CHAPTER 5

PREPARATION, ISOLATION AND CHARACTERIZATION OF NIOSOMES

- Preparation of calibration curves
- Preparation and isolation of Niosomes
- Characterization of Niosomes
- Results and Discussion

5.1.1 Experimental:

5.1.1.1 Analytical method for Estimation of Rifampicin in PBS pH 7.4

For Rifampicin many methods have been reported, such as spectrophotometric methods, chromatographic methods, Microbiological methods, fluorometric determination and volumetric methods. From the above methods, the Spectrophotometric method has been used for invitro estimation and high pressure liquid chromatographic method for invivo estimation of Rifampicin

Apparatus:

Spectrophotometer(Hitachi U 2000 UV-visible Spectrophotometer and systronics UV-visible Spectrophotometer 108) Single pan weighing Balance (Dhona 200D) Digital pH meter (Elico Pvt. Ltd. Hyderabad).

Principle :

UV-VIS Spectrophotometry has been used for structural determination of various rifamycins to obtain specific information on the chromophoric part of the molecule. In particular, the VIS maximum, which undergoes a hypochromic effect and a small hypsochromic shift with strong acids, is characteristic of the naphthohydroquinone form carrying the acidic ionizable function and the auxochromic effect on the same VIS maximum depends on the nature of the substituents in position[149]

Phosphate Buffer Saline pH 7.4

Dissolved 1.38 of disodium hydrogen phosphate, 0.19 g of Potassium dihydrogen phosphate, and 8.0g of Sodium chloride in sufficient water to produce 1000 ml.

Stock Solution of Rifampicin[147]

A 1mg/ml solution of drug was prepared in methyl alcohol. From this, 1ml was taken and it was made upto 100ml with Phosphate Buffer Saline (PBS) pH 7.4 (10µg/ml)

Method :

Appropriate aliquots of the stock solution of the drug (1,2,3,4.....10ml) were transferred to 10ml of volumetric flask and it was diluted to 10ml with PBS pH 7.4 Determined the absorbance of solution in 1cm cells relation to the blank at the wave length of λmax equal to 475nm, with the aid of Spectrophotometer. The analysis was repeated for three times with the drug. The mean absorbance values are shown in Table 2 and the lines of regression is shown in Fig No.1.

5.1.1.2 ANALYTICAL MEDTHODS FOR ESTIMATION OF RIFAMPICIN IN LUNG, LIVER, KIDNEY EXTRACT AND SERUM

The Supernatant from successive extracts of an organ from each rat was collected and the drug content was analysed by High pressure liquid chromatography (HPLC) method[148], as described below.

Analysis of Rifampicin using HPLC

The extracts were collected and filtered through membrane filter (pore size 0.2 μ) and the filtrate was analysed. The sample solution was injected into HPLC using microlitre syringe. The drug contents were calculated from the area of chromatographic peak using the standard graph.

HPLC

Solvent	(HPLC Grade)
Mobil phase	: 100% Methanol (HPLC Grade)
Column	: ODS column
Flow rate	: 1 ml. per minute.
Pressure	: 1 x 100 kgf / cm^2
Column length	: 4.6mm ID x 25 cm(main column) and
	4mm 1D x 1cm (guard column)

Detector Wave length 254nm.

5.1.1.3 ANALYTICAL METHOD FOR ESTIMATION OF GENTAMICIN IN DISTILLED WATER

For Gentamicin sulphate many methods have been reported, such as microbiological assay, Radioimmunoassay, fluoroimmunoassay, Radio enzyme assay and high pressure liquid chromatography and spectrophotometry. From the above, the spectrophotometric method has been used, for invitro, invivo as well as for assay of Gentamicin sulphate.

APPARATUS

1. Spectrophotometers (Hitachi U2000 UV-visible-Spectrophoto meter and systronics UV-visible spectrophotometer 108).

2. Single pan weighing Balance (Dhona 200D)

3 Digital pH meter (Elico Pvt Ltd. Hyderabad)

Principle[150]:

The primary or secondary amine sections of the aminoglycoside and the hydroxyl group of the enol form of acetylacetone, formaldehyde reacting with the ketoform. With the loss of several water molecules from the primary amine reaction and ROH from the action of the secondary, the result is an aminoglycoside derivative of dihydrolutidine absorb in the ultraviolet

Derivatisation reagent:

0 8 ml of acetylacetone, 1.7ml of 36% formaldehyde and 10ml of the buffer pH 2 5. It is to be freshly prepared and used within 24 hrs.

Buffer pH 2.6.

12.2g of Boric acid, 12.0g of aceticacid and 19.6g of phosphoric acid were added in one litre (1000 ml) of water and the pH being adjusted to 2.6 by using 1M Sodium hydroxide.

Stock solution of Gentamicin sulphate:

A 1mg/ml solution of drug was prepared in distilled water.

Method:

Appropriate aliquots of the stock solution of the drug (0.1, 0.2, 0.3, 0.4, 0 5, 0.6, 0.7, 0.8ml) were transferred to 10ml of volumetric flask and it was diluted to 10ml with distilled water. The freshly prepared derivatisation reagent was then mixed in a 1:1 ratio with the drug solution and placed in the boiling water bath for 25 min, cooled to room temperature to determine the absorbance of solution in 1 cm cells relation to the blank at the wavelength of λmax equal to 356nm with the aid of a spectrophotometer. All the analysis was repeated three times for the drug. The mean absorbance values are shown in Table No.3 and the lines of regression is shown in Fig No 2.

5.1.2 Preparation and Separation of Rifampicin Niosomes

APPARATUS

Rotary flash evaporator (Superfit), Voltage regulator (Auto variance), Centrifuge (C-852), Refrigerated Centrifuge (C-24), Cyclomixer (CM-101), (Remi); Microscope (OPTIK, Olympus B 201); Vaccum pump (DLF Universal Ltd.), Probe Sonicator (Ralsonics); Round bottom flask with Quick fit neck 20/22 (Borosil); Dialysis tubing (Sigma), Single pan balance (Dhona 200 D), Spectrophotometer (Hitachi U2000); Digital pH Meter (Elico Pvt. Ltd.)

5.1.2.1 METHOD OF PREPARATION OF RIFAMPICIN NIOSOMES:

Niosomes were prepared from sorbitan esters namely Sorbitan Monopalmitate (Span 40), Sorbitan monosterate (Span 60), Sorbitan Mono Oleate (Span 80), Sorbitan trioleate (Span 85) Sorbitan Monolaurate (Span 20) and also from polyoxyethylene Sorbitan esters namely polysorbate 40 (Tween 40), Polysorbate 80 (Tween 80) Polysorbate 60 (Tween 60) and Polysorbate 20 (Tween 20) and Niosomes were prepared by the thin film hydration technique using Rotary flash evaporator.

They were prepared by the method as described by Baillie et al[50] with suitable modifications. Spans, Spans and cholesterol, Tweens, Tweens and Cholesterol were used as the lipid ingredients. The lipid ingredients and drug in the ratios as shown in Table No.4 were accurately weighed and dissolved in 10ml of Chloroform in a 100ml round bottom flask, and solvent mixture was evaporated under vaccum (20" Hg) at R.T. (≅25°C) to deposit a smooth dry film on the walls of the flask by using Rotary flash evaporator at 180 R.P.M.

The film was hydrated with 2ml of Phosphate Buffered Saline for a period of 1hr at room temperature.

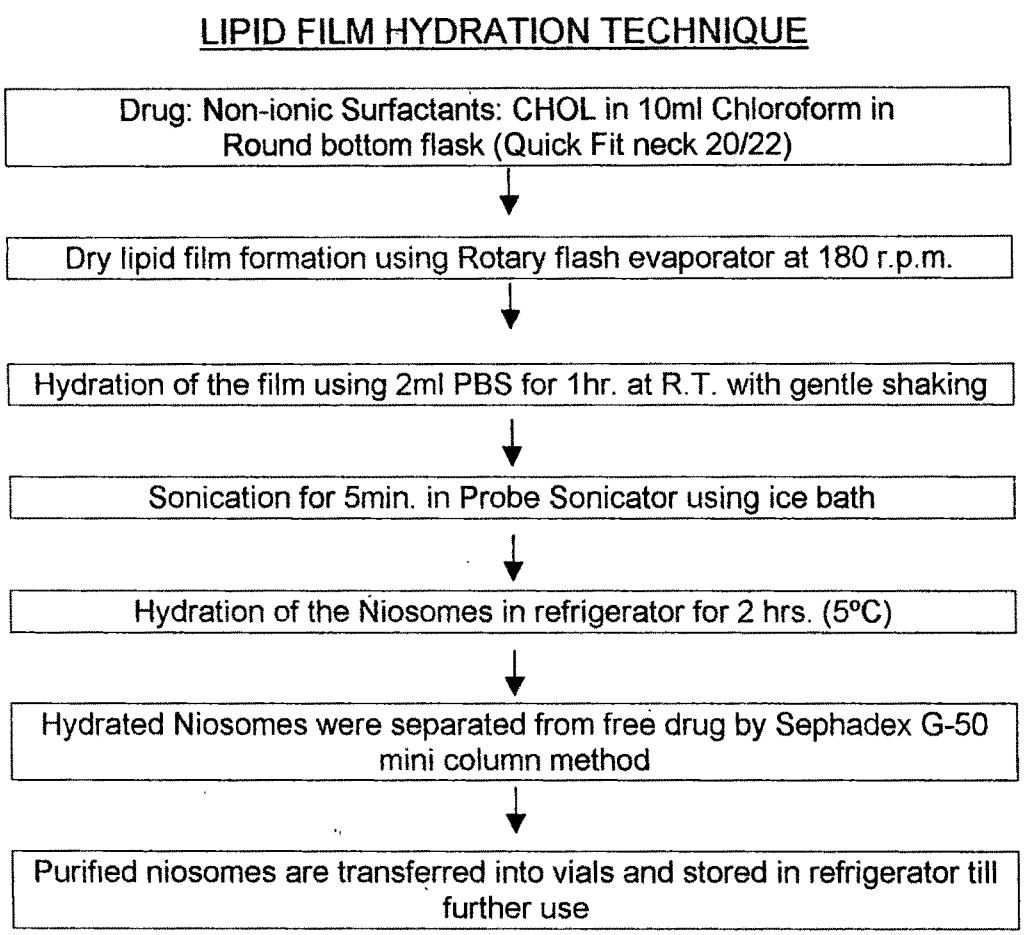
The formed niosomal Suspension was sonicated by probe sonicator for 5 mins, to get desired niosomal size.

It was then hydrated for 2hr. under refrigerated condition.

The niosomes were separated from unentrapped drug and stored in a vials till further use.

The entire process of preparation of niosomes is shown by the flow diagram given below.

Flow diagram showing formulation Technique for Preparing Niosomes of Rifampicin



5.1.2.2 SEPARATION OF UNENTRAPPED DRUG:

Techniques which are generally used to separate unentrapped solute from niosomes include dialysis, centrifugation and gel filtration. The method adopted in the work was gel filtration using Sephadex G-50 column.

Separation of free drug from Niosomal Rifampicin by Mini column Centrifugation method[66]. Sephadex G-50 column was prepared follow as:

Sephadex G-50 (10g) was allowed to swell in 120 ml of phosphate Buffer Saline for 5-6 hrs. at room temperature and then filled in the column.

Removed the plungers from 1ml disposable plastic syringe (one for each sample) and plug each barrel with a whatman filter pad and the rest in each barrel of 13 x 10mm centrifuge tube. Filled the barrels to the top with hydrated gel, using a pastear pipette with the tip removed.

Placed the tubes containing the columns in a centrifuge and rotated at 2000 r.p.m. for 3 min. to remove excess buffer solution. Emptied the eluted saline from each collection tube.

