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FORMULATION DEVELOPMENT AND INVITRO EVALUATION OF RIFAMPICIN LIPOSOME FORMULATIONS

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ABSTRACT
Rifampicin liposomes (F) were prepared by solvent injection method using different ratios of soya lecithin and Cholesterol. Characterization of liposomes was done by using optical microscopy, scanning electron microscopy and particle size distribution. Evaluation for liposomes are done by drug entrapment efficiency, in vitro diffusion studies, ex vivo diffusion studies, statistical analysis, optimization, validation of the experimental design. Stability studies were done for liposomes on storage at 2-8 °C and room temperature. From the results of physicochemical parameters, rifampicin liposomal dispersion F-8 was selected as optimized formulation. Further F-8 was formulated into pharmaceutical pastilles dosage forms for buccal drug delivery. Evaluation for FP-8 was done by average weight, drug content, swelling, erosion, moisture absorption, pH, bio adhesion time, bio adhesive strength, in vitro drug release, stability studies and in vivo pharmacokinetic studies using rabbit model. Rifampicin liposomal pastilles FP-8 are uniform in size, flexible and found to have uniform drug content.

INTRODUCTION
Liposomes are microscopic vesicles containing aqueous volume enclosed by lipid bilayer membrane (Bangham et al., 1965). A.D. Bangham and R.W. Thorne first described about liposomes in 1964 when observed under electron microscope while analysing phospholipids dispersion in aqueous environment (Betageri et al., 1993). They observed spontaneous arrangement of phospholipids into “bag-like” circular structures. Gerald Weissman, one of the colleagues of Bangham suggested the structures as liposomes (Deamer, 2010). This discovery helped as a multipurpose tool in several fields like biology, biochemistry and medicine. Liposomes gained popularity in vesicular research due their attributes of biocompatibility and similar structural features of biological cells.

Several reports showed the applicability of liposomes for the safe and effective administration of therapeutic molecules of different classes like antitubercular, anticancer, antifungal, antiviral, antimicrobial, antisense, lung therapeutics, skin care, vaccines genes etc. (Abdus, 2007). Liposomes have proven their commercial importance from the first product ‘Doxil’, a PEGylated doxorubicin liposomal formulation (Barenholz, 2012 and Fassas, 2005) to the latest ‘Marqibo’
Rifampicin is a member of the class of rifamycins that is a a semisynthetic antibiotic derived from Amycolatopsis rifamycinica (previously known as Amycolatopsis mediterranei and Streptomyces mediterranei). It has a role as an EC 2.7.7.6 (RNA polymerase) inhibitor, a DNA synthesis inhibitor, an antitubercular agent, a leprostatic drug, an Escherichia coli metabolite, a protein synthesis inhibitor, a neuroprotective agent, an angiogenesis inhibitor, a pregnane X receptor agonist, an antineoplastic agent, an antiamoebic agent and a geroprotector.

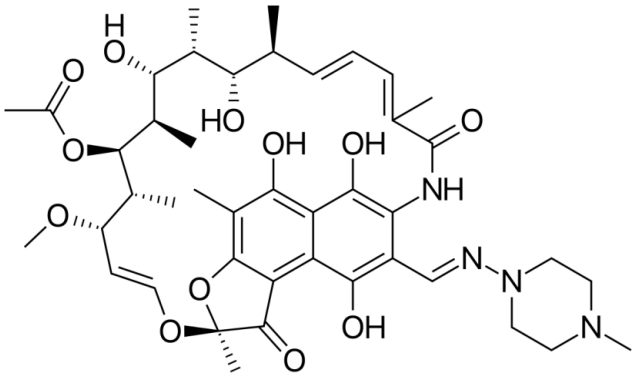

Figure 1: chemical structure of Rifampicin.

EXPERIMENTAL WORK
Materials and instruments: Rifampicin was gift sample from sai life sciences ltd Hyderabad, Soya Lecithin, Cholesterol ER, Chloroform AR, Gelatine, Carbopol, Glycerol AR, Hydrochloric Acid ER, Sodium hydroxide, Potassium di-hydrogen Phosphate was purchased from from B.M.R.Chemicals, Hyderabad and equipment and instruments like Electronic Weighing

Balance from Shimadzu Corporation Tokyo, Japan, UV-Vis Spectrophotometer (T60) from PG Instrument, FTIR Spectrophotometer from Shimadzu Corporation Tokyo, Japan, Dissolution Apparatus from LAB India, Magnetic stirrer from Remi industries, Kerala.

METHODOLOGY

Calibration Curve of Rifampicin by UV-Visible Spectroscopy

There are different analytical methods reported by colorimetry, UV-Visible spectrophotometer and HPLC for rifampicin estimation (Sushruta and Bharath, 2017). In the present investigation we used modified spectrophotometric method for rifampicin estimation and absorbance measured at wave length 475 nm using phosphate buffer pH 7.4 (Sarbjit and Raman, 2010).

Preparation of Stock Solution

Accurately weighed 50mg of rifampicin and transferred in to a 50 ml volumetric flask. To this flask containing rifampicin, 10 ml of ethanol was added and shaken thoroughly to dissolve the drug. Then 20 ml of phosphate buffer of pH 7.4 was added and mixed well. The mixture was kept under sonication in laboratory bath sonicator (M/s. Remi, India) for 15 minutes. After sonication the flask is kept aside to get equilibration and then volume made to 50 ml with using buffer. The resulting solution of concentration 1000 µg/ml was used as stock solution.

Preparation of Standard Solutions

From rifampicin stock solution, suitable dilutions were done by using phosphate buffer pH 7.4 to obtain concentrations from 10µg/ml to 50µg/ml. The absorbance of these standard solutions was measured at 475 nm using UV spectrophotometer (UV-1800 Shimadzu). The experiment was triplicated.

