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Itraconazole loaded ethosomal gel system for efficient treatment of skin cancer

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Abstract

Topical application of Itraconazole for the treatment of Basal cell carcinoma represents a new hope in dermatology. Itraconazole for treatment of Basal cell carcinoma is an alternative therapeutic approach for the efficacious treatment of non melanoma skin cancer. Itraconazole loaded ethosomes were prepared and characterized by vesicular shape, vesicular size, entrapment efficiency. Ethosomal gel were prepared and characterized by pH, viscosity, wash ability, spreadibility, drug content, drug release study, stability study, in vivo skin tolerability and antiproliferative activity. Transmission Electron Microscopy (TEM), and Dynamic Light Scattering (DLS) characterize ethosomes as spherical, unilamellar structures having low polydispersity (0.384 ± 0.037) and nanometric size range (169.0 ± 49.0), % Entrapment efficiency of Itraconazole in ethosomal carrier was found to be 82.00 ± 1.78. Ethosomal gel were prepared by using Carbopol.The viscosity was found to be in the range of 1600±1.72 to 1740±1.73 cps. The spread ability was found to be in the range of 6.4g.cm/sec. The drug content of the ethosomal gel formulations ranged from 0.384-0.386 mg/gm. The value of steady-state trans dermal flux was observed to be 54.2 ± 1.46 μg/h/cm with a lag time of 1.2 hrs with formulation EG1. Stability studies revealed no noticeable changes in drug release profile occurred. Skin irritation study on rabbit skin suggested that ethosomal gel may offer a suitable approach for trans dermal delivery of Itraconazole. The further, antiproliferative study shows that Itraconazole loaded ethosomes is significantly more toxic than the free drug on BCC1/KMC cell line, thus making it a potential alternative to the standard therapy

Keywords: Ethosomal gel; Itraconazole; Basal cell carcinoma; Antiproliferative study; skin irritation study

Introduction

Basal cell carcinoma (BCC) is the most common non-melanoma skin cancer accounting for 80% of non-melanoma skin cancer (NMSCs) [1]. Worldwide incidence of BCC is increasing gradually by about 10% per annum [2]. The approximately 30% risk to develop BCC during lifetime of the Caucasian population. [3]. The most significant risk factor of BCC is genetic predisposition and exposure to ultraviolet radiation (UVR) [4]. UVR influence BCC development by DNA damaging and immunosuppression [5]. UVB rays is primary established risk factor in the development of BCC and is a more potent and responsible cause of cutaneous damage and is believed to be the [6].

The typical BCC can be identified by pearly pink or shiny colored pa pule with telangiectasia or cystic nodule. Thus, a skin biopsy is performed for histopathologic analyses to diagnose basal cell carcinomas. A shave biopsy is most common method under local anesthesia. BCC originate from basaloid epithelia located in the bulges' region of the hair follicle, in the epidermal stem cells of the outer root sheath and the follicular matrix cells, and in specific basaloid cells of the interfollicular epidermis [7]. BCC cells also express keratins type 5, 6 [8] and 14 [9] and also α-2 and β-1 integrins [10]. BCC is an invasive epithelial skin tumor having stratum corneum of a median thickness of 0.3 mm [11]. Stratum corneum influence drug uptake and

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penetration into a tumor, therefore, affect the efficacy of topical treatment.

Itraconazole widely uses asoral anti fungal drug. Now a day it has been proven that Itraconazole inhibits activation of the Hedgehog pathway therefore it has anti BCC activity. [12]. Hedgehog signal ling pathway is key promoter of BCC. Inhibition of SMO activation by PTCH1 relieves by binding of Hedgehog protein to PTCH1 receptor.SMO signaling occurs constitutively due to mutation in the PTCH1 receptor. Itraconazole directly interact with SMO lead to inhibition of expression of transcription factors glioma-associated (Gli) causes inhibition of SMO signaling.

Transdermal drug delivery for BCC offers many advantages as compared to traditional drug delivery systems because it avoids systemic side effects and directly administered drug on the site of action. Transdermal drug delivery increases patient acceptability (non invasiveness), avoid gastrointestinal disturbances which the drug permeates through various layers of skin, via a passive diffusion pathway. However, stratum corneum is the most formidable barrier to the passage of most of the drugs. Ethosomes have the potential of overcoming the skin barrier and have been reported to enhance permeability of drug through the stratum corneum barrier.

