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Cyclosporine A (CsA) is a fat soluble cyclic undecapeptide antibiotic produced by the fungus Tolypocladium inflatum. Cyclosporine A is the only Cyclosporine analog to have been extensively used in the clinical practice. It is the prototype of a new generation of the immunosuppressive agent that selectively suppresses the activation of T lymphocytes, primarily by impairing the autocrine production of T lymphocytes growth factors.

Low oral bioavailability of systemically acting drugs is often associated with variable plasma concentrations and poorly controlled pharmacologic and toxic effects. In addition, incomplete oral bioavailability results in the wasting of much of an oral dose, and adds to the cost of drug therapy, especially when the active drug substance is expensive. Therefore, the maximization of oral bioavailability is a common goal during drug selection and development, and in clinical therapy. Cyclosporine belongs to Class IV category drug under BCS. Either low solubility or poor membrane permeation or presystemic metabolism could cause low oral bioavailability.

The CsA is known to be very poorly soluble in water and are also, known P-gp substrate, thus exhibit low therapeutic index with poor and variable bioavailability when administered orally. Furthermore, P-glycoprotein (P-gp), an ATP-dependent efflux transporter has been proposed as a factor in the poor absorption of CsA via various experiments, including single nucleotide polymorphism of P-gp. It is also the case that blood/blood-serum cyclosporine levels achieved using available dosage systems exhibit extreme variation between peak and trough levels. That is, for each patient, effective cyclosporine levels in the blood vary widely between administration of individual dosage forms. This variation in patient response has been found to be attributable to a significant extent to variation in the availability of naturally occurring surfactant components, e.g. bile acids and salts, within the gastro-intestinal tract of the subject treated.

In order to enhance bioavailability and to reduce inter-subject variability of cyclosporine by improving solubility, permeability and reducing the efflux transportation of cyclosporine from intestinal membrane, an attempt has been made to
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develop the surfactant based formulations for improved bioavailability of CsA. It is also anticipated that the application of nonionic surfactant in formulations would render CsA to get absorbed efficiently by overcoming various hindering factors as mentioned above since nonionic surfactants has ability to enhance intestinal permeation and to increase quantum of solubilized drug at absorption site. The preparations of CsA available in the market contains cremophor and other solvents which is reported to be potentially toxic and therefore it has been envisaged that having a formulation of CsA devoid of cremophor and solvents would be more desirable. It is also envisaged that the market formulations are not independent of bile flow and its absorption and bioavailability is affected by variability in bile flow within gastrointestinal tract. Nonionic surfactant based delivery systems are delivery systems, in which nonionic surfactants (spans and tweens) are used as an in-active ingredients, the basic reason for using nonionic surfactants in formulations is due to the fact that, they are safe, and inert in nature, and other inactive ingredients are selected based on physiological function of immune suppression carried out by CsA which is regulated by bile salt (sodiumdeoxycholate or sodium taurocholate) secretion and synthesis in the body.

The physical appearance and melting point of the CsA under investigation complied fully with the pharmacopeial specifications. CsA was received as white odorless powder with a bitter taste. Melting point of CsA was observed to be 149°C, which is in conformity with the reported range of 148°C-151°C.

Solubility of CsA in various solvents was carried out and was found to be in conformation with pharmacopoeial standards. Solubility of CsA, in distilled water was 9.41±0.21 µg/ml and in isotonic buffer (pH 7.4) was found to be 8.67±0.29µg/ml indicating that solubility of CsA is pH independent. CsA was found to be soluble in methanol, acetone and acetonitrile and solubility profile is presented in Table 8 CsA showed a solubility of greater than 300µg/ml in 30% PEG-200 Isotonic buffer pH 7.4. The octanol/water partition coefficient of CsA was found to be 129 complying with the reported value of 120. The CsA solution in PEG-200 solution of isotonic phosphate buffer pH 7.4 was scanned in the spectrophotometric range of 200-300 nm giving
maximum absorption at 211 nm, which is in agreement with the reported value of 210 nm. FTIR showed that there is no interaction between the drug and excipients used.

The HPLC method for estimation of CsA include mobile phase comprising TDW: ACN in the ratio of (55:45) and CN column maintained at 65°C using column oven and detection at 210nm. The calibration curves were linear in the range of (100ng-2000ng) in blood and solvent system acetonitrile (25ng-450ng). The reproducibility (precision) and accuracy of the method were determined by intra and inter assay variation reproducibility (precision) and accuracy of the developed method was within the acceptable limits and the same method is used for routine analysis of cyclosporine in formulations, for samples obtained from in-vitro release studies and in vivo studies.

