# CHAPTER 6

## INVITRO RELEASE STUDIES FROM NIOSOMES CONTAINING DRUGS

- Invitro Release study for Rifampicin Niosomes
- Invitro Release study for Gentamicin Niosomes
- Invitro affinity study for Niosomes
- Results
- Discussion

## 6.1 EXPERIMENTAL:

**Preparation of Diffusion Cell:**

Cellophane membrane (250-9U, Sigma) 200μm in thickness, pH 5 8 to 8, breaking strength 2.75 kg.f/cm and porosity 0.45μm was used as an artificial membrane for preliminary invitro studies because of simplicity, homogenicity and uniformity. The membrane was hydrated in pH 7.4 Phosphate Buffer Saline (PBS) and 1% EDTA (Ethylene diamine tetra acetic acid) for 24 hours, prior to a permeation run, PBS was prepared according to procedure mentioned in I.P. (1996).

**Design of diffusion cell**

For the present study a vertical type of membrane diffusion system was developed. The system consists of a hollow glass tube open at both ends with an outer diameter of 2.2 cm and a length about 6 cm To one end of the tube the membrane was tied with a nylon string and this acts as a donar compartment. This tube was dipped flush on the surface of a 500 ml beaker containing 250 ml of PBS. The receptor solution was stirred at 100 r.p.m. using a magnetic liner (length=2.5 cm, d=0.5 cm) and a magnetic stirrer. The temperature of the bulk of the solution was maintained at 37°C(±0.5). The donar compartment was stirred with a triple blade stirrer with plastic blades, at 50 r.p.m.

## 6.1.1. VALIDATION OF DIFFUSION CELL:

The hydrodynamic characteristics of the diffusion cell were established using the benzoic acid disc method.

A dual half circular mould was used in the preparation. The mould was placed on a smooth tile, benzoic acid was fused and then poured into the mould. After solidifying, the disc was removed and reduced to proper size by sand-papering it. In the present investigation a disc of 18mm diameter and 5 mm thickness was mounted between the donar and receptor compartment of the diffusion cell. The receptor compartment was filled with 250ml of PBS solution and the dissolution study of benzoic acid was calculated at 37 (±0.5)°C. 2ml aliquots were withdrawn at predetermined time intervals and benzoic acid concentration was determined spectrophotometrically at 266 nm after suitable dilution. The receptor compartment was replaced with 2ml drug free PBS solution immediately after the sample withdrawal.

The rate of dissolution of benzoic acid can be described by the following relationship.

$dc/dt = (kmA/V)(C_s - C_t)$----------(1)

$k_m$ = Mass transfer co-efficient

A = Effective area of disc = 25.45 $cm^2$

V = Volume of diffusion medium = 250 ml.

$C_s$ = Saturation solubility of benzoic acid in PBS at 37°C = 13.50 mg/ml.

$C_t$ = Conc of benzoic acid at time t

Integration of the equation at $C_t = 0$ and $C_t = t$ gives

$\ln C_s/(C_s - C_t) = km\ (A/V)^t$ (ii)

The plot of in $C_s/(C_s - C_t) = km\ (A/V)^t$ is linear and the slope gives the mass transfer co-efficient km.

The thickness of the hydrodynamic boundary layer is given by the equation.

$h_D = D/km$----------(III)

Where D = effective diffusivity in the dissolution medium.

$= 0\ 55 \times 10^{-5}\ cm^2$ / sec at 37°C

The permeation apparatus under study has got a mass transfer co-efficient km = 0.0135 and thickness of the hydrodynamic layer $h_D$ = 4.074. The thickness was measured at different places and the values were compared with those quoted in the literature for franz diffusion cell[175]. The values were found to be statistically similar to the reported values.