Ethsomesare soft, malleable lipid vesicles made of water, phospholipids and alcohol (ethanol, isopropyl alcohol or polyol) in relatively high concentration(20-45%). Ethanol act as a permeation enhancer and stabilizer. Ethanol fluidizes lipid bilayer of stratum corneum by intercalating into intercellular lipids and decreases the density of the lipid multi layer thus allowing the soft, malleable ethosomal vesicles to penetrate the disorganized lipid bilayer of stratum corneum [13]. Malleability of Ethosomal vesicles are increases because ethanol increases the fluidity of ethosomal lipids thus ethanol facilitate permeation of ethosomes smoothly inside the deep skin layers, where it got fused with skin lipids and releases the drugs into the deep layer of a tumor.

The carbopol is a polymer of acrylic acid. It is a hydrophilic polymer and its volume increases in the presence of water because it has a large amount of carboxyl groups on the polymer backbone ionize, resulting in repulsion between the negative charges, which adds to the swelling of the polymer. The carbopol molecules change their configuration and increase the viscosity of the liquid in the presence of water and form a gel. Carbopol swell in water up to 1000 times of their original volume to form a gel when exposed to a pH environment above 4.0 to 6.0. Because the pKa of these polymers is 6.0 to 0.5. [14]. Carbopol polymers are very well suited to aqueous formulations of the topical dosage forms because it is safe & effective use in topical gels, creams, lotions, and ointments. It is non-sensitizing with repeat. Carbopol polymers provide an excellent vehicle for drug delivery due to their extremely high molecular weight, they cannot penetrate the skin or affect the activity of the drug. Carbopol polymers have received extensive review and toxicological evaluation. Carbopol has been classified as Category 1 GRAS (Generally Recognized As Safe) materials.

In this work, Itraconazole loaded ethosomal gel were prepared and characterized as a topical formulation for the treatment of BCC. Physicochemical and technological features of Itraconazole loaded ethosomal gel were investigated, and the anticancer activity of Itraconazole loaded ethosomal gel was assayed in comparison with the free drug both as cellular death and proliferation using a human skin BCC cell lines, which represent the malignant progression and differentiation of BCC [15].

Material and Methods

Materials

Phospholipon 90G(PL-90G) (93.0 ± 3.0% phosphatidylcholine), it was used without any purification. Absolute ethanol, Isopropanol, Itraconazole (HPLC purity P99%). RPMI-1640 were purchased from Sigma Aldrich. BCC1/KMC cell line was purchased by ATCC catalog no.TE 354. T (ATCC CRL-7762^{TM}). Double distilled pyrogen-free water was used throughout experimental investigations. Carbopol 934 were purchased from Sigma Aldrich, triethanolamine, phosphate buffer pH 6.8. All other materials were of analytical grade.

Methods

Preparation of ethosome

Ethosomes were prepared by the cold method as reported and described earlier [16]. Briefly, ethosomal suspensions were made up of 2% w/v PL-90G and 30% absolute ethanol. PL-90G and Itraconazole were dissolved with absolute ethanol, and prepared lipid suspension was then hydrated with water, added slowly dropwise with help of micro pipette under a constant mixing with a magnetic stirrer at 700 rpm (Midi MR1 Digital IkamagR; IKA-WERKE). Prepared ethosomes were kept at room temperature for 30 min under continuous stirring (Orbital Shaker KS 130 Control, IKA-WERKE).

Ethosome size determination

Mean size and size distribution of ethosomes were determined in triplicates at 25 ± 2^0 C using a Zetasizer Nano ZS (Malvern Instruments Ltd.) light scattering apparatus. Ethosomal suspension was diluted with the same ethanol/water volume ratio

used for their preparation, previously filtered through 0.22 μm polypropylene filter (Whatman, Clifford). Size distribution and mean size data of various ethosomal formulations are the mean of three different batches ± standard deviation.

