

IN VITRO RELEASE AND IN VIVO TISSUE LOCALIZATION OF 5-FLUOROURACIL LOADED MICROSPHERES

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ABSTRACT

5-Fluorouracil (5-FU) is an antimetabolite with a broad spectrum activity against solid tumors. However, its very short half-life in plasma circulation greatly limited the *in vivo* antitumor efficacy and clinical application of the drug. The current research work aimed to solve this problem by preparing sustained release microspheres where the drug encapsulated into bovine serum albumin, a biocompatible, nontoxic and non immunogenic drug carrier.

Microspheres were prepared by heat denaturation method and the process was optimized. Effect of polymer concentration, stirring rate during emulsification, viscosity of oils were observed. The physical properties, tissue distribution studies and stability studies of drug loaded microspheres were carried out. *In vitro* drug release study was carried out. *In-vivo* tissue distribution study carried out by using albino rats. Transmission electron microscopy (TEM) was done to know surface morphology of prepared microspheres.

Drug contents are almost same for freshly prepared and freeze dried products. Release rate of freshly prepared microsphere and freeze dried microspheres are 88.30% and 87.86 % respectively on 7th day. Studies revealed that after I.V. administration of DLM (Drug loaded microspheres), maximum amount of drug was detected in liver and adequately remained in spleen at least up to 24 hours.

Keywords: Albumin, Heat denaturation, *In-vitro* release, 5-Fluorouracil, *In-vivo* tissue distribution.

INTRODUCTION

A well designed controlled drug delivery system can overcome some of the problems of conventional therapy and enhance the therapeutic efficacy of a given drug. To obtain maximum therapeutic efficacy, it becomes necessary to deliver the agent to the target tissue in the optimal amount in the right period of time there by causing little toxicity and minimal side effects. There are various approaches in delivering a therapeutic substance to the target site in a sustained controlled release fashion. One such approach is using microspheres as carriers for drugs. Microspheres are characteristically free flowing powders consisting of proteins or synthetic polymers which are biodegradable in nature and ideally having a particle size less than 200 μm [1].

Microspheres can also offer advantages like limiting fluctuation of drug concentration within therapeutic range, reducing side effects, decreasing dosing frequency and improving patient compliance. The use of natural biodegradable polymer to deliver drugs continues to be an area of active research, despite the advent of synthetic biodegradable polymers. Some of the materials taken from nature for microspheric preparation include lipids and waxes, proteins like albumin [2] and gelatin, polysaccharides like alginate [3, 4] and chitosan [5]. Albumin is a widely distributed natural protein. It is considered as a potential carrier of drug or proteins (for either their site specific localization or their local application into anatomical discrete sites). It is being widely used for the targeted drug for the targeted drug delivery to the tumors cells [6]. Preparation of uniformly sized Albumin Microspheres (AMS) was first reported in the late 60's and early 70's [7, 8]. AMS have received wide attention during the recent decades due to their specificity [9], biodegradability [10] and other desirable characteristics such as non-toxicity and biocompatibility [11], as an ideal drug carrier.

Fluorouracil (5-FU) is a pyrimidine analog which is used in the treatment of cancer. It is a suicide inhibitor and works through irreversible inhibition of thymidylate synthetase. It belongs to the family of drugs called antimetabolites. 5-Fluorouracil (initially 7-12 mg/kg iv for 4 days), a cell-cycle-phase-specific anti neoplastic agent, is indicated in colon, rectal, breast, ovarian, cervical, gastric, oesophageal, bladder, liver, and pancreatic cancer. 5-FU is a commonly applied anticancer drug in the treatment of colon cancer. At present, the standard regimen is an intravenous bolus injection of 5-FU modulated by folinic acid (leucovorin) [12, 13].

MATERIALS AND METHODS

Bovine serum albumin was obtained as a gift sample from Loba Chemie, Mumbai, India, and olive oil was purchased from Ashwin Chemicals, Mumbai, India, and used as obtained. 5-FU was obtained from Hoffmann La Roche, USA. Ether and liquid paraffin were procured from SD Fine Chemicals Ltd., Mumbai, India, and all other chemicals and reagents used were of analytical grade.

