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Design, Development and Characterization of Pyrazinamide Niosomal Dosage Form

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ABSTRACT

Pyrazinamide being a 1st line of defense against tuberculosis is very effective for the first 2 to 3 months of treatment and helps to eradicate a major portion of the strain. But due to its increased use, there are adverse effects such as hepato-toxicity and other dose related side effects. The amount of drug used in formulation is high i.e. 500mg to 1gm. Another major disadvantage is resistance of the bacteria leading to DR-TB (drug resistant tuberculosis). The main objective of this study is to decrease the amount of drug needed for formulation and to avoid hepato-toxicity. Pyrazinamide drug formulation was prepared into a niosomal dosage form by modified ether injection method, using various concentrations of polymer and keeping cholesterol content constant. Characterization was carried out and vesicle size determination showed that the formulated vesicles were in the range of 110-350nm. FTIR results showed that drug and polymers were compatible. Drug content and entrapment efficiency were calculated using UV-spectroscopy at 268nm. In-vitro release studies were carried out for all formulations and it was seen that Span-80 formulation had the highest percentage release when compared to other formulations. The drug release was subjected to various kinetic models and it was observed that all formulations followed zero order kinetics. It can be concluded that all these polymers can be used for the successful formulation of Pyrazinamide niosomes and the surfactant that is most apt was found to be Span-80 in the ratio 1:3 as compared to other polymers in various ratios.

Keywords: Pyrazinamide, tuberculosis, hepato-toxicity, drug resistant tuberculosis, niosomes.

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INTRODUCTION

Niosomes are microscopic vesicles which have been formed by the hydration of synthetic non-ionic surfactant with or without the inclusion of cholesterol. They are similar to liposomes. They act as active carriers for both amphiphilic and lipophilic drugs. The main difference between the two systems is that niosomal bilayer is formed by non-ionic surfactant and liposomal bilayers are made up of phospholipids[1]. Niosome have hydrophilic ends exposed outwards while hydrophobic ends facing each other forming bilayered surfactant. The size ranges is between 10 to 1000nm. Along with cholesterol, the addition of small quantity of anionic surfactant (dicetyl phosphate) stabilizes the niosomal vesicles. Higher chemical stability of surfactants is found in niosomes than phospholipids present in liposomes which are easily hydrolyzed. They are a promising drug delivery system which is effective and also helps in targeting the organs or tissues[2].

Tuberculosis, or TB (short for tubercle bacillus) is a common, and lethal, infectious disease. TB is caused by various strains of mycobacteria, usually Mycobacterium tuberculosis[3]. Tuberculosis in general attacks the lungs and may even affect other parts of the system. Spreading of this infectious disease takes place through the air. Treatment for TB is challenging and necessitates administration of multiple antibiotics over an extended period of time. When a person is infected with a strain of TB that is resistant to medication, then it called as Primary resistance. Secondary (acquired) resistance occurs when a person having a full susceptible TB, develops due to inadequate therapy or treatment, not following the regimen that is prescribed or may also be called as lack of compliance, may also be due to usage of low-standard medication. A serious issue in many developing countries that the public face is Drug-resistant strains of TB because the treatment is more time consuming and extremely expensive drugs are required for its treatment. The term MDR-TB is as stated because the strains are resistant against three of the most effectual first-line drugs used in TB that is pyrazinamide, isoniazid and rifampicin.

MATERIALS AND METHOD

Materials used

Pure drug pyrazinamide (Sun Pharma) for preparation of niosomes, Brij-35, Span-80, Tween-80, cholesterol, diethyl ether, chloroform, n-propanol used were of analytical grade procured from HIMEDIA, LOBA Chemie, s d fine-CHEM limited and NICE chemicals.

Preparation of calibration graph

The UV absorbance of the working standard solution of pyrazinamide in water was obtained at

268 nm against distilled water as blank. Calibration graph was generated by plotting the absorbance values against corresponding concentrations of pyrazinamide.

