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Liposomes for systematic delivery of vancomycin hydrochloride to decrease nephrotoxicity: Characterization and evaluation



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ABSTRACT

Vancomycin hydrochloride (VANH), the first glycopeptide antibiotic, is a water-soluble drug for the treatment of acute osteomyelitis. Liposomal formulations of VANH have already been manipulated and characterized, which was a mean of increasing their therapeutic index, reducing their toxicity and altering drug biodistribution. One of the challenges for preparing VANH-Lips is their low encapsulation efficiency (EE). In the present study, we aim to improve the liposomal formulation of VANH for higher EE, longer systemic circulation, reduced nephrotoxicity and enhanced antimicrobial activities. Vancomycin hydrochloride-loaded liposomes (VANH-Lips) were formulated by the method of modified reverse phase evaporation. Based on the optimization of formulation with orthogonal experimental design, the average drug encapsulation efficiency and the mean particle size of VANH-Lips were found to be $40.78 \pm 2.56\%$ and 188.4 ± 2.77 nm. In vitro drug release of VANH-Lips possessed a sustained release characteristic and their release behavior was in accordance with the Weibull equation. After intravenous injection to mice, the mean residence time (MRT) of VANH-Lips group was significantly prolonged in vivo and the AUC value was improved as well compared with the vancomycin hydrochloride solution (VANH-Sol) group. Furthermore, the biodistribution results in mice showed that VANH-Lips decreased the accumulation of VANH in kidney after intravenous injection. In conclusion, VANH-Lips may be a potential delivery system for VANH to decrease nephrotoxicity in the treatment of osteomyelitis.

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1. Introduction

Osteomyelitis is an infection of the bone and bone marrow, which is attributed to bacteria contamination result from trauma, surgery, orthopedic fixation device implantation. In consequence it brings huge challenges to orthopedic surgeons [1]. Regular treatment includes thorough debridement, continuous perfusion, and intravenous application of sensitive antibiotic for 4–6 weeks. Based on complete removal of the lesion, effective systemic application of antibiotics is the necessity of the treatment of chronic osteomyelitis. Antibiotics are administered to control inflammation and to prevent exacerbation in chronic infections [2]. However, the efficacy of antibiotics is severely limited by its poor penetration. Long-term and high-dose strategy is, thus, required to improve the therapeutic outcome [3]. Although there are plenty of antibiotics and the current surgical techniques are promising, patients having osteomyelitis are still suffered from high recurrence rate. This is due to drug resistance induced by frequent administrations of sensitive antibiotics. To be treated clinically, therefore, osteomyelitis has been normally considered as one of the most obstinate orthopedic and postoperative complications [4].

As the first glycopeptide antibiotic, VANH was developed in the 1950s [5]. Even if the concentration of vancomycin is low, it is still valid against the majority of Gram-positive bacteria, for example, *Staphylococcus aureus* and other staphylococcus species [5]. *S. aureus* is one of the most frequent pathogenic bacterium of osteomyelitis [6]. Although it works specifically on bacterial cell wall peptidoglycan, but, the side effects, including most notably nephrotoxicity, ototoxicity, and neuromuscular blockade, appeared in the early course of its application. These side effects limit its wide application [5,7].

Renal elimination of vancomycin is mainly through glomerular filtration and partly mediated by active tubular secretion [8]. Most of VANH is removed unchanged in the urine [9]. Renal dysfunction is a major omen of vancomycin treatment failure [10]. Gene expression profiling studies in animals with high-dose VANH suggest that cellular necrosis resulted from VANH accumulation in proximal tubular cells may be the underlying mechanism of nephrotoxicity [11]. Many of previous reports has demonstrated a highly significant relationship between vancomycin dose and the time to nephrotoxicity [12,13]. In-patients who suffer from chronic osteomyelitis often have low immunity. Therefore it easily leads to chronic kidney disease and acute kidney injury. In addition, Clinical failure reasons have also been reported owing to its slow, time-dependent bactericidal activity, poor intracellular penetration into macrophages [14]. Therefore, merely increasing the parenteral treatment doses of vancomycin offers an essentially increased risk of drug toxicity that is not offset by any appreciable benefit [15].

