Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 353 (2008) 243–250
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Preparation and evaluation of N_3-*O*-toluyl-fluorouracil-loaded liposomes

Weitong Sun, Na Zhang *, Aiguo Li, Weiwei Zou, Wenfang Xu

The Pharmaceutical College, Shandong University, Ji'nan, China

Received 3 July 2007; received in revised form 16 October 2007; accepted 10 November 2007
Available online 17 November 2007

Abstract

This study was aimed at developing a liposome delivery system for a new and potential antitumor lipophilic prodrug of 5-fluorouracil (5-Fu)–N_3-*O*-toluyl-fluorouracil (TFu), intended to improve the bioavailability and therapeutic efficacy of 5-Fu by oral and intravenous administration. TFu-loaded liposomes were prepared by a modified film dispersion–homogenization technique, the formulation and manufacture parameters were optimized concerning the drug encapsulation efficiency. TFu-loaded liposomes were characterized according to particle size, size distribution, zeta potential, drug entrapment efficiency, drug loading and physical stability, respectively. In vitro release characteristics, in vivo pharmacokinetic properties and bioavailabilities were also investigated. The formulated liposomes were found to be relatively uniform in size (400.5 ± 9.6 nm) with a negative zeta potential (-6.4 ± 0.8 mV). The drug entrapment efficiency and loading were ($88.87 \pm 3.25\%$) and ($8.89 \pm 0.19\%$), respectively. The physical stability experiments results indicated that lyophilized TFu-loaded liposomes were stable for at least 9 months at 4 °C. In vitro drug release profile of TFu-loaded liposomes followed the bi-exponential equation. The results of the pharmacokinetic studies in mice indicated that the bioavailability of TFu-loaded liposomes was higher than the suspension after oral administration, and was bioequivalent comparing with TFu 50% alcohol solution after intravenous (i.v.) administration. These results indicated that TFu-loaded liposomes were valued to develop as a practical preparation for oral or i.v. administration.

Keywords: Liposomes; N_3-*O*-toluyl-fluorouracil (TFu); Prodrug; Bioavailability

1. Introduction

5-Fluorouracil (5-Fu) has been widely used in the therapy of different solid tumor types, such as cancers of the stomach, liver, intestine, etc. In order to achieve therapeutic drug levels, 5-Fu is currently administered weekly at 400–600 mg/m^2, however, tumor cells are only exposed to the formation-rate limited active metabolites for a brief time, due to the short half-life of 5-Fu locally within tissues, as well as systemically (15–20 min) (Fraile et al., 1980), moreover, 5-Fu may cause the following adverse effects: bone marrow depression, gastrointestinal tract reaction, or even leucopenia and thrombocytopenia (Yu et al., 2003). Therefore, it is essential to design a valuable formulation to improve the therapeutic index of 5-Fu. It has been shown that liposomes could act as biocompatible, biodegradable, non-immunogenic drug carriers, the advantages of liposomal-encapsulated drugs are prolonged duration of exposure, selective delivery of entrapped drug to the site of action (Zamboni, 2005), improved therapeutic index, and potentially overcoming resistance associated with the regular anticancer agent. Nevertheless, as a water-soluble molecule, 5-Fu diffuse rapidly through liposome bilayers (Immordino et al., 2004), which limited the shelf life and clinical utility of these liposomes. Synthesis of a more lipophilic prodrug is an effective method for lipophobic drugs to form stable liposome.

N_3-*O*-toluyl-5-fluorouracil (TFu) is the intermediate metabolite of Atofluding (ATFu), the latter has shown remarkable antitumor activity and under phase III clinical study in China (Zhao and Xu, 1986). The results of phase II clinical studies of ATFu indicated that ATFu had definite effects against large intestine carcinoma, gastric carcinoma and esophageal carcinoma, while its clinical application might be limited due to the instability both in vitro and in vivo (Li et al., 2001). Thereby, TFu was synthesized and evaluated in our group according to the metabolism mechanism of ATFu. ATFu was metabolized

* Corresponding author at: The School of Pharmaceutical Science, Shandong University, 44 Wenhua West Road, Ji'nan, Shandong Province, China. Tel.: +86 531 88382015; fax: +86 531 88382548.
E-mail address: zhangnancy9@sdu.edu.cn (N. Zhang).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.11.017

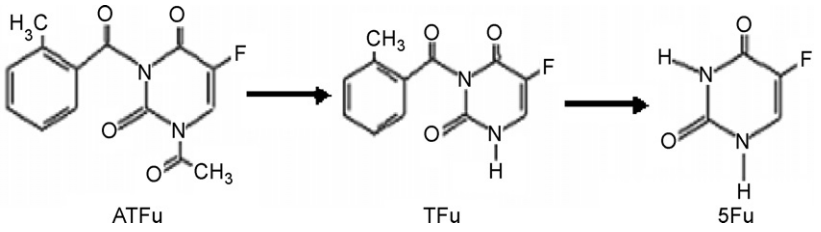

Fig. 1. Scheme of ATFu and TFu degradation process in vivo.

to TFu very fast in vivo, then TFu metabolized to 5-Fu slowly (Liu et al., 2006; Yan, 2004) (Fig. 1). The stability of TFu is better than ATFu, and as a prodrug of 5-Fu, TFu is expected to exhibit the tumor target effect due to the higher level of amidase activity in the tumor cells, and release 5-Fu in tumor tissues selectively. Thus, both the therapeutic index of TFu and the compliance of the patients could be enhanced. Also, the strong anticancer activity of TFu had been demonstrated in animals (a patent application is during the procession).

