

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/341998689>

# DESIGN AND CHARACTERIZATION OF NIOSOMES OF ETHIONAMIDE FOR MULTI DRUG RESISTANCE TUBERCULOSIS \*Corresponding Author

Article in World Journal of Pharmaceutical Research · April 2019

DOI: 10.20959/wjpr20196-14815

CITATION

1

READS

102

4 authors, including:



**Piyushkumar Sadhu**

Sumandeep Vidyapeeth University

10 PUBLICATIONS 6 CITATIONS

[SEE PROFILE](#)



**Saisivam Srinivasan**

N. R. Vekaria Institute of Pharmacy, Junagadh, Gujarat, India

35 PUBLICATIONS 513 CITATIONS

[SEE PROFILE](#)



**Sujit Kumar Debnath**

Indian Institute of Technology Bombay

17 PUBLICATIONS 132 CITATIONS

[SEE PROFILE](#)

Some of the authors of this publication are also working on these related projects:



losartan potassium [View project](#)



antibiotic isolation [View project](#)

**DESIGN AND CHARACTERIZATION OF NIOSOMES OF  
ETHIONAMIDE FOR MULTI DRUG RESISTANCE TUBERCULOSIS****Piyushkumar K. Sadhu<sup>\*1,2</sup>, S. Saisivam<sup>1</sup> and Sujit Kumar Debnath<sup>1,3</sup>**

<sup>1</sup>N. R. Vekaria Institute of Pharmacy, C. L. College campus, Bilkha road, Junagadh, Gujarat - 362 001, India.

<sup>2</sup>Department of Pharmacy, Sumandeep Vidyapeeth Deemed to be University, Pipariya, Vadodara, Gujarat-391 760, India.

<sup>3</sup>Nanobios Lab, Department of Biosciences and Bioengineering, IIT, Bombay, India.

Article Received on  
27 Feb. 2019,

Revised on 19 March 2019,  
Accepted on 08 April 2019

DOI: 10.20959/wjpr20196-14815

**\*Corresponding Author****Prof. Piyushkumar Sadhu**

N. R. Vekaria Institute of  
Pharmacy, C. L. College  
campus, Bilkha road,  
Junagadh, Gujarat - 362 001,  
India.

**ABSTRACT**

Ethionamide is a second line anti-TB agent selective for MDR-TB. In the present study, Ethionamide niosomes were prepared by thin film hydration technique for I.V. administration in which non-ionic surfactants (Span-40, Span-60 and Span-80) and Cholesterol were used in various ratios. From that span 40 was selected. Solvents like Chloroform and Methanol were used. Prepared niosomes were characterized for Percentage Entrapment efficiency (% EE), particle size, zeta potential, polydispersity index and sterility testing. Further studies like scanning electron microscopy (SEM), release kinetics and stability studies were performed for the optimized formulation F12 which was prepared using drug ethionamide(25mg), cholesterol(75mg)

and span 40(50mg) (1:3:2 respectively). The result showed that the entrapment efficiency was 88.9%, mean particle size was 124.4nm, polydispersity index was 1.294 and zeta potential was -39.71 mV. F12 formulation showed drug release of 94.89% (p value=0.04) after 24 hours in phosphate buffer 7.4 pH. The results suggest that the ethionamide niosomes formulated using span and cholesterol by thin film hydration technique might be a better choice for intravenous delivery of ethionamide for the treatment of multi drug resistance tuberculosis.

**KEYWORDS:** Niosomes, Ethionamide, MDR-TB (multi drug resistance tuberculosis), Span, Sustained ability.

## INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis*, typically affects the lungs (pulmonary TB) but can affect other sites as well (extrapulmonary TB). It is contagious and airborne disease as well.<sup>[3]</sup> Ethionamide (ETH) (166.2 gm/mole)<sup>[4]</sup> is a second line anti TB agent that is better choice of selection for MDR-TB by inhibiting mycolic acid synthesis of bacterial cell wall.<sup>[5]</sup> ETH is a nicotinic acid derivative related to isoniazid. ETH has very low protein binding (30%)<sup>[6]</sup> and is rapidly excreted from the body ( $t_{1/2}$  – 2-3 hour).<sup>[5]</sup> Hence, large dose is essential for anti TB activity. ETH has been linked to transient, asymptomatic elevations in serum aminotransferase levels and in uncommon instances of acute liver injury, which can be severe.<sup>[7]</sup> So, to overcome such problems ETH containing niosomes is better choice of selection. It improve the therapeutic performance of the drug molecules by delayed clearance from the circulation<sup>[8]</sup>, the vesicles may act as a depot and releasing the drug in a controlled manner<sup>[9]</sup> and also overcome drug resistance, to shorten the treatment course, and to reduce drug interactions. Hence, improving patient compliance and efficacy of treatment, and reduce drug related toxicity.<sup>[10]</sup> Niosomes are non-ionic bilayer surfactant vesicles in Nano-sized.<sup>[11]</sup> In the targeted tissue, it may allow drug release to sustain for prolonged time. This gives an advantage in enhanced therapeutic efficacy and minimized side effects due to the lower concentrations of the free drug in the blood.<sup>[11]</sup> It interest in delivery applications including nasal, parenteral, pulmonary, transdermal and ophthalmic delivery. Niosomes should be better choice for selection as they resolve all disadvantages of liposomes like stability, storage, expensiveness. Moreover, they can solubilize poorly soluble compounds, providing a tangible strategy to resolve formulation problems of many therapeutic molecules.<sup>[2]</sup>

