Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(1):408-415

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Preparation of lomustine-iohexol thermosensitive compound liposomes and study on the *in vitro* release characteristics

Shuoye Yang[1*] and Yun Guo[2]

[1]College of Bioengineering, Henan University of Technology, Zhengzhou, P. R. China
[2]Zhengzhou health school, Zhengzhou, P. R. China

ABSTRACT

To prepare lomustine-iohexol thermosensetive compound liposomes, and evaluate the properties and in vitro release behaviors of two drugs enveloped. Lomustine-iohexol thermosensetive compound liposomes were prepared by reverse evaporation method, HPLC assays for determination of lomustine and iohexol in vitro were developed, and their release property was studied by using dislysis method. The mean diameter of thermosensitive liposomes was 266.4 nm, Zeta potential was -21.8mV, and phase-transition temperature was 41℃. The HPLC assays developed were specific, rapid and reliable, which can be used to determine lomustine and iohexol in vitro accurately. In vitro release tests showed that lomustine and iohexol were released more entirely at 41℃. The release profile of iohexol from liposomes at 41℃ fitted to First-order kinetics model with the equation of ln(100-Q)=-1.066t-0.0498 (r=0.9993), whereas the release profile of lomustine from liposomes at 41℃ fitted to Weibull model with the equation of lg[-ln(100-Q)]=1.1069lgt-0.0976 (r=0.9923). The formulation and preparation method can be successfully prepared lomustine-iohexol thermosensitive compound liposomes with good thermal-sensitive release behaviors of both drugs.

Keywords: lomustine; iohexol; thermosensitive liposomes; phase-transition temperature; releasing in vitro

---

INTRODUCTION

In the clinical treatment for cancer, thermochemotherapy that combined thermotherapy and chemotherapy has became one of the best curing. Specifically, the new thermochemotherapy can be play a better synergistic effect of thermotherapy and chemotherapy, thus increase the drug concentrations in tumor site, lead to improved anti-tumor effects while decrease toxic effects from chemotherapeutics on normal tissues that are not be heated [1, 2]. Thermosensitive liposomes are the important branch of liposomes preparation, can carry and release drugs at relatively higher temperature, and make the chemotherapeutics to be targeting released by heating up the diseased region [3]. Nowadays, thermosensitive liposomes are becoming the best drug carriers for thermochemotherapy; for we can use them to improve therapeutic effects and reduce the toxicity by effectively utilize the advantage of both liposomes and thermotherapy.

To prepare the thermosensitive compound liposomes in which lomustine and iohexol are both enveloped, we can use this lipid vectors as efficient tool for thermochemotherapy. Tumor cells could be killed by localized hyperthermia in vitro, and the two drugs would be targeting released simultaneously in tumor site, therefore, lead to improved significantly therapeutic effect and reduced side effect. Further, tumor areas could be defined by visualization with iohexol. In the former studies, we had prepared lomustine-iohexol compound liposomes, optimized the lipid formulation and developed entrapment efficiency determination method. In this study, we prepared lomustine-iohexol thermosensitive compound liposomes with L-α-dipalmitoyl phosphatidylcholine (DPPC) as thermal-sensitive material, and investigated their in vitro release characteristics in details, to provide elementary

experiment reference for the practical use in clinic.

EXPERIMENTAL SECTION

Chemicals and reagents

Lomustine was purchased from Shandong Weifang Pharmaceutical Factory Co., Ltd, iohexol was purchased from Zhejiang Starry Pharmaceutical Co., Ltd, DPPC was purchased from Sigma-Aldrich (St. Louis, MI, USA). Protamine Sulfate injection was provided by Shanghai No.1 Biochemical Pharmaceutical Co., Ltd, vitamin E (V_E) was purchased from Guangzhou rekon Food Chemicals Co., Ltd. Trichloromethane was purchased from Tianjin concord Technology Co. Ltd. Methanol and acetonitrile were of chromatographic pure and purchased from Fisher Scientific International Inc. All other reagents were of analytical grade.

