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Research Paper

Enhanced Oromucosal Delivery of Progesterone Via Hexosomes

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Purpose. Formulation and characterization of progesterone loaded hexosomes employing a novel method for oromucosal delivery.
Method. Hexosomes were prepared employing a method in which ethanolic solution of lipid phase (monolein and oleic acid) was vortexed with aqueous phase (surfactant solution) and characterized for particle size, morphology and internal structure. FT-IR and confocal laser scanning microscopy (CLSM) were performed to investigate the possible mechanism and penetration pathway of hexosomes within the mucosa.
Results. Hexosomes exhibited anisotropy, hexagonal shape and nanometric size (251.2±1.8 nm). Internal structure confirmed by X-ray diffraction peaks with spacing ratio of √1:√3:√4 proved two-dimensional hexagonal arrangements. Entrapment efficiency of system was greater than 95%. *In vitro* release studies revealed an enhanced transmucosal flux (4.67±0.14 μg cm^{-2} h^{-1}) and decreased lag time (1.54 h) across albino rabbit mucosa. FT-IR and CLSM of treated mucosa shows lipid extraction phenomena as well as structural irregularities within intercellular lipids respectively. These irregularities can function as 'virtual channels' facilitating hexosome's penetration.
Conclusion. Developed hexosomes formulation exhibited high entrapment efficiency, high permeability and better stability on storage, thus proposing itself a novel carrier for enhanced oromucosal delivery of progesterone.

KEY WORDS: hexosomes; oromucosal delivery; permeation enhancement; progesterone.

INTRODUCTION

To reach the drug into systemic circulation buccal route is an attractive approach of administration. It provides a much milder environment for drug absorption as well as protects drugs from undergoing gastrointestinal degradation and hepatic first-pass metabolism (1). The mucosal lining of the oral cavity is richly vascularized and more accessible for the administration and removal of a dosage form (2). The membrane of oral cavity have 170 cm^2 total surface area for drug absorption (3), including ~50 cm^2 of non-keratinized tissues (4). Buccal mucosa represents low enzymatic activity as compared to other mucosal routes (5) which seems to be advantageous while administering proteins and peptides. Additionally, buccal drug delivery has a high patient acceptability compared to other non-oral routes of drug administration.

Progesterone, named for its progestational role in maintaining pregnancy, is traditionally regarded as a "female hormone", playing a key role in generating female-typical behaviors such as lordosis and maternal behavior. In females, the ovary (and/or placenta) is the primary source of the dynamic levels of plasma progesterone that exist during the estrous cycle and over the course of pregnancy and lactation (6,7). It has also shown its therapeutic efficacy in treatment of many disorders including hormone replacement therapy in the menopausal woman, as well as a natural agent in establishment and maintenance of pregnancy (8,9).

Being so efficacious, the major problem with its therapeutic use is selection of a proper route of administration. Oral intake is still the most convenient approach but requires large doses, an appropriate vehicle, and an increased surface area (micronization) to achieve reasonable blood levels (10), however rapid and extensive intestinal metabolism prevents adequate absorption (11). Alternative routes of administration of progesterone include intramuscular, vaginal and topical. Several studies indicate that topically applied progesterone is absorbed transdermally (12,13) although, there remains considerable uncertainty as to the resultant progesterone levels in target tissues (14,15). O'Leary *et al.* (16) showed no change in plasma concentration of topically administered progesterone, while salivary progesterone levels were elevated in both pre- and post-menopausal women. Thus, topically applied progesterone appears to exert biological activity, there remains uncertainty about its pattern of uptake, metabolism and subsequent tissue distribution (17). Vaginal progesterone delivery has also been proposed but changes in epithelial thickness during menstrual cycle may change its bioavailability (18).

