SUMMARY AND CONCLUSION

Viral and immunological disorders of the eyes are the common disorders of the eyes like bacterial infections. Viral infections such as Herpes Zoster and Herpes simplex Infections (herpetic keratitis), cytomegalovirus (CMV) infection in immunocompromised subjects and viral conjunctivitis which is a contagious infection and inflammation of the conjunctiva (Daniels et al., 2011). In case of immunological diseases of eyes, Posterior blepharitis, ocular rosacea, post-LASIK dry eye, contact lens intolerance, atopic keratoconjunctivitis (Dry eye disease), graft-versus-host disease, and herpetic stromal keratitis are the common ocular disorders in the autoimmune mediated inflammatory manifestations occur (Rao, 2011; Durrani et al., 2011). Antiviral and immunosuppressive drug delivery to the ocular surface is a challenging problem and is a subject of interest to scientists working in the multidisciplinary areas pertaining to the eye, including pharmaceutical, chemical, biochemical, medical, clinical, and toxicological sciences. A major problem in ocular therapeutics is the attainment of an optimal drug concentration at the site of action. Poor bioavailability of drugs from ocular dosage forms is mainly due to the tear dynamics, transient residence time in the cul-de-sac, non-productive drug absorption, and the relative impermeability of the cornea (Ludwig, 2005). Currently, Conventional oral and topical medications are available for these drugs. To treat and manage the local ophthalmic disorders Topical ocular delivery of drugs in the form of liquid eye drop is valuable, Which is the most common route and desirable dosage form respectively when considering convenience of administration, the rapid local effect , accessibility of the ocular tissue, relative safety and clinical compliance of the patients. However, due to the high hydrophilic and lipophilic character of GCV and CYA respectively, unique physiological structures of eye and the rapid elimination,
the conventional topical applications usually have quite limited therapeutic benefits due to the poor bioavailability over and into the ocular tissues. The poor bioavailability is due to a) quick dilution of the formulation after instillation by the tear film, b) exhausted into the nasolacrimal channel, c) limited capacity of cul-du-sac (30 μL), d) presence of compact barrier in the form of tear film, corneal and conjunctival epithelia that prevent the penetration and absorption into the intraocular region (Akhter et al., 2011). Development of nano-sized novel formulations is worthwhile in such cases of ophthalmic delivery as they are expected to prolong the pre-ocular retention and increase the ocular bioavailability.

Drug enclosed in the vesicles and oily nano-droplets allows for an improved partitioning and transport through the cornea. Moreover, vesicles offer a promising avenue to fulfil the need for an ophthalmic drug delivery system that has the convenience of a drop, but will localize and maintain drug activity at its site of action (Kaur et al., 2000; Sahoo et al., 2008). However, the untailored formulated nano-lipidic systems are normally negative and neutral charged that may only improve the ocular bioavailability to some extent.

Thus, an optimum delivery system should be the one which can be delivered in the form of eye drops, causing no blurred vision or irritability, and would need not more than one to two administrations per day. It is expected that positively charged nano-carriers may enhanced the drug corneal retention, permeation and subsequently the ocular bioavailability than the neutral and negatively charged systems due the result of interaction of positively charged vesicles with the polyanionic corneal and conjunctival surfaces due to presence of mucin (Fresta et al., 1999; Tian et al., 2012). So, on the basis of these concepts, we proposed novel Chitosan based GCV-
niosomal dispersion and mucoadhesive cyclosporine A nanoemulsion as ocular delivery systems for this study.

In the initial of the research after extensive literature review, we developed the analytical methodology for GCV and CYA for its quantification and pharmacokinetic study.

A rapid and sensitive UHPLC method for the determination of GCV in rabbit aqueous humor was developed and validated. Short analysis time and simple extraction provided a reliable method which would help to carry out GCV pharmacokinetics in rabbit aqueous humor.

