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Research Article

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Preparation of lomustine-iohexol compound liposomes and the determination of entrapment efficiency

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ABSTRACT

To prepare lomustine-iohexol compound liposomes, and evaluate the properties, determine the entrapment efficiency. Liposomes were prepared by reverse evaporation method; orthogonal design was used to optimize the formulation. Free drugs and liposomes were separated using protamine aggregation method and entrapment efficiency was determined by HPLC. The optimal formula was as follows: soybean phospholipid 80, proportion of phospholipid and cholesterol was 2:1, lipid contents was 33 mg/ml； protamine aggregation method can be well used to entrapment efficiency determination. The mean diameters of compound liposomes were 237.3nm, Zeta potentials were -43.9 mV, and the entrapment efficiency of lomustine and iohexol were 75% and 65%, respectively. The formulation and preparative method can be used to prepare compound liposomes with high entrapment efficiency; protamine aggregation was effective and rapid and can be used to determine the entrapment efficiency accurately.

Keywords: lomustine; iohexol; compound liposomes; entrapment efficiency; protamine aggregation method

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INTRODUCTION

Lomustine is one of the nitrosourea alkylating agents, has the potent anti-tumor activity of broad spectrum. In clinic, lomustine are mainly used for encephaloma, malignant lymphoma, lung cancer and melanoma, however, its practical use is limited largely by the adverse effect, such as gastroenteric reaction and late medulla-suppression [1]. Iohexol is a widely used nonionic contrast medium and is highly water-soluble, low viscosity and osmotic pressure, which is mainly used for spinal marrow, blood vessel visualization, computerized tomography (CT), urinary system and vascellum lymphaticum visualization.

To prepare the compound liposomes that lomustine and iohexol are both enveloped, we can define the tumor area through visualization, and make the chemotherapeutics to be targeting released simultaneously in tumor site, so as to improve therapeutic efficacy and decrease the side effects from general chemotherapy. Furthermore, lomustine is highly lipid soluble and iohexol is water soluble, thus they will not compete with each other for space in lipid carriers [2-4]. Nowadays, there are few studies in literatures about the compound liposomes in which two drugs are enveloped together in the field of pharmaceutics at home and abroad. In the present study, we prepare lomustine-iohexol compound liposomes, and developed the protamine aggregation method to determine the entrapment efficiency, to provide a means of further study for thermosensetive compound liposomes.

EXPERIMENTAL SECTION

Chemicals and reagents

Lomustine was purchased from Shandong Weifang Pharmaceutical Factory Co., Ltd, iohexol was purchased from Zhejiang Starry Pharmaceutical Co., Ltd, soybean phospholipid (SP) was purchased from Shanghai Tywei

Pharmaceutical Co., Ltd, and cholesterol was purchased from Shanghai Xuhui chemical agent supply station. 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. 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 compound liposomes

Compound liposomes were prepared by reverse phase evaporation method (RPE) [5]. In brief, a lipid mixture of lomustine, soybean phospholipid, cholesterol and V_E were dissolved in aether and the solution was evaporated at 25 ℃ to form dried film, redissolved by aether. Meantime iohexol was dissolved in purified water. After putting both the solutions in 0 ℃ water bath and shaking, iohexol solution was dropped in 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 25 ℃.

Finally the liposome suspension was further disrupted by using ultrasonic probe. Resulting liposomes were sterilized by extruding through a 0.45 μm sterile filter. 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.

Study and optimum design on formula of compound liposomes

Orthogonal design was employed to optimize lipid formulation. Three parameters that have significant effect on encapsulation efficiency were selected to investigate; they were the kind of phospholipid (A), charge ratio of phospholipid to cholesterol (B), and the total lipid contents (C, mg/ml). Three levels were chose for each factor, which were cautiously defined on the basis of result of single factor experiment and feasibility of preparing liposome samples at the maximum and minimal levels. Encapsulation efficiencies (EE) of lomustine and iohexol were used as assessment index and the weight of each EE was defined as 0.5.Design of factors and levels were described as Table 1.

Table 1. Factors and levels

level	A	B	C
1	SP95	9:1	90
2	SP80	5:1	67
3	phosphatidylcholine	2:1	33

EE determination-study on protamine aggregation method

Protamine aggregation method was developed to separate free drugs and liposomes; EE was determined by using high performance liquid chromatography (HPLC).

