Combinatorial therapeutic drug delivery of riboflavin and dexamethasone for the treatment of keratoconus affected corneas of mice: Ex vivo permeation and hemolytic toxicity

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Abstract
The aim of this study was to formulate the thermoreversible gel containing riboflavin and dexamethasone to treat keratoconus. Gel was prepared by the cold method by mixing the poloxamer 407 as a thermosensitive polymer and HPMC as viscosity enhancing agent. The formulation was evaluated for pH, clarity, isotonicity, visual appearance. Also, gelation temperature, drug content, ex vivo permeation study, bioadhesive strength, hemolytic toxicity, rheological properties have also been evaluated. All results for this formulation were found to be in an acceptable range. Bioadhesive strength increased with HPMC concentration. The pH of the formulation was found to be 6.1 to 6.51. The gelation temperature was found to be in the range 30–38°C. F5 formulation showed maximum drug release of 99.77% and 98.8% for riboflavin and dexamethasone, respectively, within 8 h. Hemolytic study revealed no change in the shape or surface of blood cells. In vitro keratoconus model was developed in mice using keratoconus and corneal fibroblast cells. The results revealed the that increased thickness of cell lines. The formulated gel played important role in the increasing thickness of cells. Further, the overall study demonstrates the safe and effective use of riboflavin and dexamethasone in the treatment of keratoconus.

1 | INTRODUCTION

Keratoconus is a hereditary disorder but how the disease is inherited is not clear yet [5]. Symptoms of this disease are variable and depends on the stages of progression of disease. Initially, there are no symptoms but it can be identified by no vision or less visual ability observed by ophthalmologist. And at progressed stage, there is significant distortion of vision resulting in visual disability. Fortunately, there occurs no total blindness in this disease. Clinical symptoms also differ depending upon the severity of disease which includes external signs as Munson’s sign (a characteristic feature of keratoconus in which there is protrusion of V-shape for the lower eyelid) and Rizzuti Phenomenon (which occurs in the nose area containing the bright reflection when light is exposed from nasal area) [6–8]. The major risk factors include atopic history, especially ocular allergy, continuous use of rigid contact lenses and rubbing of eyes frequently. Keratoconus appears spontaneously and only few cases are from genetic transmission. The disease generally occurs at puberty stage and lasts for three to four decades or may occur at any stage of life [8]. Depending on the morphology classification, keratoconus is classified as: nipple – the diameter of cone is less than 5 mm which is observed in central or paracentral of the cornea, oval – the cone diameter is more than 5 mm which is observed in paracentral location, keratoglobus – the cone is observed in 75% of the cornea. Depending upon disease evolution, keratoconus classification was proposed by
Amsler Into four different classes as: frustrre or subclinical form, early form, moderate form and severe form [4].

Causes: Many investigations had been done for the pathogenesis of this disease but its development is poorly understood. Amongst them, few investigations on etiology are as follows.

Genetics: Genetic studies have been carried out to know the nature of keratoconus. And so, family studies, twin studies were performed.

Biochemical factors: Due to loss of corneal structural components corneal thinning occurs.

Biomechanical factors: Aldehyde dehydrogenase and superoxide dismutase enzymes have a very important role in different species. The levels of both of these enzymes decrease in keratoconic condition. So accumulation of reactive oxygen tends to saturate the malondialdehyde and peroxynitrites making it responsible for damaging the corneal tissue.

Related disease: It is explained with the help of many symptoms or diseases. Investigation was done and it was found that 0.5 to 15% of patients suffer the Down’s syndrome only because of keratoconus. Other diseases or disorders have also been reported in association with keratoconus like connective tissue disorder [4].

Management and treatment: keratoconus management depends upon the severity of the disease. Various therapies are used for the management of this disease. Spectacles, contact lenses, surgical procedures such as penetrating keratoplasty, deep lamellar keratoplasty, radial keratotomy, and photorefractive keratotomy are applied for the treatment of keratoconus [4].

