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Formulation and evaluation of coencapsulated rifampicin and isoniazid liposomes using different lipids

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Abstract

An attempt has been made to design suitable co encapsulated liposomal drug delivery system for rifampicin and isoniazid and for the evaluation of these systems *in vitro* and *in vivo*. A modified lipid layer hydration method was employed to prepare these vesicular carriers. The formulated systems were characterized for size distribution analysis, drug entrapment, drug release profiles and vesicular stability carried out. *In vivo* drug kinetics and histopathology study were carried out on normal healthy albino rats. For co encapsulated liposome of rifampicin and isoniazid various pharmacokinetic parameters were determined. Coencpsulated liposomal formulations elevated plasma elimination half life and decreased elimination rate constants for rifampicin and isoniazid, *in vivo* studies suggested that co encapsulation retard the release of drug from circulation compared to free drug due to slow drug release into systemic circulation. A three fold increase in the area under plasma rifampicin and isoniazid concentration-time curve for encapsulated drug. This formulation also reduces the accumulation of drug in the liver kidney and lungs. It is evident from this study that liposomes could be promising delivery systems for rifampicin and isoniazid with prolonged drug release profiles and reasonably good stability characteristics.

Key words: Liposome, rifampicin, isoniazid, tuberculosis.

Introduction

Tuberculosis (TB) is a chronic communicable disease caused by the bacterium *Mycobacterium tuberculosis* that infects over 1.8 billion people world wide and is responsible for 1.5 million deaths annually (Facci 2001). Although an effective therapeutic regimen is available, patient non-compliance (because of need to take tubercular drugs (ATDS) daily or several times a week) results in treatment failure as well as improved by the use of ATD formulations, which reduce the dosing frequency of the drugs. For this purpose, ATDs encapsulated with microspheres and liposomes have been successfully used as injectable preparations in experimental tuberculosis models (Deol and Khuller 1997). Liposomes are simple microscopic vesicles composed of relatively biocompatible and biodegradable material and they consist of an aqueous volume entrapped by one or more bilayer of natural and/ or synthetic lipids. Drugs with widely varying lipophilicities can be encapsulated. Liposomal entrapment has considerable effect on the pharmacokinetic and tissue distribution of the administered drugs. With respect to normal tissues, liposomal encapsulation of most drugs resulted in reduced accumulation in the intestinal wall, kidney and cardiac muscles. It is likely to reduce the drug uptake in these selected areas by means of liposomal entrapment, which may be a useful means for decreasing the systemic toxicity of drugs. Rifampicin and Isoniazid are the most effective drugs for the treatment of TB. Rifampicin, like Isoniazid should never be used alone for this disease, because of the rapidity with which resistance may develop.

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The combination of Rifampicin and Isoniazid is probably as effective, for sensitive microorganism, as regimens that utilize three or more agents (Goodman and Gillman 1985). In the present study rifampicin and isoniazid are co-encapsulated in the same liposomal formulation. All liposome formulations were compared for encapsulation percentage, release properties, *in vivo* drug release and toxicity studies.

Method and Materials

Materials

Rifampicin and Isoniazid obtained as gift sample from Zydus cadila Ahmedabad,India, Diplamitoylphosphatidylcholine (DPPC), Eggphosphatidylcholine (EPC), cholesterol, obtained from Sigma Chemicals, USA. All the other chemicals and reagent were of analytical grade.

Preparation of co encapsulated of rifampicin and isoniazid liposomes by hand shaking method.

The liposomes were prepared using the hand shaking method (lipid layer hydration) as described by Azmin et al. (1985). Rifampicin was dissolved in few drops of chloroform in a round bottom flask. Eggphosphatidylcholine (EPC) / Diplamitoylphosphatidylcholine (DPPC), and cholesterol were dissolved in diethyl ether in a 100 mL round bottom flask. The organic solvent was removed by rotating the flask at a temperature of 37°C under reduced pressure. The slight trace of the solvent was removed with oxygen-free nitrogen for about 10 min. The dried lipid film was then hydrated using PBS pH (7.4), containing isoniazid for about 10 min using vortex mixer to get uniform suspension.

Size distribution analysis

The known amount of drug was dispersed in purified water dispersed using spinix shaker and this suspension was used for particle size analysis was carried out by using Horiba light scattering particle size analyzer (Horiba Ltd, Japan).

