Drug Development and Industrial Pharmacy, 25(6), 781–788 (1999)

COMMUNICATION

Feasibility of an In Vitro Microbiological Model as an Alternative to the Rabbit Eye Model

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ABSTRACT

Ocular inserts of gentamicin sulfate with polyvinyl alcohol (PVA) 1.5%, 2.0%, and 2.5% and a combination of methyl cellulose 2% and Eudragit NE 30D 30%, 35%, and 40% w/w of methyl cellulose were fabricated by a casting technique. The inserts were sterilized by gamma radiation at 25 kGy and tested for sterility. The microbiological efficacy of the ocular inserts against Staphylococcus aureus *ATCC 6538P and* Pseudomonas aeruginosa *NCIM 2200 was evaluated by developing an in vitro microbiological model and an in vivo noninvasive rabbit eye model. Parameters of the in vitro microbiological model were varied, and the results correlated with a noninvasive rabbit eye model. The in vitro model proved to be a viable alternative to the rabbit eye model in evaluating the microbiological efficacy of gentamicin sulfate ocular inserts.*

INTRODUCTION

Animal experimentation is an essential part in the research and development of ocular drug delivery systems (1–5). The rabbit is by far the most commonly used animal model in the evaluation of ocular formulations. Although several different animal species are used in ocular drug response studies, animals other than rabbits rarely have been used in ocular experiments. Other possible animals for ocular pharmacokinetic studies include cats, dogs, rats, and mice. However, the eyes of small rodents are too small for ocular testing of different drug delivery

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systems, whereas dogs and cats prove to be too expensive for ocular studies (6). Several rabbit eye models with bacterial (7–9), viral (10,11), and fungal infections have been reported in the literature.

Adequate care and humane treatment of laboratory animals are major concerns of the visual science community, and alternatives to animal experimentation are sought continuously. Hence, the objective of the present investigation was to evaluate the feasibility of an in vitro microbiological model as an alternative to the rabbit eye model.

Gentamicin sulfate, a potent aminoglycoside antibiotic, is widely used in the treatment of blepharitis, conjunctivitis, keratitis, and corneal ulcers (12). Gentamicin sulfate ocular inserts were therefore formulated to evaluate the feasibility of an in vitro microbiological model in comparison with the reported rabbit eye model.

MATERIALS AND METHODS

Materials

Gentamicin sulfate was obtained as a gift sample from Schering Plough Research Institute (NJ). Polyvinyl alcohol (PVA) was purchased from Loba Chemie (Mumbai, India). Methyl cellulose and Eudragit NE 30D were obtained as gift samples from Colorcon Asia (Mumbai, India) and S. Zhaveri and Company (Mumbai, India), respectively. The microbiological media were purchased from Hi Media (Mumbai, India). All other reagents were analytical grade and were used without further purification.

Animals

Male New Zealand albino rabbits free of any signs of ocular inflammation or gross abnormality weighing between 2 and 3 kg were obtained from Burroughs Wellcome (Mumbai, India) and were used throughout the study. The animals were individually housed in cages and had free access to food and water throughout the study.

Microorganisms

Staphylococcus aureus ATCC 6538P, the most common ocular pathogen, and *Pseudomonas aeruginosa* NCIM 2200, the most opportunistic ocular pathogen, were used as model organisms in the study. Freshly cultivated cells on nutrient agar were scraped off the agar after 18 hr of incubation, which corresponded to the active log phase of the organism. The cells were suspended in saline (13), and the optical density (OD) was adjusted to 0.1 on a spectrophotometer (Photoelectric colorimeter 112) at 660 nm. A colony count of the 0.1 OD cell suspension was performed by serial dilution using the pour plate technique (14,15), and colony-forming units were counted on plates containing 30–300 colonies after incubation on nutrient agar.

METHODS

Ocular Inserts

Drug-loaded films were obtained from 1.5%, 2%, and 2.5% aqueous solutions of PVA and gentamicin sulfate, containing propylene glycol as a plasticizer, and methyl cellulose 2%, with Eudragit NE 30D 30%, 35%, and 40% w/w of methyl cellulose, with glycerol as plasticizer. The solutions were cast on siliconized glass petri plates and dried at 45°C for 24 hr. Inserts, each containing 250 μg of the drug, were punched out of the film dried under vacuum, were individually weighed, and were packed in heat-sealed polyethylene lined aluminum pouches to avoid diffusion of oxygen before irradiation. The polymeric inserts were subjected to 25 kGy of gamma irradiation at BARC (Mumbai, India) using a cobalt source at a dose rate of 0.7 kGy/hr.