Preparation of microspheres

Albumin microspheres were prepared by protein gelation process [14]. Bovine serum albumin was dissolved in distilled water. This solution was added drop wise in olive oil to make an emulsion. The emulsion so formed was added drop wise into the preheated olive oil ($125\pm 5^\circ\text{C}$), stirred at 1600 g. After heat stabilization time of 10 min, the preparation was cooled to 25°C , centrifuged at 2500 g, and supernatant was decanted. Microspheres thus obtained were washed with liquid paraffin and twice with ether to get a free flowing and discrete product. The same were then suspended in anhydrous ether and stored at 4°C in an airtight container. Number of variables was studied affecting size, shape and entrapment efficiency of microspheres. Separate batches were prepared and minimum of 100 particles were observed under optical microscope using oil immersion lens to optimize the process.

Optimization of process variables

Variables such as concentration of albumin, stirring rate during emulsification [15, 16], viscosity of oils and drug concentration were studied by preparing series of batches. Other factors such as emulsion drop rate, heat stabilization temperature, stirring rate during heat stabilization of microspheres and heat stabilization time were studied and observations were recorded. Three batches of microspheres in olive oil at 105°C , 125°C and 145°C were prepared keeping other variables same as described.

Analysis of drug content

Surface drug

To a portion of ether suspension of microspheres equivalents to 5 mg of 5-FU, Tween 80 (2 drops) was added and the suspension was gently vortexed. Ether was then evaporated and 1 ml of 4.7 pH acetate buffer was added, centrifuged at 4000 g for 5 min and this supernatant, the first washing was analysed for drug content. The

same way, second and third washings containing 5-FU in the supernatant were analysed by spectrophotometer at 266 nm.

Entrapped drug

Microspheres obtained after three washings were digested in 10ml of 4.7 pH buffer overnight. This suspension was then sonicated for 5 min. and centrifuged to get a clear supernatant which was suitably diluted with 4.7 pH buffer and assayed for 5-FU at 266 nm by spectrophotometer. The same homogenate was redigested and analyzed for drug content.

Determination of *in-vitro* release of 5-FU from microspheres prepared at different temperature

Drug release was determined with the help of modified USP XXI dissolution rate model [17] apparatus. A 250 ml beaker was placed in the vessel. Plastic tube of diameter 17.5 mm opened from both the ends was tied at one end with treated cellophane membrane and dipped into the beaker containing dissolution media. Paddle type stirrer was attached in the center of the beaker and the speed was maintained at 100 rpm. The beaker was filled with 90 ml acetate buffer (pH 4.7) and temperature was maintained at $37 \pm 1^\circ\text{C}$. Drug loaded microspheres were suspended in 10 ml of phosphate buffer. Samples were withdrawn periodically for 8 h and concentration was determined spectrophotometrically at 266 nm.

In vivo tissue distribution

In vivo tissue distribution studies were carried out in albino rats weighing $250 \pm 20\text{gm}$ to find the survival time of 5-FU loaded albumin microspheres. Plain drug containing 500 μg was administered I.V. in a group of 12 rats, through tail vein. The same amount of drug loaded albumin microspheres was given through I.V. route.

Three rats from both the groups were scarified at the end of 1hr, 3hr, 6hr and 24 hr. Various organs such as Liver, spleen, lungs and small intestine were isolated, washed with chloroform, blotted and

weighed. All the organs were homogenized in 1:4 (g:ml) ratio with chloroform and centrifuged at 4000 g for 10min. Supernatant liquid obtained was further diluted and analyzed by HPLC. Results are shown graphically in Fig 7 and 8.

HPLC method for analysis of 5- FU from tissue samples

Tosh (PX – 8010) HPLC model with a UV detector (tosoh UV-8010) operating at 254 nm [20] utilizing a C18 reverse – phase column (25cm X 4.8 cm) was employed.

The procedure used for detection of 5-FU from tissue samples was similar as developed by youcef M. Rustum [21]. The mobile phase used was phosphate buffer containing 2.5% methanol at pH 6.9.

Freeze drying of drug loaded albumin microspheres

Drug loaded albumin microspheres were freeze dried by lyophilizer (Toshniwal Lyophilizer, Bombay, India).

