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Research paper

Melatonin loaded ethanolic liposomes: Physicochemical characterization and enhanced transdermal delivery

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Abstract

The current investigation aims to evaluate the transdermal potential of novel ethanolic liposomes (ethosomes) bearing Melatonin (MT), an anti-jet lag agent associated with poor skin permeation and long lag time. MT loaded ethosomes were prepared and characterized for vesicular shape and surface morphology, vesicular size, entrapment efficiency, stability, *in vitro* skin permeation and *in vivo* skin tolerability. Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), and Dynamic Light Scattering (DLS) defined ethosomes as spherical, unilamellar structures having low polydispersity (0.032 ± 0.011) and nanometric size range (122 ± 3.5 nm). % Entrapment efficiency of MT in ethosomal carrier was found to be 70.71 ± 1.4. Stability profile of prepared system assessed for 120 days revealed very low aggregation and growth in vesicular size ($7.6 \pm 1.2\%$). MT loaded ethosomal carriers also provided an enhanced transdermal flux of 59.2 ± 1.22 μg/cm^2/h and decreased lag time of 0.9 h across human cadaver skin. Fourier Transform-Infrared (FT-IR) data generated to assess the fluidity of skin lipids after application of formulation revealed a greater mobility of skin lipids on application of ethosomes as compared to that of ethanol or plain liposomes. Skin permeation profile of the developed formulation further assessed by confocal laser scanning microscopy (CLSM) revealed an enhanced permeation of Rhodamine Red (RR) loaded formulations to the deeper layers of the skin (240 μm). Further, a better skin tolerability of ethosomal suspension on rabbit skin suggested that ethosomes may offer a suitable approach for transdermal delivery of melatonin.

Keywords: Melatonin; Ethosomes; Confocal laser scanning microscopy; Skin irritancy

1. Introduction

Melatonin (*N*-acetyl-5-methoxy tryptamine, MT) acts as a synchronizer of biological rhythms in several animal species including humans. It is a neurohormone, secreted primarily by the pineal gland in the hours of darkness, and it plays an important role as a regulator of sleep. Other organs like retina, lens, ovary, testis and bone marrow in a circadian rhythm also produce MT. It can also mediate several cellular, neuroendocrine and physiological processes. Moreover, the free radical scavenging activity of MT established it as an endogenous antioxidant [1].

For such relevant biological properties associated with extremely low toxicities, MT can be utilized for its therapeutic potential, especially in patients suffering from delayed sleep phase syndrome, sleep disturbances in blind people. It also facilitates adaptation to jet lag in travelers, cosmonauts and shift workers [2,3]. Further several clinical and preclinical studies are in progress in order to justify the therapeutic efficacy of this neurohormone especially in the fields of cancer treatment and topical protectant.

Clinically, MT has a very short plasma half-life ($t_{1/2} < 30$ min), variable oral absorption and undergoes extensive first pass effect, making an oral mode of MT administration less preferable [4]. Transdermal delivery systems for MT that avoid extensive hepatic clearance and low bioavailability may have the potential to more closely mimic

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the normal endogenous plasma MT profile. Thus, transdermal administration of MT may be feasible, resulting in sustained plasma MT levels that can be tailored to the normal physiological range and avoid the first pass effect [5–7].

In spite of several advantages offered by transdermal route, only a few molecules are administered transdermally because of formidable barrier nature of stratum corneum (SC). To overcome the difficulties of poor skin permeability, various vesicular approaches have been proposed including elastic liposomes [8–13] and ethosomes. Ethosomes, the high ethanol containing vesicles, are able to penetrate to the deeper layers of the skin and hence appear to be vesicles of choice for transdermal drug delivery. Ethosomes present interesting features correlated with ability to permeate through the human skin due to their high malleability. The physicochemical characteristics of ethosomes allow this vesicular carrier to transport wide variety of active substances more efficiently across the skin barrier and into systemic blood circulation [14–18].

The long-term objective of this project is to develop efficient transdermal delivery system for melatonin. Earlier we have reported the feasibility of elastic liposomal system for transdermal delivery of MT thereby eliminating the limitations of long lag time and poor skin permeation associated with it [8]. In the next step of our research, we aimed to develop an ethosome based transdermal delivery system for MT in context of obtaining better transdermal parameters and investigating the possible mechanism of penetration of these ethosomal carriers.