## MATERIALS

Ethionamide (162 ±1°C) (IUPAC - 2-Ethylpyridine-4-carbothioamide) was gifted from Shiro Pharma Chem Pvt. Ltd., New Mumbai, Surfactant (Span-40, Span-60, Span-80), Cholesterol, TritonX-100 and Dialysis membrane 110LA395 (MW cutoff range 12000-14000) were purchased from Hi media lab, Mumbai. Chloroform and Methanol were purchased from Merk Millipore, Mumbai.

## METHODOLOGY

Niosomes were prepared by adopting the Thin Film Hydration technique. Briefly, an accurately weighed quantity of cholesterol and surfactant were dissolved in chloroform (5 ml)

and drug ETH was dissolve in Methanol (1 ml). Both the solvent mixture were mixed in 150 ml capacity round bottom flask. The solvent mixture was evaporated by rotary vacuum evaporator (Macro Scientific work Pvt. Ltd., Delhi) at 40°C by maintaining vacuum pressure of 380-420 mm/Hg to evaporate the solvent leaving a thin film (lipid phase) on the wall of the flask at a rotation speed of 100 rpm. All criteria for rotary vacuum evaporation were selected from prior studies. The complete removal of residual solvent can be ensured by applying vacuum. The dry lipid film was hydrated with 10 ml Phosphate Buffer 7.4 pH at a temperature of 50 to 60°C for 1 hour. The selected hydration time and volume was based on preliminary studies. The resultant niosomal suspension was kept aside for 2 hour at room temperature, to allow the vesicle's membrane to anneal. Niosomal suspension is then sonicated at 50% 3 cycle for 10 minute by probe sonicator (Bandelin soloplus, Germany). The formed niosomes were stored in a 4°C±2°C for subsequent analyses. Composition of all formulations was tabulated in table 1.

**Optimization of the formulation:** The molar ratio of non-ionic surfactant to cholesterol was optimized based on entrapment efficiency and zeta potential as well as mean particle size. Niosomal formulations using various surfactants (Span 40, Span 60 and Span 80) were prepared by thin film hydration technique. The best formulation was chosen among them.

**Table 1: Composition of Ethionamide niosomes formulations.**

Sr. no.	Ingredient	Selection of Surfactant							Optimization of Drug:CH Ratio			Optimization of Drug:CH: Span Ratio			
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14
1	Drug (mg)	25	25	25	25	25	25	25	25	25	25	25	25	25	25
2	Cholesterol (mg)	25	25	25	50	75	50	75	50	75	100	75	75	75	75
3	Span 40 (mg)	25	-	-	50	75	-	-	50	75	100	25	50	75	100
4	Span 60 (mg)	-	25	-	-	-	50	75	-	-	-	-	-	-	-
5	Span 80 (ml)	-	-	25	-	-	-	-	-	-	-	-	-	-	-

### Characterization of Ethionamide Niosomes

#### Determination of Entrapment efficiency of drug<sup>[1]</sup>

The untrapped drug was removed from niosomal suspension by dialysis method. Niosomal suspension was placed in 3cm x 8cm dialysis bag having MW cut off was 12,000 – 14,000. The dialysis bag was then placed in 250 ml beaker containing 200 ml Phosphate Buffer of pH 7.4 with constant stirring. Dialysis was carried out for 30 minutes by replacing the Phosphate Buffer of pH 7.4 with fresh at the end of every 15 minutes. The dialysis was completed where

constant ethionamide concentration could be detected in the solution. The drug content was determined by spectrophotometric method at 288.0 nm against Phosphate Buffer 7.4 pH as a blank. Amount of entrapped drug was obtained by subtracting the amount of unentrapped drug from the total drug incorporated. Furthermore, the entrapment efficiency was verified by lysis of vesicle with Triton X-100. For that, 10ml of dialyzed sample was taken and 0.5 ml of 0.1% triton X – 100 was added and incubated for 1 hour. Triton X–100 was added to lyse the vesicles in order to release the entrapped drug. Then it was diluted with distilled water and filtered through whatman filter paper. The filtrate was measured by UV method and the concentration of entrapped drug was estimated.

$$\text{Percentage Entrapment efficiency} = \frac{\text{Entrapped drug (mg)}}{\text{Total Drug Used (mg)}} \times 100$$

### Particle Size

Particle size of niosome vesicles determined by dynamic light scattering method using a zeta size analyzer (Nikisso microtrac USA – Department of pharmaceutics, Saurashtra Uni., Rajkot, Gujarat). An average dimensional distribution was then calculated referring to the mode.

