With increasing life expectancy, many women will spend more than one-third of their life span in the postmenopausal state. Increasing awareness of the problems of postmenopausal osteoporosis and cardiovascular disease, together with recognition of the lessened risk of endometrial hyperplasia when sequential progestogen is coadministered with estrogen, has led to a reevaluation of the risk-benefit ratio of estrogen replacement therapy.

It has been suggested that the transdermal estradiol in combination with a sequential progestogen might also offer a better-tolerated means of contraception than oral estrogens.

Growing evidence demonstrates that the transdermal route offers several potential advantages over conventional routes for systemic medication e.g. avoidance of first pass elimination, extended duration of therapeutic activity with reduced side effects and patient compliance. Research has been persuaded to enhance the permeability of drug across the stratum corneum and to achieve higher systemic concentration of drugs.

Several types of transdermal therapeutic systems, which utilize the rate controlled drug delivery technologies to modulate the transdermal systemic delivery of therapeutic agent have been successfully developed and commercialized.

Although a few vesicular systems like liposomes and niosomes have been studied for contraception through transdermal routes, their unstable nature limits their use in TDDS. Therefore, in order to increase the stability of liposomes, concept of proliposomes has been proposed. In a similar approach some of the researchers studied niosomes, which exhibit superior stability and are free from other limitations of liposomes like fusion, drug leakage etc.

The literature survey revealed that no investigation had been reported to date on the use of proniosomes to achieve a combined delivery of estradiol plus levonorgestrel and ethinylestradiol plus levonorgestrel from the same unit of proniosomal transdermal therapeutic system.

This project was designed to investigate the possibility of manufacturing proniosomes as drug carriers for transdermal delivery of drugs. Therefore it was thought worthwhile to incorporate proniosomes in TDDS for contraception and hormone replacement therapy. This is possible because optical anisotropic proniosomes seems to convert into the niosomes in situ by absorbing water from the skin.
Estimation of drugs was done in-vitro by UV spectrophotometric method. The analysis of estradiol, ethinylestradiol, and levonorgestrel was performed individually and in combined formulations by multicomponent mode. The scanning of these drugs in 40% v/v PEG 200 solution showed the absorption maxima at 280, 281, and 247 nm respectively. The standard curves of the drugs for spectrophotometric estimation were prepared in a range of 1-10 µg/ml in 40% v/v PEG 200. For combined formulations of these drugs, two combination of standard solution were formed and multicomponent mode was followed for the analysis of drugs. The method was found suitable for determination of drug content in combined formulations containing 2-20 µg/ml concentration of each drug in 40% v/v PEG 200 solution. The amount found experimentally by the multicomponent mode was in the same range as was taken.

The interference of formulation additives in the estimation of drugs was also checked. The absorbance of drug solution was recorded in the presence of maximum concentration of additives used in formulations. No or negligible influence on the absorbance of drug solutions was observed at 280, 281 and 247nm for estradiol, ethinylestradiol and levonorgestrel respectively.

The drug samples were identified by the I. R. spectroscopy. The I. R. spectrum of estradiol, ethinylestradiol, and levonorgestrel were found to be similar to those reported in B P 1998 and confirmed the identity of these drugs.

In preformulation studies, the solubility determination of estradiol, ethinylestradiol and levonorgestrel was performed in common aqueous and organic solvents. Estradiol, ethinylestradiol, and levonorgestrel showed good solubility in alcohol, ether, PEG-200, acetonitrile and they were found to be sparingly soluble in chloroform and insoluble in water.

The partition coefficients of estradiol, ethinylestradiol, and levonorgestrel in n-octanol : water system were found to be 2.062, 1.074, and 0.893 respectively, which are indicative of lipophilic nature of drugs. The lipophilic nature was found in following order estradiol > ethinylestradiol >levonorgestrel.

The preliminary formulations of proniosomal gels were designed using combinations of spans only. These formulations showed maximum release rate upto 60-65%. Therefore in order to optimize the drug release, the proniosomal gel formulations were designed by using the combinations of span with tweens in addition to
span-span combination for achieving the desired transdermal flux of estradiol, ethinylestradiol and levonorgestrel.