PROCESSING OF SAMPLES

0 2ml of Niosomal suspension (undiluted) was loaded on the top of the gel bed and rotate the column at 2000 r.p.m. for 3 min. in a centrifuge to expel the void volume containing the niosomes into the centrifuge tube. Applied 0.25 ml of PBS to each column and centrifuged to remove elutes as described previously. Various fractions were

collected which include the initial PBS fraction, followed by niosomal fraction and then again the PBS fraction. Niosomal fractions were collected, mixed and stored in a vial till further investigation.

Formula: 1 Composition of different batches of Niosomes prepared using Spans and Tween series of non-ionic Surfactants.

Ingredients		
Batch Number	1a 1b 1c	2a 2b 2c
I) Span 80	0.07 ml (150µmol)	0.07 ml (150µmol)
Cholesterol (CHOL)	--	58 mg (150µmol)
Rifampicin	10mg	10mg
PBS pH 7.4	2 ml	2 ml
	3a 3b 3c	4a 4b 4c
II) Span 20	0.05 ml (150µmol)	0.05 ml (150µmol)
Cholesterol (CHOL)	--	58 mg (150µmol)
Rifampicin	10mg	10mg
PBS pH 7.4	2 ml	2 ml
	5a 5b 5c	6a 6b 6c
III) Span 60	64.65 mg (150µmol)	64.65mg (150µmol)
Cholesterol (CHOL)	--	58 mg (150µmol)
Rifampicin	10mg	10mg
PBS pH 7.4	2 ml	2 ml
	7a 7b 7c	8a 8b 8c
IV) Span 40	60.39 mg	60.39 mg (150µmol)
Cholesterol (CHOL)	--	58 mg (150µmol)
Rifampicin	10mg	10mg
PBS pH 7.4	2 ml	2 ml

	9a 9b 9c	10a 10b 10c
V) Tween 40	0.18 ml (150µmol)	0.18 ml (150µmol)
Cholesterol (CHOL)	--	58 mg (150µmol)
Rifampicin	10mg	10mg
PBS pH 7.4	2 ml	2 ml
	11a 11b 11c	12a 12b 12c
VI) Tween 80	0.18 ml (150µmol)	0 18 ml (150µmol)
Cholesterol (CHOL)	--	58 mg (150µmol)
Rifampicin	10mg	10mg
PBS pH 7.4	2 ml	2 ml
	13a 13b 13c	14a 14b 14c
VII) Tween 20	0.15 ml (150µmol)	0 15 ml (150µmol)
Cholesterol (CHOL)	--	58 mg (150µmol)
Rifampicin	10mg	10mg
PBS pH 7.4	2 ml	2 ml
	15a 15b 15c	16a 16b 16c
VIII) Tween 60	0.18 ml (150µmol)	0.18 ml (150µmol)
Cholesterol (CHOL)	--	58 mg (150µmol)
Rifampicin	10mg	10mg
PBS pH 7 4	2 ml	2 ml

5.1.2.3. CHARACTERIZATIONS OF NIOSOMES CONTAINING RIFAMPICIN

1. Particle size analysis : Niosomes of the prepared batches were subjected to particle size analysis using OPTIK microscopic (least count 2 5 µm) at a magnification of 100x, with the aid of optical - stage micrometer.

The arithmetic mean diameter for each batch was calculated.(2) and is as shown in Table 6,7,8 & 9 . The frequency Vs Particle size curve of selected niosomal batches is as shown in Fig No. 3.

2. Drug content Analysis

Percent drug entrapment : 0 2ml of niosome suspension was placed in a 15ml centrifuge tube. With it, it was added with 0.08 ml of Triton-X-100 till the solution became clear. The volume was made upto 10 ml with phosphate buffer saline pH 7.4. The remaininig procedure was as described in section 5.1.1 1 of Chapter 5. For blank plain niosomes of the same cholesterol, non-ionic surfactant ratio were used. Sample were analysed in triplicates The mean percent drug entrapment is as shown in Table No 5.

3. Physical Observation:

Microscopic Examination : All the batches of the niosomes prepared were viewed under a OPTIK, microscopic to study their size, shape and lamellarity. The representative batches (Rifa 17 & Rifa 20) of niosomes were photomicrographed with OLYMPUS B201

microscope with/without appropriate dilution and photograph obtained is shown in plate no.2.

Scanning Electron Microscopic Study[151]:

Approximately 1µl of the redispersed suspension of Niosomes was transferred to a cover glass, which in turn was mounted on a specimen stub made up of Aluminium Grid. Dried samples were obtained by removing water molecule. Dried samples were coated with gold to a thickness of 100 A° using Hitachi Model No.HUS-5GB vaccum evaporator and the same ones were viewed and photographed in Hitachi H 450 SEM., operated at 20 K.V. accelerating voltage.

5.1.2.4 PREPARATION OF NIOSOMES CONTAINING GENTAMICIN SULPHATE

APPARATUS

Rotary flash evaporator (Superfit), Voltage regulator (Auto variance), Centrifuge(C-852), Refrigerated Centrifuge (C-24), Cyclomixer (CM-101), (Remi); Microscope (OPTIK, Olympus B 201); Vaccum pump (DLF Universal Ltd.); Probe Sonicator (Ralsonics); Round bottom flask with Quick fit neck 20/22 (Borosil); Dialysis tubing (Sigma), Single pan balance (Dhona 200 D); Spectrophotometer (Hitachi U2000); Digital pH Meter (Elico Pvt. Ltd)

Method

Niosomes were prepared using the lipid film hydration technique using rotary flash evaporator (Plate No.1) which was described by Baillie et al (1985) with suitable modifications.

Non-ionic surfactants (Tweens or Spans) and Cholesterol in the ratios as shown in Table No.10 were accurately weighed and dissolved in 10ml of chloroform in 100ml round bottom flask, and the solvent mixture was evaporated under vaccum(20"Hg) at R.T.(≅25°C) to deposit a smooth dry film on the walls of the flask by using rotary flask evaporator at 180 R.P.M.

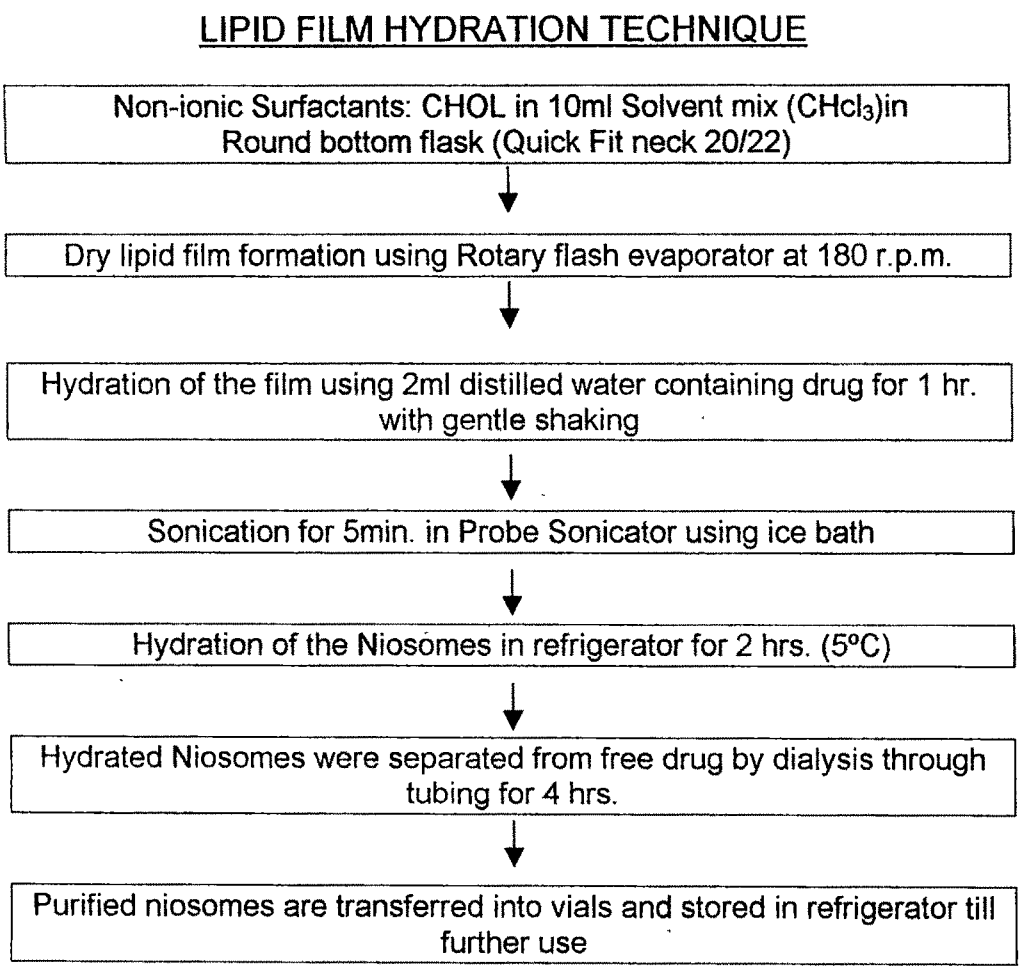
The film was hydrated with 2ml of distilled water which contains drug and then it is added with glass beads (0.5 to 3mm in diameter) for gentle agitation for a period of 1 hour at room temperature in rotary flash evaporator until a niosomal dispersion was obtained. The formed niosomal suspension was sonicated by probe sonicator for 5 mins. by using ice bath to get desired niosomal size. It was then hydrated for 2 hrs. under refrigerated condition.

5.1.2.5 SEPARATION OF UNENTRAPPED DRUG[14]:

In order to purify niosomes from free drug the preparation was then filled in a 10 cm long dialysis tubing, knotted from one end. This tubing was suspended into 250 ml distilled water in a beaker, and subjected to stirring with a magnetic stirrer at room temperature for a period of 4 Hrs.

Finally, the products were transferred to vials sealed and stored in refrigerator for further investigation. The entire process of preparation of niosomes is shown by the Flow diagram given below.

Flow diagram showing formulation Technique for preparing niosomes of Gentamicin Sulphate.



Formula: 2 Composition of different batches of Niosomes prepared using Spans and Tweens.

Ingredients

Batch Number	1a 1b 1c	2a 2b 2c
I) Tween 60	0.18 ml (150µmol)	0.18 ml (150µmol)
Cholesterol (CHOL)	--	58 mg (150µmol)
Gentamicin Sulphate	10mg	10mg
Distilled Water	2 ml	2 ml
	3a 3b 3c	4a 4b 4c
II) Tween 80	0.18 ml (150µmol)	0.18 ml
Cholesterol (CHOL)	--	58 mg
Gentamicin Sulphate	10mg	10mg
Distilled water	2 ml	2 ml
	5a 5b 5c	6a 6b 6c
III) Span 60	64.65 mg (150µmol)	64.65mg
Cholesterol (CHOL)	--	58 mg
Gentamicin Sulphate	10mg	10mg
Distilled water	2 ml	2 ml
	7a 7b 7c	8a 8b 8c
IV) Span 40	60 39 mg	60.39 mg
Cholesterol (CHOL)	--	58 mg
Gentamicin Sulphate	10mg	10mg
Distilled water	2 ml	2 ml

	9a 9b 9c	10a 10b 10c
V) Tween 40	0.18 ml (150µmol)	0.18 ml
Cholesterol (CHOL)	--	58 mg
Gentamicin Sulphate	10mg	10mg
Distilled Water	2 ml	2 ml

	11a 11b 11c	12a 12b 12c
VI) Tween 20	0.15 ml (150µmol)	0.15 ml
Cholesterol (CHOL)	--	58 mg
Gentamicin Sulphate	10mg	10mg
Distilled water	2 ml	2 ml

	13a 13b 13c	14a 14b 14c
VII) Span 20	0.05 ml (150µmol)	0.05 ml (150µmol)
Cholesterol (CHOL)	--	58 mg
Gentamicin Sulphate	10mg	10mg
Distilled water	2 ml	2 ml

	15a 15b 15c	16a 16b 16c
VIII) Span 80	0.07 ml (150µmol)	0.07 ml
Cholesterol (CHOL)	--	58 mg
Gentamicin Sulphate	10mg	10mg
Distilled water	2 ml	2 ml

5.1.2.3. Characterizations of Niosomes containing Gentamicin

1. Particle size analysis : Prepared batches of niosomes were subjected to particle size analysis using OPTIK microscopic (least unit 2.5 um) at a magnification of 100x, with the aid of optical - stage micrometer.