Preparation of lomustine-iohexol thermosensitive compound liposomes

Thermosensitive compound liposomes were prepared by reverse phase evaporation method (RPE) [4]. In brief, a lipid mixture of lomustine, DPPC and V_E were dissolved in trichloromethane. Meantime iohexol was dissolved in purified water. After putting both the solutions in 45 ℃ water bath and shaking, iohexol solution was dropped into lipid solution to form an emulsification automatically, followed by further sonication for 2 min. Subsequently the organic solvent was dried under degree of vacuum lower than 0.05 Mpa through attaching to a rotary evaporator at 45 ℃.

Finally the liposome suspension was further disrupted by using ultrasonic probe. Resulting liposomes were sterilized by extruding through a 0.45 μm sterile filter. Thermosensitive compound liposomes were prepared the day before the experiment, stored overnight at 4℃.

Particle size and zeta potential measurement

For determination process, each sample was dispersed in deionized water to a final volume of 10 ml, their particle size and zeta potential were analyzed using Laser particle analyzer (Malvern Zetasizer 3000HS, Malvern, UK). Volume-weighted Gaussian size distribution was fit to the autocorrelation functions and particle size values were obtained.

Encapsulation efficiencies determination

Protamine aggregation method was used to separate free drugs and liposomes and determine the encapsulation efficiencies.

Differential scanning calorimetry (DSC)

1 ml thermosensitive compound liposomes were taken and mixed with protamine sulfate, after centrifugating at 3000 $r \cdot min^{-1}$ for 5 min the precipitate was collected and weighed. The settled liposomes were put on crucible for DSC measurements using a METTLER TOLEDO STAR-DSC (Mettler-Toledo, Switzerland). The heating scan was from 20–60 ℃ at the rate of 5 ℃/min [5]. DSC curves were analyzed by using the fitting program.

Development of releasing assay method for lomustine

Specificity. The reference solution of lomustine, demulsification solution of blank liposomes (no lomustine contained) and demulsification solution of thermosensitive compound liposomes were taken and injected for HPLC analysis (the chromatography operational condition would be reported in another paper), respectively.

Linearity. The lomustine testing solutions of different concentrations were prepared. The calibration curve samples were assayed in triplicate, using concentration (C) as abscissa (X) and peak area as ordinates (Y).

Recovery. The proper amounts of blank liposomes were mixed precisely with different volumes of stock solution, after filtering through a 0.45 μm sterile membrane, filtrate was collected for determination. Recoveries were calculated by comparing the mean concentration obtained from the tested solutions with that of the neat standard samples.

Development of releasing assay method for iohexol

Specificity. The reference solution of iohexol, demulsification solution of blank liposomes (no iohexol contained) and demulsification solution of thermosensitive compound liposomes were taken and injected for HPLC analysis (the chromatography operational condition would be reported in another paper), respectively.

Linearity. The iohexol testing solutions of different concentrations were prepared. The calibration curve samples were assayed in triplicate, using concentration (C) as abscissa (X) and peak area as ordinates (Y).

Recovery. The proper amounts of blank liposomes were mixed precisely with different volumes of stock solution, after filtering through a 0.45 μm sterile membrane, filtrate was collected for determination. Recoveries were calculated by comparing the mean concentration obtained from the tested solutions with that of the neat standard samples.

Study on the in vitro releasing of iohexol in thermosensitive compound liposomes

Assay method. Dislysis method was applied to determine release rate [6-8]. 1000 ml release medium was taken to dissolution glass at predetermined temperature, 1 ml thermosensitive compound liposomes were precisely transferred into the semipermeable membrane with leak sealing ends and located in release solvent. Release medium was agitated by stirring blades and sampled at 10, 20, 30 min, 1, 2, 4, 6, and 8 hours after experiment initiating. 5 ml sample was collected and filtered through a 0.45 μm membrane, filtrate was selected to determine at 37 ℃ and 41 ℃, respectively.

Iohexol content at each time point was determined by HPLC analysis, meanwhile, the proper amounts thermosensitive compound liposomes was demulsificated by methanol for the total drug amounts (*W*), the accumulative release amounts and release percent were calculated according to the formula:

$$Q_n = C_n V_0 + \sum_{i=0}^{n-1} C_i V_i \qquad \text{Accumulative release percent}(\%) = Q_n / W \times 100\%$$

Noting: Qn was the accumulative release amounts at each time point, Cn was the measured concentration at each time point, V_0 was the bulk volume of release medium, V_i was the sampling volume, C_i was the measured concentration at time point *i*, W was the total drug amounts in liposomes.