Ethosome shape and surface morphology determination

Transmission Electron Microscope (TEM) (Philips CM12 Electron Microscope) were used for ethosomal vesicles visualizing. Different formulations were centrifuged at 20,000 rpm for 90 min at 4 ^{0}C by using an ultra centrifuge (RM-12C, Remi Pvt. Ltd.). Samples were dried on the carbon-coated grid and negatively impregnated with an aqueous solution ofphosphotungstic acid. After drying the specimen was observed under the microscope at 10–100 k-fold enlargements at an accelerating voltage of 100 kV.

Entrapment efficiency

The entrapment efficiency of Itraconazole in ethosomes was evaluated using the ultracentrifugation method. Ethosomal suspensions (1 ml) were transferred into 2 ml polypropylene centrifuge tube and centrifuged at 20,000 rpm for 90 min at 4 ^{0}C by using an ultra centrifuge (PR-23, Remi Pvt. Ltd.). The supernatant was separated from the pellet and analyzed by using high performance liquid chromatography (HPLC) (LC-2010HT, Shimadzu Pvt. Ltd.) apparatus than pellet was dissolved in cold methanol (HPLC grade) and immediately analyzed by HPLC [17]. The entrapment efficacy (EE) of ethosomes was expressed according to the following equations:

$$\% EE = [D_T - D_S] / D_T \times 100$$

And

$$\% EE = [D_E / D_T] \times 100$$

Where, D_T is the total amount of drug was added during the preparation of ethosomes, D_S is the amount of drug was determined in the supernatant, and D_E is the amount of drug encapsulated in the ethosomal pellet. The values of entrapment efficiency obtained using the two different equations differed by less than 3%.

Preparation of ethosomal gel

On the basis of the results of ethosomal vesicle evaluation, the best achieved ethosomal vesicles formulation was prepared into gel formulation by incorporated into carbopol gel (1%, 1.5%, 2% w/w). The carbopol 934 powder was slowly added to distilled water and kept at 100^{0}C for 20 min and triethanolamine was added to it drop wise as shown in table 1. [18]

Measurement of pH

The values of pH of all the ethosomalgel formulations were measured by using digital pH meter (ELICO.LI 610 pH meter) at the room temperature. 1gm of gel dissolved in 30ml of distilled water (pH 7.0). A glass rod was dipped into solution of ethosomal gel in a beaker. The readings were taken for average of 3 times. [16]

Measurement of Viscosity

The viscosity of the developed ethosomal gel formulations were determined by using

A Brookfield digital viscometer (Model DV-II, USA) equipped with spindle V-72. The apparent viscosity was mea- sured at shear rate 50 rpm at room temperature, after a 3-min rest time. [18]

Wash ability

A small quantity of gel was applied on the skin and washed with water in order to check the wash ability of gel.

Spreadability

Spreadability of gel was determined by using modified wooden block and glass slide apparatus. A measured amount of ethosomal gel was placed on fixed glass slide; the movable pan with a glass slide attached to it and was placed over the fixed glass slide, such that the gel was sandwiched between the two glass slides for 5min. The weight was continuously removed. Sp readability was determined using the formula. [19]

$$S = M/T$$

Where, S is the Spreadability in g/s, M is the mass in grams & T is the time in seconds.

Drug content

One gram of ethosomal gel was dissolved in a100 ml of phosphate buffer pH 6.8 stirred constantly for 2 days by using magnetic stirrers. The resultant solution was filtered and content was analysed by U. V spectrophotometer. The release profiles of Itraconazole loaded ethosomes were measured using vertical

Drug release study

Franz diffusion cells (EMFDC-06, Orchid scientific Pvt. Ltd.) having a receptor compartment with a nominal volume of 4.75 ml, a diffusion surface membrane area of 0.75 cm^2, and a donor compartment having a maximum volume of 2 ml. A synthetic poly carbonate membrane with a molecular cut-off of 20,000 daltons was placed between donor compartment and receptor compartments. Ethosomal gel of 200 μl was added to the donor

Table 1 Composition ofdifferent ethosomal gel formulation

Gel formulation	Itraconazole loaded ethosomal suspension (ml)	Carbopol 934 (% w/w)	Triethanolamine (ml)	Phosphate buffer (pH 6.8)
EG1	20	1	0.5	q.s.
EG2	20	1.5	0.5	q.s.
EG3	20	2	0.5	q.s.