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Our present study focuses on preparation and characterization of hexosomes employing a newer method for mucosal delivery of progesterone and studying the possible mechanism of penetration of these carriers. The reversed hexagonal phase (honeycomb structure) comprises hexagonally close-packed infinite water layers covered by a surfactant monolayer (19,20). Earlier, lyotropic liquid crystalline phases bearing various therapeutic agents were prepared and they were shown to exhibit sustained release properties (21–23).

Hexosomal dispersion were prepared and characterized for their particle size, morphology, stability, entrapment efficiency, release profile and internal structure. confocal laser scanning microscopy (CLSM) and FT-IR were performed for studying the extent of penetration and hexosomal–mucosal interaction and thus proposing mechanism of penetration of hexosomal dispersion.

MATERIALS AND METHODS

Materials and Reagents

Monoolein 99% [GMO], was purchased from Sigma-Aldrich (St. Louis, MO USA), Oleic acid, from CDH (India), Pluronic F-68, from Himedia (India) and Rhodamine B (RB) from Molecular Probes (Eugene, Oregon, USA). Progesterone (99%) was received as a gift sample from M/s Sun Pharmaceuticals Ltd. (Vadodara, India). All chemicals used were of AR grade, solvents were of HPLC grade and triple distilled water was used for all experiments.

Preparation of Dispersion

The method employed in preparation of hexosome dispersion was a slight modification of procedure reported by Spicer *et al.* (24). It involves dilution of an isotropic liquid made up of 50% lipid phase (GMO and oleic acid, 60:40 *w*/*w*) and 50% ethanol with a polymer solution (1%) in water and vortexing at 3,200 rpm for 120 s to form a colloidal dispersion of hexosome. The final concentrations were 5% lipid, 5% ethanol and 1% Pluronic F-68. To attain the equilibrium, dispersion was stored undisturbed in dark at room temperature for about 2 weeks.

For drug-loaded dispersion, the desired amount of progesterone (1% *w*/*w* wrt lipid) was dissolved in absolute ethanol and mixed with hydrophobic part whilst for RB loaded dispersion, the dye was dissolved in diluting solvent.

Characterization of Dispersion

Visual Inspection

After one week of preparation the dispersions were assessed optically for color, turbidity, and presence for aggregates.

Light Microscopy

A Polarizing microscope (Nikon Eclipse LV 100, Japan) was used with and without crossed polarizer or differential interference contrast at magnification of ×100 and ×450 attached with Q imaging micro-publisher, 3.3 R TV camera.

Photon Correlation Spectroscopy

The particle size of hexosomal dispersion was investigated using a Zetasizer DTS ver. 4.10 (Malvern Instruments, UK). The sample of dispersions was diluted 1:9 *v*/*v* with deionized water. Measurements were carried out at 25°C at an angle of 90° with a run time of 60 s. The mean hydrodynamic diameter was evaluated by method of cumulant. The viscosity and refractive index were assumed the same as pure water (0.89 cp and 1.330, respectively).

Transmission Electron Microscopy (TEM)

Transmission electron microscope (Philips Morgagni 268, Eindhoven Netherlands) with MegaView III FW camera was used as a visualizing aid for hexosomes. Samples were negatively stained with 2% aqueous solution of uranyl acetate. The sample was placed on the 400-mesh carbon coated grids and allowed to stand at room temperature for 90 s. Excess sample was removed by using filter paper. Then 10 µl of staining solution was placed on the grid, allowed to stand for 60 s and drained. The specimens were viewed under the microscope at 10–100 k-fold enlargements at an accelerating voltage of 60.0 kV.

Nephelometric Measurements

Turbidity of hexosome formulations were determined using a nephelometer (Superfit, Ambala, India) taking phosphate buffer saline (pH 6.8) as blank.

X-ray Diffraction (XRD) Measurements

The internal structure of hexosomes was investigated by small angle XRD. To obtain a clear XRD pattern the dispersion had to be concentrated by centrifugation. In this method 1 mm internal diameter quartz capillary was filled with dispersion and sealed with epoxy resin. X-ray generator (FLCU 4KE, Bruker, Germany) operating at 40 kV and 45 mA used to produce Cu Kα X-rays (1.542 Å). Three hours of exposure time was taken to analyze sample. X-ray diffraction data was obtained by general area detector diffraction system at 25°C.