To obtain the best chromatographic separation and sensitivity in a short time, different ratios of aqueous TFA and acetonitrile were systematically investigated. The best separation was achieved with a mixture of 0.1% w/v aqueous TFA and acetonitrile (95:5%, v/v) as the mobile phase. Protein precipitation with acetonitrile and direct injection into the chromatographic column enables high recovery of GCV. Using the chromatographic conditions described, rapid elution of GCV from aqueous humor was achieved at 0.928 min. The method was found to be selective, precise, accurate and robust. The method enabled the LOQ for GCV to be 10 ng/mL employing only 50 µL aqueous humor, which was more sensitive than the results from the previous studies. Moreover, the results of the stability studies showed that GCV was stable in aqueous humor at 20°C for 24 hrs, at 4°C for 5 days and at −20°C for 30 days. The percentage recovery of GCV was 95.8±2.2%, 96.7±2.9 and 98.4±3.1 at 20°C for 24 hrs, at 4°C for 5 days and at −20°C for 30 days, respectively. The freeze-thaw data indicated that three cycles can be tolerated without losses greater than 10% w/v.
CYA (immunosuppressing agent) with very poor therapeutic index and significant intra- and inter-individual variability in the pharmacokinetic characteristics, the therapeutic concentrations of this drug must be routinely monitored. Therapeutic drug monitoring of CYA is advocated because of its low solubility (6.6µg/ml), variable bioavailability, metabolism, and excretion among individuals (Mahalati et al., 1996; Cantarovich et al., 1999; Grevel et al., 1989). Despite routine monitoring, many patients experience adverse effects like nephrotoxicity, hepatotoxicity, neurotoxicity and gingival hyperplasia (Critical issues 1995; Schutz et al., 1998; Mahalati et al., 2000). HPLC is considered the method of choice for monitoring liver transplant recipients because of the accumulation of CYA metabolites in trough concentration blood samples (Oellerich et al., 1995). Despite these concerns, HPLC in its current form is incapable of providing routine service to a busy transplant center, and the situation is likely to become more difficult because recent clinical studies have suggested that measurement of CYA concentrations at single or multiple time points in the early period (0–6 h) after CYA administration might improve clinical outcome compared with traditional trough (pre-dose) measurements (Mahalati et al., 1999; Cantarovich et al., 1999; Grevel et al., 1989). So, first time we report a simple but very sensitive and efficient versatile bio-analytical method for the determination of Cyclosporine A in rabbit aqueous humor, cornea, conjunctiva and blood using UPLC method with Q-TOF-MS/MS detection.

For the bio-analytical methodology, mobile phase was optimized as Acetonitrile, Water and Formic Acid (0.1% v/v) in the ratio of 45:45:10 %v/v. Other solvents studied were ethyl acetate, methanol and water, but either the recoveries were low or interferences were observed.
The MS full scan spectra showed protonated precursor \([M+H]^+\) ions at 1225.1764 at the optimum collision energies of 13.0 eV. Capillary voltage of 3.2 KV was used for monitoring the precursor ions \((m/z \ 1113.0751)\). Quantification was done on the basis of main product ions.

The linearity of the detector response for CYA was evaluated by injecting a total of seven calibration (working) standard solutions (1–50 ng/mL for aqueous humor and conjunctiva, 5-50 ng/mL for plasma and 5-50 ng/mL for cornea covering the working range of the assay. The calibration curves were constructed by plotting peak area of CYA against corresponding concentrations. The correlation coefficient for the calibration regression line for aqueous humor, plasma, conjunctiva and cornea was found to be 0.9998, 0.9993, 0.9991 and 0.9996 respectively. The retention time of CYA was 2.01 min and no other interfering peak was observed either by matrix or by the formulation constituents, at the retention time of the drug, demonstrating method’s selectivity. LOD and LOQ were experimentally estimated by the analysis of aqueous humor, plasma, conjunctiva and cornea samples spiked with serially diluted CYA standard until the signal-to-noise ratio reached 3 and 10, respectively. LOD and LOQ were found to be 0.27 ng/mL & 1 ng/mL for Aqueous humor, 1.34ng/mL & 5.0 ng/mL for Plasma, 0.31 ng/g & 1.0 ng/g for Conjunctiva and 3.31 ng/g & 10 ng/g for Cornea respectively.

Precision and accuracy were determined by duplicate analysis of aqueous humor samples spiked with CYA at concentrations of 1.0 ng/mL & 25 ng/mL, plasma samples spiked with CYA at concentrations 5 ng/mL & 25 ng/mL, conjunctiva samples spiked with CYA at concentrations 1 ng/g & 25 ng/g and cornea samples spiked with CYA at concentrations 10 ng/g & 30 ng/g respectively followed by their comparison with the calibration curves prepared on the same day and on three
different days. Precision was expressed as the percentage coefficient of variation of measured concentrations for each calibration level, whereas accuracy was expressed as percent recovery (amount found/nominal amount×100) of drug added to the blank aqueous humor. Furthermore the low value of percentage RSD (< 1.74) obtained after making small changes in the developed method indicated the robustness of the method.

For the effective topical delivery of GCV, we develop the Chitosan coated biocompatible nano-sized niosomal dispersion loaded with GCV and evaluated the developed mucoadhesive nano-system for its controlled release and corneal permeation (in-vitro) and in-vivo performance evaluated in rabbit model for corneal retention by Gamma scientigraphy and ocular pharmacokinetic of the developed mucoadhesive Chitosan coated GCV niosomes.