Characteristics of freshly prepared and freeze dried drug loaded albumin microspheres

Freeze dried drug loaded albumin microsphere were studied for their shape and size, drug content, release rate and stability at 4°C in a manner similar to freshly prepared microspheres and observations were recorded.

RESULTS AND DISCUSSION

5-FU obtained from Hoffmann La Roche was analysed for its drug content by titrimetric method. Albumin microspheres containing 5-FU were prepared by simple emulsion technique. Various studies have been performed to obtain desirable product. Albumin concentration was studied due to the fact that albumin plays a vital role as emulsifying agent, thus affects the droplet size and stability [18]. Higher albumin concentrations produced smaller microspheres. However, it was not possible to increase the concentration of albumin beyond 400mg/ml (Table.1).

Table 1: Optimum variables for the preparation of 5-FU loaded albumin microspheres

Variables	Ideal condition
Albumin concentration (mg/ml)	Aqueous solution of bovine serum albumin (400 mg/ml)
Drug concentration	Solution containing 40mg/ml of 5-FU
Emulsification power	1200 rpm
Emulsification time	5 min
Stirring rate during stabilization.	1600 rpm
Heat stabilization Temperature	125°C
Oil	Olive oil
Emulsion drop rate	80 ± 5 drops/min
Heat stabilization time	10 min

Similarly appropriate drug concentration was obtained by making batches at different concentration of drug and observing the size of the microspheres under microscope. Increasing the concentration from 5.0 % to 7.5% and 10% gradually increased the size of the microspheres as well as percent drug incorporated. Drug concentration more than 10% increased the size of the microspheres beyond limit (more than 9.0μ). Thus 40mg /400 mg (drug / albumin) ratio was selected for the preparation of microspheres.

It has been reported that the power of emulsification and emulsification time affect the droplet size of emulsion [19]. Longer duration of emulsification and greater emulsification power though resulted in smaller microspheres, stirring speed higher than 1600rpm and emulsification time more than 10min resulted in frothing. Fixed oils with different viscosities viz olive, coconut and groundnut were used to obtain optimum size of microspheres. Olive oil with viscosity 120.6 cp produced smallest size of microspheres (average particle size 4.56) whereas groundnut oil (viscosity 169.0 cp) and coconut oil (viscosity 130.1 cp) had produced microspheres of average particle size 5.57 and 5.37 μ respectively.

Effect of heat stabilization temperature on microsphere size was studied by conducting experiments at various temperatures such as 105, 125 and 145° . A heat stabilization temperature of 105°C

produced larger particle size with some water trapped in the albumin matrix. At temperature 125°C , microspheres obtained were of desirable size. Microspheres prepared at 145°C were harder with very slow drug release rate. Other variables studied were heat stabilization time and emulsion drop rate. By doing heat stabilization for more than 10 min and drop rate lesser than 80 ± 10 drops per min resulted in charring of microspheres. Observations are summarized in (Table.1).

Studies of Microspheres

Transmission electron microscopic study of Microspheres

Microspheres prepared at 125°C were stained by 1% w/v solution of phosphotungstic acid and photographs were taken (Fig.1).

Drug release kinetics

Release of 5-Fluorouracil (plain drug) from microspheres prepared at 125°C and 145°C were studied at pH 4.7 and shown graphically in (Fig. 2). Fig. 3 to 6 represent the graphs obtained by plotting 5-FU released from albumin microspheres prepared at 125°C according to zero order, first order, planner matrix and spherical matrix mechanism respectively. These models can be employed in the study of influence of different formulation factors on the drug release in order to obtain the desired characteristics.

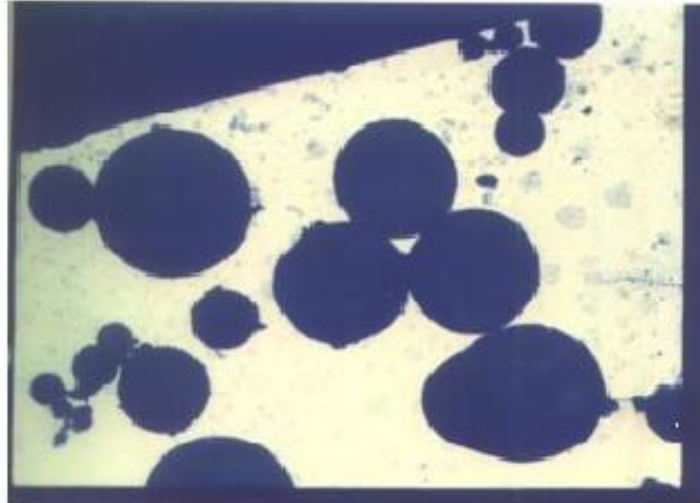


Fig.1: TEM photograph of 5-FU microspheres (Stained by 1% w/v solution of phosphotungstic acid).

