

# Statistically optimised ethosomes for transdermal delivery of tolterodine tartrate

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**Abstract:** The aim of the current investigation is to optimize ethosomes statistically for enhancing transdermal potential of Tolterodine Tartrate (TT). Ethosomes bearing TT were prepared by cold method and characterized for various parameters like vesicle size, vesicle shape, surface morphology and % drug entrapment. Microscopic examinations suggest ethosomes as spherical unilamellar vesicles with a smooth surface. Optimized ethosomal vesicles were of  $890 \pm 2.67 \text{ nm}$  size and showed  $79.83 \pm 3.18\%$  drug entrapment. Ex-vivo permeation studies across rat skin resulted in increased flux of  $4.69 \pm 0.24 \mu\text{g}/\text{cm}^2/\text{hr}$  and decreased lag time of  $0.13 \pm 0.05 \text{ hr}$  when compared with drug solution ( $0.546 \pm 0.05 \mu\text{g}/\text{cm}^2/\text{hr}$ ,  $3 \pm 0.2 \text{ hr}$ ). This shows enhancement of transdermal delivery by 8.82 times. Anatomical changes in skin samples due to vesicle-skin interaction were observed on histological examination. Optimized formulation on storage at  $4^\circ\text{C}$  for 120 days showed insignificant growth in vesicular size revealing low aggregation of vesicles. The results collectively suggest ethosomes as carriers for accentuated transdermal delivery of TT.

**Keywords:** Ethosomes, transdermal delivery, tolterodine tartrate, taguchi robust design.

## INTRODUCTION

Tolterodine tartrate (TT); a synthetic tertiary amine with antimuscarinic activity, is a genitourinary antispasmodic used for the treatment of overactive bladder (OAB). OAB is associated with voiding such as urge urinary incontinence, urgency, and frequency (Ruscin and Morgenstern, 1999). According to the International Continence Society, overactive bladder disorder is characterized by involuntary detrusor contractions (Hampel *et al.*, 1997). OAB of neurogenic origin is referred as hyperflexic disorder and one that is nonneurogenic is known as unstable disorder. TT is used for the management of symptoms associated with both neurogenic and nonneurogenic OAB (Guay, 1999).

TT is available as tablets and extended release capsules. On oral administration it undergoes hepatic metabolism to active metabolite which acts similarly as the parent on receptors (Nilvebrant *et al.*, 1997). But its oral administration is limited due to gastrointestinal obstructive disorder and dose-related adverse side effects. Its physico-chemical properties like molecular weight (475.6), half-life (1.9-3.7), log P value (1.51 at a pH of 7.4), low dose (2mg twice daily) and aqueous solubility (12g/L at room temperature) make it an ideal drug candidate for transdermal delivery (Vinay Pandit *et al.*, 2009; Ligang Zhao *et al.*, 2009). Transdermal delivery is a potential route in providing sustained levels of drug for longer periods and bypassing hepatic metabolism, but limitation is barrier property of skin. Vesicles as carrier systems have been studied for better skin permeability. Ethosomes are soft, malleable vesicles consisting of phospholipids, water and high concentrations of ethanol

(Elsayed *et al.*, 2007). These are novel lipid carriers for transdermal delivery, recently developed by Touitou *et al.* (2000 a, b). Research studies reported, ethosomes entrapping hydrophilic, lipophilic, or amphiphilic drug molecules (Bhalaria *et al.*, 2009).

Taguchi Robust design is a statistical technique for characterizing complicated process. The experimental condition with least variability can also be determined as optimum condition. Factor difficult to control is known as 'noise factor' and factor easy to control is known as 'control factor'. Variability is expressed as S/N ratio (signal to noise) and optimal condition is the experimental condition with maximal S/N ratio (Kim *et al.*, 2004).

The present work is to design ethosomes bearing TT using Taguchi robust design. Ethosomes were evaluated for vesicle size, surface morphology, % drug entrapment, permeability through rat skin and stability.