Liposomes have been proven to be a hopeful drug delivery system for a variety of drugs including antibiotics [16]. As their lipid bilayer are made up of phospholipid and cholesterol, which is similar to the cell membrane and can readily fuse with infectious microbes, Liposomes are considered to be a non-toxic and biodegradable drug carrier for sustained and targeted delivery. They are also the most widely used

antimicrobial drug delivery vehicles [17]. Encapsulation of antibiotics in liposomes is known to enhance their antimicrobial activities while minimizing their toxic effects [7]. In addition, the sustained release of antibiotics from the liposomes may prolong the half-lives of these drugs in the body.

Encapsulation of VANH into liposomes has been attempted by our group as well as several other investigators as a mean of increasing their therapeutic index, reducing their toxicity and altering drug biodistribution [14,15,18–20]. Nevertheless, due to the highly hydrophilic property, one of the challenges preparing VANH-Lips is their low encapsulation efficiency. If so, this will require high amount of liposomal formulations to achieve satisfactory therapeutic dose, a reason that prone to drug resistance. In the present work, several methods have been attempted for this challenging endeavor. The EE of the liposomes prepared by Andrew Pumerantz [15] and Krishna Muppidi [14] are both 9%. The EE for vancomycin hydrochloride into liposomes prepared by thin film dispersion method is 2.34% [19]. Yang DM [20] prepared the multivesicular liposomes, whose mean EE and particle size is 24.9% and 3.3 μm , respectively. The EE of cationic liposomes prepared by Tao Ma [18] is 7.58%. The objective of this study was to prepare vancomycin-encapsulated liposomes with relatively high encapsulation efficiency and performed an in vivo study using healthy mice to compare their pharmacokinetic and bio-distribution profiles with those of mice treated with the standard vancomycin solution. The optimal liposomes prepared by modified reverse phase evaporation methods were composed of injectable soybean phosphatides, cholesterol and VANH. The composition of the formulation is non-toxic, and the preparation process is simple and controllable. The optimal formulations with appropriate drug encapsulation percentage and homogenous particle size distribution were selected to investigate the pharmacokinetics and bio-distribution after intravenous administration, utilizing mice as animal models compared with free vancomycin hydrochloride, in order to provide the basic knowledge for further development and evaluation of liposome-encapsulated VANH formulation.

2. Materials and methods

2.1. Materials

Soybean lecithin (Injection grade, phosphatidylcholine accounts for 95% pH 5.0–7.0) was provided by Shanghai Taiwei Pharmaceutical Co., Ltd, Shanghai, China. Cholesterol was purchased from Shanghai Medical Chemical Reagent Co., Ltd. (China). VANH was purchased from Shanghai ziyi Reagent Co., Ltd. (China). The other chemicals were of analytical reagent grade or higher.

Animal: Kunming strain mice (weighed between 18 and 22 g, female or male were provided by the Medical Animal Test Center of Shandong University) were used for the in vivo pharmacokinetics and biodistribution studies. The animals were fasted 12 h before drug administration. The animal experiment protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Shandong University.

2.2. Preparation of VANH-Lips

Formulation of VANH-liposomes were prepared using various methods.

2.2.1. Freeze-thaw method [21]

Pre-formed empty liposomes were prepared by the film-hydration method. Briefly, lipid (soybean lecithin and cholesterol) was mixed with chloroform and evaporated using a rotary evaporator (Shanghai Yarong Instrument Co., Shanghai, China) at an elevated temperature (40 °C). During evaporation, a low vacuum was applied initially to avoid bursting of the lipid solution. The dried lipid was placed under vacuum overnight to remove residual solvent. Then VANH-Sol was added to hydrate for 10 min and the mixture was frozen at –20 °C for 20 min. The liposomes were then sonicated at 5 min followed by two freeze-thaw cycles (–20 °C for 20 min, 45 °C for 10 min).

2.2.2. Proliposome method

Defined amounts of phospholipids and cholesterol were weighed into a round-bottom flask and dissolved in chloroform, forming a transparent solution, which was described by Isailovic et al. [22]. Prescribed amount of sorbitol powder was added in the solution. The organic solvent was then removed under reduced pressure by the rotary evaporator to form proliposome. Afterwards, VANH-Sol was poured into the proliposome solution, and the mixture was sonicated to form uniform liposome.