Encapsulating anticancer drugs in liposomes enables drug target to tumor tissues and prevents damage to the normal surrounding tissues (Kshirsagar et al., 2005). Liposomal formulation could improve the systemic absorption of drugs after oral administration and get higher bioavailability (Fielding, 1991), and it had been demonstrated the insoluble drug molecules could pass through the G.I. tract when encapsulated in liposomes (Arien and Dupuy, 1997). Liposomes are commonly manufactured by the thin film hydration method. However, this method is considered only suitable in laboratories, thereby, high-pressure homogenization technique is frequently employed for the preparation of large-scale production of liposomes now (Brandl et al., 1998; Pupo et al., 2005). The liposomes prepared by this technique also had smaller particle diameter and narrow size distribution (Muller and Peters, 1998).

The purpose of the present study was to synthesize TFu, and develop a liposomal formulation of it for oral and intravenous administration. TFu-loaded liposomes were prepared by the film dispersion–homogenization techniques. The entrapment efficiencies were determined by protamine aggregation method and the stabilities as well as the drug release characteristics of TFu-loaded liposomes were investigated. Furthermore, in vivo pharmacokinetic characteristics and bioavailabilities were evaluated in mice after oral or intravenous administration of TFu-loaded liposomes.

2. Materials and methods

2.1. Materials

Injectable soya lecithin was provided by Shanghai Taiwei Pharmaceutical Co. Ltd. (Shanghai, China). Cholesterol was obtained from Shanghai Medical Chemical Reagent Co. Ltd. (Shanghai, China). The powdered 5-Fu was a gift from Institute of Medicinal Chemistry of Shandong University. Protamine sulfate was purchased from Sigma (China). All the other chemicals and reagents used were of analytical purity grade or higher, obtained commercially.

2.2. Synthesis of N_3-O-toluyl-5-fluorouracil (TFu)

Forty-eight milliliters anhydrous pyridine solution of 5-Fu (0.2 mol, 2.6 g) was added dropwise to a mixture of 12 ml anhydrous pyridine and 7.8 ml *p*-toluoyl chloride (0.06 mol). The reaction was maintained under stirring at 10–15 °C for 3 h, then the mixture was poured into ice water, and extracted with benzene thrice. The combined extract was washed with distilled water, dried over anhydrous Na_2SO_4, and the solvent was removed by reduced pressure distillation. The resulting oily residue was washed with *n*-hexane, and purified by fractional crystallization, and the white crystals of TFu (yield, 78.2%; mp, 156.5–157.5 °C) were obtained.

2.3. Preparation of TFu-loaded liposomes

The thin film hydration method was selected to prepare the crude liposomes suspension (Pupo et al., 2005). The parameters such as weight ratio of drug to lipids (lecithin and cholesterol) (*D*/*L* ratios), weight ratio of soya lecithin to cholesterol (SL/*C* ratios) and the volume ratio of ether to phosphate buffer (*O*/*W* ratios) were optimized each at three or four levels taking the entrapment efficiency as index. When one factor was under investigated, the other two were fixed and the fixed parameters (*D*/*L* ratios, SL/*C* ratios and *O*/*W* ratios) were 1:10, 8:1 and 2.5:1, respectively. The TFu-loaded liposomes were obtained from the crude liposomes suspension by homogenization (Brandl et al., 1990). The formulations and the manufacturing parameters were optimized concerning both drug capsulation efficiency and particle size.

The optimal formulation was as follows: soya lecithin, cholesterol (8:1, w/w) and TFu (drug-to-lipids ratio, w/w of 1:10) were dissolved in ether (the concentration of lipids was 20 mg/ml), organic solvent was evaporated to form a thin lipid film (RE52-98 Shanghai Yarong Biochemistry Instrument Factory) under vacuum in water bath at 40 °C for 30 min, then the lipid film was exposed to vacuum at room temperature for 2 h to remove traces of ether. The resulting thin film was hydrated in pH 7.4 phosphate buffer (PBS, lipid-to-PBS, 5:1, v/v) to make the crude liposomes suspension. The crude liposomes suspension was homogenized (Niro Soavi Co., Italy) for five cycles at a pressure of 80 bar. Sufficient quantum of mannitol was added into the homogeneous liposomes suspension (ratio of mannitol to lipids, 1:1, w/w), filtered through a membrane with 0.45 μm pore size (Phenomenex, 25 mm filter, CA, USA) and lyophilized (LGJ0.5, Beijing Four-Ring Scientific Instrument Co., China).

2.4. Determination of entrapment efficiency and drug loading

Liposome entrapment efficiency was determined using the protamine aggregation method (Sun et al., 2006). Briefly, 0.1 ml liposomes was mixed with an equal volume of protamine solution (10 mg/ml) on a vortex mixer for 60 s, then 3 ml normal saline (N.S.) was added and the mixture was centrifuged at 3000 rpm for 30 min (80-2, Yuhua instrument Co. Ltd). Drug content in the supernatant was measured at 258 nm by RP-HPLC using an SPD-10Avp Shimadzu pump and an LC-10Avp Shimadzu UV–vis detector. Samples were chromatographed on a 4.6 mm × 250 mm reverse phase stainless steel column packed with 5 μm particles (Venusil XBP C-18, Agela, China) eluted with a mobile phase consisting of 50:50 (v/v) mixture of acetonitrile and water at a flow rate of 1 ml/min.