The arithmetic mean diameter for each batch was calculated.(2) and is shown in Table 12,13,14 & 15. The frequency Vs Particle size curve of selected niosomal batches is shown in Fig No.4.

2. Drug content Analaysis

1. Percent drug entrapment : 0.2ml of niosome suspension was placed in a 15ml centrifuge tube. To it was added 0.08 ml of Triton-X-100 till the solution became clear. The volume was made upto 10 ml with phosphate buffer saline pH 7.4. The remaininig procedure was as described in section 5.1.1.3 of chapter 5. For blank plain niosomes of the same cholesterol, non-ionic surfactant ratio was used. Samples were analysed in triplicates and the mean percent drug entrapment is shown in Table No.11

Analysis of Free Drug Content

In order to separate free drug from niosomes, all niosomal products were subjected to dialysis for 4 hrs. The recepient compartment comprised of 250 ml of distilled water from which 10ml of distilled water was withdrawn and it was subjected to the analysis of

drug using the method described in section 5.1.1.3 chapter 5. The fresh recepient medium was replaced after the withdrawal of each sample.

3. Physical Observation:

1. Microscopic Examination : All the batches of the niosomes prepared were viewed under a OPTIK. Microscopic to study their size, shape and lamellarity. The representative batches (Rifa 17 & Rifa 20) of niosomes were photomicrographed with OLYMPUS B201 microscope with/without appropriate dilution and photograph obtained is shown in plate no.3.

3. Scanning Electron Microscopic Study[151]

Approximately 1 µl of the redispersed suspension of Niosomes was transferred to a cover glass, which in turn was mounted on a specimen stub made up of Aluminium Grid. Dried samples were obtained by removing water molecule. Dried samples were coated with gold to a thickness of 100 A° using Hitachi Model No HUS-5GB vaccum evaporator and the same ones were viewed and photographed in Hitachi H 450 SEM., operated electron microscope at 20 K.V. accelerating voltage.

5.1.2.4 OPTIMIZATION OF FORMULATION

Various batches of niosomes were prepared by the same method described earlier for optimization of formula.

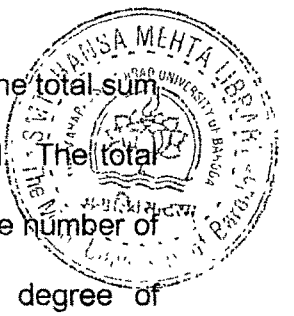
Niosomes were prepared as per formula given in section 5.1.2.2. The different batches were evaluated with respect to their drug entrapment efficiency and were taken up for further evaluation.

Study of the effect of process variables on drug entrapment efficiency by factorial analysis (Yates treatment)

The overall objective of factorial analysis is to obtain a general picture of how the results are affected by changing the parameters of factors The factorial design was estimated based on the theoretical principles described by Philippe and Chatfield[152]. In the present study four process variables were taken viz. Solvent, sonication time, volume of hydrating medium and hydration time. All the four factors were investigated at two levels. Sixteen batches were prepared as shown in Table 16 & 17. For simplicity an alphabet was written when the corresponding factor was in a high level and without the alphabet when it was in the low level. When all of the factors were in low level, it was written as number one. Generally, with n factors at two levels. 2^n treatment combinations were possible. To obtain an estimation of the error, each factor was determined in duplicate. The sum of the duplicate values was analysed using Yates method, which is a systematic method for estimating the individual effects and performing the analysis of variance.

Yate's treatment

In the first column, all the treatment combinations are listed in a systematic way; the results are determined in duplicate for each factor and are expressed as entrapment efficiency (column-2); their sum is listed in column three. According to Yates, with four factors, three columns have to be calculated (columns 4-6). Each column is generated from the preceding column in the same way. The first two numbers (relating to experiments(1) and (A) are added together (117.54 and 143.92) the result 261.46 put into the first row of the column headed 'column 4'. The next two are then added (experiments B and AB, 25.58, 49.99) and the result (75.57) is put in the second row of the column 4. Similar procedure is carried out for the next two pairs (C and AC, BC and ABC, D and AD, BD and ABD, CD and ACD, BCD and ABCD). Then the differences between adjacent pairs are calculated (A-(1), AB-B, AC-C, ABC-BC, AD-D, ABD-BD, ACD-CD, ABCD -BCD) and these are placed from ninth to the sixteenth rows of column 4. The process is then repeated using the numbers in column 4, and the results are placed in column 5. Thus the first number in column 5 is 337.03 obtained by adding together the first two rows in column 4, namely 261.46 & 75.57. The difference between these two numbers-185.89, forms the ninth row of column 5. The identical process is repeated yet again on the number in column 5, and the results are being placed in column 6. In this way, the final column headed column 6 gives the total effect corresponding to the particular treatment combination. With these estimates, an analysis



of variance is performed. This analysis consists of splitting the total sum of squares into sums of squares for the factors considered. The total effect of each factor is squared (column 8) and divided by the number of observations (2^4x 2), with each factor possessing one degree of freedom The estimate of the residual variation, i.e., the variance of the experimental error, is calculated by the sum of squares of the variance between the duplicates, divided by 2 x 16. The significance of F values are assessed by comparing them with the tabulated values.

5.1.2.5 RESULTS

TABLE 1

ULTRAVIOLET ABSORPTION OF RIFAMPICIN

λ_{max} nm	ε
237	33.200
255	32,100
334	27,000
475	15,400

TABLE No.2

CALIBRATION CURVE FOR THE ESTIMATION OF RIFAMPICIN IN PBS pH 7.4

Conc. µg/ml	Mean Absorbance ± SEM (Ref)
1	0.012
2	0.023
3	0 036
4	0.048
5	0 061
6	0.073
7	0.085
8	0.096
9	0 110
10	0.122

* n=6

r= 0.999

Equation of the regression line is y = 0.0123x -0.0008

TABLE NO.3

CALIBRATION CURVE FOR GENTAMICIN SULPHATE IN DISTILLED WATER

Concentration (µg/ml)	Mean Absorbance
10	0.123
20	0.234
30	0.352
40	0.451
50	0.582
60	0.702
70	0.784
80	0.904

*n=6 r=0 999 Equation of the regression line is y= 0 0112x + 0.013

b = 0.0112

R^2 = 0 999

Table No.4

Composition of the various batches of Rifampicin Niosomes with respect to Span 60 : CHOL : Drug Ratios.

Sl. No.	Batch No.	Drug (µmol)	Span 60 : CHOL (µmol)	% entrapment
1	Rifa 8a*	12.15	1 : 0	10
2	Rifa 8b*	12.15	1 : 0.49	22
3	Rifa 8c*	12.15	1 : 1	25
4	Rifa 8d	12.15	0.50 : 0	39.8
5	Rifa 8e	12.15	0.50 : 0.50	35
6	Rifa 8f	12.15	0.50 : 1	28
7	Rifa 8g	12.15	0.50 : 3	12
8	Rifa 8h	12.15	1 : 0	65.2
9	Rifa 8i	12.15	1 : 0.50	32
10	Rifa 8j	12.15	1 : 3	42.5
11	Rifa 8k	12.15	1 : 1	72
12	Rifa 8l	12.15	3 : 0	30
13	Rifa 8m	12.15	3 : 0.50	28
14	Rifa 8n	12.15	3 : 1	23
15	Rifa 8o	12.15	3 : 3	21
16	Rifa 8p	12.15	1 : 1	71.89
17	Rifa 8q	12.15	1 : 1	72
18	Rifa 8r	12.15	1 : 1	71.52
19	Rifa 8s	12.15	1 : 1	71.99
20	Rifa 8t	12.15	1 : 1	71.95
21	Rifa 8u	12.15	1 : 1	72

***Drug was added into the hydrating medium.**

Preliminary Studies conducted: CHOL(µmol) : 0, 0.50, 0.75, 1, 2, 3
Span60(µmol): 0.5, 0.75, 1, 2, 3

Factors 1) Span 60 : CHOL (Molar Ratio)

Response : Entrapment Efficiency.

Selected range: CHOL : 0-3µmolar
Span 60: 0-3 µmolar

TABLE NO.5.

Entrapment Efficiency of Different formulations of Rifampicin Niosomes

Batch No	Composition	Amount in mg. Mean ± SD	Percentage Mean ±SD
Rifa 1	Span 80	0.5041	5.04
Rifa 2	Span 80 + CHOL	0.9105	9.10
Rifa 3	Span 20	1.7236	17.236
Rifa 4	Span 20 + CHOL	3.2033	32
Rifa 5	Span 40	1.6260	16.26
Rifa 6	Span 40 + CHOL	2.7561	27.56
Rifa 7	Span 60	6.892	68.92
Rifa 8	Span 60 + CHOL	7.187	71.87
Rifa 9	Tween 20	0 1382	1.38
Rifa 10	Tween 20 + CHOL	0.5285	5.30
Rifa 11	Tween 80	0.3496	3.50
Rifa 12	Tween 80 + CHOL	0.6016	6.02
Rifa 13	Tween 40	0.8049	8.05
Rifa 14	Tween 40 + CHOL	1.0244	10.24
Rifa 15	Tween 60	0 3415	3.41
Rifa 16	Tween 60 + CHOL	0.7479	7.48

TABLE NO.6

PARTICLE SIZE ANALYSIS OF RIFA 2 BATCH

Mean (d)	No. of Particles(n)	nd
3.75	15	56.25
4.25	20	85.00
4.75	15	71.25
5.25	35	183 75
5.75	60	345.00
6.25	48	300.00
6.75	50	337.50
8.75	270	2362.50
		3741.25

$$\frac{\Sigma nd}{\Sigma n} = \frac{3741.25}{500} = 7.4825\mu m$$

TABLE NO.7

PARTICLE SIZE ANALYSIS OF RIFA 8 BATCH

Mean (d)	No. of Particles(n)	nd
3.75	15	56.25
4.25	12	51.0
4.75	10	47.50
5.25	35	183.75
5.75	58	333.50
6.25	54	337.50
6.75	82	553.50
8.75	250	2187.50
		3750 50

$$\frac{\Sigma nd}{\Sigma n} = \frac{3750.50}{500} = 7.501\mu m$$

TABLE NO.8

PARTICLE SIZE ANALYSIS OF RIFA 4 BATCH :

Mean (d)	No. of Particles(n)	nd
3.75	25	93.50
4.25	10	42.50
4.75	22	104.50
5.25	35	83.75
5.75	58	333.50
6.25	55	343.75
6.75	50	337.50
8.75	290	2537.50
		3976.75

$$\frac{\Sigma nd}{\Sigma n} = \frac{3976.75}{500} = 7.95\mu m$$

TABLE NO.9

PARTICLE SIZE ANALYSIS OF RIFA 6 BATCH

Mean (d)	No. of Particles(n)	nd
3.75	18	67.5
4.25	12	51.0
4.75	15	71.25
5.25	38	199.50
5.75	60	345.00
6.25	54	337.50
6.75	85	573.75
8.75	265	2318.75
		3964.25

$$\frac{\Sigma nd}{\Sigma n} = \frac{3964.25}{500} = 7.92\mu m$$

Table No.10

Composition of the various batches of Gentamicin Sulphate Niosomes with respect to Tween 60 : CHOL : Drug Ratios.