### 6.1.2 Method

After separating the unentrapped drug, the niosomal suspension containing drug equivalent to 2mg of drug diluted to 10ml with PBS pH 7 4, was taken up in the donor compartment by a vertical diffusion cell designed for the purpose. The diffusion cell designed consists of a donor compartment and a receptor compartment separated by an artificial membrane. The receptor compartment holds specific volume of the PBS at 37°C ± 0.05°C agitated constantly at 100 rpm Comparative

diffusion studies were carried out for pure drug and niosomal suspension, using 250 ml of PBS pH 7 4 in the receptor compartment. 5ml of sample from the receptor compartment was withdrawn at definite time interval and equivalent amount of fresh PBS pH 7.4 was replaced. Absorbance of the drug in sample was determined using UV-visible Spectrophotometric method using the calibration curve (Fig No.1) for Rifampicin and calibration curve (Fig No.2) for Gentamicin. All diffusion runs and sample analysis were carried out in triplicate. The mean values along with standard errors are recorded in Table No.22 & 23 and these are depicted graphically in Fig 7 & 8

### 6.1.3 INVITRO AFFINITY STUDY:

#### 6.1.3.1 Preparation of two different Liposomal formulation containing Rifampicin and Gentamicin Sulphate.

**Method:**

Liposomes were prepared using the lipid film hydration technique using rotary flash evaporator. The drug (Rifampicin) phosphatidyl choline (PC) and cholesterol (CHOL) in the molar ratios of 1 : 09.0 : 09.0 (25 mg, 225.0mg, 225.0mg) were accurately weighed and dissolved in 10ml of solvent comprising of Chloroform : Methanol · Water (6.3 6 : 0 4), in a 250 ml round bottom flask. The solvent mixture was evaporated under vaccum (20"Hg) at R.T. (≅25°C) to a smooth dry film. The film was hydrated with 2ml of Phosphate Buffer Saline pH 7.4.

The same procedure was followed for the preparation of Gentamicin Sulphate Liposomes except the 2 alteration. First one is

instead of using Rifampicin, Gentamicin sulphate was used in this method and another one is the dried film was hydrated with 2ml of Distilled water.

The entire process of film formation and its subsequent hydration was carried out under nitrogen atmosphere. The formed liposomal suspension was sonicated, by probe sonicator for 15 mins. to get desired particle size of 7-8μm and was then hydrated for 2 hrs under refrigerated condition.

In order to purify liposomes containing Rifampicin, mini column centrifugation method, as described in the chapter 5 section 5.1.2.2 was used For liposomes containing Gentamicin Sulphate, Dialysis method as described in the chapter 5 section 5.1 2 5 was used.

Finally the prepared liposome products were transferred to vials purged with nitrogen, sealed and stored in refrigerator.

### 6.1.3.2. Collection and Targetting to Macrophages.

Heat sterilized thioglycollate medium was stored in dark for 1 week before use. Rats were injected with 2ml of thioglycollate medium intraperitoneally. Three days later, midline incision was made and the peritoneal fluid was collected by lavaging with 5 x 10 ml heparinized phosphate buffered saline (PBS pH 7.4). The peritoneal fluid was withdrawn by suction into a collecting vessel on ice and supernatant was discarded. The cells were suspended in RPMI 1640 medium supplemented with 20% fetal calf serum. After this the cells were washed thrice with the same medium. Total counts were performed in

hemocytometer chamber using Phosphate Buffered Saline (pH 7.4) containing trypan blue. More than 95% of the cells were viable.

In vitro macrophage up take of Liposoms containing Rifampicin (LR) Liposomes containing Gentamicin Sulphate (LG), Niosomes containing Rifampicin (NR) and Niosomes containing Gentamicin Sulphate (NG) were studied on rat peritoneal derived macrophages in the ratios of 25 : 1, 50 : 1, 100 : 1 (vesicles:macrophages) and phagocytosis was conducted for 24 hrs.

In order to investigate the uptake of Liposomal and Niosomal product by macrophage culture, the vesicles were incubated with macrophage suspension and observed in random microscopic fields in the three different vesicle to macrophage ratios (25 : 1, 50 : 1, 100 : 1).