Ten miroliters of the liposome dispersion was dissolved in 5.0 ml methanol, and the drug content in the liposome dispersion was detected by the same HPLC method described above. The drug entrapment efficiency (EE) and drug loading (DL) were calculated from Eqs. (1) and (2), respectively:

$$\mathrm{EE}\% = \frac{W_{\mathrm{total}} - W_{\mathrm{free}}}{W_{\mathrm{total}}} \times 100 \quad (1)$$

$$\mathrm{DL}\% = \frac{W_{\mathrm{total}} - W_{\mathrm{free}}}{W_{\mathrm{lipids}}} \times 100 \quad (2)$$

W_{free} is the analyzed weight of free drug in the supernatant; W_{total} the analyzed weight of drug in the liposomes dispersions; W_{lipids} the total weight of lipids.

2.5. Characterization of TFu-loaded liposomes

The morphology of TFu-loaded liposomes was examined by transmission electron microscopy (JEM-1200EX, Japan). Samples were prepared by placing a drop of fresh prepared liposomes suspension onto a copper grid and air-dried, following negative staining with a drop of 3% aqueous solution of sodium phosphotungstate for contrast enhancement.

The average diameter, polydispersity index and zeta potential were determined by laser light scattering (Zetasizer 3000SH, Malvern Instruments Ltd, England). The lyophilized TFu-loaded liposomes were suspended with PBS (pH 7.4) before measured.

2.6. Differential scanning calorimeter (DSC) analysis

Thermal behaviour of the TFu-loaded liposomes were analyzed using differential scanning calorimeter (CDR-4P, Shanghai, China) and data were collected on a computer. Approximately 10 mg of TFu-loaded liposomes were analyzed in the open aluminium pans. A scan rate of 10 °C/min was employed in the 25–500 °C temperature range. To evaluate the internal structure modifications after drug incorporation, analysis was performed on TFu, cholesterol, soya lecithin, their physical mixtures, mannitol and the lyophilized TFu-loaded liposomes.

2.7. Stability studies

The freeze-dried TFu-loaded liposomes were stored at 4 °C for 9 months under a sealed condition. The mean particle size and drug entrapment efficiency were determined at fixed time intervals, respectively.

2.8. The drug release experiment in vitro

The in vitro release of TFu from liposomes was performed using the dialysis bag diffusion technique (Avgoustakis et al., 2002), according to the dissolution test apparatus of China pharmacopoeia (2005 edition, paddle method). Phosphate buffer (PBS, pH 7.4) was used as dissolution medium. The dialysis bags (molecular weight cutoff 12,000–14,000 Da, Sigma) were soaked in deionized water for 12 h before use. Two milliliters of TFu-Sol (100 μg/ml, 50% of acetonitrile, equivalent to 200 μg TFu) and 2 ml of diluted TFu liposomal dispersion (100 μg/ml, equivalent to 200 μg TFu) were placed in dialysis bags with the two ends fixed by thread (the distance of the two nodes was 4 cm, the total exposed surface area was 16 cm^2), respectively. Each bag was put into the flask containing 200 ml of dissolution medium. The flasks were placed into water bath at 37 ± 0.5 °C with paddle rotation at 100 rpm. Aliquots of the dissolution medium (5 ml) were withdrawn at each time interval and the same volume of fresh PBS was added to the flask to maintain the constant volume. Drug concentrations in the dissolution medium were finally analyzed using the RP-HPLC method as described previously. The release experiments were carried out in triplicates. The release rate was calculated from Eq. (3) and the results are expressed as means ± standard deviation:

$$Q_n = C_n V_0 + \sum_{i=0}^{n-1} C_i V_i \qquad \text{release rate } (\%) = \frac{Q_n}{W} \times 100\% \quad (3)$$

Q_n is the accumulative drug release mass; C_n the drug concentration in the release medium of each time interval; V_0 the total volume of the release medium; V_i the volume of the withdrawn medium; C_i the drug concentration in the release medium at time; W the total drug content of the release sample.

2.9. The bioavailability studies in mice

The bioavailability studies were complied with the requirements of the National Act on the use of experimental animals (People's Republic of China). Kunming mice (28 ± 1 g, supplied by the Medical Animal Test Center of Shandong University) were used in the experiments. The mice were housed under normal conditions, and fasted overnight before the experiment with free access to water. Animals were randomly assigned into four groups of 55 mice/group. Mice in group 1 were orally administered with TFu suspension (equivalent to 100 mg/kg of TFu, 168 mg TFu were suspended in 30 ml PBS, pH 7.4, 0.5 ml/mice) and the group 2 was orally administered with TFu-loaded liposomes suspensions (equivalent to 100 mg/kg of TFu, 3.36 g lyophilized TFu-loaded liposomes were suspended in 30 ml

PBS, pH 7.4, 0.5 ml/mice). Similarly, TFu 50% alcohol solution (equivalent to 100 mg/kg of TFu, 210 mg TFu were dissolved in 15 ml 50% of alcohol, 0.2 ml/mice) and TFu-loaded liposomes (equivalent to 100 mg/kg of TFu, 4.2 g lyophilized TFu-loaded liposomes were suspended in 15 ml PBS, pH 7.4, 0.2 ml/mice) were intravenously administered to the other two groups, respectively. All the samples were filtered through a 0.45 μm pore size Phenomenex filter before administration. Blood samples were collected from the postorbital venous plexuses of mice and placed into heparinized test tubes according to the designed time interval (Gutierro et al., 2002). The plasma samples were harvested after centrifugation (15 min, 4000 rpm) and stored at −20 °C until analysis.