Sl. No.	Batch No.	Drug (µmol)	Tween : CHOL	% entrapment
1	Genta 2a*	22.25	1 : 0	12
2	Genta 2b*	22.25	1 : 0.50	28
3	Genta 2c*	22.25	1 : 1	22
4	Genta 2d	22.25	0.50 : 0	28.5
5	Genta 2e	22.25	0.50 : 0.50	31.2
6	Genta 2f	22.25	0.50 : 1	14.2
7	Genta 2g	22.25	0.50 : 3	12.5
8	Genta 2h	22.25	1 : 0	55
9	Genta 2i	22.25	1 : 0.50	35
10	Genta 2j	22.25	1 : 1	65
11	Genta 2k	22.25	1 : 3	32
12	Genta 2l	22.25	3 : 0	18
13	Genta 2m	22.25	3 : 0.50	15.7
14	Genta 2n	22.25	3 : 1	12.4
15	Genta 2o	22.25	3 : 3	24.2
16	Genta 2p	22.25	1 : 1	64.52
17	Genta 2q	22.25	1 : 1	65
18	Genta 2r	22.25	1 : 1	64.25
19	Genta 2s	22.25	1 : 1	64.20
20	Genta 2t	22.25	1 : 1	64.50
21	Genta 2u	22.25	1 : 1	65

***Drug was added with CHOL / Surfactant Solvent mix.** n**= 3

Preliminary Studies conducted: CHOL(µmol) : 0, 0.5, 0.25, 1, 2, 3

Tween60(µmol): 0.5, 0.75, 1, 2, 3

Factors 1) Tween 60 : CHOL (Molar Ratio)

Response : Entrapment Efficiency.

Selected range: CHOL : 0-3µmolar

Tween 60: 0.5-3 µmolar

TABLE NO.11.

Entrapment Efficiency of Different formulations of Niosomes Containing Gentamicin

Batch No.	Composition	Gentamicin Entrapped	
		Amount in mg. Mean ± SD	Percentage Mean ±SD
Genta 1	Tween 60	6.0	60
Genta 2	Tween 60 + CHOL	6.5	65
Genta 3	Tween 80	2.6428	26.42
Genta 4	Tween 80 + CHOL	3.0	30
Genta 5	Span 60	2.7321	27.42
Genta 6	Span 60 + CHOL	2.9285	29.28
Genta 7	Span 40	1.0178	10.17
Genta 8	Span 40 + CHOL	1.1071	11.07
Genta 9	Tween 40	2.25	22.5
Genta 10	Tween 40 + CHOL	2.5535	25.5
Genta 11	Tween 20	2.7857	27
Genta 12	Tween 20 + CHOL	3.125	31
Genta 13	Span 20	0.625	6.25
Genta 14	Span 20 + CHOL	0.6785	6.78
Genta 15	Span 80	1.714	17.14
Genta 16	Span 80 + CHOL	1.9107	19.11

n* = 3

TABLE NO.12

PARTICLE SIZE ANALYSIS OF GENTA 2 BATCH

Mean (d)	No. of Particles(n)	nd
3.75	12	45.00
4.25	16	68.00
4.75	8	38.00
5.25	47	246.75
5 75	62	356.50
6.25	56	350.00
6.75	78	526.90
8.75	238	2082.90
		3713 65

$$\frac{\Sigma nd}{\Sigma n} = \frac{3713.65}{500} = 7.4273\mu m$$

TABLE NO.13

PARTICLE SIZE ANALYSIS OF GENTA 4 BATCH

Mean (d)	No. of Particles(n)	nd
3.75	6	22.50
4.25	12	51.0
4.75	10	47.50
5.25	20	105.00
5.75	52	299.00
6.25	46	287.50
6.75	37	249.75
8.75	270	2362.50
		3424.75

$$\frac{\Sigma nd}{\Sigma n} = \frac{3424.75}{500} = 6.8\mu m$$

TABLE NO.14

PARTICLE SIZE ANALYSIS OF GENTA 10 BATCH

Mean (d)	No. of Particles(n)	nd
3 75	5	18.75
4.25	10	42.50
4.75	22	104.50
5.25	20	105.00
5.75	50	287.50
6.25	48	300.00
6.75	30	202.50
8.75	250	2187.50
		3248.25

$$\frac{\Sigma nd}{\Sigma n} = \frac{3248.25}{500} = 6.4965\mu m$$

TABLE NO.15

PARTICLE SIZE ANALYSIS OF GENTA 12 BATCH

Size range	Mean (d)	No. of Particles(n)	nd
3.5-4	3.75	5	18.75
4-4.5	4.25	10	42.50
4.5-5	4.75	13	61.75
5-5.5	5.25	20	105.00
6-6.5	6.25	40	250.00
6.5-7	6.75	45	303.75
7.5-10	8.75	250	2187.50
			3314.25

$\Sigma n = 500$ $\Sigma nd = 3314.5$

$$\frac{\Sigma nd}{\Sigma n} = \frac{3314\ 5}{500} = 6.628\mu m$$

TABLE No.: 16

ANALYSIS OF VARIANCE TABLE FOLLOWING YATE'S TREATMENT (RIFAMPICIN NIOSOMES)

Source of variation	Entrapment efficiency		Column (3) sum	Column 4	Column 5	Column 6	Column 7 Degree of freedom	Column 8 sum of squares $\frac{(\text{column 6})^2}{2 \times 16}$	Column 9 variance	Column 10 F	Column 11 significant level F_{16} %
1	58.92	58.62	117.54	261.46	337.03	625.85	1	--	--	--	
A	72	71.92	143.92	75.57	288.82	188.66	1	1112.27	1112.27	7628.72	
B	12.48	13.10	25.58	214.59	32	136.73	1	584.22	584.22	4006.99	P>0.05
AB	25.04	24.95	49.99	74.23	156.66	-52.42	1	85.87	85.87	588.96	P>0.001
C	38.68	38.50	77.18	17.12	50.79	-326.25	1	3326.22	3326.22	22813.58	P>0.01
AC	68.92	68.49	137.41	14.88	85.94	-55.5	1	96.26	96.26	660.22	
BC	12.32	11.94	24.26	104.96	1.78	-54.22	1	91.87	91.87	630.10	
ABC	25.02	24.95	49.97	51.7	-54.2	-52.42	1	85.87	85.87	588.96	
D	3.15	2.95	6.1	26.38	-185.89	-48.21	1	72.63	72.63	498.15	
AD	4.98	6.04	11.02	24.41	-140.36	124.66	1	485.62	485.62	3330.72	
BD	5.12	3.89	9.01	60.23	-2.24	35.15	1	38.61	38.61	264.81	
ABD	3.02	2.85	5.87	25.71	-53.26	-55.98	1	97.93	97.93	671.67	
CD	32.14	31.60	63.74	4.92	-19.7	-45.53	1	64.78	64.78	444.31	
ACD	20.22	21	41.22	-3.14	-34.52	-51.02	1	81.35	81.35	557.96	
BCD	20.62	21.07	41.69	-22.52	1.78	-14.82	1	6.86	6.86	47.05	
ABCD	5.03	4.98	10.01	-31.68	-54.2	-55.98	1	97.93	97.93	671.67	

TABLE No: 17

ANALYSIS OF VARIANCE TABLE FOLLOWING YATE'S TREATMENT (GENTAMICIN NIOSOMES)

Source of variation	Entrapment efficiency		Column (3) sum	Column 4	Column 5	Column 6	Column 7 Degree of freedom	Column 8 sum of squares $\frac{(\text{column 6})^2}{2 \times 16}$	Column 9 variance	Column 10 F	Column 11 significant level F_{16} %
1	44	44.02	88.02	218.01	326.08	562.48	1	--	--	--	
A	65	64.99	129.99	108.07	236.4	206.87	1	1337.35	1337.35	8915.66	P>0.05
B	22.56	23.01	45.5	169.7	32.31	136.5	1	582.25	582.25	3881.66	P>0.001
AB	31	31.5	62.5	66.7	174.56	-72.43	1	163.94	163.94	1092.93	P>0.01
C	24.52	24.92	49.5	12.04	58.90	-212.94	1	1416.98	1416.98	9446.53	
AC	60	60.2	120.2	20.27	77.6	-38.11	1	45.38	45.38	302.53	
BC	15.10	14.80	29.9	110.45	7.21	-88.84	1	246.64	246.64	1644.26	
ABC	18.25	18.55	36.8	64.11	-79.64	-16.21	1	8.2113	8.2113	54.74	
D	2.12	2.18	4.30	41.97	-109.94	-89.68	1	251.33	251.33	16575.53	
AD	3.62	4.12	7.74	16.93	-103	142.25	1	632.35	632.35	4215.6	
BD	4.25	4	8.25	70.7	8.23	18.7	1	10.92	10.92	72.8	
ABD	5.92	6.10	12.02	6.9	-46.34	-86.85	1	235.71	235.71	1571.4	
CD	35	36	71	3.44	-25.04	6.94	1	1.505	1.505	10.03	
ACD	19.65	19.80	39.45	3.77	-63.8	-54.57	1	93.06	93.06	620.4	
BCD	27.25	27.85	56.10	-31.55	0.33	-38.76	1	46.95	46.95	31.3	
ABCD	3.92	4.09	8.01	-48.09	-16.54	-16.87	1	8.9	8.9	59.29	

Table No. 18

Optimization of Formulation and Process Variables (Rifampicin Niosomes).
[Drug : Span 60 : CHOL
12.15 µmol : 150 µmol : 150 µmol]

Sl. No.	Batch No.	Sol-vent (ml)	Variable parameters			Response parameters		
			Sonica - tion Time (min)	Volume of Hydrat-ing Medium (ml)	Hydra tion Time (hr)	% drug entrap-ment ±SEM	% drug unentra -pped ±SEM	Mean Size ±SEM (µm)
1	Rifa 8k1	10	5	2	1	58.92	41.08	6.25
2	Rifa 8k2	10	5	2	2	72	28	7.6
3	Rifa 8k3	10	5	10	1	12.48	87.52	6.50
4	Rifa 8k4	10	5	10	2	25.04	74.96	6.95
5	Rifa 8k5	10	10	2	1	38.62	61.38	3.18
6	Rifa 8k6	10	10	2	2	68.62	31.98	2.0
7	Rifa 8k7	10	10	10	1	12.32	87.68	1.8
8	Rifa 8k8	10	10	10	2	25.02	74.98	2.0
9	Rifa 8k9	15	5	2	1	3.15	96.85	7.25
10	Rifa 8k10	15	5	2	2	4.98	95.02	6.95
11	Rifa 8k11	15	5	10	1	5.12	94.88	6.50
12	Rifa 8k12	15	5	10	2	3.02	96.98	7.14
13	Rifa 8k13	15	10	2	1	32.14	65.83	1.9
14	Rifa 8k14	15	10	2	2	20.22	79.78	3.24
15	Rifa 8k15	15	10	10	1	20.62	70.38	2.0
16	Rifa 8k16	15	10	10	2	5.03	94.97	1.75

Factors:	Response:
1) Solvent (ml)	1) Entrapment efficiency
2) Sonication Time (min)	2) Particle Size
3) Volume of hydrating medium (ml)	Levels: Two : High & Low
4) Hydration time (hrs)	

Experiments $2^4 = 16$	***I Preliminary Studies Conducted***	
		Range
Factor 1) Low level = 10 ml High level = 15 ml	Factor 1	5 - 15 ml
Factor 2) Low level = 5 min. High level = 10 min.	Factor 2	5 - 15 ml
Factor 3) Low level = 2 ml. High level = 10 ml.	Factor 3	2 - 10 ml
Factor 4) Low level = 1 hr. High level = 2 hr.	Factor 4	1 - 14 hrs.

Table No.19

Analysis of Variance Table following Yate's treatment (Rifampicin Niosomes).