Entrapment efficiency liposomes

Unentrapped rifampicin and isoniazid was separated from liposomes by gel filtration on sephadex G- 50 powder kept in double distilled water for 48 h for swelling. One ml of prepared liposome suspension was placed on the top of the column and elution was carried out using normal saline. Liposomes encapsulated elutes out first as a slightly dense, white opalescent suspension followed by free drug. Vesicles were ruptured using triton-x100 and drug amount of the was measured by using HPLC. Unentapped drug amount was also determined using HPLC.

HPLC Method for estimation of Rifampicin

HPLC system (Milford, USA) with 2695 separations module with auto sampler and column oven was used. Kromasil C18 (250 nm x 4.6 mm i.d μ) column was used as stationary phase and acetonitrile: 20 mM disodium hydrogen orthophosphate (pH adjusted to 3.5 with phosphoric acid) in the ratio of 40:60 v/v as mobile phase. The mobile phase was filtered through 0.45μm membrane filter (Sartorius, Germany) and degassed before analysis. The flow rate was 1mL/min and the column effluent was monitored at 254nm.

HPLC Method for estimation of Isoniazid

Waters Alliance HPLC system (Milford, USA) with 2695 separations module with auto sampler and column oven was used. Kromasil C18 (250 nm x 4.6 mm i.d 5 μ) column was used as stationary phase and Buffer: Acetonitrile. Buffer (20 mM ammonium acetate, pH adjusted to 6.0 with acetic acid) in the ratio of 99:1 v/v as mobile phase. The mobile phase was filtered through a 0.45 μm membrane filter (Sartorius, Germany) and degassed before analysis. The flow rate was 1ml/min. and the column effluent was monitored at 280nm.

In vitro release studies coencapsulated rifampicin and isoniazid liposomes

The liposomes that were separated as mentioned were resuspended in PBS pH (7.4) and filled in a dialysis tube to which a sigma dialysis sac was attached to one end. The dialysis tube was suspended in PBS pH 7.4, stirred with a magnetic stirrer and samples were withdrawn at specific time intervals and

analyzed using HPLC. To maintain a constant volume, an amount of medium equivalent to the volume of sample withdrawn was added.

Stability studies

Coencapsulated rifampicin and isoniazid liposomes

The formulation containing only entrapped drug (unentrapped drug was removed as mentioned above) was divided in to equal portions. Each portion was filled in to amber colored ampoules and sealed with nitrogen atmosphere. The formulation were kept for both long term stability studies at 5±3°C for one year and accelerated stability at 30±2 °C and 65±5% RH. The samples were withdrawn at predetermined time intervals was determined. At the end of the study period the vesicles were examined for change in size under the microscope.

In vivo evaluation

Animals

The albino rats obtained from animal housing facility, Department of pharmacology, Kasturba Medical College, MAHE, Manipal. (An approved and registered facility under CPCSEA 1998; Registration number 94/CPCSEA/1998). The animals were maintained under the controlled conditions of temperature and humidity in propylene cages filled with sterile paddy husk. They were fed with balanced diet (obtained from Lipton India Ltd.for animals use) and water ad libitum.

Pharmacokinetics evaluation

The twenty animals were taken and divided in to two groups containing ten animals each. Group I is reserved as control and Group II with 12 mg/kg rifampicin and 10mg/kg isoniazid body weight of drug loaded liposomes.Before injecting to the animals, prepared liposomes were screened using 5µm membrane filters (Millipore, India Ltd) to obtain liposomes < 5µm in the size. The suspension was injected intravenously (through tail vein). The blood samples were collected periodically by puncturing of retro orbital plexus using heparinzed capillaries and collected in heparinzed centrifugation tubes with aseptic precautions. Plasma was separated by centrifugation and stored at-20°C in vials prior to the analysis (Jain et al.1995).

Calibration curve of rifampicin in plasma

Blank plasma of 60 µL was spiked with 40 µL of standard solutions of rifampicin (10 µg/mL) in a micro centrifuge and 1 mL of menthol was added. The tubes were vortexed and then centrifuged at 4000 rpm for 5 min, after centrifugation, aliquot of 20 µL supernatant solutions injected to HPLC. Procedure was repeated to prepare resultant concentrations of 2-20 µg/mL.