Riboflavin and ultraviolet type A rays are the photosensitizing substances used in the technique of corneal cross linking in combined action with stromal collagen fibres. This technique consists of photopolymerization of stromal collagen fibres and riboflavin which increases the rigidity of corneal collagen. Riboflavin gives the shielding effect, as it has a dual function of photosensitizer and produces the free oxygen radicles and induces physical cross linking of collagen. The use of riboflavin in keratoconus is a conventional approach. The main aim is to avoid keratoplasty. The use of riboflavin finds the many clinical and scientific basis. Recently, the use of cross linking of corneal collagen with riboflavin has emerged as a favourable treatment in mild to moderate keratoconus.

For ocular drug delivery system, there are many difficulties as human eye is a very complex organ of human body due to its anatomy, biochemical properties and also its physiological properties. Eye is an organ which is almost resistant to all molecules including drugs as well. It restricts the entry of drug at target site of action. Cornea is the main way for drug absorption which possess five different layers. Ocular drug delivery also has different route of administrations like local, topical and ocular and selection is promising and depends on the target tissue. Due to continuous precorneal secretion, there is loss of drug and only 1–3% of the total applied drug is penetrated through the cornea and reaches the target site. There are various barriers for ocular drug delivery system, like blood ocular barrier, which prevents the entrance of hydrophilic drug at target site. Lacrimal fluid eye barrier absorption is limited by corneal epithelium which is present in eye. Ocular drug delivery has limitations such as interference in vision, placement of dosage forms, itching of eyes [5, 6]. The drug concentration of the ocular drug delivery should be effective in order to get therapeutic effect for long period of time hence, the dosage form should have the mucoadhesive property [7]. There are different polymers used for ocular drug delivery system. Some natural polymers and synthetic polymers can be used to sustain the action of the drug. Colloidal also has better acceptance in ocular drug delivery as it lessens the dose administration and avoids eye irritation [8]. The problems which are associated in the ocular drug delivery can be overcome by formulating the thermoreversible gel or in situ gel. When this is instilled into the eyes it will convert to phase transition and forms gel. It is effective for prolonged action [9, 10].

Biochemical properties of cornea depend upon the collagen fibre, its structure, and bonding. The resistance of cornea of keratoconus patients is less than that of a normal cornea. Cross linking stabilizes the stromal collagen and increases the stability of cornea [11–13]. The main purpose of using cross linking stabilizers means that riboflavin UVA is to reduce the progression of keratoconus and present the visual and refractive changes [14–16]. In this study, thermoreversible gel of riboflavin in combination of dexamethasone was formulated for the treatment of keratoconus in order to overcome the problem of precorneal elimination and poor bioavailability [13].

2 MATERIALS AND METHODS

2.1 Materials

Riboflavin and dexamethasone were obtained from Shouguang Fukang Pharmacy Factory (Shandong, China), poloxamer 407 and HPMC K100 were purchased from Sigma Aldrich USA. Methyl Paraben, Propyl Paraben, Ethanol, Tween 80, and other solvents and reagents were of analytical grade.

2.2 Methods

2.2.1 Formulation of thermoreversible ophthalmic in situ gel

Table 1 represents the composition of all formulations. Cold method was applied for the preparation of in situ gel. Poloxamer 407 was added to 15 ml of water, and the solution was stirred at about 400–500 rpm for about 2 h. In this preparation a temperature of 4°C was maintained. The solution was kept in the refrigerator overnight. The next day we calculated the amount of HPMC K 100 and methyl paraben and propyl paraben were added to the above solution. The drug solution was prepared in tween 80 and ethanol (1:2) and then mixed in poloxamer dispersion. The final volume was made up with water and the pH was adjusted by triethanolamine. The solution of sodium chloride was used in order to adjust tonicity of the solution.
TABLE 1 Composition of in situ thermoreversible gel of riboflavin and dexamethasone