Release medium investigation. The in vitro releasing feature of iohexol was assessed using purified water and phosphate buffer (PBS, pH7.4) as release solvent, respectively, with the stirring blades rotation speed of 50 $r \cdot min^{-1}$.

Rotation speed investigation. The in vitro releasing feature of iohexol was assessed with the stirring blades rotation speed of 50, 75 and 100 $r \cdot min^{-1}$, respectively, using purified water as release solvent.

Releasing of iohexol at different temperature. The accumulative release percent of iohexol was determined at 37 ℃ and 41 ℃, respectively, with purified water as release solvent and rotation speed of 50 $r \cdot min^{-1}$.

Mathematical model fitting. The in vitro releasing data of iohexol at 41℃ was processed by using zero-order kinetics, first-order kinetics, Higuchi, Ritger-Peppas and Weibull equations, and the data obtained from all the time points set was selected to simulate.

Study on the in vitro releasing of lomustine in thermosensitive compound liposomes

Lomustine degradation kinetics investigation. Given the instability of lomustine in water solution, its contents would be clearly decrease with time, and the degradation rate increases as the temperature increases, thus lomustine degradation kinetics at 37℃ and 41℃ was studied in details [9-10]. Experiment was performed as follows: the proper amounts of lomustine was weighed and dissolved by methanol to the final concentration of $10\mu g \cdot ml^{-1}$. 99 ml purified water was taken to small dissolution glass at predetermined temperature, 1 ml lomustine solution was precisely located into dissolution glass. Agitated by and sampled at 10, 20, 30 min, 1, 2, 4 and 6 hours, 5 ml sample was collected for HPLC analysis to determine lomustine contents in water solution at different time points. Degradation rate of lomustine was evaluated by calculating undegradated percents at each time point comparing with the drug content at 0 min.

Releasing of lomustine at different temperature. Dislysis method was applied to determine release rate. 100 ml purified water was taken to small dissolution glass at predetermined temperature, 1 ml thermosensitive compound liposomes were precisely transferred into the semipermeable membrane with leak sealing ends and located in release solvent. Release medium was agitated by stirring blades with the rotation speed of 50 $r \cdot min^{-1}$and sampled at 10, 20, 30 min, 1, 2 and 4 hours after experiment initiating. 2 ml sample was collected and filtered through a 0.45 μm membrane, filtrate was selected to determine at 37℃ and 41℃, respectively.

Lomustine content at each time point was determined by HPLC analysis, meanwhile, the proper amounts thermosensitive compound liposomes was demulsificated by methanol for the total drug amounts (*W*). While calculating the accumulative release amounts, the degradated lomustine should also been included, thus the formula was revised as:

$$C_n^0 = \frac{C_n}{e^{k_1 t + k_2}} \qquad Q_n = C_n^0 V_0 + \sum_{i=0}^{n-1} C_i^0 V_i \qquad \text{Accumulative release percent}(\%) = Q_n / W \times 100\%$$

Noting: Qn was the accumulative release amounts at each time point, Cn was the measured concentration at each time point, Cn^0 was the practical concentration including that had been degradated, V_0 was the bulk volume of release medium, V_i was the sampling volume, C_i was the measured concentration at time point *i*, W was the total drug amounts in liposomes.

Mathematical model fitting. The in vitro releasing data of lomustine at 41℃ was processed by using zero-order kinetics, first-order kinetics, Higuchi, Ritger-Peppas and Weibull equations, and the data obtained from all the time points set was selected to simulate [11-13].

RESULTS AND DISCUSSION

Characterization of thermosensitive compound liposomes

The EE, particle size and zeta potential determination results of thermosensitive compound liposomes were shown in Table 1.

