized conditions in single cage with free access to pelleted food and drinking water.

Topical administration and sampling

Vancomycin 50 mg/ml in Tears Naturale II™ and chitosan solution divided into 2 groups. Group I stored at 2-8 °C and group II stored at 30 °C. Group I and group II instillation into the rabbit eye at the day of preparation and then at day 7, 14, 21, 28.

After instillation into the lower conjuctival sac of eye of one drop containing 25 µl of vancomycin 50 mg/ml with care to avoid spillage, tear samples obtained by using 2.0 µl calibrated glass capillaries (microcaps Drummond™). Tear samples were collected after instillation. Each formulation was tested on 6 rabbits.

- **Determination of vancomycin concentration in tears**

Concentrations of vancomycin in tears samples were determined by fluorescent polarization immunoassay (TDx-FLx system Abbott, USA). Samples with known concentrations of vancomycin, provided by the manufacturer, will be included in each run for quality control.

Statistical analysis

Different significant percentages of the labeled amounts between day 0 and days 3, 7, 10, 14, 21 and 28 at 30^{0}C and 2-8^0 C were determined by using an SPSS 12.0 for Windows One-Way Anova and Dunnett Test. Different significant area under the curve between Tears Naturale II™ and other diluents were determined by using an SPSS 12.0 for Windows One-Way Anova and Tukey Test. Results with $p<0.05$ were considered to be statistically significant.

RESULTS

Physicochemical properties of the chitosan

Physicochemical properties of the chitosan used were characterized in terms of its moisture content, degree of deacetylation (DD) and viscosity-average molecular weight (Figures 3-5) which were found to be 13.50%, 94.0%, 1.45 x10^6 respectively.

Assay Validation Report

The absorbance of blank and the absorbance of vancomycin are shown in Figures 7-10.

The calibration curve of standard vancomycin was linear over the range of 75.0-225 μg/ml (Table 3-4, Figure 11-20).

Precision and accuracy

The lower limit of detection (LLQ) was 75 μg/ml (Table 4) and the average interassay and intraassay coefficient of variations of vancomycin in chitosan solution were 1.12% and 0.72% and in Tears Naturale II™ were 1.37% and 0.67% respectively (Table 6-9). The mean recoveries of vancomycin in chitosan solution were 98.24%, 101.85% and 99.42% and vancomycin in Tears Naturale II™ were 99.96%, 99.34% and 100.35% at the drug concentrations of 100.0, 175, 212.5 μg/ml respectively . The overall mean recovery of vancomycin in chitosan solution was 99.84% and in Tears Naturale II™ was 99.88% (Table 10, 11).

Stabilities of chitosan solution

The chitosan 0.1% solution was found to be more stable if stored at 2-8°C rather than 30°C (Figure 6).

Stability studies

The percentage of the labeled amount and pH of vancomycin 50 mg/ml eye drops in Tears Naturale II™ and chitosan in the eye drops stored at 2-8°C were 107.71-108.63% and 107.78-108.75% respectively (Table 15).

The percentage of the labeled amount and pH of vancomycin 50 mg/ml eye drops in Tears Naturale II™ and chitosan in the eye drops stored at 30°C were 102.07-108.82% and 99.52-108.94% respectively (Table 15).

Minimum inhibition concentration analysis

On examining the minimum inhibitory concentrations (MIC) used broth dilution showed in Table 15, 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 50 mg/ml eye drops in Tears Naturale II™ and chitosan solution were 0.5-2.0 μg/ml (Table 16)

***In vivo* pharmacokinetic studies on rabbits**

In vivo pharmacokinetic studies on rabbits were studied on 0, 30, 60, 90 and 120 min respectively. The concentration of vancomycin in solutions showed in Table 17-20, Figure 21-24 and area under the curves showed in Table 21, Figure 25. The half life of vancomycin in solutions showed in Table 22.