### Zeta Potential

Zeta potential is a key indicator for evaluating the stability of dispersed colloid system. The higher of the absolute value of zeta potential, the bigger the electrostatic repulsion between each niosomes in suspension and can result in more stable niosomes in suspension and maintains a homogeneous, transparent and stable state for a long time without deposition of nanoparticles. For Zeta potential determination, the niosomal suspensions were suitably diluted with distilled water and dropped into the Zeta sizer electrophoretic cell which was carried out using a zeta potential analyzer (Nikisso microtrac USA – Department of pharmaceutics, Saurashtra Uni., Rajkot, Gujarat).

### Polydispersity Index

Polydispersity index was done for lyophilized nanoparticles. Polydispersity (non-uniform size distribution) was calculated by the following formula.

$$\text{Polydispersity Index} = (D_{0.9} - D_{0.1}) / D_{0.5}$$

Where  $D_{0.9}$ ,  $D_{0.5}$  and  $D_{0.1}$  are the particle diameters determined at the 90th, 50th and 10th percentile of undersized particles respectively. High polydispersity index value

indicates the high level of non-uniformity and is used to characterize the nanoparticles as monodisperse, homogeneous and heterogeneous systems.

### **Surface Morphology**

Scanning electron microscopy is an excellent tool for physical observation of morphological features of niosomes and helpful to examine the shapes and for qualitative assessment of morphology of niosomes. The surface characteristics of the niosome vesicles were studied by scanning electron microscopy (SEM). The samples were observed for morphological characterization using a gaseous secondary electron detector (SEM analyzer – ZEISS, Agriculture University, Junagadh, Gujarat).

### ***In-vitro* release study for niosomal formulations and analysis by UV method<sup>[12]</sup>**

The release was studied using membrane diffusion technique. 2ml of dialyzed niosomal suspension of ethionamide was placed in a glass tube having diameter 2.5cm, an effective length of 8cm. It was previously covered with soaked osmosis cellulose membrane, which acts as a donor compartment. The glass tube was placed in a beaker containing 100ml of phosphate buffer solution 7.4 pH, which act as receptor compartment. It was assembled in such a way that the lower end of the tube containing suspension was just touching (1-2mm deep) the surface of diffusion medium. The temperature of receptor medium was adjusted and maintained at  $37 \pm 10^{\circ}\text{C}$  and agitated at 100rpm speed using magnetic stirrer. 5ml of sample were withdrawn periodically and after each withdrawal same volume of medium was replaced and collected samples were analyzed at 288nm in Double beam UV-Visible spectrophotometer using phosphate buffer (pH 7.4) as blank.

### **Stability Study<sup>[13]</sup>**

The physical stability of the prepared niosomes against aggregation and leakage of the payload was studied over one month at two different temperatures;  $4^{\circ}\text{C}$  temperature and room temperature. More specifically, the effects of ageing and storage temperature on the physical stability were evaluated.

### **Sterility Testing**

Sterility testing was carried out by Sterility medium: sterility testing medium-A (M017 Himedia, Mumbai). The medium was sterilized by autoclaving at  $121^{\circ}\text{C}$  at 15 lb for 20 min. The sterility medium was cooled and 25 ml of the aliquot was transfer to three pre-sterilised test-tube. The test-tube was tested for positive control, negative control, and for sample

testing. In positive control, inoculation of microorganism was carried out. In negative control, there was only sterility medium was kept and for testing of formulation, 2.5 ml of final optimized niosomal formulation was added in sterility medium. The sample quantity added in sterility medium was taken according to IP 2010. Then, tube was kept in incubator at 37°C for 24 hour. After 24 hr, check whether turbidity was produce or not.

Incubation temperature: 37°C

Incubation period: 24 Hours

Sterility medium: Sterility testing medium-A (M017 Himedia, Mumbai)

### Statistical analysis

All the experiments were conducted in triplicate. The student's t test was used to analyze the stability study and pharmacokinetic properties. The significant level (P) was set as 0.05.

## RESULT AND DISCUSSION

**FT-IR Analysis:** The FT-IR spectra was carried out for pure drug as shown in figure 1. The results are summarized as follows.

Briefly, a pellet of the drug and KBr (Spectroscopic grade) was prepared using hydraulic pellet press at a pressure of 7-10 tones. FT-IR was scanned from 400 - 4000  $\text{cm}^{-1}$ . Following peaks were observed which was compared with the standard spectra of ethionamide drug (fig.2). It signifies that the drug sample, procured from shiro pharma chem pvt. Ltd. Mumbai, was pure Ethionamide.