2. Materials and methods

Soya phosphatidylcholine (99%) and phosphotungstic acid were purchased from Sigma, St. Louis, MO, USA. Rhodamine Red-X 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine trimethylammonium salt (RR) was purchased from Molecular Probes (Eugene, Oregon, USA). Melatonin (99%) was received as a gift sample from Aristo Pharmaceuticals Ltd., Mumbai, India. All other solvents were of HPLC grade and triple distilled water was used wherever required.

2.1. Preparation of melatonin loaded vesicles

The method reported by Jain et al. [14] and Touitou et al. [17] was followed for preparation of ethosomes. The ethanolic vesicular system investigated here was composed of 2.0% w/w of Soya phosphatidylcholine (PC), 30% w/w of ethanol, drug (melatonin, 1.0% w/w) or probe (Rhodamine Red, 0.03% w/w). Soya phosphatidylcholine was dissolved along with drug or probe (RR) in ethanol. Triple distilled water was added slowly in a fine stream with constant mixing at 700 rpm with a mechanical stirrer (Remi Equipment, Mumbai, India) in a house built closed container. Mixing was continued for additional 5 min. The system was maintained at 30 ± 1 °C during the preparation and then left to cool at room temperature for 30 min.

Conventional liposomes were prepared by Cast film method [19]. Briefly, Soya PC (2.0% w/w) and MT (1% w/w) were dissolved in ethanol in a clean, dry round-bottomed flask. Ethanol was then removed by rotary vacuum evaporator above the lipid transition temperature (Rotary Evaporator, Superfit, Ambala, India). Finally, the traces of solvent were removed from the deposited lipid film under vacuum overnight. Lipid film was then hydrated with triple distilled water by rotation (60 rpm, 1 h) at the corresponding temperature. Thirty percent of hydroethanolic solution of drug was also prepared to be used as a control.

2.2. Vesicular shape and surface morphology

Transmission Electron Microscope (TEM) (Philips CM12 Electron Microscope, Eindhoven, Netherlands) was used as a visualizing aid for ethosomal vesicles. Samples were dried on carbon-coated grid and negatively stained with aqueous solution of phosphotungstic acid. After drying the specimen was viewed under the microscope at 10–100 k-fold enlargements at an accelerating voltage of 100 kV.

Scanning Electron Microscopy (SEM) was also conducted to characterize the surface morphology of the ethosomal vesicles. One drop of ethosomal system was mounted on clear glass stub, air dried and coated with Polaron E 5100 Sputter coater (Polaron, UK) and visualized under Scanning Electron Microscope (Leo-435 VP, Cambridge, UK).

2.3. Vesicular size and size distribution

The size distribution of ethosomes was measured in two sets of triplicates, in a multimodal mode, by Dynamic Light Scattering (DLS) technique using a computerized Malvern Autosizer 5002 inspection system (Malvern, UK). For vesicle size measurement, vesicular suspensions were mixed with the appropriate medium (PBS, pH 6.5) and the measurements were taken in triplicate.

2.4. Storage-physical stability of ethosomes

The ability of vesicles to retain the drug (i.e., drug-retentive behavior) was assessed by keeping the ethosomal suspensions at different temperatures, i.e., 4 ± 2, 25 ± 2 (room temperature, RT), 37 ± 2 and 45 ± 2 °C for different periods of time (1, 20, 40, 60, 80 and 120 days). The vesicular suspensions were kept in sealed vials (10 ml capacity) after flushing with nitrogen. The stability of ethosomes was also assessed quantitatively by monitoring size and morphology of the vesicles over time using DLS and TEM.

2.5. Entrapment efficiency

Prepared ethosomal vesicles were separated from the free (unentrapped) drug by a Sephadex G-50 minicolumn centrifugation technique [20,21]. The method was repeated

at least three times with a fresh syringe packed with gel each time until the fraction collected was free from unentrapped drug. The vesicles were lysed by Triton X-100 (0.5% w/w) and entrapped drug was estimated using HPLC.

2.6. Calorimetric studies

The transition temperature (T_m) of vesicular lipids was measured in triplicate using modulated differential scanning calorimetry (DSC TA, Instrument 2910) with programmed heating rate of 10 °C/min, temperature modulation amplitude from ±0.01 to ±10 °C, under constant nitrogen stream within a range of −50 to +50 °C. Sample weights were 20 ± 5 mg.

