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Development of ethosomes with taguchi robust design-based studies for transdermal delivery of alfuzosin hydrochloride

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ABSTRACT

In the present investigation efficiency of ethosomes as novel lipid carriers for transdermal delivery of Alfuzosin Hydrochloride (AH) has been evaluated. Taguchi robust design method was used for optimization of ethosomal formulations. Phospholipid type, concentration of phospholipid and concentration of ethanol was selected as independent variables and their effect on the dependent variables (entrapment efficiency and flux) was studied. Ethosomal formulation (EA8) with soya phosphatidylcholine (3%) and ethanol 20% were optimized. Vesicles were spherical, unilamellar with smooth surface. The optimized formulation exhibited vesicle size (6.85 ± 1.35µm), zeta potential (-8.14 ± 0.62mv), entrapment efficiency (91.86 ± 3.25%), flux (27.42 ± 0.04µg/cm^2/hr), lag time (0.26±0.20hr) and skin deposition (298.01 ± 15.4µg/g). Transdermal flux was enhanced by 6.92 times over drug solution. Vesicle skin interaction studies showed fatty change in the dermis. The formulations were stable at 4°C for 120 days. Results suggested ethosomes as efficient carriers for AH transdermal delivery.

***Key Words*:** DOE, vesicles, ethanolic vesicles, permeation enhancement, BPH.

INTRODUCTION

Transdermal drug delivery (TDD) is the potential route for delivering systemic drugs. But the greatest challenge is the barrier nature of stratum corneum (Aqil *et al.*, 2007). Many techniques have been aimed to disrupt or weaken the barrier property of skin. One method is the use of vesicle formulations as skin delivery systems (Honeywell-Nguyen and Bouwstra, 2005). Intensive research over the past two decades led to the development of novel carriers, the ethanolic liposomes that have been termed ethosomes (Touitou *et al.*, 2000).

Ethosomes are novel lipid vesicles embodying high concentration (20-45%) of ethanol and are prepared by dissolving the lipids and drug in ethanol (Dayan and Touitou, 2000). Several studies have reported that ethosomes were able to improve in vitro and in vivo skin delivery of various drugs, both under occlusive and non-occlusive conditions (Prasanthi and Lakshmi, 2012).

The aim of the present study was to statistically optimize the vesicular formulations (Ethosomes) for enhanced skin delivery of a model drug Alfuzosin hydrochloride (AH), α-adrenoreceptor antagonist used for benign prostatic hyperplasia. It has low oral bioavailability of about 64% and its physico-chemical properties like molecular weight (425.9), half-life (3-5), log P value (1.604), and low dose (2.5 to 10 mg/day) makes it an ideal drug candidate for TDD (Martindale, 1993). AH has been previously identified as a promising candidate for TDD (Prasanthi *et al.*, 2010).

Taguchi robust design is a statistical technique which studies all levels of input parameters with fewer experiments and optimizes the experiment having least variability. The variability of a property is expressed by signal to noise ratio (S/N Ratio) (Kim *et al.*, 2007).

In the present study, ethosomes bearing AH was prepared by cold method and optimized by Taguchi

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robust design method. They were evaluated for permeation enhancement over pure drug solution and the optimized formulation was evaluated for vesicle skin interaction studies and stability studies.

MATERIAL AND METHOD

Alfuzosin hydrochloride (AH) was obtained as a gift sample from Dr. Reddy's Laboratories Ltd (Hyderabad, India). Phospholipon 90 H and phospholipon 80 H was obtained as a gift sample from Lipoid GmbH (Germany). Soyaphosphatidyl choline (SPC) was purchased from Otto Chem. Ltd. (India). Ethanol was purchased from S. D. Fine-Chem. Ltd. (India).