Compatibility Study (FT-IR analysis)

IR-spectroscopic analysis was carried out using a FTIR spectrophotometer and the spectrum was recorded in the wavelength region of 4000–400 cm^{-1}. The procedure consisted of dispersing the samples in KBr and gentle grinding followed by compression using hydraulic press with a pressure of 100 kg/cm^2 to prepare pellets.

Preparation of pyrazinamide niosomes

Modified ether injection method[4]

In this method, cholesterol and non-ionic surfactant (Span-80, Tween-80, Brij-35) were dissolved at various ratios in 5ml of diethyl ether/chloroform which was injected slowly at a rate of 0.25 ml/min through 16 gauges needle into 10ml of hydrating solution of distilled water containing Pyrazinamide as the aqueous phase. The solution was stirred on magnetic stirrer by maintaining the temperature at 60°C. The formulations of the prepared niosomes are shown in Table 1.

Table 1. The formulation code for the prepared pyrazinamide niosomes

Code	Amount of Drug(mg)	Contents	Ratios	Amount of Cholesterol(mg)	Amount of Surfactant(mg)
N1	10	Cholesterol : Brij-35	1 : 1	10	10
N2	10		1 : 2	10	20
N3	10		1 : 3	10	30
N4	10		1 : 4	10	40
N5	10	Cholesterol : Span-80	1 : 1	10	10
N6	10		1 : 2	10	20
N7	10		1 : 3	10	30
N8	10		1 : 4	10	40
N9	10	Cholesterol : Tween-80	1 : 1	10	10
N10	10		1 : 2	10	20
N11	10		1 : 3	10	30
N12	10		1 : 4	10	40

CHARACTERIZATION OF NIOSOMES

Photomicrographs[5]

Photographs of the prepared formulations were taken at 40X magnification using 2 megapixel version of Motic's popular B1 microscope connected to a computer from which the shape of the prepared vesicles was confirmed.

Scanning Electron Microscopy

The suspension of niosome was freeze dried and the shape and topography of the niosomes were

studied using SEM. A drop of hydrated niosome was transferred onto a silver tape on aluminum stub. The stubs were stored under vacuum and then placed into the instrument and was scanned.

Vesicle size determination[6]

Vesicle size determination of Pyrazinamide niosomes were carried out using Malvern zeta-sizer nano series (Nano-S90) instrument. A quantity of 2 ml, prepared niosomes were taken into the cuvette and exposed to laser light diffraction at an angle of 90°.

Determination of Density

Density of each preparation was carried out by using specific gravity bottle. The weight of water and formulations were measured and substituted in the formula given below.

$$Density\ of\ sample\ (\rho_2) = \frac{w_2}{w_1} \times \rho_1$$

Where,

w_1 - weight of water

w_2 - weight of sample

ρ_1 - density of water (0.998)

ρ_2 - density of sample

Determination of Viscosity

Viscosity of the formulation was determined using Ostwald Viscometer. The time taken for water and formulation to flow from point A to B was calculated and substituted in the formula and the viscosity was calculated

$$Viscosity\ of\ sample\ (\nu) = \frac{\rho_1 \times t_1}{\rho_2 \times t_2} \times \nu_2$$

Where,

ρ_1 - density of sample

ρ_2 - density of water

ν_1 - viscosity of sample

ν_2 - viscosity of water

t_1 - time taken for sample to flow from point A to B

t_2 - time taken for water to flow from point A to B

Zeta Potential[7]

Zeta potential or ions on the slipping plane of vesicles is measured based on laser Doppler electrophoresis technique using Malvern zeta-sizer nano series (Nano-S90) instrument. Zeta potential is the electrical potential in the interfacial double layer at the location of the slipping

plane versus a point in the bulk fluid away from the interface. A value of 25 mV (positive or negative) can be taken as the arbitrary value that separates low-charged surface for the highly charged surfaces.