2.7. Confocal laser scanning microscopy (CLSM)

Depth and mechanism of skin penetration of RR loaded ethosomes was investigated using confocal laser scanning microscopy (CLSM). The probe-loaded vesicles were first passed through the Sephadex G-50 minicolumn to separate the unentrapped probe and thereafter formulation was applied non-occlusively for 8 h to the dorsal skin of 5- to 6-week-old nude albino rat (Sprague–Dawley strain). Treated animal was kept in a separate cage and maintained under laboratory conditions. Food and water were allowed *ad libitum*. All investigations were performed as per the protocol approved by the Institutional Animals Ethical Committee of Dr. H.S. Gour University, Sagar, India. The rat was sacrificed by heart puncture; dorsal skin was excised and washed with distilled water. The excised skin was then placed on aluminium foil and the dermal side of the skin was gently teased off any adhering fat and/or subcutaneous tissue. The skin was sectioned into the pieces of 1 mm^2 size and evaluated for depth of probe penetration. The full skin thickness was optically scanned at different increments through the *z*-axis of a CLS microscope (LSM 510 with an attached universal Zeiss epifluoroscence microscope). Optical excitations were carried out with a 488 nm Argon laser beam and fluorescence emission was detected above 560 nm for RR.

2.8. Skin permeation studies

The *in vitro* skin permeation of melatonin loaded ethosomal formulations was studied using locally fabricated Franz diffusion cell with an effective permeation area and receptor cell volume of 1.0 cm^2 and 10 ml, respectively. The temperature was maintained at 32 ± 1 °C. The receptor compartment contained 10 ml PBS (pH 6.5) and was constantly stirred by magnetic stirrer (Expo India Ltd., Mumbai, India) at 100 rpm. Dermatomed (500 μm thickness) human cadaver skin from abdominal areas was obtained from District Hospital, Sagar, India, and stored at −20 °C. The dermatomed skin was mounted on a receptor compartment with the stratum corneum side facing upward into the donor compartment. The ethosomal formulation (200 μl) was applied on the skin in donor compartment, which was then covered with a parafilm to avoid any evaporation process. Samples (200 μl) were withdrawn through the sampling port of the diffusion cell at predetermined time intervals over 24 h and analyzed for drug content by HPLC. The receptor phase was immediately replenished with equal volume of fresh diffusion buffer. Triplicate experiments were conducted for each study. Similar experiments were performed with liposomal formulation and hydroethanolic MT solution. Sink conditions were maintained throughout all the experiment.

The amount of melatonin retained in the skin was determined at the end of the *in vitro* permeation experiment (24 h). The skin was washed 10 times using a cotton cloth immersed in methanol. A sample of skin was weighed, cut with scissors, positioned in a glass homogenizer containing 1 ml of methanol, and homogenized for 5 min with an electric stirrer. The resulting solution was centrifuged for 10 min at 7000 rpm. The supernatant was analyzed for drug content by HPLC.

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.

2.9. FT-IR analysis of human cadaver skin

The prepared human cadaver skin as mentioned above was treated with 200 μl of ethosomal, 30% hydroethanolic and liposomal solution for 6 h. The treated skin samples were washed with water and blotted dry. The FT-IR spectrum of the cadaver skin was recorded in the range of 3000–1000 cm^{-1} using FT-IR multiscope spectrophotometer (Perkin-Elmer, Buchinghamshire, UK). The FT-IR spectrum of the control human cadaver skin was also recorded [22].

2.10. Skin irritancy studies

The skin irritancy (erythema) potential of ethosomes, liposomes and hydroethanolic solution (30% v/v ethanol) was evaluated as reported elsewhere [12,23]. Briefly, irritancy of different formulation was determined in male albino rabbits (1.9–2.0 kg). The animals were housed in an air-conditioned room (20 °C) and hair at the back was trimmed short, 24 h before the beginning of assay. The animals were divided in four groups of six each. First, second, and third groups received ethosomal, liposomal and hydroethanolic solution, respectively. Three squares were drawn on each side of the back of each rabbit, and 200 μl formulation was applied on each square. After exposure for 32 h, the test substance was removed and exposed skin was scored depending on the degree of erythema, as follows: no erythema – 0, very slight erythema (barely perceptible- light pink) – 1, well-defined erythema (dark pink) – 2, moderate to severe erythema (light red) – 3.

2.11. HPLC assay of melatonin

The quantitative determination of melatonin was performed by HPLC using methanol:water (50:50) as mobile phase at a flow rate of 0.5 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 223 nm using SPD-M10A vp diode array UV detector (Shimadzu, Japan), melatonin peaks were separated with a retention time of 5.5 min.