Estimation of rifampicin from the plasma samples

A plasma sample (100 µL) was taken in a micro centrifuged tubes and 1mL of methanol was added. The tubes were vortexed, then centrifuged at 4000 rpm for 5 min. After centrifugation, an aliquot 20 µL supernatant solution was injected to HPLC. Same procedure was repeated for 30 min, 1, 2, 3, 4, 6, 8, 10, and 24 h. Pharmacokinetic data were obtained by using PK Solution 2 non compartmental software.

Calibration curve of isoniazid in plasma

A blank plasma of 60 µL was spiked with 40 µL of standard solutions of Isoniazid (10 µg/mL), 20 µL nicotinamide (µg/mL), 200 µL of acetonitrile was taken in a micro centrifuge. To this 100 µL of methanol and 700 µL of buffer were added. The tubes were vortexed and then centrifuged at 4000 rpm for 5 min. After centrifugation, aliquot of 20 µL supernatant solution injected to HPLC. Procedure was repeated to prepare resultant concentrations of 2-20 µg/mL.

Estimation of Isoniazid from the plasma samples.

A plasma sample (100 µL) was taken in a micro centrifuged tubes and 20 µL Nicotinamide (20 µg/mL), 200 µL of acetonitrile was taken in a micro centrifuge. To this 100 µL of methanol and 700 µL of buffer was added. The tubes were vortexed and then centrifuged at 4000 rpm for 5 minutes. After centrifugation, an aliquot 20 µL supernatant solution was injected to HPLC. Same procedure was

repeated for 30 minutes, 1, 2, 3, 4, 6, 8, 10, and 24 h. Pharmacokinetic data were obtained by using PK solution 2 non compartmental software.

Histopathology study

Duration of this study was one month. 30 male albino rats weighing, 200-250 g were taken for the study. The rats were divided in to three groups. The first group served as control. Second group received the marketed oral dosage form as per protocol human dosex5x0.018 mg/kg the formulation of co encapsulated Rifampicin (12 mg/kg) and Isoniazid (10 mg/kg) injected for group 3. Animals were sacrificed at the end of fourth week and vital organs (liver, kidney) were isolated. Histopathological investigations were carried out on the isolated tissue samples and any abnormal changes in the tissues were reported.

Results and Discussion

Size of the vesicles

In this study we observed that size of the vesicles varied for coencapsulated rifampicin and isoniazid Table 1. The method used for the preparation of the liposomes give large multilamelar vesicles with mean diameter and particle size distribution depending on the type of the lipid used. It was found that as the acyl chain increases the liposomal size decreases. It was also clear that the presence of CHOL did not influence the particle size of the liposomes, but it gave a lower particle size indicating more ordered structure (Al-Angary et al.1996).

Table1. Characterization of liposomal vesicles containing isoniazid.

Influence of phospholipids and cholesterol on liposomal entrapment efficiency

Drug	Lipid composition	Molar ratio	Entrapment Efficiency (%)	Particle Size
Coencapsulated Rifampicin and Isoniazid EPC:	EPC: CHOL	50:50	68.25 and 57.85	3.165±0.002
	CHOL	40:60	69.23 and 59.36	2.895±0.001
	DPPC: CHOL	50:50	70.92 and 61.11	2.8075±0.001
	DPPC: CHOL	40:60	72.98 and 63.22	2.7452± 0.005

Drug entrapment

In the study the entrapment efficiency of coencapsulated rifampicin and isoniazid liposomes was as shown in the Table 1. The entrapment increased in drug encapsulated with lipids like DPPC: CHOL than lipids like EPC: CHOL. This is due to increase in the acyl chain length of phospholipids (Puglisi et al.1995, Al-Angary et al. 1996). Also, from this study we found that entrapment of rifampicin encapsulated with DPPC: CHOL is higher than isoniazid encapsulated with DPPC: CHOL. This is due to lipophilic and hydrophilic nature of the drug molecules. In this case, the encapsulation of drug is completely dependent upon the volume of aqueous phase encapsulated during liposome formation. An increase in chain length of fatty acid and inclusion of cholesterol results in an increase in the encapsulation efficiency. This data indicate the importance of molecular structure and liphophilicity with respect to their encapsulation in liposomes. The drug molecules may also influence liposome formation by an ability to bind water molecules and form liquid crystal structures in aqueous solutions (Alliga et al. 1979).