Preparation of ethosomes

Ethosomal formulations were prepared by the cold method (Verma and Pathak, 2012) using Taguchi robust design. The ethanolic vascular system was composed of phospholipid (2.0% to 4.0% with/Vol), ethanol (20% to 40% Vol/Vol), drug (AH, 0.5% wt/Vol) and double distilled water to 100% (Vol/Vol). Phospholipid was dissolved along with the drug in ethanol. This mixture was heated to 40°C ± 1°C and a fine stream of double-distilled water was added slowly, with constant mixing at 700 rpm with a mechanical stirrer (Remi Instruments, Vasai, India) in a house-built closed container. Mixing was continued for an additional 5 minutes, while maintaining the system at 40°C ± 1°C. The preparation was left to cool at room temperature for 30 min and then it was sonicated at 4°C for five cycles of 3 minutes each with a 1-minute rest between cycles using a probe sonicator (Sonics-Vibra Cell). Nine formulations were prepared according to Taguchi L9 orthogonal array design shown in Table 2. Ethosomes were compared with thirty percent of hydroethanolic solution of drug and drug solution in distilled water.

Table 1: Three-factor, three-level Taguchi L9 Orthogonal array experimental design.

Factors	Levels		
	1	2	3
A. Type of phospholipid	80H	90H	soya phosphatidyl choline
B. Concentration of phospholipid (%)	2	3	4
C. Concentration of ethanol (%)	20	30	40

Response : Entrapment efficiency and Flux (J_{ss}, μg/cm²/hr).

Experimental design

A three-factor, three-level Taguchi L9 orthogonal array experimental design was constructed using MINITAB 16 software (Minitab Inc., PA, U.S.A). The independent variables selected were the type of phospholipid, concentration of phospholipid and concentration of ethanol. Their corresponding levels and the dependent variables are shown in Table1.

Evaluation of ethosomes

Vesicular shape and surface morphology

Scanning electron microscopy (SEM) was used to characterize the surface morphology of the prepared vesicles. One drop of ethosomal suspension was mounted on a clear-glass stub, air-dried, gold coated with Polaron E5100 sputter coater (Polaron, United Kingdom), and visualized under scanning electron microscope (Jeol 5400, Japan).

Table 2: Observed responses in Taguchi Robust Design for AH ethosomal formulation (EA).

Formulation code	Independent variables			Dependent variables	
	A	B (%)	C (%)	Y_1 (%)	Y_2(μg/cm²/hr)
EA1	1	1	1	65.73±6.14	18.41±0.41
EA2	1	2	2	82.86±4.20	26.71±0.15
EA3	1	3	3	74.5±3.37	24.03±0.21
EA4	2	1	2	60.03±5.75	15.98±0.24
EA5	2	2	3	79.1±3.57	24.54±0.19
EA6	2	3	1	63.96±4.56	21.60±0.16
EA7	3	1	3	84.50±4.05	25.93±0.16
EA8	3	2	1	91.86±3.25	27.42±0.04
EA9	3	3	2	76.00±2.28	23.18±0.41

A: Type of phospholipid; B: Concentration of phospholipid (%); C: Concentration of ethanol (%);
Y_1:Entrapment efficiency (%); Y_2 :Flux (μg/cm²/hr). Values represent mean ± SD (n=3).

Table 3: Characterization of various formulations of AH.

Parameters	Ethosomes (3% SPC, 20% EtOH)	Hydroethanolic solution	Drug solution
Shape and surface morphology	Spherical, unilamellar	-	-
Particle size (μm)*	6.85 ± 1.35	-	-
Zeta potential (mV)*	-8.14 ± 0.62	-	-
Entrapment efficiency (%)*	91.86 ± 3.25	-	-
Transdermal flux (μg/cm²/hr)*	27.42 ± 0.04	6.66 ± 0.17	3.96 ± 0.13
Permeability co-efficient (cm/hr)×10^{-3} *	5.48 ± 0.009	0.13 ± 0.03	0.079 ± 0.02
Lag time (hr)*	0.26 ± 0.20	3.03 ± 0.5	4.06 ± 0.25
Enhancement ratio	6.92	1.68	-
Skin deposition (μg/g)*	298.01 ± 15.4	120.36 ± 8.0	86.33 ± 11.99

*-Values represent mean ± SD (n=3). EtOH: ethanol; SPC: soya phosphatidylcholine.

Vesicle size and zeta potential

The vesicle size and zeta potential were measured by photon correlation spectroscopy (Delsa Nano, Beckman Coulter Inc. UK).