## 6.2 RESULTS

### Table No. 22

### Diffusion of drug from selected batches of Rifampicin across cellophane Membrane

| Time | √t | Batch Rifa 8 (Span 60 +Rifa) % release | Batch Rifa 2 (Span80+ Rifa) %release | Batch Rifa 6 (Span40+ Rifa) %release | Batch Rifa 4 (Span 20 + Rifa) %release |
|---|---|---|---|---|---|
| 1 | 1 | 3.04 | 9.17 | 10.88 | 17.31 |
| 2 | 1.41 | 5.13 | 12.40 | 15.26 | 21.73 |
| 3 | 1.73 | 7.16 | 14.47 | 18.64 | 24.82 |
| 4 | 2 | 7 8 | 16 | 20.69 | 28.71 |
| 5 | 2.23 | 9.24 | 17.56 | 21.75 | 33.74 |
| 6 | 2.44 | 10.09 | 18.62 | 24.2 | 37.20 |
| 7 | 2.64 | 11.12 | 20.69 | 26.91 | 39.82 |
| 8 | 2.82 | 12.40 | 21.75 | 28.98 | 42.07 |
| 9 | 3 | 13.36 | 22.78 | 31.06 | 44.71 |
| 10 | 3.16 | 14.25 | 24.85 | 33.11 | 48.17 |
| 12 | 3.46 | 16 | 26.95 | 36.20 | 56.86 |
| 24 | 4.89 | 23 | 39 | 55.61 | 80.45 |

### Invitro release of Rifampicin Pure Drug

| Time | √t | Absorbance | Concentration | Amount | Correction factor | Total Amount | Cumulative % release |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 0.030 | 2.4390 | 0.6098 | - | 0.6098 | 30.49 |
| 2 | 1.41 | 0 046 | 3.7398 | 0.9349 | 0.9349 + (2.4390x0.005) | 0.9471 | 47.36 |
| 3 | 1.73 | 0.064 | 5.2033 | 1.3008 | 1.3008 + (3.7398x0.005) | 1.3195 | 65.98 |
| 4 | 2 | 0.072 | 5.8537 | 1.4634 | 1.4643 + (5.2033x0.005) | 1.7976 | 74.47 |
| 5 | 2.23 | 0.087 | 7.0731 | 1.7682 | 1.7682 + (5.8567x0.005) | 1.7976 | 89.88 |
| 6 | 2.44 | 0.098 | 7.9675 | 1.9919 | 1.9716 + (7.0731x0.005) | 2.0069 | 100.345 |

## Table No.23

## Diffusion of drug from selected batches of Gentamicin Sulphate across cellophane Membrane

| Time | √t | Batch Genta 4 (Tween 80 +Genta) % release | Batch Genta 12 (Tween20+ Genta) %release | Batch Genta 10 (Tween40+ Genta) %release | Batch Genta 2 (Tween60 + Genta) %release |
|---|---|---|---|---|---|
| 1 | 1 | 9.2 | 16.86 | 10 | 4.46 |
| 2 | 1.41 | 12 | 20.45 | 14.78 | 7.67 |
| 3 | 1.73 | 13.64 | 23.84 | 18.15 | 9.93 |
| 4 | 2 | 15.6 | 25.02 | 20.45 | 12.20 |
| 5 | 2.23 | 18 | 28.39 | 22.72 | 14.36 |
| 6 | 2.44 | 20.42 | 30.69 | 23.89 | 15.2 |
| 7 | 2.64 | 22 | 32.97 | 25.82 | 16 |
| 8 | 2.82 | 23 | 35.25 | 28.39 | 18.8 |
| 9 | 3 | 26 00 | 38.63 | 30.69 | 20.2 |
| 10 | 3 16 | 27.12 | 39.82 | 32.97 | 21 |
| 12 | 3.46 | 30.65 | 44.31 | 35.25 | 23.84 |
| 24 | 4.89 | 45 | 65 | 55 | 35 |