Sterility

The PVA inserts were dissolved in sterile water, and the sterile aqueous solutions were filtered through a 0.22-μ membrane filter. For methyl cellulose–Eudragit NE 30D, gentamicin sulfate was extracted from the inserts by two extractions with 50 ml water each over 48 hr. The solutions were filtered through a 0.22-μ membrane filter. After sufficient washings with sterile water, the cut membrane filters were inoculated in sterile thioglycollate medium and soy bean casein digest medium for 7 days. Positive and negative controls were maintained throughout the study.

In Vitro Microbiological Study

A 0.1 OD suspension of *S. aureus* and *P. aeruginosa* was prepared. Aliquots of 1, 1.5, 2.0, 2.5, and 3.0 ml of the 0.1 OD suspension were inoculated into nutrient broth, and the volume was made up to 5 ml with nutrient broth. Sterile inserts were introduced into these solutions, and the plugged tubes were placed at 37°C in a modified USP dissolution apparatus (16), which reciprocated horizontally with an amplitude of 4 cm and a frequency of

30 cycles/min. Aliquots of 500 µl were withdrawn at the end of 6, 12, 24, 30, 36, and 48 hr and were replaced with sterile nutrient broth. The withdrawn aliquots were inoculated into sterile nutrient broth and incubated at 37°C for 24 hr. A challenge study with the microorganisms was also performed. Challenging was done at 3 hr from the start of the study by further inoculation with 25% v/v of the initial inoculum of *S. aureus/P. aeruginosa*. Challenging with 25% v/v of the initial inoculum volume was repeated at 24 hr only for PVA inserts with the *P. aeruginosa*. Both positive and negative controls were maintained throughout the study.

In Vivo Rabbit Eye Study

Six rabbits were used throughout the study, with 25, 50, 75, and 100 µl of the 0.1 OD suspension of *S. aureus* and *P. aeruginosa* instilled in both the eyes of each rabbit. After allowing the growth to proceed to the log phase (i.e., at 18 hr), swabs from both the eyes were taken at 6, 12, 24, 30, 36, and 48 hr and inoculated into sterile nutrient media and checked for growth. The study was conducted to arrive at an inoculum dose that was capable of maintaining a growth up to 48 hr for conducting the in vivo study. The selected inoculum dose was then instilled in the right eye of the rabbit, and the left eye served as a positive control. The sterile inserts were inserted in the cul-de-sac of the right eye, and cotton swabs from both the eyes were taken at 6, 12, 24, 30, 36 and 48 hours. Both single dose and repeated dose study was performed.

Single-Dose Study

One insert was introduced in the cul-de-sac of the rabbit eye, and the effect observed over 48 hr.

Repeated-Dose Study

One insert was introduced in the cul-de-sac of the rabbit eye. At the end of 24 hr, a fresh insert was introduced, and the effect observed.

The cotton swabs were inoculated into Vogel Johnson agar and cetrimide agar for *S. aureus* and *P. aeruginosa*, respectively. Negative controls were also maintained. A 10-day washout period was allowed between experiments.

RESULTS AND DISCUSSION

Sterility Testing

Sterility testing was carried out to confirm the sterility of the inserts and to avoid contamination or interference in the subsequent study. All the inserts were sterile.

In Vitro Microbiological Study

The 0.1 OD suspension of *S. aureus* revealed a count of 1.18×10^8 organisms/ml, while a 0.1 OD suspension of *P. aeruginosa* indicated a count of 3.5×10^8 organisms/ml.

The microbiological effectiveness of the inserts was tested at varying volumes of the inoculum (Tables 1–4). A challenge study, the introduction of fresh inoculum at the time point at which the tubes revealed no growth, was performed to simulate growing organisms. When the microorganism was *S. aureus*, tubes were challenged at 3 hr for PVA and methyl cellulose–Eudragit NE 30D ocular inserts. When *P. aeruginosa* was the microorganism, challenging was carried out at 3 hr for methyl cellulose–Eudragit NE 30D ocular inserts and only at 24 hr for PVA inserts as tubes revealed growth until 24 hr.