EXPERIMENTAL METHODS

Factorial Experimental Design

Response Surface Methodology aims to establish the relative importance of two or more factors and also to indicate whether or not interaction occurs between the factors and thereby affects the magnitude of response. The three level two (3^2) factorial design was selected to study variables effect on physicochemical properties of liposomes. Amount of soya lecithin (X_1) and cholesterol (X_2) were selected as two independent variables which were varied at three levels (Table 4.2) i.e. low level (−1), medium level (0) and high level (+1). Amount of rifampicin and chloroform were kept constant. Particle size (Y_1), percentage entrapment efficiency (Y_2), percentage drug release using dialysis membrane [DM] (Y_3) and percentage drug release using porcine buccal mucosa [PBM](Y_4) were selected as dependent variables. Design-Expert® 9 software was used to design and evaluate (Shah and Pathak, 2010).

Table 1 3^2 Factorial Design for the Preparation of Rifampicin Liposomes.

S.No.	Formulation	Levels of Independent variables		X_1:X_2 (mg)
		Soya Lecithin (mg) (X_1)	Cholesterol (mg) (X_2)	
1.	F-1	-1	-1	5:3
2.	F-2	-1	0	5:4
3.	F-3	-1	+1	5:5
4.	F-4	0	-1	10:3
5.	F-5	0	0	10:4
6.	F-6	0	+1	10:5
7.	F-7	+1	-1	15:3
8.	F-8	+1	0	15:4
9.	F-9	+1	+1	15:5

Preparation of Rifampicin Liposomes

Rifampicin liposomes (F) were prepared by solvent injection method using different ratios of soya lecithin (SL) and cholesterol (CH) (Ajay et al., 2010; Sudhakar et al., 2014) as per formulae given in Table 4.2. Accurately weighed 50 mg of lecithin, 30 mg of cholesterol were transferred into a 25 ml beaker and dissolved by adding 5 ml of chloroform. To this mixture 50 mg of accurately weighed rifampicin was added and dissolved with gentle stirring at 60 ºC. In another beaker 10 ml of phosphate buffer pH 7.4 (aqueous phase) was taken and kept under stirring at 500 rpm on thermostatically controlled magnetic stirrer (Remi Magnetic Stirrer, Model: LBMS-5886) using a teflon coated magnetic bead at a 60 ºC. To the aqueous phase of buffer, the drug containing lipid phase was injected directly in a single jet by using glasssyringe with 14 gauze needle. The mixture was continued stirring for about 2 h to obtain uniform dispersion and was subjected to evaporation at 60 °C for 1 h to remove excess chloroform. Phosphate buffer pH 7.4 was added to adjust the volume of final liposomal dispersion to 10 ml. The final dispersion was stored in tightly closed light resistant container and kept in refrigerator at 2-8 ºC until for further use. Three different batches of rifampicin liposomes were prepared in each formulation by using the same procedure to obtain reproducibility. All the liposomal formulations as per formulae given in Table 4.2 were prepared using the same procedure as mentioned above.

Characterization and Evaluation of Liposomes

Optical Microscopy

The prepared liposomes were viewed under optical

microscope (Lawrence and Mayo) for observing the vesicle formation and discreteness of dispersed vesicles. A slide was prepared by placing a drop of liposome dispersion on a glass slide and cover slip was placed over it and this prepared slide was viewed under optical microscope at 100X magnification. The images were captured by using digital camera.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy was used to characterize the surface morphology of the prepared vesicles. One drop of liposomal dispersion was mounted on a clear-glass stub, air-dried, coated with Polaron E 5100 sputter coater (Polaron, Watford, United Kingdom), and visualized under a scanning electron microscope (Leo-435 VP; Leo, Cambridge, United Kingdom (Batzri and Korn, 1973; Boulmedarat et al., 2005).

Particle Size Distribution

The particle size and size distribution of liposome dispersion was determined by using dynamic light scattering technique by zetasizer 300HSA (Malvern Instruments, Malvern, UK). Photon correlation spectroscopy (PCS), also known as quasielastic light scattering (QELS), was used to determine particle size and polydispersity of samples (Chu and Liu, 2000). Samples are transferred into cuvette for scattering. The analysis was carried for about 60 sec at a scattering angle of 90° for detection. Polydispersity Index (PI) was determined for the measurement of particle size homogeneity of prepared liposomes (Mugabe et al., 2006). Zeta potential was also determined for optimized liposomal formulation F-8.

Drug Entrapment Efficiency

The entrapment efficiency (EE) was determined by ultracentrifugation method which gives quantity of drug in three regions of the vesicular system (Sudhakar et al., 2014; Demel et al., 1972). Those three regions are the quantity adsorbed on vesicular membrane, quantity incorporated in the internal core phase and the quantity incorporated in to the vesicle membrane bilayer. To find out the entrapment efficiency the liposomal dispersions were centrifuged (M/s. Remi CPR-30, India) at 5 ºC at 18,000 rpm for 1h. The supernant portion of the dispersion (unentrapped drug content) was diluted and estimated by using UV visible spectrophotometer (Shimadzu, UV1800) at 475 nm wave length. Entrapment efficiency of all formulation calculated by using following formula.

Average Weight and Weight Variation

Twenty pastilles were taken and their weight was determined individually and collectively on a digital weighing balance. The weight variation was calculated using the following formula (Peck et al., 1989).

$$\text{Weight Variation} = \frac{\text{Individual weight} - \text{Average weight}}{\text{Average weight}} \times 100$$

Drug Content Estimation

Drug content estimation was done by dissolving one pastille in sufficient quantity of phosphate buffer pH 7.4 and diluted up to 100 ml in volumetric flask. The sample was filtered and diluted suitable. The absorbance of the resulting solution was measured using UV-Visible spectrophotometer at 475 nm against phosphate buffer pH 7.4. From the observed absorbance drug content in

Stability Studies of Rifampicin Liposomes

The stability of rifampicin liposomes in different formulations was studied at two storage conditions. One sample is kept at 2-8 °C refrigeration condition and other at 25 °C room temperature for three months. The samples analysed for liposomal dispersion physical stability.

