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Original Research Article

Formulation Development and Evaluation of Ethosomal Gel of Amphotericin B for Treatment of Fungal Infections

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ABSTRACT

Ethosomes are novel vesicular delivery system which has great potential for topical and transdermal delivery of various drugs. Amphotericin B is a polyene antifungal drug used intravenously for systemic fungal infections. It has severe and potentially lethal side effects, therefore, it has been limited use in clinical. Ethosomes were formulated using phospholipid, cholesterol, ethanol, polyethylene glycol and purified water by cold method. Ethosomes having for evaluated like shape, vesicle size, optical microscopy, entrapment efficiency and *in-vitro* release study. F3 have better drug entrapment efficiency than the other formulation. The best formulation (F3) was used to prepare gel by using carbopol 934 as a gelling agent. The ethosomes were entrapped in gel matrix of carbopol 934 in different concentration 0.5%, 1.00% and 1.5% w/w. FT-IR studies revealed no interaction between the drug and excipients. The formulated gel formulation was evaluated with parameter pH, viscosity, spreadability, *in-vitro* release test, washability, extrudability study and stability studies. The formulation EG2 have better *in-vitro* drug release profile which contains carbopol 934 concentration 1%w/w. The present work also focuses on making the formulation more pharmaceutically acceptable.

Key words: Fungal infections, Ethosomal gel, Amphotericin B, Franz diffusion cell.

INTRODUCTION

Fungal infections are spreading on a serious note and are approximated to occur in billions of people every year (Hsu *et al.*, 2011). These infections affect different parts of body including skin, nails, hair, scalp and eye (Brown *et al.*, 2012; Fungal Research Trust, 2014). More than 20–25% of the world population is suffering from diverse type of surface fungal infections (Havlickova et al., 2008). Physiological conditions of skin (temperature around 30^0C along with the presence of moisture) provide an ideal environment for fungal growth. Skin fungal infections are more prevalent in sub-tropical countries like India and Africa where the climatic conditions are hot and humid that further accelerates the occurrence and spread of dermatomycoses. Furthermore, due to increase in the number of immunocompromised individuals in disease

conditions like AIDS/HIV, diabetes and cancer, the rate of surface fungal infections (cutaneous candidiasis) is currently on the rise (Hsu *et al.*, 2011; Baddley *et al*., 2011; Silva, 2010). Due to decreased immunity and ongoing treatments with antiretroviral and other immunosuppressant drugs, it is quite difficult to treat these patients with systemic antifungal therapy. Almost 90% of people suffering from AIDS develop at least one fungal infection over the course of disease, out of which 10–20% of infections prove fatal (Benedict and Colagreco, 1994). Thus, effective topical delivery of antifungal drug is required clinically for the effectual management of multiple disease conditions and at the same time to increase the patient compliance (Atiyeh et al., 2005). Amphotericin B (AmB), a potent antimycotic polyene macrolide, is considered as the gold standard in the treatment of fungal infections. Due to its broad spectrum activity, almost all the fungi are susceptible to AmB, which has made it the mainstay of treatment for serious fungal infections. Also, it is the first line treatment for burn-related fungemia attributed to Candida species as well as many moulds (Sanchez et al., 2014). Not only this, it is also the drug of choice for the treatment of disseminated mycosis in AIDS patients, organ transplants and cancer chemotherapy (Hsu *et al.*, 2011). This drug is also found to be clinically effective for the treatment of cutaneous leishmaniasis (Santos et al., 2012). Two types of topical formulations of AmB are available in the market, one is conventional lotion or cream (FungizoneTM, Bristol-Myers Squibb, NY) and other is liposomal carrier based gel (FungisomeTM, Life care innovations, Gurgaon, India). Conventional marketed formulations suffer from the serious drawback of clinical ineffectiveness in many of the cases due to very poor skin permeation (Santos et al., 2012). Although liposomes based formulation was found to increase the skin permeation and deposition of AmB to some extent. Still proficient carrier-based formulation is needed with good skin permeation and deposition potential so that clinical potency of AmB can be increased and AmB could be used for the effective treatment of surface fungal infections. Therefore, reliable drug delivery systems providing better drug penetration can result in better efficacy and also help in the prevention of development of resistance. The aim of the present study was to statistically optimize the ethosomal gel for enhanced skin delivery of AmB, which was effective candidate for the treatment of fungal infection.