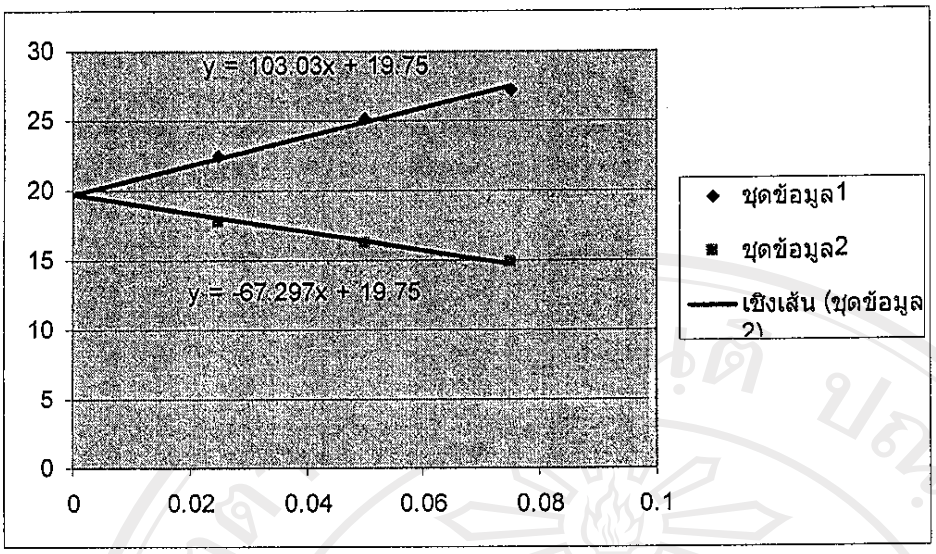


Figure 3 Viscosity-average molecular weight, ($\overline{M_v}$) = 1.32x10^6

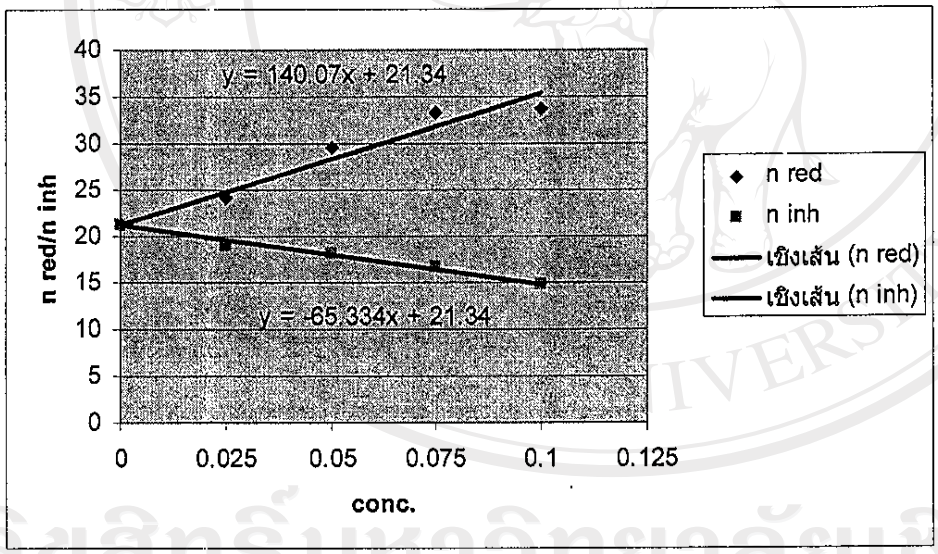


Figure 4 Viscosity-average molecular weight, ($\overline{M_v}$) = 1.47x10^6

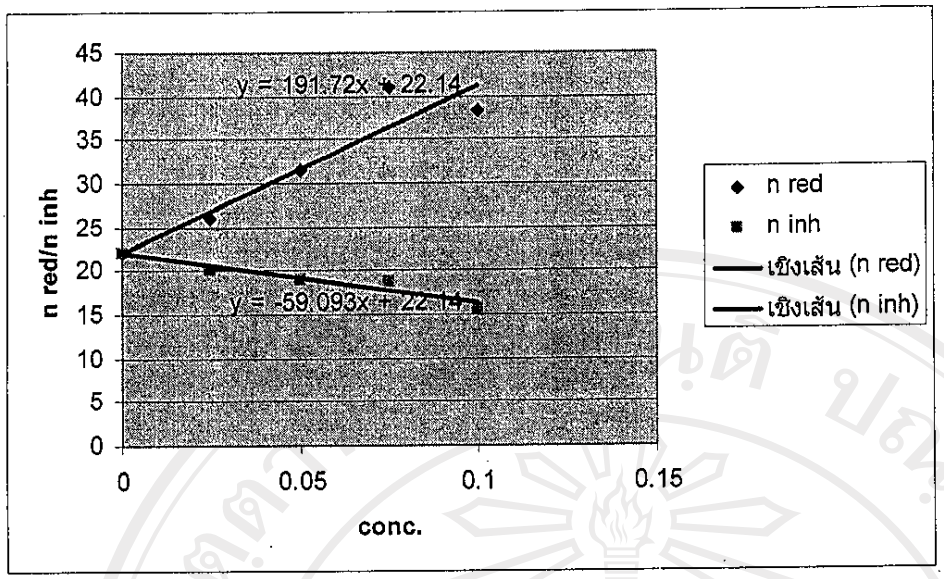


Figure 5 Viscosity-average molecular weight, ($\overline{M_v}$) = 1.55×10^6

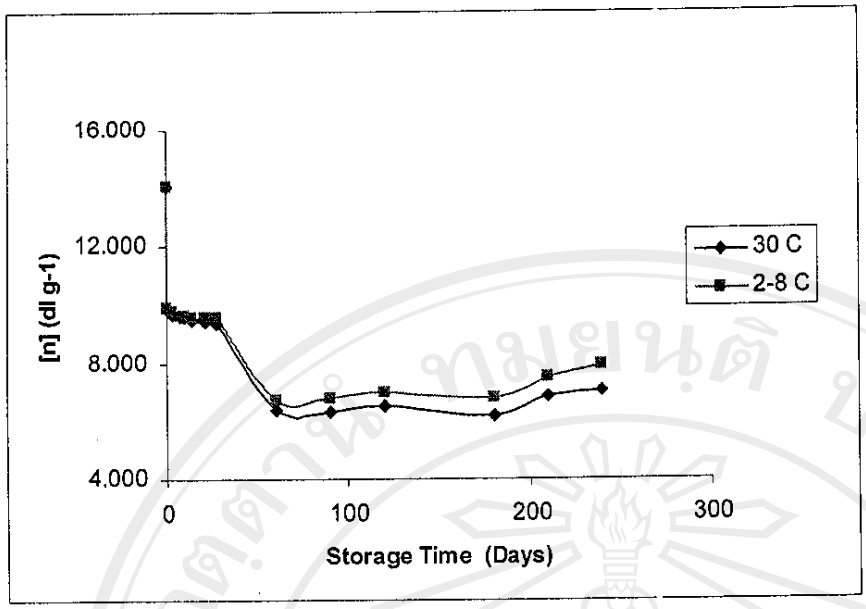


Figure 6. Variation in intrinsic viscosity,[η] of the 0.1% w/v chitosan solutions

Table 2 Broth dilution of vancomycin

Day	Inoculum(ml)	Conc (µg/ml)
1	0.5	125.0
2	0.5	62.5
3	0.5	31.25
4	0.5	15.63
5	0.5	7.80
6	0.5	3.90
7	0.5	2.00
8	0.5	0.98
9	0.5	0.50
10	0.5	0.24
11	0.5	0.14
Positive	0.5	-
Negative	-	125.0