Assay & Entrapment efficiency[8]

1ml niosomal preparation of Pyrazinamide was taken into a 10ml volumetric flask and was lysed with 9 ml of propane-1-ol by continuous shaking. From this solution, 1 ml was drawn into a 250ml volumetric flask and it was subsequently diluted. Absorbance was measured at 268nm and the drug content was estimated from the calibration curve.

$$Entrapment\ Efficiency\ (\%) = \frac{C_e}{C_t} \times 100$$

Where,

C_e - the amount of entrapped drug

C_t - the initial amount drug

***In vitro* release studies**[9]

In-vitro release was carried out using Himedia dialysis membrane 50 with the molecular weight cut-off range from 12000 – 14000 daltons which has the capacity of holding 1.61 ml/cm. Dialysis bag which acts as a donor compartment was soaked in warm water for 10min and closure clips were used to close the dialysis bag on both the sides to prevent the leakage of formulation during the release study.

Centrifugation of niosomal dispersion was done at 9000 rpm for 30 minutes. The sediment which has the entrapped drug was taken for the release study after reconstituted with 1ml of PSB pH 7.4. The dispersed entrapped drug was taken in the dialysis bag and it was closed using closure clips. Dialysis bag was placed in 100ml of PSB pH 7.4 which acts as the receptors compartment. The medium was stirred by using magnetic stirrer at 100rpm in room temperature. At each one hour interval 5ml of sample were withdrawn and after each withdrawal, same volume of medium was replaced. Then the samples were assayed spectrophotometrically, at 228nm using PSB pH 7.4 as blank.

$$Cumulative\ \%\ release = \frac{Concentration\ (\mu g) \times Bath\ Volume}{1000 \times Drug\ Content} \times 100$$

Determination of drug release kinetics[10]

To study the release kinetics from the formulation, data obtained from in-vitro drug release studies were plotted in various kinetic models: zero order (Equation 1) as cumulative percentage of drug released vs. time; first order (Equation 2) as log cumulative the percentage of drug

remaining vs. time; Higuchi's model (Equation 3) as cumulative percentage drug release vs. square root of time.

$$C = K_0 t \quad (1)$$

Where, K_0 is the zero order rate constant expressed in units of concentration/time and t is time in hour. A graph of concentration vs. time would yield a straight line with a slope equal to K_0 and intercept the origin of the axis.

$$Log\ C = \frac{Log\, C_0 - Kt}{2.303} \quad (2)$$

Where C_0 is the initial concentration of the drug, K is the first order constant and t is the time.

$$Q = K t_{\frac{1}{2}} \quad (3)$$

Where, K is the constant reflecting the design variable of the system and t is the time in hour. Hence, drug release rate is proportional to reciprocal of the square root of time.

RESULTS AND DISCUSSION

Compatibility Study (FT-IR analysis)

The FT-IR spectrum of a physical mixture of drug and polymer (brij-35) is given in Figure 1. From the spectra it was observed that there were no significant changes observed in the spectra of the corresponding pure drug and the polymers. The characteristic peaks of pyrazinamide was observed

- Stretch in primary amides (-NH2) gives two bands 3413 (peak 1) and 3162 cm^{-1} (peak 3) respectively.
- -C=O stretch of primary amides occurs at 1610 cm^{-1} (peak 10).
- -N-H bending occurs at 1580 cm^{-1} (peak 11).
- Absorptions at 1610 (peak 10), 1580 (peak 11) and 1523 (peak 12) show the presence of aromatic ring.