## MATERIALS AND METHODS

### Materials

Tolterodine tartrate (TT) was obtained as a Gift sample from RA Chem Ltd (Hyderabad, India). Phospholipon 80 H and Phospholipon 90 H was obtained as a Gift sample from Lipoid GmbH (Germany). Soyaphosphatidyl choline (SPC) was purchased from Otto Chem. Ltd. (India). Cholesterol and Ethanol were purchased from S. D. Fine-Chem. Ltd. (India).

### Preparation of TT loaded vesicles

Cold method was followed for preparation of Ethosomal formulations (Touitou *et al.*, 2000a). Phospholipid along with drug (0.07%) was dissolved in ethanol which was maintained at  $40^\circ\text{C} \pm 1^\circ\text{C}$ . Fine stream of Double-distilled

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water was added slowly, under constant mixing at 700 rpm (Remi Instruments, Vasai, India). For an additional 5 min mixing was continued at  $40^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . The preparation was left for 30 min to cool at room temperature. Later the preparation was sonicated at  $4^{\circ}\text{C}$  for five cycles of 3 minutes each with a 1-minute rest between cycles using a probe sonicator (Sonics-Vibra Cell). Composition of various ethosomal formulations were according to Taguchi L9 Orthogonal array design given in table 2.

Cast film method was followed for preparation of Conventional liposomes (Vaibhav Dubey *et al.*, 2006). Drug (0.07% w/v), Soya Phosphatidylcholine (3% w/v) and Cholesterol (1% w/v) were dissolved in a mixture of methanol: chloroform (2:1) in a round-bottomed flask. Using rotary flash evaporator (SuperVac), organic solvent was removed above transition temperature of lipid. The flask was left under vacuum overnight for removal of traces of solvent. The resulting film was hydrated with double-distilled water for 2hr at  $37^{\circ}\text{C}$ . The preparation was sonicated at  $4^{\circ}\text{C}$  for five cycles of 3 minutes each with a 1-minute rest between cycles using a probe sonicator.

Thirty percent of hydroethanolic solution of drug and pure drug solution were prepared for comparison.

#### Taguchi robust design

Taguchi L9 Orthogonal array experimental design was constructed with 3 factors and 3 levels. The three factors (independent variables) are: Type of phospholipid, Concentration of phospholipid and Concentration of ethanol. Their corresponding levels and the responses (dependent variables) are shown in table1. MINITAB 16 software (Minitab Inc., PA, USA) was used for the generation and evaluation of the statistical experimental design.

#### Characterization of ethosomal formulations

##### Surface morphology and Vesicle shape of ethosomal vesicles

Surface morphology and vesicle shape was characterized by scanning electron microscopy. One drop of formulation was placed on a clear-glass stub, then it was air-dried and coated with gold by Sputter coater (Polaron E5100, Watford, United Kingdom). Later, it was visualized under scanning electron microscope (Jeol 5400, Japan).

#### 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

Ultracentrifugation technique was used to determine free untrapped drug within ethosomal formulations. 2 ml of the suspension was diluted with distilled water up to 5 ml and centrifuged at 20,000 rpm for 45 min at  $4^{\circ}\text{C}$  using a cooling centrifuge (Eltek centrifuge). Following centrifugation, the supernatant and sediment were recovered. The ethosomal pellet (sediment) was lysed using methanol and filtered through a  $0.45\mu\text{m}$  nylon disk filter. The concentration of TT in the supernatant and sediment was analysed by UV-VIS Spectrophotometer (Chemito Spectrascan UV 2600). Using following equation, percentage drug entrapment was calculated:

$$\% \text{ Drug entrapment} = \frac{(\text{Amount of entrapped drug})}{(\text{Total drug taken})} \times 100$$

The determination of entrapment efficiency was repeated three times per sample at  $25^{\circ}\text{C}$ .

#### Skin permeation studies

Skin permeation studies were approved by the Institutional Animal Ethical Committee (IAEC). A Keshary Chein diffusion cell, locally fabricated was used for diffusion studies. The diffusion medium (20 ml of PBS 7.4) was taken in receptor compartment and was continuously stirred with a magnetic stirrer and equilibrated at  $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$  with a recirculating water bath. The study was conducted using dermatomed and prepared rat skin. The dermatomed skin was mounted such that stratum corneum faced towards donor compartment. 1 ml of ethosomal formulation equivalent to 0.7mg was placed in the donor compartment occluded with parafilm. 1ml Samples were withdrawn by sampling port at predetermined intervals over 24 hr and analysed for drug content by Spectrophotometry. Similar studies were conducted for liposomes, drug solution and hydroethanolic solution.

