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Provesicular Transdermal Drug Delivery System of Ethinylestradiol and Levonorgestrel for Contraception and Hormone Replacement Therapy

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Transdermal drug delivery of ethinylestradiol and levonorgestrel for contraception and hormone replacement therapy was attempted from the proniosomal gel formulations prepared by coacervation phase separation technique. The proniosomes were hydrated with saline solution to form niosomes. Proniosomal gels were prepared from nonionic surfactants e.g. Span 20, 40, 60 and 80 with egg lecithin, Brij 58, dicetyl phosphate, soya lecithin and cholesterol. These gels were characterized and evaluated for particle size distribution, spontaneity, entrapment efficiency and stability. *In vitro* drug diffusion was studied through cellophane membrane and rat skin. The estrogenic activity in immature albino rats was also studied. The formulations prepared using Span 20 and Span 40 (3:1) have shown better *in vitro* and *in vivo* performance. This study indicates that proniosomal gel preparation of contraceptive hormones may be used as an effective means for population control programme.

General means of contraception used by women include pills, injections (depot), intrauterine devices (IUD), intra vaginal rings (IVR), subdermal implants (Norplant), nasal spray, vaginal jellies, foaming tablets and diaphragms, and those used by men include condoms and coitus interuptus, while sterilization can be done in either of two[1]. The significant failure rates related to user compliance and associated side effects are major limitation in successful use of above means[2]. 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[3], their unstable nature limits their use in transdermal drug delivery system (TDDS). In order to increase the stability of niosomes, concept of proniosomes has been proposed. These exhibit superior stability and are free from other limitations of liposomes and niosomes like fusion and drug leakage. Optical anisotropic proniosomes seems to convert into the niosomes *in situ* by absorbing water from the skin.

Ethinylestradiol is frequently used in menopausal symptoms, female hypogonadism, and treatment of prostate cancer, as the estrogenic component of combined oral contraceptive preparation and with progesterone for menstrual disorders[4]. The daily dose of ethinylestradiol is 20 μg to 50 μg[5]. Levonorgestrel is a potent progestogen and has been widely prescribed as a contraceptive steroid for female fertility regulation. But when progestin alone was used as a contraceptive, it produced various untoward effects like episodes of irregular and unpredictable spotting. These most frequently encountered side effects were the major reasons why women discontinued its use[6]. The daily dose of levonorgestrel is 30 to 37.5 μg per day[4]. Progestin alone is less efficacious than combination oral contraceptives[6]. Therefore, for the present study, combination of ethinylestradiol and levonorgestrel was used to make an effective contraceptive formulation. The use of proniosome to achieve a combined delivery of levonorgestrel and ethinylestradiol from the same unit of a provesicular transdermal therapeutic system has been attempted.

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MATERIALS AND METHODS

Ethinylestradiol and levonorgestrel were procured as gift samples from Wyeth Laboratories Ltd., Mumbai. Span 20, 40, 60 and 80 were purchased from Koch Light Laboratories Ltd., Colnbrook Bucks, England. Soya lecithin, egg lecithin and dicetyl phosphate were procured from Sigma Chemical Co. St. Louis, Mo, USA and cholesterol was obtained from Central Drug House Pvt. Ltd., Mumbai. All other chemicals and reagents used were of analytical grade. Due permission was obtained from Institutional Animal Ethics Committee for conduct of animal experiments reported under *in vivo* studies in this paper.