Initially the proniosomal gel were developed with combinations of tweens with or without cholesterol as per method reported by Perrett et al. (1990). But these combinations fail to produce proniosomal gel. Freely soluble non-ionic surfactant such as tween can form the micelles on hydration and in the presence of cholesterol above 30% concentration of the selected ratio of tweens, it produced only liquid crystals either square shape or needle shape. This is because the vesicles cannot be formed by tweens only.

Proniosomal gels were prepared by drop wise addition of water to lipid dissolved in alcohol by heating. The addition of water resulted in the precipitation of lipid in the form of hydrated bilayers. Similarly proniosomal gels were also prepared by addition of water to warmed solution of surfactants (Span + Tween) in alcohol. An initial proniosomal gel was prepared consisting of lipid or surfactant: alcohol: water in the proportion of surfactant (300mg Span 40: 100 mg Tween20): alcohol (0.75 ml): water (9 drops). This proniosomal gel was used as a control formulation.

All these formulations were evaluated for their visual and microscopic appearance under cross polarizer microscope for optical anisotropic structure, and from amongst them optimized formulations were utilized for the characterization like consistency, crystal appearance, shape and size, encapsulation efficiency, release rate through cellophane membrane and rat skin. Niosomes derived from proniosomes were also characterized for their size and size distribution after hydration with or without agitation and with sonication. The niosomes were also evaluated for polydispersity index, and drug entrapment efficiency.

These formulations were developed with span 20 (3) with tween 20, 40, 60, 80 (1), span 40 (3) with tween 20, 40, 60, 80 (1), span 60 (3) with tween 20, 40, 60, 80 (1), span 80 (3) with tween 20, 40, 60, 80 (1), span 40 (3) with tween 20 (1) containing different alcohols, span 40 (3), tween 20 (1), and soya lecithin, egg lecithin, dicetylphosphate, cholesterol (1). All of these formulations were containing ethinylestradiol. The same formulations were developed with estradiol and levonorgestrel.

These proniosomal gels were chosen on the basis of (a) optical anisotropic structure observed under the cross polarizer equipped microscope and (b) formation of vesicles spontaneously upon hydration. More than 100 proniosomal formulations using
combinations of surfactants (Spans & Tweens) and alcohols were prepared in the same way. Out of these, three formulations prepared using the combination of Span 40 and tween 20, Span 40, tween 20 and lecithin, and Span 40, tween 20 with cholesterol were selected for further studies because they showed the uniform vesicle size with good entrapment efficiency.

Niosomes derived from proniosomes indicated that the process of dissolution may occur by progressive hydration of surfactant on the surface of proniosomes taking the form of niosomes as "budding off" from the outer surface of proniosomal gel. So long as the hydration was taking place, the formation of vesicles was continued. This may be due to the presence of tween with span that could produce the hydration environment for more water absorption. It seems likely that the comparatively uniform size of niosomes formed under this relative static condition may result in the absence of shear force normally present during conventional hydration procedure. The size of vesicles formed was larger in case of hydration without agitation while it was smaller in case of hydration with agitation. This may be attributed to breaking of vesicles due to shear force of agitation. The diameter of most niosomes appears to lay in the range of 1.52 μm to 15.44 μm; little variation of size was seen between the batches of niosomes of given composition. The diameter of niosomes in formulation of span40: span20 was in the range of 8.84 μm to 15.44 μm, and in formulations ST22, ST24, ST26, ST28 was 3.01 μm to 3.82, in ST42, ST44, ST46, ST48 was 5.44μm to 6.63, and in formulations ST62, ST64, ST66, ST68 it was 6.02μm to 6.84 μm, and in ST82, ST84, ST86, ST88 was 4.12 μm to 5.22 μm. The size of niosomes prepared using different lipids was in range of 4.33 μm to 7.99 μm. However the size of vesicles formed from proniosomal gel with cholesterol was uniform and very small. The polydispersity index was always found to be very low showing that this method of niosome formation results in vesicles of highly uniform in size. In the case of span-span combinations (HLB value 7.08 to 7.65) the vesicle size was larger (8.44 μm -15.44 μm) in comparison to formulation prepared using span tween combination (HLB value 8.75 to9.15). But span 40 + tween 20 produced medium size (5.44 μm to 6.63 μm) uniform vesicles. Therefore formulations containing span 40 with tweens were considered to be optimum because incorporation of tween had reduced the size of vesicles.
The phase transition temperature of span 40 is higher in comparison to span 20, which affects the permeability of bilayers. Therefore, as the proportion of span 40 was changed, the permeability also changed. Thus the size was found to decrease with increasing HLB value of surfactant mixture but drug entrapment efficiency also decreased in the same order.