Skin deposition studies were used to determine drug (TT) retained in skin. After 24 hr of permeation study, skin was removed from diffusion cell and washed with methanol. Sample of skin was homogenized and the mixture was centrifuged at 7000 rpm for 30min. The supernatant was

**Table 1:** Factors and their corresponding levels for the construction of Taguchi L9 orthogonal array experimental design

Factors		Levels		
		1	2	3
A	Type of Phospholipid	Soya Phosphoti-dyl choline	90H	80H
B	Concentration of Phospholipid(%)	2	3	4
C	Concentration of Ethanol (%)	20	30	40

Response: Entrapment efficiency, Particle size and Flux ( $J_{ss}$ ,  $\mu\text{g}/\text{cm}^2/\text{hr}$ ).

**Table 2:** Observed responses in Taguchi L9 Orthogonal array design for TT Ethosomal formulation

Formulation code	Independent variables			Dependent variables		
	A	B (%)	C (%)	Y <sub>1</sub> (%)	Y <sub>2</sub> (μm)	Y <sub>3</sub> (μg/cm <sup>2</sup> /hr)
ET1	1	1	1	57.23±3.12	1±0.8	2.84±0.11
ET2	1	2	2	79.83±3.18	0.890±0.16	4.69±0.24
ET3	1	3	3	66.36±3.52	4±0.9	3.53±0.12
ET4	2	1	2	73.46±3.11	10±1.2	2.31±0.30
ET5	2	2	3	63.12±4.28	8±0.9	2.52±0.09
ET6	2	3	1	52.67±3.50	7±0.86	2.46±0.33
ET7	3	1	3	58.49±3.64	4±1.1	3.82±0.46
ET8	3	2	1	48.07±2.30	11±2.1	2.05±0.17
ET9	3	3	2	63.36±2.89	6±0.95	3.89±0.13

A, Type of phospholipid; B, Concentration of phospholipid (%); C, Concentration of ethanol (%); Y<sub>1</sub>, Entrapment efficiency (%); Y<sub>2</sub>, Particle size (μm); Y<sub>3</sub>, Flux (μg/cm<sup>2</sup>/hr). Values represent mean ± SD (n=3).

filtered through Whatmann filter paper and analysed by spectrophotometer.

### Histopathological studies

To determine local toxicity, rats were treated with optimized formulations. The treated rats were sacrificed after 6hrs by cervical dislocation. Treated skin was excised and stored in 50% formalin solution. The samples were fixed by routine fixation, rehydration and dehydration with graded alcohols, paraffin block processing and stained with haematoxylin-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, % drug entrapped and zeta potential were measured using the method described earlier.

### Data analysis

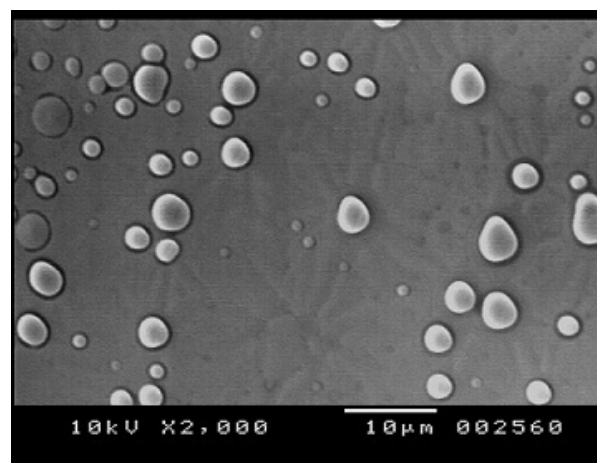
All the experiments were performed in triplicate unless specified, and the data are expressed as mean values ± standard deviations. Statistical significance was performed using one-way ANOVA at confidence limit of *P* 0.05. (GraphPad PRISM, Version 6.01, San Diego, CA).