2.12. Statistical analysis

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

3. Results and discussion

Ethanol is known as an efficient penetration enhancer and is commonly believed to act by affecting the intercellular region of the stratum corneum, thus enhancing penetration [24,25]. This penetration enhancing effect of ethanol can be attributed to two effects: (a) increase in thermodynamic activity due to evaporation of ethanol known as 'Push effect' and (b) pull effect in which penetration of drug molecule is increased due to reduction in barrier property of SC by ethanol [22,26]. Embodying high concentration of ethanol in liposomes led to the formation of ethosomes, which are very potent transdermal drug delivery system, having high entrapment efficiency for wide range of molecules including lipophilic drugs and are effective at delivering drugs to and through the skin. Ethosomes prepared from 2% Soya PC and 30% ethanol when examined by TEM appeared as unilamellar vesicles with a predominant spherical shape (Fig. 1a). Surface morphology and three-dimensional nature of ethosomes were further confirmed by SEM, justifying the vesicular characteristics possessed by this novel carrier (Fig. 1b).

In terms of vesicular characterization, the vesicular size of 122 ± 3.5 nm presents an ample opportunity to the MT loaded ethosomal system to attain a better skin permeation profile, as the vesicular size has been reported to affect the permeation parameters [27]. An optimum polydispersity index (0.032 ± 0.011) of ethosomal formulation could better justify the homogeneous nature of the prepared MT formulation (Table 1).

The melting temperatures of phospholipid in the ethosomal systems (T_m) were evaluated by DSC as a measure of demonstrating lipid bilayers' fluidity. DSC scans showed transition temperature (T_m) of −8.9 and 16.6 °C, respectively, for ethosomes and liposomes. Calorimetric studies

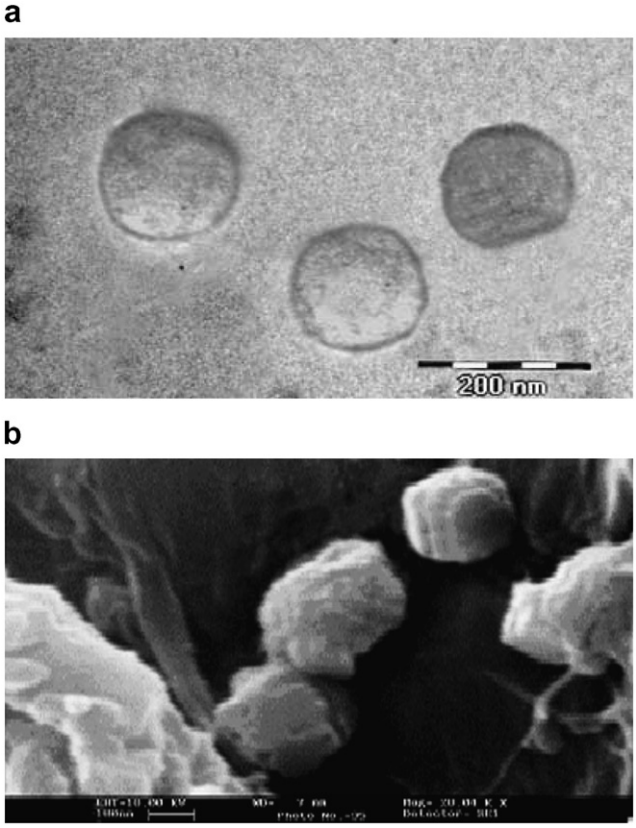

Fig. 1. (a) Visualization of ethosomes by Transmission Electron Microscopy (X 1,10,000). (b) Visualization of ethosomes by Scanning Electron Microscopy (bar 100 nm).

demonstrated low T_m values for ethosomal system as compared to liposomes suggesting a fluidizing effect of ethanol on phospholipid bilayers. Thus, ethosomes could be considered as soft, liquid state vesicles with fluid bilayers. Further, T_m of MT (1.0% w/w) loaded ethosomal system was similar to that of empty ethosomal systems, suggesting presence of MT in ethosomal core.