Entrapment efficiency

Prepared ethosomal vesicles were separated from the free (unentrapped) drug by ultracentrifugation technique (Verma and Pathak, 2012). 2 ml of the ethosomal suspension was diluted with distilled water up to 5 ml and centrifuged at 20,000 rpm for 45 min at 4^0C using a cooling centrifuge (Eltek centrifuge). After centrifugation, the supernatant and sediment were recovered, and sediment was lysed using methanol and filtered through a 0.45 μm nylon disk filter. The concentration of AH in the supernatant and sediment was analysed by UV-VIS double beam spectrophotometer (Chemito Spectrascan UV2600, India) at 245nm. The percentage drug entrapment was calculated using the following equation:

$$\%\text{Drug entrapment} = \frac{\text{Amount of entrapped drug recovered}}{\text{Total amount of drug}} \times 100$$

The determination of entrapment efficiency was repeated three times per sample at 25°C.

Skin permeation studies

Institutional animal ethical committee (IAEC) approved skin permeation studies. Locally fabricated Keshary Chein diffusion cell was used for permeation studies. Studies were conducted using dermatomed and prepared rat skin. 20 ml of PBS 7.4 was taken in receptor compartment and was continuously stirred with a magnetic stirrer and equilibrated at 37°±1°C with a recirculating water bath. The dermatomed skin was mounted with stratum corneum facing upward into the donor compartment. 1 ml of ethosomal formulation was taken in donor compartment and covered with parafilm to avoid any evaporation process. 1 ml samples were withdrawn through the sampling port at predetermined intervals over 24 hr and analysed for drug content by UV-VIS double beam spectrophotometer (Chemito Spectrascan UV2600, India) at 245nm. Similar studies were performed with hydroethanolic solution and drug solution.

Skin deposition studies

The amount of AH retained in the skin was deter-

Table 4: Stability of AH ethosomes.

Parameters	Initially	After 120 days (4±1°C)
Vesicle size (μm)	6.85 ± 1.35	6.99 ± 3.56
Zeta potential (mV)	-8.14 ± 0.62	-7.86 ± 1.23
Entrapment efficiency (%)	91.86 ± 3.25	90.98 ± 2.87

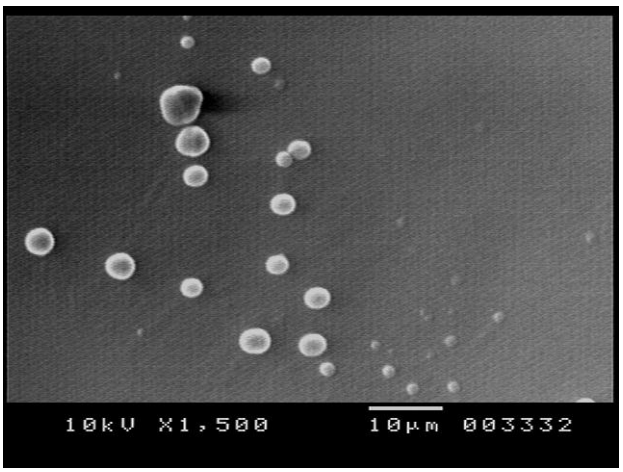

Figure 1: Scanning electron microscope photomicrograph of optimized ethosomal formulation EA8.

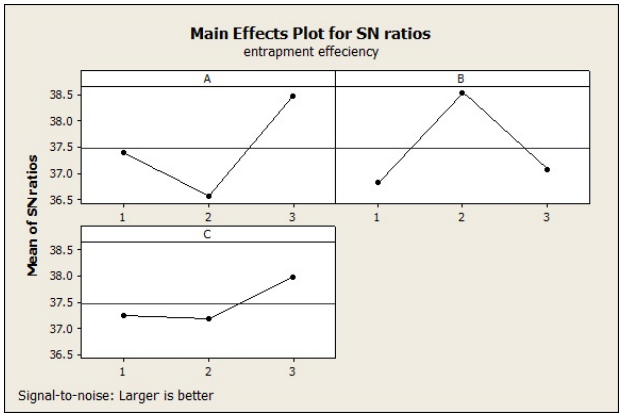

Figure 2: Main effects plot for SN ratios of A) type of phospholipid, B) concentration of phospholipid and C) concentration of ethanol for entrapment efficiency.