For inducing the mucoadhesive characteristic to the niosomal formulation, chitosan concentration firstly optimized. Forces of detachment of the chitosan solutions were measured using a texture analyzer apparatus for evaluation of mucoadhesive strength. Force of detachment of CH was significantly increased with the increased concentration of CH (varied from 0.1% w/v to 1% w/v). Chitosan, being a cationic mucoadhesive biopolymer interacts with the negative charged mucin present over the cornea is responsible for the mucoadhesion of chitosan solution. 1% w/v of chitosan solution showing the strongest and desirable mucoadhesion (0.153N) among the test concentration which is significantly less than the normal blinking force required for the eyes. Therefore, 1% w/v of chitosan solution was further used to produce the mucoadhesive characteristic in the developed formulations for the further study.

For the composition optimization of the GCV-niosomal formulation, Box-Behnken statistical design with 3 factors, 3 levels and 15 runs was selected using the software
Design Expert® 8.0, a polynomial equation (quadratic model) was generated for experimental design the formulation.

\[ Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \]

Where, \( Y_i \) is the dependent variable; \( b_0 \) is the intercept; \( b_1 \) to \( b_{33} \) are the regression coefficients; \( X_1, X_2 \) and \( X_3 \) are the independent variables that were selected on the basis of pilot experiments.

Theoretically, experimental design consists of a set of points lying at the midpoint of each edge and the replicated center point of the multidimensional cube. 15 batches of niosomes prepared by both the surfactants i.e. Span60 (S60-01 to S-60-15) and Span40 (S40-01 to S40-15) were analyzed for particles size and put in the response column in the experimental design. A correlation between the different factors and formulation size is established using the quadratic polynomial generated using the Box-Behnken design with the help of Design Expert® 8.0 software. From the prepared runs we selected three formulations from both S40 (SN40-1, SN40-2SN40-3) and S60 (SN60-1, SN60-2 and SN60-3) were selected and coating was done with chitosan.

Overall the factors affecting the formulation development and characterization parameters of Span40 and Span60 based selected formulation are summarized as follow:

The particle size distribution of selected Span40, Span60-based niosomes and their chitosan coated form were found to be varied from 121.3nm-181.8nm (Span40-uncoated), 139.1nm-207.7nm (Span 60-uncoated) and 147.9nm-211.3nm (Span40-CH coated) and 166.7nm-237.9nm (Span 60-CH coated). Average particle size was found to be 121.3nm nm with PDI 0.114 for optimized formulation SN40-3.
Regarding PDI, a value of zero indicates an entirely mono-disperse population and a value of 1 indicates a completely poly-disperse population. Results of entrapment efficiency showed that the incorporation of Cholesterol into niosomes significantly increased the drug entrapment efficiency up to an optimum ratio of Span60: Cholesterol and Span40: Cholesterol. These results were supported by the fact that Cholesterol alters the fluidity of chains by reducing the transition of gel to liquid phase of surfactant bilayer and hence providing the transition state leading to the high drug entrapment \((\text{Uchegbu et al., 1995; Devaraj et al., 2002; Hao et al., 2002})\).

Moreover, it also increased the microviscosity of niosomal membrane conferring more rigidity, resulting in a higher stability which leads to the greater drug retention.

In addition, the length of the alkyl chain influences the HLB value of the surfactant mixture that directly affects the drug entrapment efficiency. The HLB values for Span40 and Span60 are 6.7 and 5, respectively, the lower value of HLB of both the surfactant are comparable enough that leads to the high drug entrapment \((\text{Guinedi et al., 2005})\).

In-vitro release study were carried out for the developed formulation, the release profile of GCV chitosan coated and uncoated niosomes showed that span 40 based niosomes have maximum GCV release in 24 h. As compared to span 60 based niosomal dispersion. Span 40 surfactant based niosomes have 67.12% (SN40-1), 79.35% (SN40-2) and 93.78% (SN40-3) of GCV release over the period of 24 h in sustained manner. In case of span 60 based formulations, maximum release was seen with SN60-3(85.69%). Chitosan coating over the same formulation increased the release of GCV as compared to the uncoated niosomes. The maximum release in case of SN40-3 after coated with chitosan was found to be 99.09% (CHSN40-3). Similarly in case of span 60 based niosomes, after coating it was found to be 90.59%. Result
shows that the increase of Cholesterol molar ratio significantly reduced the efflux of Ganciclovir, showing Cholesterol membrane stabilizing ability and space filling action (Raja Naresh el al., 1994, Namdeo and Jain, 1999). Furthermore, Cholesterol is known to increase the rigidity of niosomes and abolish the gel-to-liquid phase transition of niosomal systems resulting in niosome formulations that are less leaky (Cable, 1989; Alsarra et al., 2005) thus decreasing the drug release from niosomes. We tried to establish the correlation between the vesicular size, maximum percentage of drug release and nature of surfactant, its molar ratio with cholesterol.