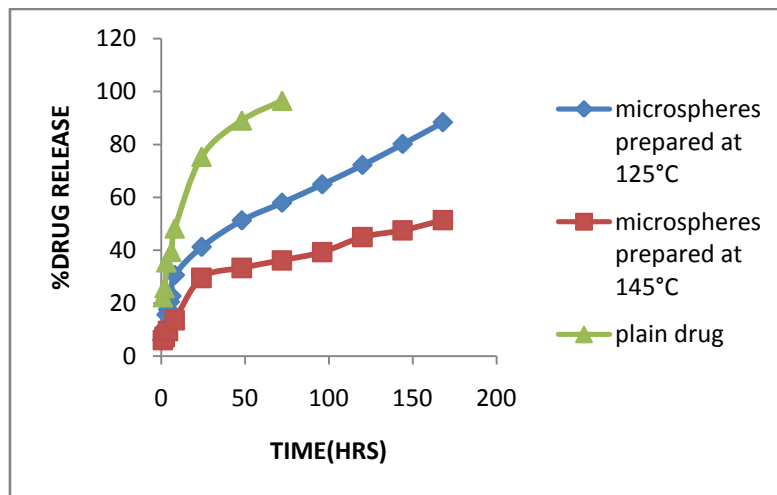


Fig.2: Release rate of 5-FU

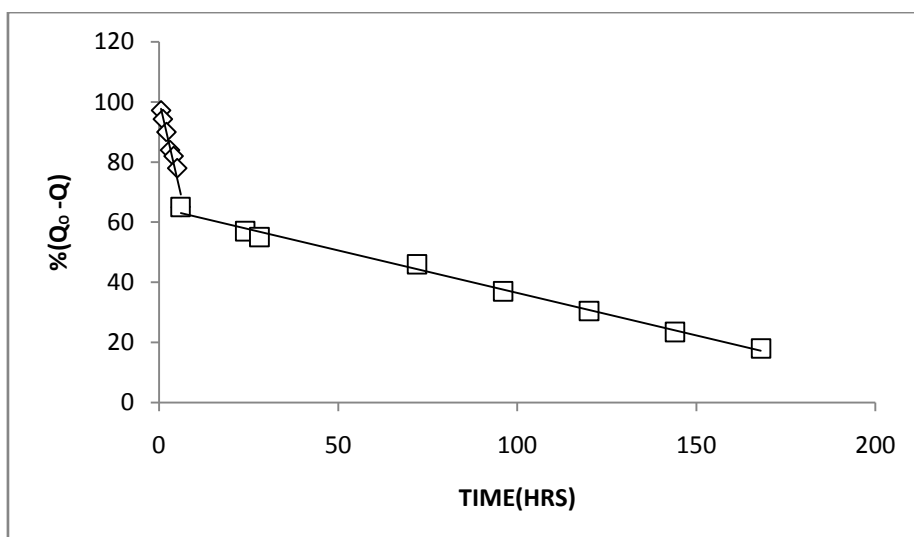


Fig. 3: 5-FU release from microspheres prepared at 125°C according to zero order mechanism.