Preparation of proniosomes:

Proniosomes were prepared using the reported methods of Perret *et al*, 1991 and Ishii *et al*, 1995 with slight modifications[7,8]. Precise quantity of surfactants in different ratio, alcohol 1 ml and 10mg of drug were taken in a clean and dry wide mouth glass test tube. After warming, all the ingredients were mixed well with a glass rod and the open end of each glass tube was covered with a lid to prevent loss of solvent and warmed over a water bath at a 60±2° till the surfactant mixture dissolved completely. Aqueous phase containing glycerol solution (0.1% w/v) was added to it and warmed on water bath till clear solution was formed, which on cooling converted into proniosomal gel. The gel so obtained was preserved in the same glass tube in dark for characterization. Different proniosomal formulations were prepared using various sorbitan esters like Span 20 (sorbitan mono laurate), Span 40 (sorbitan mono palmitate), Span 60 (sorbitan mono stearate) and Span 80 (sorbitan mono oleate) with Brij 58 (polyoxyethylene 20 cetyl ether), egg lecithin, soya lecithin, dicetyl phosphate, cholesterol, isopropyl alcohol, butanol, propanol and absolute alcohol (Table 1). All these formulations were evaluated for their visual and microscopic appearance and drug loading by phase contrast and cross polarizer.

Size distribution analysis:

The proniosomal gel was hydrated using saline solution (0.154 M NaCl)[9] with occasional shaking for 15 min. The dispersion was observed under optical microscope at 100X magnification. Size and size distribution of 300 vesicles were noted using calibrated ocular micrometer. After hydration without agitation and with agitation in a cavity slide, the size was noted for niosomes formed spontaneously from proniosomal gel.

Spontaneity of niosomes formation:

Spontaneity can be defined as a number of niosomes formed after hydration of proniosomes for 15-20 min[10]. Definite amount (10 or 20 mg) of proniosomal gel was hydrated

TABLE 1: FORMULATION CODES AND COMPOSITION.

Name assigned	Surfactants	Ratio	Alcohols
SKR_1	$S_{20}+S_{40}$	1:1	IPA
SKR_2	$S_{20}+S_{40}$	2:1	IPA
SKR_3	$S_{20}+S_{40}$	3:1	IPA
SKR_4	$S_{20}+S_{40}$	4:1	IPA
SKR_3B	$S_{20}+S_{40}$	3:1	AA
SKR_3P	$S_{20}+S_{40}$	3:1	IPA
SKR_3I	$S_{20}+S_{40}$	3:1	P
SKR_3A	$S_{20}+S_{40}$	3:1	B
SKS_1	$S_{20}+S_{60}$	3:1	IPA
SKS_2	$S_{20}+S_{60}$	3:1	IPA
SKS_3	$S_{80}+S_{40}$	3:1	IPA
SKS_4	$S_{80}+S_{60}$	3:1	IPA
SKM_1	$S_{20}+S_{40}$ + Brij 58	3:1	IPA
SKM_2	$S_{20}+S_{40}$ + DCP	3:1:1	IPA
SKM_3	$S_{20}+S_{40}$ + SL	3:1:1	IPA
SKM_4	$S_{20}+S_{40}$ + EL	3:1:I	IPA
SKM_5	$S_{20}+S_{40}$ + CHL	3:1:1	IPA
LP	$S_{20}+S_{40}$ + LN	3:1	IPA
LSL	$S_{20}+S_{40}$ + SL + LN	3:1	IPA
LDCP	$S_{20}+S_{40}$ + DCP + LN	3:1	IPA
LEL	$S_{20}+S_{40}$ + EL + LN	3:1	IPA
COM1	$S_{20}+S_{40}$ + EE + LN	3:1	IPA
COM2	$S_{20}+S_{40}$ + SL + EE + LN	3:1:1	IPA

IPA: Isopropyl alcohol, AA: Absolute alcohol, B: Butanol, P: Propanol, EL: Egg Lecithin, SL: Soya lecithin, DCP: Dicetyl Phosphate, CHL: Cholesterol, EE: Ethinylestradiol. LN: Levonorgestrel, S: Span (Sorbitan esters), Brij 58: polyoxyethylene 20 cetyl ether.

using saline solution (0.154 M NaCl)[9] and allowed to stand for about 20 min. Then a drop of solution was placed on Neubaurs chamber (Fein-Optik, Germany) to count the number of vesicles.