Percent Drug Loading

The amount of progesterone loaded in to the dispersion was determined with Centricon® (YM-100, Millipore Corp., Bedford, MA) as described previously by Chung *et al.* (25) and the amount of drug present in the filtrate was assayed through HPLC.

$$\text{Percent drug loading} = \frac{\text{Entrapped drug (mg)}}{\text{Total drug added (mg)}} \times 100$$

HPLC Analysis

The high performance liquid chromatography technique described by Pucci *et al.* (26) was used to separate and estimate progesterone. The quantitative determination of

progesterone was performed by HPLC using a mixture of 2-propanol-phosphate buffer (pH 2.5; 30 mM) with an apparent pH of 3.0 (1:1, *v*/*v*) as mobile phase at a flow rate of 2.0 ml/min by LC 10-AT vp pump (Shimadzu, Japan). Twenty microliters of injection volume was eluted in LUNA 54, C18, 4.6×150 mm, column (Phenomenex, USA) at room temperature. The column eluant was monitored at 245 nm using SPD–M10A vp diode array UV detector (Shimadzu, Japan), progesterone peaks were well separated from that of internal standard (Indomethacin) with a retention time of 4.8 min. A calibration curve with a concentration range from 50–500 ng/ml was used to measure the progesterone concentration of samples and to validate analytical technique. Internal standard was utilized to better resolve the peaks obtained in chromatogram.

Physical Stability of Dispersion

The ability of hexosomal dispersion to retain the drug (i.e., drug-retentive behavior) was assessed by keeping the dispersion at 25±2°C (room temperature; RT) for a period of 16 weeks (27). The dispersion was kept in sealed ampoules (10 ml capacity) after flushing with nitrogen. Samples were withdrawn periodically and analyzed for the drug content by HPLC. Stability of hexosome was also assessed by the measurement of size and structure over time using photon correlation spectroscopy along with TEM visualization after negative staining with uranyl acetate as described above.

Viscous Vehicle Preparation

A weighed amount of Carbopol 934P (1% *w*/*w*) was dispersed in deionized water and stored (12 h) for swelling. Then adequate amount of triethanolamine up to pH 7 was added to produce gel (27). The obtained gel was diluted with suitable amount of hexosomal dispersion loaded with progesterone and progesterone–water suspension. Final ratio of dispersion and gel was 2:1 *w*/*w* and final drug concentrations in the formulations were 0.066%.

Ex Vivo Studies

FT-IR Study

Infrared spectroscopy was performed on FT-IR 8400 S (Shimadzu, Columbia USA) was employed as a tool to study the transbuccal permeation of formulation. The rabbit was sacrificed; buccal mucosa was excised and washed with normal saline. The underlying tissue was removed from the mucosa with surgical scissors, and epithelium was separated carefully from most of the connective tissue using a scalpel. The slice thickness was around 700 μm. All investigations were performed after approval by the Institutional ethical committee of Dr. H. S. Gour University, Sagar.

For FT-IR study, separated mucosa was incubated for 4 h with 500 mg formulation, washed with PBS and dried in a desiccator. Dried mucosa (3 mm^2) was mixed with potassium bromide and compressed into a disc ready for analysis. The obtained spectrum was normalized by using the quantitative software of the system. For comparison, the lipid extracted mucosa was obtained by treating it in chloroform/methanol solutions (1:1) for 2 h and then solvents were evaporated (28).