The different vesicular systems (niosomal and bilosomal) were developed by using variety of surfactants and to prepare vesicles hand shaking method was employed as the method is simple, less tedious, easily possible in the laboratory facilities and less expensive. Span 60 shows the maximum entrapment efficiency of 97 ± 2.1% followed by Span 40 showing entrapment of 91.03 ± 1.8% and Span 80 showing entrapment of 75.02 ± 5.5%. This could be attributed to the structure, orientation and packing behavior of surfactant. The viscosity of various formulations (without addition of CsA) was recorded using (Bohlin Visco 88). It has been observed that there was gradual increase in viscosity of the formulations starting from Span 40 to Span 60 and when different molar ratios of spans: cholesterol: stearylamine are recorded for viscosity, it has been observed that there is consistent increase in viscosity, where there is increase in the molar ratio of span and decrease in the cholesterol quantity. The average vesicle size of optimized formulation was found in the range of 800nm and 2μm. When optimized NV2 formulation was viewed through cross polarizer clear birefringes could easily be seen which peculiar characteristics of liquid crystalline bilayers structure. Vesicles (niosomes) stability against solubilization by micelle forming detergents (such as bile salts) has been studied using turbidimetry method. In all studied formulation, transferring from phase I to phase II did not show the sharp increase in turbidity. The concentration of bile salt solution at the onset of phase II in the case of Span 80, Span 20, and Span 40 was 2.5, 10, and 15 mM, respectively. Span 60 niosomes did not display any abrupt
change in turbidity at the studied detergent concentrations; we postulated this is due to high gel to liquid crystal transition temperature and rigidity of Span 60 bilayers. It has been observed that on incorporation of bile salt as integral component upto 10mM, the turbidity was maximum (92%), on exposure of 15mM bile salt showing optimum stability. On the contrary, if we omit incorporation of bile salt in vesicles the turbidity was lowest (68%) which showed least stability.

The amount of CsA release from (NV2a, NV2b, NV2c, NV2 and BL was 49.13, 55.78, 57.11, 68.90 and 70.15% respectively after 24hrs. The optimized composition of surfactants (NV2a, NV2b, NV2c and NV2), were evaluated for in-vitro release studies, containing different compositions of Span 60, cholesterol and stearylamine. No significant difference in release profile was observed when sodium deoxycholate (SDC) was encapsulated as integral component. All the formulations were showing controlled release profile. When several models were used to fit for release study of CsA, it has been found that developed formulations found to be close to Higuchi model compared to other formulations having regression value >0.8 for optimized NV2 and BL formulations.

The proniosomes were prepared using sorbitol as a water soluble carrier upon which the mixture of surfactants (optimized concentration) was coated. The flow properties of proniosomes were found to be within limits. The results reveal that the angle of repose of the proniosomes (26.56°) was smaller than that of sorbitol powder (30.96°). As the angle of repose indicates the surface irregularity and roughness, the lower angle of repose of proniosomes confirms that the surfactant coating of sorbitol powder has been achieved, resulting in the smoothening of surface. The in-vitro release rate of CsA from (Proniosomes) niosomal dispersion and conventional niosomes was found to be similar in release rate. In case of niosomes and proniosomes a clear sustained release profile was achieved releasing about 30-40% in 6 hours. In both formulations the CsA release was evident even after 20 hours (55-64%) showing better performance of formulation. The stability studies selected niosomal optimized (NV2) and bilosomal (BL) formulations were carried out at 28 ± 2°C (room temperature) and 4 ± 2°C for 2 months. The formulations were found to be
stable at 4 ± 2°C for two months and significant changes were observed in entrapment efficiency, vesicle size and its structural properties.

In the preparation of microemulsion, surfactants namely labrasol, glyceryl monostereate, tween-80 and PEG-400 and co-surfactant namely plurol oleique were selected. Formation of microemulsion systems (the spotted area) was observed at room temperature. Phase behavior investigations of this system demonstrated the suitable approach to determine the water phase, oil phase, surfactant, and co-surfactant concentrations for which the transparent, one-phase, low-viscous systems form. Various microemulsion systems i.e. ME1, ME2, ME3, ME4, ME5 were prepared. The microemulsion (ME1) containing IPM, labrasol, plurol oleique was found to function as optimized composition. There was no change in the phase behavior and microemulsion area of phase diagrams when CsA 1mg/ml was incorporated in the formulations which were expected as the formation of stable microemulsion which is not affected by the pH and or ionic strength. The selected formulations were subjected to different thermodynamic stability by using heating cooling cycle, centrifugation and freeze thaw cycle stress tests. The formulation ME1a, ME1b, ME2a, ME3a and ME4a were found to pass the various stress conditions. Formulations in group ME1, ME2 and ME3 that passed dispersebility test grade A or B were taken for further study, as grade A and B formulations will remain as microemulsions when dispersed in GIT. The optimized microemulsion system was found to be stable in terms of globule size, drug content etc at room temperature for two months.