Liquid–liquid extraction was performed prior to the analysis the contents of TFu in plasma by RP-HPLC method mentioned above. Briefly, 200 μl of NaH_2PO_4 (0.5 mol/l) and 2 ml acetic ether were added into 200 μl plasma sample, respectively, the mixture was vortexed for 3 min, then centrifuged (3000 rpm, 5 min). One milliliter supernatant was transferred to a clean test tube thereafter and evaporated under nitrogen at 60 °C. The resulting residue was reconstituted with 500 μl of mobile phase (acetonitrile–water, 50:50, v/v) and filtered through a 0.22 μm Phenomenex filter for RP-HPLC determination. Pharmacokinetic parameters were evaluated by Drug And Statistics (DAS 2.0 program, Mathematical Pharmacology Professional Committee, China).

3. Results and discussion

3.1. Synthesis of N_3-O-toluyl-5-fluorouracil (TFu)

TFu was obtained by *N*-benzoylation of 5-Fu (Fig. 2). TFu is a white lamellar crystal, mp 156.5–157.5 °C, IR (KBr) cm^{-1}: $\nu_{=CH}$ (3196.9), ν_{-NH} (3094.7), ν_{CH_3} (2956.8), $\nu_{C=O}$ (1773.2, 1714.5, 1660.7), $\nu_{C=C}$ (1249.4, 1230.1), γ_{Ar-H} (778.7, 749.3, 730.5). ^{1}H-NMR (300 MHz, DMSO) δ 11.49 (1H, s, H-1), 8.04 (1H, m, H-6), 7.83 (1H, d, J = 7.8 Hz, H-6′), 7.61 (1H, t, J = 7.8 Hz, H-4′), 7.45 (1H, d, J = 7.8 Hz, H-3′), 7.36 (1H, t, J = 7.8 Hz, H-5′), 2.58 (3H, s, CH_3). MS m/z 249 ($M+1^+$). Obviously, TFu increased lipophilicity of 5-Fu due to *N*-benzoylation.

3.2. Preparation of TFu-loaded liposomes

The effects of the three influential factors, *D*/*L* ratios (X_1), SL/*C* ratios (X_2), *O*/*W* ratios (X_3), on encapsulation efficiency (EE) of liposomes were shown in Table 1. The results showed that the maximal value of EE could be reached when *D*/*L* ratio was set at 1:10 or 1:15. The optimized SL/*C* ratio and *O*/*W* ratio were 8:1 and 5:1, respectively.

Table 1
The levels of experimental factors and the liposomes entrapment efficiencies

	X_1 (w/w)	EE (%)	X_2 (w/w)	EE (%)	X_3 (V/V)	EE (%)
1	1:5	70	4:1	69	1:1	74
2	1:7	73	8:1	81	1.5:1	71
3	1:10	81	10:1	74	2.5:1	76
4	1:15	80			5:1	86

X_1: weight ratio of drug to lipids (*D*/*L* ratios). X_2: weight ratio of soya lecithin to cholesterol (SL/*C* ratios). X_3: volume ratio of ether to phosphate buffer (*O*/*W* ratios).

The drug loading could be enhanced with the increase of *D*/*L* ratio, while a higher lipids ratio (the weight ratio of drug to lipids was 1:15 to 1:10) improved EE in this study. Moreover, EE value increased when SL/*C* ratio increased until SL/*C* ratio reached to 8:1. Certain concentration of cholesterol could reduce the bilayer permeability, increase the stability of liposomes and cause high EE (Gregoriadis, 1993), while beyond a certain ratio, cholesterol could disrupt the regular structure of the liposomal membrane, result in lower EE (El-Samaligy et al., 2006). It was very hard to form liposomes when SL/*C*h ratio was 1:1, and the stability was low when SL/*C*h ratio was 4:1. Furthermore, EE of the hydrophobic drugs depends on the volume of hydrophobic bilayers, the volume of phosphate buffer solutions hydrating lipids could affect the ratio between the aqueous interior and hydrophobic bilayers of the vesicles. In our case, the higher *O*/*W* ratio (5:1) caused higher EE and better stability in vitro.

The homogenization could result in more homogeneous liposome dispersions with higher surface area (Lippacher et al., 2002). The influences of homogenization pressure on the particle size and encapsulation efficiency of TFu-loaded liposomes were investigated by varying the homogenization pressure (Table 2). The high homogenization pressure caused high shear forces to shatter the liposomes, the mean particle size significantly decreased when the homogenizing pressure increased, while the entrapment efficiency reduced at the same time (Tan and Nakajima, 2005). Since the entrapment capacity of liposomes for drugs strongly diminishes with decrease of liposome size, larger liposomes were considered more promising pharmaceutical carriers (Torchilin et al., 2001). Therefore, the optimized homogenization process was five cycles under a pressure of 80 bar. The optimized formulation was repeated in triplicates. The average entrapment efficiency and the average drug loading of TFu-loaded liposomes were (88.87 ± 3.25%) and (8.89 ± 0.19%), respectively.