Table 1. Determination results of lomustine-iohexol thermosensitive compound liposomes

Batch	EE of lomustine/%	EE of iohexol/%	Particle size/nm	Zeta potential/mV
1	66.7	54.3	275.1	-21.8
2	62.9	56.6	266.4	-17.4
3	70.2	52.7	258.7	-23.9

DSC measurements

The DSC scanning result was shown in Fig 1, endothermic peak was detected at 39-43℃ and the peak value at 41℃. As the absorption amounts of heat of phospholipids would suddenly increase as they transform from gel to liquid crystal state, the phase-transition temperature of thermosensitive compound liposomes was defined at 41℃, which was consistent with that of DPPC [14, 15].

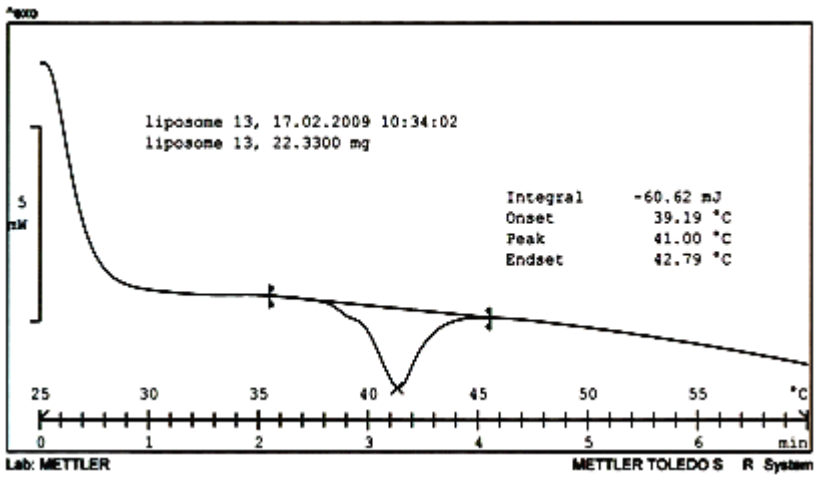

Fig1. DSC of thermal compound liposomes

Development of releasing assay method for lomustine

The HPLC chromatograms of lomustine were shown in Fig 2, it was indicated that the retention time (RT) of lomustine was about 7.7min, iohexol and other pharmaceutical necessities were eluted within 3 min, without interfering the determination.

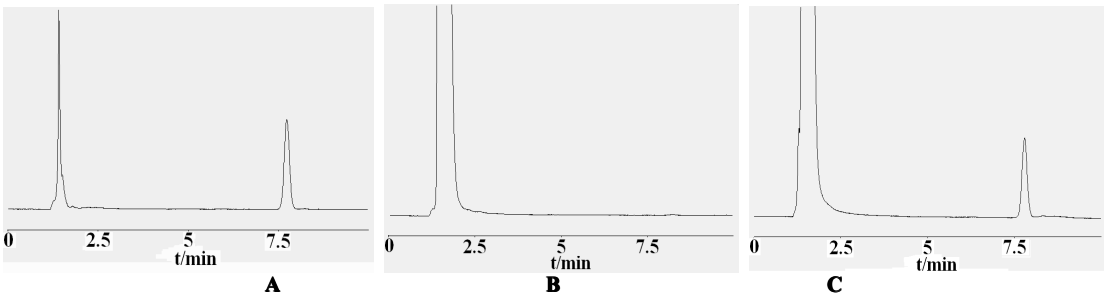

Fig 2. Chromatograms of lomustine standard (a), blank liposome (b) and thermal compound liposomes (c)

Further, the calibration curve of lomustine was calculated as: A=1.6046×10^{4}C+1839.3 (r=0.9999), indicating the good linear relationship from 1.0 to 20.0μg·ml^{-1}. The recovery were 98.4%, 97.5% and 98.6%, with RSD of 2.14 %, 1.58% and 2.03%(n=5).

Development of releasing assay method for iohexol

The HPLC chromatograms of iohexol were shown in Fig 3, it was indicated that the retention time (RT) of iohexol was about 10 min (peak 2), chromatographic peak of lomustine and other pharmaceutical necessities were not be detected, without interfering the determination.