It was observed that, for 1.5% and 2% PVA and all the methyl cellulose–Eudragit NE 30D formulations at 1 ml, 1.5 ml, and 2.0 ml of the inoculum for *S. aureus*, one insert inhibited growth of the microorganism for 24 hr despite challenging at 3 hr (Tables 1 and 4). However, for PVA 2.5% at volumes greater than 1 ml and for PVA 1.5% and 2.0% for volumes greater than 2 ml of the inoculum of *S. aureus* and similarly for methyl cellulose–Eudragit NE 30D formulations for volumes greater than 2 ml, two inserts (one/24 hr) over a period of 48 hr were required to inhibit the microorganisms (Tables 2 and 4).

In the case of *P. aeruginosa* for all the PVA formulations at all volumes of inoculum and a 24-hr challenge, one insert over 24 hours for a 48-hr period inhibited the growth of microorganisms (Table 3). Similar to all methyl cellulose–Eudragit NE 30D formulations at inoculum volumes of greater than 1.5 ml with a 3-hr challenge, one insert over 24 hr for a 48-hr period inhibited the growth of the microorganisms (Tables 5 and 6).

In Vivo Rabbit Eye Study

Selection of the Inoculum Dose

Varying aliquots of the 0.1 OD suspension of *S. aureus* and *P. aeruginosa* (25, 50, 75, and 100 µl) were instilled in both eyes of the rabbit and checked for growth over a period of 48 hr. After an 18-hr incubation period, swabs from both the eyes were taken and checked for growth. It was observed that the time period of growth was directly proportional to the inoculum volume of the organisms. For 25 µl of the inoculum, growth was observed over 12 hr; for 50 µl, it was observed over 24 hr;

Table 1

In Vitro Microbiological Effectiveness of PVA Ocular Inserts (S. aureus)

0.1 OD Suspension	Formulations PVA	3 hr[a]	6 hr	12 hr	24 hr	30 hr	36 hr	48 hr
1 ml	1.5%	−6	−6	−6	−6	−6	−6	−6
	2.0%	−6	−6	−6	−6	−6	−6	−6
	2.5%	−6	−6	−6	−6	−6	−6	−6
1.5 ml	1.5%	−6	−6	−6	−6	−6	−6	−6
	2.0%	−6	−6	−6	−6	−6	−6	−6
	2.5%	−6	−6	−6	−6	+4 (−2)	+6	+5 (−1)
2.0 ml	1.5%	−6	−6	−6	−6	−6	−6	−6
	2.0%	−6	−6	−6	−6	−6	−6	−6
	2.5%	−6	−6	−6	−6	+6	+6	+6
2.5 ml	1.5%	−6	−6	−6	−6	+1 (−5)	+4 (−2)	+6
	2.0%	−6	−6	−6	−6	+6	+6	+6
	2.5%	−6	−6	−6	+6	+6	+6	+6
3.0 ml	1.5%	−6	−6	−6	−6	+6	+6	+6
	2.0%	−6	−6	−6	−6	+6	+6	+6
	2.5%	−6	−6	−6	+6	+6	+6	+6

n = 6 tubes; +, growth; −, no growth.
[a] 3-hr challenge.

for 75 μl, it was seen over 36 hr; and for 100 μl, it was observed over 48 hr. Therefore, 100 μl was selected as the inoculum dose for further studies.

Single-Dose Study

For both *S. aureus* and *P. aeruginosa*, growth was observed in the single-dose study over 48 hr after instillation of the insert for all the formulations. Hence, a repeated-dose study was carried out.

Repeated-Dose Study

Introduction of a fresh insert at the end of 24 hr inhibited growth over the next 24 hr for all the PVA and methyl cellulose–Eudragit NE 30D ocular inserts, thus

Table 2

In Vitro Microbiological Effectiveness of PVA Ocular Inserts (S. aureus)

0.1 OD Suspension	Formulations PVA	3 hr[a]	6 hr	12 hr	24 hr[b]	30 hr	36 hr	48 hr
1.5 ml	2.5%	−6	−6	−6	−6	−6	−6	−6
2.0 ml	2.5%	−6	−6	−6	−6	−6	−6	−6
2.5 ml	1.5%	−6	−6	−6	−6	−6	−6	−6
	2.0%	−6	−6	−6	−6	−6	−6	−6
	2.5%	−6	−6	−6	−6	−6	−6	−6
3.0 ml	1.5%	−6	−6	−6	−6	−6	−6	−6
	2.0%	−6	−6	−6	−6	−6	−6	−6
	2.5%	−6	−6	−6	−6	−6	−6	−6

n = 6 tubes; +, growth; −, no growth.
[a] 3-hr challenge.
[b] Instillation of a fresh insert.