In the case of different combinations of span and tween the size of vesicle was decreased with increasing HLB value. But the drug entrapment efficiency was more in case of span-span combination while it was less in case of combination of span with tween.

These results indicated that the drug entrapment (estradiol, ethinylestradiol and levonorgestrel) was more in the case of surfactants combination of low HLB value (hydrophobic) and decreased as the HLB value of surfactant combination was increased. This may be accounted for the hydrophobic nature of drug having more affinity towards a hydrophobic surfactant span.

The entrapment efficiency was higher in case of spans combination and overall entrapment efficiency was in decreasing order of SS42 > ST42 with lipid > ST22 > ST42 > ST 62 > ST82. (where S= span, T=tweens, 2 = 20, 4= 40, 6= 60, 8=80).

The microscopic study of niosomes revealed that the vesicle size was in decreasing order of SS42 > ST42 with lipid > ST62 > ST82 > ST 42 > ST22. This again may be attributed to hydrophilic nature of tweens forming small vesicles on hydration with water.

From different compositions of lipids, the vesicle size in niosomes was found to be in the increasing order of ST42CHL < ST42DCP < ST42 EL < ST42 SL. This may be attributed to the varying factors like change in solubility, effect of bilayer integrity and their intrinsic composition. In the case of egg and soya lecithin, a proper conclusion could not be made. The entrapment efficiency of niosomes prepared using lipids was found in the increasing order of ST42EL < ST42SL < ST42 DCP < ST42 CHL. This order reflected the rigidization effects of various additive lipids. The lipid causing the highest rigidization of bilayer i.e. cholesterol shows highest entrapment efficiency. The study of vesicle size and percent (%) drug entrapment of different proniosomal formulations revealed that in case of large vesicle percent (%) of drug entrapment was more as compared to small vesicles.
Vesicles formed from different alcohols were of different sizes. They followed the increasing order in size as Isopropyl alcohol < Absolute alcohol < Propanol < Butanol. Vesicles with isopropyl alcohol results in smallest size which may be due to branched chain present in it and larger size was found with other homologues alcohols.

From the different proniosomal formulations of ethinylestradiol, release rate across cellophane membrane was determined after optimization of formulation. Their permeation rate was also determined through rat skin using locally fabricated Keshary-chein type diffusion cell. The drug release rate was found to follow zero order. On the basis of these release rate observations, formulation ST42 (T1) was selected for further studies in order to see the effect of different amount of drugs, spans, phospholipids, alcohols and sonication time, on drug permeation profile studied. Similarly the release rate of estradiol and levonorgestrel for permeation was also performed in the same manner. After optimization of proniosomal formulation containing estradiol, ethinylestradiol, and levonorgestrel and permeation through cellophane membrane, two combined formulations S1 and T1 were developed and their permeation profile through rat skin was determined by using locally fabricated Keshary-chein type diffusion cell.

All these formulations showed linear correlation between spans and tweens in the proniosomal gel systems for transdermal flux. The transdermal flux pf proniosomal formulation was in following order for mixture of spans and tweens, i.e. ST22 > ST82 > ST42 > ST62. The reason for the increased drug release may be the increase in HLB value with the addition of tween 20, 40, 60, & 80. From all these formulations of span 20 with tween 20 and 80 were found to be in the liquid state while composition of span 40 & 60 with span 20 were in ordered gel state which delayed the drug release.