MATERIALS AND METHODS

Ethanol, chloroform and carbopol-934 purchased from CDH chemical Pvt. Ltd. New Delhi. Dialysis membrane of Mol Wt cutoff 1200 was purchased from Himedia Laboratory, Mumbai. Demineralized and double distilled water was prepared freshly and used whenever required. All other reagents and chemicals used were of analytical grade.

Determination of λ_{max} of Amphotericin B

Accurately weighed 10 mg of drug was mixed in 10 ml of 7.4 pH buffer solution in 10 ml of volumetric flask. The resulted solution 1000µg/ml and from this solution 1 ml pipette out and transfer into 10 ml volumetric flask and volume make up with 7.4 pH buffer solution. Make appropriate

dilution to make it to a concentration range of 5-25µg/ml. The spectrum of this solution was run in 200-400 nm range in U.V. spectrophotometer (Labindia-3000+). A graph of concentration Vs absorbance was plotted.

Preparation of Amphotericin B loaded ethosomes

The ethosomal preparation was set up as per the technique reported by Touitou et al., 2000. The ethosomal method arranged were made out of phospholipid, ethanol, sedate, propylene glycol and water to 100% w/w. Phospholipid and drug were broken up in ethanol/propylene glycol blend; the blend was warmed to 30^0C in a water shower. The double distilled water warmed to 30^0C was included gradually in a fine stream, with consistent blending (mechanical stirrer) at 700 rpm in a shut vessel, blending was proceeded for extra 5 min. The system was kept at 30ºC throughout preparation. The Light Scattering method (DLS) (Malvern Zetamaster, ZEM 5002, Malvern, UK).

Zeta potential

The zeta potential was calculated according to Helmholtz–Smoluchowsky from their final milky solution of ethosomes was left to cool at room temperature. The preparation was homogenized by using vertex shaker for 15 min. In the ethosomal formulation, the lipid: ethanol ratio was optimized by taking their different ratio and all other parameters were kept remain constant. The prepared formulations were optimized on the basis of average particle size and % entrapment efficiency. Six ethosomal formulae were presented in Table 1.

Evaluation of Amphotericin B loaded ethosomes

Microscopic observation of prepared ethosomes

An optical microscope (cippon, Japan) with a camera attachment (Minolta) was used to observe the shape of the prepared ethosomes formulation.

Surface charge and vesicle size

The vesicles size and size distribution and surface charge were determined by Dynamic electrophoretic mobility. For measurement of zeta potential, a zetasizer was used with field strength of 20 V/cm on a large bore measures cell. Samples were diluted with 0.9% NaCl adjusted to a conductivity of 50 lS/cm.

Table 1 Different composition of Amphotericin B ethosomes formulation

F. Code	Drug (mg)	Phospholipid (mg)	Cholesterol (mg)	Ethanol (ml)	PEG (mg)	Water (ml)
F1	50	50	50	10	10	100
F2	50	50	100	10	10	100
F3	50	50	150	10	10	100
F4	50	50	50	10	10	100
F5	50	100	50	10	10	100
F6	50	150	50	10	10	100

Entrapment efficiency

Entrapment efficiency was determined by measuring the concentration of unentrapped free drug in aqueous medium. About 1 ml of the drug loaded ethosomes dispersion was placed in the Ependorf tubes and centrifuged at 17000 rpm for 30 min. The ethosomes along with encapsulated drug were separated at the bottom of the tubes. Plain ethosomes without drug was used as blank sample and centrifuged in the same manner. In order to measure the free drug concentration, the UV absorbance of the supernatant was determined at 284 nm. The entrapment efficiency was determined by the following equation, EE% = (Total drug) - (free drug)/ Total drug X 100.

Preparation of ethosomal gels

The incorporation of the drug loaded ethosomes (equivalent to 3%) into separate 10gm gels was achieved by slow mechanical mixing at 25 rpm for 10 minutes. The optimized formulation was incorporated into three different carbapol gel concentration 0.5, 1 and 1.5% w/w.