It was found that niosomes prepared using Span60 were slightly larger in size (139.1 to 207.7) than those prepared using Span40. The result was in accordance with the previous finding (Manconi et al., 2005; Guinedi et al., 2005). The increased size of the developed niosomes may be due to the presence of Span60 which has a longer saturated alkyl chain as compared with Span40 (Manosroi et al., 2003). Furthermore, the larger vesicles are formed when the hydrophilic portion of the molecule is decreased relative to the hydrophobic portion (Uchegbu et al., 1997), it may also be attributed to the fact that the increase in alkyl chain length as the series is ascended from the C12 to the C18 ester would result in an increase in the value of the critical packing parameter (Uchegbu et al., 1995). Moreover, it was seems that the increase of Cholesterol ratio into niosomes significantly decreases the drug release. Cholesterol decreased the fluidity and diffusion of chains by reducing the transition of gel to liquid phase of surfactant bilayer so, this feature may consider as the factor responsible for release effect.

Further in-vitro transcorneal permeation study were performed for the developed formulations, the steady-state flux calculated for optimized niosomal formulation [SN40-09 (SN40-3)] was 14.7-fold higher than those for GCV solution (control).
Moreover, transcorneal flux were improved after the chitosan. The enhanced hydrophilic characteristic over the surface of lipophilic surfactant bilayer can be considered as reason for this effect. These results correlated well with the \textit{in vitro} release data suggesting that the corneal permeation process of GCV was dependent on its release characteristics. Similar results were obtained from literature using lipid based delivery systems such as liposomes and solid lipid nanoparticles (SLN) using excised rabbit cornea and bioengineered human cornea, respectively.

In-vivo study of the developed formulations was carried out in two segments. The precorneal clearance of the optimized GCV non mucoadhesive and CH-coated mucoadhesive niosomal formulation and control (GCV- sol) were monitored using γ-scientigraphy in rabbits. For the study, GCV niosomal dispersion and GCV solution was radiolabeled with radionuclide Tc-99m. GCV niosomal dispersion and GCV solution was instantaneously labeled with Tecnetium-99m with good labeling efficiency (≥ 95%) and less number of colloids (≤ 5%). Gamma scintigraphic dynamic images of whole-body of rabbit for first 30 minutes after administration of the formulations and the control and the radioactivity remaining in the ROI on dynamic images (radioactivity remaining Vs time profile) over period of 30 min are obtained. Percentage of radioactivity remaining at different time point, Log % activity remained, AUC of activity of niosomal and mucoadhesive niosomal dispersion as compared to the GCV solution are calculated. As expected, the drainage of the solution was fast, and it was detectable in the stomach and rectum of the rabbit at the end of the monitoring period. The drainage profiles observed for GCV-NDs were similar to the biphasic pattern of the solution, but with significantly less rapid initial clearance. The poor performance of the GCV-NDs during the drainage study could be attributed to lack of mucoadhesion with the cornea (Akhter \textit{et al.}, 2011).
However, unlike solution, radioactivity was not detectable in the stomach and rectum of the rabbit following topical administration of GCV-NDs. This could be explained if we corroborate our finding with earlier theory in support of the enhanced ocular bioavailability of topically applied drug by negative charged niosomal dispersion. This suggested that negatively charged niosomes could be more effective to improve permeation through the cornea than neutral ones, although the negatively charged nature of the epithelial corneal surface.

Additionally, this could be due to colloidal nature of the vesicles which slows down nasolacrimal drainage and most of radioactivity loss could be due to rapid tear turnover rate. In contrast, the GCV-MNDs system was retained on the ocular surface significantly longer (P<0.05) than solution and GCV-NDs. The AUC for the retained activity of GCV-MNDs over the ocular surface is 39505.5 as compare to the GCV-Sol which is only 10033.5. These findings suggest that the presence of chitosan in the GCV-MNDs system may serve to prolong the retention. A possible explanation for this could be the interactions of chitosan with the mucin of cornea. Another possible reason behind the longer retention of the GCV-MNDs system could be its positive zeta potential that could favour the attachment of the nano systems onto the negatively charged ocular surface. Moreover, uniform and spherical large surface area increased the spreading and contact time over corneal surface subsequently enhanced the corneal retention.