Effect of cholesterol in drug entrapment

From the above it was observed that, as the cholesterol content in the vesicles increased, the incorporation of the drug in the vesicles also increased. Cholesterol is known to increase the

rigidity of the liposomal membrane. This resulted in more rigid liposomes containing cholesterol during the incorporation process. Cholesterol also increases the separation between the choline head groups in the liposomal membrane Table1.

In vitro drug release

In case of *invitro* release kinetics of liposomes 78 % (F2), 70% (F3), 65 %(F4) and 50% (F5) are released for 10h .After that sustained release was observed till 20h. Incase of isoniazid it is 82% (F7), 75 % (F8), 65 % (F9) and 50 % (F10) for 10h. After wards sustained release was observed for till 20 h. The drug release from the rifampicin and isoniazid encapsulated liposomes and coencapsulated rifampicin and isoniazid liposomes using different polymers vary.

This difference may be due to the transition temperature values of phospholipids used to prepare liposomes. Since DPPC has higher transition value than EPC the drug release from DPPC: CHOL liposomes was slower than EPC: CHOL liposomes (Gürsoy et al.2004). It was clear that increase in the length of lipid acyl chain length was accompanied by a decrease in the release rate liposomal preparation. The release rates of rifampicin and isoniazid decreased with increase in transition temperature of liposomes. Cholesterol in bilayers increases the phospholipids transition temperature modulates membrane fluidity by restricting the movement of relatively mobile hydrocarbon chains; this reduces the permeability (Betegeri et al.1992).In general, the release of isoniazid was greater than that of rifampicin. This is due to hydrophilic character of isoniazid, which is a major determinant for leakage. However, the release of rifampicin is controlled by liposome structure due to the lipophilic property of this drug (Betegeri et al.1992).The formulation of rifampicin, isoniazid, combination of rifampicin and isoniazid with DPPC: CHOL (50:50) showed better release than other formulation, because of the presence of cholesterol in the membrane which imparts rigidity to the bilayers.

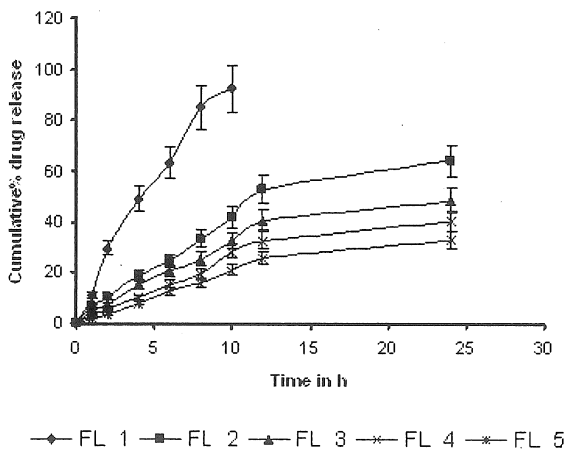


Figure 1. In vitro release profile of rifampicin from coencapsulated rifampicin and isoniazid liposomes and free rifampicin in PBS pH (7.4).

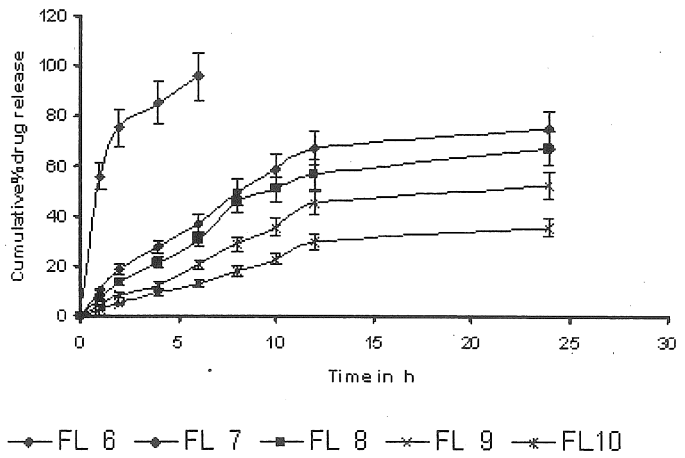


Figure 2. In vitro release profile of isoniazid from coencapsulated rifampicin and isoniazid liposomes and free isoniazid in PBS pH (7.4).