The applicability of various nonionic surfactant based formulations has been evaluated by first studying its absorption efficacy through intestinal membranes in-vitro. Various methods like everted gut sac technique, in-situ permeability and efflux studies have been carried out. In order to ascertain the effect of various segments of intestine, regional permeability study has been carried out. It has been observed that all nonionic surfactant based formulations (BL, ME and NV2), were effective in improving absorption compared to control (CsA dissolved in oil). However the BL formulation was found to be more effective than ME and NV2 formulations when studied using whole intestine through everted gut sac technique. Therefore the
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Improved absorption is due to contribution by whole intestine. The low absorption phenomenon could be due to variety of reasons i.e. low solubility, low permeability, metabolism or efflux through specific transporters like P-gp. Therefore permeability of CsA was studied in different segments of intestine like duodenum, jejunum, and ileum. Since it has been reported that majority of absorption inhibitory phenomena exist in these segments like, metabolism, P-gp efflux etc. The control formulation showed the time dependent reduction in apparent permeability coefficient (P_{app}), in all segments of intestine, while this effect was more pronounced in ileum region indicating the phenomena of efflux mediated by P-gp.

The apparent permeability coefficient (P_{app}) of BL formulation was found to be much higher at all time points compared to control. It was interesting to note that fraction of CsA was effluxed in BL formulation as well, but the degree of permeation was much higher indicating better absorption by BL formulation. The permeation profile of all the formulations was found in order of BL>ME>NV2>Control. It is evident that BL formulation is able to enhance permeation which could be due to reduction in efflux and improvement in solubility and permeability of CsA. These results are supported by (Nathalie et al, 1997), who have reported improvement in CsA absorption using taurodeoxycholate/monolein micellar solutions.

Although everted gut sac technique is simple and reliable, but to have more insight with regard to performance of formulation, in-situ studies was conducted using both whole and segmented intestines. Similar to previous results, a reduction in percent absorption was observed on increasing the residence time in case of control formulation indicating the efflux of drug into the lumen. The absorption of all formulations (BL, ME and NV2) was improved than control. However the absorption of CsA through BL formulation was found to be maximum. The mechanism of absorption was evident when in-situ regional permeability study was conducted. The absorption of CsA from control formulations was found to be maximum, in duodenum while it was lowest in ileum after 120min, indicating absorption of CsA in first phase, while efflux in the later phase.

Other nonionic surfactant based formulations (BL, NV2 and ME) were able to improve absorption, in the order of BL>ME>NV2. The data indicates that the
formulations are able to prevent P-gp mediated efflux and moreover formulations especially BL are contributing in improving solubility and permeability. As per reported literature various nonionic or anionic surfactants have been investigated as intestinal permeation enhancers.

It has been revealed from whole study that P-gp mediated efflux is a contributory factor in limiting bioavailability of CsA, a study was conducted to assess the changes in degree of efflux by different formulations. It has been observed that order of efflux in all formulations was in order of ileum>jejunum>duodenum. This data reflects that P-gp is playing its vital role in effluxing CsA back into GI lumen, but the formulations are able to push CsA from lumen to portal blood. The BL formulation was found to be highly effective in increasing absorption and thus it is apparent that the BL formulation is not inhibiting P-gp function, but rather rendering availability of larger fraction of solubilized drug at absorption site by which the degree of efflux would have been reduced.

These studies however do not ensure that, the formulation will exhibit improved oral bioavailability, since the effects of formulations are related to its concentration of excipients along with CsA at the site of absorption. Both CsA and formulation should be delivered to the absorption site simultaneously and a sufficient concentration of CsA must be achieved and maintained there. This could be subject to the influence of both formulations and physiological variables. Therefore any improvement in bioavailability could be assessed by investigating its efficacy in animals.

