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# *LIGAND CONJUGATED LIPOSOMAL DRUG DELIVERY SYSTEM FOR ENHANCED BRAIN UPTAKE OF AMPICILLIN*

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**ABSTRACT**

Targeting of antimicrobial agents by means of liposomes may be of great value in the treatment of intra or extracellular infections compare to conventional forms of antimicrobial therapy. In the present study Ampicillin loaded non-targeted Polyethylene glycolated liposomes and targeted Glutathione Polyethylene glycollated liposomes of about 132.14 nm size were prepared with 80 % of drug entrapment. Prepared liposomes were evaluated for *in vitro, in vivo* release profile and brain uptake studies. Results of these studies revealed more absorption of drug than standard Ampicillin solution and non targeted liposomes ($Auc_{0\text{-}6h}$ 1858.908 µg h/ml) and 3.5 times increase in brain uptake. Incorporation of polyethylene glycol in the liposomes increased the drug concentration and circulation time in plasma as well as in the extracellular fluid of brain thus improved therapeutic availability of Ampicillin trihydrate.

## INTRODUCTION

The blood brain barrier (BBB) makes the brain practically inaccessible for lipid insoluble compounds such as most polar molecules and small ions [1]. Nanomedicine, the application of nanotechnology to health care holds great promise for revolutionizing medical treatments, imaging, faster diagnosis, drug delivery, and tissue regeneration [2]. It can be effectively utilized to overcome the diagnostic and neuron therapeutic challenges of neuron degenerative and neurological diseases [3]. Novel drug delivery which includes the use of small colloidal particles (such as nanoparticles and liposomes) has emerged an ideal approach of drug targeting to brain. Due to their steric phenomenon they conceal themselves from opsonisation and achieve targeting to the brain. They provide a controlled profile of drug release as well as selected targeting mechanism [4-8].

Ampicillin trihydrate is commonly used to treat bacterial infections such as bacterial meningitis which is a life threatening infection of the meninges. The major hurdle in treating such CNS diseases is the inability of molecules to pass the BBB and the blood cerebro spinal fluid barrier. Targeting of antimicrobial agents by means of liposomes may be of great value in the treatment of intra or extracellular infections that prove refractory to conventional forms of antimicrobial therapy. Liposomal technology is with better site specific action. The brain distribution of long circulating liposomes can be modulated by conjugation of appropriate targeting vectors such as monoclonal antibody (mAb to anti-transferrin receptor, mAb to insulin receptor), cationized proteins (cationized human serum albumin), endogenous peptides or plasma proteins. The basic mechanism by which these liposomes achieve brain concentration by crossing BBB is by coupling with brain drug transport vector through transcytosis. These sterically stabilized liposomes are surface modified by coating them with chemical substances which imparts stealthness to them [9-11].

Objective of the present study was to prepare and evaluate Ampicillin loaded non-targeted polyethylene glycolated liposomes and targeted glutathione polyethylene glycollated

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liposomes for better brain uptake. Rationale of GSH-PEG liposomes includes the safety of the liposomal constituents, the ability to encapsulate compounds without modification, the prolonged plasma exposure, and the possibility to enhance drug delivery to the brain. This confirms that GSH-PEG liposomes improved the therapeutic availability of Ampicillin trihydrate, i.e. a lower dose and lower dosing frequency can be used to obtain an effective brain concentration.

## MATERIALS AND METHOD

### Materials

Ampicillin trihydrate was obtained as a gift sample from Macleods Pharmaceutical Ltd. (Mumbai). Egg phosphatidylcholine (LIPOID E PC S) and Distearoyl phosphatidyl ethanolamine-polyethylene glycol 2000 (DSPE-PEG 2000) (LIPOID PE 18:0/18:0-PEG 2000) was gifted by LIPOID GMBH (Germany). Cholesterol was obtained from SRL Pharma (Mumbai). Reduced glutathione and tris buffer composition were obtained from Loba Chemicals (Mumbai).

### Preparation of Polyethylene glycolated (PEG) liposomes

The phospholipid, cholesterol and DSPE-PEG 2000 in equal proportion were dissolved in a mixture of chloroform and methanol (2:1) in round bottom flask. The solvent was evaporated and a thin film was formed using a rotary flash evaporator. The film was hydrated using tris buffer solution (pH 7.4) containing drug and hand shaken till the film is completely removed and a milky suspension of multi lamellar vesicles resulted. The size of liposomes were further reduced by high speed homogeniser to form small unilamellar vesicles. It was then filtered through a 0.45 µm membrane to separate the liposomes [12,13].