Preparation of Liposomal Pastilles

Pastilles were prepared for optimized liposomal formulation F-8. Each pastille is prepared with dose of 10 mg rifampicin. The pastilles were prepared with modified method reported in British Pharmaceutical Codex, 1907. Accurately weighed 1.5 g of gelatine was transferred into 25 ml glass beaker. To gelatine 5 ml phosphate buffer pH 7.4 was added and heated at 60 ºC on water bath to get complete dissolution. Accurately weighed 445 mg of Carbopol and 1 g of sugar were added while stirring and heated until they dissolve in gelatine base. Glycerol of 1 g, sodium saccharin 5 mg was weighed and added to the above mixture. To this mixture liposomal dispersion containing 50 mg drug was added under stirring. Finally the mixture was transferred into 5 moulds of diameter 15 mm and cooled in freezer condition for solidification. Pastilles were also prepared with pure rifampicin (PRP) as control by similar procedure mentioned above. The pastilles are stored in tightly closed container in refrigerator at 2-8 °C until further use.

Figure 1: Rifampicin Liposomal Pastilles.

Evaluation Tests for Rifampicin Liposomal Pastilles (FP-8) and Pure Rifampicin Pastilles (PRP)

Dimensions of Pastilles

The diameter and thickness for prepared FP-8 and PRP were determined by vernier caliper.

pastilles was calculated.

Swelling and Erosion Studies

Swelling and erosion studies for pastilles were determined gravimetrically in phosphate buffer of pH 6.8 which was attached to pre-weighed glass *petri* dish and supported using adhesive sealant. Three pastilles were weighed individually (W_1) and immersed separately in phosphate buffer of pH 6.8. The pastille was removed after 1 h from *petri* dish and excess water present on surface was removed using blotting paper. The swollen pastilles were then reweighed (W_2) and swelling index (SI), erosion were calculated using formula given in below equation (Chinna Reddy et al., 2011a; Shivanand et al., 2011; Vishnu et al., 2007).

Moisture Absorption Study

The moisture absorption study indicates the relative moisture uptake of the polymers used in the pastilles formulation and also the integrity of pastilles after absorption of moisture. Moisture absorption studies have been performed using 5% w/v agar in distilled water, which while hot was transferred to *petri* plates and allowed to solidify (Khurana et al., 2000). Then pastille was weighed and placed in desiccators overnight prior to the study to remove moisture and placed on the surface of agar plate for 2 h. The pastilles were weighed again and percentage of absorbed moisture was calculated using the formula.

$$\% \text{ Moisture Absorbed} = \frac{W_2 - W_1}{W_1} \times 100$$

Where, W1 = initial weight of the pastille W2 = final weight of the pastille

Study of pH

One pastille was taken and dissolved in 100ml of 37 °C distilled water. The pH was measured by using pH meter and allowed it to equilibrate for one minute (Bottenberg et al., 1991).

Bio Adhesion Time

The mucoadhesion time was studied after application of the pastille on freshly cut porcine buccal mucosa. The fresh porcine buccal mucosa was tied on the glass slide. One side of the pastille was wetted with 1 drop of phosphate buffer pH 6.8 and placed on the porcine buccal mucosa by applying a light force with a fingertip for 30 seconds.The glass slide was then put in the beaker, which was filled with 200 ml of the phosphate buffer pH 6.8 under magnetic stirrer at 300 rpm to maintain homogeneous condition. The time was recorded as mucoadhesion time to detach the pastille from porcine buccal mucosa (Vishnu et al., 2007).

Bioadhesive Strength

Bioadhesive strength of the pastille was measured on the "modified physical balance method" (Ramana et al., 2007) using porcine buccal membrane as the model mucosal membrane. The fresh porcine buccal mucosa was cut and washed with phosphate buffer pH 6.8. A piece of mucosa was tied to the glass slide which was moistened withphosphate buffer pH 6.8. The pastille was stuck to the lower side of another glass slide with glue. The both pans were balanced by adding an appropriate weight on the left hand pan. The glass slide with mucosa was placed with appropriate support, so that the pastille touches the mucosa. Weights were added slowly on the right hand pan until the pastille detach from the mucosal surface. The weight required to detach the pastille from the mucosal surface gave the bioadhesive strength. The experiment was performed in triplicate and average value was calculated (Ramana et al., 2007).

***In vitro* Drug Release Studies**

The *in vitro* drug dissolution studies were performed by using USP dissolution test apparatus II (paddle method). The dissolution test was conducted in 500 ml of phosphate buffer pH 7.4 as dissolution media (United States Pharmacopoeia, 2007). The speed of the paddle was 75 rpm. The temperature was main 37±0.5 ºC. One pastille was added in a dissolution medium. The aliquots of 5 ml were withdrawn at the time interval of 10, 20, 30, 45 and 60 minutes and replaced with equal volume of fresh dissolution medium. The samples were suitable diluted and analysed for amount of drug at 475 nm in a UV-visible spectrophotometer. The percentage drug release at various time intervals was calculated. The study was conducted in triplicate.

Fourier Transform Infrared Spectroscopy

The FTIR spectroscopic studies were carried for pure drug (rifampicin), soya lecithin, cholesterol, gelatin, carbopol, liposomal dispersion and pastille formulation using FTIR spectrophotometer (M/s. BRUKER, Model Alpha). Potassium bromide pelted method was used for all solid samples and nuzol method was used for all liquid and semisolid samples. In KBr pellet method sample preparation includes grinding a small quantity of the sample with a potassium bromide which helps to remove scattering effects from large crystals. The powder mixture was then crushed in a mechanical die press to form a translucent pellet through which the beam of the spectrometer willpass. The pressed sample was carefully removed from die and was placed in the FTIR sample holder. In nuzol method the sample was triturated with liquid paraffin. The mixture was applied between two sodium chloride cells and was placed in the FTIR sample holder. The IR spectrum was recorded from 4000 cm^{-1} to 400 cm^{-1}.