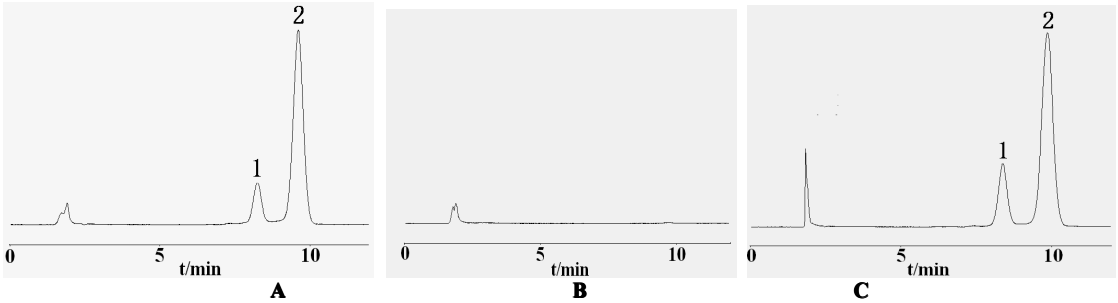

Fig 3. Chromatograms of iohexol standard (a), blank liposome (b) and thermal compound liposomes (c)

Further, the calibration curve of iohexol was calculated as: A =1.9934×10^{4}C-2416.9 (r=0.9999), indicating the good linear relationship from 6.0 to 60.0μg·ml^{-1}. The recovery were 99.2%, 98.5% and 100.6%, with RSD of 0.88%, 0.67% and 1.44%(n=5).

Study on the in vitro releasing of iohexol

Release condition investigation. As shown in Fig 4, iohexol was released more rapidly within 1 h in PBS, but became relatively slower in subsequent time. Therefore, purified water was selected as release medium in order to make complete release.

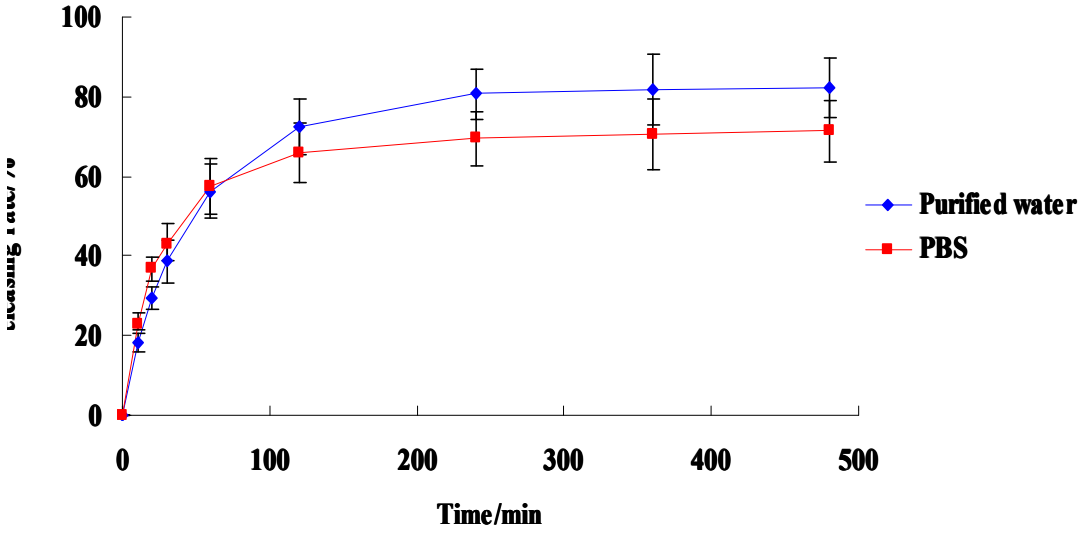

Fig 4. Effects of mediums on releasing of iohexol (n=3)

As shown in Fig 5, the releasing rate of iohexol increased as rotation speed increase, thus the speed of 50 r·min^{-1} was selected so as to compare release characteristics of thermosensitive compound liposomes more adequately.