Stability Studies

From the present investigation we found that co encapsulated liposomal preparation were satisfactorily stable up to 6 months at accelerated conditions of 30 ± 2° C and 65 ± 5% RH. The results of this study indicate that the liposomal formulations of rifampicin are expected to be stable for 6 months under shelf conditions. The results of stability studies of rifampicin in the liposomal formulation at 5 ± 3° C are shown in Table 2, 3.

Table 2. Stability studies of rifampicin from coencapsulated of rifampicin and isoniazid liposomal formulation at 30 ± 2° C and 65 ± 5% RH.

Time (Months)	Drug content (%)	Morphology
0	100±0.0	-
1	95.28±0.1	-
2	79.45±0.1	-
3	51.45±0.1	-
4	24.29±0.1	-
5	10.15±0.1	+
6	2.15±0.1	++

Deg Const: 6.17 $x10^{-1}$ $month^{-1}$

- = No change in morphology of the formulation; +=mild change in morphology formulation characteristics (aggregation/discoloration/increased vesicle diameter) ++ = Moderate to severe change in morphology formulation characteristics (aggregation/discoloration/increased vesicle diameter)

Drug content values are expressed as Mean±SD (n=3)

Table 3. Stability studies of rifampicin from coencapsulated of rifampicin and isoniazid liposomal formulation at 5±3°C.

Time (months)	Drug content (%)	Morphology
0	100.00±0.0	-
1	99.98± 0.1	-
2	99.80± 0.1	-
4	99.79± 0.1	-
6	98.72± 0.1	-
8	97.98± 0.1	-
10	97.89± 0.2	-
12	97.53± 0.2	+

The degradation constants for rifampicin from the liposomal formulations were 6.17 $x10^{-1}$ $month^{-1}$ at 30 ± 2° C and 65 ± 5% RH and 2.533 $x10^{-3}$ $month^{-1}$ at 5 ± 3° C for rifampicin. The results of stability studies of isoniazid in the liposomal formulation at 5 ± 3° C are shown in Table 4, 5. The degradation constants for isoniazid from the liposomal formulations were 7.87 $x10^{-1}$ $month^{-1}$ at 30 ± 2° C and 65 ± 5% RH and 2.533 $x10^{-3}$ $month^{-1}$ at 5 ± 3° C for isoniazid.

Table 4. Stability studies of isoniazid from coencapsulated of rifampicin and isoniazid liposomal formulation at 30 ± 2° C and 60 ± 5% RH.

Time (Months)	Drug content (%)	Morphology
0	100.00±0.0	-
1	93.18±0.1	-
2	65.45±0.1	
3	39.52±0.1	-
4	22.23±0.1	-
5	6.15±0.1	+
6	1.18±0.1	++

Table 5. Stability studies of isoniazid from coencapsulated of rifampicin and isoniazid liposomal formulation at 5 ± 3° C.

Time (Months)	Drug content (%)	Morphology
0	100.00±0.0	-
1	99.97±0.1	-
2	99.80±0.1	-
4	99.42±0.1	-
6	98.62±0.1	-
8	97.83±0.1	-
10	97.52±0.1	-
12	97.32±0.1	+

Deg Const: 2.533 $x10^{-3}$ $month^{-1}$

Pharmacokinetic Evaluation

In case of free drug concentration of rifampicin and isoniazid was rapid as indicated by high peak plasma concentration 281.2 ± 0.20 µg/mL and 311.2 ± 0.18 µg/mL (C_{max}). After administration of coencapsulated rifampicin and isoniazid, liposome the C_{max} was 261.5 ± 0.14 µg /mL and 241.6 ± 0.20 µg/mL respectively. Though the drug concentration was higher above the minimum inhibition concentration level (0.01 to 0.02µg/mL) in plain drug administration, the drug concentration in plasma remained high for longer period with liposomal system

(Shivani et al.2002). The biodistribution of the plain drug and the co encapsulated liposomal formulation appeared to follow the biphasic clearance Fig.3, 4.