## Invitro release of Gentamicin Sulphate Pure Drug

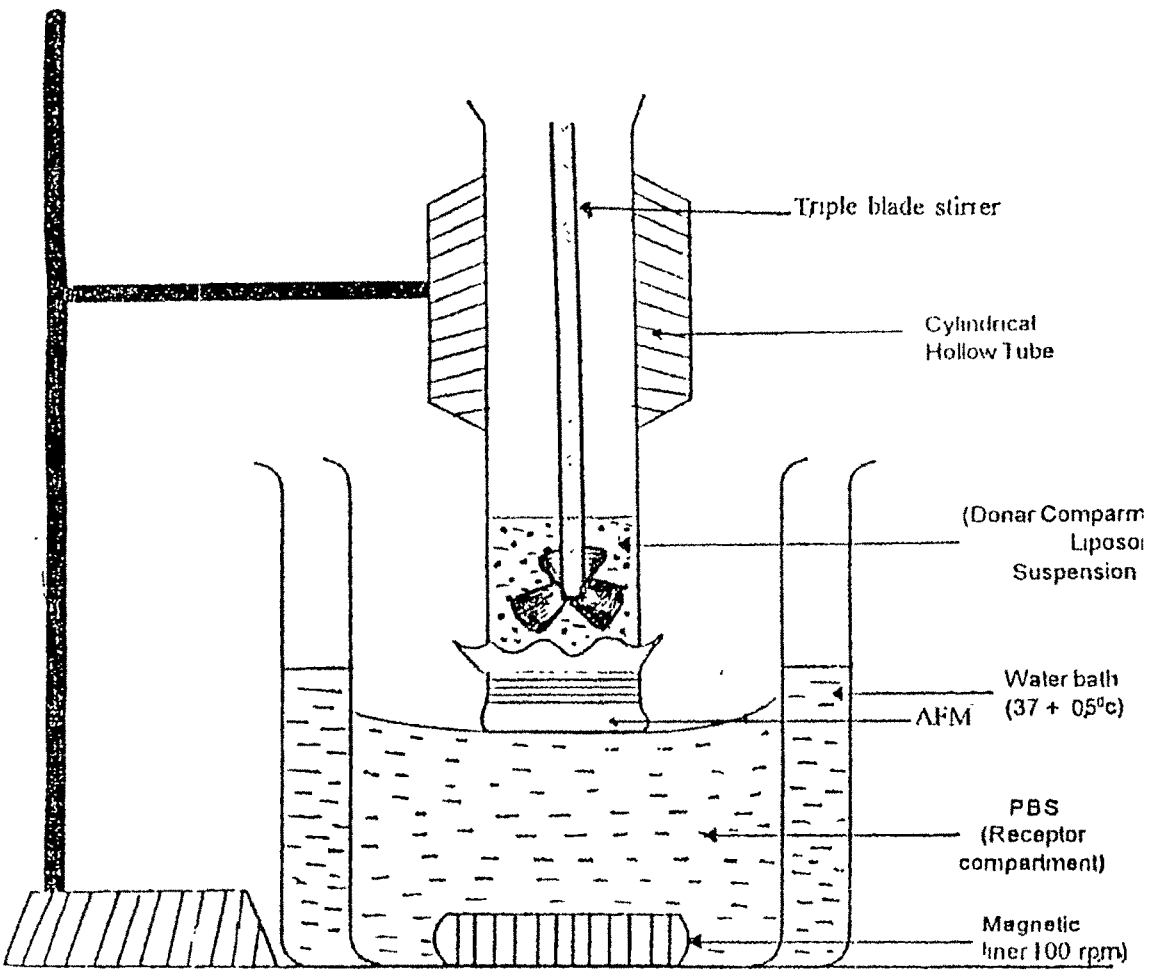
| √t | Absorb -ance | Concen Tration | Amount | Correction factor | Amount | % release |
|---|---|---|---|---|---|---|
| 1 | 0.032 | 2.8571 | 0.7143 | -- | 0.7143 | 35.72 |
| 1.41 | 0 053 | 4.7321 | 1.1830 | 1.1830+(2.8571x0.005) | 1.1973 | 59.86 |
| 1.73 | 0.076 | 6.7857 | 1.6964 | 1.6964+(4.7321x0.005) | 1.7201 | 86.01 |
| 2 | 0.088 | 7.851 | 1.9643 | 1.9643+(6.7857x0.005) | 1.9982 | 99.91 |
| 2.23 | 0.047 | 4.1964 | 1.0491 | 1.0491+(7.8571x0.005) | 1.0884 | 54.42 |
| 2.44 | 0.034 | 3.0357 | 0.7589 | 0.7589+(4.1964x0.005) | 0.77988 | 38.99 |

TABLE NO.24

## PHAGOCYTOSIS OF LIPOSOMAL AND NIOSOMAL DRUG LOADED VESICLES BY MACROPHAGES

| Formulation | Visual Assay | | | |
|---|---|---|---|---|
| | Initial count Millions/ml | Number of cells after Phagocytosis (millions / ml) | | |
| | | 25 : 1 | 50 : 1 | 100 : 1 |
| LR | 11.5 | 1.8 (P<0.001) | 2.2 (P<0.001) | 4.5 (P<0.001) |
| LG | 10.4 | 2.6 (P<0.001) | 3.8 (P<0.001) | 4.8 (P<0.001) |
| Rifa 8k2 | 12.3 | 10.6 (P>0.001) | 11.3 (P>0.001) | 11.8 (P>0.001) |
| Genta 2j2 | 13.2 | 11.7 (P>0.001) | 12.4 (P>0.001) | 12.8 (P>0.001) |

