CHAPTER 4

SUMMARY AND CONCLUSION
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4.1 SUMMARY

Conventional, liquid ophthalmic formulations show low bioavailability because of constant lachrymal secretion and rapid nasolachrymal drainage. Normal drainage of an instilled dose commences immediately upon instillation and is essentially completed within 5 min. Typically, ophthalmic bioavailability of only 1-10% are achieved due to the short precorneal residence times of ophthalmic solutions. Consequently, there is a need for frequent instillation of concentrated solutions to achieve the desired therapeutic effect. Moreover, systemic absorption of the drug drained through the nasolachrymal duct may result in some undesirable side effects. To overcome these problems various ophthalmic vehicles, such as suspensions, ointments, inserts and aqueous gels, have been investigated in attempts to extend the ocular residence time of medications for topical application to the eye. These ocular drug delivery systems offer some improvement over a conventional liquid dosage form but because of blurred vision (e.g. ointments) or lack of patient compliance (e.g. inserts), they have not been universally accepted. As a result, good ocular bioavailability following topical delivery of a drug to the eye remains a challenge yet to be satisfactorily resolved.

Conjunctivitis is an acute inflammation of the conjunctiva (the outermost layer of the eye and the inner surface of the eyelids), most commonly due to an allergic reaction or an infection. A purulent discharge, papillary conjunctival chemosis and itching is more frequently associated with conjunctival allergy. Hazing of the anterior chamber, infiltrative keratitis in the visual axis or decreased vision are harbinger of imminent visual loss and require prompt microbiological studies for organism's identification and proper antibiotic selection. Similar fastidious cultures of the lids or conjunctiva are important for any chronic conjunctivitis. Fluoroquinolones (ciprofloxacin, moxifloxacin, sparfloxacin and levofloxacin etc) eye drops are used in the management of conjunctivitis.

From the point of view of patient acceptability, a liquid dosage form that can sustain drug release and remain in contact with the cornea of the eye for extended periods of time is ideal. If the precorneal residence time of a drug could be improved, then we can achieve better bioavailability with reduced dosing frequency and improved patient acceptability. This relatively modest improvement can be achieved by novel delivery systems based on the concept of hydrogel formations i.e. in situ gel, pHEMA gel, nanosuspension, nanoparticles laden in situ gel, nanoparticles laden pHEMA gel. Such delivery systems consist of various delivery mechanisms i.e. in situ gel is based on phase transition systems that are instilled as...
a liquid form and shift to the gel or solid phase once it reaches in the cul-de-sac of the eye, whereas nanoparticles due to their nano size particles easily retain on the eye. *In situ* gel and nanoparticles have there own advantages and disadvantages. *In situ* gel stay only for 12 hours and PLGA nanoparticles are non mucoadhesive and drained out quickly. So, in our novel approach we have tried to combine both these formulations as nanoparticles laden *in situ* gel. Nanoparticles provide sustained release and chitosan *in situ* gel coat over nanoparticles to make them mucoadhesive to get a long acting sustained release formulation. The principal advantage of this formulation is the possibility of administering accurate and reproducible quantities, in contrast to already gelled formulations, and more over promoting precorneal retention. Another hydrogel approach is developing pHEMA hydrogels. It is also used as contact lens. These are liquid monomers which are polymerized to swellable disc like transparent structure. pHEMA gels have tendency to absorb solution and swell to release drug slowly. Hence we also try this strategy by simple soaking the disc in drug solution or encapsulating nanoparticles in the pHEMA matrix as nanoparticle laden pHEMA gel.

The procured drug gift samples of sparflloxacin and levoflloxacin (Micro Labs Ltd. Chandigarh, India) were characterized for various pharmacopoeial and non-pharmacopoeial tests to cross-examine their authenticity. Chemical tests for sparflloxacin and levoflloxacin gave results in conformity with the literature. An IR spectrum of the provided drug was found to be in concordant with reference IR spectral peaks. UV absorption maxima were found to be 290 nm and 288 nm for sparflloxacin and levoflloxacin, respectively. Comparison of the XRD and DSC scans of the drug samples with corresponding reference standards (R.S.) showed that the spectra were super-imposable, confirming the identity of the drug samples.