Table 3

In Vitro Microbiological Effectiveness of PVA Ocular Inserts (P. aeruginosa)

0.1 OD Suspension	Formulations PVA	6 hr	12 hr	24 hr[a]	30 hr	36 hr	48 hr
1 ml	1.5%	+6	+6	+6	−6	−6	−6
	2.0%	+6	+6	+6	−6	−6	−6
	2.5%	+6	+6	+6	−6	−6	−6
1.5 ml	1.5%	+6	+6	+6	−6	−6	−6
	2.0%	+6	+6	+6	−6	−6	−6
	2.5%	+6	+6	+6	−6	−6	−6
2.0 ml	1.5%	+6	+6	+6	−6	−6	−6
	2.0%	+6	+6	+6	−6	−6	−6
	2.5%	+6	+6	+6	+6	+6	+6
2.5 ml	1.5%	+6	+6	+6	+6	−6	−6
	2.0%	+6	+6	+6	+6	−6	−6
	2.5%	+6	+6	+6	+6	−6	−6
3.0 ml	1.5%	+6	+6	+6	+6	−6	−6
	2.0%	+6	+6	+6	+6	−6	−6
	2.5%	+6	+6	+6	+6	−6	−6

n = 6 tubes; +, growth; −, no growth.
[a] 24-hr challenge and instillation of a fresh insert.

Table 4

In Vitro Microbiological Effectiveness of Methyl Cellulose–Eudragit NE 30D Ocular Inserts (S. aureus)

0.1 OD Suspension	Formulations[a]	3 hr[b]	6 hr	12 hr	24 hr	30 hr	36 hr	48 hr
1 ml	30%	−6	−6	−6	−6	−6	−6	−6
	35%	−6	−6	−6	−6	−6	−6	−6
	40%	−6	−6	−6	−6	−6	−6	−6
1.5%	30%	−6	−6	−6	−6	−6	−6	−6
	35%	−6	−6	−6	−6	−6	−6	−6
	40%	−6	−6	−6	−6	−6	−6	−6
2.0 ml	30%	−6	−6	−6	−6	−6	−6	−6
	35%	−6	−6	−6	−6	−6	−6	−6
	40%	−6	−6	−6	−6	−6	−6	+1 (−5)
2.5 ml	30%	−6	−6	−6	−6	−6	+1 (−5)	+2 (−1)
	35%	−6	−6	−6	−6	−6	+1 (−5)	+2 (−4)
	40%	−6	−6	−6	−6	−6	+2 (−4)	+1 (−5)
3.0 ml	30%	−6	−6	−6	−6	+1 (−5)	+2 (−5)	+2 (−5)
	35%	−6	−6	−6	−6	−6	+2 (−4)	+2 (−4)
	40%	−6	−6	−6	−6	+2 (−4)	+2 (−4)	+2 (−4)

n = 6 tubes; +, growth; −, no growth.
[a] Methyl cellulose–Eudragit NE 30D formulations.
[b] 3-hr challenge.

Table 5

In Vitro Microbiological Effectiveness of Methyl Cellulose–Eudragit NE 30D Ocular Inserts (P. aeruginosa)

0.1 OD Suspension	Formulations[a]	3 hr[b]	6 hr	12 hr	24 hr	30 hr	36 hr	48 hr
1 ml	30%	−6	−6	−6	−6	−6	−6	−6
	35%	−6	−6	−6	−6	−6	−6	−6
	40%	−6	−6	−6	−6	−6	−6	−6
1.5 ml	30%	−6	−6	−6	−6	−6	−6	−6
	35%	−6	−6	−6	−6	−6	−6	−6
	40%	−6	−6	−6	−6	−6	−6	−6
2.0 ml	30%	−6	−6	−6	−6	−6	−6	+1 (−5)
	35%	−6	−6	−6	−6	−6	−6	+1 (−5)
	40%	−6	−6	−6	−6	−6	−6	+2 (−4)
2.5 ml	30%	−6	−6	−6	−6	−6	−6	+2 (−4)
	35%	−6	−6	−6	−6	−6	−6	+2 (−4)
	40%	−6	−6	−6	−6	−6	+1 (−5)	+2 (−4)
3.0 ml	30%	−6	−6	−6	−6	+1 (−5)	+2 (−5)	+2 (−5)
	35%	−6	−6	−6	−6	−6	+2 (−4)	+2 (−4)
	40%	−6	−6	−6	−6	−6	+3 (−3)	+2 (−4)

n = 6 tubes; +, growth; −, no growth.
[a] Methyl cellulose–Eudragit NE 30D formulations.
[b] 3-hr challenge.

indicating that one insert per 24 hr over a 48-hr period was capable of inhibiting the growth of microorganisms (Tables 7 and 8).