In order to see the effect of alcohols, the formulation ST42 was prepared with different alcohols and applied to treated cellophane membrane. The apparent steady state transdermal flux was highest for the formulation containing isopropyl alcohol. Inclusion of absolute alcohol, and propanol, and butanol into the formulation also led to the enhanced drug release but the effect was less than isopropyl alcohol. This may be due to the branched chain structure of isopropyl alcohol, which acts as a cosurfactant and might have reduced the bilayer packing with resultant increase in the flux value. The presence of tweens that can easily equilibrate with the alcohols for hydration also enhanced the transdermal flux value. The effect of alcohols on the release rate of drugs from plain
formulation (ST42) prepared with different alcohols was observed in the following decreasing order.

ST42 (Isopropyl alcohol) > ST42 (Absolute alcohol) > ST42 (Propanol) > ST42 (Butanol).

Effect of sonication time on the selected formulation (ST42) the flux was maximum after 120 seconds of ultrasonication. The flux was found to increase with the sonication time. This may be due to the membrane disruption reducing vesicle size and energy mediated mixing of vesicle compositions and skin lipids.

In case of proniosomes prepared using mixture of spans the release rate was found to be in increasing order if the amount of span20 was increased SS24 (ratio 1:1) < SS24 (ratio 2:1) < SS24 (ratio 3:1) < SS24 (ratio 4:1) and on increasing the ratio of span 40 the release rate was found in the order of SS42 (ratio 1:1) > SS42 (ratio 2:1) > SS42 (ratio 3:1) > SS42 (ratio 4:1). Among all the combinations of spans; span 40 & tweens the release rate was found to be in the following decreasing order in case of T20, 40, 60, 80 with span 20 > T20, 40, 60, 80 with span 80 > T20, 40, 60, 80 with span 40 > T20, 40, 60, 80 with span 60. This was in accordance with the phase transition temperature of the surfactant mixtures. The effect of different spans on the release rate showed that the maximum flux was in the case of span 20 and span 80 because span 20 and 80 has low phase transition temperature. The minimum flux was obtained in the case of span 40 and span 60. Further the slow release may be due to the reduction in surface free energy that caused the formation of larger size vesicles resulting in smaller surface area exposed to receptor medium and membrane or skin. The low phase transition temperature of span 20 combinations allowed the faster release than span 80 combinations.

All the formulations containing span and tween combinations released significantly less estradiol, ethinylestradiol than phospholipid formulation. The release rate was in the following decreasing order of:

ST42SL > ST42DCP > ST42 EL > STCHL > ST24.

In the case of formulation with varying composition of lipids the drug release was maximum in soya lecithin. The dicetylphosphate formulation showed intermediate release because its vesicle are charged which are responsible for increasing the curvature and decreasing the size of vesicle with the increase in surface area. Both the commercial soya lecithin and egg lecithin were effective penetration enhancers. The drug release rate was more in soya lecithin formulation than egg lecithin. Higher skin permeability may be due to an increase in partition coefficient between vehicle and skin or direct effect of
lecithin on the skin thereby reducing the skin resistance to permeation of the drugs. Both soya and egg lecithin contain phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and polyunsaturated acids including linoleic acid and linolenic acid. These polyunsaturated fatty acids are relatively more in soya lecithin that may be the reason for faster release rate than egg lecithin. The cholesterol increases the rigidity of bilayers hence slowest release rate was observed in formulations containing cholesterol.

On the basis of these release rate studies, formulation ST42 was found to exhibit optimum drug release and permeation through the membrane. The formulation containing estradiol, ethinylestradiol, and levonorgestrel were prepared separately with or without tweens and lipids and evaluated for drug release studies. The effect of soya lecithin, egg lecithin, dicetylphosphate and cholesterol on the drug release rate from the formulations was also studied.

After optimization of these formulations containing single drug, two formulations of span: span combination (span 40 and span 20) were prepared containing drug combination i.e. estradiol with levonorgestrel (1mg+1mg) and ethinylestradiol with levonorgestrel (1mg+1mg) and coded as S-1 and S-2. The formulations of span 40 with tween 20 were prepared containing same drug combinations and coded as T-1 and T-2. The release rate was found higher in the case of T-1 and T-2. This may be due to the higher transdermal flux of span and tweens formulation in comparison to spans combination only. This may be due to the better hydration of proniosomal gel with resultant smaller vesicles in presence of tween.