UPLC assay showed that the drug samples of sparflloxacin and levoflloxacin complied with the specifications of 98-101%.

Solubility of the drugs was determined in various solvents at room temperature. It was observed that the drugs were soluble in organic solvent i.e. chloroform, ethanol and methanol, and practically insoluble in water. The drugs were found to be hydrophobic. In order to estimate sparflloxacin and levoflloxacin in the experimental protocols, calibration curves of the drug were prepared in PBS (pH 7.4) and simulated tear fluid.

*Analytical quantification* of the model drugs (sparflloxacin and levoflloxacin) was done by developing suitable ultra performance liquid chromatographic (UPLC) methods for accurate
quantification. The developed methods were validated according to the ICH guidelines, ICH Q2A (R1), for linearity, accuracy, precision, recovery, reproducibility, specificity, LOD, LOQ, ruggedness and robustness. Recovery of the sparfloxacin and levofloxacin was observed to be in the range of 99.1 to 100.5% and 98.7 to 100.9% respectively from different formulations. The LOD and LOQ for developed UPLC methods were found to be 0.02 μg/ml and 0.06 μg/ml for sparfloxacin and 0.015 μg/ml and 0.05 μg/ml for levofloxacin respectively. The developed stability indicating UPLC methods were found to be robust and can be used in present research for quantification of the drugs. The methods were completely validated showing satisfactory data for all the parameters tested. The methods exhibited an excellent performance in terms of sensitivity and speed. Moreover, since the methods do not involve any sample preparation step, a rapid determination of drug i.e. sparfloxacin or levofloxacin can be performed.

Drug-excipient compatibility studies were carried out by IR, DSC and XRD analysis of the ingredients individually and in mixture. No additional peak or change in existent peak was recorded which confirms that the formulation ingredients were compatible with each other. It can be inferred that, no physicochemical reactions or incompatibility was found among drug and excipients and hence they can be used further in the formulations development and optimization.

In situ gel of sparfloxacin and levofloxacin were prepared using chitosan and ion activated polymer (Gellan gum/Sodium alginate). Chitosan was used in combination with gellan gum and sodium alginate respectively for sparfloxacin and levofloxacin gel formulations. Various placebo formulations were developed using different combination of chitosan and gellan gum/sodium alginate and evaluated for their physicochemical characteristics like physical appearance and viscosity at formulation pH (6) and at ocular pH (7.4). Based on the above observation, formulation SG12 (chitosan 0.5%w/v and 0.3% w/v gellan gum) and LG12 (chitosan 0.5%w/v and sodium alginate 0.3% w/v) were selected for further studies. Formulations SG12 and LG12 remains clear or become very slightly turbid at pH 7.4, so that the vision would not hampered. Methylparaben at concentration 0.1% w/v was added as a preservative and sodium chloride was added to make the formulation isotonic with eye. For therapeutic action, a dose of 0.3%w/v of sparfloxacin and 0.5%w/v of levofloxacin was prescribed. The same dose was used for the developed formulations.

pHEMA gel was formed by thermal polymerization. The pHEMA hydrogels were synthesized by free radical solution polymerization of HEMA monomer. Formulation SC3 and LC3 were
optimized and taken further for evaluation. pHEMA is an extremely stable hydrogel and variations in temperature, pH, and tonicity have relatively little effect on its water content. Therefore, it has a definite advantage over other polymers in contact lens manufacture. The water content was found to be near 45%, which is a characteristic of HEMA monomer.

**PLGA nanoparticles** were prepared by nanoprecipitation method. Acetone was used as a solvent and PVA as stabilizer. The selection of solvents was done on the basis of previous literature and experience. Different drug:polymer ratios were evaluated for particle size, encapsulation efficiency, nanoparticles recovery, zeta potential and polydispersibility index etc to obtain low particle size with maximum encapsulation efficiency for both sparfloxacin and levofloxacin. A drug (sparfloxacin, levofloxacin) readily precipitated in aqueous medium and gets encapsulated by the PLGA matrix preventing its diffusion in external phase. Among all formulations, SN4 and LN4 were found appropriate for further analysis. The particle size of these formulation were found below 200 nm with an encapsulation efficiency of >85%. Nanoparticles are usually stabilized by lyophilization, but during lyophilization, nanoparticles tend to agglomerate and form clumps or pellets. Hence to ensure the complete and easy re-dispersion of freeze dried nanoparticles, mannitol (1.5%w/v) was used as a cryoprotectant. The quantity of nanoparticles was calculated on the basis of drug loading and release.