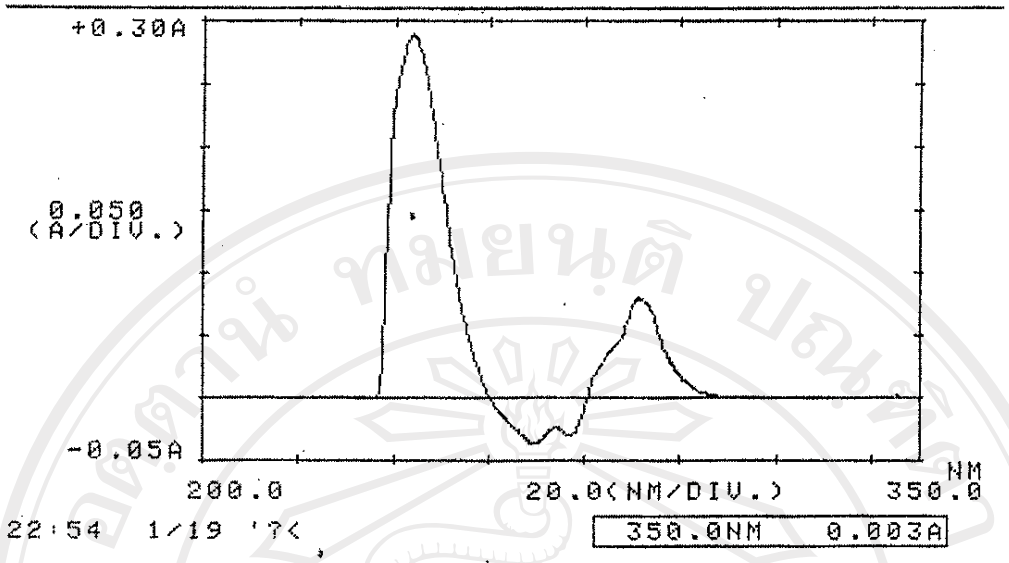


Figure 7 Absorbance of vancomycin in chitosan

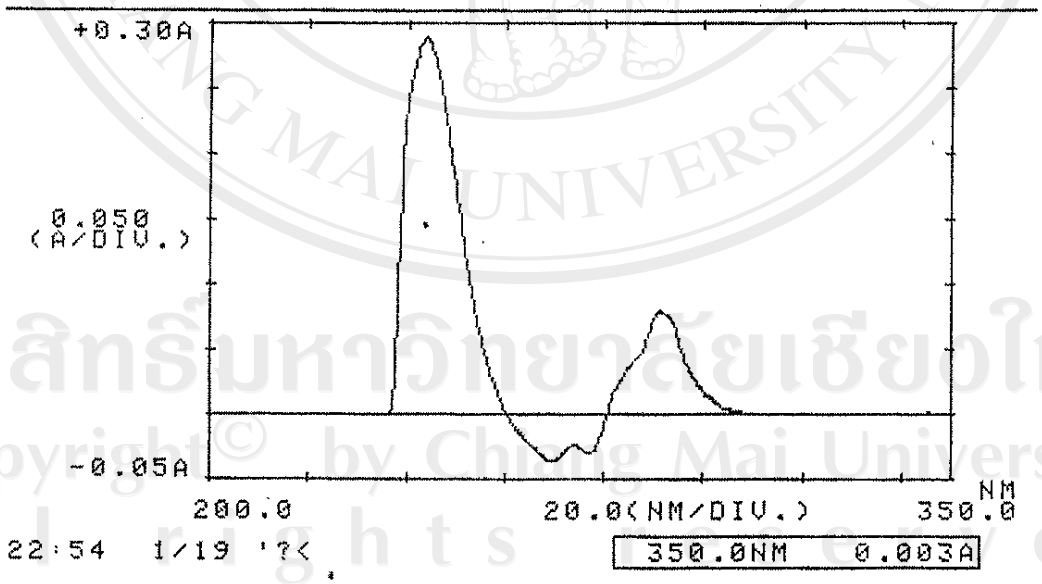


Figure 8 Absorbance of vancomycin in Tears Naturalle II™

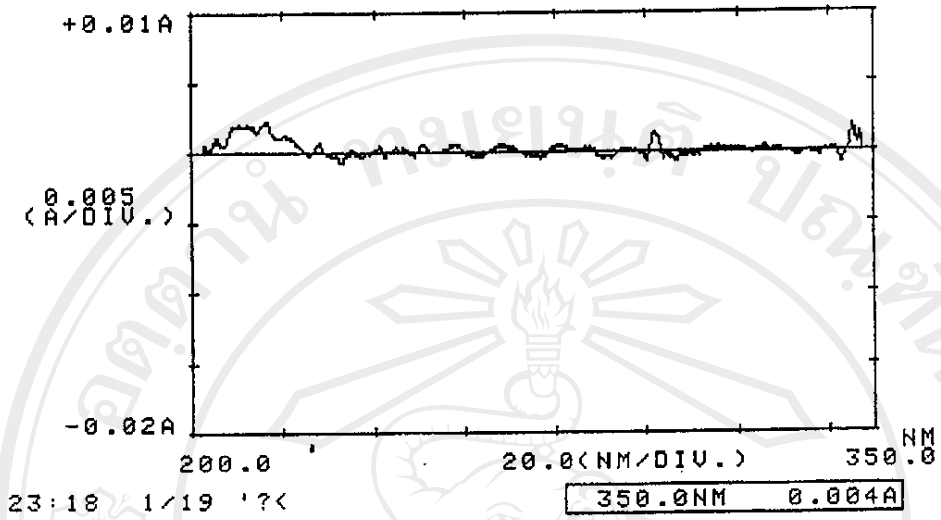


Figure 9 Absorbance of chitosan

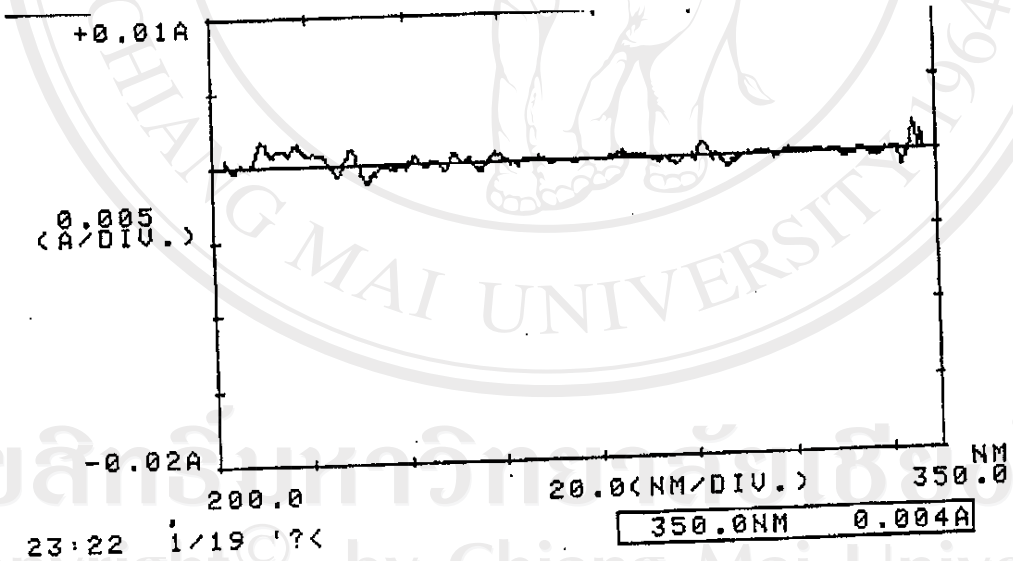


Figure 10 Absorbance of Tears Naturalle II™

Table 3 Calibration of vancomycin in Tear Naturale II

Conc Vancomycin(ug/ml)	In Tear Naturale II absorbance				
75	0.269	0.288	0.288	0.295	0.269
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	0.790	0.884	0.882	0.874	0.790
r^2	0.9985	0.9995	0.9998	0.9997	0.9989

Table 4 Calibration of vancomycin in chitosan

Conc Vancomycin(ug/ml)	In chitosan absorbance				
75.0	0.242	0.286	0.259	0.268	0.242
87.5	0.308	0.338	0.318	0.320	0.308
112.5	0.406	0.446	0.441	0.413	0.388
125.0	0.428	0.492	0.471	0.486	0.428
162.5	0.570	0.639	0.620	0.599	0.570
225.0	0.777	0.870	0.828	0.843	0.777
r^2	0.9964	0.9995	0.9969	0.997	0.9990