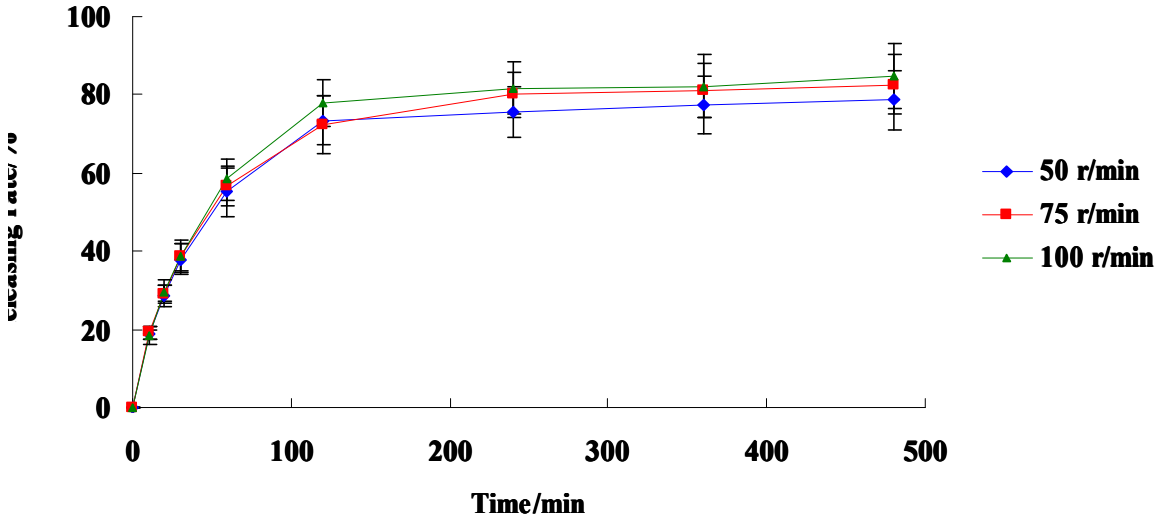

Fig 5. Effects of rotate speed on releasing of iohexol (n=3)

Releasing of iohexol at different temperature. As shown in Fig 6, iohexol was released more rapidly at 41℃ than 37 ℃, the total release amounts was also higher at 41℃, with accumulative release amounts reaching 93.75% in 8 h. As Table 2 listed, the best fitting for iohexol releasing from liposomes at 41℃ was first-order kinetics model, with the equation of ln(100-Q)=-1.066t-0.0498 (r=0.9993) [16].

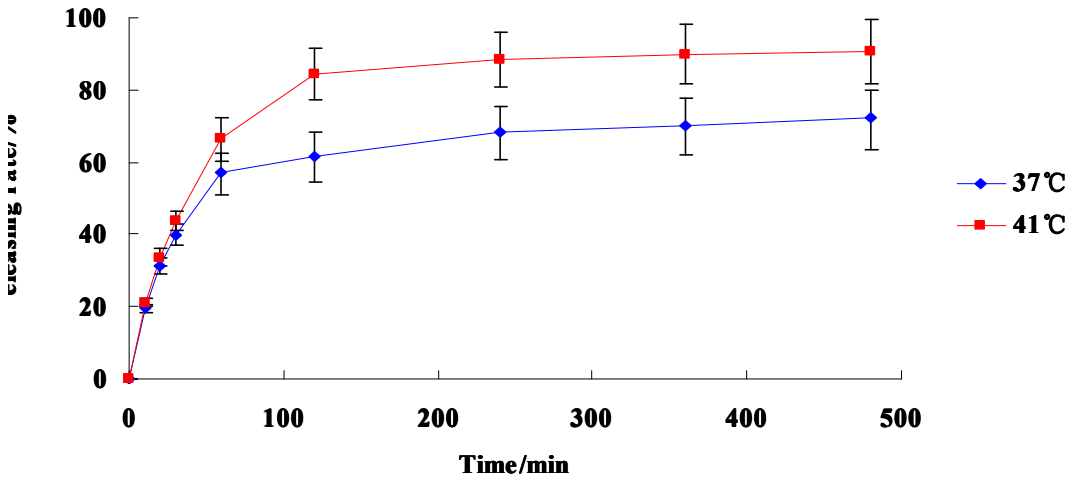

Fig 6. Releasing of iohexol in thermal compound liposomes at 37 and 41℃

Table 2. The different release models of iohexol in thermal compound liposomes in vitro