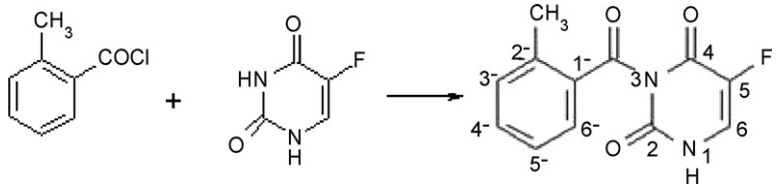

Fig. 2. The synthesis route of TFu.

Table 2
The liposomes entrapment efficiencies under different homogenization pressures (five circles) (mean ± S.D., $n = 3$)

	Pressure (bar)							
	0	10	80	200	300	600	900	1500
EE (%)	88.5 ± 3.57	88.5 ± 3.01	88 ± 3.25	85.6 ± 3.67	75 ± 3.98	78.0 ± 4.57	68 ± 3.99	53 ± 4.08
Size (nm)	532 ± 16.3	530 ± 7.21	400 ± 9.84	320 ± 9.26	307 ± 6.25	180 ± 3.79	175 ± 2.02	101 ± 2.89

3.3. Characterization of TFu-loaded liposomes

The liposomes with spherical or hexagonal in shape (Fig. 3), the hexagonal-shaped liposomes might be formed via microfluidization through high shear forces of homogenization, similar liposomes shape had been reported by other group (Brandl et al., 1998). In general, a high homogenization pressure ensures a good microfluidization process and therefore leads to smaller particles with a satisfactory span value. The mean diameter of TFu-loaded liposomes was (400.5 ± 9.6 nm) the polydispersity index was 0.093 and the zeta potential was (−6.4 ± 0.8 mV).

3.4. Differential scanning calorimetry (DSC)

In this study, DSC was used as a useful technique to ascertain the physicochemical status of drug inside the excipients, assess the interaction amongst different components during the fabrication process. Fig. 4 exhibited the DSC thermograms of TFu, cholesterol, soya lecithin, their physical mixtures, mannitol and the lyophilized TFu-loaded liposomes. Pure TFu showed a single sharp endothermic peak of melting at about 173.5 °C, while no TFu peak was observed in the curve of the lyophilized TFu-loaded liposomes, only a wide peak (130.8–173.6 °C) was presented. This showed that TFu successfully encapsuled into liposomes, was not in a crystalline state but in an amorphous state (Dubernet, 1995). It might be explained that the lipids inhibited the crystallization of TFu during the formulation fabrication process. Similar results were reported by other groups (Venhateswarlu and Manjunath, 2004; Hou et al., 2003). Polymorphs are different states of a drug that may have different physicochemical properties, such as melting point, solubility, morphology, density and bioavailability. The amorphous forms are associated with higher energy with increased surface area, subsequently higher solubility, dissolution rates and bioavailability (Morissettea et al., 2004). The encapsulated amorphous drug was likely to have higher energy and therefore showed increased solubility, dissolution rates and higher bioavailability (Corrigan et al., 2003).

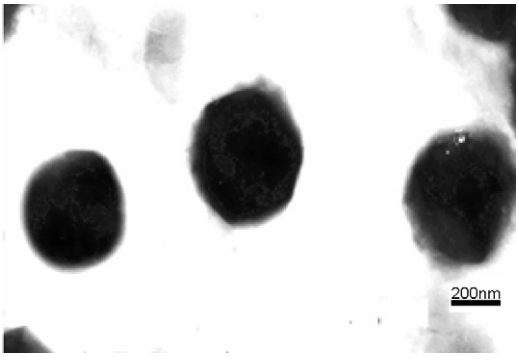

Fig. 3. Transmission electron photomicrograms of TFu-loaded liposomes.

3.5. Stability studies

The stability test of the freeze-dried TFu-loaded liposomes at 4 °C indicated that the mean particle size and drug entrapment efficiency had no significant change during 9 months (Table 3).

3.6. In vitro drug release

The release experiment was conducted under sink conditions and the dynamic dialysis was employed for separation of free drug from TFu-loaded liposomes. The release of TFu from TFu-loaded liposomes followed the bi-exponential equation and could be expressed by the following equation: $100 - R = 98.18e^{-0.2735t} + 40.89e^{-0.028t}$, $r_\alpha = 0.9990$, $r_\beta = 0.9963$. The initial fast release of around 50% of the drug from the TFu-loaded liposomes was observed in the first 2.5 h, which could be probably due to the portion of the drug that leaked out of liposomes and the unloaded drug (Glavas-Dodov et al., 2005). Subsequently, the release of drug from liposomes was slower and a release plateaux was obtained from 4 to 48 h and less than 10% of the drug remained encapsulated in liposomes after 48 h of dialysis (Fig. 5). The delayed release

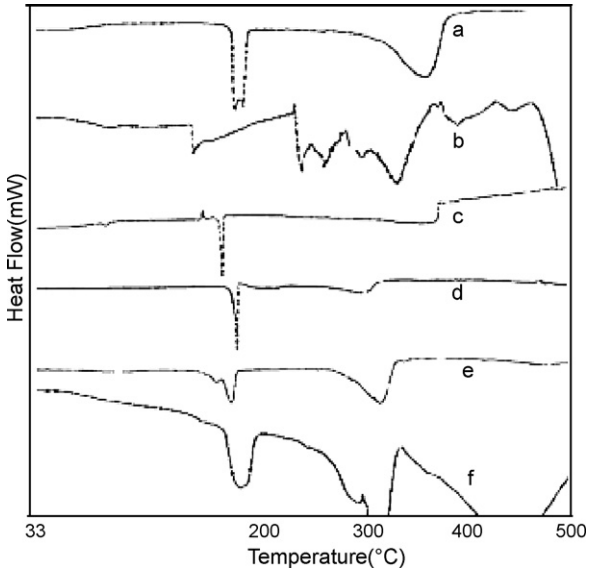

Fig. 4. DSC thermograms of (a) mannitol, (b) soya lecithin, (c) cholesterol, (d) TFu, (e) lyophilized TFu-loaded liposomes, and (f) physical mixture of TFu, soya lecithin and cholesterol.