Drug entrapment efficiency:

An accurately weighed quantity of proniosomal gel (100 mg) was dispersed into the saline solution and warmed slightly to form the niosomes. The niosomal dispersion was placed into cellophane dialysis tubing (Sigma Chemical Co. St. Louis, Mo, USA) and dialyzed exhaustively against 400 ml saline solution (0.154 M NaCl)[9] thrice at 4° for 24 h. After dialysis, the vesicles were resuspended in 30% v/v PEG 200 (in saline solution) and to it 1 ml of 0.1 % v/v Triton-X 100 solution was added to disrupt the vesicles. The resulting solution was filtered and analyzed spectrophotometrically at 281 nm and 247 nm for ethinylestradiol and levonorgestrel respectively against 30% v/v PEG-200 as blank. In this multicomponent mode of analysis no interference was encountered with common excipients i.e. span 20, 40, 60, 80, soya lecithin, egg lecithin, dicetyl phosphate, cholesterol, glycerol and alcohols like isopropyl alcohol, butanol, propanol and absolute alcohol. The entrapment efficiency was expressed as percent of total drug entrapped in vesicles.

Fabrication of transdermal patch:

The circular ring of flexible fiber (thickness 0.9 mm, outer diameter 1.35 cm and inner diameter 1.25 cm) was stuck on the adhesive tape. A circular aluminum foil of diameter 1.50 cm was used as backing membrane and kept on the ring with the little pressure to prepare reservoir cavity. The inner diameter of cavity was 1.20 cm (corresponding to 1.13 cm^2 area). The proniosomal gel was evenly spread into this reservoir cavity and covered with a thin nylon mesh (Johnson & Johnson, Mumbai). Finally round shaped patch was stuck on a polythene foil, which was to be removed before use. The transdermal patches of ethinyestradiol alone as well as ethinyestradiol with levonorgestrel in combined formulation were prepared and stored in airtight container for further study.

Drug diffusion studies through cellophane membrane:

A locally fabricated and calibrated Keshary-Chien type permeation system was used for *in vitro* permeation studies of levonorgestrel and ethinylestradiol. Freshly treated cellophane membrane was mounted between the half-cell of Keshary-Chien permeation cell[11]. The donar half cell which faced the upper surface of patch containing proniosomal formulation with 10 mg drug was adhered to the outer side of the cellophane membrane, while the receptor half cell, which faced the lower side, had a solution containing 30% v/v PEG 200 (in saline solution) to maintain the sink condition[12,13]. The receptor compartment was surrounded by a water jacket for maintaining the temperature 37±2° for 24 h, using a thermostatically controlled magnetic stirrer. The top of the donor compartment was open for air circulation. The capacity of receptor compartment was 10 ml and area of donor compartment was 1.31 cm^2.

At predetermined time interval of 45 min, 5 ml sample was taken from receptor compartment, which was replaced with the same volume of 30% v/v PEG 200 (in saline solution). Samples so collected were analyzed spectrophotometrically at 281 nm for ethinylestradiol and at 247 nm for levonorgestrel against respective blank. The amount of drug diffused through the membrane at various time intervals was calculated. The samples for drug diffusion from combined formulation patches were also analyzed by multicomponent mode in the same manner. The steady state transdermal flux, permeability coefficient, slope of straight line, regression coefficient for the transport of ethinylestradiol and levonorgestrel were determined (Table 1).

Drug diffusion studies through rat skin:

The drug diffusion study was performed using fresh and shaved skin of female Sprauge-Dawley rats weighing 200±10 g[13]. Locally fabricated Keshary-Chien permeation cell was used. The patch containing proniosomal formulation with 1.0 mg drug was adhered to the skin. This patch applied on skin was mounted and clamped between the donar and receptor compartment with outer (epidermal) side facing upward (donar side). Samples (5 ml) were withdrawn from sampling port at intervals of 1 h and were replaced with same volume of the fresh receptor fluid every time. The samples so withdrawn were analyzed spectrophotometrically against respective blank as described earlier.