| Sr. no | Ingredients % w/v | F1   | F2   | F3   | F4   | F5   |
|--------|-------------------|------|------|------|------|------|
| 1      | Riboflavin        | 0.35 | 0.35 | 0.35 | 0.35 | 0.35 |
| 2      | Dexamethasone     | 0.2  | 0.2  | 0.2  | 0.2  | 0.2  |
| 3      | Poloxamer 407     | 8.0  | 10.0 | 12.0 | 14.0 | 16.0 |
| 4      | HPMC K 100        | 0.25 | 0.35 | 0.45 | 0.55 | 0.65 |
| 5      | Methyl paraben    | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| 6      | Propyl paraben    | 0.03 | 0.3  | 0.03 | 0.03 | 0.03 |
| 7      | Ethanol           | 6    | 6    | 6    | 6    | 6    |
| 8      | Tween 80          | 3    | 3    | 3    | 3    | 3    |
| 9      | Distilled water (ml) | q.s | q.s  | q.s  | q.s  | q.s  |

3 | EVALUATION OF PREPARED IN SITU GEL

3.1 | Visual appearance and clarity [17, 18]

By visual inspection against black and white the appearance and clarity of the gel was determined.

3.2 | pH determination [19]

pH was determined by pH meter. Calibration was done before determination. Three readings were taken and the average was calculated.

3.3 | Drug content [17]

2 ml of sample was taken in volumetric flask and dilution was done with the help of simulated tear fluid having pH 7.4 for getting the approximate concentration of 10 mg/ml. The solution was then mixed by shaking the volumetric flask for a few minutes. Absorbance was measured at maxima of 280 nm by using UV spectrophotometer. The percentage of the drug content was then calculated.

3.4 | Rheological properties

For ophthalmic preparations, rheological properties are a very important parameter. From the various literature surveys it was noted that gel must have the viscosity of 5 to 1000 cps and after administration into the eyes it should be 50 to 50,000 cps. With the help of Brookfield synchro electric viscometer, viscosity of the formulations was carried out and it was carried out at different speed conditions like 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5.

3.5 | Gelling capacity [17]

Gelling capacity was determined for the in situ gels to investigate whether the composition is suitable for use as in situ gel system. For determination of gelling capacity, simulated tear fluid was used. Gel was mixed in simulated tear fluid which was taken in the proportion of 25:7. Gel volume was taken as 25 μl and 7 μl of tear fluid was taken. This mixture was visually observed for gel formation and time was noted for dissolution of gel completely.

3.6 | Gelation temperature [19]

Gelation temperature was maintained by taking the sample into a test tube and placing the test tube in a water bath having the temperature 37 ± 50°C. Test tube was dipped in water bath for 2 min. The solution was converted into gel. The temperature needed for the conversion of solution to gel was noted down which is called gelation temperature and was noted down by placing the thermometer in the test tube.

3.7 | Determination of bioadhesive strength [18]

The bioadhesive strength was determined by using texture analyser (CT3 Brookfield, USA). For this corneal mucosa was utilized. The tissue was fixed on holder before the fixing of tissue equilibration for 10 min at 37°C. The probe was lowered at a rate of 0.3 mm/s until contact with tissue membrane was made. A contact force was made for about a minute and probe was simultaneously removed at a rate of 0.5 mm/s to a distance of 20 mm. The force which is required separating the probe from tissue can be detected by texture analyser software.

3.8 | Isotonicity evaluation [21]

Isotonicity was determined by mixing the formulation with few drops of blood and observed under 45X magnification of microscope. The effect of formulation was determined on red blood cells like cremation, swelling, bursting.
3.9 | Ex vivo diffusion study through cornea [21, 22]

Ex vivo permeation study was done on cornea which was obtained from the porcine eyes. These freshly collected eyes were checked for any visual defects and stored in normal saline solution. Franz diffusion cell was used where donor and acceptor compartment was available. The temperature was maintained at 35°C. The porcine cornea and human cornea resemble each other, only porcine cornea is thicker than latter. Three corneas were used and then same step was carried out in in vitro release study. The phosphate buffer was used to fill the receptor compartment which was having pH 6.8 at 35°C and was stirred continuously. The samples were withdrawn from receptor compartment with time intervals 30 min, 1 h, 2 h, 3 h, 4 h, upto 8 h. Every time phosphate buffer was replaced to maintain the sink condition. The amount of KT that permeated across porcine cornea was determined by HPLC method.