Fig. No. 5




Design of Diffusion Cell

Fig No. 6

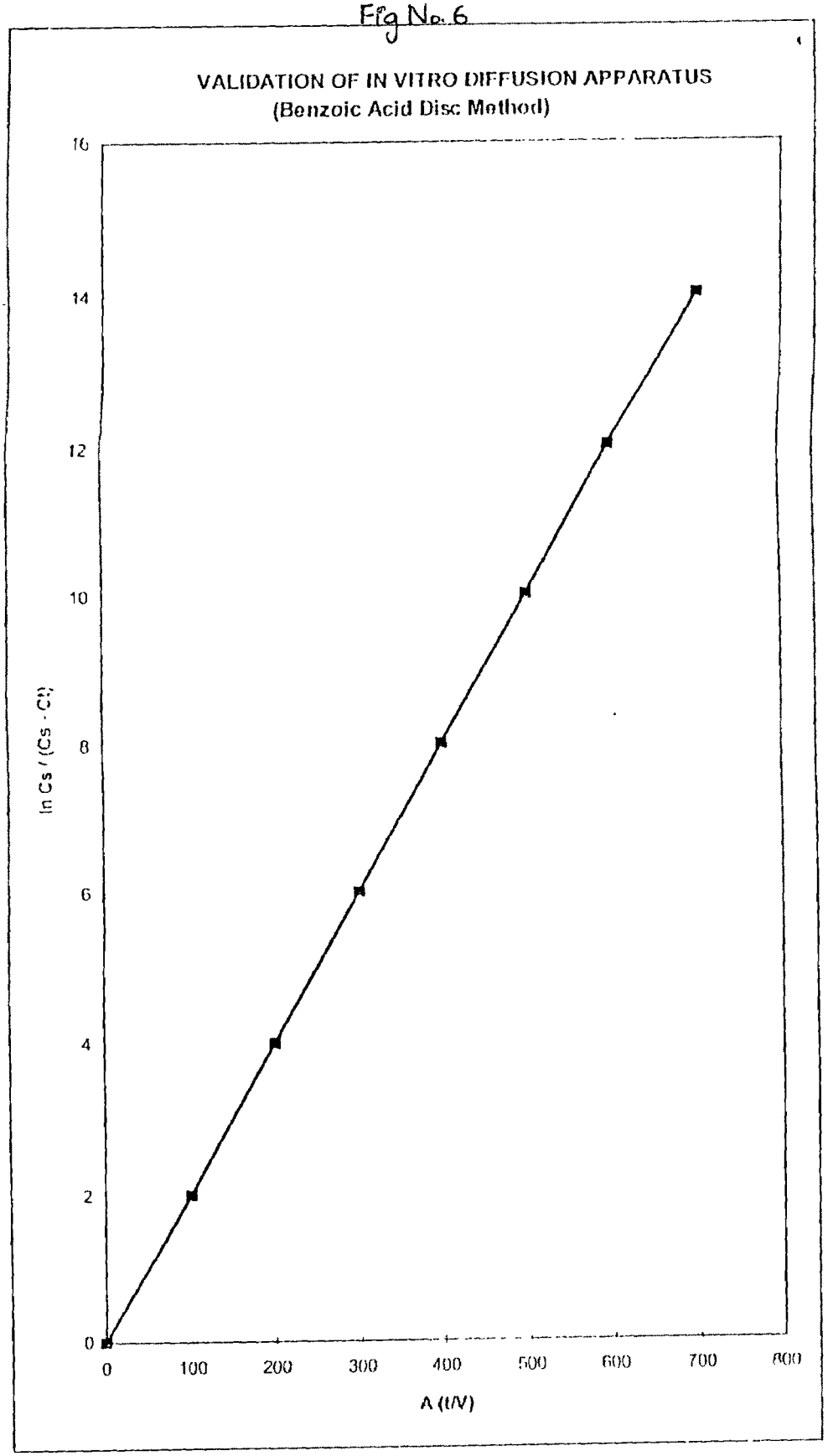

VALIDATION OF IN VITRO DIFFUSION APPARATUS
(Benzoic Acid Disc Method)
16
14
12
10
8
6
4
2
0
ln Cs / (Cs - Ct)
0
100
200
300
400
500
600
700
800
A (t/V)