Stability Studies of Liposomal Pastilles on Storage

The stability of FP-8 monitored and compared for best formulation at 2-8 °C and 25 °C/60% RH for six months. Physicochemical properties of pastilles were determined during the stability studies.

RESULTS AND DISCUSSION

Table 2: Calibration Data for the Estimation of Rifampicin by UV.

Concentration (µg/ml)	e ± SD at 475nm(n=3)
10	0.157
20	0.322
30	0.458
40	0.621
50	0.779

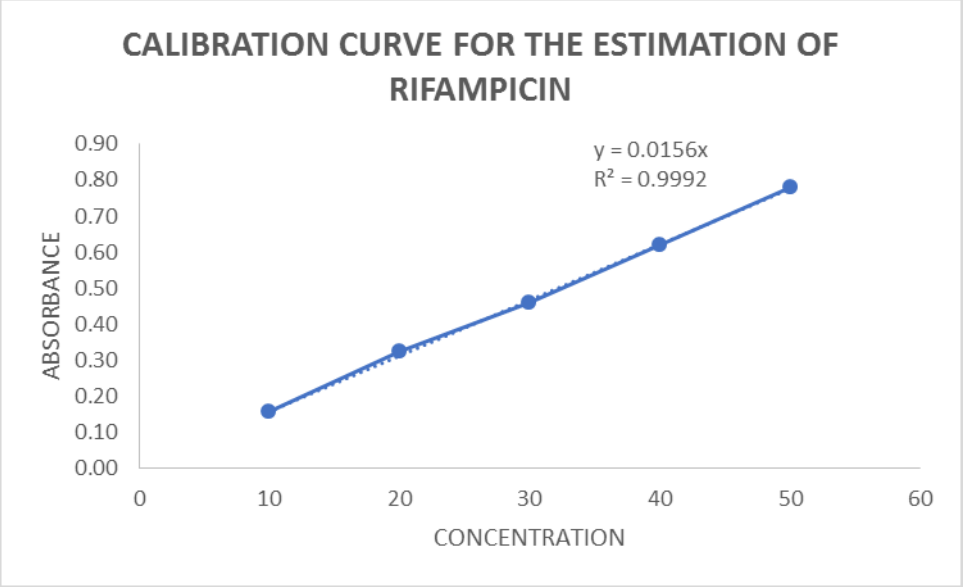

Figure 2: Calibration Curve for the Estimation Rifampicin by UV.

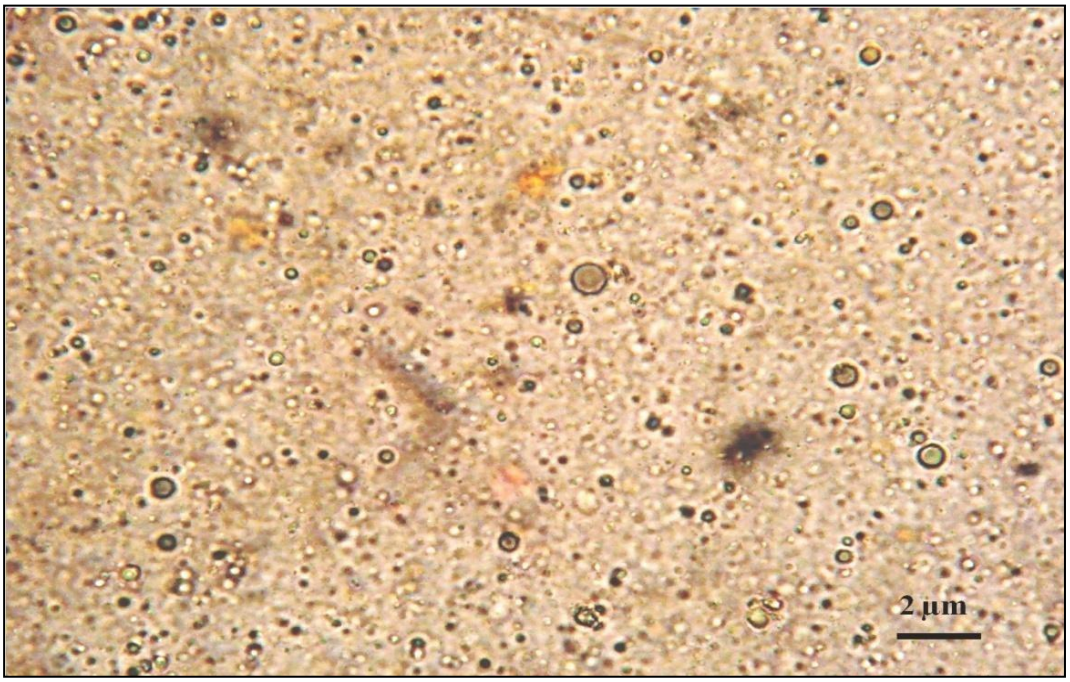

Figure 3: Optical Microscopic Image of F-8.