**Table 2: Integration of the FTIR of Ethionamide.**

Sr. No.	Group	Type of Vibration	Wave number ( $\text{cm}^{-1}$ )	Identification
1	C-C	Streching	1419.66	Aromatic
2	C-H	Streching	2964.69	Aromatic
3	C=S	Streching	1151.54	Thioamide
4	C-N	Streching	1207	Amine
5	C-H	Streching	2877.89	Alkyle
6	N-H	Streching	3270	Amine
7	N-H	Bending	1595.18	Amine

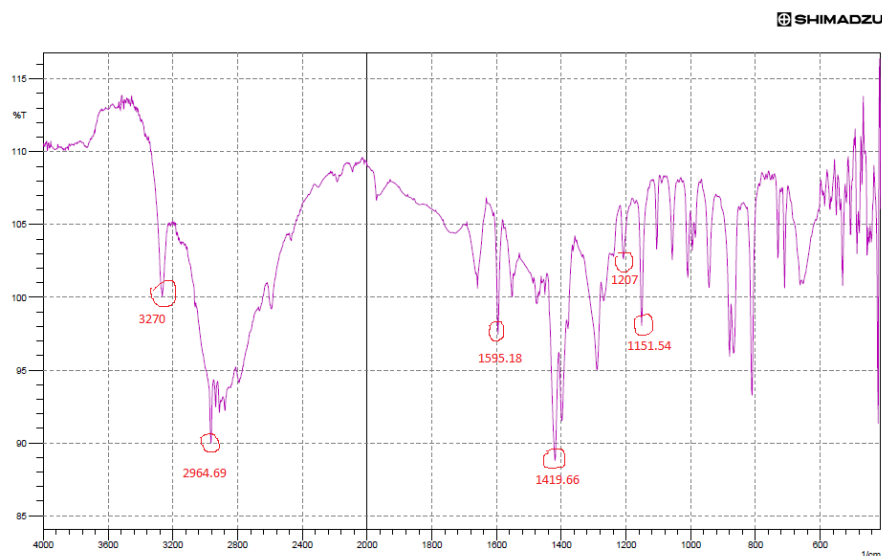
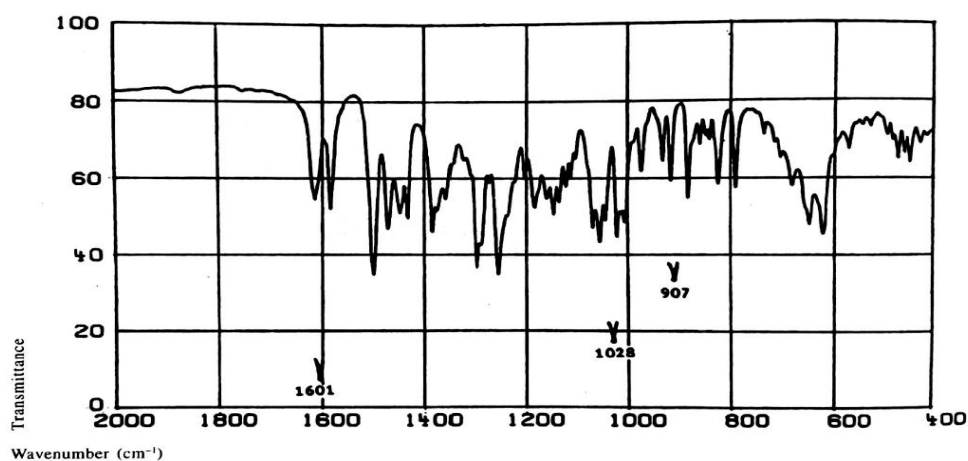


Figure 1: FTIR Spectra for Ethionamide pure sample.