### Preparation of Glutathione polyethylene glycolated (GSH-PEGylated) liposomes

GSH-PEGylated liposoms with Ampicillin trihydrate were prepared by dissolving phospholipids and cholesterol in ethanol at $55^0$C and adding to tris buffer (pH 7.4) containing drug with stirring and heated to the same temperature. Reduced GSH and DSPE-PEG 2000 were incubated at 1:1 molar ratio at room temperature for 2 h before adding GSH-PEG micelles to the liposomes. Liposomes and micelles were incubated at $60^0$C for 30 min. The mixture was homogenized at 6000 rpm for 10 min and filtered through 0.45 µm membrane filter to separate liposomes [14,15].

### Characterization of Liposomes:

Obtained liposomes were evaluated for entrapment efficiency, particle size and zeta potential measurement, i*n-vitro* drug release, pharmacokinetic and brain uptake study.

### Entrapment Efficiency

The liposomal suspension was centrifuged at 10,000 rpm for 30 min. Obtained bottom residue was separated from supernatant. Supernatant was analysed for entrapped drug by validated HPLC method with pump: Jasco HPLC system, detector: Jasco UV detector, column: Hypersil C18, length : 25cm, inner diameter: 5µ, mobile phase A:B (60:40) where A consisted of Acetic acid (0.5 ml), 0.2 M dihydrogen phosphate buffer (50 ml ), Acetonitrile ( 50 ml ) and water to make up volume to 1000 ml. B is Acetic acid (0.5 ml), 0.2 M dihydrogen phosphate buffer (50 ml ), Acetonitrile (400 ml) and water to make up volume to 1000 ml [16]. Entrapment efficiency was calculated by following formula

$$\text{Entrapment efficiency } (\%) = \frac{(\text{Total drug} - \text{Free Drug})}{\text{Total Drug}} \times 100$$

### Particle size and zeta potential measurement

The average particle size, size distribution, polydispersity index and zeta potential of liposomal formulation was determined by using Zetasizer Ver. 6.34 (Malvern Instruments Ltd.) using software Nanophox Particle Size Analysis Windox 5. Prior to the measurements sample was diluted with double distilled water to produce a suitable scattering intensity.

### *In-vitro* drug release from liposomes

Release studies were carried out by dialysis method. The liposomal suspension (5 ml) was filled in the dialysis bag. Both ends were closed with clips and suspended in 200 ml of phosphate buffer saline (pH 7.4) at $37^0$C. Samples were withdrawn at intervals of 0, 0.5, 1, 2, 3, 4, 5 and 6 hr. After suitable dilution the amount of drug released was quantified by validated U.V. method at 196 nm [17,18].

### Pharmacokinetic studies

*In vivo* studies were carried out in order to study bio-availability and brain uptake of non-targeted PEGylated and targeted GSH-PEGlyated liposomes of Ampicillin trihydrate. The protocols for the animal studies were approved by Institutional Animal Ethics Committee (IAEC) and all experiments were conducted as per the norms of the committee

for the purpose of control and supervision of experimental animals (CPCSE), India, protocol no: 131409/KMKCP/IAEC. Sprague-Dawley rats of either sex weighing 250-300 g were housed at 25 ± 2 $^0$C and 50-60 % RH. Rats were supplied with food and water. Animals were divided into three groups (2 test and 1 standard) comprising 12 animals in each. Group one two and three were given non-targeted PEGlyted liposomes, targeted GSH-PEGlyted liposomes and Ampicillin sodium injection (40 mg/kg or equivalent dose) intra-peritoneally respectively.

Blood samples (1.5-2 ml) were withdrawn from the retro-orbital plexus at time intervals of 0.5, 1, 2, 4 and 6 h after administration. Blood samples were collected in eppendorffs tube containing 0.4 % di-sodium EDTA solution and centrifuged at 8000 rpm for 10 min at of 4$^0$C to separate the plasma. Obtained plasma was evaluated for drug concentration by validated HPLC method (pump: Jasco HPLC system, detector: Jasco UV detector, column: Hypersil, C18 Length 25cm, Inner diameter: 5µ, mobile phase acetonitrile (60) : dihydrogen sodium phosphate buffer pH 3.5 (40) with flow rate 1 ml/min [19,20].