3.10 | Hemolytic toxicity [21–24]

For the determination of hemolytic toxicity, suspension of red blood cell was dispersed in the doubled distilled water and also in normal saline solution. This solution was considered as the control as distilled water is 100% hemolytic and normal saline solution is non-hemolytic. For this determination aqueous solution of the drug was prepared and added into the 5 ml of normal saline solution or 5 ml of distilled water and it was reacted with RBC suspension. The gel formulated with and without (Plain gel) riboflavin and dexamethasone was kept stable. The hemolysis data was compared with plain gel and drug loaded gel. This study was determined on increased concentration of gel in blank and drug loaded gel. Results for the plain and drug loaded gel was observed separately. The above mixtures were centrifuged and supernatant liquid was analysed for UV spectrophotometric assay. In normal saline solution, absorbance was taken at 445 nm against blank and hemolysis was calculated in percentage with the help of absorbance factor of 100% hemolytic sample in distilled water.

3.11 | Effect of gel of riboflavin and dexamethasone in keratoconus mice model

In this study, in vitro keratoconus model was developed in mice using keratoconus and corneal fibroblast cells (CFCs). CFCs were isolated from the mice corneas and stroma was separated from corneal epithelium and endothelium by scraping. This stroma pieces were separated into 6 well plates and Eagles medium which is 10% fetal bovine serum. This was cultivated into the medium and after cultivation into it, these cells were passed through 120 mm cell culture plate. The KCs having the characteristic morphology with actin microfilaments and also containing high level of α smooth muscle actin microfilaments are different from the healthy CFCs. Both these cells were mounted on 6 well plates which contain polycarbonate membrane, inserted into the 0.4 μm pores at a density of 10^6 cells/ml. These cells were cultured in the 10% FBS and 0.5 mM vitamin C. These cultures were grown for 4 weeks where some agents were added like 0.1 ng/ml TGF-β1 (T1) + riboflavin + dexamethasone formulation or TGF-β3 (T3) + riboflavin+dexamethasone formulation. Control test was considered for cell cultures without growth factors. The cultures were examined for its morphology by using TEM or immunofluorescence which is the specific marker for stromal cells like smooth muscles actin (SMA), type I collagen, type III collagen, and fibronectin.

4 | RESULTS AND DISCUSSION

4.1 | Visual appearance and clarity [17, 18]

The clarity of the gel of all formulations was found to be clear. Only at the time of terminal sterilization by autoclaving, solutions were found to be hazy occurring because of precipitation of HPMC at increased temperature. This haziness was regained by keeping solutions overnight. The results are represented in Table 2.

4.2 | pH determination [19]

The pH of all formulation was observed in the range 6.1 to 6.51 which is acceptable for opthalmic preparations. Results are represented in Table 2.

4.3 | Drug content [17]

In formulations F1–F5 drug content of riboflavin was found to be in the range 94.66% to 104.21% which is an acceptable range and for dexamethasone it was 93% to 99.32%. So it can be concluded that in all formulations, drug is equally distributed which results in acceptable drug content. Results are represented in Table 2.

4.4 | Rheological properties

The rheological property determination is an important parameter. From the various literatures it was found that formulations before gel must have the viscosity 5 to 1000 cps and after gelling in the eye, they must have the from viscosity 50 to 50,000 cps. Rheological study of the formulation is represented in Table 3.

4.5 | Gelling capacity [17]

Viscosity and gelling capacity having important characteristic in the opthalmic preparations especially in in situ gel systems. The formulation should have the optimum viscosity so that it
## TABLE 2 Evaluation of gel