2.2.3. Remote loading method by ammonium sulfate gradients

This method included two steps [23]. The first step was the formation of empty liposomes. The liposomes were prepared by dissolving phospholipids/cholesterol in chloroform. The organic solvent was then removed in a 40 °C water bath under reduced pressure by the rotary evaporator to form lipid membrane. The lipid membrane was then hydrated in ammonium sulfate solution. And the mixture was sonicated to form translucent solution. Taken suitable amounts of liposomes in dialysis bag, and dialysis in distilled water for 12 h at room temperature, and the empty liposomes were prepared. The second step was drug loading. Briefly, take empty liposomes, adding to VANH-Sol, and incubate in water bath, then the drugs could across the lipid bilayer and were entrapped into the vesicles.

2.2.4. pH-gradient method

Suitable amounts of phospholipids and cholesterol were weighed into a round-bottom flask and dissolved in chloroform, as described by Zhou [24]. Then chloroform was removed in a 40 °C water bath under reduced pressure by the rotary evaporator to form lipid membrane. The lipid membrane was then hydrated in the citric acid solution (300 mmol/L) to produce multivesicular vesicles. The liposomes were then homogenized at 800–1000 bar. In order to form the transmembrane pH-gradient between the external phase and internal phase of liposome, Na₂HPO₄ solution (0.5 mol/L) was added into the system to adjust the external pH of liposomes to 6.5. Then, the system was incubated under 50 °C for 10 min.

2.2.5. Reverse phase evaporation method

Appropriate amounts of phospholipids and cholesterol were weighed into a round-bottom flask and dissolved in chloroform [25]. Subsequently, VANH-Sol was directly added to the dispersed solution of phospholipids, and the mixture was sonicated 3 min at room temperature in an ultrasonic bath (Kun Shan Ultrasonic Instruments Co., Ltd). The organic solvent was then removed in a 40 °C water bath under reduced pressure by the rotary evaporator. Sufficient amounts of water was added to hydrate for an additional 10 min. Then, the liposomal emulsion was maintained under control temperature to make the liposome structure more stable.

2.2.6. Modified reverse phase evaporation-rehydration method

Similar to reverse phase evaporation method, except for the difference that VANH-Sol was dispersed with a microinjector (Kd Scientific 781100, Fabrique' Auxetats-Units Co. Ltd., USA) into the dispersed solution of phospholipids. And the mixture was sonicated to form a primary emulsion at room temperature in an ultrasonic bath.

2.3. Optimization of formulation with orthogonal experimental design

On the basis of preliminary test, modified reverse phase evaporation method reached the highest EE. Four selective formulation factors that mainly affected EE were chosen as research objects, including (A) the ratio of cholesterol to lecithin (w/w), (B) the ratio of drug to lipids (w/w), (C) the ratio of water phase to oil phase (v/v) and (D) hydration temperature. In this place, water phase is VANH-Sol and oil phase is the chloroform solution of phospholipid and cholesterol. Nine formulations were designed for test according to L₉ (3⁴) orthogonal experimental design, in order to screen the optimal formulation. On the basis of size plus EE as the evaluation index, the factors and levels of the orthogonal experimental design are listed in Table 1.

2.4. Freeze-drying of VANH-Lips

In the freeze-drying process, mannitol (5%, w/v) was used as a cryoprotectant. First the fresh prepared VANH-Lips with 5% mannitol were pre-frozen using an ultra-cold freezer (MDF-382E, Sanyo Electric Co., Ltd., Osaka, Japan) for 24 h at –80 °C. Then, the resultant samples were transferred to the lyophilizer

Table 1 – Factors and levels of the orthogonal experimental design.

Levels	Factor			
	A	B	C	D
1	1:2	1:10	1:2	35
2	1:4	1:15	1:3	45
3	1:6	1:20	1:4	55

(A) the ratio of cholesterol to lecithin (w/w), (B) the ratio of drug to lipids (w/w), (C) the ratio of water phase to oil phase(v/v) and (D) hydration temperature(°C).

(FD5-2.5, GOLD SIM, Issaquah, WA) at $-50\text{ }^{\circ}\text{C}$ for 48 h. The lyophilized powder was collected for further experiments.