Model	Equation	k	C	r
Zero-order kinetics	Q%=kt+C	0.4081	0.1568	0.9424
First-order kinetics	ln(100-Q)=kt+C	-1.066	-0.0498	0.9993
Higuchi equation	Q%=kt1/2+C	0.6523	-0.0177	0.9955
Ritger-Peppas equation	lnQ=klnt+C	0.4888	-0.5378	0.9740
Weibull equation	lg[-ln(100-Q)]=klgt+	0.851	0.0411	0.998

C 7 4

Study on the in vitro releasing of lomustine

Lomustine degradation kinetics investigation. The degradation curve of lomustine at 37 and 41℃ was shown in Fig 7. After data processing, we found that lomustine degradation fitted first order linear relation; the fitting results were as follows:

37 ℃: lgC/C_0=-0.2395t-0.2634, R＝0.9943

41 ℃: lgC/C_0=-0.2778t-0.2674, R＝0.9967

Noting: C represented the undegradated lomustine amounts at each time point, C_0 represented the lomustine contents at 0 min, R was the coefficient correlation.

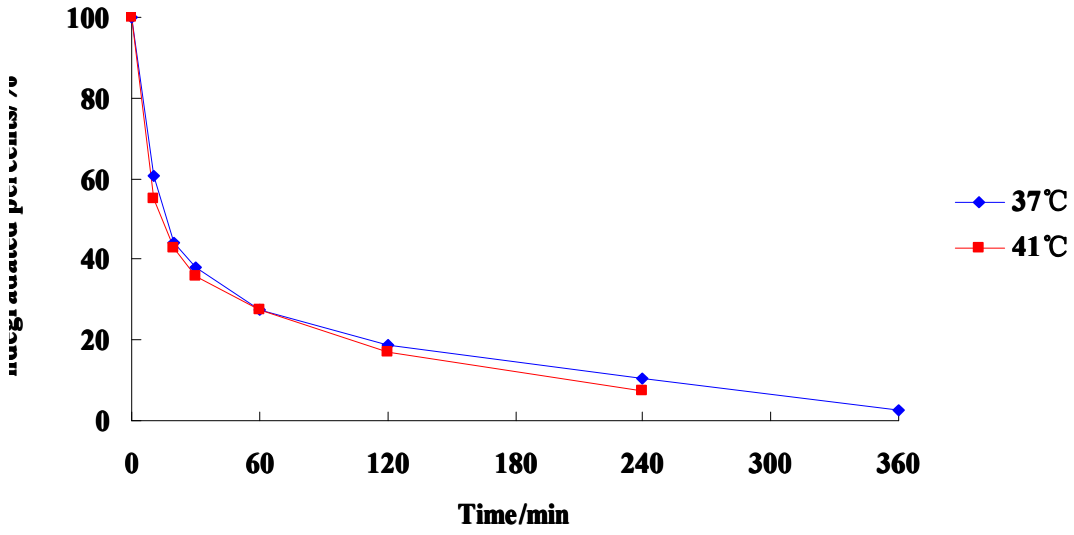

Fig 7. Degradation curve of lomustine solution at 37 and 41℃

Releasing of lomustine at different temperature. As shown in Fig 8, lomustine was released more rapidly at 41 ℃ than 37 ℃, and the accumulative release amounts reached 95.03% in 4 h. As Table 3 listed, the best fitting for lomustine releasing from liposomes at 4l℃ was Weibull model, with the equation of

lg[-ln(100-Q)]=1.1069lgt-0.0976 (r=0.9923).

