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Formulation and *in vitro* Evaluation of Metronidazole loaded Ethosomes for Antibacterial activity

Muskan Lashkari*, Kaushelendra Mishra, Priyanka Namdeo

Lakshmi Narain College of Pharmacy, Raisen Road, Bhopal-462022, Madhya Pradesh, India.

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ABSTRACT: Background: Ethosomes are non-invasive Novel modified lipid carriers composed of phospholipids, high concentration of ethanol, and water, used for dermal and transdermal delivery of molecules. **Aim:** The present study was aimed to prepare and evaluate the Metronidazole loaded ethosomes. **Method:** Ethosomes entrapping metronidazole were prepared using cold methods and the effect of varying concentration of ethanol was considered for obtaining an optimized formulation. Lecithin (2 % w/w) was used as the phospholipid to provide the structure to vesicles and propylene glycol (10 %) was used as the permeating agent. The prepared ethosomes were evaluated for shape, size, entrapment efficiency, *in vitro* drug release, and stability studies. **Results:** The vesicles were found to be of spherical to irregular shape ranging from 3.248 ± 0.733µm to 6.032 ± 2.883 µm in size. The drug entrapment in the ethosomes was studied by analyzing the amount of drug after breaking the vesicles with Triton X100 and it was found that the maximum entrapment efficiency was found to 94.43% for formulation F5 and minimum 86.24 % for formulation F1, respectively. The *in vitro* release study suggested that the maximum amount of drug released from the ethosome was 97.4 % for F1 while the least release was 91.2 % from F5 in 12 h. **Conclusion:** It could be concluded that the optimized formulation of Metronidazole loaded Ethosomes was successfully prepared which could show effective antimicrobial activity.

Corresponding author:
Ms. Muskan Lashkari
Research Scholar
Lakshmi Narain College of Pharmacy,
Raisen Road, Bhopal, 462022, India.
Tel: +91-9131913675
E. Mail ID: muskan03081999@gmail.com

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INTRODUCTION:

Transdermal drug delivery offers many advantages as compared to traditional drug delivery systems, including oral and parenteral drug delivery systems [1]. Advantages claimed are increased patient acceptability (non-invasiveness), avoidance of gastrointestinal disturbances which the drug permeates through various layers of skin, via a passive diffusion pathway. However, this limits the basic potential of these systems, as stratum corneum is the most formidable barrier to the passage of most of the drugs, except for highly lipophilic, low molecular weight drugs. To overcome the stratum corneum barrier, various

mechanisms have been investigated, including use of chemical or physical enhancers, such as iontophoresis, sonophoresis, etc. Liposomes, niosomes, transfersomes and ethosomes also have the potential of overcoming the skin barrier and have been reported to enhance permeability of drug through the stratum corneum barrier [2]. The vesicles have been well known for their importance in cellular communication and particle transportation for many years. Researchers have understood the properties of vesicle structure for use in better drug delivery within their cavities, which would tag the vesicle for cell specificity. One of the major advances in vesicle research was the finding of a vesicle derivative, known as an Ethosomes [3,4].

Ethosomes are non-invasive delivery carriers that enable drugs to reach the deep skin layers and/or the systemic circulation. These are soft, malleable vesicles tailored for enhanced delivery of active agents. They are composed mainly of phospholipids, (phosphatidylcholine, phosphatidylserine, phosphatidic acid), high concentration of ethanol and water [5,6]. The high concentration of ethanol makes the ethosomes unique, as ethanol is known for its disturbance of skin lipid bilayer organization; therefore, when integrated into a vesicle membrane, it gives that vesicle the ability to penetrate the stratum corneum. Also, because of their high ethanol concentration, the lipid membrane is packed less tightly than conventional vesicles but has equivalent stability, allowing a more malleable structure and improves drug distribution ability in stratum corneum lipids [7]. The Ethosomes were found to be suitable for various applications within the pharmaceutical, biotechnology, veterinary, cosmetic, and nutraceutical markets. These soft vesicles represent novel vesicular carriers for enhanced delivery to/through skin. The size of Ethosomes vesicles can be modulated from tens of nanometers to microns. Ethosomes were designed to enhance the delivery of drugs into the deep layers of the skin and through the skin. The enhanced delivery of actives using ethosomes over liposomes can be ascribed to an interaction between ethosomes and skin lipids. A possible mechanism for this interaction has been proposed. It is thought that the first part of the mechanism is due to the ethanol effect, whereby intercalation of the ethanol into intercellular lipids increases lipid fluidity and decreases the density of the lipid multilayer. [8] This is followed by the ethosome effect, which includes inter lipid penetration and permeation by the opening of new pathways due to the malleability and fusion of ethosomes with skin lipids, resulting in the release of the drug in deep layers of the skin [9,10].