2.5. Characterization of VANH-Lips

2.5.1. Visualization of liposomes by transmission electron microscopy (TEM)

TEM (H-7000, Hitachi, Japan) was used as a visualizing aid for vesicles. Samples were negatively stained with a 2% aqueous solution of phosphotungstic acid. Vesicular suspension samples were dried on a carbon-coated grid for staining. The excess solution was removed by blotting. After drying, the specimen was viewed under the TEM.

2.5.2. Measurement of particle size and pH value

Measurements were performed using a Beckman Delsa™ Nano C Particle Analyzer (Beckman Coulter A53878, Otsuka Electronics Co. Ltd., USA). Samples were placed in plastic disposable cuvettes and equilibrated at $25\text{ }^{\circ}\text{C}$. Particle size measurements included z-average, PDI, and PDI width. The pH value of VANH-Lips was determined with a digital pH meter (FE20, Mettler Toledo, Greifensee, Switzerland). Each measurement was made at least in triplicate at $25\text{ }^{\circ}\text{C}$.

2.5.3. Determination of encapsulation efficiency (EE)

The EE of VANH-Lips were determined by ultrafiltration-HPLC method using an Agilent G1310A pump and an Agilent G1314A variable wavelength detector set at 230 nm. VANH was monitored at a wavelength of 230 nm with the column, InertSustain®-C18 column (4.6 mm \times 250 mm) (Shimadzu-GL, JAPAN). The mobile phase was composed of 0.05 mol/L potassium phosphate monobasic monopotassium phosphate solution (pH 3.2) and methanol (spectroscopic grade) (78:22, ml/ml) at a flow rate of 1.0 ml/min. As follows, 0.1 ml of liposome was mixed with 2 ml methanol, adding distilled water volume to 10 ml. The total drug contents in liposomes were measured by HPLC. 0.1 ml of liposome was mixed with 0.5 ml distilled water into ultrafiltration centrifuge tube (MILLIPORE, UFC901024 15 M 10 K), and was centrifuged at 4000 rpm for 40 min (3K30, Sigma, Germany). Then another 200 μl distilled water was added into the mixture and centrifuged at 4000 rpm for 20 min again. The free drug content in the outer tube was also measured by HPLC. The EE and DL were calculated by using under-mentioned equations (1) and (2) respectively.

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

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

where w_{total} was the analyzed weight of drug in liposomes, w_{free} was the analyzed weight of free drug, w_{lipid} was the weight of lipid added in system.

2.5.4. The drug release experiment in vitro from VANH-Lips

The in vitro release study of VANH-Lips was carried out at $37\text{ }^{\circ}\text{C}$ using dynamic dialysis technique [26,27]. The liposomes with encapsulation efficiency 40.31% were used for this analysis. Briefly, the VANH-Lip dispersion (2 ml, donor

solution) was kept in a dialysis membrane (MD34, 8000–14,000, AMERICA) with a molecular weight cut-off of 8000–14,000 Da and this system was immersed in 30 ml of pH 7.4 buffer solution. The medium was kept at $37\text{ }^{\circ}\text{C}$ under continuous magnetic stirring of 100 rpm. At a regular interval of time, 0.5 ml of receiver solution was withdrawn and same volume of fresh medium was replaced. The VANH-Sol obtained from a dialysis test was used as control. The percentage of drug released was determined using the HPLC conditions mentioned previously. The mean calculated values were obtained from 3 replicates. The cumulative fraction of release rate was calculated from the following equation and the results are expressed as mean \pm standard deviation (SD):

$$\text{Release rate } (\%) = \frac{c_n v_0 + \sum_{i=0}^{n-1} c_i v_i}{c_0 v_0} \times 100\%$$

c_n is the drug concentration in the release medium of each time interval, v_0 is the total volume of the release medium, v_i is the volume of the withdrawn medium, c_i is the drug concentration in the release medium at time.