Finally, therapeutic application of the developed mucoadhesive niosomal formulation was demonstrated by comparing aqueous humor bioavailability of GCV following topical instillation of GCV solution, GCV-MNDs, and GCV-NDs to rabbit eye. In the group treated with the GCV solution, low ocular bioavailability ($AUC_{0-\infty, 692.0\pm26.2 \text{ ng.h/mL}}$) was observed and the aqueous humor levels of the drug were undetectable.
after 2 hrs, attributed to rapid pre-corneal loss. Topical instillation of GCV-MNDs and GCV-NDs provided ocular bioavailability of 19402.5±40.4 and 2763.9±46.1 ng.h/mL, respectively. The bioavailability of GCV-MNDs was approximately 28 fold greater than the GCV solution and nearly 7 fold higher than the GCV-NDs. As determined by UPLC method, GCV-MNDs provided significantly higher ocular bioavailability as compared to GCV-NDs and GCV solution. This could be attributed to (a) increased pre-corneal retention of the GCV -MNDs due to presence of chitosan that (b) sustained release of entrapped drug and (c) increased corneal penetration of nano-sized vesicles. Furthermore, presence of chitosan also favor in transcorneal permeation as chitosan is known to open tight cellular junctions. (d) GCV-NDs and GCV-MNDs follow all the possible permeation pathways wiz paracellular, transcellular and endocytosis of drug loaded vesicles.

For the effective management of immune disorder of eyes like dry eye disease, we developed thermodynamically stable Chitosan- based muco-adhesive nanoemulsion (nanocapsules) of Cyclosporine A (CYA- MNEs).

Being a moderately lipophilic drug, it was very important to find out an appropriate solvent to dissolve cyclosporine A, because only the dissolved state of drug in moderately lipophilic carrier facilitates the drug permeation (Akhter et al., 2008). In order to screen appropriate solvent/s for the preparation of NE, the solubility of cyclosporine A in various oils, surfactants and co-surfactants was measured. After performing solubility study in different oils, it was found that cyclosporine A exhibited maximum solubility in the oleic acid (21.79±0.93 mg/mL). Based on preliminary solubility studies, the surfactants; polysorbate 20 (Tween 20) (1.16±0.19 mg/mL), polysorbate 80 (Tween 80) (1.13±0.15 mg/mL), polyethoxylated castor oil (Cremophore EL) (1.50±0.41mg/mL) and caprylocaproyl macrogol- 8-glyceride
(Labrasol) (2.50±0.70 mg/mL) and co-surfactants; Transcutol P (Transcutol P) (2.73±0.41 mg/mL), PEG 200 (2.15±0.45 mg/mL), PEG 400 (2.68±0.39 mg/mL) and propylene glycol (1.30±0.40 mg/mL) showing comparable solubility of drug, were chosen for further optimization of NE formulations.

After studying the result, it was found that maximum nanoemulsion region or points were obtained with Transcutol P and hence Transcutol P was chosen as cosurfactant for nanoemulsion formulation. Finally, this is inference here that Tween 20 and Cremophore EL showed maximum formation of NE with Transcutol P as cosurfactant. So, for the preparation of nanoemulsion formulation oleic acid was selected as oil, Tween 20 and Cremophore EL as surfactants (to validate the effect of different Smix combination on phase behavior) and Transcutol P as cosurfactant. It was observed, when Tween 20 and Cremophore EL was used alone without Transcutol P (Smix ratio 1:0), very low amount of oleic acid could be solubilized at high concentration (>55% w/w) of polysorbate 20. This could be attributed to the fact that transient negative interfacial tension and fluid interfacial film is rarely achieved by the use of single surfactant, usually necessitating the addition of a co-surfactant (Lawrence et al., 2000, Akhter et al., 2008, Akhter et al., 2011). At equal amounts of surfactants and Transcutol P (Smix ratio 1:1), the NE region in the phase diagram increased significantly compared to that obtained at Smix ratio 1:0. The presence of Transcutol P (cosurfactant) decreases the bending stress of interface and makes the interfacial film sufficiently flexible to take up different curvatures required to form NE over a wide range of compositions (Kawakami et al., 2002). However, when concentration of Transcutol P with respect to surfactants was increased (Smix ratio 1:2 and 1:3) the NE area was decreased compared to Smix ratio 1:1. The decrease in the NE area is possibly due to presence of low
concentration of surfactant which reduces the amount of micelles and consequently decreases the solubilisation capacity of NE (Yuan et al., 2008). Moreover, NEs formed at Smix ratio 1:3 were unstable and showed phase separation within 24 h. More particularly, as compared to the Tween 20, Cremophore EL as surfactant showed lesser phase area at all the studied Smix. With further increase in Transcutol P concentration (Smix ratio 1:4), not a single NE point was found in both the cases. In contrast to this, when concentration of Transcutol P with respect to polysorbate 20 and Cremophore EL was decreased (Smix ratio 2:1), the NE area was increased compared to their respective Smix ratio 1:1. However, at further lower Transcutol P concentrations (Smix ratio 3:1 and 4:1), the NE area was decreased, it was because Transcutol P is a polar solvent with the tendency to highly incorporate into water, and the relatively lower Transcutol P content in the NE system decreases the hydrophilicity of the Smix, so the area of o/w NE was decreased.