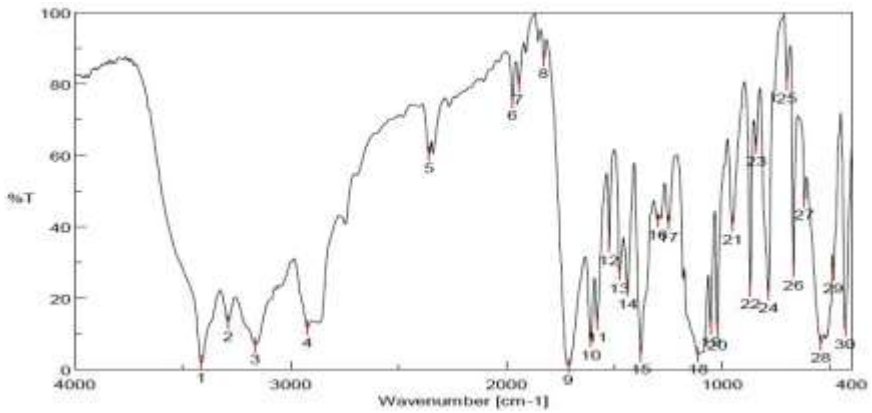

Figure 1. FT-IR spectrum of physical mixture of drug and polymer (Brij-35)

Physical Characterization

Photomicrographs

From the following observations, it was found that formulation N1 (Brij: Drug = 1:1) had fewer yields of niosomes when compared to the other concentrations for Brij-35 formulations. In the case of Span-80, formulation N8 (Span-80: Drug = 4:1) had lower yield of niosomes when compared to the other formulations. Whereas for Tween-80, formulation N9 (Tween-80: Drug = 1:1) had irregular shape formation when differentiated with the other formulations of Tween-80 polymer. So from the following observations, formulations N1, N8 and N9 were discarded from the 12 formulations and the remaining 9 formulations were selected.

Out of the 12 formulations that were prepared using Brij-35, Span-80 and Tween-80 in concentrations 1:1, 1:2, 1:3, 1:4, nine were selected for further evaluation. The selected concentrations are 1:2, 1:3 and 1:4 formulations in Brij-35 category. Concentration 1:1 was not chosen as the number of vesicles formed was less compared to the other 3 formulations. The concentrations of formulations selected in Span-80 are 1:1, 1:2, 1:3 where 1:4 was not selected as the number of vesicles formed was comparatively less. Concentration of Tween-80 formulations that were selected are 1:2, 1:3, 1:4 and 1:1 was omitted as there was no formation of vesicles. Thus the 9 selected formulations were used for further analysis and evaluation.

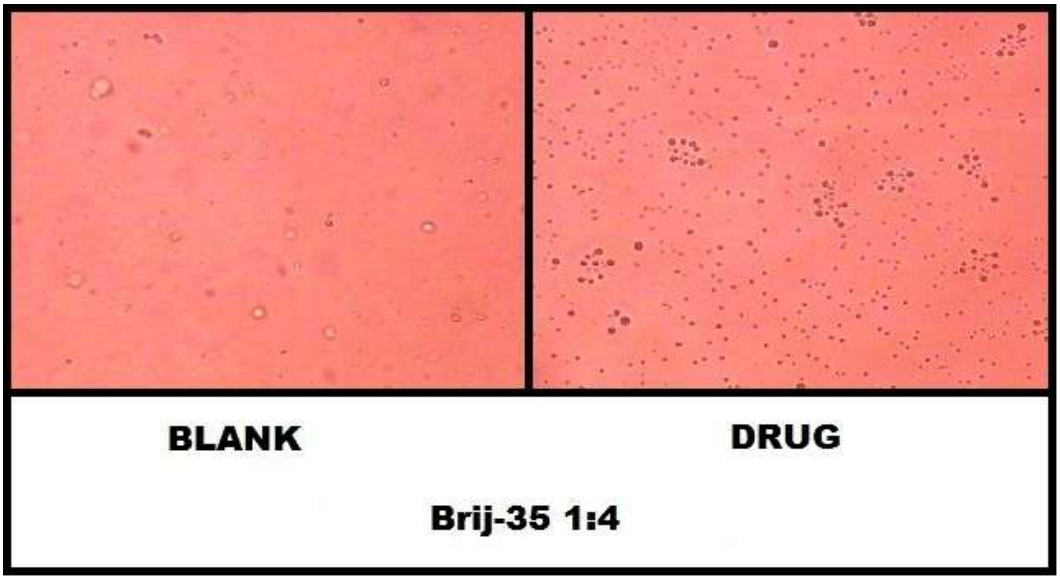

Figure 2. Photomicrograph comparing N4 niosomes and its blank

Scanning Electron Microscopy

The SEM images of pyrazinamide niosomes (N10) are shown in Figure 3. As the niosomes were prepared in the form of suspension, it was freeze dried. Thus the image depicts particles with increased surface area. The images also shows drug particles embedded into the non-ionic

surfactant. Clumping of niosomes can be observed due to freeze drying. The image shows that the prepared niosomes after freeze drying can be found in an amorphous state and thus can also be converted into a lyophilized form.