Effect of protamine dosage on separation for liposomes

The proper amounts of protamine sulfate injection (10 mg/ml) was taken and diluted by 2, 4, 8, 16, 32, 64, 128 and 256 fold, which signed as 0 (no diluted), 1, 2, 3, 4, 5, 6, 7, and 8 dilution, respectively. 100 μl liposomes and 100 μl dilution 0 were taken and mixed, placed for about 3 min, centrifugated with the relative centrifugal force 350×g for 15 min after diluting with 5 ml purified water precisely. The supernatant was selected to measure absorbance by using an ultraviolet spectrophotometer at 540 nm, signed as A_1. Another 100 μl liposomes were mixed with purified water directly, centrifugated and collected the supernatant to determine turbidity, signed as A_0, the clarity was calculated according to the formula: $(A_0-A_1)/A_0 \times 100\%$. By the same method, the clarities of supernatant from 1-8 dilutions by separate liposomes were determined.

Effect of protamine dosage on EE of lomustine

100 μl liposomes and 100 μl protamine sulfate were taken and mixed; the free drugs and liposomes were separated as described above. The supernatant was collected for HPLC analysis. The chromatography separation was performed with a Diamonsil^{TM}C$_{18}$ Column (200mm×4.6mm, 5μm), the flow rate was 1.0 ml·min^{-1}, with column temperature of 25℃, the mobile phase was acetonitrile-water (65:35), the drug was detected at 230 nm for determining the content of lomustine. By the same method, EE of lomustine from 1-6 dilutions by separate

liposomes were determined.

Effect of protamine dosage on EE of iohexol

The free drugs and liposomes were separated as described above. The chromatography separation was performed with the mobile phase methanol-water (10:90), the drug was detected at 244 nm for determining the content of iohexol. By the same method, EE of iohexol from 1-6 dilutions by separate liposomes were determined.

Determination of recovery of lomustine

Different concentrations of lomustine solutions (80%, 100% and 120%) were prepared with chloroform, N_2 gas was used to remove the residuary solvent. The proper amounts of blank liposomes were mixed with lomustine solutions, 100 μl mixtures was taken to separate by protamine sulfate and centrifugation. The supernatant was collected for HPLC analysis to determine the recovery.

Determination of recovery of iohexol

Different concentrations of iohexol solutions (80%, 100% and 120%) were prepared with purified water; the recovery was determined by HPLC as described above.

Determination of EE of lomustine

100 μl compound liposomes were taken precisely and mixed with 100 μl protamine sulfate, after centrifugation the supernatant was collected for HPLC analysis to determine the amounts of free drug (W_1). Meanwhile, another 100 μl compound liposomes were taken and dissolved by 5 ml methanol; samples were taken to determine the total amount of lomustine (W_0). EE was calculated according to the formula: $EE=(1-W_1/W_0)\times100\%$.

Determination of EE of iohexol

The liposomes samples were processed as described above, 100 μl supernatant was taken and diluted by 25 ml mobile phase, 20 μl dilution was collected for HPLC analysis to determine the amounts of free drug (W_1). Meanwhile, another 100 μl compound liposomes were taken and dissolved by 10 ml methanol, 100 μl dilution was dissolved again by 10 ml mobile phase, samples were taken to determine the total amount of lomustine (W_0). EE was calculated according to the formula: $EE=(1-W_1/W_0\times1.25)\times100\%$.

RESULTS

Result of formulation design

According to orthogonal designs, a total of nine tests containing five replicates at the center point were carried out in random order (Table 2). Analysis of variance for the experimental results was shown in Table 3. The results showed that all the factors had no significant effects on EE of lomustine and iohexol ($p>0.05$), the optimal grouping were $A_2B_3C_3$, which were SP80,charge ratio of phospholipid to cholesterol of 2:1, the total lipid contents of 33 mg/ml.