Fig. No.7

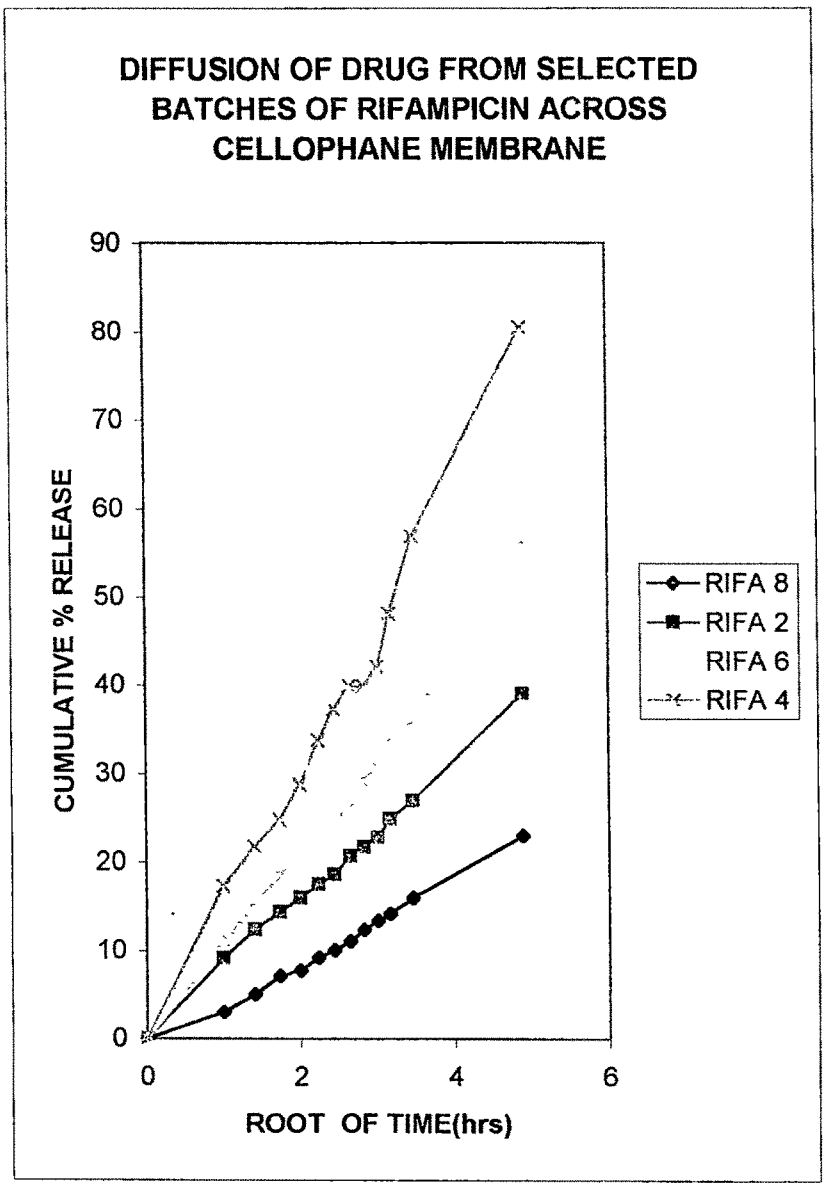
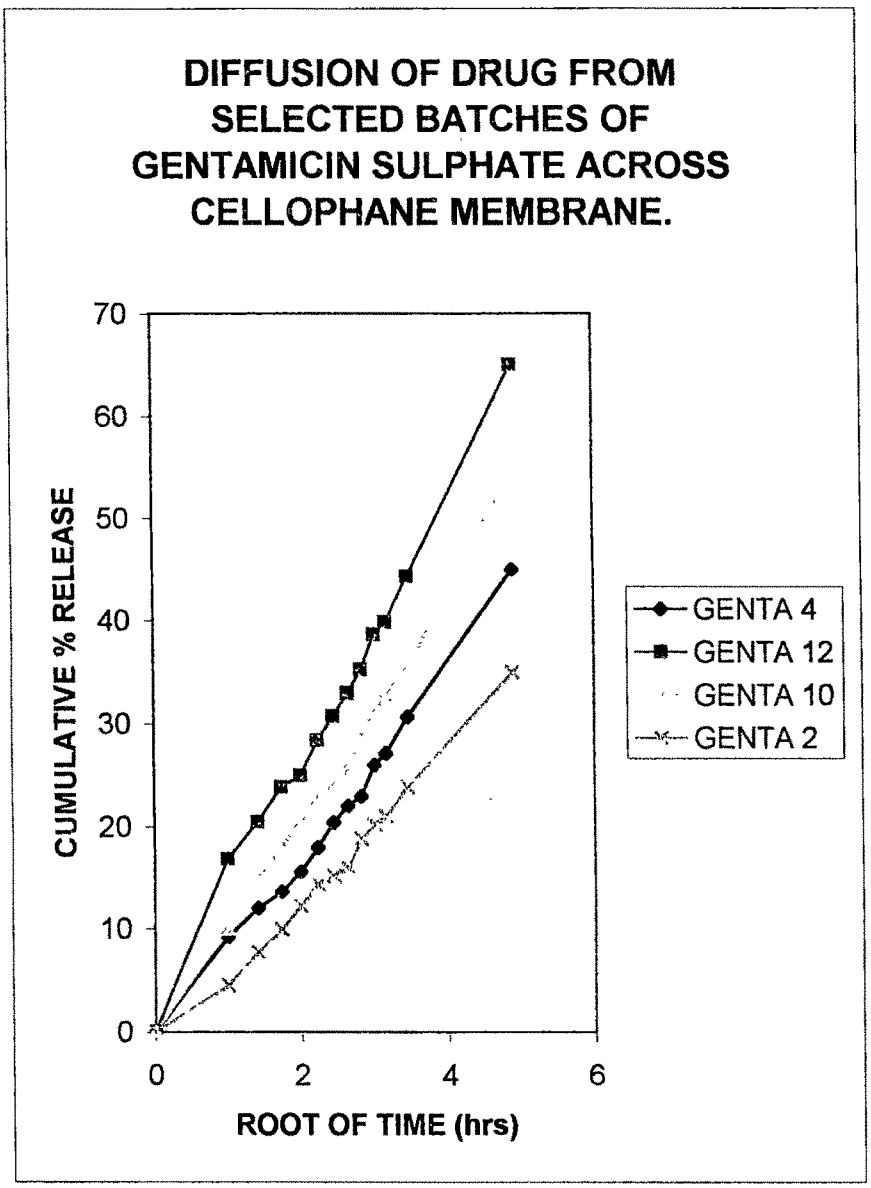