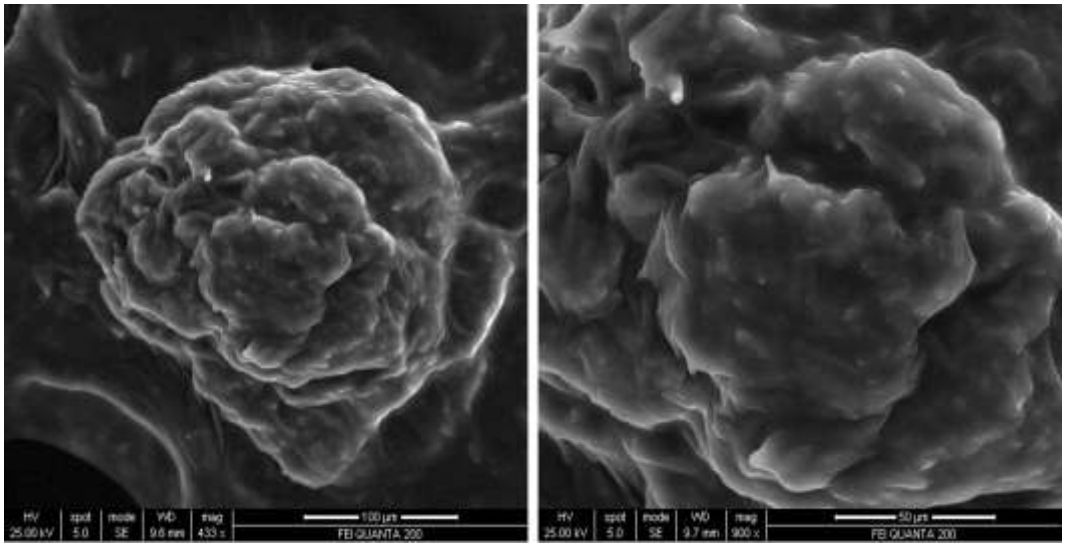

Figure 3: SEM images of N10

Vesicle Size Determination

From the obtained results for the average size shown in Figure 4 and Table 2, it was found that formulation N10 (tween-80 ratio 1:2) has the lowest size of 114.2 nm and N6 (span-80 ratio 1:3) has maximum size of 348.9 nm. It is also observed that there is a proportional increase of the vesicle size as the concentration of the polymer is increased. It is seen in the case of Brij-35, Span-80 and Tween-80 where the vesicle size increases from 238.2 nm to 269.9 nm, 247.5 nm to 263.0 nm, 114.2 nm to 289.4 nm respectively.