**Brain uptake studies**

Animals were sacrificed and brains were removed at time intervals of 0.5, 2, 4 and 6 h after administration from 3 animals at each interval. Brains were cleaned in cold saline and homogenized immediately, centrifuged at 8000 rpm at 10 min at 4$^0$C. The supernatant obtained was stored at -20$^0$C until analysis.The concentrations of Ampicillin trihydrate in rat plasma and homogenized brain tissue was determined by a validated bio-analytical HPLC method [21].

## RESULTS AND DISCUSSION

**Drug Entrapment Efficiency**

Encapsulation of hydrophilic active pharmaceutical ingredient into liposomes presents unique challenges during formulation design and processing. Due to the aqueous solubility of hydrophilic drugs they are dissolved in the external aqueous phase during liposome preparation and become entrapped in the aqueous compartments within the formed liposomes. In film hydration method, multilamellar vesicles were formed after removing the organic solvents under vacuum using rotary evaporator. These vesicles were hydrated with buffer solution containing drug for 24 hrs which eventually increased the encapsulation of drug in the aqueous part of liposomal bilayers. Multilamellar vesicles were further size reduced to small unilamellar vesicles using probe sonicator. The entrapment efficiency was found to be 80.0 %.

**Particle size and zeta potential measurement**

The mean size of optimized liposomal formulation was found to be 132.14 nm and the polydispersity index was 0.09. This reveals that the particles had a narrow size distribution and a uniform size. The nanoparticles had higher absolute value of zeta potential indicating a better stability of this colloid system. PEGylating agent DSPE-PEG 2000 imparts negative charge to the formulation. Zeta potential of liposomes was found to be -87 mV. Image of particle size analysis and zeta potential measurement is shown in fig. no.1 and 2.

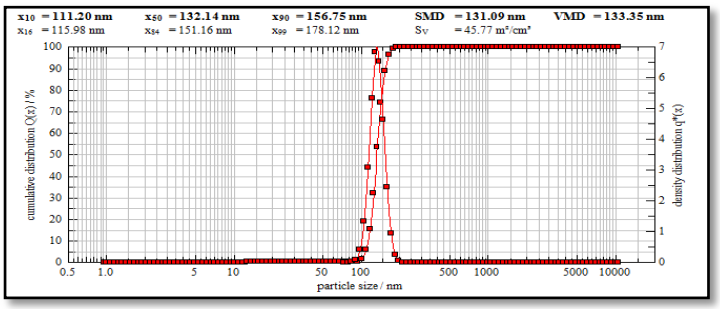


**Fig. 1: Particle size determination**

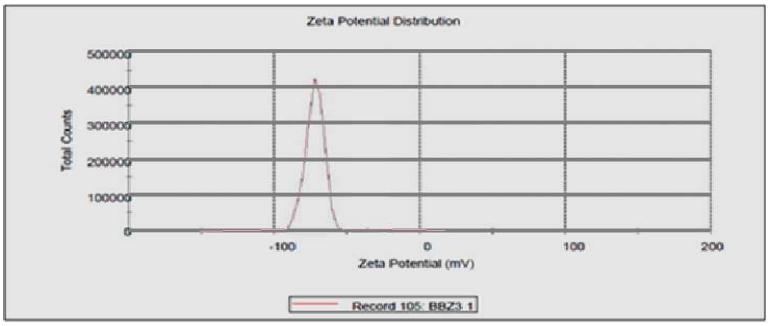


**Fig 2: Zeta potential measurement**

***In vitro* drug release studies**

The bulk-equilibrium dialysis technique was used to investigate Ampicillin trihydrate release from non-targeted PEG liposomes, targeted GSH-PEG liposomes and pure drug solution in saline phosphate buffer (pH 7.4). Comparing the release curves of targeted and non-targeted liposomes with the standard, targeted and non-targeted liposomes showed no burst release at initial stages which indicates absence of attached drug to the surface of the particles. Release profile for 6 h was done in phosphate buffer pH 7.4 by both targeted and non-

targeted liposomes. Targeted liposomes showed higher release up to 80 % than the non-targeted one due to presence of glutathione which imparts redox action. *In vitro* release profile is shown in fig. no 3.