compartment, and the receptor was filled up with phosphate buffer of pH 6.8 at the same v/v ratio used for the preparation of the different vesicular colloidal systems. The receptor fluid was stirring continuously at 600 rpm using a magnetic anchor (1 mm length), and the temperature kept at 37.0 ± 0.5 ^{0}C. [20]

The experiment was carried out up to 24 hrs, and 1 ml of the receptor fluid was withdrawn at predetermined times using a syringe and then immediately replaced with the same volume of fresh fluid. These samples were immediately analyzed by HPLC (LC-2010HT, Shimadzu Pvt. Ltd.). The experiment was performed in triplets and results were expressed as the average of three different experiments ± standard deviations. [21]

The release kinetics of Itraconazole loaded ethosomes were determined by the best fit of the experimental data by applying the Higuchi release model equation:

$$M_t / M_\infty = K_h\, t^{1/2}$$

Where, M_t is the fraction of drug released at each time points (t), M_∞ is the total amount of drug present in ethosomal suspension and $K_h$represents the Higuchi release kinetic constant. This equation can be simplified for a zero-order release kinetic inthe following form:

$$M_t / M_\infty = K_0\, t$$

where $K_0 represents the zero-order release kinetic constant.$

Stability Studies

Stability study was carried out for ethosomal gel preparation at two different temperature i.e. refrigeration temperature (4 ± 2° C) room temperature (27 ± 2° C, RH ± 5 RH) for 8 weeks (as per ICH guidelines). The gel formulation was subjected to stability study and stored in borosilicate container to avoid any sort of interaction between the ethosomal gel preparation and glass of container, which may affect the observations. Sample was collected for every 2 weeks and content was analyzed at 262 nm in U. V spectrometer.

Skin irritancy studies

The skin irritancy or erythema potential of ethosomes, was evaluated as reported elsewhere [22]. Briefly, skin irritancy of the formulation was determined in male albino rabbits (1.9–2.0kg). The animals were housed at 20^{0}C in an air-conditioned room and hair at the back was trimmed short, 24 h before the beginning of assay. The animals were divided in two groups of six each. First, groups received ethosomal gel and the second group was control (0.9% w/w NaCl solution). Each rabbit subjected to draw three squares on each side of the back, and 200 µl formulation was applied on each square. The test substance was removed after exposure for 32 h, and exposed skin was scored depending on the degree of erythema, as follows: no erythema — 0, very slight erythema (light pink) — 1, well-defined erythema (dark pink) — 2, moderate to severe erythema (light red) —3.

Anti-proliferative activity

The cytotoxicity of Itraconazole loaded ethosomal vesicle was determined by MTT assay in BCC1/KMC cell line on 96-well plates. Cells were seeded in a dilution rate of 1 × 10^4 each well at 37^{0}C and 5 % CO_2 by using RPMI-1640 medium containing 10 % fetal bovine serum and 1 % penicillin/streptomycin. They were allowed to attach for 48 h. Thereafter culture media was removed and cells were treated with free Itraconazole and ethosome loaded Itraconazole at concentrations of 6.383, 3.596, 1.585, 0.792, 0.391 and 0.162 µM. The drug-containing culture was removed after 24 hrs and 80 µL MTT solutions (0.5 mg/mL, pH 7.4) was added to each well and incubated for 2 hrs Later, MTT was discarded and to dissolve the formazan crystals, 100 µl isopropanol 100 % was added. Finally, absorbance was read at 540 nm in an Elisa reader (Bio Tek Instruments). IC_{50} was measured by statistical package Pharm-PCS software.

Results & Discussion

Preparation and evaluation of Ethosomes

Ethosomes were prepared by cold methods having vesicle size 169.0 ± 49.0 nm shows ethosomes had optimum size. Dynamic light scattering analysis showed that physicochemical properties including mean size, size distribution, and zeta-potential were depended on the composition of the vesicle. Ethanol and PL 90G influenced vesicular size distribution. Experimental findings showed that a narrow size distribution with a polydispersity index (PDI) of 0.384 ± 0.037 was obtained for ethosomalvesicles. Prepared ethosomes were shown 82.00 ± 1.78 % of drug entrapped inside the vesicles this is good percent of drug pene-

tration. Prepared ethosomes were examined by TEM appeared as unilamellar vesicles with a predominant spherical shape (Figure 1).