In brief, Nanoemulsion system at Smix ratio 2:1 formed large isotropic NE region than the systems at other Smix ratios with both the surfactants. NEs formation behaviour of Tween 20 is significantly larger than the Cremophore EL with Transcutol P as cosurfactant. Moreover, at Smix ratio 2:1 and 3:1 of Cremophore EL with Transcutol P produces larger number of NEs gel form with is not suitable for the topical solution delivery in eyes. Such phase behaviour is commonly seen with polyethylene glycol ethers of castor oil like Cremophore EL (Akhter et al., 2008; Akhter et al., 2011). After extensively evaluating the phase behaviour of Tween 20 and Cremophore EL we concluded here that among the surfactant, Tween 20 is best for the development of nanoemulsion formulation with Transcutol P as the cosurfactant. So, for the further study, we selected nanoemulsion formulation from the different phase ratios of Tween 20 and Transcutol P. The selected components
for the nanoemulsion formulation development have appropriate features considering for the topical ocular drug delivery. Oleic acid (oil phase) is a well-known permeation enhancer, itself the integral part of corneal lipids and nontoxic and considered to be safe for ophthalmic drug delivery. Tween 20 being a nonionic surfactant and Transcutol P as cosurfactant are non-irritant and effective corneal permeation enhancers \((\text{Li et al., 2008; Liu et al., 2006})\).

Following criteria were chosen for the selection of formulations:

- The oil and Smix concentration should be such that it solubilises the single dose of the drug and give the final concentration of 3% w/v.
- Formulations with Smix ratio 1:3 and 4:1 were not selected because of the presence of Nanoemulsion is very less in number and such formulation required very high surfactant concentration (>50%). Nanoemulsion from phase diagram of Smix ratio 4:1 showed high viscosity as well as high surfactant were required to develop the nanoemulsion system.
- High concentration of surfactant which might cause ocular irritation. So, we restrict our formulation selection upto the Smix concentration of 35% from the selected phase diagram. Moreover at these Smix ratios the area of NE isotropic region was comparatively small.

Based on these set principal, four Smix ratios 1:0 (A1, A2), 1:1 (B1, B2), 1:2 (C1, C2), 2:1 (D1, D2) and 3:1 (E1, E2) were selected for the thermodynamic stability testing. All these selected formulations have equal Smix concentration i.e 30% and 35% for their first and second formulations respectively.

The selected formulations were studied for thermodynamic stability study, results showed that nanoemulsion of Smix ratio 1:0 (A1 and A2) does not tolerated any stage of stability testing. Absence of cosurfactant which is required to create a suitable HLB
value and stable flexible film for the stable NE system may be the rationale behind the instability of the NE of Smix ratio of 1:0 (V/V). The selected formulations were subjected to different thermodynamic stability by using heating cooling cycle, centrifugation and freeze thaw cycle stress tests. Those formulations, which survived thermodynamic stability tests, were taken for characterization with different physiochemical attributes.

The optimized chitosan concentration (1% w/v) of chitosan solution showing the strongest and desirable mucoadhesion (0.153N) among the test concentration (chapter 5.3) was selected for imparting the mucoadhesive characteristic in the optimized nanoemulsion (B1).

The developed formulations were characterized for different characterization parameters vis. Droplet size distribution, zeta potential and viscosity. Mean droplet size of formulation A1, A2 (A: 1:1), B1, B2 (B: 1:2) and C1, C2 (C: 2:1) were 36.10±1.97nm, 39.02±1.46nm, 18.92±1.03nm, 23.05±1.17nm, 42.30±3.30nm and 55.55±3.50nm respectively. Their zeta potential were varied from -14.3± 2.53mV – (-31.3±1.54) mV. The viscosities of the formulations are low as expected for the o/w type nanoemulsion. The viscosities of the formulations (A1-C2) were varied from 29.35±1.92mP - 132.67±3.54mP. The lowest viscosity was found for the formulation B1 (Smix; 1:2) which is 29.35±1.92mP.