**Ethionamide**

**FTIR**

Phase: KBr disc



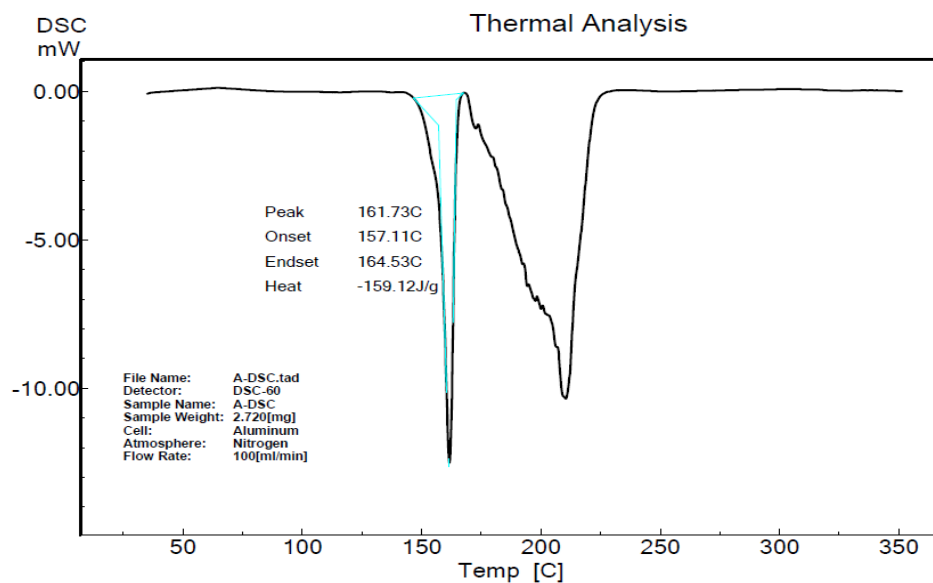
330

Figure 2: FTIR Spectra of Ethionamide Standard.<sup>[4]</sup>

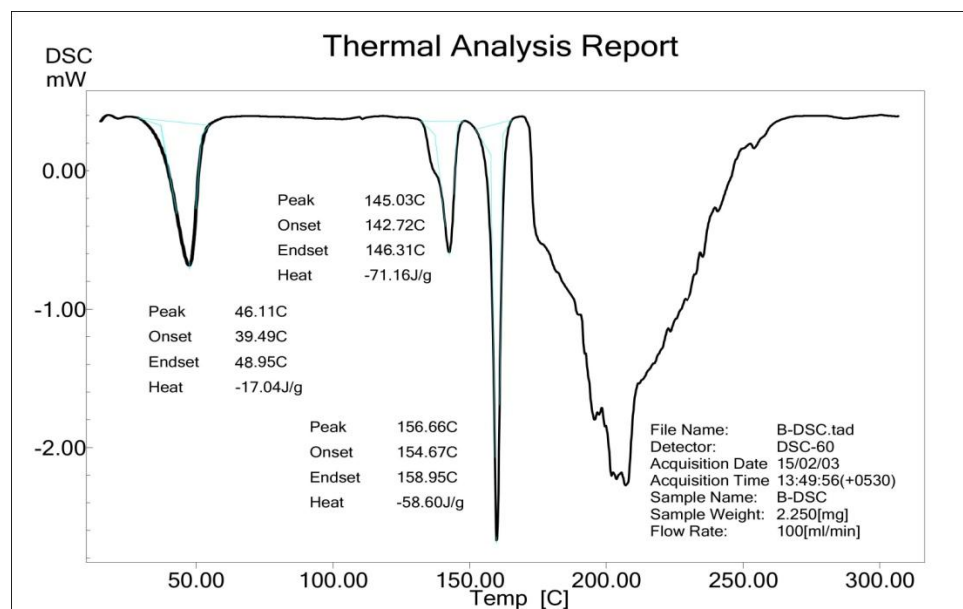
### Drug and Excipients Compatibility Study

Compatibility study was carried out by differential scanning calorimetric method. In the present study, Figure 4 shows the combined peaks of physical mixture of cholesterol, span 40 and ethionamide. The span 40 shows peak at 46.11°C, the cholesterol shows peak at 145.03°C whereas the Ethionamide shows its presence at 156.66°C. It was observed that there was no any significant change in main peak of pure ethionamide (161.73°C) as shown in figure 3. Hence, DSC confirmed that there was no any physical interactions between drug ethionamide and other excipients used in the formulations.





**Figure 3: DSC of Pure Ethionamide.**



**Figure 4: DSC of Physical mixture of Ethionamide, Span 40 and Cholesterol.**

### Optimization Process

**A. Selection of surfactant:** As per Table 1, the formulations were prepared and evaluated for the particle size, Zeta potential, and PDI. F1 formulation (177.0 nm) shows the least particle size in comparison to F2 (228.3nm) and F3 (263.6nm). On the other hand, F2 formulation (68.54%) shows the highest drug entrapment in comparison with than F1 (25.16%) and F3 (47.84%) formulation. Here, span 80 was excluded as it showed least drug entrapment and higher particle size and hence by excluding the Span 80, further studies were carried out with Span 40 and Span 60. Different ratios of span 40 and span

60 were taken and with least particle size and higher drug entrapment, formulation F5 (span 40) was selected for further studies.

**B. Optimization of Drug & Cholesterol concentration:** After selection of span 40, different formulations (Table 1) were formulated for the optimization of drug and cholesterol concentration. Formulations coded F9 (88.80%) and F10 (92.20%) shows higher entrapment efficiency as compare to F1 (25.16%) and F8 (67.60%). Hence, F9 & F10 formulation were selected for further studies but it was shown that the zeta potential of F10 formulation was 10.51 mV and there would be a more chances of instability of niosomal suspension. As per the literature, suspension having zeta potential in between -30 to +30, particles may get aggregate and size is increases <sup>2</sup>. Hence F9 formulation was chosen for further studies.