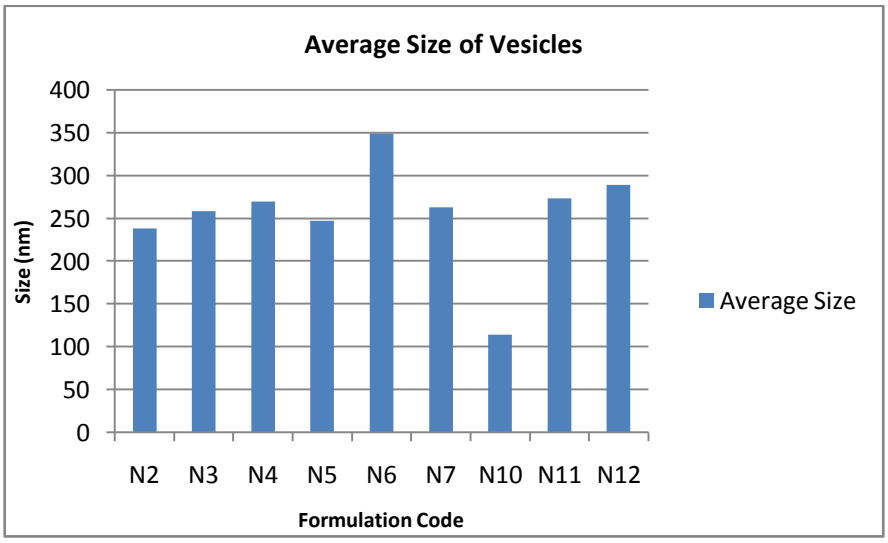

Figure 4. Comparative Average Vesicle Size of Niosomal Preparations

Determination of density

The densities of various formulations of Brij-35, Span-80 and Tween-80 were calculated. Maximum density was found to be 0.9977 mg/ml for formulation N11 (Tween-80 1:2) whereas minimum density was found to be 0.9902 mg/ml for formulation N5 (Span-80 1:1) as shown in Table 2.

Determination of viscosity

Formulation N7, prepared form Span-80 with a concentration of polymer three times to that of drug and cholesterol, has the highest viscosity, where as the lowest viscosity was found out to be for formulation N10, prepared from Tween-80 at concentration 1:1. It was also observed that there was a proportional increase in the viscosity of the formulation, shown in Table 2, with respect to the increase in polymer concentration.

Zeta Potential

The zeta potential that was calculated, given in Table 2, and it was found that N7 had the highest zeta potential of -21.5 whereas the lowest was found to be for N6 which is -5.77. Zeta potential is a very important factor which plays a role in the stability of the niosomal dosage form. Charge inducing characters such as dicetyl phosphate (positive) or sterylamine (negative) must be added to the formulation to increase the charge of the formulation. The charge of the niosomal dosage form must be ±25 mV so as to get a stable formulation. Since no charge inducing substances were added to the formulation, the zeta potential that is calculated solely depends upon the formulation alone.

Table 2. Physical Characterization of prepared niosomes

S.No:	Formulation Code	Average Size (d.nm)	Density (mg/ml)	Viscosity (cP)	Zeta Potential (mg)
1	N2	238.2	0.9958	1.3434	-8.93
2	N3	258.4	0.9944	1.4090	-7.13
3	N4	269.9	0.9926	1.4792	-7.14
4	N5	247.5	0.9902	1.3771	-17.4
5	N6	348.9	0.9919	1.8050	-5.77
6	N7	263.0	0.9945	1.9657	-21.5
7	N10	114.2	0.9908	1.3367	-11.3
8	N11	273.5	0.9977	1.4189	-9.23
9	N12	289.4	0.9932	1.5060	-12.5

Assay & Entrapment efficiency

The drug content present in the niosomes prepared by modified ether injection method ranged from 6.48 – 9.58mg. It was observed that formulation N3 has highest drug content which was found to be 9.58mg (95.8 %) and the lowest drug content was found to be for formulation N7

which is 6.48mg (64.5 %). It was found that there is a decrease of drug content as we increase the concentration of polymer in the case of Brij-35 and Span-80 formulations, whereas there was a minor increase in drug content in the case of Tween-80 formulations.

Table 3. Assay & Entrapment efficiency of niosomal preparations

S.No:	Formulation	Drug Content (mg)	Entrapment Efficiency (%)
1	N2	8.20	82
2	N3	9.58	95.8
3	N4	7.06	70.6
4	N5	7.34	73.4
5	N6	8.78	87.8
6	N7	6.48	64.8
7	N10	8.01	80.1
8	N11	7.74	77.4
9	N12	7.78	77.8