**Nanoparticle laden in situ gel** is prepared by taking calculated freeze dried nanoparticles of sparfloxacin/levofloxacin (40/60mg) and dispersing it in their respective 1 ml in situ gel base. The slight turbidity found can be due to suspension form of nanoparticles in in situ gel. After mixing there is not much difference found in gelation pH and viscosity of the formulations. It is well in concordant with plain in situ gel formulation.

**Nanoparticle laden pHEMA gel** was prepared by dispersing the above amount of SN4 and LN4 nanoparticles in HEMA monomer and then polymerized as per previously optimized protocol C3. We observed a slightly lower transmittance then pHEMA without nanoparticles; this could be due to the nanoparticles dispersed and hindering the light. Whereas we do not observe any visible particle or hinderance in these developed pHEMA gel SNC and LNC.

**In vitro drug release** profile of all the formulations i.e. SG12, LG12, SN4, LN4, SNG, LNG, SNC, LNC were determined in simulated tear fluid (pH 7.4) using dialysis technique and the release profiles were compared with marketed formulations of sparfloxacin and
levofoxacin. Nearly 97% of the drug from the conventional eye drops was released within 4-6 hours. The drugs from the formulation SG12 and LG12 released up to 95% and 98% in 12 hours respectively. The in situ gel follows a matrix pattern of drug release. The drug from nanosuspension SN4 and LN4 was released up to 86% and 89% in 24 hours respectively. The drug soaked pHEMA hydrogel SC3 and LC3 released 98.29% and 98.38% of drug within 8 hours. This seems to show a similar pattern like marketed drops. Due to soaking, the drug will mainly be available on the surface of the pHEMA gel and released as soon as it comes in contact with tear buffer. The formulations SN4 and LN4 seem to follow the peppas model of drug release. Formulations SNG and LNG shows a slow release, 65.23% and 68.43% drug in 12 hours and 98.86 % and 98.83% in 48 hours. This seems to give a prolonged sustained release pattern. The nanoparticles laden pHEMA gel SNC and LNC show negligible release of drug i.e. 14.64% and 14.50% in 24 hours and 28.60% and 28.30% in 48 hours. This seems a very slow and inadequate release as per our objectives. On the above observations of in vitro release profile of formulations, the following pattern of release was observed.

Marketed > SC3, LC3 > SG12, LG12 > SN4, LN4 > SNG, LNG > SNC, LNC
The formulations which seemed promising were SG12, LG12, SN4, LN4, SNG, LNG and SNC.

In in vitro transcorneal permeation studies, various developed formulations i.e. SG12, LG12, SC3, LC3, SN4, LN4, SNG, LNG, SNC and LNC were compared. The drugs sparflaxacin and levofoxacin were able to permeate 41.26 and 41.85% from marketed formulations through cornea in 8 hours whereas, the drugs permeated 75.13 and 74.06% in 8 hours from in situ gel formulations SG12 and LG12; drug soaked pHEMA hydrogel SC3 and LC3 permeated up to 34% of drug in 8 hours; nanosuspensions SN4 and LN4 are able to permeate the drug up to 57.73% and 56%; nanoparticles laden in situ gel system SNG and LNG permeated 61.79% and 61% drug respectively, whereas the nanoparticles laden pHEMA gel SNC and LNC were not able to permeate considerable amount with in 8 hours i.e. 3.84% and 3.83% of drug respectively. The above observation shows that the formulation follows the following sequence in drug permeation through cornea:

SG12, LG12 > SNG, LNG > SN4, LN4 > Marketed > SC3, LC3 > SNC, LNC
On the basis of in vitro drug release and in vitro transcorneal permeation, SC3, LC3, SNC, LNC were discarded and formulations SG12, LG12, SN4, LN4, SNG and LNG were found optimum for further evaluation.