Factor of interaction	Experi-ment	Degree of freedom	Mean Square	F^{1}_{16}	
Solvent	A	1	1112.27	7628.72	
Sonication Time	B	1	584.22	4006.99	
Solvent x Sonication Time	AB	1	85.87	588.96	P>0.05
Vol. of hydrating medium	C	1	3326.22	**22813.58**	P>0.001
Solvent x Vol. of hydrating medium	AC	1	96.26	660.22	P>0.01
Sonication Time x Vol. of hydrating medium	ABC	1	91.87	630.10	
Solvent x Vol. of hydrating medium	BC	1	85.87	588.96	
Hydration Time	D	1	72.63	498.15	
Solvent x Hydration Time	AD	1	485.62	3330.72	
Sonication Time x Hydration Time	BD	1	38.61	**264.81**	
Solvent x Sonication Time x Hydrating Time	ABD	1	97.63	671.67	
Vol. of hydrating medium x Hydrating Time	CD	1	64.78	444.31	
Solvent x Vol. of Hydrating medium x Hydrating	ACD	1	81.35	557.96	
SonicationTime x Vol. of hydrating medium x Hydrating Time	BCD	1	6.86	347.05	
Solvent x Sonication Time x Vol. of hydrating medium x hydration Time	ABCD	1	97.93	671.67	

Table No. 20

Optimization of Formulation and Process Variables(Gentamicin Niosomes).
[Drug : Tween 60 : CHOL
22.25 µmol : 150 µmol : 150 µmol]

Sl. No	Batch No.	Solvent (ml)	Sonication Time (min)	Volume of Hydrating Medium (ml)	Hydration Time (hr)	% drug entrap-ment ±SEM	% drug unentra-pped ±SEM	Mean Size ±SEM (µm)
1	Genta 2j1	10	5	2	1	44	58	6.25
2	Genta 2j2	10	5	2	2	65	35	7.5
3	Genta 2j3	10	5	10	1	22.56	77.44	7.25
4	Genta 2j4	10	5	10	2	31	69	7.30
5	Genta 2j5	10	10	2	1	24.52	75.48	1.8
6	Genta 2j6	10	10	2	2	60	31	2.2
7	Genta 2j7	10	10	10	1	15.10	84.9	1.7
8	Genta 2j8	10	10	10	2	18.25	81.75	2.2
9	Genta 2j9	15	5	2	1	2.12	87.88	6.6
10	Genta 2j10	15	5	2	2	3.62	86.38	6.8
11	Genta 2j11	15	5	10	1	4.25	95.75	7.3
12	Genta 2j12	15	5	10	2	5.92	94.08	7.2
13	Genta 2j13	15	10	2	1	35	85	2.2
14	Genta 2j14	15	10	2	2	19.65	80.35	1.8
15	Genta 2j15	15	10	10	1	27.25	93.75	2.0
16	Genta 2j16	15	10	10	2	3.92	96.08	1.8

Factors:
1) Solvent (ml)
2) Sonication Time (min)
3) Volume of hydrating medium (ml)
4) Hydration time (hrs)

Response:
1) Entrapment efficiency
2) Particle Size

Levels: Two : High & Low

Experiments $2^4 = 16$

I Preliminary Studies Conducted

		Range
Factor 1) Low level = 10 ml High level = 15 ml	Factor 1	5- 15 ml
Factor 2) Low level = 5 min. High level = 10 min.	Factor 2	5 - 15 ml
Factor 3) Low level = 2 ml. High level = 10 ml.	Factor 3	2 - 10 ml
Factor 4) Low level = 1 hr. High level = 2 hr.	Factor 4	1 - 14 hrs.

Table No.21

Analysis of Variance Table following Yate's treatment (Gentamicin Niosomes)

Factor of interaction	Experi-ment	Degree of freedom	Mean Square	F^{1}_{16}
Solvent	A	1	1337.35	8915.66
Sonication Time	B	1	582.25	3881.66
Solvent x Sonication Time	AB	1	163.94	1092.93
Vol. of hydrating medium	C	1	1416.98	**9446.53**
Solvent x Vol. of hydrating medium	AC	1	45.38	302.53
Sonication Time x Vol. of hydrating medium	ABC	1	246.64	1644.26
Solvent x Vol. of hydrating medium	BC	1	8.2113	54.74
Hydration Time	D	1	251.33	1675.33
Solvent x Hydration Time	AD	1	632.35	4215.6
Sonication Time x Hydration Time	BD	1	10.92	72.8
Solvent x Sonication Time x Hydrating Time	ABD	1	235.71	1571.4
Vol. of hydrating medium x Hydrating Time	CD	1	1.505	**10.03**
Solvent x Vol. of Hydrating medium x Hydrating	ACD	1	93.06	620.4
SonicationTime x Vol. of hydrating medium x Hydrating Time	BCD	1	46.95	31.3
Solvent x Sonication Time x Vol. of hydrating medium x hydration Time	ABCD	1	8.9	59.29

Fig. No.1

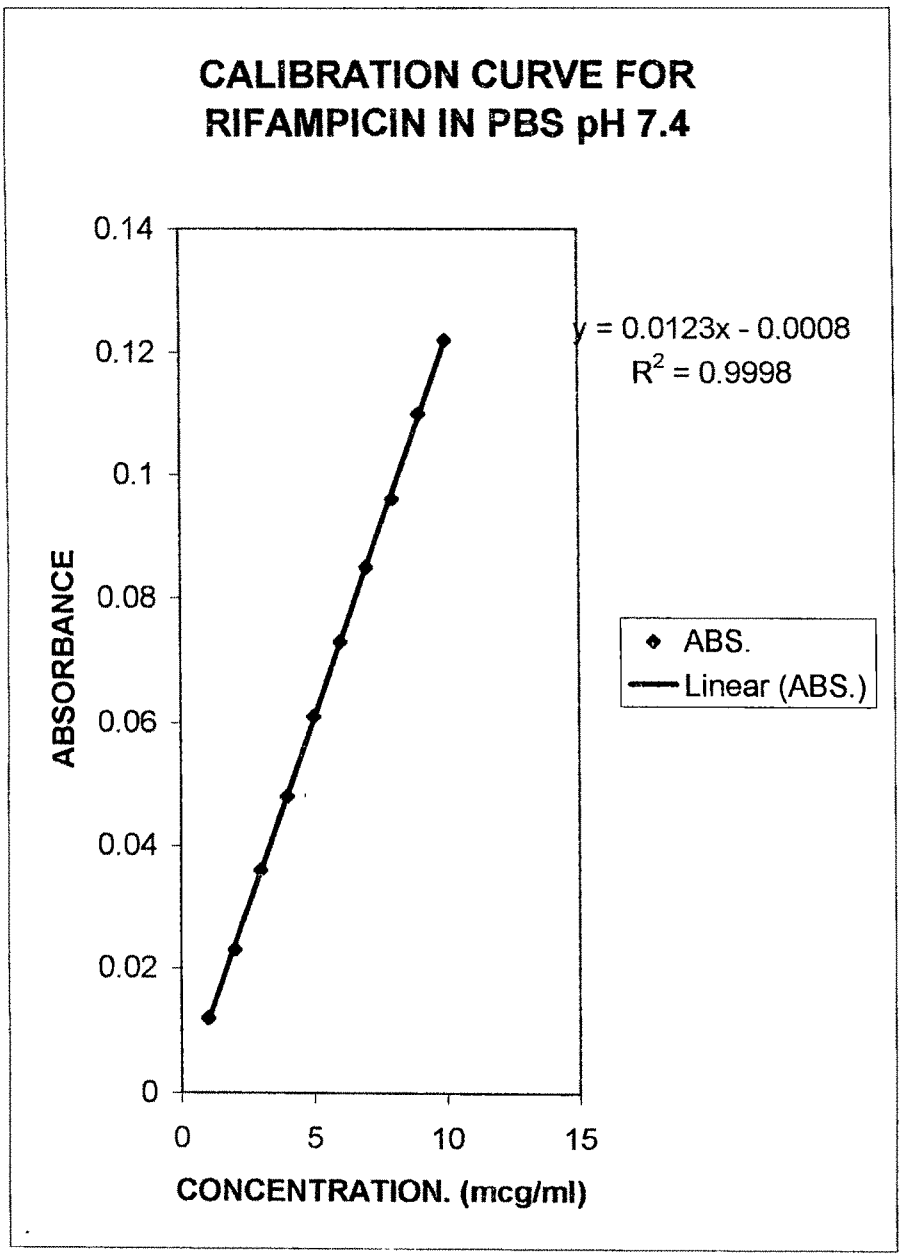

CALIBRATION CURVE FOR
RIFAMPICIN IN PBS pH 7.4
y = 0.0123x - 0.0008
R2 = 0.9998
ABS.
Linear (ABS.)
ABSORBANCE
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
5
10
15
CONCENTRATION. (mcg/ml)

Fig. No.2

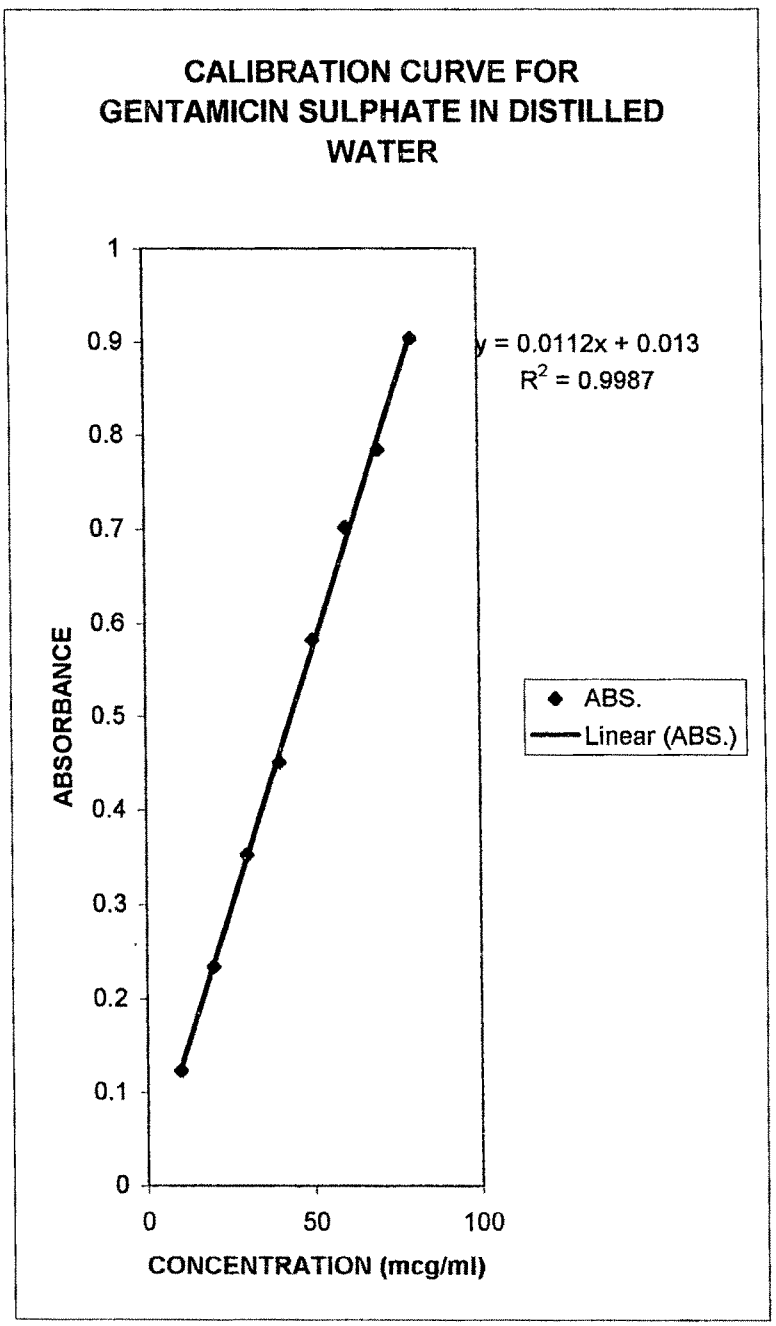

CALIBRATION CURVE FOR GENTAMICIN SULPHATE IN DISTILLED WATER
y = 0.0112x + 0.013
R2 = 0.9987
ABS.
Linear (ABS.)
ABSORBANCE
CONCENTRATION (mcg/ml)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
50
100