Table 4. Cumulative Percentage Release data for niosomal formulations

Time (min)	Cumulative Percentage Release (%)								
	Drug : Brij-35			Drug : Span-80			Drug : Tween-80		
	N2	N3	N4	N5	N6	N7	N10	N11	N12
0	0	0	0	0	0	0	0	0	0
30	3.22 ± 0.23	5.68 ± 0.5	3.90 ± 0.91	4.51 ± 0.3	3.12 ± 0.09	4.32 ± 0.81	3.98 ± 0.12	4.96 ± 0.64	4.23 ± 0.51
60	7.16 ± 0.72	9.70 ± 0.18	6.17 ± 0.3	9.80 ± 0.2	7.29 ± 0.11	8.63 ± 0.39	7.26 ± 0.91	8.58 ± 0.44	8.12 ± 0.23
120	12.86 ± 0.35	15.35 ± 0.21	10.24 ± 0.08	15.85 ± 0.72	15.62 ± 0.32	17.85 ± 0.52	13.59 ± 0.08	16.73 ± 0.5	12.80 ± 0.45
180	19.01 ± 0.64	25.56 ± 0.37	17.61 ± 0.19	22.39 ± 0.28	19.71 ± 0.55	29.83 ± 0.1	19.75 ± 0.15	23.01 ± 0.09	18.35 ± 0.25
240	25.21 ± 0.13	32.69 ± 0.3	23.87 ± 0.27	31.48 ± 0.44	29.04 ± 0.83	42.15 ± 0.67	25.02 ± 0.19	30.19 ± 0.93	22.72 ± 0.53
300	34.46 ± 0.3	44.90 ± 0.81	34.33 ± 0.56	40.78 ± 0.64	41.42 ± 0.4	49.49 ±0.78	36.52 ± 0.09	39.37 ± 0.27	33.79 ± 0.63
360	44.61 ± 0.13	50.05 ± 0.48	43.59 ± 0.05	49.17 ± 0.73	47.50 ± 0.22	55.19 ± 0.64	43.11 ± 0.89	48.06 ± 0.69	41.97 ± 0.44
420	51.76 ± 0.08	57.17 ± 0.57	49.95 ± 0.33	54.51 ± 0.43	56.88 ± 0.62	60.26 ± 0.22	51.20 ± 0.11	55.83 ± 0.36	46.40 ± 0.49
480	56.06 ± 0.81	63.73 ± 0.22	56.01 ± 0.5	61.65 ± 0.88	60.13 ± 0.51	68.74 ± 0.24	57.64 ± 0.13	62.61 ± 0.42	53.54 ± 0.62
1440	79.11 ± 0.07	86.24 ± 0.32	78.40 ± 0.52	84.31 ± 0.85	81.07 ± 0.45	89.29 ± 0.27	75.62 ± 0.73	85.01 ± 0.25	72.28 ± 0.41

***In vitro* release studies**

The in vitro dissolution data of pyrazinamide niosomes prepared by modified ether injection method in distilled water is shown in Table 4. The drug release studies were carried out for 24hrs. It was noticed that all the prepared formulations had a release of 50-70% by the end of 8th

hour. At the end of the first hour, maximum release was obtained for formulations N3 and N5 where as a sustained effect was observed for formulations N10 and N6. The release data shows that the drug release was found to be approximately linear. For all the formulations, we can also notice that after the 8th hour till the 24th hour, the release of the drug is in a sustained manner, which can be studied from the graph no: 20.

It was also found from the release data that formulation N7 (Span-80 1:3 ratio) showed the best release of 89.29% when compared to the other formulations. This was followed by formulations N3 (Brij-35 1:3 ratio) and N11 (Tween-80 1:3 ratio) which had a release of 86.24% and 85.01% respectively. It was also noticed that for all the three polymers used in the study, the concentration of the polymer at ratio three times to the drug and cholesterol was optimum and obtained maximum release in each category. The lowest release or sustained effect was observed for N12 formulation having a release percent of 72.28%.