Stability studies:

The selected proniosomal formulations were stored at 4±2° and 27±2° for 12 w. After 3, 6, 9 and 12 w, they were observed visually and under optical microscope for the change in consistency, optical anisotropic structure and appearance of drug crystals upon storage. Niosomes formed from proniosomes were characterized after 3, 6, 9 and 12 w storage for size and size distribution after hydration with agitation and without agitation[14].

***In vivo* studies:**

The method reported by Hebborn[15] was used to per-

TABLE 2: EFFECT OF DIFFERENT PERMEATION PARAMETERS ON DRUG PERMEATION THROUGH CELLOPHANE MEMBRANE.

Name assigned	Steady state Transdermal flux[a] ($\mu g/cm^2$). h	Permeability Coefficient[b] ($\mu g.cm/h$)	Percentage Release[c]
Formulation with different ratio of span 20 and span 40			
SKR_1	6.23±0.25	$1.17x10^{-2}$	19.84±9.9
SKR_2	8.94±0.22	$2.46x10^{-2}$	28.48±10.1
SKR_3	13.43±0.17	$3.69x10^{-2}$	42.69±5.6
SKR_4	14.67±0.15	$4.04x10^{-2}$	46.73±6.1
Formulation with different Alcohols			
SKR_3B	12.99±0.30	$3.57x10^{-2}$	41.44±1.1
SKR_3P	11.04±0.25	$3.04x10^{-2}$	35.18±8.0
SKR_3I	13.43±0.17	$3.69x10^{-2}$	42.79±5.6
SKR_3A	9.93±0.24	$2.73x10^{-2}$	31.63±7.5
Formulation with different span mixtures			
SKS_1	11.50±0.10	$3.16x10^{-2}$	45.61±8.2
SKS_2	20.68±0.23	$5.67x10^{-2}$	65.86±7.3
SKS_3	14.32 ±0.25	$3.94x10^{-2}$	36.17±4.7
SKS_4	13.09±0.09	$3.64x10^{-2}$	41.70±3.0
Formulation with other surfactants			
SKM_1	21.02±0.21	$5.79x10^{-2}$	66.96±6.6
SKM_2	14.52±0.25	$4.00x10^{-2}$	46.36± 9.4
SKM_3	16.52±0.16	$4.55x10^{-2}$	52.63±5.4
SKM_4	11.85±0.78	$3.16x10^{-2}$	60.29±4.8
SKM_5	11.48±0.29	$3.16x10^{-2}$	36.58±9.3
Formulation with levonorgestrel			
LP	8.24±0.05	$2.26x10^{-2}$	26.04±3.2
LSL	13.57±0.44	$3.73x10^{-2}$	44.04±3.9
LDCP	15.94±0.15	$4.39x10^{-2}$	50.65±5.3
LEL	13.39±0.28	$3.68x10^{-2}$	42.60±8.5
Optimized formulation containing levonorgestrel and ethinylestradiol			
COM1	3.96±5.62	$1.09x10^{-2}$	14.48±10.7
COM2	5.23±7.25	$1.44x10^{-2}$	21.34±10.1

A= amount of drug/time x area of the patch, b= amount of drug/saturation solubility of drug in receptor fluid and c= Total amount of drug released/Total amount of drug in formulationx100

form *in vivo* study, which has been approved by the Institutional Animal Ethics Committee. Eighteen female Sprauge-Dawley rats (23-24 d old), each weighing 100±5 g were divided into three groups. The fur on right dorsal side was removed and the skin was properly cleaned. All the 18 rats were estrus synchronized (a treatment which would bring a large percentage of the breeding herd into estrus all at once) by injecting 5 ml of 0.1% copper acetate solution intraperitoneally. After 24 h, the formulations were applied in the form of transdermal patch having a diameter of 1.2 cm corresponding to 1.13 cm^2 area. The blank patch (without drug loading) was applied to control group (group I). The group II was applied with patch of formulation COM 1, containing span 20, span 40 (3:1) as surfactant and the group III was applied with patch of formulation COM 2 containing span 20, span 40 and soya lecithin (3:1:1) as surfactant, both formulations COM 1 and COM 2 contained 1 mg each of ethinylestradiol and levonorgestrel and 1ml of isopropyl alcohol. All these patches were applied at neck area and then fixed using dressing tape and rats of each group were kept in separate cages, conditioned and maintained on laboratory rat feed.