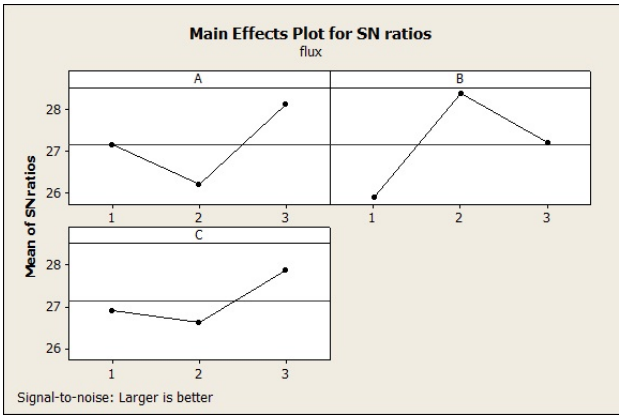

Figure 3: Main effects plot for SN ratios of A) Type of phospholipid, B) concentration of phospholipid and C) concentration of ethanol for Flux.

mined by skin deposition studies. At the end of permeation studies (24hr), the skin was washed 10 times with a cloth immersed in methanol. A sample of skin was weighed and homogenized with methanol for 5 min using an electric stirrer. The resulting solution was centrifuged at 7000 rpm for 10min and supernatant was analysed for drug content by UV-VIS double beam spectrophotometer (Chemito Spectrascan UV2600, India) at 245nm.

Vesicle-skin interaction studies

Interaction studies were studied on the basis of structural changes in stratum corneum, epidermis and dermis (Jain *et al.*, 2007). The formulations were applied topically to the dorsal portion of rats for 8hrs. Later, the rats were killed by cervical dislocation; the skin was excised and fixed by immersion in 50% neutral formalin solution in saline for 24hrs. The samples were subjected to histological processing, by dehydration and rehydration with graded alcohols, paraffin block processing and stained with haemotoxyllin-eosin. Microscopic evaluation using dark-light microscope was performed by a blinded assessor.

Stability studies

Vesicles were stored at 4°C± 0.5°C for 120 days. To determine their stability vesicle size, zeta potential and entrapment efficiency of the vesicles was measured using the method described earlier.

Data analysis

All the experiments were performed in triplicate unless specified, and the data are expressed as mean values ± standard deviations. Statistical significance was performed using one-way ANOVA at confidence limit of $P<0.05$. (MINITAB 16 software (Minitab Inc., PA, U.S.A)).

RESULTS AND DISCUSSION

Ethosomal formulations were prepared by the cold method according to Taguchi L9 orthogonal array design given in Table 2. On characterization spherical, unilamellar vesicles with smooth surface were observed under scanning electron microscope (Figure 1).

Ethosomes were optimized by varying phospholipid type, concentration of phospholipid and concentration of ethanol. Effect of independent variables on dependent variables was evaluated by SN ratio plots (Figure 2 & Figure 3). Main effects plot of SN ratios showed phospholipid type had no significant effect on entrapment and flux ($P> 0.05$). But maximum permeation was observed with soya phosphatidylcholine as it offers rigidity to the layers thereby reducing vesicle fusion resulting in decreased particle size and better permeation through skin pores.

Concentration of phospholipid (2% to 4%) effected entrapment efficiency and flux. Previous studies reported increases in phospholipid, increased entrapment (Patel and Patel, 2009; Dubey *et al.*, 2010). From the SN ratios plot it has been observed entrapment efficiency and flux increased with increase in phospholipid concentration up to 3%, but further increase decreased entrapment efficiency