## RESULTS

In the present investigation ethosomes were prepared according to Taguchi L9 Orthogonal array design. Particle size and % drug entrapped play decisive role in enhancing transdermal flux. Varying phospholipid type, concentration of phospholipid (2.0 to 4.0%w/v) and ethanol concentration (20% to 40% v/v) led to entrapment efficiency from 48.07 % to 79.83% and vesicular size ranging from 890nm to 11.36 μm (table 2).

Ethosomes examined by SEM appeared as unilamellar, spherical shape with smooth surface possessing vesicular characteristics (fig. 1). The zeta potential of all the

ethosomal vesicles was of higher magnitude than liposomal vesicles (table 3).



**Fig. 1:** SEM photomicrograph of optimized ethosomal formulation ET2.

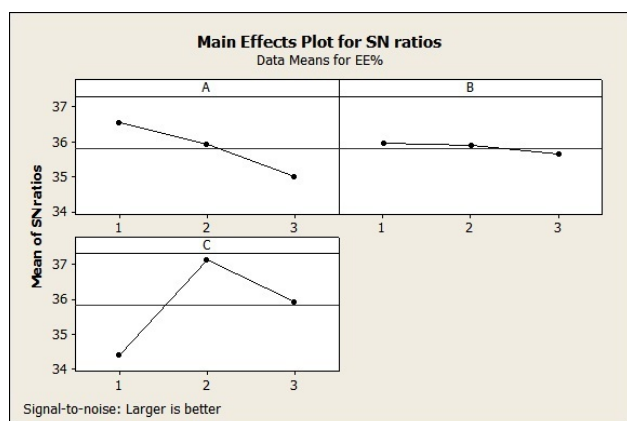
*Ex-vivo* permeation studies across rat skin were performed and amount permeated was calculated. Flux was calculated from the slope of linear portion of curve obtained by plotting cumulative amount permeated per unit area against time. From x-intercept of the linear portion lag-time was calculated. Formulation ET2 containing soya phosphatidyl choline 3% and ethanol 30% showed highest permeation (112.04±10.31 μg/cm<sup>2</sup>), flux (4.69±0.24 μg/cm<sup>2</sup>/hr) and lowest lag time (0.13±0.05 hr).

Conventional liposomes, pure drug solution and hydroethanolic solution provided lower flux values and longer lag times of 1.28±0.86 μg/cm<sup>2</sup>/hr, 1.5±0.18 hr; 0.546±0.05 μg/cm<sup>2</sup>/hr, 3.0±0.2 hr and 1.84±0.06 μg/cm<sup>2</sup>/hr, 1.4±0.2 hr respectively (fig. 5). Ethosomes also facilitated better skin deposition (151.91±7.70 μg/g) as compared with liposomes (80.36±4.36 μg/g), pure drug solution (21.16±1.94) and hydroethanolic solution (35.63±5.16 μg/g). The results are shown in table 3.

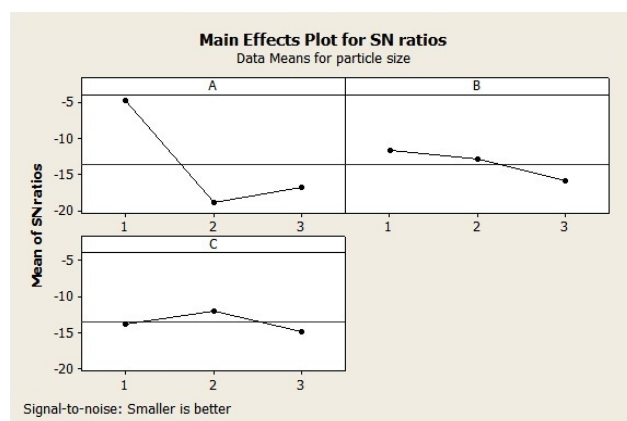
**Table 3:** Comparison of different parameters of TT loaded formulations