Table 2 Different composition of AmB gel formulation

S. No.	F. Code	Carbopol gel
1	EG1	0.5%
2	EG2	1%
3	EG3	1.5%

Evaluation of gel

Physical characteristic

The **physical** characteristic was checked for gel formulations (homogeneity and texture).

Determination of pH

The pH of the gels was determined by digital pH meter. One gram of gel was dissolved in 25 ml of distilled water and the electrode was then dipped in to gel formulation for 30 min until constant reading obtained. And constant reading was noted. The measurements of pH of each formulation were replicated two times.

Washability

Formulations were applied on the skin and then ease and extent of washing with water were checked manually.

Extrudability study

The gel formulations were filled into collapsible metal tubes or aluminium collapsible tubes. The tubes were pressed to extrude the material and the extrudability of the formulation was checked.

Spreadability

An important criterion for gels is that it must possess good spreadability. Spreadability is a term expressed to denote the extent of area to which the gel readily spreads on application to skin. The therapeutic efficacy of a formulation also depends on its spreading value. A special apparatus has been designed to study the spreadability of the formulations. Spreadability is expressed in terms of time in seconds taken by two slides to slip off from formulation, placed between, under the application of a certain load. Lesser the time taken for the separation of two slides, better the spreadability. It is determine by formula given below.

$$\text{Spreadability} = \frac{m.l}{t}$$

Where, S=Spreadability (gcm/sec)

m = weight tied to the upper slide (20 grams)

l= length of glass slide (6cms).

t = time taken is seconds.

Viscosity

The measurement of viscosity of the prepared gel was done using Brookfield digital Viscometer. The viscosity was measured using spindle no. 6 at 10 rpm and 25^0C. The sufficient quantity of gel was filled in appropriate wide mouth container. The gel was filled in the wide mouth container in such way that it should sufficiently allow to dip the spindle of the Viscometer. Samples of the gels were allowed to settle over 30 min at the constant temperature (25 ±/1^0C) before the measurements.

***In-vitro* drug release studies using the prehydrated cellophane membrane**

The prepared ethosomal gel was evaluated for *in vitro* drug release. *In vitro* diffusion study was carried out in a Franz diffusion cell using cellophane membrane. The cellophane membrane was mounted on the Franz diffusion cell. Formulation was applied through donor compartment on the dialysis membrane. Reservoir compartment was filled with 25 ml phosphate buffer of pH 7.4 The study was carried out at 37 ± 1°C and at a speed of 100 rpm for 8 h. Samples were withdrawn from reservoir compartment at 1 h interval and absorbance was measured spectrophotometrically at 284.0 nm. Each time the reservoir compartment was replenished with the same quantity of 7.4 pH phosphate buffer (Thomas, 2019; Mishra, 2018).

Drug content

Accurately weighed equivalent to100 mg of topical ethosomal gel was taken in beaker and added 20 ml of phosphate buffer pH 7.4. This solution was mixed thoroughly and filtered using Whatman filter paper no.1. Then 1.0 ml of filtered solution was taken in 10 ml capacity of volumetric flask and volume was made upto 10 ml with phosphate buffer pH 7.4. This solution was analyzed using UV spectrophotometer at λ_{max} 284 nm.

Release kinetics

In order to elucidate mode and mechanism of drug release, the *invitro* data was transformed and interpreted at graphical interface constructed using various kinetic models. The zero order release Eq. (1) describes the drug dissolution of several types of modified release pharmaceutical dosage forms, as in the case of transdermal systems, matrix tablets with low soluble drugs, coated forms, osmotic systems etc., where the drug release is independent of concentration.

$Qt = Qo + Kot$ (1)

Where, Qt is the amount of drug released in time t, Qo is the initial amount of the drug in the solution and Ko is the zero order release constant

The first order Eq. (2) describes the release from the system where release is concentration dependent e.g. pharmaceutical dosage forms containing water soluble drugs in porous matrices.

$\log Qt = \log Qo + K1\ t /2.303$ (2)

Where Qt is the amount of drug released in time t, Q is the initial amount of drug in the solution and K1 is the first order release constant.

Higuchi described the release of drug from insoluble matrix as a square root of time as given in Eq. (3)

$Qt = KH \sqrt{t}$ (3)

Where, Qt is the amount of drug released in time t, KH is Higuchi's dissolution constant (Gadakh et al., 2012).