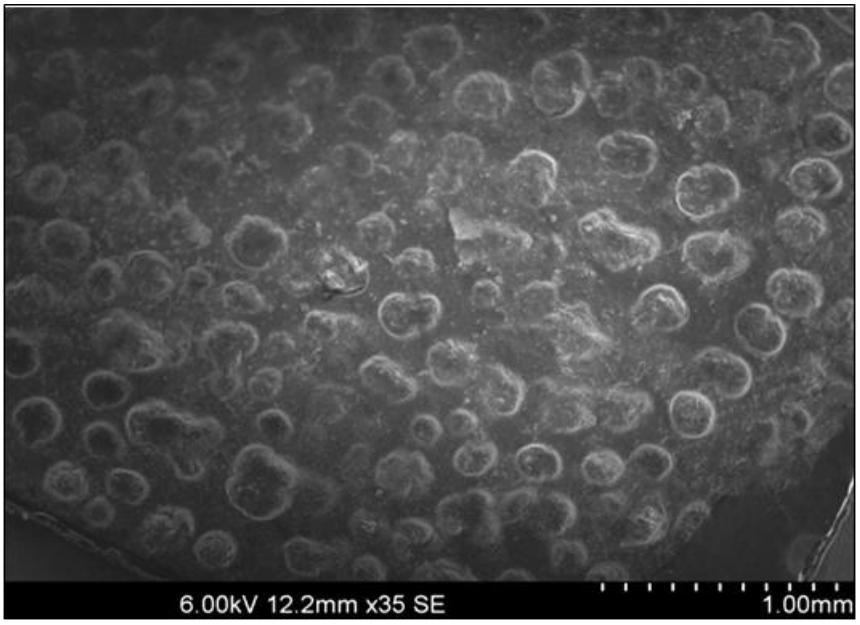

Figure 4: Scanning Electron Microscopic Image of F-8.

Table 3: Physicochemical Parameters of Different Rifampicin LiposomalFormulations.

Formulation	ParticleSize (nm)	Drug Content (%±SD)	EntrapmentEfficiency (%±SD)	*In vitro*drug release (%w/w±SD)
F-1	302	92.89±0.3	58.7±0.6	54.2±0.9
F-2	321	93.44±0.2	64.2±0.5	52.3±0.5
F-3	247	94.26±0.1	68.5±0.5	51.5±0.8
F-4	269	92.99±0.5	64.2±0.6	55.8±0.6
F-5	325	92.22±0.7	68.2±0.9	56.2±0.7
F-6	236	96.81±0.6	70.4±0.5	61.0±0.7
F-7	244	93.12±0.5	67.1±0.7	59.5±0.7
F-8	272	98.94±0.4	72.1±0.5	66.0±0.6
F-9	255	95.41±0.3	70.8±0.5	63.9±0.8

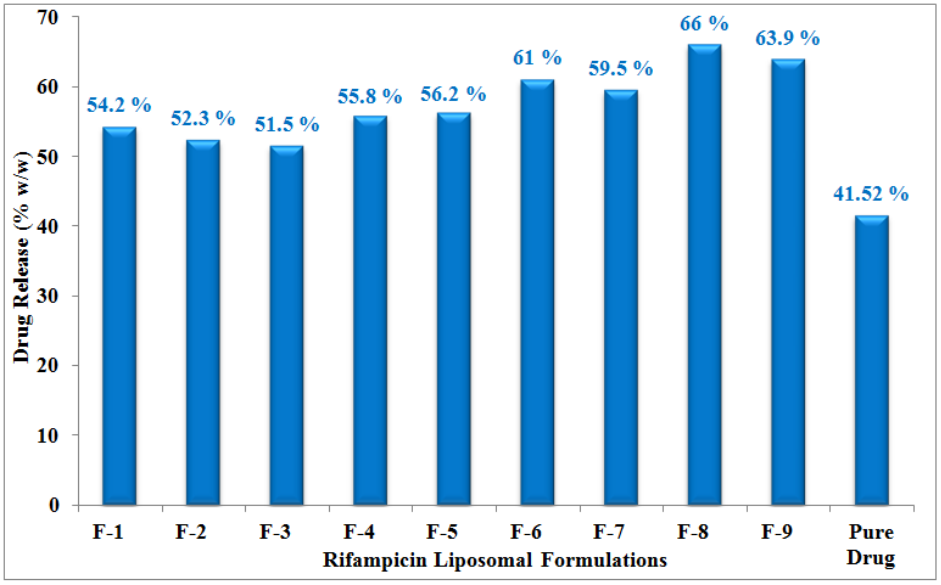

Figure 4.8: Comparative *In Vitro* Drug Diffusion Profiles of RifampicinLiposomes.

Table 4: Statistical Data for Physicochemical Parameters of RifampicinLiposomes.

Parameter	Particle Size	Entrapment Efficiency (EE)	*In Vitro* Percentage drug release(DM)	*Ex Vivo* Percentage drug release(PBM)
Model	Quadratic	Quadratic	Linear	Linear
ANOVA (p<0.05)	0.0273 (Significant)	0.0096 (Significant)	0.0047 (Significant)	0.0019 (Significant)
Std. Dev.	14.95	0.97	2.39	2.19
Mean	274.56	67.17	57.87	62.49
C.V. %	5.45	1.45	4.14	3.50
PRESS	4168.14	32.04	88.26	77.00
R-Squared	0.9012	0.9798	0.8329	0.8765
Adj R-Squared	0.8025	0.9462	0.7771	0.8354
Pred R-Squared	0.5396	0.7728	0.5709	0.6693
Adeq Precision	7.523	16.127	9.208	11.655

Table 5: Relative Error Data for Various Physicochemical Properties ofRifampicin Liposomal Formulations.

Formulations		F-1	F-2	F-3	F-4	F-5	F-6	F-7	F-8	F-9
Experimental Results	ParticleSize	302	321	247	269	325	236	244	272	255
	Entrapment Efficiency	58.71	64.27	68.54	64.22	68.12	70.41	67.11	72.19	70.89
	*In Vitro*Drug Release	54.24	52.36	51.57	55.85	56.25	61.04	59.56	66.03	63.90
	*Ex Vitro*Drug Release	57.53	55.73	56.58	59.76	63.37	66.35	63.80	70.17	69.10
PredictedResults	ParticleSize	305	323	246	272	306	246	239	290	246
	Entrapment Efficiency	58.51	64.88	68.14	63.77	68.62	70.37	67.76	71.10	71.33
	*In Vitro*Drug Release	51.50	52.65	53.79	52.72	57.87	59.01	61.94	63.09	64.23
	*Ex Vitro*Drug Release	55.12	56.94	58.77	60.66	62.48	64.31	66.20	68.02	69.85
Relative Error (%)	ParticleSize	0.87	0.46	0.40	0.98	6.20	4.06	2.23	6.04	03.65
	Entrapment Efficiency	0.34	0.92	0.59	0.70	0.72	0.06	0.95	1.54	00.62
	*In Vitro*Drug Release	5.31	0.54	4.12	5.92	2.78	3.43	3.84	4.66	01.06
	*Ex Vitro*Drug Release	4.36	2.14	3.73	1.49	1.41	3.16	3.62	3.14	01.06

Table6: Physicochemical Parameters of FP-8 and PRP.