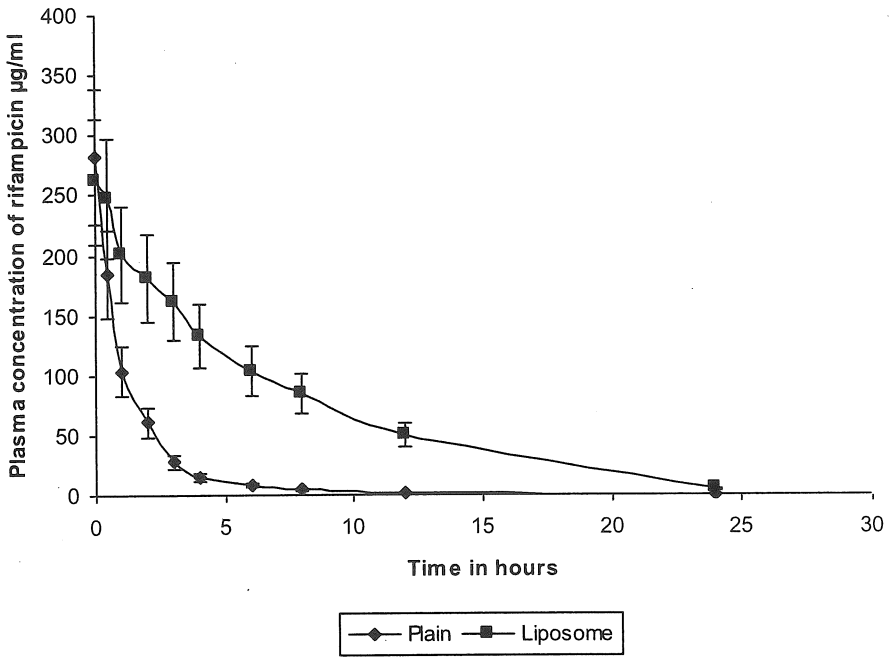


Figure 3. Plasma concentration of rifampicin from coencapsulated liposome and plain drug.

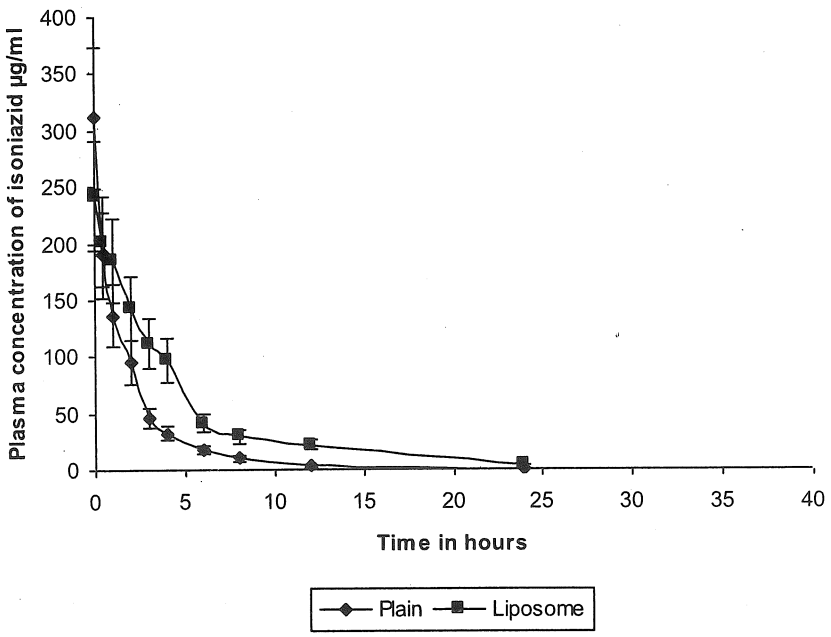


Figure 4. Plasma concentration of isoniazid from coencapsulated liposome and plain drug administration.

The biphasic clearance may be accounted for the slower release of the drug in liposomal delivery systems. The calculated parameters also indicated that the biological half-life ($t_{1/2}$) of coencapsulated rifampicin and isoniazid liposomal formulation was prolonged to 3.370 ± 0.26 h and 4.531±0.16 h respectively. Hence the drug administered through the liposomes systems will remain in the body for a longer period and thus will exert a sustained action with significantly less elimination rate constants (K_e) 0.032±0.001 h^{-1} and 0.028±0.001 h^{-1} and further it showed high mean residential time (MRT) 4.6±0.35 h and 5.4±0.23 h values ($p<0.05$). The results further supported the sustained action of drug from the liposomal systems. Although, the C_{max} was significantly less with co encapsulated rifampicin and isoniazid liposomal system, the AUC of coencapsulated rifampicin and isoniazid were 2379.0±3.12 µg-h/mL and 1059.5±5.75 µg-h/mL was higher when compared to free drug administration. These values were significantly higher when compared to the intravenous administration of plain drug ($p<0.05$), which could be due to maintenance of concentration of drug within the pharmacologically effective range for longer period of time from the coencapsulated liposomal systems. The significantly high AUC values were observed with coencapsulated liposomal system. It also indicated that increase in the bioavailability of drug from these systems compared to free drug ($p<0.05$). The results indicated the slow and steady clearance of the coencapsulated drug in liposomes Table 6, 7.