| Code | Appearance | Clarity | pH | Ribo (% content) | Dexa (% content) | Isotonicity | Gelation temperature (°C) | Gelling capacity | Strength (dyne/cm²) |
|------|------------|---------|----|------------------|------------------|-------------|--------------------------|-----------------|------------------|
| F1   | Pale yellow | Clear   | 6.10 | 94.66            | 93.00            | Isotonic    | 30                       | +               | 884 ± 5.23       |
| F2   | Pale yellow | Clear   | 6.13 | 95.8             | 94.32            | Isotonic    | 32                       | ++              | 932 ± 4.56       |
| F3   | Pale yellow | Clear   | 6.21 | 99.6             | 95.12            | Isotonic    | 35                       | ++              | 1003 ± 6.21      |
| F4   | Pale yellow | Clear   | 6.42 | 100.45           | 98.94            | Isotonic    | 36                       | +++             | 1034 ± 7.44      |
| F5   | Pale yellow | Clear   | 6.51 | 104.21           | 99.32            | Isotonic    | 38                       | +++             | 1343 ± 2.23      |

* indicates the gel with less gelling capacity
++ indicates the better gelling capacity than +
+++ means quick gelation and remains for few hours.
++++ means quick gelation and remains for extended period.

## TABLE 3 Rheological properties of all formulations

| RPM | F1  | F2  | F3  | F4  | F5  |
|-----|-----|-----|-----|-----|-----|
| 1   | 432 | 877 | 1245| 1876| 2550|
| 1.5 | 335 | 811 | 1176| 1812| 2432|
| 2   | 321 | 745 | 1122| 1798| 2233|
| 2.5 | 298 | 711 | 1043| 1745| 1988|
| 3   | 222 | 654 | 1011| 1532| 1922|
| 3.5 | 197 | 553 | 943 | 1421| 1865|
| 4   | 156 | 511 | 911 | 1326| 1765|
| 4.5 | 144 | 432 | 832 | 1299| 1676|

will instill into the eyes and convert into the sol to gel transition. The gelling capacity is represented in Table 2.

### 4.6 Gelation temperature [19, 20]

The gelation temperature of all the formulations was observed in the range 30 to 38°C. Results are represented in Table 2.

### 4.7 Determination of bioadhesive strength [18]

The bioadhesive strength of gel increases with the increase in the concentration of the polymer. Hence, formulation F5 showed more strength. It is related to the hydrogen bonding between gel and mucosal membrane. Bioadhesion depends on the strong bonding of polymer with membrane and it varies from polymer to polymer. The values are observed in the range of 884 to 1343 dyne/cm². The results are represented in Table 2.

### 4.8 Isotonicity evaluation [21]

All the formulations were found to be isotonic which is a very much important parameter and must maintain ophthalmic delivery system to avoid the tissue damage or irritation of the eye.

### 4.9 Ex vivo diffusion study through cornea [21-23]

In this study, the amount of drug diffused from each formulation is represented in Table 4. The in vitro diffusion study was performed in phosphate buffer 6.8. The results for the diffusion study, drug diffused from each formulation is represented in Table 4. Tiwari et al. also studied ocular film of riboflavin acetate in the treatment of keratoconus where they developed and characterized riboflavin lipid conjugate. In this formulation, they have used single riboflavin drug and studied permeation profile [24]. From the result of the present study, it can be concluded that F5 formulation was showing the maximum drug release i.e. 99.77% and 98.8% for riboflavin and dexamethasone respectively within 8 h as compared to the other formulations (Figure 1). In minimum time maximum amount of drug is released; hence, F5 is the best formulation because of its maximum and rapid release.

### 4.10 Hemolytic toxicity [21, 25]

In this study, it was found that there was no change in the shape of RBCs with respect to the shape, no bulging or shrinkage. This indicates no adverse reaction of formulation with blood cells. So this study reveals that this gel system of formulation exhibited hemolysis as a function of concentration. Formulations which contain drug are less toxic with an equivalent concentration of gel than plain gel. In blank formulation, there are free terminal amino groups which interact with blood cells and leads to hemolysis.