**C. Optimization of Drug, Cholesterol & Surfactant concentration:** After fulfilling the selection of surfactant and cholesterol amount, the third optimization process for determining drug, cholesterol and span concentration for getting best niosomal suspension formulation was carried out. In this optimization process, four formulation were formulated and evaluated. There were no any significant difference in Percentage entrapment efficiency of all four formulations. While comparing formulations in favour of particle size, PDI and zeta potential, Formulation F12 (124.4nm) had least particle size than the F11 (228nm), F13 (182.5nm) and F14 (501nm). Zeta potential of Formulation F13 was found to be 10.51mV which was signifies that higher possibility of aggregation of the niosomes causing bigger size of niosomal suspension. Hence, formulation F12 with 88.9% EE, 124.4nm particle size, 1.294 PDI and -39.7mV zeta potential, was selected for final optimized batch.

**Table 2: Data shows %EE, Zeta Size, PDI and Zeta Potential.**

Results	Selection of Surfactant							Optimization of Drug:CH Ratio			Optimization of Drug:CH: Span Ratio			
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14
% EE	25.16 %	68.54 %	47.84 %	67.60 %	88.80 %	82.40 %	71.20 %	67.60 %	88.80 %	92.20 %	92.00 %	88.90 %	88.60 %	90.90 %
Particle Size (nm)	177	228.3	263.6	205.2	182.5	1544	371	205.2	182.5	143.4	228	124.4	182.5	501
PDI	0.903	3.32	1.137	1.364	1.426	3.38	1.469	1.364	1.426	2.067	1.364	1.294	2.067	3.32
Zeta potential (mV)	-36.21	-49.51	21.13	44.08	41.77	37.61	33.12	44.08	41.77	10.51	44.08	-39.71	10.51	41.44

### Scanning Electron Microscopy

SEM image (Fig. 5a, 5b) showed that the optimized ethionamide niosomes were spherical in shape. Most of the particles size was of 124.4 nm, which was suitable for the intravenous delivery.

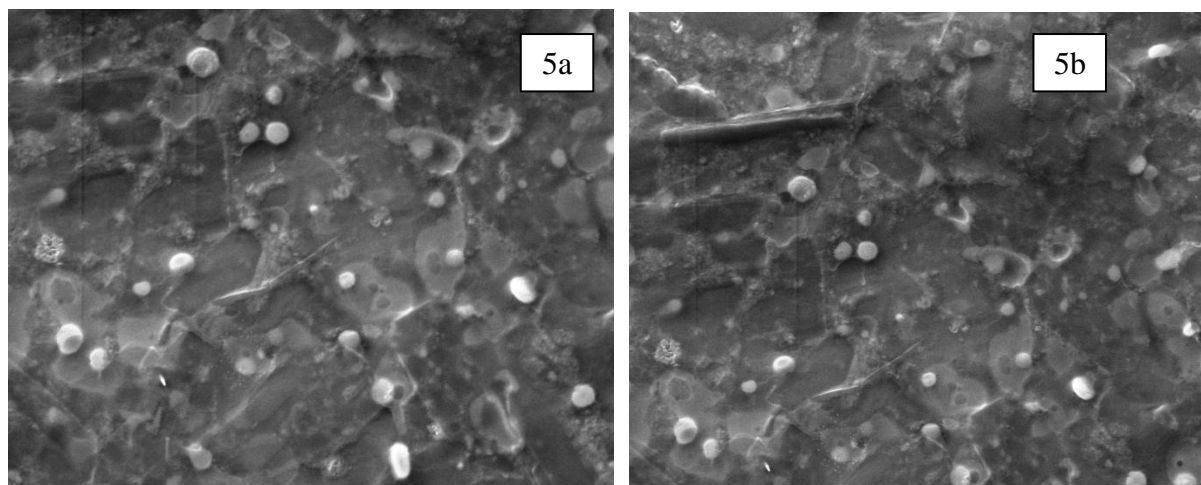


Figure 5 – SEM analysis of Optimized formulation.

### In-Vitro release study for Final optimized formulation

The optimized ethionamide niosomal suspension (F12 batch) was subjected for *in vitro* drug release behaviour.

Figure 6 shows release profile of ETH from the prepared optimized niosomes. After performing in-vitro release study for final optimized formulation it was found that 94.89% of drug ETH was released from the niosomes at the end of 24 hr. The partitioning of ETH molecules through the bilayer membranes of the niosomes was the rate-controlling step in the release process. Hence, an appreciable concentration gradient was maintained.