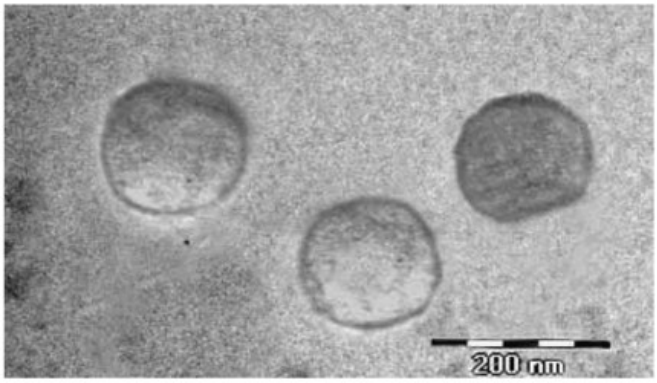

Figure 1 Visualization of ethosomesby Transmission Electron Microscopy (X 1,10,000).

Preparation and evaluation of ethosomal gel

Itraconazole loaded ethosomal gel have been prepared and evaluated for following parameters.

Measurement of pH

The pH measurement of the developed ethosomal gel carried out by using digital pH meter at room temperature. Results are shown in Table 2. The pH value of the developed ethosomal gel was found to be in the range of 6.80±0.05 to 6.84±0.08 which demonstrate that the prepared gels will irritation free to the skin.

Viscosity

The viscosity measurement results of the developed ethosomal gel, carried out by using Brookfield digital viscometer at room temperature. The results are shown in Table 2. The viscosity of the developed ethosomal gel was found to be in the range of 1600±1.72 to 1740±1.73 cps.

Washability

Prepared ethosomal gel is easily washable without leaving any residue on thesurface of the skin.

Spreadability

The spreadability of the ethosomal gels was found to be in the range of 6.4g.cm/sec reveal that its spread smooth and uniformly.

Drug content

The drug content of the ethosomal gel formulations ranged from 0.384-0.386 mg/gm. The values obtained from the drug content are shown in Table 2, it was concluded that there was no loss of drug during the gel preparation process.

Table 2 Results of ethosomalgel evaluation.

Formulation	pH	Viscosity (CPS)	Drug content (mg/gm)
EG1	6.83 ± 0.16	1600 ± 1.72	0.384 ± 0.021
EG2	6.80 ± 0.51	1689 ± 1.45	0.386 ± 0.016
EG3	6.84 ± 0.46	1740 ± 1.68	0.386 ± 0.034

Values represent means ± SD (n = 3).

Drug release study

Experimental findings showed that Itraconazole loaded ethosomal gel (formulation EG2) permeated through the dialysis membranes faster than the Itraconazole suspension. The ability of ethosomal gel to deliver Itraconazole was investigated by determining the flux of Itraconazole across dialysis membrane. The cumulative amount of Itraconazole permeated per unit area across dialysis membrane via formulation E5 was plotted as a function of time (Figure 2). The value of steady-state transdermal flux for Itraconazole loaded ethosomal formulation was observed to be 54.2 ± 1.46 μg/h/cm with a lag time of 1.2 hrs with formulation EG1 and 51.3 ± 1.46 μg/h/cm with a lag time of 1.22 hrs with formulation EG2.

Kinetic analysis of release mechanism

The drug release data of optimized formulation EG1 and EG2 were fitted to models representing Higuchi's, zero order, first order, Banker - lonsdale and Korsmeyer's- peppas equation kinetics to know the release mechanisms with the help of software Sigma plot (v12).

Where,

Q_t: Amount of drug released in time t

Q_0: Initial amount of drug in the tablet

k_0 K_1 K_H Ks: release rate constants

n: Release exponent (indicative of drug release mechanism)

W: Initial amount of drug in dosage form,

Wt: Remaining amount of drug in dosage form at time t

Ks: A constant incorporating the surface–volume relation.

It was observed that the correlation coefficient values are higher (r^2 = 0.97) with Higuchi model. Higuchi proposed this

Table 3 Equation and obtainedregression coefficient value for different model.