The rats of all groups were kept undisturbed and on first, fourth and tenth day, two rats each of all the three groups were sacrificed by excessive inhalation of chloroform. The animal was dissected and uterus and ovary were cut and the fatty materials were removed. After dehydration with absolute alcohol, uterus horns and ovaries were weighed using electronic digital pan balance. The ovaries were also examined visually for the presence of ovulation point or bleeding points. Then organs were subjected to over night treatment with the saturated solution of xylene to make the tissue hard. After 24 h, tissues were washed, dehydrated, cleared and blocks were made by embedding the tissue in matured wax. After forming these blocks, sections of 10μ thickness were cut using a Microtome and the frozen section was mounted on a slide and examined for histological changes in the uterine horns and ovaries after staining with Ehrlich's hematoxylin and eosin. The estrogenic activities were checked on the basis of following parameters.

1. In the study of four-day uterine weight assay[16,17], the transdermal patches were applied on the neck portion of rats. The uteri were separated from the vagina by cutting through the cervix, the surrounded tissue was stripped off and the uterotubal junction severed. The uteri were then washed, and by pressing on moistened blotting paper, the intrauterine fluid was removed. They were weighed immediately after dehydration by absolute alcohol.

2. Normal female rat at maturation can be observed to go through stages of vaginal opening [16,17]. The patch was applied on the neck of 21 d old rats and time of complete opening and diameter of vagina was observed as a sign of estrogenic activity.

3. In the study of vaginal cornification, holding the animal ventral side up, a drop of water was inserted into the vagina with a Pasteur pipette. Then a drop of vaginal fluid was withdrawn after 5 min and transferred to a microscopic slide, which was placed in absolute alcohol for 5 s and allowed to dry. It was stained with a 5% aqueous methylene blue solution and observed using the low power of microscope.

4. In endometrial assay[18,19], the morphological changes produced by the direct action of combined formulation on the uterine endometrium of the rat were recorded. The uterine endometrium thickness, lumen, myometrium and effect on villi formation in each group of rats were determined. Both the area of the mucosal and glandular part of the endometrium was also determined (Table 3).

RESULTS AND DISCUSSION

Proniosomes were prepared with Span 20, Span 40 and cholesterol produced compact niosome having white semi-

TABLE 3: EFFECT OF FORMULATIONS ON UTERINE WEIGHT, VAGINAL OPENING AND VAGINAL CORNIFICATION.

Animals Groups	Uterine weight	Vaginal opening	Vaginal cornification	Mucosal thickness
Group I	104.4±5.55	0%	0%	14.1 μ
Group II	224.6±7.64	100%	100%	51.6 μ
Group III	199.3±6.72	60%	80%	74.2 μ

Group I- Control, Group II- COM 1 (S_{20}+S_{40}+EE+LN), Group III- COM 2 (S_{20}+S_{40}+SL+EE+LN).