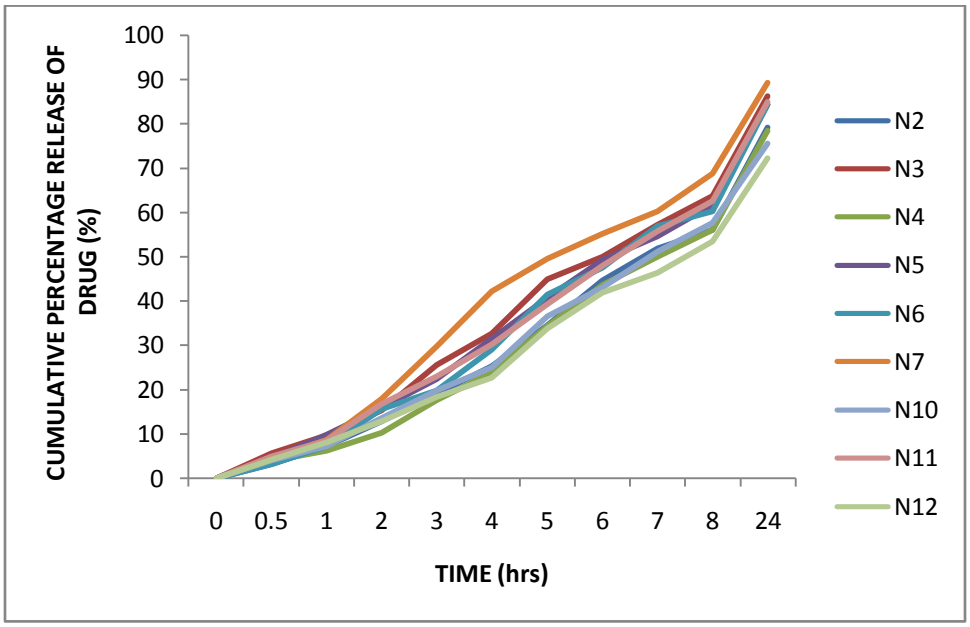

Figure 6. Comparative Cumulative Percentage Release of Niosomal Preparations

Drug Release Kinetics

The regression coefficient [r^2] value obtained for all the formulations are shown in table 5. The regression coefficient obtained for Zero order kinetics was found to be higher [r^2 = 0.9588 to 0.9808] than that compared to First order kinetics [r^2= 0.0004 to 0.0011] and Higuchi kinetics [r^2= 0.0.775 to 0.9324]. These values indicate that the drug release from the formulations was dependent upon the concentration. It also signifies that the release of drug is in a controlled manner.

Table 5. Drug release kinetics

Batch	Zero Order Kinetics		First Order Kinetics		Higuchi Kinetics	
	r^2	k	r^2	k	r^2	K
N2	0.9633	0.1191	0.8344	0.0005	0.9312	1.7366
N3	0.9743	0.0858	0.8166	0.0009	0.9264	4.4164
N4	0.9588	0.1543	0.8346	0.0005	0.9262	5.0085
N5	0.9722	0.1398	0.8194	0.0006	0.775	3.3090
N6	0.978	0.1388	0.8659	0.0005	0.9103	5.2518
N7	0.9808	0.1996	0.8278	0.0011	0.9077	3.6205
N10	0.9752	0.1055	0.8801	0.0005	0.9137	5.0833
N11	0.9713	0.1358	0.8121	0.0006	0.9324	5.0522
N12	0.9648	0.1363	0.8719	0.0004	0.9235	1.8462

CONCLUSION

The performed work aimed to form stable niosomes, using modified ether injection method without addition of charge inducing agents, having vesicle size in the range of 110 – 350 nm. Drug entrapment was highest for Brij-35 formulation 1:3. As no charge inducing agent was added, the charge of the niosomes were below 25 mV and the highest charge was found to be for Tween-80 formulation 1:4. Maximum release was obtained for Span-80 1:3 having 89.29% and extended in-vitro release was obtained for Tween-80 1:4 having 72.28%. Drug Release Kinetic studies revealed that all formulations follow zero order kinetics and release of drug is in a controlled manner. It can be concluded that all three polymers can be used for the successful formulation of pyrazinamide niosomes and the surfactant most apt was found to be Span-80 in the ratio 1:3 according to the work presented.

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