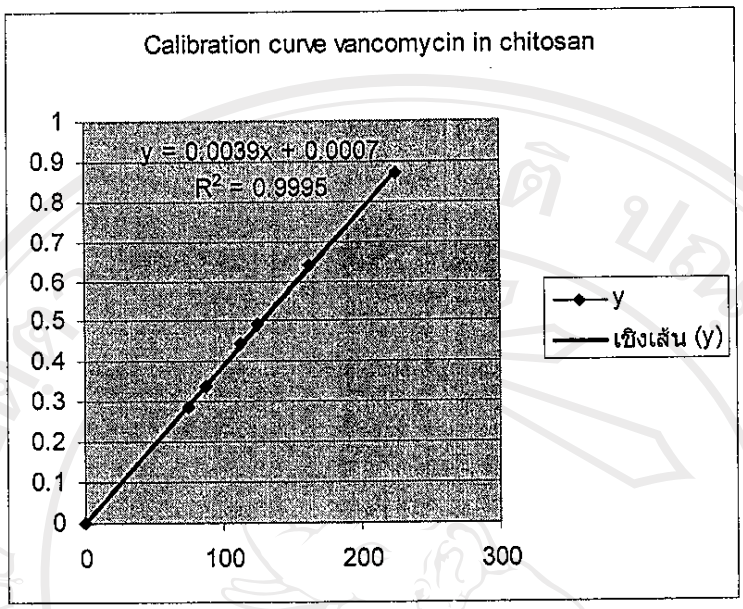


Figure 11 Calibration curve vancomycin in chitosan no. 1

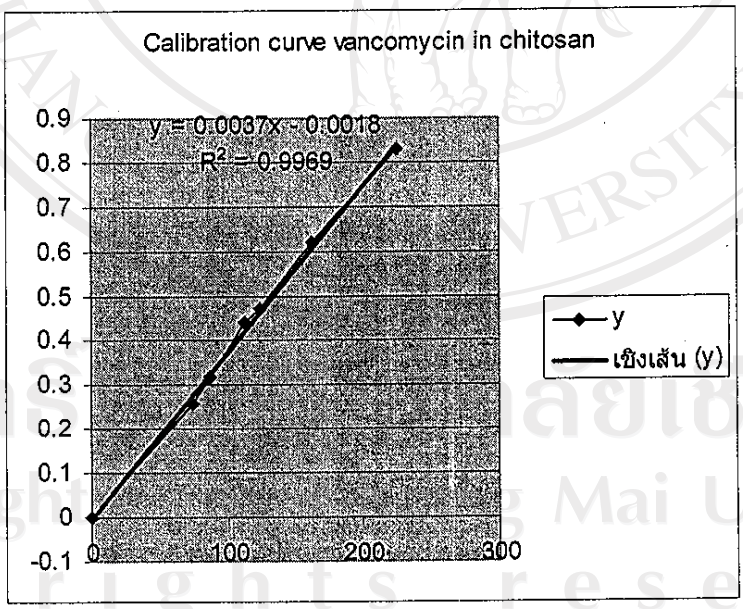


Figure 12 Calibration curve vancomycin in chitosan no. 2

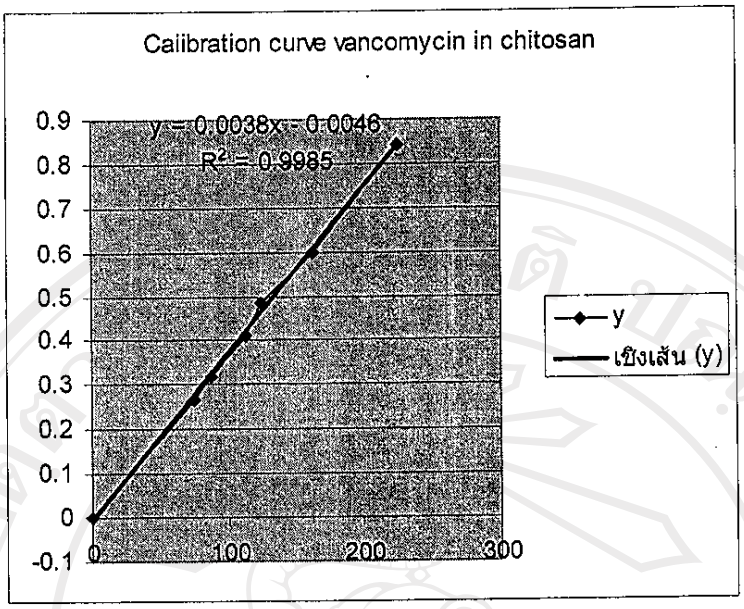


Figure 13 Calibration curve vancomycin in chitosan no. 3

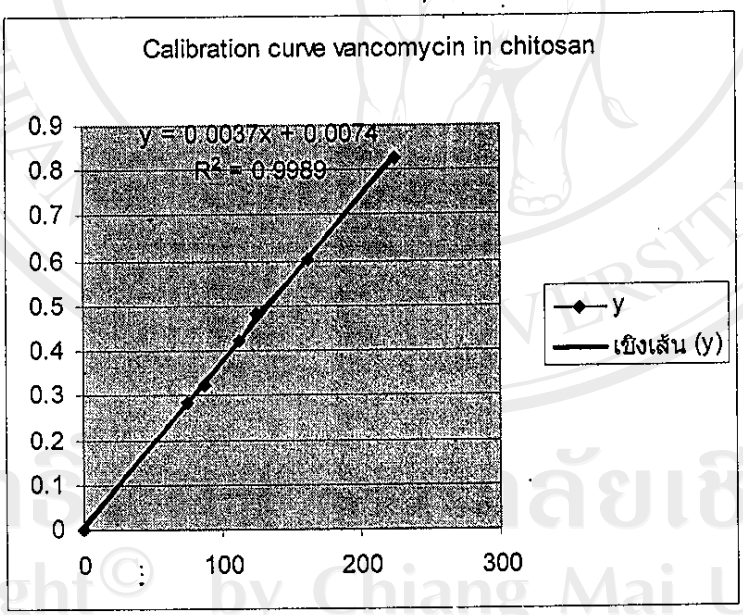


Figure 14 Calibration curve vancomycin in chitosan no. 4

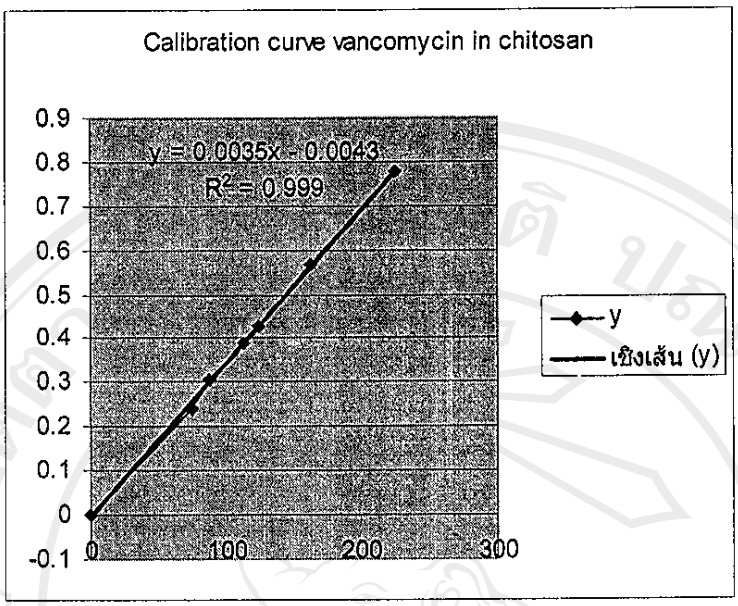


Figure 15 Calibration curve vancomycin in chitosan no. 5

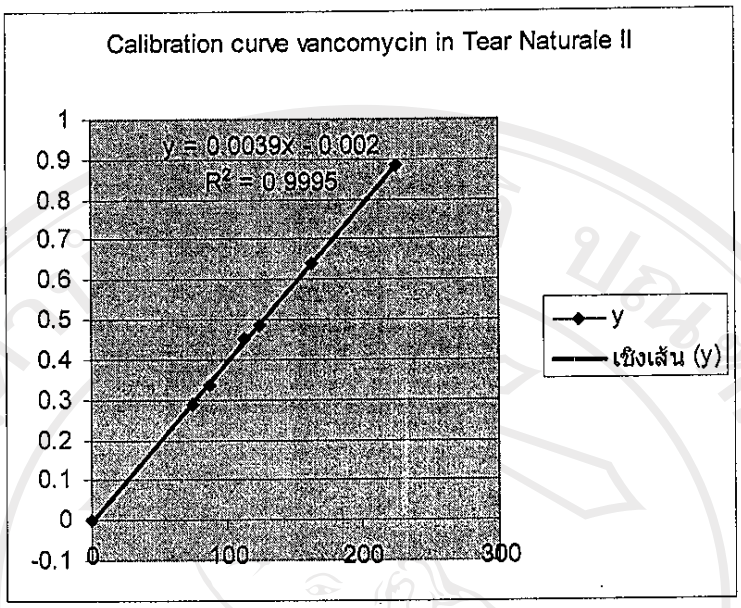


Figure 16 Calibration curve vancomycin in Tears Naturale II™ no. 1

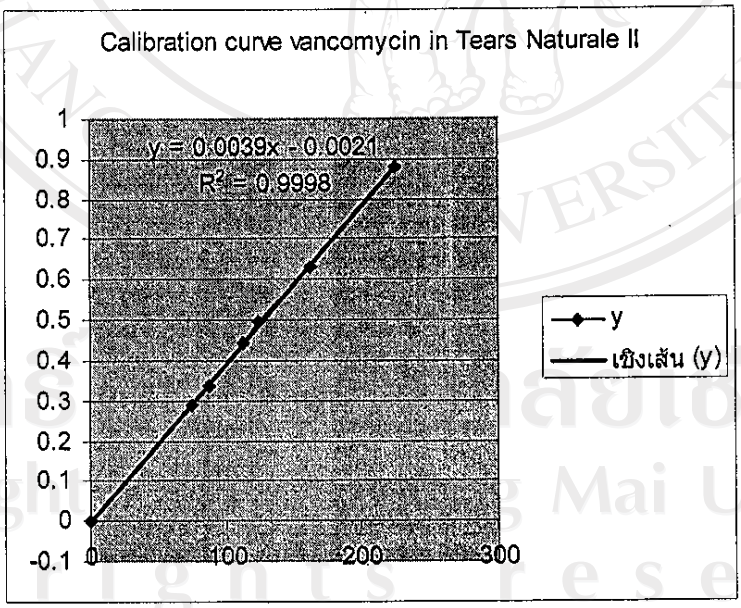


Figure 17 Calibration curve vancomycin in Tears Naturale II™ no. 2

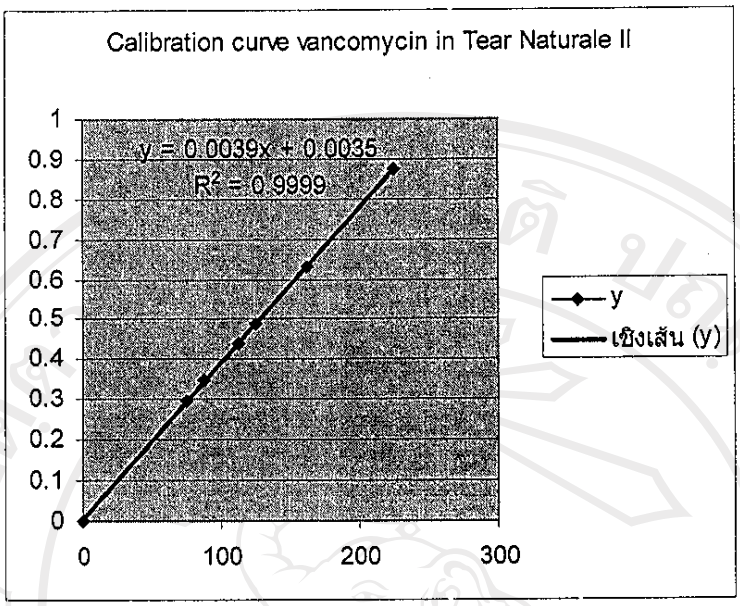


Figure 18 Calibration curve vancomycin in Tears Naturale II™ no. 3

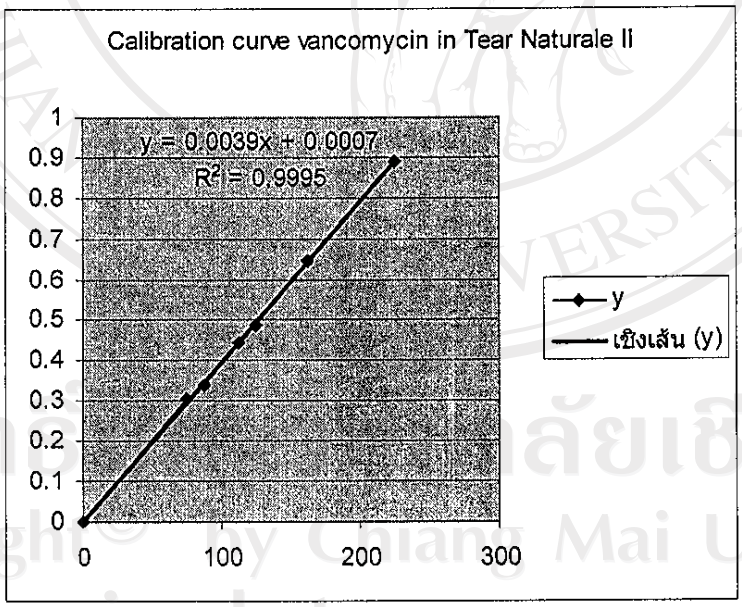


Figure 19 Calibration curve vancomycin in Tears Naturale II™ no. 4

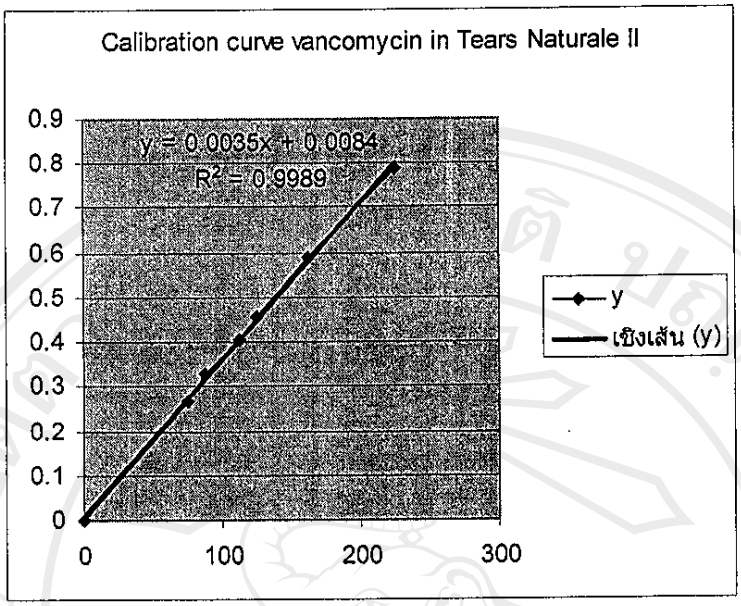


Figure 20 Calibration curve vancomycin in Tears Naturale II™ no. 5

Table 5 Lower limit of quantitation of vancomycin 75 µg/ml in chitosan and Tears Naturale II™.

NO	Tears Naturale II™	%Recovery In Tear Naturale II™	In Chitosan Absorbance	%Recovery In Chitosan
1	0.295	99.66	0.288	98.22
2	0.308	104.10	0.300	102.32
3	0.289	97.61	0.299	101.98
4	0.301	101.71	0.290	98.91
5	0.297	100.34	0.292	99.59
6	0.297	100.34	0.283	96.51

$$\% CV^{\bullet} \text{ (Coefficient of variation)} = \frac{SD}{mean} \times 100\%$$

Table 6 Interday assay validation of vancomycin in chitosan