Confocal Laser Scanning Microscopy

The penetration pathway of RB loaded hexosomes through mucosa was assessed using CLS microscopy. The probe loaded dispersion was separated from unentrapped probe by utilizing Centricon® and then about 100 mg of hexosomal dispersion was applied for 8 h on the mucosal surface of 5–6 week old albino rabbit. The rabbit was sacrificed; buccal mucosa was excised and washed with normal saline. The excised mucosa was gently teased off any adhering tissue and cut out in the pieces of 1 mm^2 and tested for probe penetration. Optical excitations were carried out with a 488 nm argon laser beam and fluorescence emission was detected above 460 nm for RB. The following parameters were set up before the experiment and were not changed throughout the measurements: pin hole size, electron gain, neutral density filters and background levels.

Ex Vivo Release Study

For Ex vivo release studies fresh samples of excised buccal mucosa of rabbit was employed and mounted on a locally fabricated Franz-type diffusion cell (effective permeation area=3.14 cm^2, receptor compartment volume=12 ml). To establish "sink condition" and permeant solubilization water–ethanol solution (80: 20 *v*/*v*) was employed and filled in the receptor compartment. The solution was stirred (500 rpm) with the help of a magnetic stirrer (Expo India Ltd., Mumbai, India) and thermostated at 37±1°C during all the experiments (29, 30). Five hundred milligrams of gel formulation was applied on exposed mucosa and donor compartment sealed to avoid evaporation. Each experiment was run in triplicate for 12 h (n=3). Samples (100 μl) were collected at different time intervals (t=0, 1, 2, 3... 12 h), and replaced with fresh solution. Progesterone content in samples was analyzed using HPLC.

Calculation of Permeation Parameters

The cumulative amount of drug permeated per unit area was plotted as a function of time, the steady-state permeation rate (Jss) and lag time (LT, h) were calculated from the slope and X-intercept of the linear portion, respectively. Diffusion coefficient (D, cm^2/h) was calculated from the following equation:

$$LT = H^2/6D$$

Where H=thickness of rabbit mucosa; the enhancement ratio (ER) was calculated from following equation:

$$ER = \frac{\text{Transmucosal flux from formulation}}{\text{Transmucosal flux from plain drug suspension}}$$

Statistical Analysis

Data are expressed as the mean±standard deviation (SD) of the mean and statistical analysis was carried out employing the Student's t test using the software Prism (Graph Pad). A value of P<0.005 was considered statistically significant.

RESULTS

Preparation of Dispersion

In this preparation ethanol served as a hydrotrope, which reduces the viscosity and increases solubility of lipid when mixed. The isotropic liquid upon dilution with polymeric solution forms liquid crystalline dispersion spontaneously by a presumed homogeneous nucleation process (24). Oleic acid had been incorporated to increase the triglyceride content and hence to alter or bring about lattice modification.

Characterization of Dispersion

The plain dispersion (without drug) was milky white, turbid and slightly translucent when inspected visually. A few aggregates in micrometer range were evident microscopically. Fig. 1 shows photographs of plain and drug loaded dispersion observed with and without cross polarizer. Fig. 1c, d (with cross polarizer) reveals that formulation is anisotropic in nature and appearance of fan like structure and angular texture (31) confers to the formation of hexosomal crystals. It was further confirmed by TEM and XRD analysis (Figs. 2 and 3).

Particle size measurement using photon correlation spectroscopy technique showed an particle size of 251.2±1.8 nm (polydispersity index, 0.324±0.004) for blank dispersion, while progesterone incorporated dispersion showed a slight increase in particle size to 257.4±2.3 nm (polydispersity index, 0.312±0.07). It is clear from the data that well-defined dispersions of submicrometer-sized particles can easily be obtained with low energy method.

The particles we prepared here, analyzed through XRD and negative staining TEM were exhibiting hexosomal conformation. TEM photomicrograph showed that particles possessed hexagonal geometry and very few vesicular structures. The H_{II} phase was characterized by three strong X-ray diffraction peaks with spacing ratios of √1:√3:√4 proving two-dimensional hexagonal arrangements. The lattice parameter was found to be 64.2±0.3 Å and peaks correspond to the (100), (110), and (200) reflections of the hexagonal structure (Fig. 3). The lattice parameter was similar to those of hexagonal dispersion reported earlier (32).