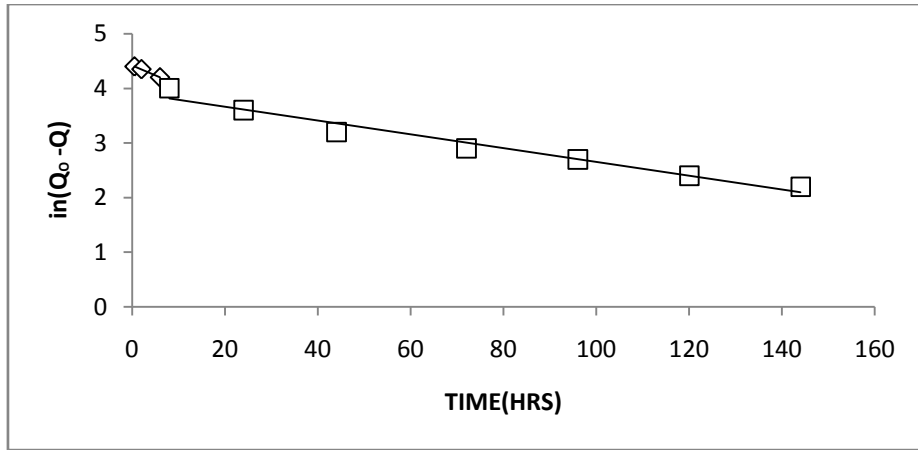


Fig. 4: 5-FU release from microsphere prepared at 125°C according to first order mechanism.

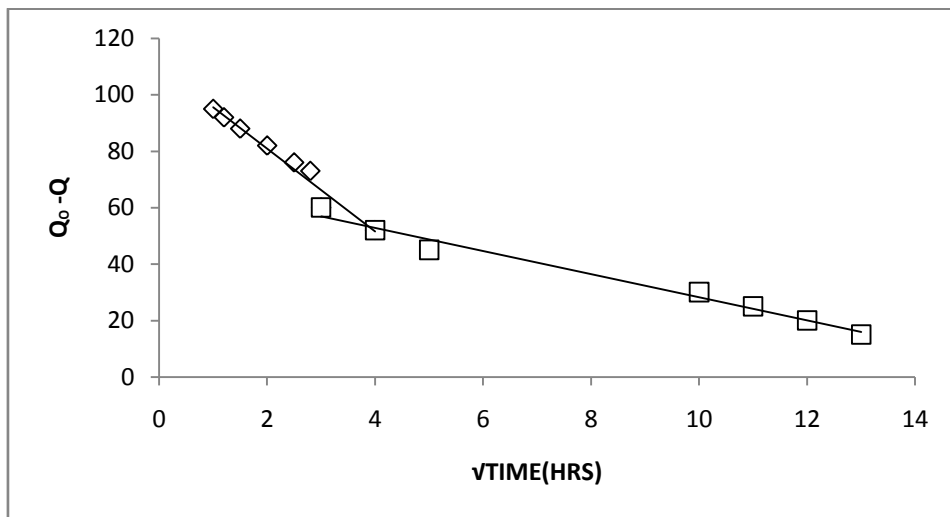


Fig. 5: 5-FU release from microsphere prepared at 125°C according to planar matrix mechanism.

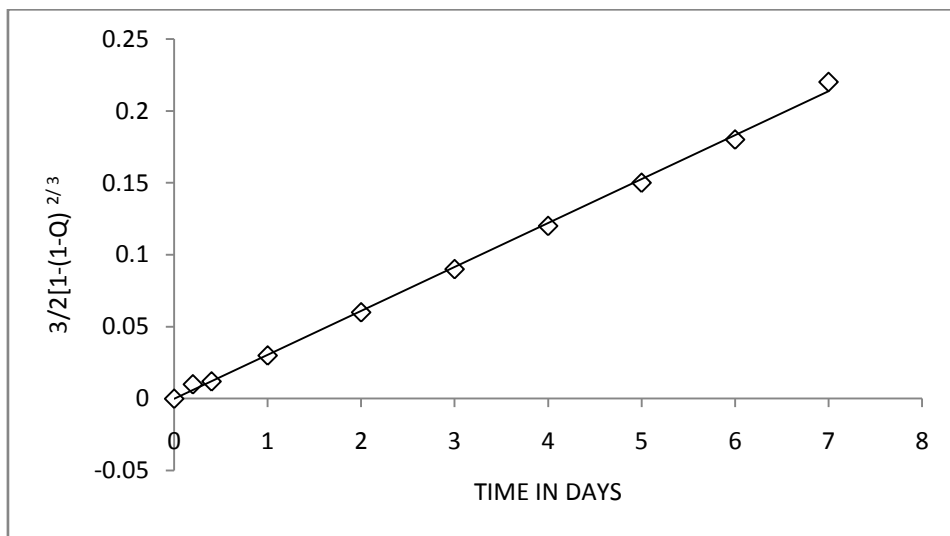


Fig. 6: 5-FU release from microsphere prepared at 125°C according to spherical matrix mechanism.