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	1.8
175	175.08	174.0	174.65	173.71	172.34	173.96	1.05	0.6
212.5	207.77	208.97	206.49	208.88	211.83	208.79	1.98	0.95
					Average %CV = 1.12			

Table 7 Interday assay validation of vancomycin Tears Naturale II™

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	1.6	1.60
175	180.73	171.71	178.80	175	180	177.38	3.94	2.21
212.5	219.10	218.68	220.43	219.10	219.32	219.32	066	0.30
					Average %CV = 1.37			

Table 8 Intraday assay validation of vancomycin in chitosan.

No.	Conc (µg/ml)	Vancomycin. (absorbance)	Conc (µg/mL)	Error(%)
1	100	0.385	98.54	-1.46
2	100	0.386	98.79	-1.21
3	100	0.377	96.49	-3.51
4	100	0.387	99.05	-0.95
5	100	0.388	99.31	-0.69
6	100	0.390	99.82	-0.18
7	100	0.388	99.31	-0.69
8	100	0.390	99.82	-0.18
9	100	0.384	98.28	-1.72
10	100	0.386	98.79	-1.21
	Mean		98.82	
	SD		0.97	
1	175	0.684	175.21	0.12
2	175	0.685	175.46	0.26
3	175	0.670	171.62	-1.93
4	175	0.675	172.90	-1.20
5	175	0.686	175.72	0.41
6	175	0.687	175.97	0.56
7	175	0.679	173.92	-0.62
8	175	0.681	174.44	-0.32
9	175	0.684	175.21	0.12
10	175	0.686	175.72	0.41
	Mean		174.62	
	SD		0.81	

No.	Conc (µg/ml)	Vancomycin. (absorbance)	Conc (µg/mL)	Error(%)
1	212.5	0.811	207.77	-2.23
2	212.5	0.809	207.26	-2.47
3	212.5	0.820	210.08	-1.14
4	212.5	0.797	204.18	-3.92
5	212.5	0.795	203.67	-4.16
6	212.5	0.790	202.38	-4.76
7	212.5	0.789	202.13	-4.88
8	212.5	0.805	206.23	-2.95
9	212.5	0.820	210.08	-1.14
10	212.5	0.816	209.05	-1.62
	Mean		206.28	
	SD		1.48	

Table 9 Intraday assay validation of vancomycin in Tears Naturale II™.