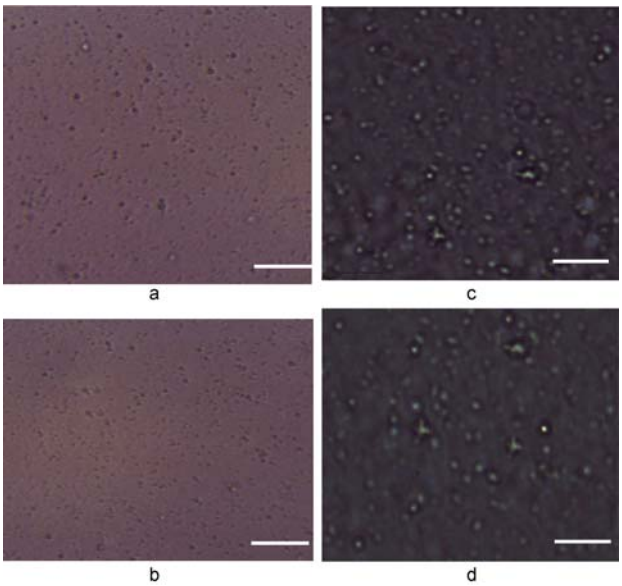

Fig. 1. Visualization of hexosome by polarizing microscope (×100). **a** Without cross-polarization (without drug). **b** Without cross-polarization (with drug). **c** With cross-polarization (without drug). **d** With cross-polarization (with drug). Bar 20 μm.

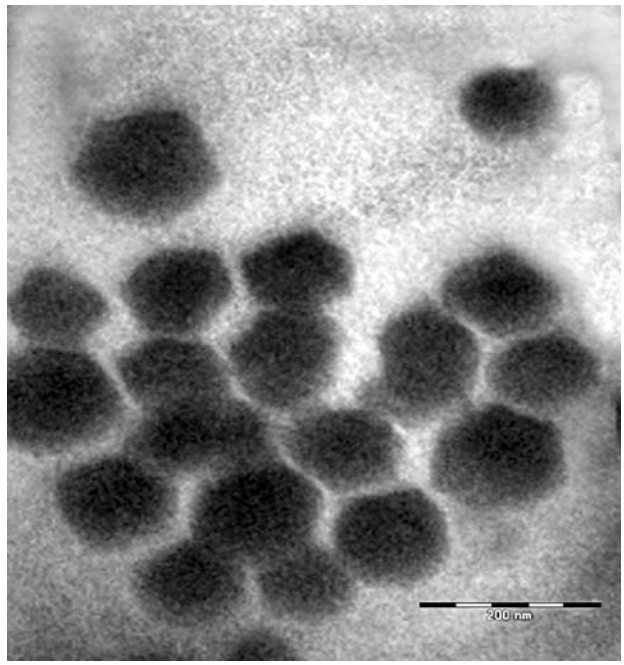

Fig. 2. Visualization of hexosomes by transmission electron microscopy (×1, 10,000). Bar 200 nm.

Physical Stability of Dispersion

The structural and organoleptic features of hexosomes do not show any significant changes on storage and remain free of phase separation phenomena. TEM photographs of hexosomes stored for 16 weeks at room temperature showed no significant difference in shape and size proving to be quiet stable on storage. Table I shows particle size of hexosomes at room temperature up to one month remained rather constant but in the succeeding months a slight increase was observed.

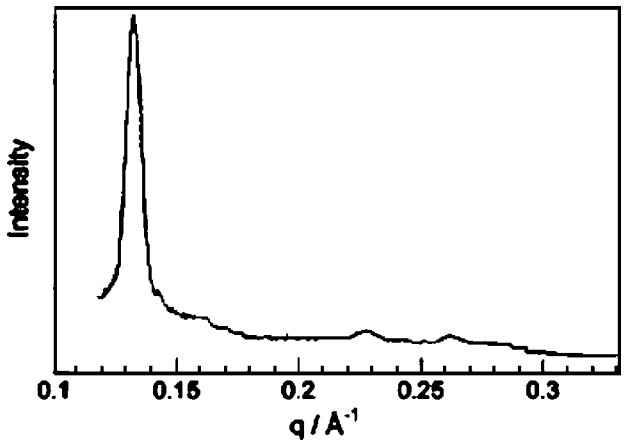

Fig. 3. X-ray diffraction pattern of hexosomes.