TEM photomicrographs of ethosomes stored for 120 days at room temperature showed no significant difference in shape and lamellarity suggesting their stability under the given conditions. Table 2 shows the size of different melatonin loaded ethosomal formulations measured over the period of 120 days. Vesicular size measurements of ethosomes stored at room temperature for various time periods showed only 7.6 ± 1.2% size increase (in 120 days) suggesting a stabilizing effect of ethanol in the formulation in terms of aggregation of vesicles by providing a net negative charge on the surface to the system thus avoiding aggregation. Our findings are in agreement with the results obtained by Lopez-Pinto et al. [15]. Liposomes without ethanol showed greater aggregation and about 30% rise in vesicular size was observed in 120 days.

The MT loaded ethosomal formulations were evaluated for substantial loss of drug at various temperatures. The results suggested greater drug loss at elevated temperatures (25 ± 2, 37 ± 2 and 45 ± 2 °C) from the system as against

Table 1
Composition and characterization of melatonin loaded formulations

S. no.	Parameters	Ethosomes (2% PC, 30% EtOH)	Liposomes (2.0% PC)	Hydroethanolic solution (30% EtOH)
1	Shape and surface morphology	Spherical, unilamellar	Spherical, multilamellar	–
2	Vesicular size (nm)	122 ± 3.5	148 ± 9.0	–
3	Polydispersity index	0.032 ± 0.011	0.042 ± 0.023	–
4	% Entrapment efficiency	70.71 ± 1.4	49.2 ± 5.0	–
5	Release rate via dialysis membrane	63.2 ± 1.48	16.9 ± 1.65	–
6	Transdermal flux (μg/cm^2/h)	59.2 ± 1.22	10.9 ± 1.65	22.43 ± 0.24
7	Lag time (h)	0.9	2.7	2.1
8	% Skin drug deposition	9.46 ± 1.52	4.12 ± 0.46	6.44 ± 1.22

Values represent means ± SD ($n = 3$).

storage at refrigerated temperature (4 ± 2 °C) (Fig. 2). It might be ascribed to the effect of temperature on the gel-to-liquid transition of lipid bilayers together with possible chemical degradation of the phospholipids, leading to defects in the membrane packing. Earlier the stability of MT in lipid-based carrier has been well proved [8].

The maximum melatonin entrapment in ethosomes and in conventional liposomes estimated using minicolumn centrifugation technique was found to be 70.71 ± 1.4% and 49.2 ± 5.0%, respectively (Table 1). The greater entrapment of melatonin in ethosomes as compared to earlier reported elastic liposomes (67.1 ± 2.2%) [8] and conventional liposomes could be attributed to the greater solubility of melatonin in ethanol present in ethosomal core.

The ability of ethanolic lipid vesicles to deliver melatonin was investigated by determining the flux of melatonin across dermatomed human skin, and skin deposition of the drug. The cumulative amount of melatonin permeated per unit area across excised human cadaver skin via various formulations was plotted as a function of time (Fig. 3). The value of steady state transdermal flux for MT loaded ethosomal formulation was observed to be 59.2 ± 1.22 μg/h/cm^2 with a lag time of 0.9 h. Hydroethanolic solution and conventional liposomes provided significantly lower flux values ($P < 0.005$) and longer lag time of 22.43 ± 0.24 μg/h/cm^2, 2.1 h and 10.9 ± 1.65 μg/h/cm^2, 2.7 h, respectively, as compared to ethosomal systems (Table 1). MT loaded ethosomal formulation also led to better skin drug deposition (9.46 ± 1.52%), possibly due to combined effect of ethanol and phospholipids thus providing a mode for sustained

Table 2
Stability of ethosomes: vesicle size (nm) over time

Days (after preparation)	Vesicular size (nm)	
	ETE2	Liposomes
1	121 ± 3.0	148 ± 2.5
15	124 ± 2.5	159 ± 3.3
30	126 ± 3.5	164 ± 1.8
60	128 ± 4.0	172 ± 3.5
90	130 ± 3.5	181 ± 2.8
120	131 ± 4.5	193 ± 3.5

Values represent means ± SD ($n = 13$).

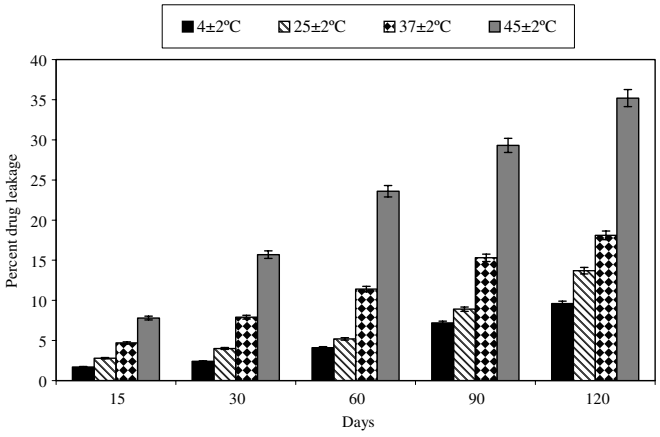

Fig. 2. Extent of MT leakage from ethosomal formulation at different temperatures during storage. Values represent means ± SD ($n = 3$).