No.	Conc (µg/ml)	Vancomycin. (absorbance)	Conc (µg/mL)	Error(%)
1	100	0.389	98.85	-1.15
2	100	0.389	98.85	-1.15
3	100	0.393	99.87	-0.13
4	100	0.381	96.79	-3.21
5	100	0.387	98.33	-1.67
6	100	0.384	97.56	-2.44
7	100	0.375	95.26	-4.74
8	100	0.379	96.28	-3.72
9	100	0.380	96.54	-3.46
10	100	0.386	98.08	-1.92
	Mean		97.64	
	SD		1.45	
1	175	0.690	176.03	0.59
2	175	0.689	175.77	0.44
3	175	0.695	177.31	1.32
4	175	0.697	177.82	1.61
5	175	0.692	176.54	0.88
6	175	0.696	177.56	1.47
7	175	0.689	175.77	0.44
8	175	0.698	178.08	1.76
9	175	0.697	177.82	1.61
10	175	0.688	175.51	0.29
	Mean		176.82	
	SD		0.57	

No.	Conc (µg/ml)	Vancomycin. (absorbance)	Conc (µg/mL)	Error(%)
1	212.5	0.840	214.49	0.94
2	212.5	0.845	215.77	1.54
3	212.5	0.838	213.97	0.69
4	212.5	0.839	214.23	0.81
5	212.5	0.841	214.74	1.06
6	212.5	0.847	216.28	1.78
7	212.5	0.845	215.77	1.54
8	212.5	0.846	216.03	1.66
9	212.5	0.847	216.28	1.78
10	212.5	0.839	214.23	0.81
	Mean		215.18	
	SD		0.43	

Table 10 Vancomycin in 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 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 12 Lower limit of quantitation of vancomycin in 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 13 Lower limit of quantitation of 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 pH of vancomycin in Tears Naturale II™ and chitosan (n=10)

Day	PH			
	Vancomycin 50 mg/ml in Tears Naturale II™		Vancomycin 50 mg/ml in chitosan	
	2-8°C	30°C	2-8°C	30°C
0	3.23	3.23	3.52	3.52
3	3.57	3.50	3.80	3.90
7	3.45	3.58	3.71	3.86
10	3.40	3.40	3.78	3.89
14	3.51	3.66	3.84	4.03
21	3.45	3.73	3.75	4.02
28	3.41	3.55	3.60	3.86

Table 15 Percentage of the labeled amounts of vancomycin in Tears Naturale II™ and chitosan (n=10)

Day	Percentage of the labeled amounts[a]			
	Vancomycin 50 mg/ml in Tears Naturale II™		Vancomycin 50 mg/ml in chitosan	
	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
3	107.88 ± 0.87	109.45 ± 1.40	107.78 ± 1.89	108.87 ± 0.46
7	107.74 ± 1.22	108.75 ± 0.93	108.94 ± 0.46	108.80 ± 1.12
10	108.07 ± 0.53	108.82 ± 1.09	108.75 ± 0.38	108.74 ±0.90
14	107.73 ± 0.76	108.74 ± 0.56	108.43 ± 0.49	108.94 ± 0.68
21	108.29 ± 0.32	108.53 ± 0.48	108.15 ± 0.67	100 .77 ± 1.00*
28	107.71 ± 0.81	102.07 ± 0.33*	107.88 ± 1.00	99.52 ± 0.80*

Table 16 Minimum inhibition concentration (MIC) of vancomycin in Tears Naturale II™ and chitosan

Day	Vancomycin in NSS (Freeze) (μg/ml)		MIC vancomycin in Tears Naturale II™ (μg/ml)				MIC vancomycin in Chitosan (μg/ml)			
			2-8°C		30°C		2-8°C		30°C	
	1	2	1	2	1	2	1	2	1	2
0	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98
3	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98
7	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.98	0.50	0.5
10	0.50	0.50	0.50	0.50	0.98	0.50	0.50	0.50	0.50	0.50
14	0.98	0.5	0.98	0.98	2.00	2.00	2.00	0.98	2.00	2.00
21	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	2.00	2.00
28	0.98	0.98	0.98	0.98	0.98	2.00	0.98	0.98	0.98	0.98

Table 17 Concentration of vancomycin in Tears Naturale II™ in tear film

Time(Min)	Conc vancomycin in Tears Naturale II™ (μg)					
	1	2	3	4	5	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

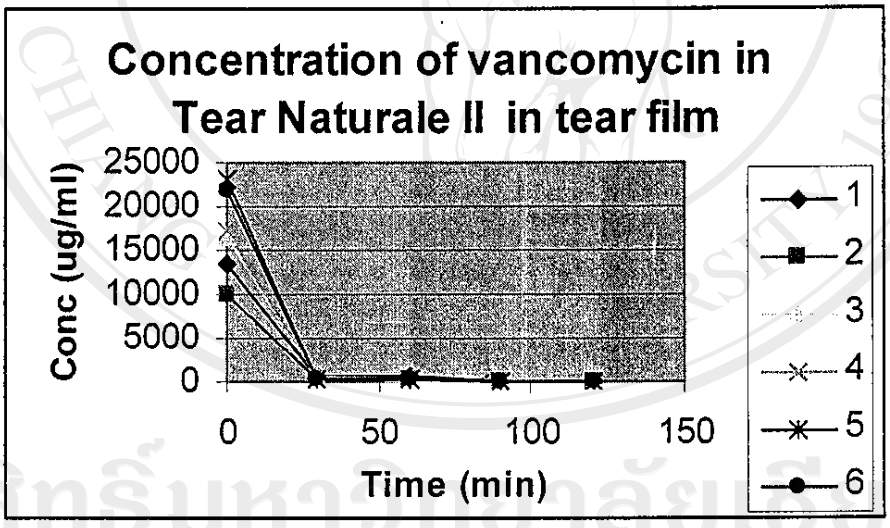


Figure 21 Concentration of vancomycin in Tear Naturale II in tear film

Table 18 Concentration of vancomycin in 0.1% chitosan in tear film

Time(Min)	Conc vancomycin in 0.1% chitosan (μg)					
	1	2	3	4	5	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

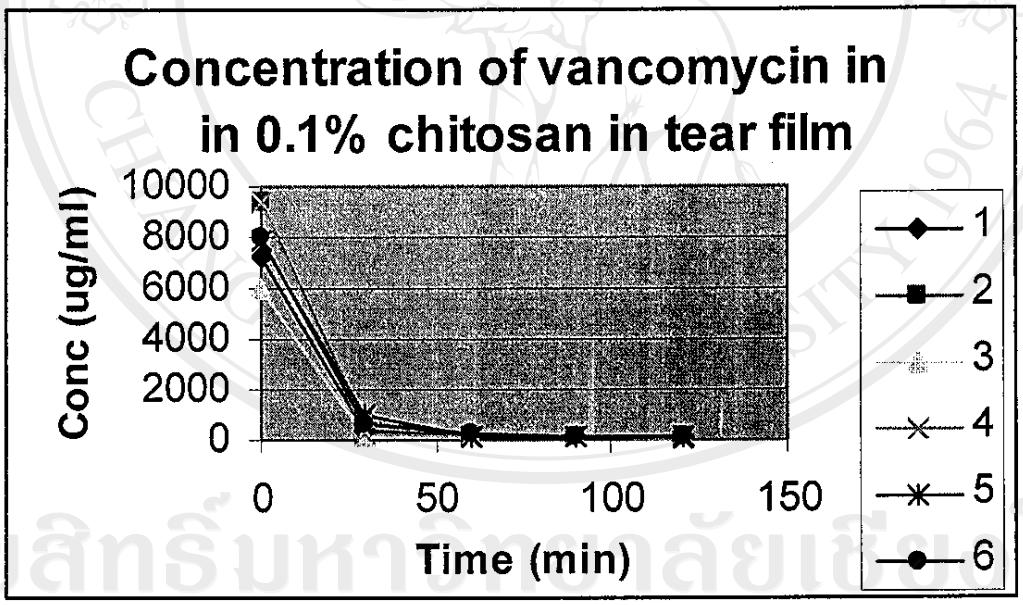


Figure 22 Concentration of vancomycin in 0.1% chitosan in tear film

Table 19 Concentration of vancomycin in 0.3% chitosan in tear film

Time(Min)	Conc vancomycin in 0.3% chitosan (μg)					
	1	2	3	4	5	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