Figure 4 shows that turbidity has decreased in first few days and then started to increase. Decrease in turbidity is a consequence of particle size reduction, as dispersion attains equilibrium. After 15 days turbidity remains approximately constant showing acceleration in particle growth is slow.

The entrapment efficacy of the system was found to be around 98.7±0.15 % (after one month) and 96.6±0.26 % (after 4 months) showing a slight leakage of the drug from the system. The absence of secondary peak in HPLC chromatogram and unchanged retention time (data not shown) suggests that the entrapped drug was not degraded after 4 months of preparation.

Ex Vivo Studies

FT-IR Study

IR technique serves as a tool to study the correlation between barrier function of buccal epithelial lipids and transbuccal drug flux that may be intimately related to epithelial lipid structure and organization. Epithelial lipid when extracted with chloroform shows a reduction of C–H stretching frequency suggesting that C–H bands corresponds to long chain lipids in buccal epithelium (Fig. 5). After incubation of mucosa with hexosomal dispersion, stretching vibrations were broadened but no significant shift was observed. The areas under the C–H symmetric stretching absorbance (over the frequency 2,862.0–2,855.6 cm^{-1} ~ range) and C–H asymmetric stretching absorbance (over the frequency 2,945–2,911 cm^{-1} ~ range) were computed and were in accordance with Golden *et al*. (33). It was shown that intensities were decreased with 4 h treatment with hexosomal dispersion when compared to intact tissue (Fig. 5).

Confocal Laser Scanning Microscopy

Confocal laser microscopy have been very good tool to investigate mechanism and extent of penetration (34–36). We utilized this to investigate possible penetration pathway through mucosa. Intact mucosa does not contain significant number of pathways of sufficient width to allow nanoparticles to pass. To penetrate the mucosa hexosomes must follow either transcellular or paracellular pathways. Pores evident in epithelium of mucosa (Fig. 6) suggest that these regions may contain structural irregularities within the intercellular lipids and these irregularities can function as 'virtual channels' through which the hexosomal dispersion can penetrate. This hypothesis was further well correlated by FT-IR study.

Table I. Particle size variation at room temperature at different time interval (n=3)

Days	Hexosomal dispersion	Particle size[a] (nm)±SD	PI±SD[b]
0	Blank dispersion	251.2±1.8	0.324±.004
	Progesterone dispersion	257.4±2.3	0.312±.07
30	Blank dispersion	253.3±1.6	0.334±0.06
	Progesterone dispersion	258.5±1.7	0.344±0.05
60	Blank dispersion	255.4±2.4	0.352±0.04
	Progesterone dispersion	261.2±1.4	0.348±0.03
90	Blank dispersion	256.4±1.8	0.392±0.08
	Progesterone dispersion	263.6±2.2	0.384±0.04
120	Blank dispersion	257.3±1.2	0.356±0.06
	Progesterone dispersion	266.1±1.5	0.361±0.02

Photon correlation spectroscopy (PCS) data were the mean of three determinations of different batches of the same type of dispersion. Dispersion were produced by a dispersed phase constituted of monoolein (with oleic acid) Pluronic F-68 in water.
SD Standard deviation
[a] Determine by PCS
[b] Polydispersity index