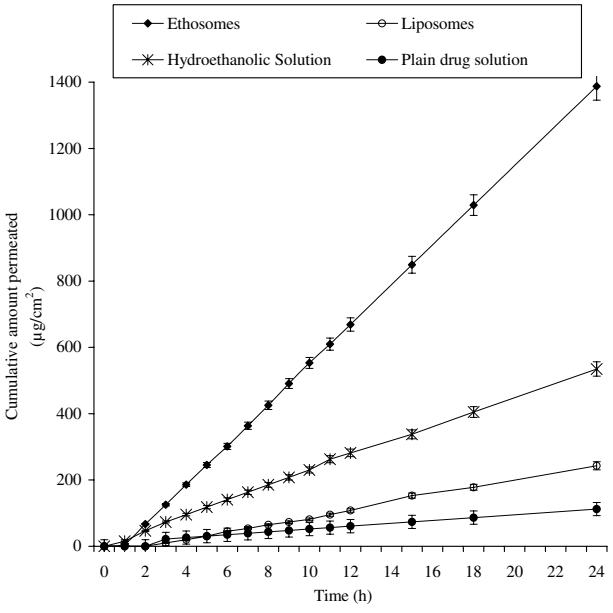

Fig. 3. Comparative cumulative amount of melatonin permeated from ethosomal formulation, liposomal formulation, hydroethanolic and plain drug solution in a 24 h study via human cadaver skin. Values represent means ± SD ($n = 3$).

delivery of melatonin (Table 1). These findings are in good agreement with previous observation of cannabidol ethosomes [28] and ammonium glycyrrhizinate ethosomes [16], which produced significant accumulation of these drugs in the skin.

The skin permeation profile of MT from the proposed novel melatonin formulation (ethosomes) is superior in all aspects than that reported by us earlier, taking elastic liposomes as a carrier for melatonin [8]. In terms of flux, ethosomal system provided a greater flux (59.2 ± 1.22 μg/h/cm^2) as compared to the earlier reported data using elastic liposomes (51.2 ± 2.2 μg/h/cm^2) as delivery module. Also a lower lag time of 0.9 h was observed with ethosomal melatonin formulation as compared to 1.1 h obtained with that of elastic liposomes. The better permeation profile from ethosomal carriers could be attributed to the better entrapment efficiency of MT in ethosomes, enhanced fluidity possessed by these carriers and a synergistic mechanism between ethanol, phospholipid vesicles and skin lipids leading to an enhanced flux and decreased lag time. Further the shortest lag time obtained, i.e., 0.9 h in this study, in comparison to those reported by Kandimalla et al. [29] and Oh et al. [30] of 5.6 and 2.2 h, respectively, is more favorable and clinically significant.

FT-IR spectral profile of stratum corneum provides a measure of the fluidity of stratum corneum lipids. On comparison of spectral profile of untreated SC, treatment of SC with liposomes, hydroethanolic solution and ethosomes resulted in a shift to a higher frequency and an absorbance broadening for both the C–H symmetric stretching absorbance frequency peak near 2850 cm^{-1} and C–H asymmetric stretching absorbance near 2920 cm^{-1} (Table 3 and Fig. 4). These results suggest that in case of liposomes, the externally applied PC can disrupt the SC lipid structure [31], whereas in case of hydroethanolic solution the ethanol could probably increase the rotational freedom of lipid acyl chains leading to increased fluidity of skin lipids. According to Harrison et al. [32], SC lipid exists as a solid gel phase at physiological temperature (~32 °C). In context to our DSC studies the ethosomal carriers exist as liquid state vesicles above −8.9 °C, suggesting their transdermal permeation potential via an interaction with solid gel phase SC lipids thus leading to disruption, extraction and fluidization of the SC lipids. Other groups have reported similar

Table 3
Effect of various formations on the C–H asymmetric and C–H symmetric stretching absorbance shifts on the acyl chains of stratum corneum lipids

Treatments	C–H symmetric stretching	C–H asymmetric stretching
No treatment	2850.24 ± 1.1	2920.14 ± 1.12
Liposomes	2851.76 ± 0.88	2921.76 ± 0.88
30% Hydroethanolic solution	2852.14 ± 1.15	2924.42 ± 0.68
Ethosomes	2854.42 ± 1.19	2925.12 ± 1.11

Values represent means ± SD ($n = 3$).