2.6. Pharmacokinetics studies and drug distribution studies in mice

Kunming strain mice, weighing between 18 and 22 g, were randomly divided into two groups. Group 1 was treated with VANH-Sol while group 2 was treated with the VANH-Lips. The concentration of VANH in liposome is 0.96 mg/ml, while the VANH was dissolved in the sterile water for injection to obtain the same concentration. Each preparation was injected through the tail vein at the VANH dose of 15 mg/kg mouse. Blood samples were taken from the terminal retro-orbital bleeding at various times (0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8 and 10 h) into micro-tubes containing sodium heparin as an anticoagulant, and centrifuged immediately (10 min, 12,000 rpm). 0.2 ml 10% zinc sulfate solution was added to an aliquot (0.2 ml) of each plasma sample and mixed for 3 min by vortex to extract VANH. Following centrifugation at 12000 rpm for 10 min, the organic phase was transferred to a glass tube and the solvent was evaporated under nitrogen stream at $40\text{ }^{\circ}\text{C}$. The dry sample was then dissolved in 100 μl mobile phase and 20 μl of the solution was injected into the HPLC column to measure VANH peak area and calculate its concentration by standard curve method.

The heart, liver, spleen, lungs, kidneys and brain of each mouse was rapidly excised following blood collections, and immediately washed twice with normal saline (0.9% NaCl), wiped with filter paper, weighed and homogenized with 1.0 ml normal saline (0.9% NaCl), except for liver (2 ml). 0.2 ml 10% zinc sulfate solution was added to an aliquot (0.2 ml) of each plasma sample and mixed for 3 min by vortex to extract vancomycin hydrochloride. After vortexing (IKA®VORTEX1, GEMANY) for 3 min, the sample was centrifuged for approximately 10 min at 12,000 rpm. The supernatant was collected and the solvent was evaporated under nitrogen gas at $40\text{ }^{\circ}\text{C}$. The dry sample was reconstituted in 100 μL mobile phase for measurement of VANH by HPLC.

The concentration of VANH was measured by the method of reversed-phase HPLC with the column, InertSustain®-C18

column (4.6 mm × 250 mm). VANH was monitored at a wavelength of 230 nm. The mobile phase was composed of 0.05 mol/L potassium phosphate monobasic monopotassium phosphate solution (pH 3.2) and methanol (spectroscopic grade) (82:18, ml/ml) at a flow rate of 1.0 ml/min. Aliquots of deposited supernatants (20 μL) were loaded on the HPLC. The detection limit of VANH was 0.4 ng. Intraday and interday variabilities were less than 3% and 5%, respectively. Mean recovery rates of each organ exceeded 80%, RSD <5%.

2.7. Statistical analysis

Data were expressed as mean ± standard deviation (SD). The statistical analysis of significance among various treatments was performed using unpaired Student's t-test with $P < 0.05$ indicating significant difference. An analysis of variance (ANOVA) test was also used if necessary. Pharmacokinetic parameters of VANH-Lip were obtained using DAS2.0 (drug and statistics for windows) program.

3. Results and discussions

3.1. Encapsulation efficiency (EE) of VANH-Lips

VANH is highly hydrophilic and water soluble. According to the results showed in previous report [14,15,18–20], the EE of liposome-loaded water-soluble drugs is low. There are many approaches for the preparation liposomal formulation of VANH including thin film dispersion method [19], double emulsion method [20] and reverse phase method [18]. So it is very difficult to prepare high EE, stable VANH-Lips by classic passive loading method due to the fact that VANH is hydrosoluble.

So we attempt different ways to improve the EE, including freeze-thaw method, proliposome method, modified reverse phase evaporation-rehydration method, remote loading methods by ammonium sulfate gradients and pH-gradient method. Modified reverse phase evaporation method, is used to prepare liposomes with a large internal aqueous space, which is advantageous to encapsulate water-soluble drugs [25]. Based on reverse phase evaporation method described by Szoka and Papahadjopoulos [25], the difference was that VANH-Sol was uniformly dispersed with a microinjector to make the liposome with better formability, emulsification time was increased to reduce the size of VANH-Lips, and the hydration time was shorten to improve the EE.

Modified reverse phase evaporation method can make the drug dispersed more uniform. Then a more uniform film was formed after rotary evaporation and the particle size was smaller after hydration. Thus we can reduce ultrasonic time after hydration to avoid leakage of drugs, making drug encapsulation efficiency improved.