In Vitro/In Vivo Correlation

The in vitro microbiological model at higher doses of inoculum (i.e., at volumes greater than 2 ml for *S. aureus* and volumes greater than 1 ml for *P. aeruginosa*) correlated well with the in vivo rabbit eye study, in which only 100 µl inoculum was used. Availability of gentamicin sulfate in the in vivo study is affected because of the multiple protective barriers imposed by the eye against the entry of drug, compared to the in vitro study, in which the entire amount of gentamicin released from the insert is available for exerting its action against the microorgan-

Table 6

In Vitro Microbiological Effectiveness of Methyl Cellulose–Eudragit NE 30D Ocular Inserts (P. aeruginosa)

0.1 OD Suspension	Formulations[a]	3 hr[b]	6 hr	12 hr	24 hr[c]	30 hr	36 hr	48 hr
2.0 ml	30%	−6	−6	−6	−6	−6	−6	−6
	35%	−6	−6	−6	−6	−6	−6	−6
	40%	−6	−6	−6	−6	−6	−6	−6
2.5 ml	30%	−6	−6	−6	−6	−6	−6	−6
	35%	−6	−6	−6	−6	−6	−6	−6
	40%	−6	−6	−6	−6	−6	−6	−6
3.0 ml	30%	−6	−6	−6	−6	−6	−6	−6
	35%	−6	−6	−6	−6	−6	−6	−6
	40%	−6	−6	−6	−6	−6	−6	−6

n = 6 tubes; +, growth; −, no growth.
[a] Methyl cellulose–Eudragit NE 30D formulations.
[b] 3-hr challenge.
[c] Instillation of a fresh insert.

Table 7

In Vivo Microbiological Effectiveness of PVA Ocular Inserts (S. aureus, P. aeruginosa) *in Rabbits*

Formulations PVA	6 hr	12 hr	24 hr[a]	30 hr	36 hr	48 hr
1.5%	+6	+6	+6	−6	−6	−6
2.0%	+6	+6	+6	−6	−6	−6
2.5%	+6	+6	+6	−6	−6	−6

$n = 6$ rabbits; +, growth; −, no growth.
[a] Instillation of a fresh insert.

Table 8

In Vivo Microbiological Effectiveness of Methyl Cellulose–Eudragit NE 30D Ocular Inserts (S. aureus, P. aeruginosa) *in Rabbits*

Formulations[a]	6 hr	12 hr	24 hr[b]	30 hr	36 hr	48 hr
30%	+6	+6	+6	−6	−6	−6
35%	+6	+6	+6	−6	−6	−6
40%	+6	+6	+6	−6	−6	−6

$n = 6$ rabbits; +, growth; −, no growth.
[a] Methyl cellulose–Eudragit NE 30D.
[b] Instillation of a fresh insert.

isms. In the in vivo study, two inserts (one per 24 hr) for 48 hr were required to inhibit the infection up to a point at which there was no growth. In the in vitro study, at lower volumes of the inoculum, one insert over 24 hr was sufficient to inhibit the growth despite the challenge, but as the volume of inoculum increased, two inserts (one per 24 hr), as seen in vivo, were essential to inhibit growth completely. The in vitro microbiological model therefore correlated well with the in vivo model at inoculum volumes of greater than 2 ml for *Staphylococcus aureus* and 1 ml for *Pseudomonas aeruginosa* in the present study.

CONCLUSION

This study demonstrates the feasibility of the in vitro microbiological model as a safe alternative to the rabbit eye model. Though further studies with different drugs are required to standardize the model, this preliminary study is important as it suggests a novel in vitro methodology as an alternative to animal experimentation.

ACKNOWLEDGMENT

The authors wish to thank the Isotopes in Industry Committee for the BRNS grant sanctioned for the project.

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