Parameters	Ethosomes (3% PC, 30% EtOH)	Liposomes (3% PC)	Hydroethanolic solution	Drug solution
Shape and surface morphology	Spherical, unilamellar	Spherical, unilamellar	-	-
Particle size ( $\mu\text{m}$ )*	0.890 $\pm$ 0.16	20 $\pm$ 4	-	-
Zeta potential (mV)*	-18.16 $\pm$ 1.2	-1.5 $\pm$ 0.3	-	-
Entrapment efficiency (%)*	79.83 $\pm$ 3.18	40.16 $\pm$ 2.30	-	-
Transdermal flux ( $\mu\text{g}/\text{cm}^2/\text{hr}$ )*	4.69 $\pm$ 0.24	1.28 $\pm$ 0.86	1.84 $\pm$ 0.06	0.546 $\pm$ 0.05
Permeability co-efficient ( $\text{cm}/\text{hr}$ ) $\times 10^{-3}$ *	6.71 $\pm$ 0.36	1.82 $\pm$ 0.52	2.64 $\pm$ 0.08	0.78 $\pm$ 0.07
Lag time (hr)*	0.13 $\pm$ 0.05	1.8 $\pm$ 0.18	1.4 $\pm$ 0.2	3 $\pm$ 0.2
Enhancement ratio	8.82	2.34	3.36	-
Skin deposition (% w/w)*	151.91 $\pm$ 7.70	80.31 $\pm$ 4.36	35.63 $\pm$ 5.16	21.16 $\pm$ 1.94

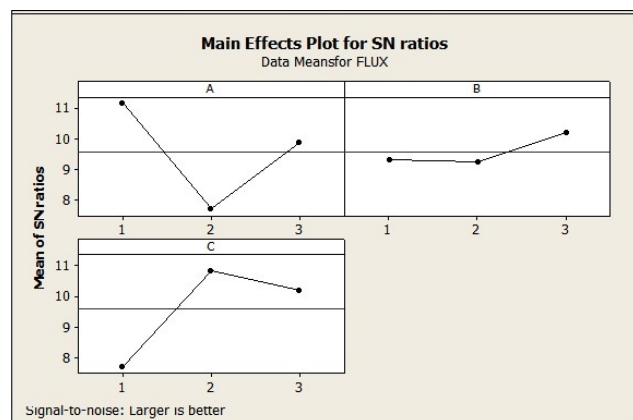
EtOH, ethanol; PC, soya phosphatidylcholine. \*Values represent mean  $\pm$  SD (n=3).



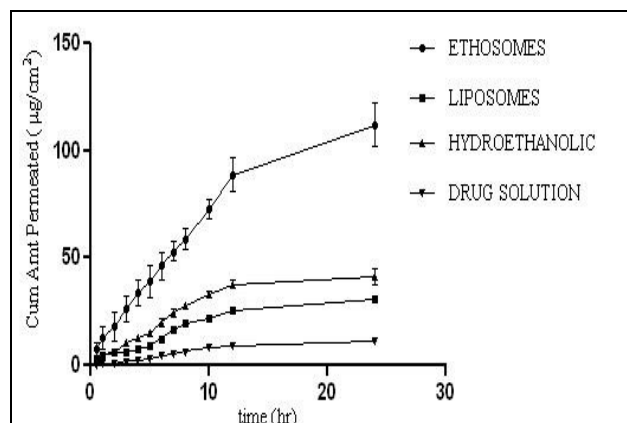
**Fig. 2:** Main effect plot for SN ratios of (A) type of phospholipids, (B) concentration of phospholipids and (C) concentration of ethanol for entrapment efficiency.



**Fig. 3:** Main effect plot for SN ratios of (A) type of phospholipids, (B) concentration of phospholipids and (C) concentration of ethanol for particle size.



**Fig. 4:** Main effect plot for SN ratios of (A) type of phospholipids, (B) concentration of phospholipids and (C) concentration of ethanol for Flux.



**Fig. 5:** Comparative cumulative drug permeated via rat skin versus time of tolterodine tartrate formulations. Values represent mean  $\pm$  SD (n=3).