The objective of the research work to develop a optimized formulation of Metronidazole loaded ethosome.

MATERIALS AND METHODOLOGY:

The pure drug (active pharmaceutical ingredient) metronidazole was obtained as gift sample from Medreich Pharmaceuticals, Bengaluru. The ethanol, lecithin, and propylene glycol were purchased from HiMedia, India. All chemicals were of analytical grade and procured from authorised dealers.

Pre-formulation Studies:

The pre-formulation studies of the drug and excipient provides a preliminary picture of the methodologies that could be adopted for any formulation. The pre-formulation study was carried out for confirming the compatibility amongst the drug and excipients, the analytical methodology to be used and the purity of the drug [11].

Organoleptic Evaluation:

The color, odor and taste of the obtained drug sample were observed with the help of the sensory organs.

Solubility study:

Solubility was determined in different solvents like water, HCl, ethanol and acetone in room temperature by UV-Visible spectroscopy method.

Melting point determination:

Melting point was determined by the open capillary method by placing a small quantity of powder into the capillary tube and placing it in the melting point apparatus. The temperature of the apparatus was gradually increased and the temperature at which the powder started to melt and the melting temperature was recorded.

Preparation of Calibration Curve in PBS pH 7.4:

Accurately weighed 10 mg of metronidazole was taken in 100 mL volumetric flask and dissolved in water and the volume was made up to the mark with PBS pH7.4. The solution was suitably diluted with PBS pH7.4 to obtain solutions of 10 to 50 ppm. The calibration curve was prepared by measuring the absorbance of the solutions at 340 nm using UV-Visible spectrophotometer [12].

Drug-excipient compatibility study:

FT-IR spectra matching approach was used for detection of any possible chemical interaction between drug and excipients. A physical mixture (1:1:1) of drug, chitosan and Pluronic F-68 was prepared. It was scanned from 4000 to 400 cm^{-1} in an FT-IR spectrometer. The IR spectrum of the physical mixture was compared with that of pure drug to detect any appearance or disappearance of peaks.

Preparation of ethosomes:

Ethosomes were prepared by cold method. In brief the lecithin (3 % w/v) was taken in a small round bottom flask and solubilized using ethanol (10 to 50 % v/v) and propylene glycol (10 % v/v) containing drug under mixing with a magnetic stirrer. The round bottom flask was covered to avoid ethanol evaporation. Distilled water was added slowly with continuous stirring to obtain the ethosomal colloidal suspensions. The final suspension of ethosomes was kept at room temperature for 30 min under continuous stirring [13]. Formulations were stored in the refrigerator.

Table 1. Composition of ethosomal formulations.

Formulation code	Lecithin (%)	Ethanol (%)	Propylene glycol (%)
F1	3	10	10
F2	3	20	10
F3	3	30	10
F4	3	40	10
F5	3	50	10

Evaluation of ethosomes:

The ethosome formulations were evaluated for vesicle size, vesicular shape, surface morphology, entrapment efficiency, *in vitro* drug permeation study and stability study.

***Shape and size*:**

An optical microscope (Magnus) with a camera attachment was used to observe the shape of the prepared ethosomes formulation. Size and size distribution were determined by dynamic light scattering (DLS) using a computerized inspection system (Malvern Zetamaster ZEM 5002, Malvern, UK).

***Entrapment efficiency*:**

Aliquots of ethosomal dispersion were subjected to centrifugation using cooling ultracentrifuge (Remi) at 12000 rpm. The clear supernatant was siphoned off carefully to separate the unentrapped metronidazole. The sediment was treated with 1 ml of 0.1 % Triton X 100 to lyse the vesicles and then diluted to 100 ml with methanol and metronidazole was analyzed by UV-Visible spectrometry. Amount of metronidazole in sediment was calculated using a calibration curve. The percent entrapment was calculated using the formula,

Entrapment (%) = (Ws/Wa) ×100 ….(1)

Where, Ws is the amount of metronidazole in sediment and Wa is the amount of metronidazole added.