The following plots were made: cumulative % drug release vs. time (zero order kinetic models); log cumulative of % drug remaining vs. time (first order kinetic model); cumulative % drug release vs. square root of time (Higuchi model).

Results and discussions

The absorption maxima of AmB were determined by running the spectrum of drug solution in double beam ultraviolet spectrophotometer (Labindia UV 3000+) using concentration range of 5-25µg/ml AmB in pH 7.4 phosphate buffers. AmB showed a linear relationship with correlation coefficient of 0.999 in the concentration range of 5-25µg/ml in phosphate buffer pH 7.4. Melting point of drug was found 170-172°C while it was 170°C reported in standard monograph. All the data of preformulation study were found similar as given in standard monograph which confirmed that the drug was authenticated and pure in form and it could be used for formulation development of AmB-loaded ethosomes.

Vesicle size and zeta potential of the ethosomes were measured by photon correlation spectroscopy using a Malvern Zetasizer and entrapment efficiency was determined by measuring the concentration of unentrapped free drug in aqueous medium by UV spectrophotometer the results shown in Table 3. Zeta potential of optimized ethosomes formulation (F3) was found -35.2 mV Table 4.

Table 3 Result for vesicle size and entrapment efficiency of amphotericin b loaded ethosomes

F. Code	Vesicle size(nm)	Entrapment efficiency (%)
F1	325±4	65±3
F2	325±5	62±4
F3	265±8	70±2
F4	220±5	76±6
F5	361±4	65±3
F6	365±2	59±4

Table 4 Vesicle size and entrapment efficiency of optimized

Formulation Code	Vesicle size (nm)	Entrapment Efficiency	Zeta potential
F3	265±8	70±2	-35.2

Results of evaluation of ethosomal gel formulation (EG1- EG3) of optimized formulation (F3) were incorporated into three different carbapol gel concentration 0.5, 1 and 1.5 % w/w respectively. Formulation EG2 was found to be good Table 5. Results of *In-vitro* drug release from optimized formulation (EG2) are given in table 6 was found 95.56 after 12 hrs. The *in vitro* drug release data of the formulation was subjected to goodness of fit test by linear regression analysis according to zero order, first order

kinetic equation and Korsmeyer's -pappas models in order to determine the mechanism of drug release. When the regression coefficient values of were compared, it was observed that 'r' values of formulation was maximum i.e 0.961hence indicating drug release from formulations was found to follow zero order model of drug release kinetics.

Table 5 Results of evaluation of gel formulation

F. Code	Homogeneity and Texture	pH*	Washability	Spreadability* (gm.cm/sec.)	Viscosity (cps)	% Assay
EG1	**Smooth**	6.85	Good	20.00±2	2352±12	98.89±0.25
EG2	**Smooth**	7.02	Good	17.14±1	2150±15	99.85±0.32
EG3	**Smooth**	6.92	Good	15.00±2	2032±14	99.75±0.12

Table 6 *In Vitro* drug release data for gel formulation

Time (hrs)	% Cumulative Drug Release		
	EG1	EG2	EG3
0.5	33.45	22.12	19.98
1	45.56	30.25	33.36
2	55.65	45.56	40.25
4	78.85	55.65	55.89
6	88.85	69.98	63.36
8	96.56	77.89	71.56
10	-	89.98	75.56
12	-	95.56	80.23

Table 7 Regression analysis data of ethosomal gel formulation

Formulation	Zero order	First order
EG1	0.837	0.948
EG2	0.961	0.952
EG3	0.914	0.958

Conclusion

Ethosomes of AmB were prepared successfully by using different concentrations of phospholipids and ethanol as well as the incorporation of the ethosomes into carbopol 934 base gel to obtain ethosomal gel formulations. The prepared formulations were characterized for various properties. The compositions of ethosomes and gels were manipulated to investigate their effects on the characteristics of final formulations. It can serve as a useful vehicle or the delivery of AmB through the affected part of the skin for extended period of time. This study also revealed that ethosomal gel (EG2) resides at targeted site for a relatively longer period of time with a zero order release profile. It signifies the improved patient compliance.

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