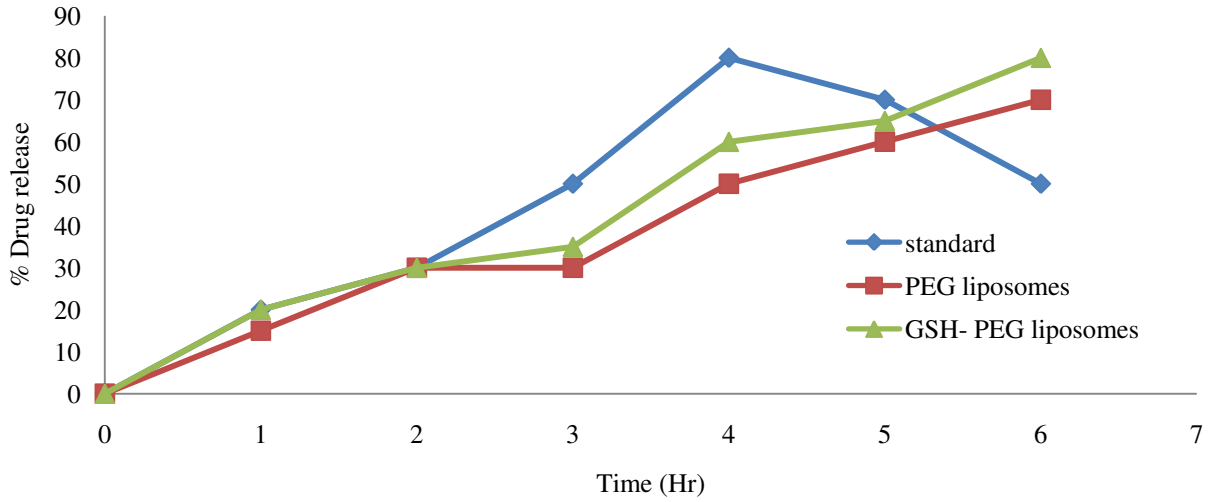


**Fig. 3: *In vitro* release profile of liposomes in phosphate buffer pH 7.4**

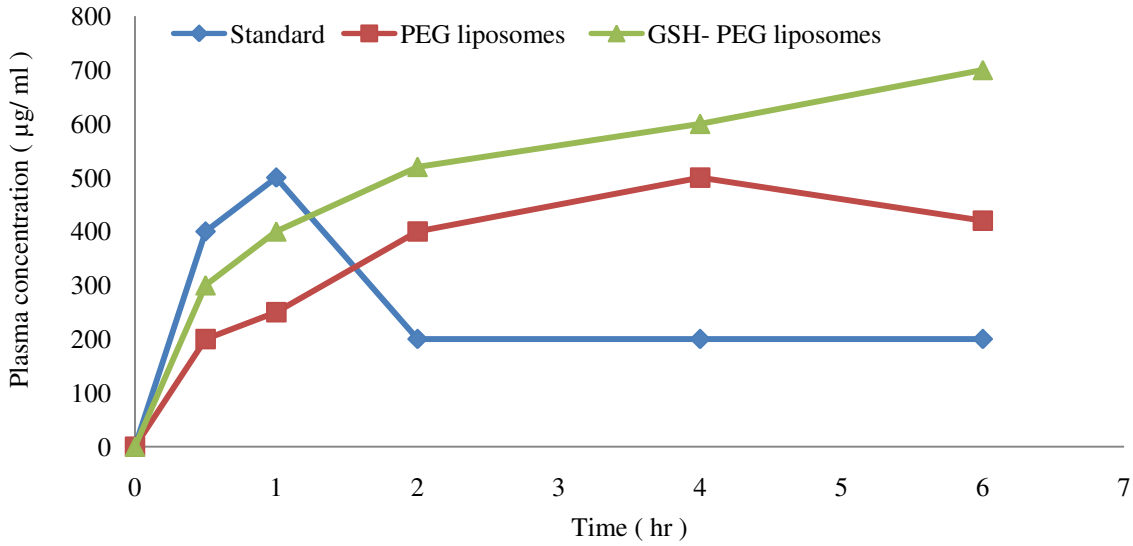


**Fig. 4: Plasma concentration vs. time profile of Ampicillin trihydrate following i. p. administration of Ampicillin sodium injection, non-targeted and targeted liposomal formulations of drug**

**Pharmacokinetic Studies**

The plasma concentration time plots in rats after i.p administration of Ampicillin sodium injection and non-targeted and targeted liposomal drug formulations are shown in fig. 4 and the calculated pharmacokinetic parameters in table 1. The $AUC_{0-6}$ h after i.p administration of Ampicillin sodium injection, non-targeted and targeted formulation showed that the plasma values of targeted and non-targeted formulations were comparable with that of the standard. The results indicate that systemic absorption of Ampicilin trihydrate was enhanced and prolonged effect was obtained by incorporating into liposomes compared with pure drug solution.