Stability study was done on proniosomal gel formulations SS 42 (S1) and ST 42 (T1) and ST42 with cholesterol (T_CH). These formulations were stored at 4°C, 25±2°C and 40±2°C in clean glass vials. Prior to hydration, the appearance of crystals and size were determined by observing under microscope. Drug crystals were not seen after 12 weeks in case of formulation SS 42 (S1) and ST 42 (T) while in case of ST42 with cholesterol (T_CH), crystals were not observed even after 16 weeks. The consistency of proniosomal gel SS42 (S1) was increased after 12 weeks storage and in case of ST42 CHL (T1_CH) consistency was increased after 16 weeks. There was no significant difference in the optical anisotropic structure of proniosomal gel formulation even after 16 weeks of storage time.
The polydispersity analysis of formulations SS42 (S1), ST42 (T1), and ST42_{CHL} (T_{CH}) was performed and niosomes were counted. The results of polydispersity index showed that the vesicle size increased and polydispersity index decreased.

*In vivo* performance of optimized proniosomal transdermal gel system was studied. The estrogenic activity was determined in terms of their effect on uterine weight, vaginal opening and cornification. Formulation T1_{(17\beta)} and T1_{(EEE)} was applied on the skin of dorsal side of the animal. In the smear of immature rats, the epithelial cells were comparatively bigger in the case of T1_{(EEE)} than T1_{(17\beta)} in comparison to control animals. After four-day application of patch, only keratinized cells without any nucleus were seen. This was clear indication that ethinylestradiol has produced full cornification in comparison to estradiol. The other formulations containing levonorgestrel, T1_{(LN)} and combined formulation of ethinylestradiol with levonorgestrel, T1_{(EEE + LN)} & estradiol with levonorgestrel T1_{(17\beta + LN)} showed mixture of leukocytes and epithelial cells. The histological studies showed that the endometrial mucosal thickness in control animals and those treated with proniosomal gel T1_{(Cont)}, T1_{(17\beta)}, T1_{(EEE)}, T1_{(LN)} and combined formulation T1_{(EEE + LN)} & T1_{(17\beta + LN)} were 16.61 \mu m, 52.62 \mu m, 58.42 \mu m, 64.52 \mu m, 70.12 \mu m and 76.32 \mu m respectively. This may be due to the stimulation of protein synthesis by these hormonal drug formulation.

The contraception studies were performed for their endometrium thickness, ovulation point and formation of corpora lutea. The percent inhibition of corpora lutea with proniosomal gel T1_{(17\beta)}, T1_{(EEE)} & T1_{(LN)} and combined formulation T1_{(EEE+LN)} & T1_{(17\beta + LN)} was 41.75\%, 49.23\%, 52.14, 89.41\% and 94.74\%, and number of ovulation point were less in both combined formulations.

In the plasma lipid studies of various groups of animals treated with transdermal proniosomal gels containing, only estradiol, levonorgestrel, ethinylestradiol, and two combined formulations containing estradiol + levonorgestrel, and ethinylestradiol + levonorgestrel produced favorable changes in lipid components of plasma in rats.

The plasma cholesterol level increased in the animals group which was treated with proniosomal gel containing levonorgestrel but decreased with proniosomal gel containing estradiol and estradiol + levonorgestrel. The lower value was found in the case of proniosomal gel containing ethinylestradiol and ethinylestradiol+ levonorgestrel. The
overall effect of drugs used in formulations on the cholesterol level was observed in decreasing order:

\[ T_1 \text{(LN)} > T_1 \text{(17\beta+LN)} > T_1 \text{(Cont)} > T_1 \text{(17\beta)} > T_1 \text{(EEE+LN)} > T_1 \text{(EEE)}. \]

The increased level of triglycerides has been identified as a risk factor for atherosclerotic disease and its management is also important in the hyperlipidaemia. In this study the plasma triglycerides level in comparison to control group decreased in all formulations in following order:

\[ T_1 \text{(Cont)} > T_1 \text{(17\beta+LN)} > T_1 \text{(EEE+LN)} > T_1 \text{(LN)} > T_1 \text{(EEE)} > T_1 \text{(17\beta)}. \]