Table 3
Physical stability of the freeze-dried TFu-loaded liposomes ($n = 3$)

	Time (days)							
	0	1	7	15	30	90	180	270
EE (%)	88.5 ± 2.65	88.2 ± 3.72	88.3 ± 4.13	88.0 ± 1.97	87.4 ± 4.29	86.8 ± 1.66	85.1 ± 2.08	84.7 ± 3.03
Size (nm)	400.3 ± 9.33	400.5 ± 7.61	401.9 ± 8.35	401.9 ± 4.12	402.6 ± 9.97	403.3 ± 8.05	405.6 ± 7.89	405.9 ± 5.75

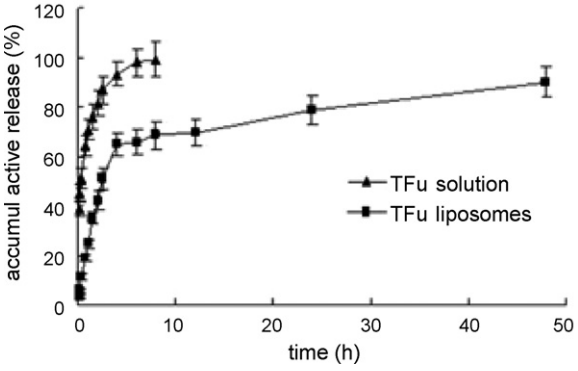

Fig. 5. In vitro release profile of TFu from TFu 50% alcohol solution and TFu-loaded liposomes in phosphate-buffered saline (pH 7.4) at 37 ± 0.5 °C ($n = 3$).

might be attributed to the lipophilic TFu that was held by the small fragment of the liposomal membrane and the drug encapsulated in lipid membrane that released mainly through dissolution and diffusion from the lipid bilayer (Cócera et al., 2000). In contrast, the release of TFu from TFu-Sol was much fast, approximately 99.3% of the drug had been dissolved in phosphate-buffered saline (pH 7.4) after 8 h. The release dynamics characteristics was fitted to first-order kinetics model and could be expressed by the following equation: $\ln(100 - R) = -0.5585t + 4.037$, $r = 0.9984$.

3.7. The bioavailability studies in mice

The analysis results of the pharmacokinetic studies data by DAS (2.0) program indicated that all the TFu formulations fitted to the open two-compartment model following i.v. and oral administration, the weight coefficient was expressed $1/c^2$. Fig. 6 showed the mean concentration in plasma versus time profile of TFu after oral or i.v. administration of liposomes and suspension (or 50% alcohol solution) at a dose of 100 mg/kg to mice, the pharmacokinetic parameters were summarized in Table 4.

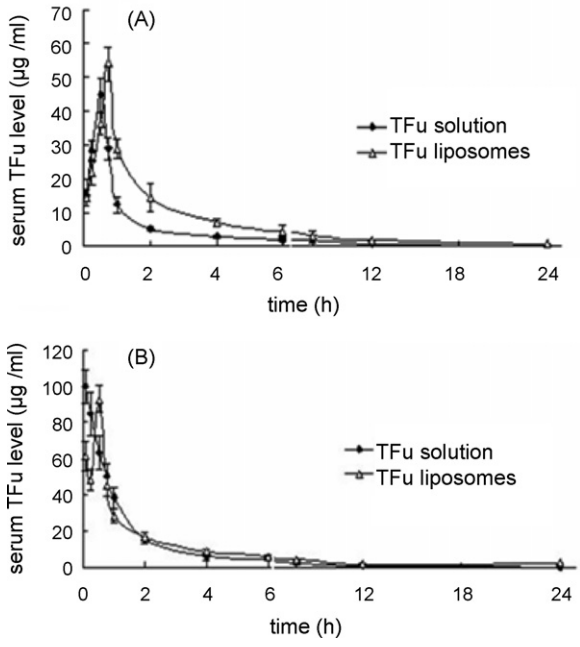

Fig. 6. Mean plasma concentration of TFu after oral administration of TFu suspension and TFu-loaded liposomes in mice (A), and after intravenous administration of TFu 50% alcohol solution and TFu-loaded liposomes in mice (B) (mean ± S.D., $n = 5$).