Table 2. Results of orthogonal experiment

Run	A	B	C		EE of lomustine （%）	EE of iohexol （%）	Response (%)
1	1	1	1	1	42.6	22.3	32.5
2	1	2	2	2	38.7	18.4	28.6
3	1	3	3	3	66.9	37.6	52.3
4	2	1	2	3	58.4	42.6	50.5
5	2	2	3	1	71.3	42.3	56.8
6	2	3	1	2	44.2	43.1	43.7
7	3	1	3	2	39.5	40.8	40.2
8	3	2	1	3	62.3	49.7	56.0
9	3	3	2	1	42.2	50.2	46.2
K1	113.25	123.10	132.10	135.45			
K2	150.95	141.35	125.25	112.35			
K3	142.35	142.10	149.20	158.75			
R	12.567	6.333	7.983	15.467			

Table 3. Analysis of variance

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	P Value
A	260.23	2	130.114	0.725	0.580
B	77.181	2	38.590	0.215	0.823
C	101.44	2	50.719	0.283	0.780
error	358.83	2	179.414		

Development of protamine aggregation method

Effect of protamine dosage on separation for liposomes

The clarities determination results were shown in Fig.1, we can conclude that free drugs and liposomes are well separated (Clarities were higher than 98%) by using very low amounts of protamine sulfate (0.016 mg).

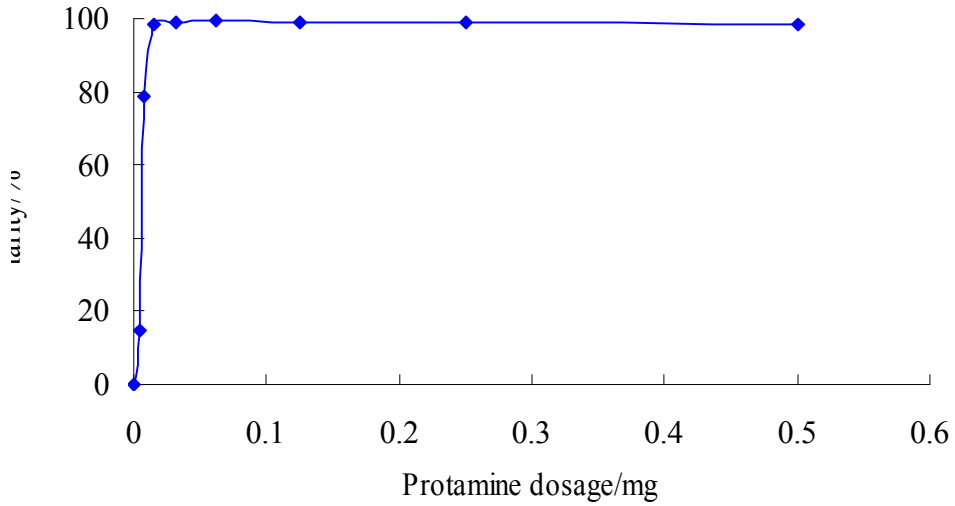

Fig 1. The effect of protamine dosage on separation

Effect of protamine dosage on EE

As shown in Fig.2 and Fig.3, while protamine dosage was between 0.016-1.0 mg, EE of lomustine and iohexol determined were 63 %-72 % and 56 %-65 %, respectively, thus there were little effects of protamine dosage on the EE of lomustine and iohexol.