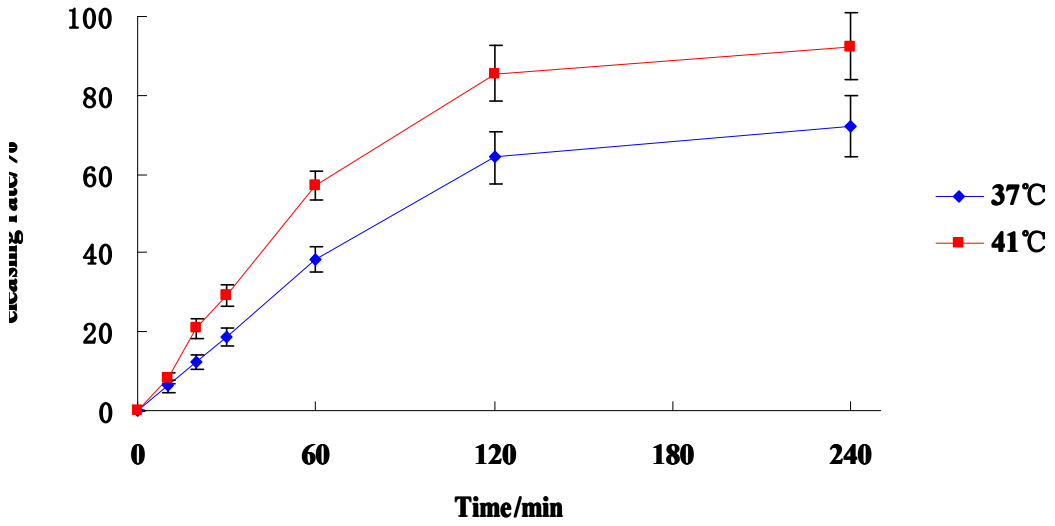

Fig 8. Releasing of lomustine in thermal compound liposomes at 37 and 41℃

Table 3. The different release models of lomustine in thermal compound liposomes in vitro

Model	Equation	k	C	r
Zero-order kinetics	Q%=kt+C	0.4294	0.0479	0.9916
First-order kinetics	ln(100-Q)=kt+C	-1.0226	0.1006	0.9911
Higuchi equation	$Q\%=kt^{1/2}+C$	0.6390	-0.1033	0.9753
Ritger-Peppas equation	lnQ=klnt+C	0.7341	-0.8046	0.9767
Weibull equation	lg[-ln(100-Q)]=klgt+C	1.1069	-0.0976	0.9923

Acknowledgment

This work was financially supported by the doctoral scientific research start-up foundation from Henan University of Technology (No: 2012BS034).

REFERENCES

[1] YD Zhang, C Ding, ZJ Zhang. *Chin J Exp Surg*. **2006,** 23: 1191-1193.
[2] L Wang, DS Zhang. *J Southeast Univ (Med Sci Ed)*. **2009,** 28: 349-352.
[3] M Hossann, T Wang, Z Syunyaeva. *J Control Release*. **2013,** 166: 22-29.
[4] YZ Huang, JQ Gao, JL Chen, WQ Liang. *Colloids Surf B Biointerfaces*. **2006,** 49: 158–164.
[5] MA Kisel, LN Kulik, IS Tsybovsky. *Int J Pharm*. **2001,** 216: 105-114.
[6] CH Huang, JP Sipe, ST Chow, RB Martin. *Proc. Natl. Acad.Sci. U S A*. **1974,** 71: 359-362.
[7] SC Semple, A Chonn, PR Cullis. *Biochemistry*. **1996,** 35: 2521-2525.
[8] L Xu, TJ Anchordoquy. *BBA-Biomembranes*. **2008,** 1778: 2177-2181.
[9] S Igarashi, Y Hattori, Y Maitani. *J.Control.Release*. **2006,** 112: 362-368.
[10] N Smyth Templeton. *Curr.Med.Chem.* **2003,** 10: 1279-1287.
[11] H Eliyahu, N Servel, AJ Domb, Y Barenholz. *Gene Ther.* **2002,** 9: 850-858.
[12] B Ma, S Zhang, H Jiang, B Zhao, H Lv. *J.Control.Release*. **2007,** 123: 184-194.
[13] CR Middaugh, RK Evans, DL Montgomery, DR Casimiro. *J.Pharm.Sci.* **1998,** 87: 130-146.
[14] L Wasungu, D Hoekstra. *J.Control.Release*. **2006**, 116: 255-264.
[15] D Hirsch-Lerner, M Zhang, H Eliyahu, ME Ferrari, CJ Wheeler, Y Barenholz. *BBA-Biomembranes*. **2005,** 1714: 71-84.
[16] M Hossann, T Wang, M Wiggenhorn. *J Control Release*. **2010,** 147: 436-443.