Sl. No.	Evaluation Test Parameters	FP-8	PRP
1.	Diameter (mm ± SD)	15±0.2	15.9±0.17
2.	Thickness (mm ± SD)	5±0.2	4.8±0.08
3.	Average Weight (g ± SD)	1.5±0.05	1.5±0.10
4.	Drug content (% ± SD)	99.0±1.15	98.6±1.00
5.	Swelling Index (% ± SD)	4.5±0.29	5±0.59
6.	Erosion (% ± SD)	28.0±0.89	26.5±0.95
7.	Moisture absorption (% ± SD)	2.95±0.15	2.65±0.27
8.	pH ± SD	6.2±0.06	6.5±0.10
9.	Bio adhesion time (h ± SD)	1.5±0.29	1.5±0.23
10.	Bio adhesive strength(g±SD)	20±1.5	15±1.2

Table 7: *In Vitro* Drug Release Data of FP-8 and PRP.

Time (Min)	Cumulative Percentage Drug Release(%w/w ± SD)	
	FP-8	PRP
10	55.7±1.44	25.5±1.50
20	73.5±1.45	42.2±1.33
30	84.5±1.56	58.9±1.39
45	92.5±1.26	71.4±1.47
60	100.1±1.23	82.8±1.29

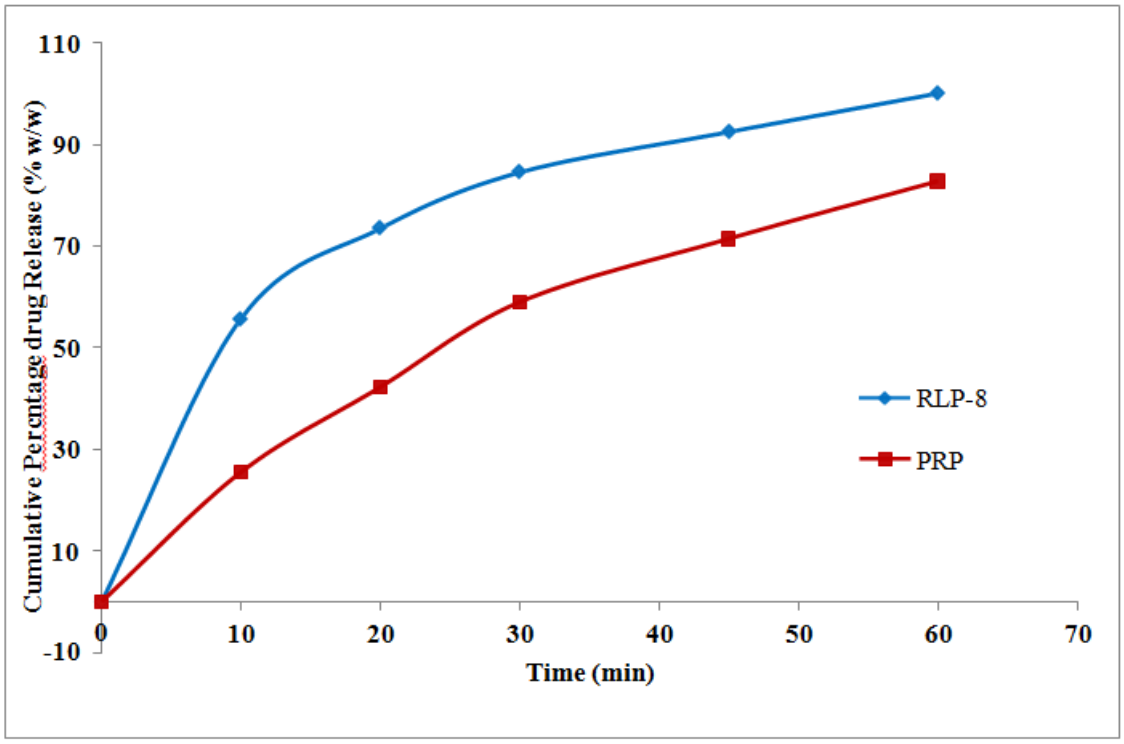

Figure: *In Vitro* Drug Release Profiles of Rifampicin Liposomal Pastilles.

Table 8: Stability studies of the FP-8 and PRP.

S. No	Parameters	Stability at2-8 °C (after 6 Months)	Stability at 25 °C, 60% RH (after 6 Months)
1.	Physical appearance	No Change	No Change
2.	Diameter (mm ± SD)	15±0.2	15.2±0.18
3.	Thickness (mm ± SD)	4.9±0.17	4.8±0.18
4.	Average Weight (g ± SD)	1.5±0.06	1.4±0.11
5.	Drug content (% ± SD)	98.6±1.19	97.6±1.11

DISCUSSION

Rifampicin calibration data and calibration curve were shown in Table 4.3 and Figure 4.3 respectively. The data indicated that rifampicin was found to be linear in concentration range of 0–50 µg/ml. The regression coefficient (R2) value is 0.9994 and the regression equation is y = 0.0155x - 0.002, which shows that the method obeyed Beer lamberts law. The following linear regression equation used for rifampicin estimation of in vitro samples.

All the liposomal formulations were examined under phase contrast optical microscope (100 X magnification) (Danilo, 1997; Yekta, 2007) after the preparation to observe the vesicle formation. The microscopic image of liposomal formulation F-8 shown in Figure 4.6 indicated uniform and discrete vesicles. The vesicles are lamellar and spherical.