According to the results showed in Fig. 1, the EE of the liposome prepared by freeze-thaw method is the highest, the second is modified reverse phase evaporation method. In the preparation of liposomes, freeze-thaw cycling is implemented to reduce the lamellarity of liposomes, form a less poly-dispersed system and/or disrupt the liposomal bilayer to allow drug molecules to diffuse into the liposome, promoting

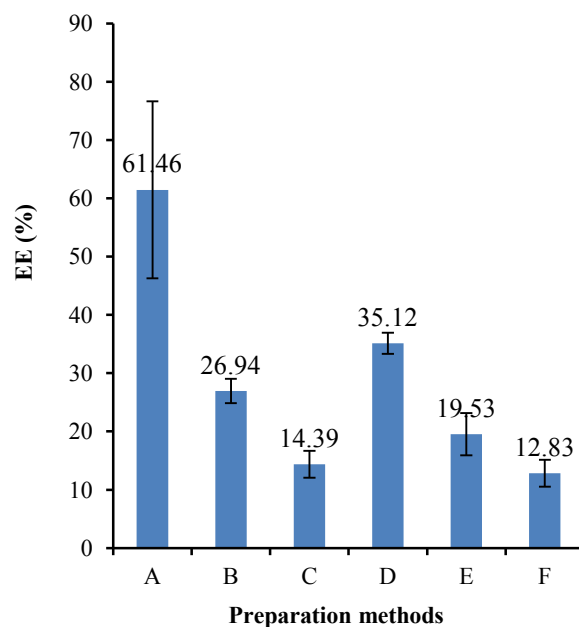


Fig. 1 – Encapsulation efficiency of VANH-Lips prepared by different methods. (A. Freeze-thaw method. B. Proliposome method. C. Reverse phase evaporation method. D. Modified reverse phase evaporation-rehydration method. E. Remote loading method by ammonium sulfate gradients. F. pH-Gradient Method).

encapsulation. Three batches of liposomes were prepared by freeze-thaw method. The particle size of the three batches were 321.6, 457.7, 489.5 nm, respectively. The mean size was 422.9 nm, and RSD is 21.09%. So the size of the liposome prepared by freeze-thaw method is difficult to control, and the reproducibility is poor. Regarding the efficiency of drug reencapsulated as shown in Fig. 1, correlating to the different preparation methods, the mean encapsulation percentage of VANH prepared by modified reverse phase techniques was elevated significantly and the particle size distribution was more homogeneous as compared to that of thin film or proliposome method. However, it should be noted the fine drug encapsulation efficiency of liposome was obtained using 1:15 M ratio of VANH and phospholipids. Therefore, modified reverse phase evaporation method is the best choice.

3.2. Results of the test of orthogonal design of VANH-Lip

Based on the preliminary experimental results, the EE can be improved through formulation and preparation optimization. On the basis of studying many documents, four selective formulation factors that mainly affected EE and size were chosen as research objects, including (A), (B), (C) and (D). Furthermore, the formulation factors affecting EE were studied in orthogonal experimental design. The results are showed in Table 2.

The results were calculated by range analysis. According to Table 2, the effect of (A) the ratio of cholesterol to lecithin (w/w) was extremely significant; the effect of other factors is

Table 2 – Results of the test of orthogonal design of VANH-Lips.

Experiment number	A	B	C	D	EE (%)	Size (nm)	Size-EE
1	1	1	1	1	37.09	372.2	335.11
2	1	2	2	2	41.96	237.5	195.54
3	1	3	3	3	35.1	192.9	157.83
4	2	1	2	3	38.14	242.0	203.89
5	2	2	3	1	42.78	182.7	139.89
6	2	3	1	2	42.25	162.0	119.78
7	3	1	3	2	28.17	163.4	135.26
8	3	2	1	3	36.45	152.9	116.45
9	3	3	2	1	36.07	172.4	136.30
K1	229.49	224.76	190.45	203.76			
K2	154.52	150.63	178.58	150.20			
K3	129.34	137.97	144.33	159.39			
R	100.16	86.78	46.12	53.57			

(A) the ratio of cholesterol to lecithin (w/w). (B) the ratio of drug to lipids (w/w). (C) the ratio of water phase to oil phase(v/v). (D) hydration temperature.