Table 4
The pharmacokinetic parameters of TFu after intravenous administration of TFu 50% alcohol solution and TFu-loaded liposomes or oral administration of TFu suspension and TFu-loaded liposomes in mice (mean ± S.D., $n = 5$)

Parameters	i.v.		Oral	
	TFu 50% alcohol solution	TFu-loaded liposomes	TFu suspension	TFu-loaded liposomes
AUC_{0-24} (mg/l h)	146.67 ± 13.82	168.62 ± 18.54	57.54 ± 8.12	114.47 ± 14.59$^{\#\#}$
MRT $_{0-24}$ (h)	1.98 ± 0.56	5.14 ± 0.77**	2.42 ± 0.54	3.84 ± 0.85$^{\#}$
CL/F (l/h/kg)	0.79 ± 0.21	0.45 ± 0.11*	2.16 ± 0.73	0.87 ± 0.25$^{\#\#}$
$T_{1/2\alpha}$ (h)	0.40 ± 0.13	1.24 ± 0.43**	0.71 ± 0.16	0.99 ± 0.15
$T_{1/2\beta}$ (h)	2.14 ± 0.39	11.95 ± 0.92**	2.44 ± 0.16	4.47 ± 0.24$^{\#\#}$
T_{max} (h)	0.083	0.500	0.500	0.750
C_{max} (mg/l)	99.72 ± 5.97	92.80 ± 3.00	44.92 ± 5.25	54.05 ± 5.68$^{\#}$

* $P < 0.05$ vs. TFu 50% alcohol solution.
** $P < 0.01$ vs. TFu 50% alcohol solution.
$^{\#}$ $P < 0.05$ vs. TFu suspension.
$^{\#\#}$ $P < 0.01$ vs. TFu suspension.

Oral liposomal administration showed a higher C_{max} compared with the TFu suspension ($P < 0.05$). The $AUC_{0-24\,h}$ following oral liposomal administration was significantly higher than TFu suspension ($P < 0.01$), the relative bioavailability was 199%. Compared with the TFu suspension, MRT and $T_{1/2\beta}$ increased by about 0.6 and 0.8 times, respectively, while CL decreased by 1.5 times. This could be explained by the increased affinity between TFu-loaded liposomes and the gut liquid, which caused better dispersibility of TFu in the gastrointestinal tract, prolonged the drug residence time in the absorption sites, and resulted in better absorption effects (Vasir et al., 2003). Compared with the i.v. administration of the TFu 50% alcohol solution, the absolute bioavailability of TFu suspension and TFu-loaded liposomes were 39.23% and 78.05%, respectively, this confirmed the better absorption effects of TFu-loaded liposomes after oral adminstration. On the other hand, TFu-loaded liposomes were bioequivalent after i.v. administrated in mice compared with TFu 50% alcohol solution (115% of absolute bioavailability).

TFu-loaded liposomes showed a lower C_{max} ($P < 0.05$) compared with the TFu 50% alcohol solution after i.v. injection. These meant that the acute toxicity might be reduced after intravenous liposomal administration compared with the TFu 50% alcohol solution. TFu-loaded liposomes were remarkably effective in prolonging the drug retention time in vivo after intravenous administration compared with the TFu 50% alcohol solution. Both the rapid uptake of liposomes by the organs of the reticuloendothelial system (RES) and the slow release due to the reservoir effect might be the reasons (Klibanov and Huang, 1992). Liposomes as circulating reservoirs in tissues probably enter the blood again, and repeatedly, achieve dynamic balance eventually. The prolonged drug circulation was consistent with the results obtained from the release in vitro.

4. Conclusion

In this study, a lipophilic prodrug of 5-Fu–TFu was successfully synthesized, and incorporated into liposomes for applying to oral and intravenous administration. The liposomes formulation did enchance the gastrointestinal absorption of TFu by oral administration, about twofold of relative bioavailability comparing to TFu suspension was observed. TFu-loaded liposomes were bioequivalent after i.v. administrated in mice comparing with TFu 50% alcohol solution, and had significantly prolonged circulation duration, which was consistent with the results obtained from the release tests in vitro. Further studies are needed to focus on the safety and efficiency of TFu-loaded liposomes to evaluate the potential clinical application value.

Acknowledgements

We thank Tao Wu (Shandong University) for the average diameter, polydispersity index and zeta potential measurements. We also thank Ms. Du (Shandong Province Medical Institute) for the transmission electron microscopy examination.

References

Arien, A., Dupuy, B., 1997. Encapsulation of calcitonin in liposomes depends in the vesicle preparation method. J. Microencapsul. 14, 753–760.

Avgoustakis, K., Beletsi, A., Panagi, Z., 2002. PLGA-mPEG nanoparticles of cisplatin: in vitro nanoparticle degradation, in vitro drug release and in vivo drug residence in blood properties. J. Control. Release 79, 123–135.

Brandl, M., Bachmann, D., Drechsler, M., Bauer, K.H., 1990. Liposome preparation by a new high pressure homogenizer Gaul in Micron Lab 40. Drug Dev. Ind. Pharm. 16, 2167–2191.

Brandl, M., Drechsler, M., Bachmann, D., Tardi, C., Schmidtgen, M., Bauer, K.H., 1998. Preparation and characterization of semi-solid phospholipid dispersions and dilutions thereof. Int. J. Pharm. 170, 187–199.

Cócera, M., López, O., Coderch, J.L., 2000. Alterations in stratum corneum lipid liposomes due to the action of Triton X-100: influence of the level of ceramides on this process. J. Control. Release 68, 387–396.

Corrigan, D.O., Healy, A.M., Corrigan, O.I., 2003. The effect of spray drying solutions of bendroflumethiazide/polyethylene glycol on the physicochemical properties of the resultant materials. Int. J. Pharm. 262, 125–137.

Dubernet, C., 1995. Thermoananlysis of microspheres. Thermochim. Acta 248, 259–269.

El-Samaligy, M.S., Afifi, N.N., Mahmoud, E.A., 2006. Increasing bioavailability of silymarin using a buccal liposomal delivery system: preparation and experimental design investigation. Int. J. Pharm. 308, 140–148.