The results of particle size analysis were in agreement with the droplet size measured by TEM photograph. In case mucoadhesive nanoemulsion (CH-B1), there were increased in the droplet size (41.70±1.15nm) and zeta potential was positive (+37.2±1.54mV). The value of zeta potential clearly favour here that the selected formulation and the mucoadhesive CH-B1 having good dispersion stability due to fair charge repulsion. It is hypothetically described that at the optimum Smix ratio (1:2 in
our study) the Transcutol P was exactly inserted into the cavities between the tween 20 molecules, causing the interfacial film to condense and stabilize, resulting in smallest droplet diameters (18.92±1.03) with lowest polydispersity value (0.125 ± 0.05). In addition, particle size analysis revealed that with increase in oil concentration the droplet size of NE increases, irrespective of the Smix ratio. Large droplet size of oil rich formulations could be attributed to the fact that the higher concentration of Smix is required to solubilise the oil phase in the aqueous phase resulting an increase in size (Chen et al., 2006). As a result of viscosity measurements, a similar behavior, as for droplet size was obtained. Viscosity of all the NE formulations was very low as expected for o/w emulsion. When formulations with different Smix ratios were compared, the minimum viscosity values were obtained for B1 formulations (29.35±1.92mP). The low viscosity may be due to the presence of low amount of tween 20 (a fatty acid polyhydric alcohol ester having high intrinsic viscosity) compared to Transcutol P (a short chain alcohol having low intrinsic viscosity) (Akhter et al., 2008). All the drug free or drug loaded NE formulations had pH values ranging from 6.7 to 6.81, favourable for topical ocular application. It was observed that incorporation of drug did not significantly affect the pH values of NEs. In the later part of the study, release profile of the selected NE formulations (A1-C2), mucoadhesive NE (CH-B1) and control formulations (Smix and oil). As expected for the NEs, fast drug release behaviour were observed due to the enhanced dissolution and spreading of the oil micellar solubilisation due to the optimized bend of surfactant and cosurfactant that developed the optimum HLB. Maximum percentage release of CYA after 12h was found to be highest (99.10±1.9nm) for B1 (Smix: 1:2). Moreover, chitosan coated formulation (CH-B1) also showed the similar release profile having the equivalent maximum percentage of
drug release (99.49±1.5). Although, all the formulations contain equal drug amount, low release rate as observed for controls, provided that concentration gradient is not a single factor affecting the rate of permeation.

- Enhanced drug release with NEs could be explained on basis of the several mechanisms:
  1) Continuous and spontaneous fluctuating stable interfaces of NE enable high drug mobility and might enhance the drug diffusion process.
  2) High solubilisation of CYA in NE resulting in high thermodynamic activity of the drug providing significant driving force for its release.

Adopting the same methodology as that of chapter 5, in-vivo study of the developed formulations was carried out in two segments. The precorneal clearance of the optimized CYA non mucoadhesive and CH-coated mucoadhesive nanoemulsion formulation were monitored using γ-sci:entigraphy in rabbits. Non mucoadhesive and CH-coated mucoadhesive nanoemulsion formulation serve here as control. CYA mucoadhesive NEs formulation (CH-B1) and non mucoadhesive formulation (B1) was radiolabeled with radionuclide Tc-99m. They were instantaneously labeled with Tecnetium-99m with good labeling efficiency (≥ 95%) and less number of colloids (≤ 5%). After administration of the radio labeled ophthalmic formulation, a good spreading was observed over the entire precorneal area.

For the dynamic imaging, percentage of radioactivity remaining at different time point, Log % activity remained, AUC of activity of CYA mucoadhesive NEs formulation (CH-B1) as compared to non mucoadhesive formulation (B1) were calculated. Results showed that the drainage of the CYA mucoadhesive NEs formulation was fast, and it was detectable in the stomach and rectum of the rabbit at the end of the monitoring period. The poor performance of the CYA NEs (B1) during the drainage study could be attributed to lack of mucoadhesion with the cornea. In contrast, the CYA mucoadhesive NEs formulation system (CH-B1) was
retained on the ocular surface significantly longer (P<0.05) than B1. The AUC for the 
retained activity of CYA mucoadhesive NEs formulation system (CH-B1) over the ocular 
surface is 69197.0 as compare to the NEs formulation system (CH-B1) which is only 
11833.5. These findings suggest that the presence of chitosan may serve to prolong the 
retention due to the charge interaction of chitosan (+ve) with mucin (-ve).