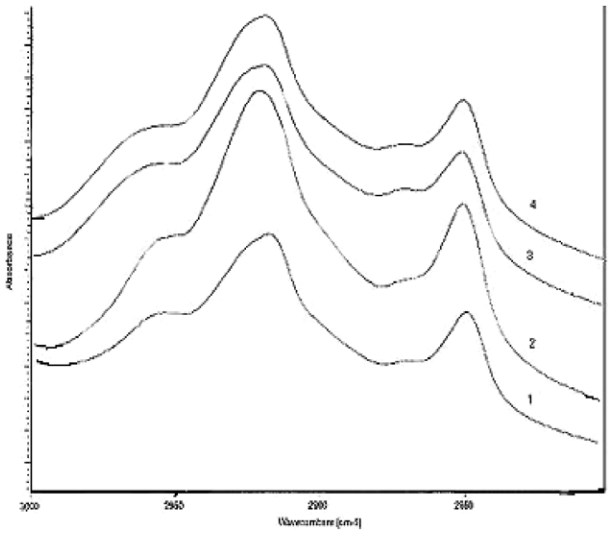

Fig. 4. FT-IR spectra of human cadaver skin after 6 h. 1. Untreated skin, 2. Liposomes, 3. Hydroethanolic solution, 4. Ethosomes.

results of better interaction of liquid state vesicles with human skin as compared to gel state vesicle earlier [33–35]. Also, a synergistic penetration enhancing effect of phospholipid and ethanol could be observed in case of ethosomes, as visualized from our FT-IR data (higher frequency shift and absorbance broadening as compared to liposomes and hydroethanolic solution), suggesting greater mobility of SC lipids on application of ethosomes. Hence it can be hypothesized that ethosomes penetrate the skin via disrupted stratum corneum's lipid bilayer organization.

To support the above hypothesis CLS microscopic studies were conducted. The extent of vesicular penetration measured by CLS microscopy after application of three systems (a) ethosomes, (b) hydroethanolic solution, (c) conventional liposomes each containing 0.03% Rhodamine Red clearly delineated the transdermal potential of ethosomal carrier. The use of RR loaded ethosomal system resulted in an increase in both the depth (up to 240 μm) (Fig. 5a and b) of penetration and in fluorescence intensity (Max FI = 160 AU) as compared to rigid liposomes that were confined to few micrometers depth only and maximum fluorescence intensity (Max FI) was found to be 40 AU with depth of penetration up to 80 μm. Hydroethanolic solution was also effective in permeating probe up to 160 μm, though very low fluorescence intensity was observed as compared to ethosomal system with a maximum fluorescence intensity of 80 AU (Fig. 6). This prominently efficient delivery of RR by ethosomal carriers suggests their enhanced penetration in the depths of the skin supporting the above-mentioned hypothesis (Fig. 5b). CLSM study also revealed the characteristics of ethosomes supporting the permeation of MT to the deeper layers of the skin.

An important characteristic to be evaluated before the proposal of a drug carrier as a potential transdermal drug