DIFFUSION OF DRUG FROM SELECTED BATCHES OF RIFAMPICIN ACROSS CELLOPHANE MEMBRANE
CUMULATIVE % RELEASE
90
80
70
60
50
40
30
20
10
0
0
2
4
6
ROOT OF TIME(hrs)
RIFA 8
RIFA 2
RIFA 6
RIFA 4

Fig. No.8


DIFFUSION OF DRUG FROM SELECTED BATCHES OF GENTAMICIN SULPHATE ACROSS CELLOPHANE MEMBRANE.
CUMULATIVE % RELEASE
70
60
50
40
30
20
10
0
0
2
4
6
ROOT OF TIME (hrs)
GENTA 4
GENTA 12
GENTA 10
GENTA 2

# 6.3. Discussion

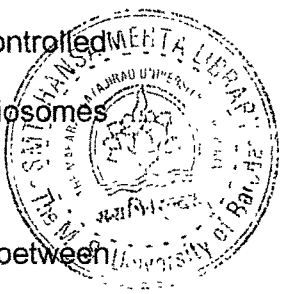
**Invitro release Study for Niosomes containing Rifampicin and Gentamicin Sulphate**

Invitro release characteristics of Rifampicin pure drug and various niosomal batches containing Rifampicin into phosphate Buffer Saline pH 7.4 at 37±0.05°C were studied using a sigma dialysis membrane and the results were represented in Table No.22

The release of drug from pure Rifampicin solution was studied upto 8 hours. The release rate was found to increase with time and almost 100% of the drug was released within 6 hours from the control solution. The release rate calculated was 106 84 $hr^{-1}$ it indicating that the release followed the first order.