Biodistribution and target efficiency *in vivo*

Distribution of drug loaded microspheres (DLM) in various organs following intravenous administration in the rats is

shown in Fig. 8 as µg/gm of tissue. The drug not metabolized was analyzed by HPLC. Blank tissue samples were also analyzed for any absorption of tissue content at 254 nm and made necessary corrections.

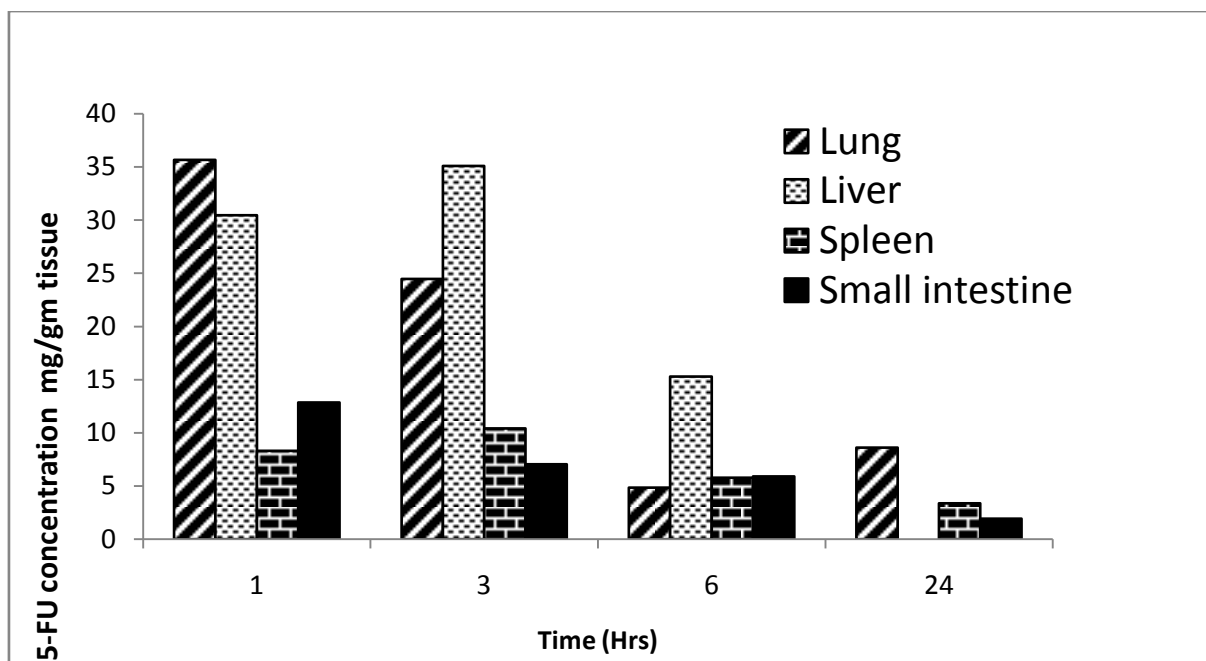


Fig. 7: 5-FU Concentration in various tissues of albino rats after IV administration of plain drug.