***In vitro* drug permeation study:**

The *in vitro* permeation study was carried out by using a modified Franz diffusion cell with egg membrane. The study was performed with phosphate buffer saline (pH 7.4). The formulation (centrifuged sediment) was placed (equivalent to 2.5 mg of drug) on the upper side of the egg membrane in the donor compartment. The temperature of the assembly was maintained at 37±2ºC. Samples were withdrawn after every hour from the receptor media through the sampling tube and at the same time, the same amount of fresh receptor media was added to make sink condition. Withdrawn samples were analyzed for metronidazole content using UV Visible spectrophotometer. [14,15]

Stability study

Optimized ethosomal formulations were selected for stability study. Formulations were stored at 4 °C/60 ± 5 % relative humidity and 25 °C/60 ± 5 % relative humidity for a period of three months. Percent drug entrapment was determined at different time intervals.

RESULTS AND DISCUSSION:

Pre-formulation studies:

The pure drug (active pharmaceutical ingredient) metronidazole was observed for its organoleptic characters. The organoleptic characters are the first step towards evaluating the identity and purity of the drug samples.

Table 2. The organoleptic features of metronidazole.

Sl. No.	Test	Observation
1	Physical appearance	White
2	Odour	Odourless
3	Melting Point	157-159°C

Table 3. The Solubility profile of metronidazole.

Sl. No.	Solvent	Solubility
1	Water	Soluble
2	Ethanol	Soluble
3	0.1N HCl	Soluble
4	Chloroform	Slightly Soluble

Table 2 presents the observations of the organoleptic features of the acquired sample of metronidazole whereas the solubility is presented in Table 3.

Calibration curve of metronidazole:

The absorption maximum of metronidazole in PBS 7.4 was found to be 340 nm and the calibration curve was prepared for a range of 10-50 µg/ml.

Table 4. The absorbance of metronidazole in PBS 7.4.

Concentration (µg/mL)	Absorbance
10	0.131
20	0.247
30	0.380
40	0.521
50	0.665

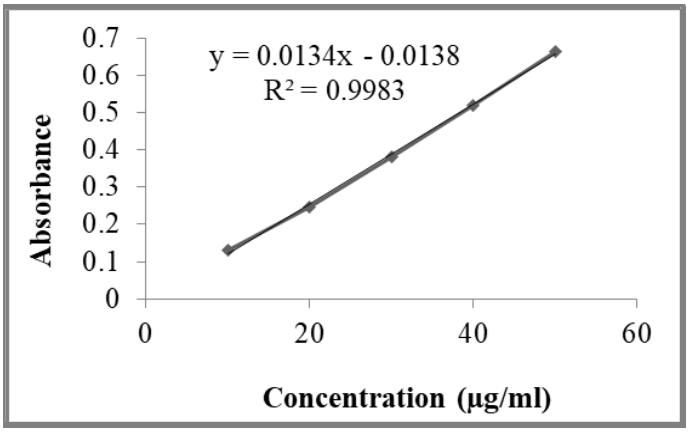


Fig 1. The standard curve of Metronidazole.

FT-IR spectrum of metronidazole:

The FT-IR spectrum of the procured sample of metronidazole was obtained using Bruker alpha spectrometer (Fig 2) and the spectrum was observed for the characteristic peaks of the functional groups present in the compound (Table 5).

Drug-Excipient Compatibility Study:

To confirm the compatibility of the drug and the excipients, a physical mixture of lecithin and metronidazole was subjected to FT-IR analysis. The spectrum was observed for the occurrence of stretching and bending vibrations.

Table 5. Characteristic peaks of metronidazole FT-IR.

Sl. No.	Peak (cm^{-1})	Functional group
1	3228	Aliphatic primary amine
2	3104, 2970	CH Stretching
3	1696	O=C-NH_2
4	695, 658	Out of plane N-H Wagging

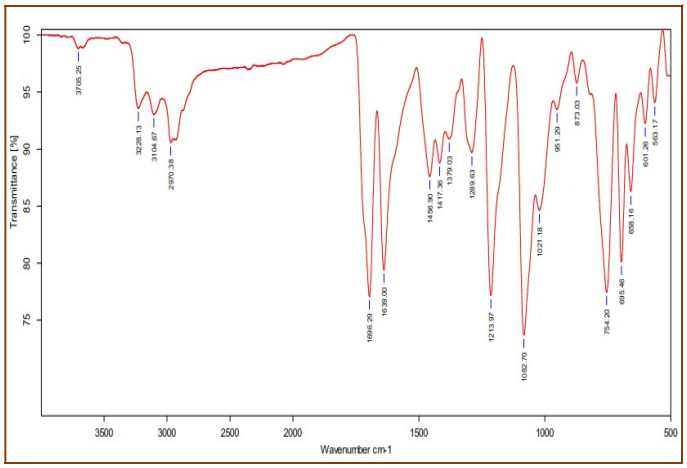


Fig 2. The FT-IR spectrum of metronidazole.