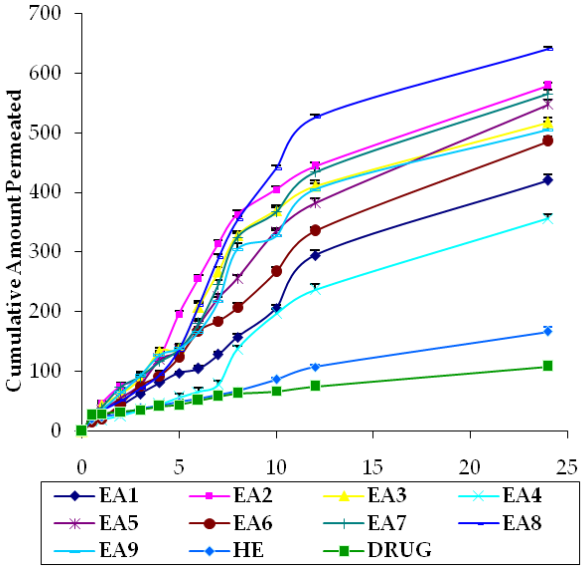

Figure 4: Cumulative amount permeated from ethosomal formulations, hydroethanolic solution (HE) and drug solution.

and flux. This effect is not significant (P>0.05). Concentration of ethanol (20% to 40%) showed linear relationship, as the concentration increased the entrapment and flux increased.

Permeation studies were performed using a rat abdominal skin. Overall ethosomes enhanced transdermal permeation by 4.03 to 6.92 times when compared with drug solution. Higher skin retention of the drug was seen with ethosomes (298.01 ± 15.4 µg/g to 1543.96 ± 40.1 µg/g). Ethosomal formulation EA8 were optimized, which showed maximum flux of 27.42 ± 0.04 µg/cm²/hr; lower lag time 0.26 ± 0.20 hr and higher entrapment efficiency of 91.86 ± 3.25% when compared with other formulations. Cumulative amount permitted by ethosomal formulations ranged from 356.68 ± 7.62 µg/cm² to 637.10 ± 2.95 µg/cm² when compared with hydroethanolic solution (167.13 ± 6.92 µg/cm²) and drug solution (108.35 ± 2.85 µg/cm²). Increased flux (15.98 ± 0.24 µg/cm²/hr to 27.42 ± 0.04 µg/cm²/hr) and lower lag time (0.26±0.20hr) was observed with ethosomes when compared with drug solution (3.96 ± 0.13 µg/cm²/hr; 4.06 ± 0.25hr) and hydroethanolic solution (6.66 ± 0.17 µg/cm²/hr; 3.03±0.5 hr). Comparison of cumulative amount permeated of various formulations is given in Figure 4. The results are given in Table 3.

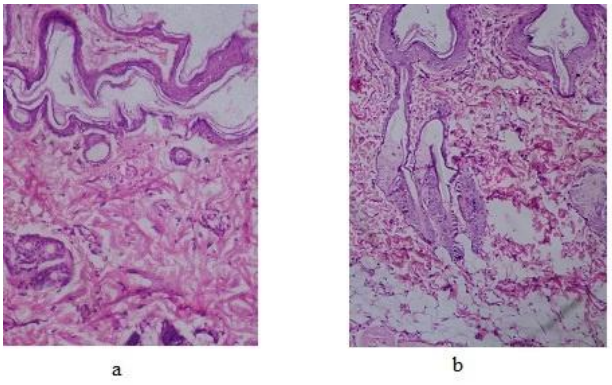

Figure 5: Histological section of skin a) control and b) treated with ethosomal formulation (EA8) under magnification ×200.

When compared to drug solution and hydroethanolic solution ethosomes showed better permeation due to the synergistic effect of phospholipids and ethanol as phospholipids in the vesicles entrap hydrophilic/lipophilic drug molecules act as carriers and deliver the entrapped drugs into or across the skin. Ethanol enhances permeation by various mechanisms such as push and pull effect, fluidization of stratum corneum lipids and vesicular lipid bilayers providing malleability to vesicles to penetrate skin pores smaller than their diameter (Panchagnula *et al.*, 2001; Kowatsu and Okada, 1996).

Vesicle-skin interaction studies (Figure 5) did not exhibit any major alterations except the slight fatty change in dermis revealing a decrease in resistance of skin as a barrier by fluidization of lipids, by ethanol. Stability studies performed at 4°C ± 1°C for 120 days showed good storage stability (Table 4) (Dubey *et al.*, 2007).

CONCLUSION

Soft malleable vesicles consisting of phospholipids and higher concentration of ethanol exhibited synergistic effect of phospholipids and ethanol on permeation proving elastic liposomes (ethosomes) are better carriers for Alfuzosin hydrochloride transdermal delivery.

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