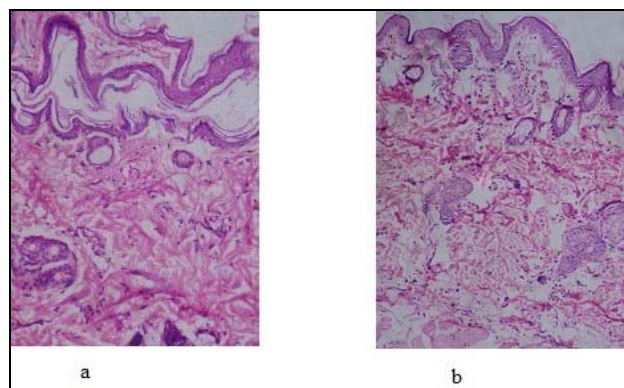
From Taguchi L9 orthogonal array method a relationship between independent and dependent variables was established by SN ratio plots which indicates ethanol

concentration significantly affected entrapment efficiency ( $P < 0.05$ ) and type of phospholipid significantly affected particle size ( $P < 0.05$ ) (figs. 2, 3, 4).

**Table 4:** Stability of ethosomes

Parameters	Initially	After 120 days 4±1°C
Vesicle size (µm)	0.890±0.16	0.896±1.3
Zeta potential (mV)	-18.16±1.2	-17.45±0.9
Entrapment efficiency (%)	79.83±3.18	77.54±2.31

Histopathological studies of treated skin sections revealed changes in the state of the skin when compared with normal untreated skin (fig. 6). The Haematoxylin and Eosin sections of skin showed fatty change in dermis and no change in the epidermis.

**Fig. 6:** Histological section of skin a control and b treated with ethosomal formulation under magnification x 200

Stability studies were performed at 4°C±1°C for 120 days. SEM studies revealed no significant difference in vesicle shape and surface morphology. Due to negative surface charge insignificant size variation ( $P>0.05$ ) (table 4) was observed.

## DISCUSSION

Vesicle size influences mechanism of penetration in transdermal delivery; consequently, smaller vesicles are able to deliver their content into deep layers of the skin (Prasanthi and Lakshmi, 2012). Previous research studies have shown increase in concentration of phospholipid increased particle size whereas increase in ethanol concentration decreased particle size (Vaibhav Dubey *et al.*, 2010). Similar effect of phospholipid and ethanol has been observed in the present study (table 2). From Taguchi Robust Design it has been concluded that type of phospholipid significantly affects particle size, this can be attributed to the fact that soya phosphatidylcholine confers greater rigidity reducing vesicle fusion.

Increase in phospholipid concentration and ethanol concentration showed increase in drug entrapment (Vaibhav Dubey *et al.*, 2010). Taguchi studies indicated ethanol concentration significantly affected entrapment

efficiency as ethanol modifies net surface charge leading to some degree of steric stabilization resulting in decreased vesicle size, increase in % drug entrapped and increase in zeta potential (Poonam verma *et al.*, 2012; Subheet Jain *et al.*, 2007).

Better Ex-vivo permeation of ethosomes over liposomes is due to presence of ethanol which decreases particle size and confers flexibility of vesicles resulting in better penetration through skin pores, much smaller than their size (Abdul Ahad *et al.*, 2012). The enhanced transdermal efficiency from ethosomes can be further attributed to the mechanism of ethanol present in ethosomes. As ethanol fluidizes vesicular lipid bilayers and stratum corneum lipids, it imparts flexibility to vesicles; it exerts push and pull effect thus improving permeability in skin layers (Komatsu and Okada, 1996; Panchagnula *et al.*, 2001; Vaibhav Dubey *et al.*, 2010).

Histopathology studies of the skin at the site of treatment showed that the application of TT ethosomes did not induce changes in the thickness of the horny layer but the fatty change was seen in the dermis which can be correlated to the action of ethanol, as ethanol fluidizes the lipids and reduces the paracellular as well as intracellular pathways resistance (Prasad *et al.*, 2007).

Stability studies indicated good storage stability as negative surface charge imparted by ethanol inhibits aggregation of vesicles due to repulsion of like charges.

The present studies revealed ethosomes as efficient carriers for transdermal delivery which has been reported by researchers (Subheet Jain *et al.*, 2007; Vaibhav Dubey *et al.*, 2007; Poonam verma *et al.*, 2012; Lopez-Pinto *et al.*, 2000).

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