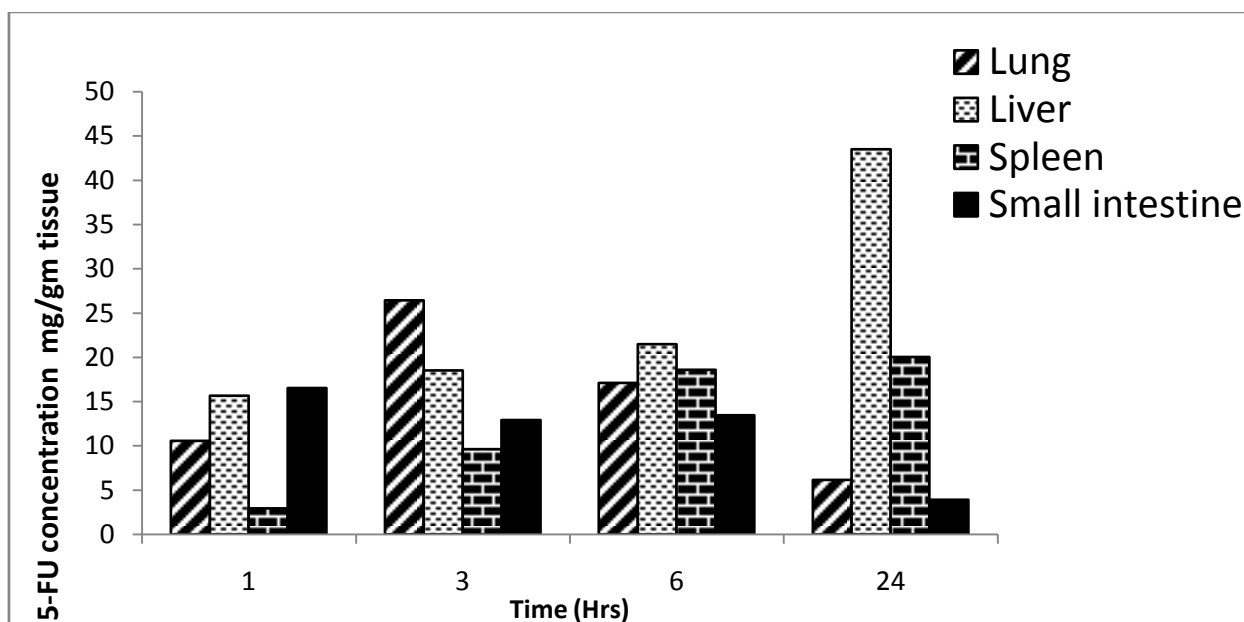


Fig. 8: 5-FU Concentration in various tissues of albino rats after I.V. administration of Drug Loaded Microspheres (DLM)

Table 4: Comparative study of freshly prepared and freeze dried drug loaded albumin microspheres

Characteristics	Freshly prepared microsphere	Freeze dried microsphere
Shape	Spherical	Spherical
Colour	Off White	Off White
Odour	Odourless	Odourless
Size	Below 9µ	Below 9µ
Drug Content	12.20µg/mg	12.12µg/mg
i) Surface drug	32.7µg/mg	32.45µg/mg
ii) Entrapped drug	41.19%	41.0%
Release Rate on	72.85%	72.2%
1 st day	88.30%	87.86%
5 st day	Stable	Stable
7 st day		
Stability at 4°C		

Administration of free drug and DLM showed considerable differences in the distribution of drug [22, 23]. A rapid elimination of free drug was observed in comparison with DLM. Maximum drug concentration was found in the liver (87.02%) followed by spleen (41.0%) after 24 hours. No drug was detected in liver after 24 hrs from the time when free drug solution was administered. Drug from DLM was gradually increasing in liver tissues and found maximum in the liver after 24 hrs and results are shown in figure 8. Similar results were obtained in the studies conducted by L.F. Lai and H.X Guo (2011).

Measurable difference was not observed in the concentration of drug in lungs and small intestine after I.V. administration of DLM.

An important aspect for the clinical use of drug loaded albumin microsphere is to remain stable for prolonged period of time and with reproducible characteristics. It is therefore necessary to increase the shelf life of drug loaded albumin microspheres so that large scale clinical studies can be conducted. This can achieve by freeze drying of the product.

Characteristics of freeze dried albumin microspheres were compared with freshly prepared microspheres and observations show quite similar results of the two products (Table no.2).

CONCLUSION

The drug loaded 5-FU microspheres could be prepared and optimized for delivery into various RES rich organs particularly Liver. The higher encapsulation efficiency and drug loading is achieved using optimum experimental design. *In vitro* drug release of DLM revealed a sustained release profile. The 5-FU loaded DLM are more stable at 4°C then at room temperature (25° to 40°C). Studies revealed that after I.V. administration of DLM, maximum amount of drug was detected in liver and adequately remained in spleen at least up to 24 hours. This study demonstrates that the drug loaded albumin microspheres could be efficiently targeted at the liver by intravenous injection.

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