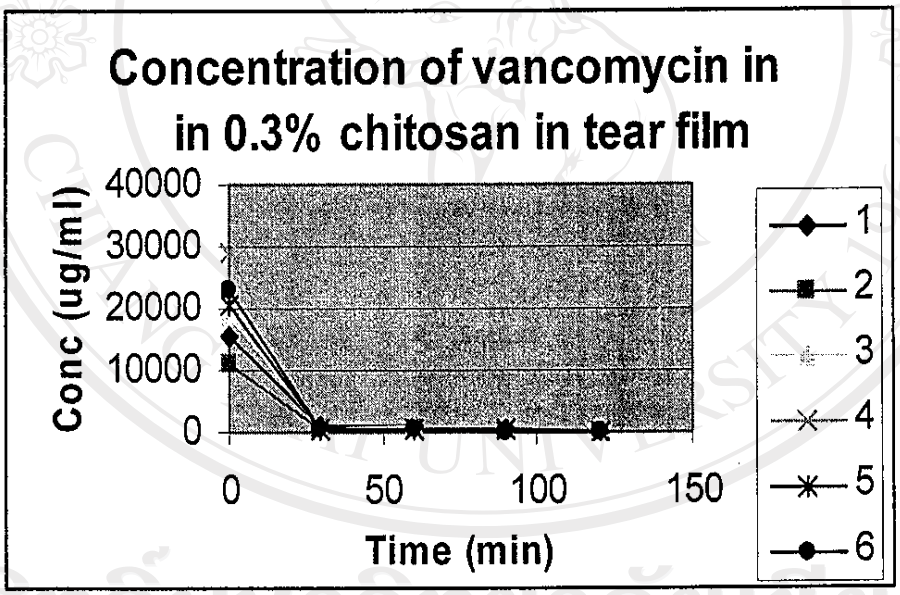


Figure 23 Concentration of vancomycin in 0.3% chitosan in tear film

Table 20 Concentration of vancomycin in normal saline solution (NSS) in tear film

Time(Min)	Conc vancomycin in NSS (μg)					
	1	2	3	4	5	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

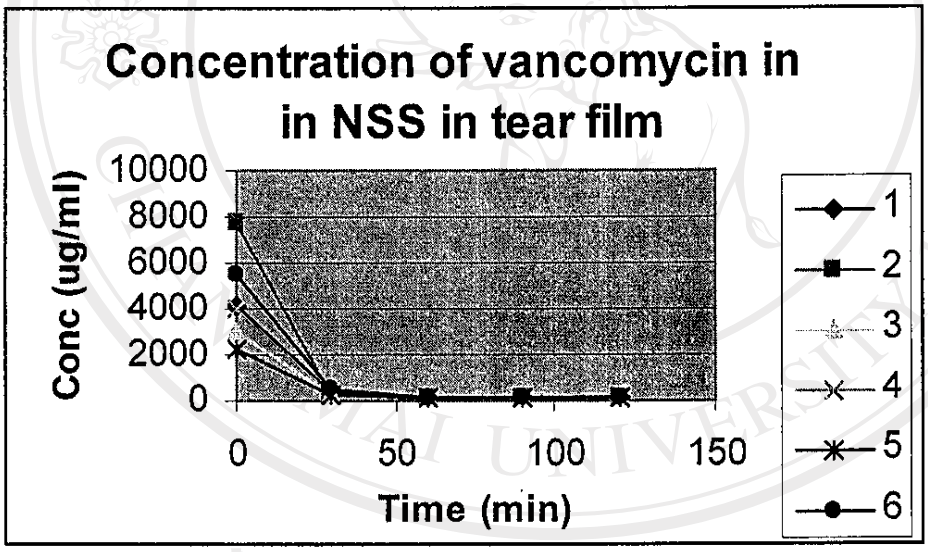


Figure 24 Concentration of vancomycin in NSS in tear film

Table 21 Area under the curve of vancomycin in diluents

NO	Area under the curve (μg.min/ml)			
	Vancomycin in Tears Naturale II™	Vancomycin in 0.1% chitosan	Vancomycin in 0.3% chitosan	Vancomycin in NSS
1	245975.0	129922.5	306015.0	86602.5
2	171322.5	171157.5	192720.0	133920.0
3	281010.0	101280.0	354487.5	62265.0
4	284887.5	155595.0	488362.5	79680.0
5	373395.0	153285.0	346447.5	52500.0
6	348202.5	150952.5	389017.5	106902.0
Mean	284132.1	143698.8*	346175.0	86978.3*
SD	66162.89	22469.06	88772.37	27243.91

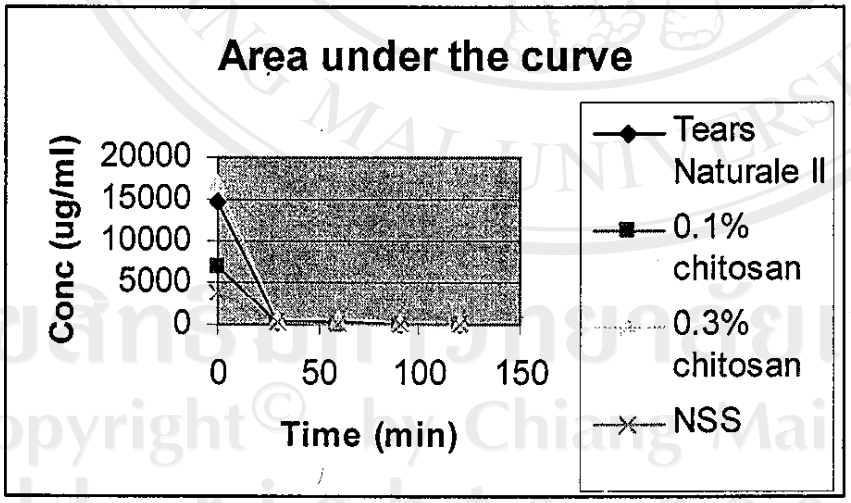


Figure 25 Area under the curve of vancomycin in diluents

Table 22 Half life of vancomycin in diluents

NO	Half life (min)			
	Vancomycin in Tears Naturale II™	Vancomycin in 0.1% chitosan	Vancomycin in 0.3% chitosan	Vancomycin in NSS
1	15.80	23.28	22.17	27.79
2	19.77	17.73	21.63	20.88
3	15.92	25.08	20.88	24.94
4	15.04	18.67	24.24	26.17
5	14.63	18.27	19.43	31.31
6	17.07	19.52	18.53	21.81
Mean	16.37	20.43	21.15	25.48
SD	1.86	3.02	2.04	3.87

DISCUSSION

In this study, attention has been focused on chitosan, a glycopeptide 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[25] . However, vancomycin hydrochloride eye drops (50 mg/ml) are not commercially available; instead they are made up by reconstitution in artificial tears[14]. 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 [7, 17]. Thus, chitosan was chosen in this study for the delivery of vancomycin.

The physicochemical properties of the chitosan used were characterized in terms of its moisture content, degree of deacetylation (DD) and viscosity-average molecular weight which were found to be 13.50%, 94.0%, 1.45 $x10^6$ respectively. The storage stability of the chitosan solution was studied at two different temperatures: room temperature (30^0C) and refrigeration temperature (2-8^0C). Stability was monitored in terms of solution viscosity which, in turn, reflected change in the chitosan molecular weight. Chitosan is well known to undergo acid-catalysed hydrolytic chain scission of the 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 decrease in solution viscosity. To a good approximation, the viscosity-average molecular weight, $\overline{M}_v$, of chitosan in dilute aqueous acid solution can be considered to be directly proportional to the intrinsic viscosity, [η], of the solution. The value of [η] can be estimated, again to a good approximation, from a single solution concentration via the Solomon-Ciuta One- Point Equation:

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

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% w/v (g dl^{-1}). The variations in [η] withstorage time (days) at 30^0C and 2-8^0C are compared in Figure 6. As the results clearly show, the main decrease in [η] is brought abount by the sterilization process during which the solutions are subjected to high temperature (120^0C). This causes rapid hydrolytic degradation of the chitosan in solution ever

only a short period of time (25 mins). Further degradation then occurs during storage, although much more slowly, especially at the lower temperature (2-8^{0}C). Thus, lowering the storage temperature increases storage stability by decreasing the rate of hydrolytic degradation.