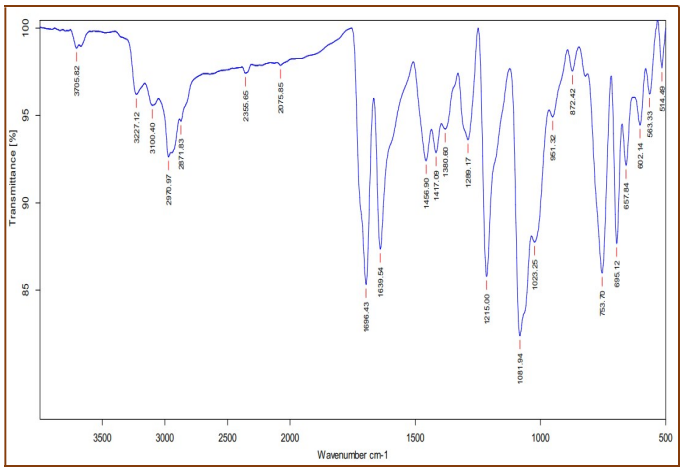


Fig 3. The FT-IR spectrum of physical mixture of metronidazole and lecithin.

The spectra of the mixture exhibited all the peaks of the pure drug as well as some peaks due to the functional groups of the excipients. No peak of the pure drug was removed though the position of the peak changed marginally due to the vibrations of the functional groups of the excipients.

Table 6. The FTIR peaks of physical mixture of metronidazole, and lecithin.

Sl. No.	Peak (cm^{-1})	Functional group
1	3705	OH stretching
2	3227	Primary amine stretching
3	3100, 2970	CH Stretching
4	1696	O=C-NH_2
5	1456	C-N strectching
6	695, 658	Out of plane N-H Wagging

Evaluation of ethosomes:

The ethosomes were evaluated for shape and size, entrapment efficiency and *in vitro* permeation through egg membrane. The results of the study are presented in the following sections.

Vesicle shape and size

The vesicles were found to be of spherical to irregular shape ranging from 3.248 ± 0.733 µm to 6.032 ± 2.883 µm in size. The smallest particle size was found to be the formulation F5 whereas the largest size was found to be of F1. The vesicles were visualized under an optical microscope and were found to be spherical in F2 and F3 whereas irregular in F1, F4 and F5. The confirmation of the shape of the vesicles was done by scanning electron microscopy. The particles were found to be clustered together with spherical and irregular particles in the same sample (Fig 4).

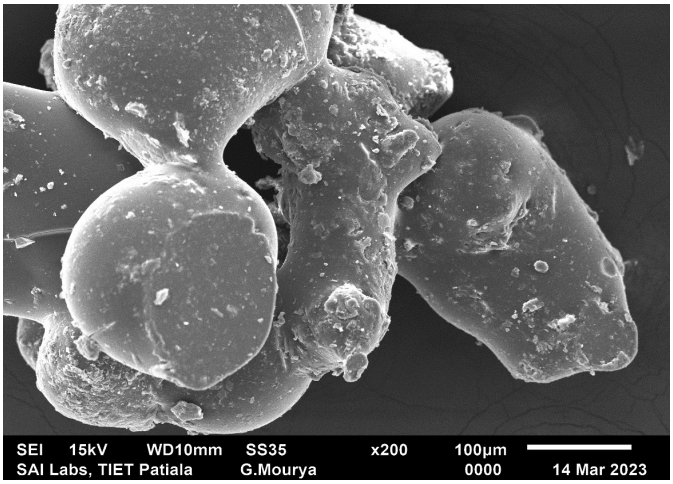


Fig 4. The Scanning electron microscope photomicrograph of F3.

The vesicular size of the ethosome (F3) was studied with the help of dynamic light scattering technique using a particle size analyzer. The particle size was found to be 1604 nm, corresponding to 3208 nm (3.208) in diameter with a polydispersity index of 0.701.