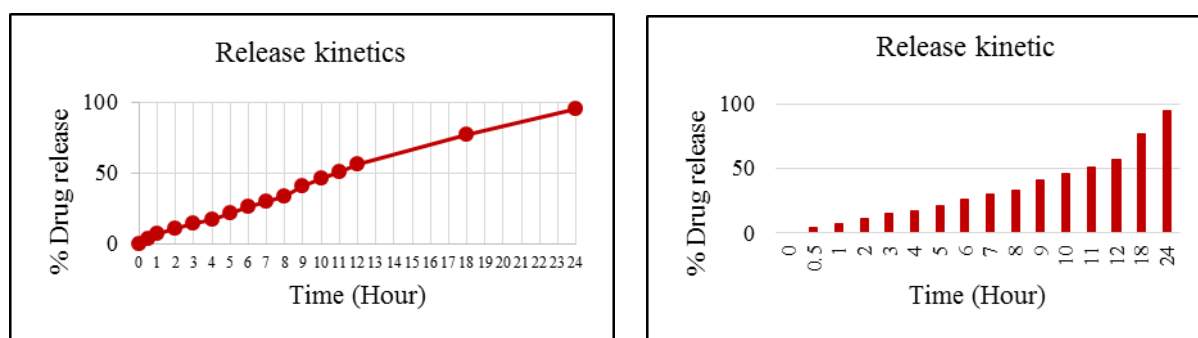
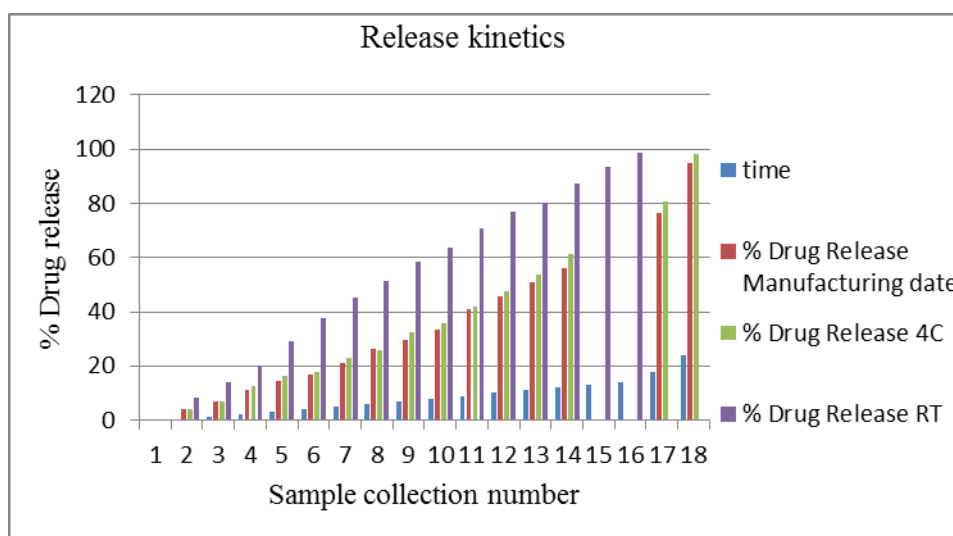


Figure 6: % Drug Release of final optimized formulation batch at a time of manufacturing date.

### Stability Study

After 30 days of storage period in two different temperature condition that was at room temperature and at 4°C temperature, the final optimized batch was evaluated for mean particle size and release kinetics. Results showed that, after 30 days of storage at room temperature, the optimized formulation coded F12 showed mean particle size of 1,141nm and about 98.79% of drug was released within 14 hours. Whereas mean particle size and %drug release after 24 hours of optimized formulation batch was found to be 197.2nm and 98.33% respectively. These results (Figure 7) suggest that the storage temperature is more likely to have a noticeable effect on the stability of the prepared ethionamide niosomes against aggregation. At higher temperature (room temperature), ethionamide niosomes were relatively unstable compared with the cold storage temperature (4°C temperature). The thermal energy imparted to vesicles increases when the temperature increases. Hence, the rate and force of collision between vesicles increases. ETH niosomes tend to aggregate and coalesce to become more energetically stable and consequently, ETH niosomes size increases. These findings suggest that the storage temperature of choice is 4°C±2°C where the prepared niosomes showed minimal changes in sizes over 30 days.