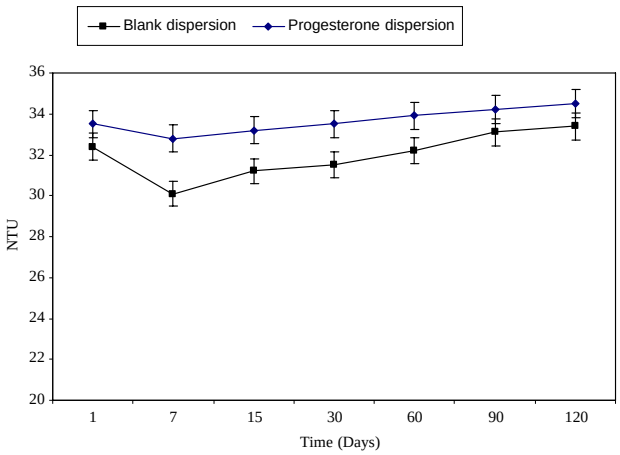

Fig. 4. Nephelometric measurement of hexosomal dispersion at different time intervals. *NTU* Nephelometric turbidity units.

Ex Vivo Drug Release

Highly drug loaded system delivered appreciable quantity of drug, there by increasing the amount of free drug available for diffusion into deeper mucosal layer (Fig. 7). Thus gel–hexosomal formulation showed increased flux (4.67±0.14 μg cm^{-2} h^{-1}) and decreased lag time (1.54 h) as compared to progesterone loaded gel (0.93±0.14 μg cm^{-2} h^{-1}, lag time 2.27 h) and plain progesterone suspension (1.13±0.13 μg cm^{-2} h^{-1}, 2.24 h; P<0.05). The observed flux was fivefold higher than that of progesterone loaded gel and nearly fourfold higher than plain progesterone suspension. The

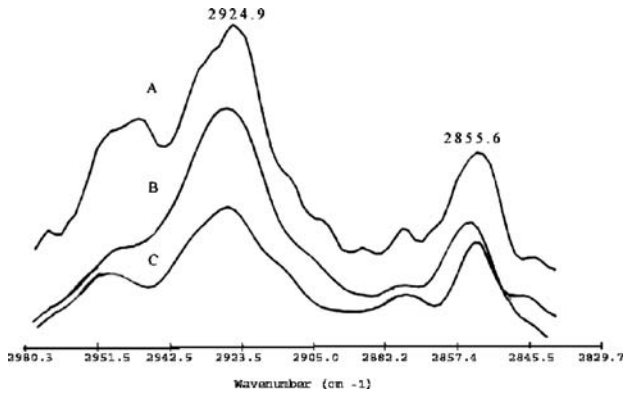

Fig. 5. FT-IR of excised rabbit buccal mucosa. *A* Intact mucosa. *B* Mucosa treated with chloroform. *C* Mucosa treated with hexosomal dispersion.

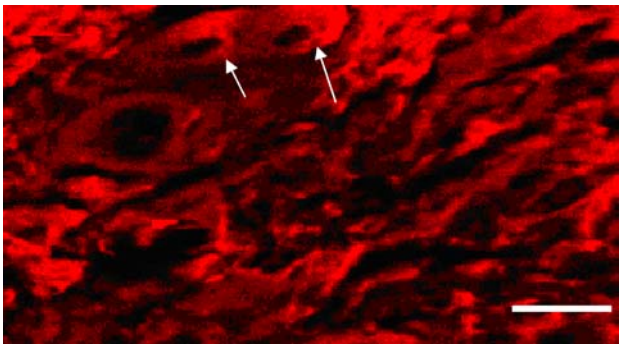

Fig. 6. Virtual channel like structures can be visualized (*white arrows* representing the stained channels) at 10-μm mucosal depth. Bar 20 μm.

very low permeability of plain drug suspension is due to its extreme hydrophobicity (log$K_{o/w}$ 3.87; 37) and low solubility in PBS (11.4 μg/ml) which in turn leads to a very low concentration gradient across the mucosa.