solid like appearance while those prepared with Span 60 and Span 80 were blackish brown translucent gel. The diameter of most niosomes lies in the range of 4.2 to 13.5 μ. Entrapment efficiency in case of proniosomal gel was found to be in the range of 89.1±1.96% for formulation SKR_4 and 95.5±0.85% for formulation SKM_5. The niosomes formed from these proniosomal formulations also exhibit good entrapment efficiency. This may be due to lipophilicity of drug. The regression coefficient for the transport of ethinylestradiol and levonorgestrel from formulation were nearly one, showing zero-order release profile for all the proniosomal formulations (Table 1). The permeation rate of drugs from the niosomes prepared from various mixtures of Span 20 and Span 40 in different ratios followed the order 4:1>3:1>2:1>1:1 (fig. 1). This may be attributed to increase in HLB value on increasing amount of Span 20. When the formulation SKR_3 (span 20 and span 40, 3:1) was prepared with different alcohols like absolute alcohol, propanol, butanol and isopropyl alcohol and applied to treated cellophane membrane, the apparent steady state transdermal flux was highest for formulation containing isopropyl alcohol. Introduction of different alcohols into the formulation also led to enhanced drug permeation. This may be due to branched chain structure of isopropyl alcohol, which acts as cosurfactant and might loosen the bilayer packing resulting in the increase of flux values. In combined formulations containing mixture of Spans, the permeation profile was found to be in order of (Span 20:Span 80) <(Span 80:Span 60)<(Span 80:Span 40)<(Span 20: Span 60). Effect of different Span combinations on the permeation rate, showed that the flux value was highest in case of Span 20: Span 60 mixture because Span 20 and Span 60 have low phase transition temperature[20]. In case of formulations with varying composition, drug permeation was maximum in Brij 58 (polyoxyethylene 20 cetyl ether) formulation, because it is hydrophilic in nature[21] and it easily diffuses into the receptor fluid disturbing membrane integrity. Formulation with DCP (SKM_3) showed intermediate permeation rate because its vesicles are charged which is responsible for increase in the curvature and decrease in the size of vesicles with the increase in surface area. The drug permeation rate in case of soya lecithin, dicetyl phosphate and egg lecithin formulations was in order of SKM_3>SKM_2>SKM_4. Soya lecithins have been reported to contain unsaturated fatty acids, oleic and linoleic acid, which

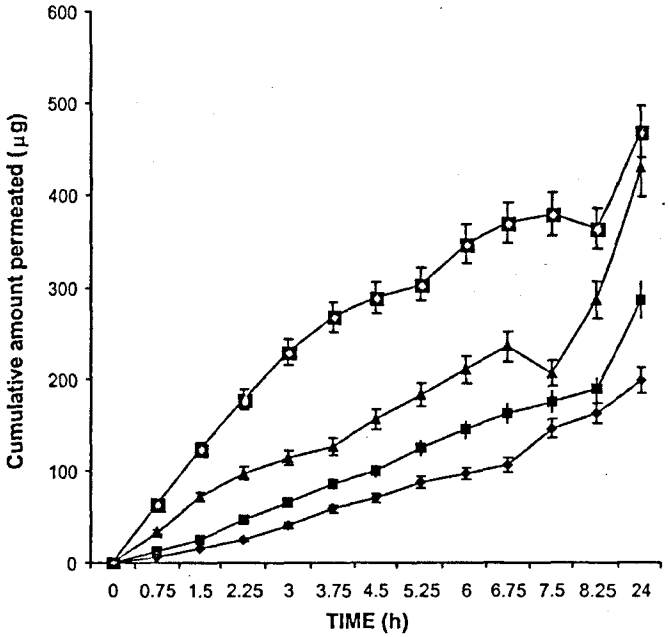

Fig. 1: Permeation profile of ethinylestradiol and levonorgestrel.

Permeation of ethinylestradiol and levonorgestrel from proniosomal formulations SKR_1 1:1 (-◆-), SKR_2 2:1 (-▫-), SKR_3 3:1 (-▲-) and SKR_4 4:1 (-●-) containing different ratio of Span 20 and Span 40.