Table 6. Pharmacokinetic parameters of rifampicin from co encapsulated liposome and plain drug after iv administration.

Parameters	Rifampicin Plain	Rifampicin Liposomes
C_{max} (µg/ml)	281.2±0.20*	261.5±0.14*
K_e (h^{-1})	0.065±0.002*	0.032±0.001*
AUC (0→24) (µg-h/ml)	374.9±5.41*	2379.0±3.12*
AUC (0→∞) (µg-h/ml)	375.1±10.21*	2399.5±6.93*
$t_{1/2}$ (h)	2.004±0.49*	3.370±0.26*
MRT (h)	2.2±0.69*	4.6±0.35*

All values are expressed as Mean±SD, n=10.

C_{max}=Maximum concentration; K_e=Elimination rate constant; AUC=Area under plasma concentration-time curve; $t_{1/2}$=Elimination half-life; MRT=Mean residential time; * significant compared to plain drug ($p<0.05$).

Table 7. Pharmacokinetic parameters of isoniazid from co encapsulated liposome and plain drug after iv administration.

Parameters	Isonoazid Plain Drug	Isoniazid Liposomes
C_{max} (µg/ml)	311.2±0.18*	241.6±0.20*
K_e (h^{-1})	0.033±0.002*	0.028±0.001*
AUC (0→24) (µg-h/ml)	771.8±6.01*	1059.5±5.75*
AUC (0→∞) (µg-h/ml)	771.9±5.05*	1081.7±6.98*
$t_{1/2}$ (h)	1.7±0.13*	4.531±0.16*
MRT (h)	1.8±0.21*	5.4±0.23*

All values are expressed as Mean±SD, n=10.

C_{max}=Maximum concentration; K_e=Elimination rate constant; AUC=Area under plasma concentration-time curve; $t_{1/2}$=Elimination half-life; MRT=Mean residential time.

* Significant compared to free isoniazid ($p<0.05$).

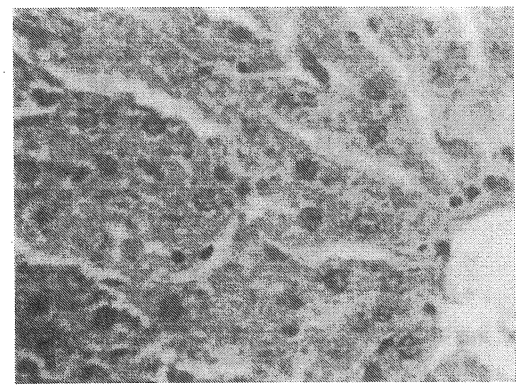
The liposomal delivery systems showed better bioavailability because of the lower particle size, stability of formulation. This may reduce the toxicity of the drugs (Srinath et al. 2000).

Histopathology Study

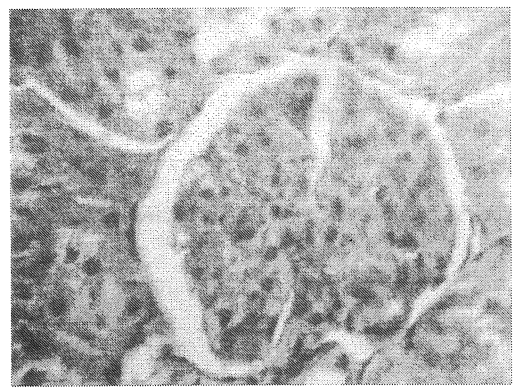
Hepatotoxicity is the most common adverse effect with antitubercular drugs. The rifampicin and isoniazid will be metabolized in the liver; the excretion of the drug will be from the kidney. In case of control, no degeration, necrosis, inflammation, congestion and edema were found. In oral dosage form, liver and kidney showed moderate inflammation. Liver showed moderate degeneration. Necrosis, congestion and edema were not found. Coencapsulated liposomes of rifampicin and isoniazid showed mild inflammation in liver and kidney. Degeneration in liver was not observed in the formulation and there was no necrosis, congestion and edema.