not significant. The range reflected the extent of each factor affecting on index and range was bigger, extent affected was greater. The effects of each factor on the EE were as follows: (A) the ratio of cholesterol to lecithin (w/w) > (B) the ratio of drug to lipids (w/w) > (D) hydration temperature > (C) the ratio of water phase to oil phase (v/v). K1, K2, K3 represented the sum value of each level. On the basis of the synthetic mean value of a certain factor (A or B or C or D), the optimized level combination of each factor was chosen. The lower the synthetic mean value, the better the level was and a higher EE can be achieved in this level. Analytical results of three factors were A: 1 > 2 > 3; B: 1 > 2 > 3; C: 1 > 2 > 3; D: 1 > 3 > 2, so the optimal parameters is A3B3C3D2. Furthermore, the encapsulation percentage was elevated accompanied with the increment of phospholipid amount. So the optimal formulation is adjusted slightly to improve drug loading efficiency, precisely the ratio of cholesterol to lecithin is 1:6.5, the ratio of drug to lipids (w/w) is 1:15, hydration temperature 45 °C, the ratio of water phase to oil phase (v/v) is 1:4. The EE of the liposome prepared by the optimum formulation reached to 40.78%. The optimum formulation is feasible and the EE of the liposome prepared by the optimum formulation is higher. Furthermore, with the optimization of formulation, the achievement of liposomes with higher EE by reverse phase evaporation method would be helpful to improve pharmaceutical and

pharmacological effects in curing Osteomyelitis. In short, the reverse phase evaporation method was superior to previous reports in encapsulating vancomycin hydrochloride.

3.3. Characterization of VANH-loaded liposome

3.3.1. Morphology and physicochemical property of VANH-Lips

The photographs of fresh-prepared VANH-Lips, the VANH-Lips freeze-dried powder and the suspension of VANH-Lips freeze-dried powder (dispersed with distilled water) are shown in Fig. 2. The particle size of the fresh-prepared VANH-Lips was 188.4 nm, indicating light blue opalescence. The lyophilized powder of VANH-Lips appeared as full, no collapse of the porous solid block. The lyophilized powder displayed good redispersibility and the reconstituted VANH-Lips with distilled water were still an opalescent colloidal solution. The morphology of VANH-Lips was observed by TEM and the observation results were displayed in Fig. 3. The TEM image shows that most liposomes were spherical particles with approximate size and uniform dispersion.

As shown in Table 3, the EE and drug loading of the fresh-prepared VANH-Lips Table 3 were 40.78% and 2.55%, which were relatively higher than that reported previously [14,15,18–20]. The pH of VANH-Lips was 5.96, which is consistent with the requirements for intravenous injection.

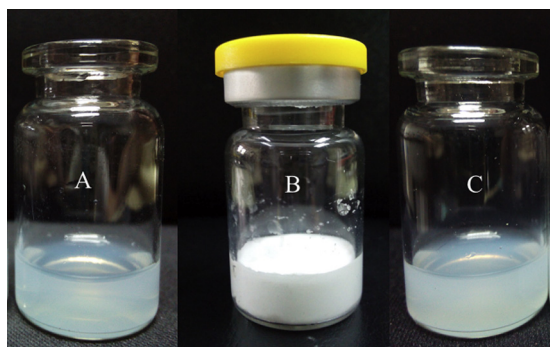


Fig. 2 – Photographs of VANH-Lips: (A) fresh-prepared, (B) lyophilized powder, and (C) the suspension of lyophilized powder.

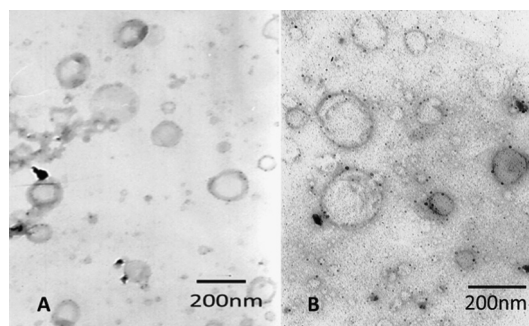


Fig. 3 – Transmission electron microscopy micrographs of VANH-Lips: (A) fresh-prepared, (B) the suspension of lyophilized powder.