Sr. no.	Model	Equation	R^2 for EG1	R^2 for EG2
1	Zero order	$Q_t = Q_0 + K_0 t$	0.9231	0.9215
2	First order	$\ln Q_t = \ln Q_0 - K_1 t$	0.8124	0.8023
3	Hixson–Crowell	$W_0^{1/3} - W_t^{1/3} = Ks\ t$	0.8164	0.8026
4	Higuchi	$Q_t = K_H\ t^{1/2}$	0.9902	0.9834
5	Korsmeyer–Peppas	$Qt / Q_\infty = K_k\ t^n$	0.6315	0.6234
6	Baker–Lonsdale	$(3/2)\ [1- (-1(Q_t/Q_\infty))^{2/3}] - (Q_t/Q_\infty) = K\ t$	0.6315	0.6146

model in 1961 to describe the drug release from matrix system. Higuchi model is based on the hypotheses that:

Initial drug concentration in the matrix is much greater than drug solubility

Drug diffusion is taking place only in one dimension (edge effect must be negligible).

Drug particles are much smaller than system thickness.

Matrix swelling and dissolution are in negligible level.

Drug diffusivity is constant.

perfect sink conditions are always attained in the release environment.

Higuchi model can be written as:

$ft = Q = K_H.\ t_{1/2}$

where,

K_H is the Higuchi dissolution constant

The data obtained were plotted as cumulative percentage drug release versus square root of time.

On comparing the release kinetics data (Table 4), formulation EFG1 were found to release the drug in accordance to Higuchi Kinetics which were indicated by the highest correlation coefficient values.

Stability Studies

The ability of vesicles to retain the drug was assessed by keeping the ethosomal gel formulation at different temperature. Optimized ethosomal gel formulation (EG1) were selected for stability studies. Ethosomal gel formulations were observed for any change in appearance for a period of 8 weeks showed no noticeable changes in drug release profile occurred. It was concluded that the ethosomal gel system is more stable at specified storage conditions.

Skin irritation Studies

Skin tolerability/irritancy is an important characteristic to be evaluated before the proposal of a drug carrier as a potential transdermal drug delivery system. Measurement of erythema scores upon exposure of hairless rabbit skin of ethosomal formulations and saline solution (0.9% w/w NaCl solution, control) revealed that saline and ethosomes showed no significant erythema (Table 5),demonstrating that ethanol present in the ethosomal formulation is unable to act as a skin erythema inducing agent, even though present in high concentration.

Values represent means ± SD (n = 3).

Anti proliferative studies

MTT assay is a valid test to evaluate the efficacy of the ethosomal formulation in vitro , and hence it was used in the present study to assess the cytotoxicity of Itraconazole on BCC1/KMC cell line. The ethosomal formulation decreased cell viability by 34 % compared with the free-form. The results showed the IC_{50} of ethosomal formulation for BCC1/KMC cell line is less than that of the standard drug. Based on the definition of IC_{50} (8.2 free drugs against 1.8 of the ethosomal formulation), the cytotoxicity of the ethosomal formulation against BCC1/KMC is about 4.6 times of that of the free drug.

Conclusions

The current investigation revealed that Itraconazole loaded ethosomal gel provided an enhanced trans dermal flux, lower lag time, good entrapment efficiency and low skin irritant potential. The ethosomal formulated drug penetrate more deeper and more effectively kill cytotoxic cells. Ethosomal gel incorporated drug has a longer half-life and stronger cytotoxic effect than the free drug form. Topical penetration of drug enhances thus, leading

Table 4 Release rate constant(k) and correlation coefficient (R^2) of formulation EG1 and EG2.

Sr. no.	Gel formulation	Zero order kinetics		First order kinetics		Higuchi kinetics	
		k	R^2	k	R^2	k	R^2
1.	EG1	2.156	0.946	1.82	0.953	0.435	0.932
2.	EG2	1.864	0.916	1.24	0.931	0.386	0.895

Table 5 Erythema scores of formulations

Rabbit no.	Erythema scores	
	Saline solution	Ethosomes
1	0	1
2	0	1
3	0	1
4	0	0
5	1	1
6	0	0
Average score	0.16	0.66

to the generic conclusion that this approach offers a suitable approach for trans dermal delivery of Itraconazole.

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