Scanning Electron Microscopy (SEM).

The morphology of F-8 was studied by using scanning electron microscopy and the image is shown in Figure 4.7. The SEM image indicated that the liposomal formulation F-8 has spherical smooth regular surface.

Particle Size Determination

The particle size distribution of rifampicin liposomes is shown in Table 4.6. The mean particle size of all the

liposomes formulation were found to be in the range of 236 nm to 325 nm. The results indicated as the concentration of lecithin:cholesterol weight ration increases the vesicle size decreased. Polydispersity index of the liposome vesicles for F-8 was found to be 0.439. Small values of P.I. (< 0.3) indicate a homogenous population, whereas high P.I. (> 0.3) is heterogeneity (Sentjurc et al., 1999; Centisa and Vermettea, 2008). The results indicated uniform size of the vesicles in the dispersion which may be due to the membrane stabilizing effect of cholesterol (Gorner et al., 1999; Douglas et al., 1984). The zeta potential for F-8 was found to be 38.1 mV. The results indicate good dispersion stability as the value is less than 50 mv.

Drug Entrapment Efficiency

The entrapment efficiency (Table 4.6) of formulations F-1 to F-9 was found to be in range 58.7±0.6% to 72.1±0.5%. Among the liposome formulations F-8, prepared by using 15:4 ratio of soya lecithin: cholesterol weight ratio showed highest entrapment efficiency of 72.1±0.5%, where as formulation F-1 prepared by using 5:3 ratio (soya lecithin: cholesterol) showed lowest percentage entrapment efficiency of 58.7±0.6%. A proportional relation was observed between the entrapment efficiency of rifampicin and the size of vesicle. Increase in phospholipid and cholesterol content resulted in significant improvement in entrapment efficiency. The results are in agreement with eaFier reports showed on increasing cholesterol the stability and drug entrapment efficiency in liposomes was increased (Gorner et al., 1999; Touitou et al., 2000).

In Vitro Diffusion Studies on Prepared Liposomes

In vitro diffusion studies of liposomes were carried using dialysis membrane for 24 h and the cumulative percent rifampicin release values are shown in Table 4.6 and Figure 4.8. The results indicated slow and continuous release of rifampicin from liposomes over a period of 24 h when compared to pure drug. The pure drug showed faster drug release of 34.8% w/w within 6 h. Following that the release became slow and stagnant and only 41.5 % release shown in 24 h. The liposome formulation showed slow and gradual release in the range of 10.1% w/w (F-1) to 19.9% w/w (F-9) in first 6 h and the drug release of 51.5% w/w (F-3) to 66.0% w/w (F-8) in 24 h. Among the prepared liposome formulations F-8 showed maximum drug release of 66.0% w/w. The slow and continuous release of rifampicin, which exhibited biphasic release pattern, may be due to gradual diffusion of free drug and encapsulated drug from the liposomal membrane through dialysis membrane (Deol et al., 1997).

Statistical Analysis for Physicochemical Parameters of Rifampicin Liposomes

Nine rifampicin liposomes formulations prepared using different ratios of soya lecithin and cholesterol as per the experimental design obtained from Design Expert® 9 software. The liposomes were evaluated for particle size, entrapment efficiency (EE) and percentage drug release at 24 hrs. All responses were fitted to linear and quadratic models by using Design Expert® 9 software. Among them quadratic model is selected for particle size, entrapment efficiency (EE) and a linear model is selected for percentage drug release (with both dialysis and porcine buccal mucosa) by using the Design Expert® 9 software. The "*P* value" for particle size, entrapment efficiency (EE) and percentage drug release at 24 hrs (with both dialysis and porcine buccal mucosa) were found to be 0.0273, 0.0096, 0.0047 & 0.0019 for all four responses and were less than 0.05 indicating that all four models are significant (Table 4.7).

The calculated R2 value in the present models for all four responses was close to 1 indicating a good model. In all cases, the adjusted R2 values are in reasonable agreement with the predicted R2 values (0.8025 and 0.5396 for particle size, 0.9462 and 0.7728 for entrapment efficiency, 0.7771 and 0.5709 for percentage drug release using DM, 0.8354 and 0.6693 for percentage drug release using PBM). "Adeq Precision" measures the signal to noise ratio and a ratio greater than 4 was desirable. In all the cases precision values for four responses were found to be 7.523, 16.127,9.208 & 11.655 indicating an adequate signal and that the model can be used to navigate within the design space (Table 4.7). The application of response surface methodology yielded the following regression equations (A: Soya Lecithin; B: Cholesterol).

Particle Size = +306.00 − 16.50A − 12.83B + 16.50AB − 47.17B2

Entrapment efficiency = +68.62 + 3.11A + 3.30B − 1.51AB − 0.63A2 − 1.55B2

Percentage drug release (DM) = +57.87 + 5.22A + 1.14B

Percentage drug release (PBM) = +62.49 + 5.54A + 1.82B

The statistically predicted results are compared with experimental results for all liposomal formulations with three different responses (Particle size determination, entrapment efficiency and diffusion studies). The observed responses for all formulations showed neaFy close with the model predictions (Table 4.8). The relative errors (%) between the predicted and experimental values for each response were calculated and the values for maximum responses were found to be within 5%. The experimental values were in reasonable agreement with the predicted values confirming the predictability and validity of the model. The response surface plots for all responses of all formulation factors are shown in Figure 4.10. The response surface plots showed that at lower levels of lecithin and cholesterol the particle size was found to be small and at higher levels of lecithin and cholesterol the drug entrapment was found to be more. The response surface plots of percentage drug release also showed similar manner to entrapment efficiency. Higher levels of lecithin and cholesterol showed highest amount of drug release for in vitro and ex vivo diffusion studies.