Antimicrobial activity was conducted for various developed formulations of sparflaxacin and levofoxacin. Agar-well diffusion assay was used to assess and compare the antibacterial efficacy of in situ gel, nanosuspension and nanoladen in situ gel for both the drugs with the
marketed preparation. The broad-spectrum antibacterial activity of different formulations of sparfloxacin and levofloxacin were observed to be similar or more sustained than the marketed eye drop preparations of respective drugs, suggesting that the different conditions employed during manufacturing of different formulations do not affect the intrinsic antimicrobial activity of loaded drugs whereas the formulations have sustained effect on the release of the medicament.

**Ocular irritation test** of the developed formulations were assessed by Hen's egg chorioallantoic membrane test which is a rapid, sensitive and inexpensive test. Different formulations were tested by this test and results were compared with control (normal saline) as this is supposed to be practically non-irritant. Sparfloxacin and levofloxacin marketed eye drops formulation were non-irritant (mean score 0) up to 8 hours, whereas the mean score obtained were found to be 0.66 up to 24 hours for the sparfloxacin and 0.33 up to 24 hours for the levofloxacin marketed drops. It can be inferred from the results that the formulation is non-irritant to mild irritant and is well tolerated. The mean score for sparfloxacin *in situ* gel (SG12) and levofloxacin *in situ* gel (LG12) was found to be 0.66 and 0.33 respectively up to 24 hours. The results showed that these formulations are mild-irritant. Further, the mean score for nanoparticles suspension formulation of sparfloxacin (SN4) and levofloxacin (LN4) was found to be 0.33 each which confirms the formulation to be non-irritant. The formulation of nanoparticle laden *in situ* gel for sparfloxacin (SNG) and levofloxacin (LNG) showed the mean score of 0.66 and 0.33 respectively up to 24 hours, it confirms that the formulation is mild-irritant and easily tolerable. The HET CAM assay shows that all the developed formulations SG12, LG12, SN4, LN4, SNG, and LNG are non-irritant to mild irritant and can be well tolerated.

**Corneal hydration studies** are generally used as an important parameter to evaluate damage to the corneal tissue. Hydration level found for sparfloxacin marketed formulation and developed formulations i.e. SG12, SN4 and SNG was found to be 78.12%, 76.56% and 76.28%, respectively, whereas hydration level found for levofloxacin marketed formulation and developed formulations i.e. LG12, LN4 and LNG was found to be 77.47%, 76.9%, 77.29% and 77.31%, respectively, which is well in acceptable range of 76-79% and indicates that formulations did not cause any damage to corneal tissue.

**Histopathological studies** showed that undamaged corneal cell images by applying the various developed formulations. No abnormalities were noted in visual and histopathological evaluation. With this evaluation, the formulations can be said to have non-irritant effect with better tolerance for 5-10 days twice daily dose regimen.
Ocular inflammation study through IR camera observation does not show noticeable increase in temperature after instillation of formulations, so all the formulations were found to be non inflammatory.

From all ocular tolerance tests, we inferred that the formulations SG12, SN4, SNG, LG12, LN4, LNG were non irritant to mild irritant with no signs of damage to corneal tissue. Formulations were non inflammatory and found in accordance with marketed eye drops. Hence all the formulations passed ocular tolerance test and proved to be safe to use.

**Gamma scintigraphic studies** are used to analyze the retention time of the formulation in cul-de-sac. Firstly, sparflloxacin and levofloxacin were labeled with radionuclide Tc-99m using stannous chloride and stannous tartarate as reducing agent respectively. The observation of the acquired gamma camera images showed a good spreading over the entire precorneal area for developed ocular formulations of sparflloxacin (SG12, SN4, SNG). Marketed formulation cleared very rapidly from the corneal region and reached in to systemic circulation via nasolachrymal drainage system as significant activity was recorded in kidney and bladder after 5 hours of ocular administration, whereas formulations SG12, LG12, SN4, LN4, SNG and LNG were retained for longer duration at corneal surface. No significant radioactivity was observed in kidney and bladder after 5 hours of administration of these formulations. The retention on cornea follows the following sequence:

*Marketed Formulation < Nanosuspension < In situ gel < Nanoparticle Laden In situ Gel*

It shows that the nanoparticles laden in situ gel retained for the longer duration in the eye giving extended release than nanosuspension and in situ gel alone, whereas in dynamic studies in gamma scintigraphy, the marketed formulation drained out of the eye with in 30min. Due to mucoadhesive property of chitosan, in situ gel based formulation cleared at slower rate as compared to nanosuspension and retained at corneal surface for longest time duration.