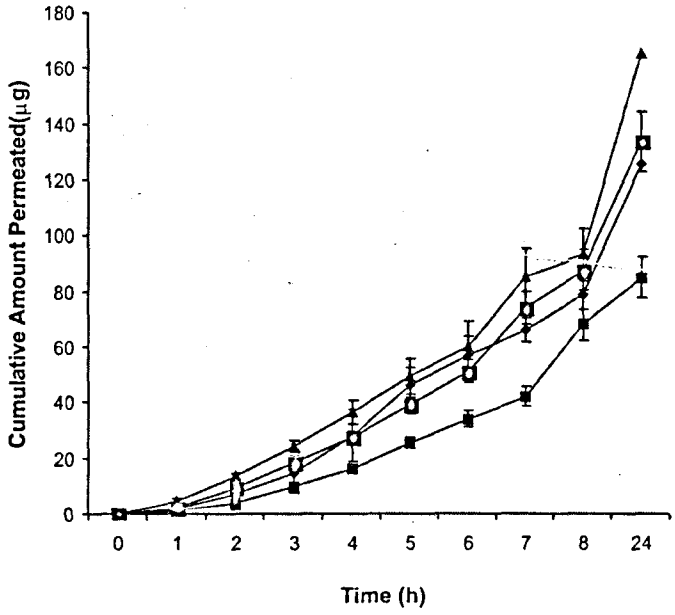

Fig. 2: Drug permeation profile from combined formulations.

Drug permeation from combined formulations such as COM 1 containing ethinylestradiol (-◆-), COM 1 containing levonorgestrel (-▫-), COM 2 containing ethinylestradiol (-▲-) and COM 2 containing levonorgestrel (-●-) through rat skin.

has penetration enhancing properties of their own as compared to egg lecithin, which contains saturated fatty acids[22,23]. These unsaturated fatty acids reverses the physiological process of barrier formation and reduction in the quantity of cholesterol in stratum corneum which results beneficial increase in the fluidity of lipid barrier. At the same time high water binding capacity of phospholipids increase the degree of hydration of stratum corneum and reduces its roughness and at the same time making it possible for high molecular weight water soluble substance to permeate through lipid barrier[23].

On the basis of these permeation rate studies, two formulations COM 1 and COM 2 were prepared containing 1 mg each of ethinylestradiol and levonorgestrel. For both formulations release of ethinylestradiol was faster than levonorgestrel, which may be due to the higher solubility of ethinylestradiol in receptor fluid. COM 1 showed slower permeation for both drugs than COM 2 formulation. This may be due to better interaction of soya lecithin with skin lipids compared to the plain formulation of Spans (fig. 2).

The consistency of proniosomal gel SKR_3 was same up to 9 w, but is case of SKM_4 after 6 w consistencies increased. The optical anisotropic structures of both the formulations after 12 w were unchanged and the crystal formation did not occur during the storage period. In formulation SKR_3 and SKM_3 drug crystals did not appear up to 12 w at 4° as well as room temperature. The characterization of niosomes formed from proniosomes revealed that with increase in vesicle size the polydispersity index decreased.

In vivo performance of the fabricated proniosomal transdermal system was studied. In immature rats, only epithelial cells were comparatively bigger than those observed in control animals, finally seen as cornified cells. After four-day application of patch, only keratinized cells without any nucleus were seen, indicating that the ethinylestradiol produced full vaginal cornification. The histological studies showed that the endometrial thickness in case of control animal and COM 1 and COM 2 treated rats was 14.1, 51.6 and 74.2 μ, respectively. In comparison to initial vaginal opening 100% vaginal opening was observed in case of proniosomal gel formulation COM 1 (Table 3).

The results of *in vivo* study show that proniosomal gel of ethinylestradiol and levonorgestrel for transdermal drug delivery system is a better choice for hormone replacement therapy and provides effective contraception by inhibiting the release of follicle stimulating hormone (FSH) and leutenizing hormone (LH) and by increasing the uterine mucosal thickness and endometrial proliferation. These studies have clearly shown that ethinyestradiol and levonorgestrel were continuously delivered within therapeutic level up to 10 d for contraception purpose.

In conclusion, the proniosomal gel systems have shown great potential, for preferred delivery of hormonal drugs for their use in hormonal replacement therapy as well as contraceptive purpose. In addition, the proposed systems has some added advantages like greater stability, higher entrapment efficiency, greater membrane permeability and enhanced penetration.

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