Table 7. The particle size, Drug entrapment and shape of ethosomes.

FC	Vesicle Size (µm) ± SD	Shape	DE (%)
F1	6.032 ± 2.883	Irregular	86.24
F2	5.104 ± 1.361	Spherical	89.58
F3	3.596 ± 1.276	Spherical	93.17
F4	3.48 ± 0.773	Irregular	94.09
F5	3.248 ± 0.733	Irregular	94.43

Data are presented as mean standard deviation (n=3), DE - Drug Entrapment, and FC –Formulation.

Entrapment Efficiency:

The entrapment efficiency of ethosomes was determined for all formulations. Effect of ethanol concentration was observed on percent drug entrapment of ethosomes. The maximum entrapment efficiency was found to be 94.43 % for formulation F5 and minimum 86.24 % for formulation F1, respectively. An increase in percent drug entrapment was observed with an increase in ethanol concentration. Improvement in aqueous solubility of metronidazole was achieved with higher concentration of ethanol, which could be due to its co-solvent effect. Therefore, the more drug amount could be accommodated in the aqueous core of the vesicles.

***In vitro* drug release:**

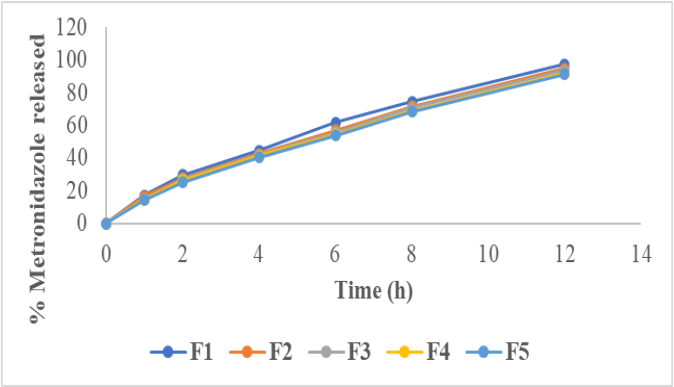
The amount of drug released from the ethosome was determined using Franz diffusion cell method employing egg-membrane as the barrier imitating skin. It was found that all the formulations were able to control the *release* of metronidazole up to 12 h of the study (Table 8).

Table 8. The *in vitro* drug release of metronidazole from ethosome.

FC	% Metronidazole released at time (h)					
	1	2	4	6	8	12
F1	17.3	29.7	44.5	61.6	74.4	97.4
F2	16.5	27.8	42.6	57.1	71.6	94.8
F3	15.1	27.1	42.1	55.8	70.7	93.6
F4	14.8	26.4	41.3	54.3	68.9	91.7
F5	14.4	25.3	40.5	53.7	68.5	91.2

FC - Formulation Code.

It was seen that the highest amount of metronidazole released from F1 (97.3 %) while the least amount released was from F5 (91.2 %) at the 12th hour of study. The drug released at a steady rate from the vesicles suggesting erosion in vesicles over time leading to the release of the drug (Fig 5).



***Stability study*:**

The best formulation (F3) with spherical shape and higher entrapment efficiency was selected for stability study at various temperatures. The formulation was stored in an amber glass container at different temperatures. The drug content after treatment with triton X100 and % residue of metronidazole was

calculated. It was observed that only about 0.72 % degradation occurred at 25 °C and the formulation was almost stable at 4 °C with only 0.58 % loss of the entrapped metronidazole thereby proving the stability of the developed system (Table 9).

Table 9. The stability of the ethosome formulation (F3) on storage.

Time (d)	Drug entrapment (%)	
	4 °C/60 ± 5 RH	25 °C/60 ± 5 RH
30	93.1 ± 0.057	93.0 ± 0.1
60	92.9 ± 0.1	92.76 ± 0.152
90	92.6 ± 0.057	92.5 ± 0.173

Data are presented as mean standard deviation (n=3).

CONCLUISION:

The use of ethosomal flexible carriers has gained popularity as a promising approach for transdermal drug delivery. Incorporation of metronidazole in the ethosomal carrier enhances the topical applicability of the drug as ethosomes can be easily incorporated into gel base and formulated as topical gels. The stability of the ethosomes and its controlled release up to 12 h make the carrier system suitable for formulation as a topical delivery system.

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