The developed nanoformulations were further evaluated in pharmacokinetic study for 
monetering of the CYA safe concentration and sustained CYA level in different 
structure of eyes for the effective therapeutic benefits. For this purpose, bio-
distribution study of CYA in cornea, conjunctiva, aqueous humor and blood were 
calculated.

The UPLC/Q-TOF-MS/MS method showed satisfactory determination of CsA in 
rabbit cornea, conjunctiva, plasma and aqueous humor for the pharmacokinetic study 
of CsA suspension (CsA-Sus), CsA nanoemulsion (B1) and mucoadhesive CsA 
nanoemulsions (CH-B1). Clinically pharmacokinetic study in ocular tissues is 
relevant as they might act as a reservoir for CsA.

Treatment with CH-B1 Showed highest concentration of drug over the period of 24 
hrs as compared to other formulations in cornea.

This result might be explained on the following basis:

- Nano droplets settle down to close contact with the cornea and a large amount of 
  inner oleic acid carrying drug might penetrate within the cornea.
- Permeation of drug carrying nano-sized droplets through cornea without NE fusion 
  and subsequent drug release as SUS here in the study showing insignificant corneal 
  deposition.
- Continuous and spontaneous fluctuating stable interfaces of NE enable high drug 
  mobility and might enhance the drug diffusion process.
High solubilisation of drug in NE resulting in high thermodynamic activity of the drug providing significant driving force for its release and permeation.

- Presence of chitosan enhances the corneal retention as well as helpful in opening of the cellular tight junction may also contribute as the driving force for permeation.

- CsA levels in the conjunctiva decreased much faster than in the cornea. Concentration of drug in conjunctiva was found to be highest for CH-B1 as compared to B1 and CsA-SUS in 24hrs of post instillation. Consequently, using the CsA-loaded CH-B1, therapeutic concentrations of CsA were maintained in the cornea and conjunctiva throughout the duration (24 hr) of the study. This suggests that these clinically relevant ocular tissues may act as a reservoir for CsA-loaded CH-B1 nanoemulsions.

The reason for this improved interaction of CH-B1 with the cornea and the conjunctiva could be found in the mucoadhesive properties of CH (Lehr et al., 1992). However, the limited effectiveness of the SUS and CYA-B1 as compared to the CH-B1 indicates that the fact that CH plays a key role in improving their interaction with the ocular surface. In fact, this facilitated interaction of the CH with the cornea and the conjunctiva has recently been visualized by confocal microscopy (Jain et al., 2011). Moreover, it is our hypothesis that it might not be the mucoadhesive character of CH molecules but the electrostatic interaction between the positively charged CH and the negatively charged corneal and conjunctival cells that is the major force responsible for the prolonged residence of CsA in these epithelia. The surface of cornea and conjunctiva is covered by a thin fluid layer called mucus film (Ludwig, 2005). The primary component of mucus is mucin, a high molecular mass glycoprotein which is negatively charged at physiological pH. Thus, the positively charged CH coating layer can provide a binding force to the eye surface. Nevertheless, the bioadhesion of chitosan is not exclusively determined by the positive charge. It could also be promoted by the presence of free amine and hydroxyl groups of...
chitosan molecules which form hydrogen bonds to the eye surface. This might explain that the CH could achieve a prolonged retention though its positive charge was limited at neutral pH.

Indeed, the systemic absorption of CsA cannot be discarded; however the blood levels observed for the three formulations tested are much below the described toxic levels (300 ng/ml; Bowers and Canafax, 1984). Moreover, the plasma concentrations were found to be least in case of CH-B1 as compared to B1 and CsA-sus during the study period.

Drug concentration in aqueous humor was also monitored. Result showed that only minimal intraocular concentrations were attained throughout the duration of the study and CsA levels in aqueous humour went down below the limit of detection at 24 h post-administration. The extremely low CsA levels at these inner ocular structures are justified by the limited intraocular penetration of free CsA, because of its retention in the corneal stroma (BenEzra and Maftzir, 1990). Nevertheless, these results also suggest that the Chitosan coated nanoemulsions do not enhance the penetration of CsA, which remains on the ocular surface. Indeed, the higher CsA levels in the aqueous humour, at 0.5 h post-administration, achieved for B1 should be attributed to lipophilic carrier of the formulation. Thereby confirming that chitosan favour the accumulation of CsA on the external tissues without compromising intraocular structures due to its mucoadhesive characteristics. Therefore, we can conclude that a selective and prolonged CsA delivery was achieved using CH-B1, without compromising inner ocular tissues and minimizing systemic drug absorption.