The relative bioavailability obtained after administration of non-targeted liposomes were found to be 1.212 whereas it was found to be 1.5925 after administration of targeted liposomes. The targeted and non-targeted liposomes thus showed a promising potential for enhancing bioavailability as well as the duration of action of hydrophilic drugs like penicillins by reducing their metabolism due to protective coating of PEG which avoids their recognition by body's RES (Reticulo

Endothelial System) and thus increases their blood circulation time.

**Table 1: Pharmacokinetic parameters after intra-peritoneal administration of Ampicillin sodium injection, non-targeted and targeted liposomal formulations of drug**

| Formulation | $C_{max}$ (µg/ml) | $T_{max}$ (min) | AUC $_{0-6h}$ (µg h/ml) |
|---|---|---|---|
| Ampicillin sodium injection | 520.90 | 60.0 | 1167.298 |
| Non-targeted PEG liposomes | 514.13 | 240.0 | 1414.816 |
| Targeted GSH-PEG liposomes | 635.13 | 240.0 | 1858.908 |

**Brain Uptake Studies**

In this study non-targeted liposomal formulation showed considerably better uptake in brain tissue as compared to standard Ampicillin sodium injection due to the lipid nature of liposomes. Targeted liposomal formulation showed about 3.5 folds increase in brain uptake (fig.no. 5) as compared to the standard.

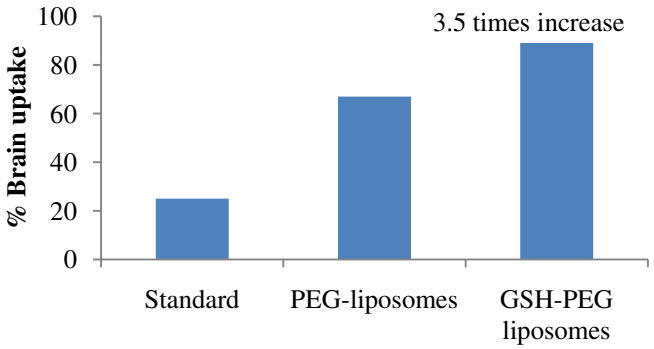


**Fig. 5: Brain uptake data**

Ampicillin trihydrate is delivered to the extracellular fluid of the brain. GSH combines with the water soluble drug at the aqueous interiors of the liposomes and imparts redox action while delivering the drug to the brain. Due to inclusion of GSH formulation has higher affinity for brain tissues and hence helped to enhance the concentration of hydrophilic Ampicillin trihydrate into the brain thus enhanced uptake of brain is observed.

## CONCLUSION

In this study non-targeted PEGylated liposomal formulation and the targeted GSH-PEG liposomes of around 132 nm with uniform particle size distribution were successfully and efficiently loaded with Ampicillin trihydrate. The process can be easily scaled up due to its simplicity. Moreover, in *vitro* release showed 80.0 % release over a period of 6 h. Absorption of Ampicillin trihydrate from non-targeted and targeted liposomal formulation was found to be significantly higher than the pure drug solution.

The results suggest that it takes time for Ampicillin trihydrate to be released once encapsulated in the liposomes because lipid bilayers are stabilized by cholesterol. Thus a depot effect was achieved using liposomes, especially in the PEGylated liposomal formulation. From brain uptake studies, it was found that Ampicillin trihydrate was delivered to the extracellular fluid of the brain when GSH was conjugated to liposomes loaded with the drug. The benefits of GSH-PEG liposomes include the safety of the liposomal constituents, the ability to encapsulate compounds without modification, the prolonged plasma exposure, and the possibility to enhance drug delivery to the brain. This confirms that GSH-PEG liposome improves the therapeutic availability of Ampicillin trihydrate at low dose and less dosing frequency in a cost effective way.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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