Fig. No. 3

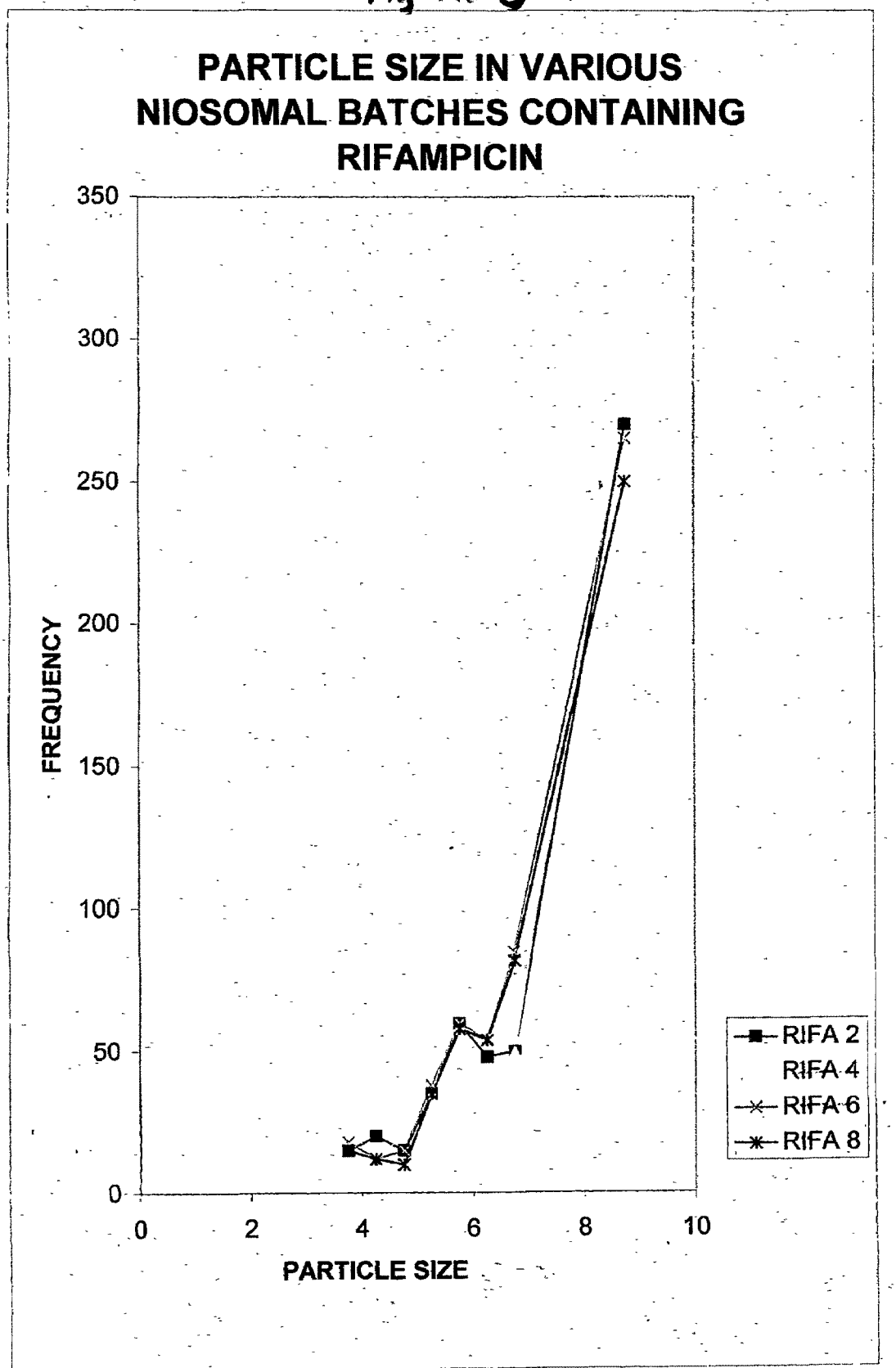

PARTICLE SIZE IN VARIOUS NIOSOMAL BATCHES CONTAINING RIFAMPICIN
350
300
250
200
150
100
50
0
FREQUENCY
0
2
4
6
8
10
PARTICLE SIZE
RIFA 2
RIFA 4
RIFA 6
RIFA 8

Fig No. 4

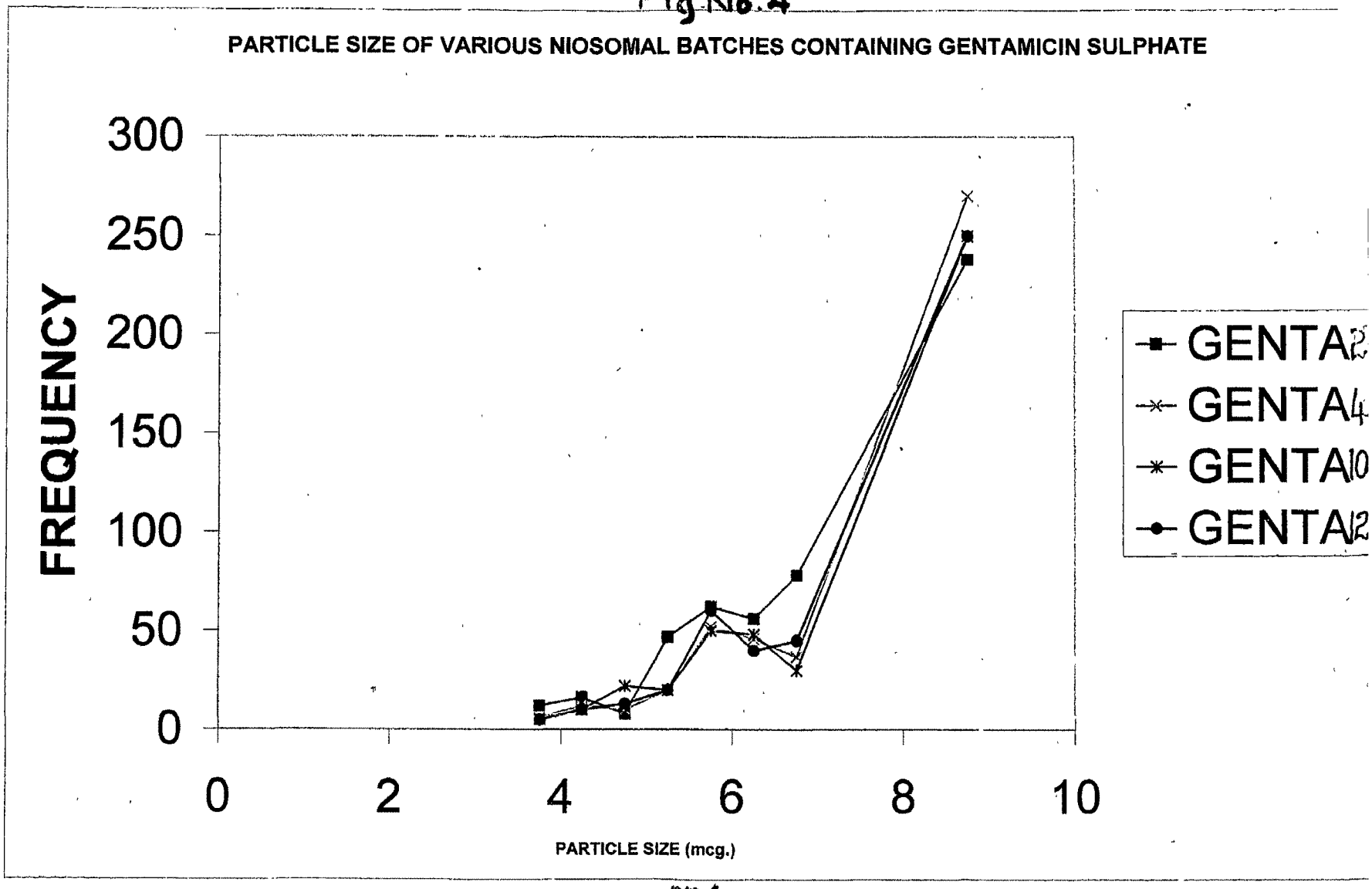

PARTICLE SIZE OF VARIOUS NIOSOMAL BATCHES CONTAINING GENTAMICIN SULPHATE
FREQUENCY
300
250
200
150
100
50
0
0
2
4
6
8
10
PARTICLE SIZE (mcg.)
GENTA2
GENTA4
GENTA10
GENTA12

Plate No.1

Photograph of Rotary flask evaporator (Super fit) used for preparing niosomes

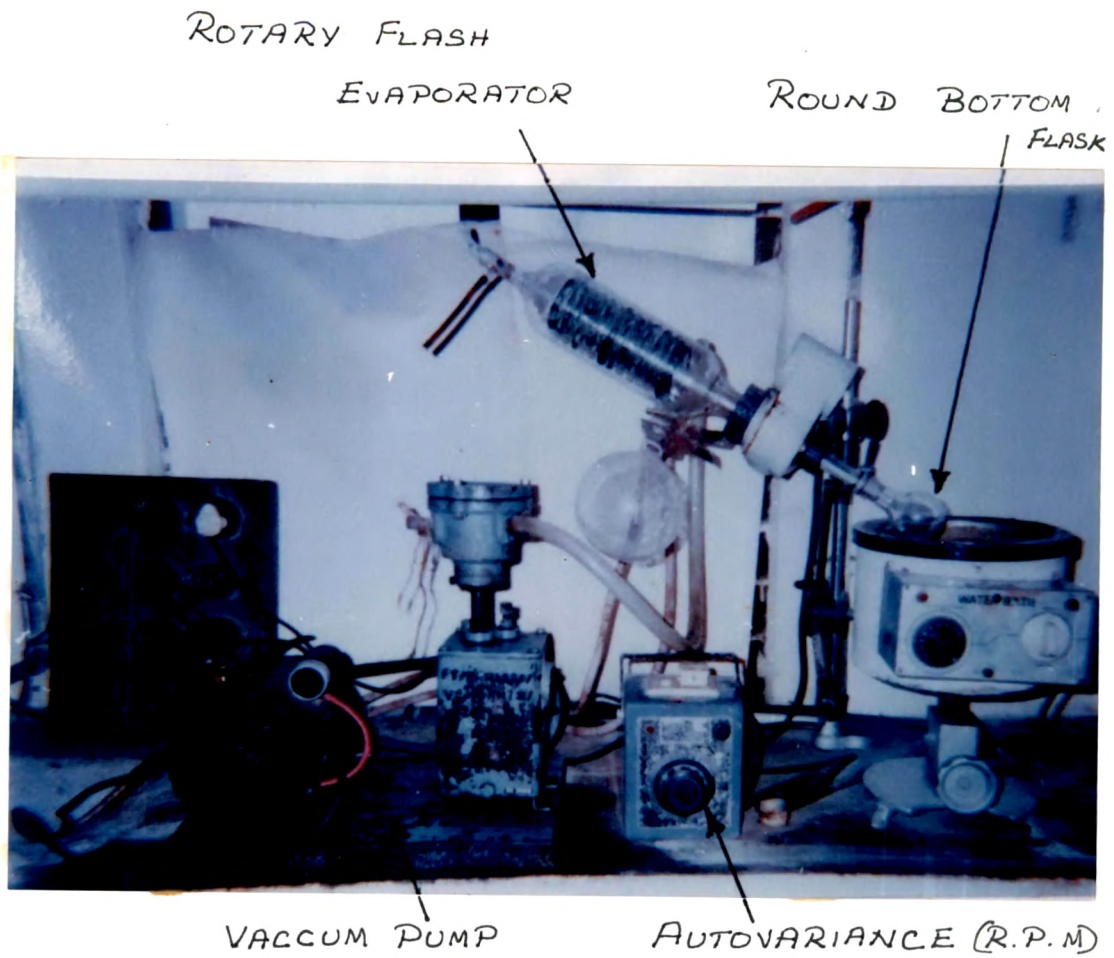


Plate No.2

Photomicrograph of optimized niosomal batches containing Rifampicin (Undiluted)