The immunosuppression studies has been carried out using two methods i.e. MTT assay and using tritiated thymidine. When studied using MTT assay the BL formulation was able to reduce T-cell proliferation by approximately 2.3 fold as compared to control. The NV2 and ME formulation showed 1.3 and 1.7 fold reduction in proliferation with respect to control. The reduction in proliferation by all formulation was in order of BL>ME>NV2>Control. Alternatively when this study was carried out using tritiated thymidine the BL formulation reduced T-cell proliferation to a degree of more than 7 fold with respect to control. The ME and NV2 formulation were also able to reduce T cell proliferation significantly which was
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found to be 4.6 and 3.2 fold as compared to control. It is evident from both the assays that, overall there a significant reduction in lymphocyte proliferation showed by all the formulations.

In vitro toxicity studies of all optimized formulations were carried by perfusing into intestinal membranes in terms of LDH release and Histopathological changes. LDH assay is related to measurement of one of the biochemical markers i.e. lactate dehydrogenase (LDH) present in cytoplasm of the cells which releases due to intestinal wall damage. When intestinal membranes are treated with plain saline buffer about 49% of LDH was detected in first 30 minutes which was reduced to 21% after 120 minutes compared to positive control. This data suggests that there may be continuous process of shedding off and revival of cells which could give rise to release of LDH to such an extent. When intestinal membranes were exposed to BL formulation the amount LDH release was 48% after 30 minutes which was reduced to 25% after 120 minutes. It is anticipated that this data do not signify toxicity as this may be related to intrinsic release of LDH. This study suggests that the components employed in the formulation are safer and none either of them is liable to cause the intestinal damage on oral administration and they are biocompatible with intestinal membranes. When studied histologically, no significant changes were observed in epithelial cells, brunners gland etc. The present work demonstrates that concentration of nonionic surfactant used in formulations did not cause much change in LDH release and the changes were minimal histologically.

When studies in animals the pattern of concentration–time curve of CsA from BL formulation was very similar to that of (marketed preparation) Neoral®, showing two maxima indicating that both of the formulations are undergoing enterohepatic circulation. The results suggested that there was no significant difference between the Tmax of BL formulation and Neoral i.e 3.5 hours in both cases while the Cmax of BL formulation is almost 1.9 times that of Neoral indicating enhanced absorption within same time. It is evident that highest relative bioavailability was achieved with BL formulation i.e. 173% compared to Neoral®. BL formulation increased Cmax and AUC0-24 to almost 1.9 and 1.7 times respectively compared to Neoral without impacting MRToral, suggesting an effect primarily during cyclosporine absorption.
The $C_{\text{max}}$ value obtained for BL and NV2 formulation was 5.48 and 4.67 $\mu$g/ml respectively while $\text{AUC}_{0-24}$ for BL and NV2 was found to be 72.8 and 24.03 $\mu$g.hr/ml respectively. The $C_{\text{max}}$, $T_{\text{max}}$ and $\text{AUC}_{0-24}$ of control formulation was found to be 2.17 $\mu$g/ml, 2.5h and 14.4 $\mu$g.h/ml respectively while for the BL formulation it was 5.48 $\mu$g/ml, 3.5h and 72.8 $\mu$g.h/ml respectively. The degree of enhancement in $\text{AUC}_{0-24}$ is more than five times for BL formulation with respect to control. This data is well correlated with absorptive $\text{Papp}$ obtained for BL formulation through in-vitro absorption studies. The order of $\text{AUC}_{0-24}$ among different formulations used was found in the order of Control < NV2 < Neoral < ME < BL. The $K_{\text{el}}$ of control, NV2, ME, BL and Neoral was observed to be 0.11, 0.217, 0.262, 0.189 and 0.071 h$^{-1}$ respectively. When compared to control formulation (in oil) the $C_{\text{max}}$ and $\text{AUC}_{0-24}$ value of NV2 formulation was two and 1.5 times respectively while there was no significant difference between MRT suggesting an effect primarily due to presence of surfactants and lipids in the vesicles. The $\text{AUC}_{0-24}$ of ME formulation was found to be 43.75 $\mu$g.h/ml which is almost three times compared to control while it is nearly equal to Neoral®. It is interesting to note that the trough concentration of CsA i.e at 24 hr with BL formulation was 1862 ng/ml while that of Neoral was 584 ng/ml which is almost three times compared to Neoral. These results indicate that absorption profile and pattern of microemulsion based formulation is entirely different from vesicular formulation.

In nutshell this work comprises the delivery CsA using various nonionic surfactant based formulations composed of pharmaceutically acceptable ingredients. The formulations NV2, ME and BL with these compositions resulted in improved bioavailability compared to control formulation. A bioavailability study in rats showed that ME formulation is equally effective with respect to marketed Neoral and exhibited similar bioavailability. However BL formulation is more effective in improving relative bioavailability to a degree of 173% with respect to marketed formulation.