The compatibility and stability studies of vancomycin 50 mg/ml eye drops in Tears Naturale II™ and chitosan solution showed that the solutions of vancomycin 50 mg/ml eye drops in both Tears Naturale II™ and chitosan solution remained clear when stored at 2-8^{0}C but precipitation occurred at day 21 and 28 respectively when stored at 30^{0}C. Vancomycin 50 mg/ml eye drops in Tears Naturale II™ and chitosan solution are less stable at 30^{0}C and should be stored in a refrigerator.

The validation results showed a linearity of the vancomycin hydrochloride calibration curve from 75.0 to 225.0 ug/ml, as seen from correlation coefficients of more than 0.99 in all assays. Linearity was established in the calibration curves for vancomycin 50 mg/ml eye drops in Tears Naturale II™ and chitosan where $y = 0.0039 + 0.0035$ and $y = 0.0039 + 0.0007$, respectively. All of the within-day and between-day precision and accuracy levels were determined as percent coefficients of variation which were less than 5%.

The percentage of the labeled amount of vancomycin 50 mg/ml eye drops in Tears Naturale II™ and chitosan in the eye drops stored at 2-8°C showed no loss of stability during 28 days. 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 chitosan solution respectively ($p<0.05$) (Table 15). The pH values of the vancomycin 50 mg/ml eye drops in Tears Naturale II™ and chitosan solution stored at 2-8 $^\circ$C and 30°C were in the ranges 3.23-3.73 and of 3.52-4.03 respectively (Table 14). The pH range 3.5-10.5 is usually tolerable by the eyes[26] . Thus the pH of vancomycin 50 mg/ml eye drops in Tears Naturale II™ is slightly lower than the tolerable range.

This study has also been concerned with the antimicrobial potency and the stability of extemporaneous preparations of vancomycin 50 mg/ml eye drops in Tears Naturale II™ and chitosan solution. 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 50 mg/ml eye drops in Tears Naturale II™ and chitosan solution were 0.5-2.0 µg/ml (Table 16). According to the National Committee for Clinical Laboratory Standards [27] , standard minimum inhibitory

concentration of vancomycin hydrochloride is 0.5-2.0 ug/ml. All positive controls without added vancomycin showed positive results. Negative controls not inoculated with *Staphylococcus aureus* ATCC 29213 showed negative results. This study showed that vancomycin 50 mg/ml eye drops in Tears Naturale II™ and chitosan solution stored at 2-8°C and 30°C resulted in no loss of MIC during 28 days.

The osmolality of the Tears Naturale II™ and the 0.1% chitosan solution were 297 mOsmol/kg and 267 mOsmol/kg respectively. The osmolalities of vancomycin 50 mg/ml eye drops in Tears Naturale II™ and chitosan solution were 334 mOsmol/kg and 310 mOsmol/kg respectively. The osmolality which can be tolerated by the human eye is 160-670 mOsmol/kg [28].

In previous study[17], it was demonstrated by gamma scintigraphy that the presence of chitosan in an ophthalmic solution always resulted in a significant increase of the precorneal residence time when compared with commercial controls. A general observation regarding vancomycin is that 0.3% chitosan solution, significantly improve vancomycin availability in tears (Table 21). The half-life of vancomycin in 0.3% chitosan solution is longer than in Tears Naturale II™ (Table 22), it showed the ability 0.3% chitosan solution to prolong the precorneal retention.

CONCLUSION

The results of this study show that a 0.3% w/v solution of chitosan in aqueous L(+)-lactic acid may be of value for the ocular delivery of vancomycin because vancomycin 50 mg/ml eye drops in the chitosan solution have a stability comparable with Tears Naturale II™ . Furthermore, chitosan offers other potential benefits as regards its bioadhesive and antimicrobial properties, particularly in the treatment of bacterial keratitis.

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APPENDIX

Pharmacokinetics

Pharmacokinetics is one of the two basic areas of pharmacology, in addition to pharmacodynamics. It deals with the quantitation of the process of 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 to predict the intensity of drug effects if the relationship exists between the drug concentrations and pharmacologic or toxic effects of drugs.

The area under concentration-time curve from administration and time (AUC_{0-t})

Area under the concentration versus time curve for data using the linear trapezoidal rule. The AUC is calculated between the time points in the time-data range. In pharmacokinetic calculations, the time points usually begin at time 0 and finish at the last quantifiable point.

Elimination half-life ($T_{1/2}$)

$T_{1/2}$ is the time taken for the amount of drug in the body to fall by half. The unit for $t_{1/2}$ is a unit of time (e.g., h, min).

VITA

ผศ.ดร.ภก. ภูริวัฒน์ ลี้สวัสดิ์

สังกัดคณะเภสัชศาสตร์ มหาวิทยาลัยเชียงใหม่

วุฒิสูงสุด Ph.D. (Industrial and Physical Pharmacy)

ประสบการณ์การวิจัยและผลงานตีพิมพ์ที่เกี่ยวข้องกับสาขาที่ทำการวิจัยในครั้งนี้

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ภญ. อนุตรา มังตระกูล

วัน เดือน ปี เกิด 15 มกราคม 2513

ประวัติการศึกษา สำเร็จการศึกษาเภสัชศาสตร์บัณฑิต คณะเภสัชศาสตร์ มหาวิทยาลัยเชียงใหม่ ปีการศึกษา 2536

สำเร็จการศึกษาวิทยาศาสตรมหาบัณฑิต (เภสัชวิทยา) คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ปีการศึกษา 2546

สถานที่ทำงาน หน่วยผลิตยาปราศจากเชื้อ งานผลิตยา ฝ่ายเภสัชกรรม โรงพยาบาลมหาราชนครเชียงใหม่ มหาวิทยาลัยเชียงใหม่

โทรศัพท์ 053-945613, 01-5956877

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Dr. Robert Molloy

Date/Place of Birth	3 August 1950/ Birmingham, UK	
Education	BSc Chemistry	
	University of Manchester (UMIST), UK	(1971)
	MSc Chemistry and Technology of Polymers	
	Aston University, Birmingham, UK	(1972)
	PhD Applied Polymer Science	
	Aston University, Birmingham, UK	(1977)
Honours/Awards	Chartered Chemist (CChem), UK	(1980)
	Fellow of the Royal Society of Chemistry (FRSC), UK	(1988)
Present Position	Foreign Special Instructor	
(since 1978)	Department of Chemistry, Faculty of Science	
	Chiang Mai University	
Work-Related Duties	Teaching in Polymer/Physical Chemistry	
	Polymer Chemistry Laboratory class supervision	
	M.S./PhD research Group	
	Member of various Department/ Faculty Committees	

Current Research/Area of Specialization

Speciality polymers for use in biomedical applications

Ring-opening polymerisation of cyclic edters

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Fibre extrusion (melt spinning and wet spinning)

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รศ.พญ. สมสงวน อัษญคุณ

สังกัดภาควิชาจักษุวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

Education: 1971-1977 Chiang Mai University

1978-1979 Mahidol University

1983-1984 Johns Hopkins University, USA

ประสบการณ์การวิจัยและผลงานตีพิมพ์ที่เกี่ยวข้องกับสาขาที่ทำการวิจัยในครั้งนี้

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