Batch : Rifa $8k_2$ magnification : 10 x

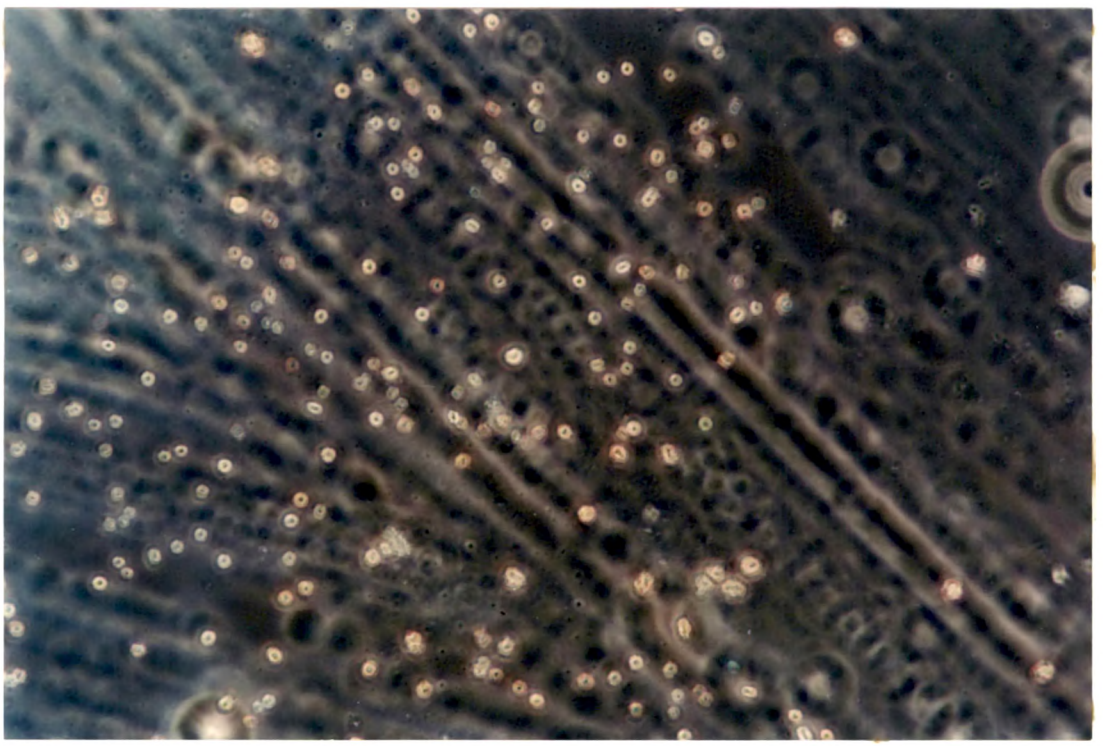


Plate No.3

Photomicrograph of optimized niosomal batches containing Gentamicin (Undiluted)

Batch : Genta $2j_2$ magnification : 10 x

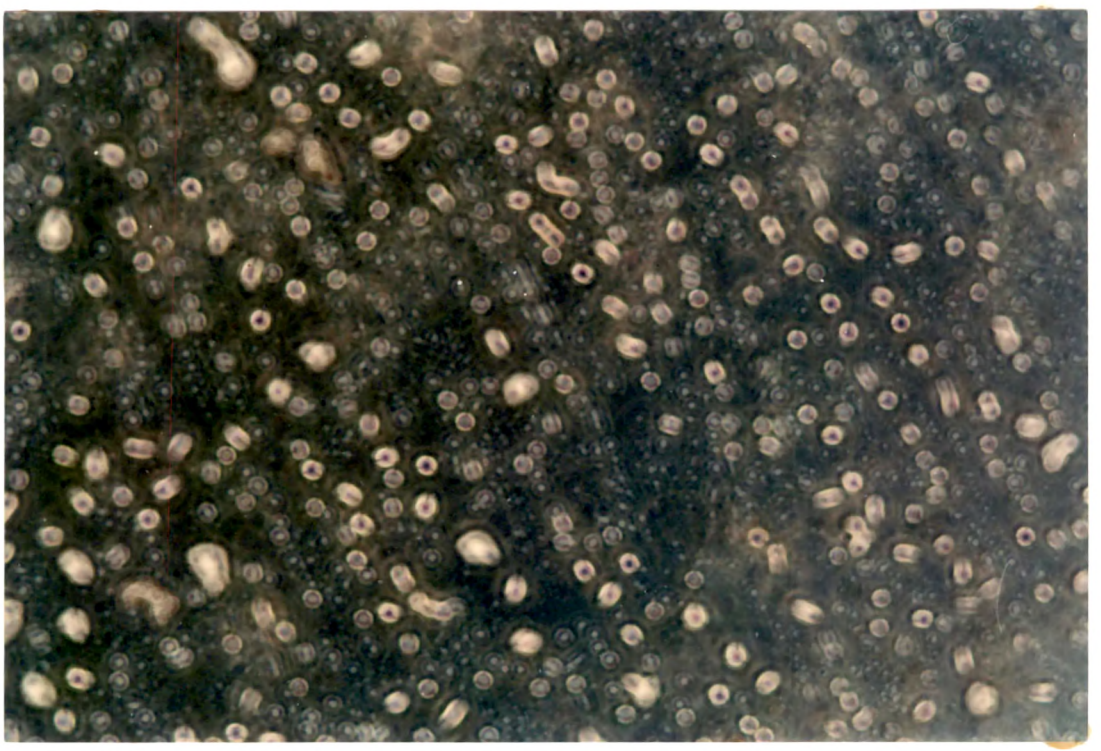


Plate No. 4

Rifampicin Niosome as seen under Scanning Electron Microscope

Batch : Rifa $8k_2$ Magnification : 5000X

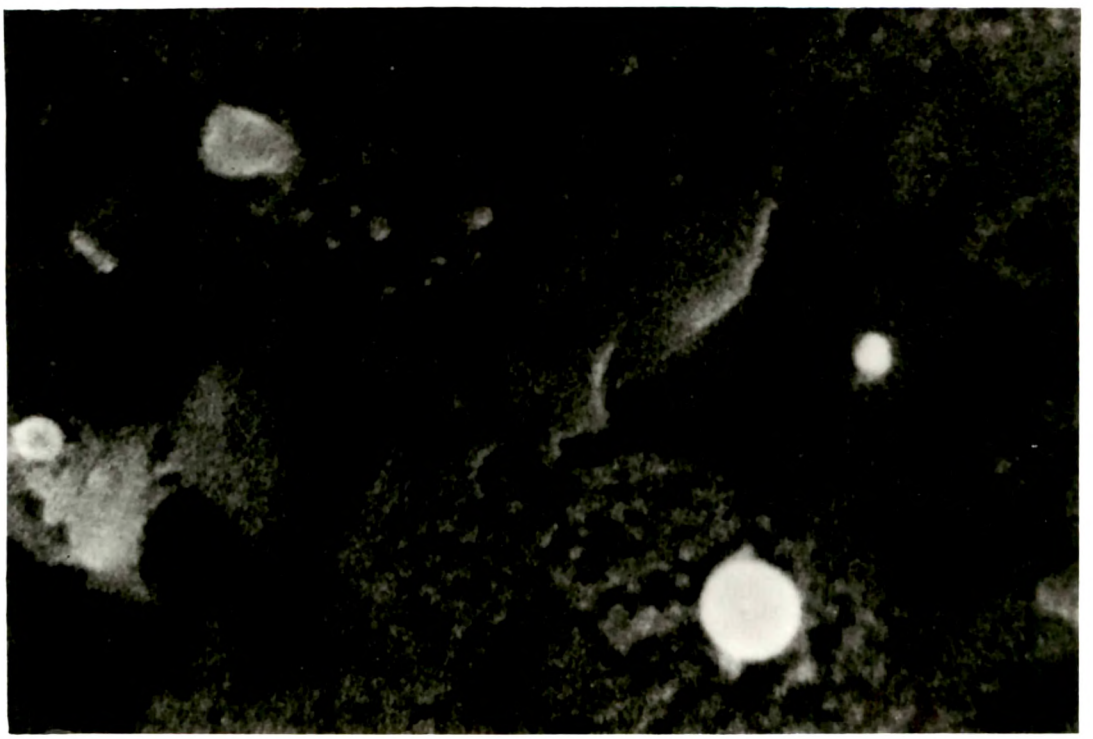
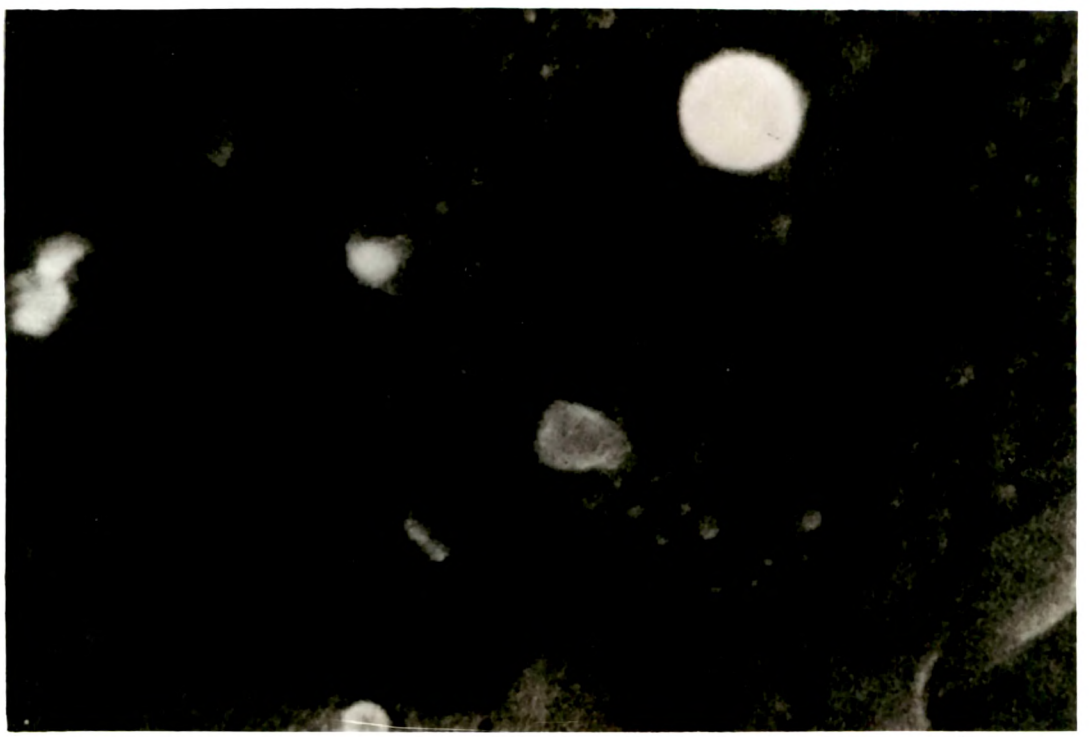


Plate No. 5

Gentamicin Niosome as seen under Scanning Electron Microscope

Batch : Genta $2j_2$ Magnification : 5000X



5.1.2.6 DISCUSSION

Preparation of Niosomes containing Rifampicin

1. Formulation and Method of preparation:

The non-ionic surfactants like Tweens (Tween 20, Tween 40, Tween 60 and Tween 80) and Spans (Span 20, Span 40, Span 60 and Span 80) were used to formulate surfactant vesicles known as niosomes. Chandra Prakash et al[155] have reported that Tween demonstrates good entrapment efficiency and N.Udupa et al[2] have reported that Spans demonstrates good entrapment efficiency.