Stability Studies of Liposomes on Storage

Physical stability for formulation is important parameter to be considered to have effective therapeutic action. The results showed rifampicin liposomal dispersions were physically stable and displayed better stability after being kept at 2-8 °C for three months. Significantly there is no change in physical appearance was seen during stability study for liposomal dispersion at 2-8 °C. But displayed poor physical stability after being kept at room temperature (25 °C) and observed separation of dispersion after one month of storage.

Evaluation Tests for Rifampicin Liposomal Pastilles

Dimensions of Pastilles

The results of dimensions for FP-8 and PRP are shown in Table 4.9. The diameter and thickness for rifampicin liposomal pastilles formulation (FP-8) were found to be 15 ± 0.2 mm and 5 ± 0.2mm respectively. The results indicated uniformity of size and shape of pastilles

Average Weight and Weight Variation

The results of average weight and weight variation for FP-8 and PRP were shown in Table 4.9. The average weight for FP-8 and PRP was found to be 1.5±0.05 g and 1.3±0.10 g.

Drug Content Estimation

The results of drug content for FP-8 and PRP was shown in Table 4.9 and found to be 99.0±1.15% w/w and 98.6±1.00.

Swelling and Erosion Studies

The results for swelling and erosion studies of FP-8 and PRP were shown in Table 4.9. The swelling index for FP-8 and PRP was found to be 4.5±0.29 % w/w and 5±0.59 % w/w. The percentage mass loss for FP-8 and PRP after swelling was found to be 28.0±0.89% and 26.5±0.95% in 2 h.

Study of pH

The pH for FP-8 and PRP in distilled water was found to be 6.2 and 6.5 (shown in Table 4.9).

Moisture Absorption, Bio-adhesive Strength and Bio-adhesion Time

The results for moisture absorption, bio-adhesion time and bio-adhesive strength were shown in Table 4.9. Moisture absorption for FP-8 and PRP was found to be 2.95±0.15% and 2.65±0.27 in 2 h. The bio-adhesion time to porcine buccal membrane for FP-8 and PRP was found to be 1.5 h. The bio-adhesive strength observed to detach FP-8 and PRP from glass slide was found to be 20 g and 15 g.

In vitro Drug Release Studies

The in vitro dissolution studies performed by using USP dissolution test apparatus II (paddle method) for 1 h and the cumulative percent rifampicin release from FP-8 and PRP are shown in Table 4.10 and Figure 4.11. The results indicated complete release of rifampicin from liposomal pastilles in one hour. The in vitro drug release for FP-8 was found to be 100.1% in one hour. The rifampicin release from pure rifampicin pastilles (PRP) was 82.8% w/w in one hour.

Stability Studies of Liposomal Pastilles

Rifampicin liposomal pastilles FP-8 displayed good physical stability after six months storage at 2-8 °C and room temperature. Results are shown in Table 4.11 and indicated no change in physical appearance, diameter, thickness, average weight and drug content during stability study at 2-8 °C and room temperature for 6 months. The results indicated that storage of liposomal pastilles at 2-8 °C is a suitable condition for long term stability.

SUMMARY AND CONCLUSION

Rifampicin liposomes (F) were prepared by solvent injection method using different ratios of soya lecithin and Cholesterol. Characterization of liposomes was done by using optical microscopy, scanning electron microscopy and particle size distribution. Evaluation for liposomes are done by drug entrapment efficiency, in vitro diffusion studies, ex vivo diffusion studies, statistical analysis, optimization, validation of the experimental design. Stability studies were done for liposomes on storage at 2-8 °C and room temperature. The prepared liposomes were found to be uniform and discrete. The mean particle size of all the liposomes formulation was found to be in the range of 236 nm to 325 nm. Among the prepared liposomal formulations, F-8 prepared by using 15:4 ratio of soya lecithin and cholesterol showed highest entrapment efficiency of 72.1±0.5%. In vitro and ex vivo diffusion studies of liposomes were carried using dialysis membrane and porcine buccal mucosa. The results indicated slow and gradual release of rifampicin from liposomes over a period of 24 h when compared to pure drug. Among the formulations F-8 prepared with soya lecithin and cholesterol weight ratio of 15:4 has showed gradual and highest in vitro and ex vivo percentage of drug release of 66.0% w/w and 70.1% w/w respectively. Pure rifampicin showed in vitro and ex vivo percentage of drug release of 41.5 % w/w and 47.5% w/w respectively.

All responses were fitted quadratic and linear models by using Design Expert® 9 software. The p-values for all responses were found to be less than 0.05 indicating that all four models are significant. The response surface plots showed entrapment efficiency was high at higher levels of lecithin & cholesterol. From the results of physicochemical parameters, rifampicin liposomal dispersion F-8 was selected as optimized formulation. Further F-8 was formulated into pharmaceutical pastilles dosage forms for buccal drug delivery. Evaluation for FP-8 was done by average weight, drug content, swelling, erosion, moisture absorption, pH, bio adhesion time, bio adhesive strength, in vitro drug release, stability studies and in vivo pharmacokinetic studies using rabbit model. Rifampicin liposomal pastilles FP-8 are uniform in size, flexible and found to have uniform drug content. The complete drug release from pastilles was observed.

Rifampicin liposomal pastilles (FP-8) displayed good physical stability at 2-8 °C and indicate that storage of liposomal pastilles at 2-8 °C is a suitable condition for long term stability.

CONCLUSION

From the results of physicochemical parameters, rifampicin liposomal dispersion F-8 was selected as optimized formulation. Further F-8 was formulated into pharmaceutical pastilles dosage forms for buccal drug delivery. Evaluation for FP-8 was done by average weight, drug content, swelling, erosion, moisture absorption, pH, bio adhesion time, bio adhesive strength, in vitro drug release, stability studies and in vivo pharmacokinetic studies using rabbit model. Rifampicin liposomal pastilles FP-8 are uniform in size, flexible and found to have uniform drug content

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