DISCUSSION

Spicer *et al*. prepared the cubosomes by using monoolein and Poloxamer 407 (Pluronic F-127), here we used oleic acid (with GMO) and Pluronic F-68 which is more hydrophobic and increases the critical packaging parameter (24,38,39). The molecular packing of the monoglyceride can be affected by the increase in the hydrocarbon chain space obtained upon solubilization of oleic acid (40) that leads to the formation of hexosomes instead of cubosomes. As oleic acid is well known to effectively increase percutaneous and transmucosal absorption rates by altering the intercellular lipid fluidity and disrupting lipid bilayers to achieve penetration enhancing effects, it serves as penetration enhancer as well as lattice modifier in the current formulation (41,42).

The drug whatever have been incorporated tends to reside in the matrix of carrier system as well as dispersion medium. The total amount of drug encapsulated in hexosomes was measured by Centricon®. The drug has no influence of its own on characteristics as well as particle size of hexososmes.

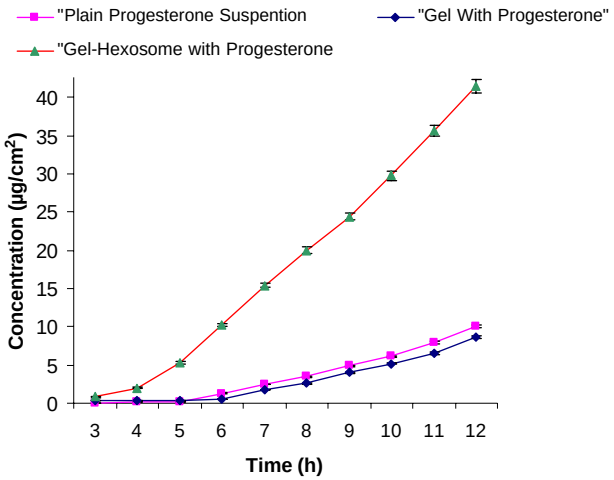

Fig. 7. Ex vivo drug release study. Comparative cumulative drug release of progesterone after 24 h from gel hexosome, gel formulation, plain drug suspension. Values represent mean±SD (n=3).

Gustafsson *et al*. (43,44) investigated the internal structure of dispersed cubic particles using cryo-TEM and XRD. The cryo-TEM images show a typical ordered cubic texture and inner periodicity which was further confirmed by XRD. The prepared hexosomes were here analyzed through negative staining TEM to observe the outer conformation and inner periodicity was confirmed by XRD. The criteria behind selecting negative staining TEM was to explore potential of this particular method to elicit the exact structure of hexosomes. The outer shape of particles found in the current experiment correctly matches with structure reported earlier (45).

Bommannan *et al*. (46) suggested that removal of the lipids corresponds to a highly permeable tissue. As we incorporated a subtle amount of oleic acid (a well-known penetration enhancer) in the formulation, it induces lipid disorder in buccal epithelium and strongly alters lipid packing of the bilayer (41,47). Thus it is reasonable to hypothesize that, incorporation of oleic acid and hence formation of hexosomes disrupts the epithelial lipids and decreases the diffusional resistance to permeants. This hypothesis was further confirmed by CLSM of mucosa treated with hexosomal formulation.

The drug release data shows that permeation through mucosa has been increased to a greater extent and high flux values were obtained using hexosomal–gel formulation. Although, gel has high viscosity it does not greatly alter the flux with respect to plain drug suspension while gel-loaded formulation containing oleic acid, increases the mucosal permeability progressively i.e., a time dependent change in lipid integrity of the barrier (42) due to which system probably enters in mucosa through virtual channels.

CONCLUSIONS

A novel method has been employed to prepare hexosomal dispersion based on less time consuming procedure. The incorporated oleic acid and hexagonal configuration simultaneously enhances penetration and delivery of progesterone through oral mucosa. FT-IR and CLSM have been utilized to explore the possible mechanism of penetration of these carriers through mucosa. The prepared formulation shows high entrapment efficiency, high permeability and better stability on storage. The formulation has been optimized and checked for its *in vitro* performance although further experimentation required exploring its performance *in vivo*.

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