Fielding, R.M., 1991. Liposomal drug delivery: advantages and limitations from a clinical pharmacokinetic and therapeutic perspectives. Clin. Pharmacokinet. 21, 155–164.

Fraile, R.J., Baker, L.H., Buroker, T.R., Horwitz, J., Vaitkevicius, V.K., 1980. Pharmacokinetics of 5-fluorouracil administered orally, by rapid intravenous and by slow infusion. Cancer Res. 40, 2223–2228.

Glavas-Dodov, M., Fredro-Kumbaradzi, E., Goracinova, K., Simonoska, M., Calis, S., Trajkovic-Jolevska, S., Hincal, A.A., 2005. The effects of lyophilization on the stability of liposomes containing 5-FU. Int. J. Pharm. 291, 79–86.

Gregoriadis, G., 1993. Liposome Technology, 2nd ed. CRC Press, Boca Raton, FL, US.

Gutierro, I., Hernandez, R.M., Igartua, M., 2002. Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres. Vaccine 21, 67–77.

Hou, D.Z., Xie, C.S., Huang, K.J., Zhu, C.H., 2003. The production and characteristics of solid lipid nanoparticles (SLNs). Biomaterials 24, 1781–1785.

Immordino, M.L., Brusa, P., Rocco, F., Arpicco, S., Ceruti, M., Cattel, L., 2004. Preparation, characterization, cytotoxicity and pharmacokinetics of liposomes containing lipophilic gemcitabine prodrugs. J. Control. Release 100, 331–346.

Klibanov, A.L., Huang, L., 1992. Long-circulating liposomes: development and perspectives. Liposome Res. 2, 321–334.

Kshirsagar, N.A., Pandya, S.K., Kirodian, G.B., Sanath, S., 2005. Liposomal drug delivery system from laboratory to clinic. J. Postgrad. Med. 51, 5–15.

Li, Q., Feng, F.Y., Han, J., Sui, G.J., Zhu, Y.G., Zhang, Y., Zhang, Z.H., Wang, P.H., Zhou, M.Z., Zhang, Y.C., 2001. Phase II clinical study of a new anticancer drug—Atofluding. Chin. J. Cancer 20, 1387–1390.

Lippacher, A., Muller, R.H., Mader, K., 2002. Semisolid SLNTM dispersions for topical application: influence of formulation and production parameters on viscoelastic properties. Eur. J. Pharm. Biopharm. 53, 155–160.

Liu, J., Xu, W.F., Zhou, Y., Yuan, Y.X., Qu, X.J., 2006. Effect of growth inhibition on tumour cells induced by N_3-*O*-toluyl-flulorouracil. Chin. Pharm. J. 37, 252–255.

Morissettea, S.L., Almarsson, O., Peterson, M.L., Remenar, J.F., Read, M.J., Lemmo, A.V., Ellis, S., Cima, M.J., Colin, R.G., 2004. High-throughput crystallization: polymorphs, salts, co-crystals and solvates of pharmaceutical solids. Adv. Drug Deliv. Rev. 56, 275–300.

Muller, R.H., Peters, K., 1998. Nanosuspensions for the formulation of poorly soluble drugs. I. Preparation by a size-reduction technique. Int. J. Pharm. 160, 229–237.

Pupo, E., Padron, A., Santana, E., Sotolongo, J., Quintana, D., Duenas, S., Duarte, C., de la Rosa, M.C., Hardy, E., 2005. Preparation of plasmid DNA-containing liposomes using a high-pressure homogenizationextrusion technique. J. Control. Release 104, 379–396.

Sun, W.T., Huang, G.H., Ye, J.S., Zhang, N., 2006. Determination encapsulation efficiencies of liposomes and nanoliposomes by protamine aggregation method. Chin. Pharm. J. 41, 43–47.

Tan, C.P., Nakajima, M., 2005. β-Carotene nanodispersions: preparation, characterizationand stability evaluation. Food Chem. 92, 661–671.

Torchilin, V.P., Levchenko, T.S., Lukyanov, A.N., Khaw, B.A., Klibanov, A.L., Rammohan, R., Samokhin, G.P., Whiteman, K.R., 2001. *p*-Nitrophenylcarbonyl-PEG-PE-liposomes: fast and simple attachment of specific ligands, including monoclonal antibodies, to distal ends of PEG chains via *p*-nitrophenylcarbonyl groups. Biochim. Biophys. Acta 1511, 397–411.

Vasir, J.K., Tambwekar, K., Garg, S., 2003. Bioadhesive microspheres as a controlled drug delivery system. Int. J. Pharm. 255, 13–32.

Venhateswarlu, V., Manjunath, K., 2004. Preparation, characterization and in vitro release kinetics of clozapine solid lipid nanoparticles. J. Control. Release 95, 627–638.

Yan, M.X., 2004. The synthesis and proclinical study of multiple-grade prodrugs of 5-fluorouracil. Shandong University post-graduate dissertation.

Yu, B.T., Sun, X., Zhang, Z.R., 2003. Enhanced liver targeting by synthesis of N_1-stearyl-5-Fu and incorporation into solid lipid nanoparticles. Arch. Pharm. Res. 6, 1096–1101.

Zamboni, W.C., 2005. Liposomal, nanoparticle, and conjugated formulations of anticancer agents. Clin. Cancer Res. 11, 8230–8234.

Zhao, B., Xu, W.F., 1986. Studies of 1-acetyl-3-*O*-toluyl-5-fluorouracil. I. Antitumor activity. Acta Acad. Med. Shan Dong 24, 43–45.