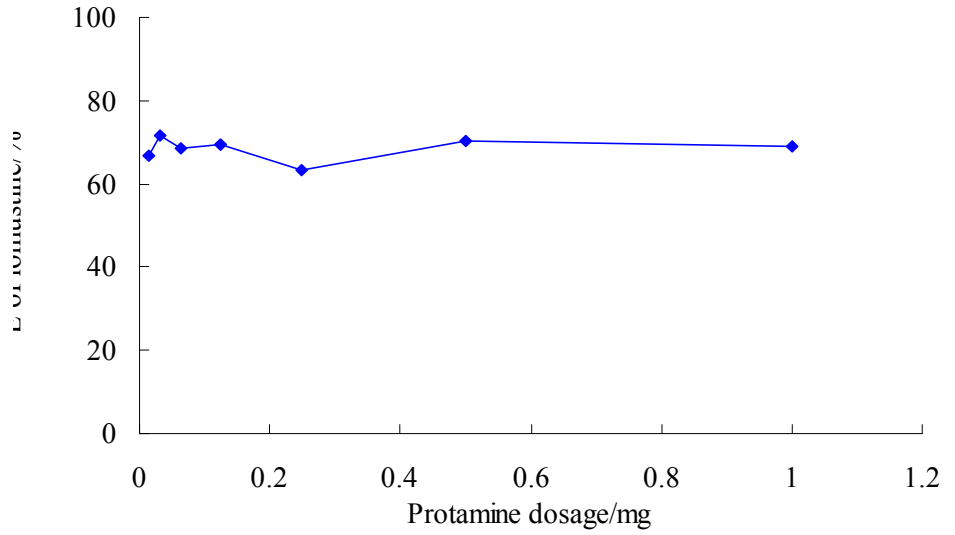

Fig 2. Relation between protamine dosage and entrapment efficiency of lomustine

Determination of recovery

Through HPLC analysis and calculation, recovery of lomustine were 97.2 %, 95.8 % and 98.1 %, RSD=1.58 % (n=3); recovery of iohexol were 98.5 %, 97.4 % and 96.8 %, RSD=1.42 % (n=3).

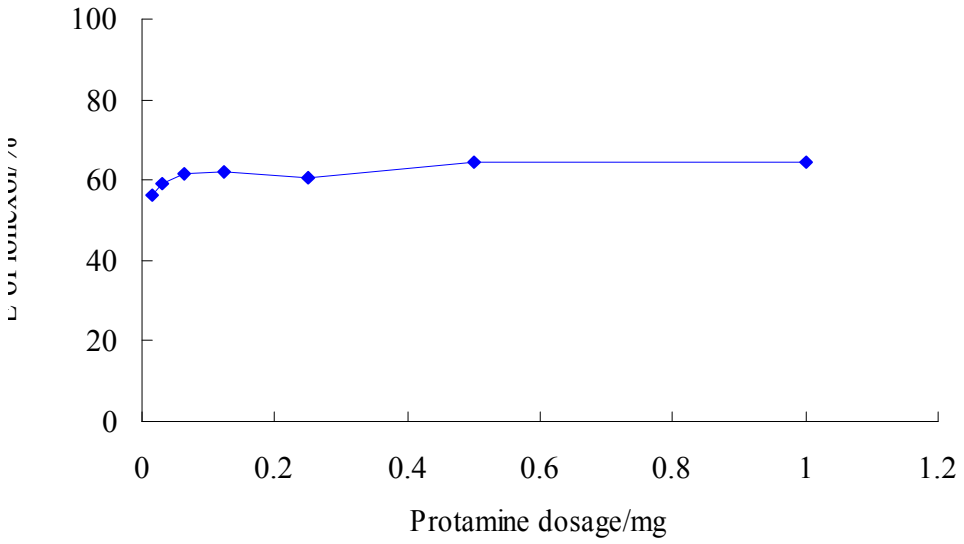

Fig 3. Relation between protamine dosage and entrapment efficiency of iohexol

Characterization of compound liposomes

The EE, particle size and zeta potential determination results of compound liposomes were shown in Table 4.

Table 4. Determination results of lomustine-iohexol compound liposomes

Batch	EE of lomustine/%	EE of iohexol/%	Particle size/nm	Zeta potential/mV
1	78.7	68.7	237.3	-38.6
2	75.4	65.4	216.4	-44.1
3	72.9	62.9	255.7	-43.9

DISCUSSION

EE is one of the most important evaluation parameters for liposomes carriers. In general, the free drugs and liposomes are separated, and the amounts of free drug as well as the total amounts are both determined to calculate EE [6-8]. Protamine sulfate is one kind of polycation macromolecule and composed of basic amino acids. After mixing with liposomes protamine sulfate can adsorb their surface through electrostatic interaction, thus the density of liposomes would be increased and may be separated effectively with free drugs by lower centrifgual force. For the advantages of quickness, simple operation and high efficiency, furthermore, the separation is based on electrostatic attraction and independent of the drugs enveloped in liposomes, thus this method is applicable for EE determination of most of the drugs.

In this study, several EE measurement methods including high speed centrifugation, equilibrium dialysis and sephadex filtration, had been applied to separate liposomes [9-12]. However, there were two drugs carried in lipid vectors, and the solubility of lomustine and iohexol differed greatly, the methods mentioned above can not to separate free drugs and liposomes effectively. Through large experimental validation and comparison with other methods, protamine aggregation method was selected to separate liposomes, which could determine EE accurately.

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