The plasma HDL-cholesterol level was decreased in the animal group that was treated with proniosomal gel containing ethinylestradiol with or without levonorgestrel. The plain levonorgestrel proniosomal gel had shown increasing effect on HDL-cholesterol level. The effect of proniosomal formulations on HDL cholesterol level was found in decreasing order as follows:

\[ T_1 \text{(LN)} > T_1 \text{(17\beta+LN)} > T_1 \text{(17\beta)} > T_1 \text{(Cont)} > T_1 \text{(EEE)} > T_1 \text{(EEE+LN)} \]

The lipoprotein subfractions i.e. plasma VLDL decreased with all the formulations of proniosomal gels containing estradiol, ethinylestradiol, levonorgestrel and their combinations. The VLDL level was found in decreasing order:

\[ T_1 \text{(Cont)} > T_1 \text{(17\beta+LN)} > T_1 \text{(EEE+LN)} > T_1 \text{(LN)} > T_1 \text{(EEE)} > T_1 \text{(17\beta)} \]

The LDL level was increased in all the treated groups of animals except estradiol /ethinylestradiol formulations in comparison to control group. The influence of different formulations was found in decreasing order as follows:

\[ T_1 \text{(LN)} > T_1 \text{(17\beta+LN)} > T_1 \text{(Cont)} > T_1 \text{(EEE+LN)} > T_1 \text{(17\beta)} > T_1 \text{(EEE)} \]

The effect of oral administration of hormonal drugs leads to various problem like endometrial cancer, breast cancer, thromboembolic disorder, hypertension. The proniosomal transdermal drug delivery thus offers a convenient and well-tolerated form of estrogen replacement therapy in post menopausal women seems to provide a week protective effect against cardiovascular disease; this effect had been associated with a decrease of serum low-density lipoprotein cholesterol.

In conclusion, the result reported here indicates that proniosomes are very promising means as drug carriers. The essential features of proniosomes are their ability to rearrange on dilution to form a stable niosome suspension, which allow faster permeation through the lipid/surfactant bilayers formed by this technique. Drugs from
proniosomes seem to pass through the skin with comparatively faster rate than free drug. The experimental results and supportive theoretical analysis suggest that either direct transfer of drug(s) from vesicles to the skin or the penetration enhancer effect of non-ionic surfactant may contribute to the faster permeation of drugs from proniosomal formulations. Thus proniosomal gel appears to efficiently deliver drugs by the transdermal route as observed in this study. A high proportional of span 40 with tweens (20, 40, 60, 80) was needed in the formulations to enhance the transdermal flux of drugs. While no significant difference was observed on comparing the transdermal flux of different formulations of span 40 with tweens. The proniosomal gels developed with cholesterol, soya lecithin, egg lecithin, and dicetylphosphate showed higher drug entrapment and release rate except with cholesterol. These findings suggest that inclusion of surfactants and lecithin in niosome vesicle derived from proniosomes may play a significant role in drug permeation than inclusion of cholesterol alone. Proniosomes may become a useful dosage form for estradiol, ethinylestradiol and levonorgestrel, specifically due to their simple, scaling-up production procedure and ability to modulate drug transfer across skin.

In this study the proniosomal transdermal replacement therapy reduced total cholesterol and triglycerides, and VLDL while HDL-cholesterol level was increased with proniosomal gel containing levonorgestrel and estradiol with or without levonorgestrel. The level of LDL was also reduced with proniosomal gel containing estradiol, and ethinylestradiol but increased with levonorgestrel. A negligible change was observed with proniosomal formulation containing estradiol/ethinylestradiol with levonorgestrel. All these results clearly indicate that the levonorgestrel alone has increasing effects on lipid profile of the treated animals and its combination with estradiol also has the similar effect but to a lesser extent. However the combination of levonorgestrel with ethinylestradiol had shown good results on the lipid profile of treated animals. Therefore, it proved to be a better combination for both contraception as well as for hormone replacement therapy when given as proniosomal gel formulation through the transdermal route. In human females, the cardiovascular problems will not occur on using proniosomal gels of estradiol, ethinylestradiol with levonorgestrel for contraception as well as for hormone replacement therapy, which are encountered on oral treatment.