So, we have chosen both Spans and Tweens as Surfactants for the preparation of Niosomes.

Baillie et al [50] had observed that stable vesicles could be formed in the absence of cholesterol and these displayed the general characteristics of niosomes but were more permeable to entrapped solutes Though stable vesicles could be prepared by using surfactant alone, the drug entrapment was found to be very poor in it. Moreover it was observed that after separation of Niosomes from unentrapped drug by gel filtration, a very dilute product was obtained. Hence, in an attempt to increase the drug entrapment efficiency, cholesterol was incorporated in the formulation of Niosomes. It was observed that stable Niosomes could be formed and entrapment efficiency increased considerably, by incorporating cholesterol

Vesicles prepared from surfactant alone showed decreased drug entrapment efficiency. But equal admixture of cholesterol and surfactant allowed higher entrapments of drug which is shown in Table No.5. Non-ionic surfactants (Tweens, Spans) and cholesterol were used in a molar ratio of 150µmol: 150µmol as reported by A.J.Baillie et al [50]. Out of various formulations prepared, Rifa 8k2 formulation was selected for further studies based on maximum drug entrapment efficiency.

Out of the many techniques reported for the preparation of niosomes, lipid film hydration technique was adopted to prepare Rifampicin niosomes as shown in Flow diagram. Many simple methods namely ether injection, Reverse Phase evaporation technique and sonication were initially tried and none of these methods gave satisfactory niosomal products. These methods resulted in extensive aggregated niosomes, poor drug entrapment or improper vesicle formation or combination of these phenomenon.

To prepare niosomes by thin film hydration technique, care was taken to avoid moisture during the preparation of dry lipid film. The vaccum of 20 inches of Hg was used to aid rapid and complete evaporation of solvent mixture. Flask was rotated at 180 r.p.m. to facilitate rapid drying and acquire uniform film formation. Use of round bottom flask of size 250 ml, resulted in a very thin film and subsequently small size niosomes and hence 100 ml. flask was used.

Dried film prepared was hydrated using 2ml of phosphate Buffer Saline (pH 7.4) solution for a period of 2 hrs. at room temperature.

In the initial efforts to incorporate the drug into the niosomes, the drug was solubilized in the hydrating medium. It gave very poor entrapment of just 10-25% of the drug added. So co-evaporation of drug and lipid from solvent mix was adopted to produce maximum Rifampicin entrapment in niosomes.

The process variables like sonication time, hydration time, volume of hydrating medium and volume of solvent were also optimized The formulation and process variables were optimized to obtain the niosomes with the maximum drug entrapment and mean niosomal size in the range of 7-7.5μm, since the controlled particle size is the essential parameters in targetting to lungs following intratracheal as well as intravenous administration.

The effect of one variable was studied at a time keeping the other variables constant and each experiment was repeated six times. The results shown in Table No.18 reveal the following conclusions.

The sonication time was increased from 5 min. (Rifa $8k_2$) to 15 min (Rifa $8k_{17}$) to bring the mean niosomal size from 7.5μm to 2μm. Further increase in sonication time had no major advantage in terms of niosomal size and resulted in reduced drug entrapment.

The volume of hydrating medium was reduced from 10ml (Rifa $8k_3$) to 2ml (Rifa $8k_2$) with the improved drug entrapment of 72% from 12 48% Further reduction in the volume of hydrating medium had no noticeable change in percent drug entrapment.

The time of hydration was reduced from 14 hrs. (Rifa $8k_{18}$) to 1 hr (Rifa $8k_5$) which remarkably improved the drug entrapment from 3.58% to 38 62% Further reduction in the hydration time had no change in drug entrapment.

The volume of solvent for lipids was altered from 5ml (Rifa $8k_{19}$) to 15ml (Rifa $8k_{14}$) which improved the drug entrapment from 9.20 to 20 22%. But reduction in the volume of solvent to 10ml (Rifa $8k_2$) had noticeable increase in the drug entrapment to 72%

Influence of the process variables on the entrapment efficiency of the Rifampicin niosomes was studied by 2^4 factorial design (Yate's treatment). Sixteen (2^4) experiments were carried out varying process variables. Two levels of each were taken at high and low values as shown in Table No.16. The composition of various batches of Rifampicin niosomes was correlated with the percent drug entrapment.

As shown in the Table No.19 'F', the values from the drug entrapment efficiency were calculated for different batches. Analysis of variance following Yate's treatment showed higher value of significance (F = 22813.58) for batches prepared with variable volume of hydrating medium and less value of significance (F = 264 81) for those with variable sonication time and hydration time combined together.

It was found that formulation of Rifa $8k_2$ with surfactant cholesterol ratio 150 µmol : 150 µmol, volume of solvent for lipids 10 ml, sonication time 5 mins., volume of hydrating medium 2ml and hydration time 2 hrs gave the maximum drug entrapment of 72%. Hence this

batch was taken as a model formulation for further Invitro and Invivo and stability studies.

When niosomes were prepared by using sorbitan esters, the surfactant film was hydrated at about 60°C temperature above the gel-liquid transition temperature of non-ionic surfactants.

Span 60 has the highest phase transition temperature of 50°C. Therefore, all vesicles were prepared at about 60°C. It was earlier tried to hydrate the film at room temperature but the thin film could not be hydrated properly might be because the temperature was below phase transition temperature.

Among non-ionic surfactants, (Tweens, Spans) Span 60 gave the promising results. Though Span 20 is less hydrophobic than Span 60, better results were obtained with Span 60. This may be due to the physical state of the surfactants Span 20 is liquid and Span 60 is solid at room temperature. Hence Span 60 was used to prepare niosome batches by altering various parameters like surfactant, cholesterol ratio, solvent for lipids, hydration time, sonication time and volume of hydrating medium.

Span 60 and Span 80 have the same head group but Span 80 has an unsaturated alkyl chain. Degier et al[153] demonstrate that the introduction of double bonds into the paraffin chain causes a marked enhancement in the permeability in niosomes, this possibly explains the lower entrapment efficiency of niosomes prepared using Span 80

Although Span 40 and Span 60 are solids in room temperature

and show higher phase transition temperature, the higher entrapment efficiency of niosomes occurs in Span 60 it may obviously due to their higher phase transition temperature than Span 40.

These results are also in confirmation with those reported by Yoshioka et al[154].

The Tween series gave very poor entrapment of the drug in the niosomes. It may be due to that entrapment efficiency increases with lipophilicity of surfactant. However, certain exceptions could be observed with data reported by Chandraprakash et al[155].

Me elintosh[156] has demonstrated using X-ray diffraction methods that cholesterol increase the width of phospholipid bilayer Accordingly increase in Rifampicin entrapment was observed when cholesterol was included in niosomes which showed in the Table No.5.

Separation of unentrapped drug from niosomes

Various techniques such as dialysis[157] centrifugation[158] and gel filtration[159] have been reported for separation of unentrapped drug from niosomes. In the present study, we used the technique involving gel filtration through Sephadex G-50 column as it is a relatively simple technique for separation, when a small volume of fluid is to be handled.

Particle size Analysis:

All the batches were subjected to microscopic examination at magnification of 100x for characterizing the size and shape of niosomes containing drug Microscopic examination revealed that size range of vesicles were 7-8μm for various batches. The mean size of

vesicles in Span 60 niosomes was found to be 7.5µm. Photomicrograph of niosomes were obtained with the Olympus 201 microscope and scanning electron microscope and they indicated a spherical multilamellar vesicles with a distinct boundary.

Preparation of Niosomes containing Gentamicin Sulphate

1. Formulation and method of preparation

In the present study, Non-ionic Surfactant (Spans, Tweens) and cholesterol were used in the molar ratio of 150µmol: 150µmol as reported by A J. Baillie et al[50]. Out of various formulations prepared, one formulation (Genta 2j2) was selected for further characterization based on its maximum drug entrapment efficiency.

In the initial efforts to incorporate the drug into the niosomes, (Genta 2a, Genta 2b, Genta 2c) the drug was solubilized in the CHOL: Surfactant solvent mix. It gave a very poor entrapment of just 12% to 22% of the added drug. So the drug was added with the hydration medium to produce niosomes with higher Gentamicin Sulphate entrapment.

The process variables like sonication time, hydration time, volume of hydrating medium, and volume of solvent were also optimized. The formulation and process variables were optimized to obtain the niosomes with maximum drug entrapment and mean niosomal size in the range of 7-8µm, since the controlled particle size of vesicles is the essential parameter in targeting to lungs following the intratracheal as well intravenous administration.

The effect of one variable was studied at a time keeping the other variables constant and each experiment was repeated six times. The results shown in Table No.20 reveals the following conclusions.

The sonication time was altered from 5 min. (Genta $2j_2$) to 15 min (Genta $2j_{17}$) to bring the mean niosomal size from 7.5µm to 3µm. Further increase in sonication time had no major advantage in terms of niosomal size and resulted in a reduced drug entrapment.

The volume of hydrating medium was reduced from 10ml (Genta $2j_3$) to 2ml (Genta $2j_1$) with improved drug entrapment of 44% from 22 56%. Further reduction in the volume of hydrating medium had no noticeable change in percent drug entrapment.

This increase in the % of the drug entrapment could be due to the reduced diffusion of drug entrapped from the niosomes during hydration.

The time of hydration was altered from 14 hrs. (Genta $2j_{18}$) to 1 hr (Genta $2j_5$) which remarkably improved the drug entrapment from 4.28% to 24.52%. Further reduction in the hydration time had no change in drug entrapment.

The volume of solvent for lipids was increased from 5ml (Genta $2j_{19}$) to 10ml (Genta $2j_2$) which improved the drug entrapment from 7 25% to 65%. But further increase in the volume of solvent to 15ml (Genta $2j_{15}$) showed noticeable decrease in drug entrapment to 27.25%.

Influence of the process variables of the Gentamicin niosomes on the entrapment efficiency was studied by 2^4 factorial design (Yate's treatment). Sixteen (2^4) experiments were carried out varying process

variables Two levels of each were taken at high and low values as shown in Table No.20.

The composition of various batches of Gentamicin niosomes were correlated with the percent drug entrapment. As shown in the Table No 21 'F' values for drug entrapment efficiency were calculated for different batches. Analysis of variance following Yate's treatment showed higher value of significance (F = 9446.53) for batches prepared with the variable volume of hydrating medium and less value of significance (F = 10.03) for those with the variable volume of hydrating medium time and hydration time combined together.

It was found that formulation of batch Genta $2j_2$ with surfactant; cholesterol ratio 150 µmol : 150 µmol, volume of solvent for lipids 10 ml , sonication time 5 mins., volume of hydrating medium 2ml and hydration time 2 hrs. gave the maximum drug entrapment of 65%. Hence this batch was taken as a model formulation for further Invitro and Invivo and stability studies.

Among Tween series, Tween 60 gave better entrapment efficiency, than those prepared with Tween 80, Tween 40 and Tween 20. This may be due to higher hydrophilicity of Tween 80, Tween 40 and Tween 20 resulting in higher permeability across niosomal membrane for a hydrophilic drug like Gentamicin sulphate and hence leakage.

The Span series gave a very poor entrapment of drug in the niosomes, may be due to its low hydrophilicity in comparison to Tweens

Particle size Analysis:

All the batches were subjected to microscopic examination at magnification of 100 x for characterizing the size and shape of niosomes containing drug. Microscopic examination revealed that the size range of vesicles were 6.5-7μ for various batches as seen in Table No 12,13,14 & 15, Fig No.4 and Plate No.3.

Vesicle size of all formulations of niosomes were compared. The mean size of vesicles in Tween 60 niosomes was found to be 7.42μm. Photomicrograph of niosomes obtained with Olympus 201 microscope and scanning electron microscope indicated the niosomes to be spherical with multilamellar vesicles with a distinct boundary.