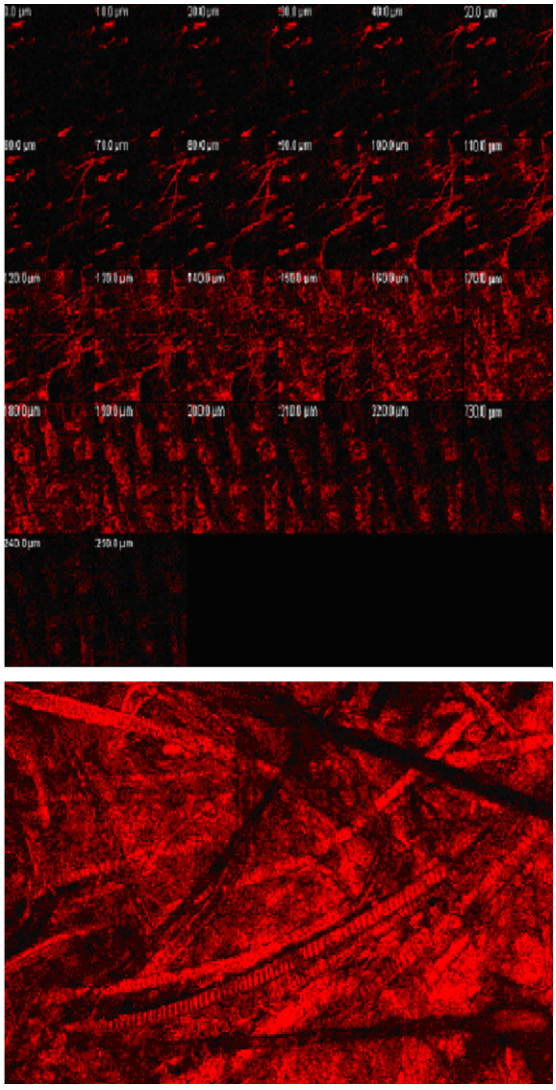

Fig. 5. (a) CLS photomicrograph of penetration of Rhodamine Red from ethosomes applied non-occlusively onto nude rat skin. (b) CLSM optical slice following skin delivery of Rhodamine Red from ethosomes applied on rat skin.

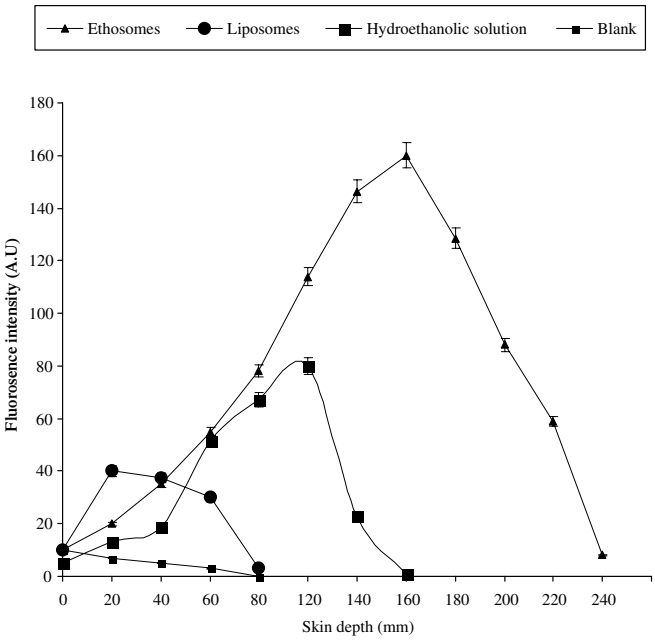

Fig. 6. Fluorescence intensity (AU) vs. skin depth (μm) studies revealing comparative skin penetration profile of ethosomes, liposomes, hydroethanolic solution and blank. AU, arbitrary units. Values represent means ± SD ($n = 3$).

Table 4
Dermal irritation scores of formulation in terms of erythema

Rabbit No.	Erythema scores			
	Saline solution	Ethosomes	Liposomes	Hydroethanolic solution
1	0	1	1	1
2	0	1	0	2
3	0	1	0	1
4	0	0	1	2
5	1	1	0	2
6	0	0	0	1
Average score	0.16	0.66	0.33	1.5

Values represent means ± SD ($n = 3$).

delivery system is its *in vivo* skin tolerability/irritancy. As, skin non-irritancy of MT is well justified by Kanninkkanan et al. [36], we conducted the skin irritancy studies of empty formulations. Measurement of erythema scores upon exposure of hairless rabbit skin to various formulations including saline solution (0.9% w/w NaCl solution, control), ethosomal formulation, liposomal and hydroethanolic solution (30% w/w ethanol) revealed that saline, liposomes and ethosomes showed no significant erythema, whereas remarkable skin erythema was observed with hydroethanolic solution (Table 4), demonstrating that ethanol present in the ethosomal formulation is not able to act as a skin erythema inducing agent, even though present in high concentration. Similar results from *in vivo* experiments on human subjects following reflectance spectrophotometry procedures were obtained by Paolino et al. [16].

4. Conclusions

The current investigation revealed that MT loaded ethosomes provided an enhanced transdermal flux, lower lag time, higher entrapment efficiency and low skin irritancy potential, thus leading to the generic conclusion that this approach offers a suitable approach for transdermal delivery of melatonin.

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