For packing, the formulations were kept in amber colored bottle closed with a cap and dropper with teat was used. The nanoparticles were packed in freeze dried form with separate supply of sterilized normal saline or in situ gel. These need to be reconstituted at the time of opening for use with in 1 month. The packaging was tested for resistance by evaluating the closure efficiency (leakage) and pourability of the formulation. Packaging passed all the quality control tests and hence was a good choice for packaging of present ocular formulations.

Sterilization of the developed in situ gel formulations (SG12, LG12) was done by autoclaving at 121°C for 20 min at 15 psig. The In situ gel is sterilized by autoclaving whereas frieze
dried nanoparticles are thermolabile, so gamma rays were used at a dose of 25 KGY. Test for sterility was performed on sterilized packaging according to IP 2007 standards. No growth/microbial contamination were observed up to 14 days of incubation. Hence the formulations passed the sterility test. It was observed that the teats didn't become sticky and caps also didn’t change in size and shape. This indicates that the packaging system is resistant to autoclaving/gamma radiation and is suitable for packaging the test formulations. In the closure efficiency test, there was no alteration observed in the volume or color of the contents. Packages passed all the tests and proved to be a good choice for packaging of present ocular formulations.

Stability Studies of the various developed formulations were done to evaluate the shelf-life of the active ingredient(s). A formulation must be stable enough, so that the active ingredient must be available to the patient throughout the expected shelf life. To calculate shelf life of the formulation, extensive stability data were collected according to ICH guidelines and Arrhenious plot method. According to ICH, samples need to be kept under climatic zone III conditions in India, so formulations were kept for accelerated stability testing at above conditions i.e. 40°C and 75% RH for 90 days. According to ICH guidelines, if long-term studies are conducted at 25°C±2°C/60% RH ±5% RH and "significant change" occurs at any time during 6 months testing at the accelerated storage condition, additional testing at the intermediate storage condition should be conducted and evaluated against significant change criteria. Since, no change was found in any of the formulations during the 6 months so the stability studies at intermediate conditions were not performed. Graph was plotted between time (in days) and drug remaining in pack. Degradation constant (K) (at 25°C) was calculated from the slope of line, which was found to be 1.19 X 10^-4 days^-1, 1.01 X 10^-4 days^-1 and 1.06 X 10^-4 days^-1 for SG12, SN4 and SNG respectively. Similarly, degradation constant (K) (at 25°C) was calculated from the slope of line, which was found to be 1.08 X 10^-4 days^-1, 1.01 X 10^-4 days^-1 and 1.13 X 10^-4 days^-1 for LG12, LN4 and LNG respectively. As the amount of drug degraded in 180 days was less than 5%, therefore a tentative shelf life of 2 year was assigned to the optimized formulation.

Arrhenious plot was drawn from 9 packages kept at 3 different temperatures 40°C, 50°C and 60°C. K values were calculated from slopes of lines and Arrhenious plot was plotted with K values of 3 different temperatures. Shelf life was calculated, which was found to be 2.35-2.85 years. Hence from both ICH guidelines and Arrhenious plot of various optimized formulations, a shelf life of 2 years could be assigned to the test formulations.
Conclusion

Novel hydrogel based ocular drug delivery systems of sparfloxacin and levofloxacin were successfully formulated and characterized for improved management of conjunctivitis. Five different ocular formulations were prepared. Of these, three formulations v.i.z. in situ gel, nanosuspension and nanoparticle laden in situ gel were found to be satisfactory based on in vitro drug release and transcorneal permeation studies. The selected formulations presented better antimicrobial efficacy, ocular tolerance, ocular retention and pharmacokinetic profile vis-a-vis marketed formulations.

Further studies are warranted to corroborate the clinical efficacy of the above laboratory formulations.