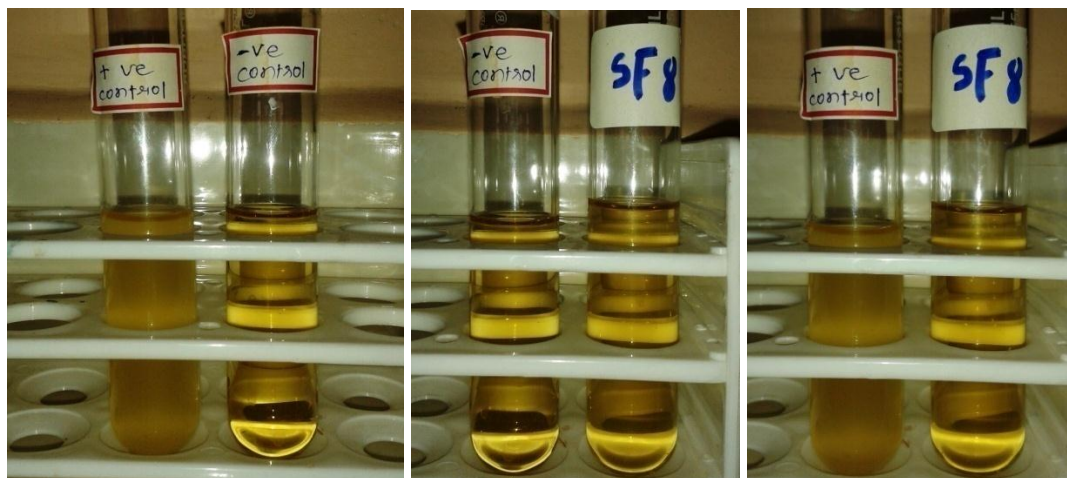


**Figure 7: % Drug Release of final optimized formulation batch at a time of manufacturing date and after 30 days of storage in two different temperature condition; 4°C temperature and Room temperature.**

### Sterility testing study

Sample test tube was compared with positive control test tube and negative control test tube. This result finds that the optimized niosomal formulation had no any microbial growth

formation as it did not show any turbidity as shown in positive control test tube during the formulation process (Figure 8).



**Figure 8: Sterility testing test tube with positive control, negative control & Formulation.**

**Note: SF<sub>8</sub> = F12**

## CONCLUSION

The study has presented the ethionamide (ETH) niosomes as a new and potential treatment for Multi-drug resistance tuberculosis prepared by thin film hydration method by which the ETH is released for a prolong period of time (94.89% within 24hrs). Along with the main attributes of niosomes of ETH as an I.V. route of administration is to prolong the uptake of ETH. This delivery can provide the pharmaceutical formulator using a surfactant-based delivery system as a potential parenteral pharmaceutical dosage form for ETH. It has been concluded that the Niosomes prepared with Span-40 (F12) has more entrapment efficiency and releases drug slowly in a sustained manner as compared to other formulations. The prolonged release of the drug from the niosomes suggests that the frequency of administration may be reduced. Further, as the particles are in nanometer size range, the bioavailability may be increased and effective targeting may be achieved. Hence, we can conclude that niosomes provide controlled release of drug and these systems are used as drug carriers for the delivery of hepatotoxic drugs with severe side effects.

## ACKNOWLEDGEMENTS

I would like to express my very great appreciation to Dr. Balwant Parchure (Technical head, Shiro Pharma Chem Pvt. Ltd., New Mumbai, Maharashtra-India) for his valuable and

constructive suggestion during this research work. I would also like to thank my friends for their valuable assistance during this work.

## REFERENCES

1. Tangri P and Khurana S. Niosomes: Formulation and Evaluation. *International Journal of Biopharmaceutics*, 2011; 2(1): 47-53.
2. Kumar A, Pal JL, Jaiswal A and Singh V. Review on Niosomes as novel drug delivery system. *International Research Journal of Pharmacy*, 2011; 2(5): 61-65.
3. World Health Organization (WHO), "Global Tuberculosis Report", 2015.
4. Indian Pharmacopoeia 2010. The Indian Pharmacopoeia Commission, Ministry of Health and Family Welfare, Ghaziabad, Vol. II, 2010: Pp. 1308.
5. National Center for Biotechnology Information: PubChem compound Database. <http://pubchem.ncbi.nlm.nih.gov/coumpound/2761171>
6. Ethionamide - 2014 available at <http://www.drugbank.ca/drugs/DB00609>
7. TB Online-2011 available at <http://tbonline.info/post/2011/24/8/ethionamide>
8. Buckton G and Harwood. *Interfacial phenomena in drug delivery and targeting*. Academic Publishers, 1995; 13: 154-155.
9. Madhav NVS and Saini A. Niosomes a novel drug delivery system. *International Journal of Research in Pharmacy and Chemistry*, 2011; 1(3): 498-511.
10. Hari BNV, Chitra KP, Bhimavarapu R, Karunakaran P, Muthukrishnana N and Samyuktha R. Novel technology: A weapon against tuberculosis. *Indian Journal of Pharmacology*, 2010; 42(6): 338-344.
11. Hu C and Rhodes DG. Proniosomes: a novel drug carrier preparation. *International Journal of Pharmaceutics*, 1999; 185: 23-35.
12. Patel GM and Parmar DR. Formulation, Optimization and Evaluation of Niosomal Gel of Alitretinoin. *American Journal of Pharmacy and Health Research*, 2013; 1(4): 66-80.
13. Gevariya HB. Ph. D. Thesis: Formulation and Evaluation of Sustained Release Ocular Drug Delivery System for an Anti-Glaucoma Drug. Saurashtra University 2013.