The release of Rifampicin from various batches of Niosomes (Rifa8, Rifa 2, Rifa 6 and Rifa 4) were studied for 48hrs. The percentage release was found to increase with time and 23% (Rifa 8), 39% (Rifa 2) 55 61% (Rifa 6) and 80.45% (Rifa 4) of the drug was released in 24 hrs Linear curves were not obtained by plotting the percent drug released vs either time or logerthem of time for all the formulations taken for the study, indicating that the release does not follow zero or first order. However plot of percent drug released vs Root T, yielded a linear

relationship, indicating the release obeys Higuchi's diffusion controlled model Diffusion of drug from selected batches of Rifampicin Niosomes are shown in Fig No.7.



Two way ANOVA was applied to analyse the difference between the cumulative release of Rifampicin in the selected four formulations at each time points. The calculated 'F' (F = 13.24) values between each formulation and between each time point was significantly higher than tabulated F (2.23) at p = 0.001.

From this study, lower drug release rate was observed in the case of batch Rifa 8 (Span 60 : CHOL : Drug), and so this batch was selected for invivo study.

Invitro release characteristics of Gentamicin pure drug and various niosomal batches containing Gentamicin into phosphate Buffer Saline pH 7.4 at 37±0.05°C were studied using a sigma dialysis membrane and the results are represented in Table No.23

The release of Gentamicin from various batches of Niosomes (Genta 4, Genta 12, Genta 10 and Genta 2) were studied for 48hrs. The release of drug from pure Gentamicin solution was studied for 8 hours. The release rate was found to increase with time and almost 100% of the drug was released within 6 hours from the control solution. The release rate calculated was 139 41 $hr^{-1}$ indicating that the release followed the first order.

The percentage release was found to increase with time and 45% (Genta 4), 65% (Genta 12), 55% (Genta 10) and 35% (Genta 2) of

the drug was released in 24 hrs. Linear curves were not obtained by plotting the 'percent drug released' vs either 'time' or 'logerthem of time' for all the formulations taken for the study, indicating that the release did not follow zero or first order. However the plot of percent drug released vs Root of time. yielded a linear relationship, indicating the release obeys Higuchi's diffusion controlled model Diffusion of drug from selected batches of Gentamicin Niosomes are shown in Fig No.8

Two way ANOVA was applied to analyse the differences among the cumulative release of Gentamicin in the selected four formulations at each time points The calculated 'F' (F = 5 33) values in each formulation and in each time point was significantly higher than tabulated F (2.23) at p = 0.001.

From this study, lower drug release rate was observed in the case of batch Genta 8 (Tween 60 : CHOL : Drug) and so this batch was selected for invivo study.

**Targeting of Niosomes and liposomes to macrophages.**

Many studies have been focused on the mechanism underlying the efficient uptake of liposomes by the RES. It is now generally understood that the mononucleophagocytes of the RES, principally , the Kupffer cells of the liver and secondarily the splenic macrophages are responsible for the clearance of liposomes from the circulation[176]. In addition parenchymal cells of the liver are also involved in liposome uptake[177]. Furthermore, serum factor(s) (opsonins) coating the liposome surface are believed to promote the specific uptake of liposomes by the

liver and the spleen, although the molecules have not been identified[178].

Macrophage targeting was carried out on the rat peritoneal derived macrophages. The number of vesicles in the diluted niosomes Genta 2j2 as wells Liposomes (LR, LG) were counted in hemocytometer chamber before and after phagocytosis with macrophages. As depicted in Table No.24, the number of liposomal vesicles (LR, LG) were significantly decreased after phagocytosis with macrophages in all the three different ratios. But for niosomal products (Rifa 8k2 and Genta 2j2), the number of vesicles were not significantly decreased after phagocytosis with macrophages in all the three different ratios

According to the values from visual examination, it was confirmed that the macrophage uptake of niosomal vesicles was less when compared to the uptake of liposome vesicles of the similar size range.

Higher uptake value for liposomal vesicles by macrophages is due to the fact that the liposomes are comparatively more lipoidal than the niosomes. This is obviously due to the presence of hydrophilic surfactant in the niosomal structure. Increase in the phagocytic capasity of macrophages has been reported [164] for bacteria with hydrophobic coat , which supports the above finding.