Figure 5

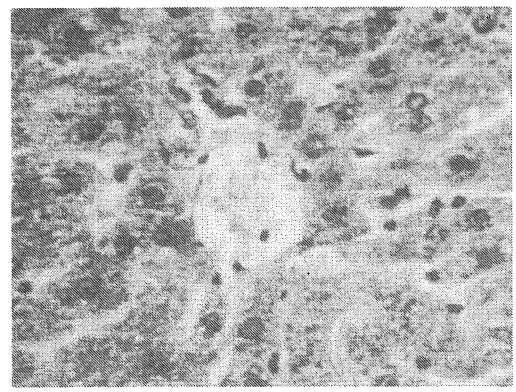
a. Control (without treatment) Liver



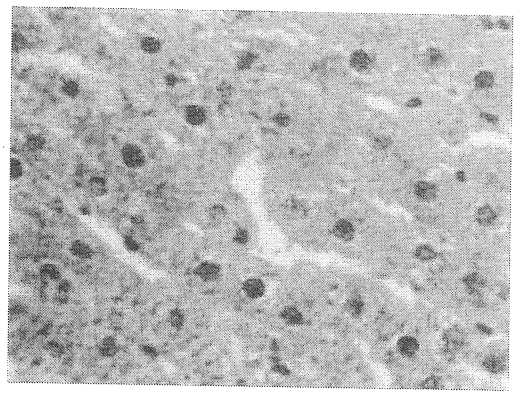
b. Control (without treatment) kidney



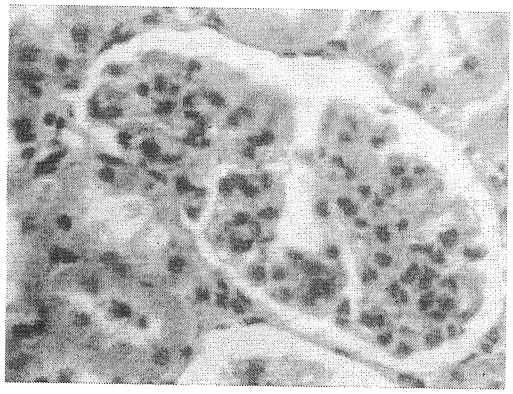
c.Oral dosage form moderate inflammation liver



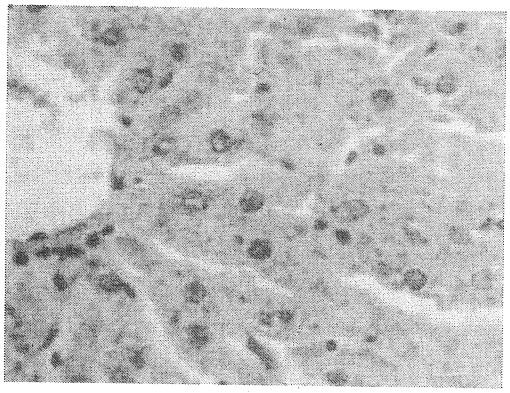
d.Oral dosage form degeneration of liver



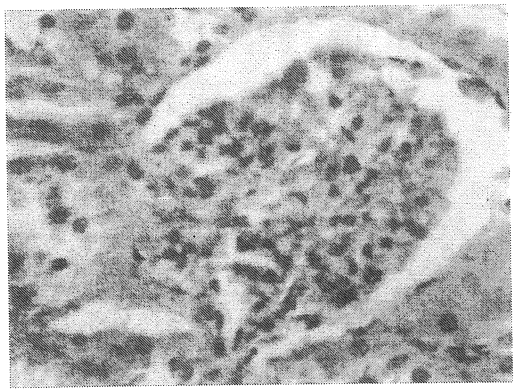
e.Oral dosage form moderate inflammation of kidney



f. Coencapsulated liposomal formulation Mild Inflammation of liver



g.Coencapsulated liposomal formulation mild Inflammation of kidney.



Conclusion

From the present investigation, it can be concluded that the prepared liposomal drug delivery system of antitubercular agents such as rifampicin and isoniazid has exceptional potential for development.

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