### 4.11 Effect of gel of riboflavin and dexamethasone in keratoconus mice model

Ostacolo et al. also studied the corneal permeation of riboflavin phosphate through transepithelial cross linking treatment. In
TABLE 4  Ex vivo diffusion study of thermoreversible gel

| Times (h) | F1 Ribo % | F1 Dexam % | F2 Ribo % | F2 Dexam % | F3 Ribo % | F3 Dexam % | F4 Ribo % | F4 Dexam % | F5 Ribo % | F5 Dexam % |
|-----------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|
| 0         | 0         | 0          | 0         | 0          | 0         | 0          | 0         | 0          | 0         | 0          |
| 0.5       | 6.5       | 5.45       | 16.2      | 9.65       | 17.8      | 17.7       | 25.12     | 21.20      | 25.92     | 22.11      |
| 1         | 11.4      | 7.9        | 17.4      | 16.6       | 22.9      | 19.9       | 45.81     | 35.4       | 47.27     | 43.90      |
| 2         | 20.23     | 12.8       | 25.12     | 19.9       | 42.8      | 36.6       | 57.35     | 45.9       | 58.79     | 55.20      |
| 3         | 51.90     | 36.20      | 58.08     | 57.4       | 54.9      | 52.9       | 68.35     | 55.9       | 72.34     | 65.40      |
| 4         | 62.92     | 51.65      | 72.27     | 68.38      | 72.18     | 68.5       | 77.71     | 68.8       | 79.65     | 75.80      |
| 5         | 82.36     | 65.17      | 87.73     | 74.4       | 81.44     | 75.5       | 86.90     | 79.8       | 87.47     | 80.32      |
| 6         | 87.33     | 78.66      | 91.90     | 83.9       | 91.0      | 90.4       | 93.21     | 88.57      | 92.11     | 85.90      |
| 8         | 91.25     | 84.9       | 96.5      | 90.76      | 96.20     | 97.23      | 98.65     | 97.27      | 99.77     | 98.8       |

FIGURE 1  Cumulative % drug released study

*P value of <0.05 was considered to be statistically significant

this study they reported corneal permeation and which was compared with standard solution of riboflavin [26]. Bottós et al. investigated the riboflavin and its absorption on corneal epithelium on ultra violet. Many studies have been done on the effect of riboflavin for corneal permeation [27]. However, no study has been found with the combination of riboflavin and dexamethasone in the treatment of keratoconus. All results of the combination of riboflavin and dexamethasone were found satisfactory. Wen et al. had formulated the in situ gel loaded with nanoparticles of dexamethasone for ophthalmic drug delivery system. Ex vivo permeation studies were performed which were compared with standard preparation [28, 29]. The present study is the combination of riboflavin and dexamethasone which gives the better drug release and permeation. Compared to other preparations, in the treatment of keratoconus, corneal transplant or corneal collagen cross linking to be done. There are various modern techniques which overcome the chances of infection. But some investigations predict the post-operative inflammation, redness and itching. Dexamethasone, an anti-inflammatory drug can be used mostly in both post-operative treatment of keratoconus. In this study, after 5 weeks, KC cells showed thickness of 8.3 μm while in CFC matrix was produced of about 19.2 μm. The comparison between mean thickness for KC and CFC treated with samples are shown in Figure 2.

T1 with formulated gel was added to the culture, the KC had increased thickness and CFC had developed thickness more than KC. After T3 stimulation, KC and CFC showed similler
thickness as with T1 (18.6 μm and 54.7 μm respectively). So, it can be concluded that KC secretes less matrix as compare to CFC. These results suggest that matrix produced after stimulation of KC cells and increased thickness means increased number of cells or matrix produced. The formulated gel played an important role in the increased thickness of cells [30-34].

4.12 Statistical analysis

All experiments were performed on three independent preparations and a mean value was determined ± SD. Statistical analysis was performed and a P value of < 0.05 was considered to be statistically significant.

5 CONCLUSION

The present study has been developed and evaluated for thermoresversible ophthalmic gel containing riboflavin and dexamethasone as main drug ingredients. As per the experimental data and analysis formulation F5 was found to be the optimized formulation containing Poloxamer and HPMC polymers to form a thermoresversible gel. All the physicochemical properties were found satisfactory considering the requirements of ophthalmic drug delivery system. This formulated ophthalmic gel has the capacity to overcome the corneal epithelial barrier resistance and the problem of precorneal elimination and poor bioavailability.

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