Table 3 – Physicochemical characteristics of VANH-Lips: (A) fresh-prepared, (B) the suspension of lyophilized powder, each data was expressed as mean \pm SD ($n = 3$).

Sample	Particle size (nm)	PDI ^a	EE ^b (%)	DL ^c (%)	pH
A	188.4 \pm 5.22	0.289 \pm 0.019	40.78 \pm 1.04	2.55 \pm 0.065	5.96 \pm 0.066
B	203.7 \pm 9.85	0.305 \pm 0.036	35.58 \pm 2.66	2.23 \pm 0.166	6.02 \pm 0.103

^a PDI means polydispersity index.
^b EE means encapsulation efficiency.
^c DL means drug loading.

After lyophilization, the EE and drug loading were slightly reduced to 35.58% and 2.23%. This is possibly because that VANH is so hydrophilic that it is easy to leak from the liposome in the freeze-drying process.

3.3.2. In vitro drug release of VANH-Lips

In order to mimic the behavior of liposomes after intravenously administration to patients, VANH-Lips were incubated using a dialysis membrane in pH 7.4 phosphate buffer solution at 37 °C. The release experiment was carried out under sink conditions. The accumulative release percentage profile versus time of VANH-Lips and VANH-Sol is shown in Fig. 4. The release profile of VANH from VANH-Lips was, further, compared with VANH-Sol. It was apparent that almost 75% of the drug released within 4 h of dialysis, when VANH-Sol was dialyzed in pH 7.4 phosphate buffer solution. The VANH release from VANH-Lips, however, did not show significant burst release. VANH was released slower from VANH-Lips than VANH-Sol obviously. The VANH release from VANH-Lips was relatively slow and controlled. The release of VANH from VANH-loaded liposomes was in accordance with the Weibull equation and can be expressed using the following equation: $\ln[1/(1-R/100)] = 0.4742\ln t - 0.9485$, $r = 0.9791$. The release of VANH from VANH-Sol was in accordance with the first-order kinetic model and can be expressed using the following equation: $\ln(100-R) = -0.2722t + 4.4234$, $r = 0.9951$. This equation can be applied to forecast the release amount of

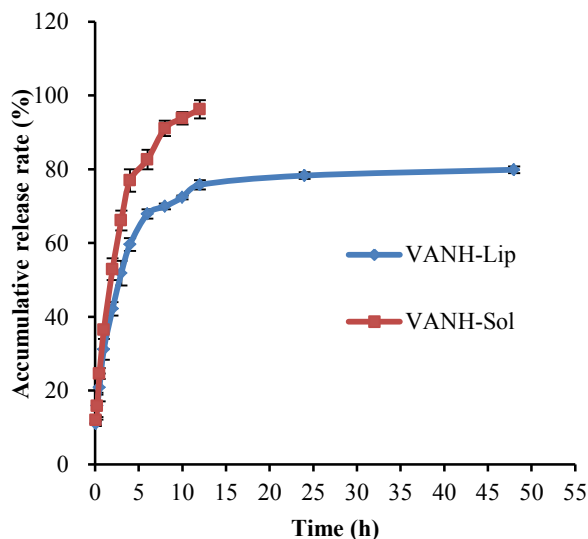


Fig. 4 – In vitro release profile of VANH-Sol and VANH-Lips ($n = 5$).

VANH at different time, or calculate the time to release a certain amount of VANH, which could predict the status of liposomes in practical applications. The release profile of VANH from liposomes was biphasic. The initial fast release of around 31% of the drug from the liposomes was observed in the first 1 h, which could probably be due to the portion of the drug that leaked out of liposomes and the unloaded drug.

3.4. Pharmacokinetics studies and drug distribution studies in mice

3.4.1. The experimental results of pharmacokinetics

At the range of 0.5–40 $\mu\text{g/ml}$, the standard curve was $A = 14.699C + 0.1388$ and the standard showed a good linearity with a correlation coefficient of 0.9999. The blood concentration–time curves of VANH in mice after intravenous administration of a single 15 mg/kg dose of VANH-Sol and VANH-Lips were shown in Fig. 5. The pharmacokinetic parameters calculated by DAS 2.0 software were shown in Table 4. Based on the analysis of the models and parameters, it was concluded that the mean blood concentration–time profiles for both VANH-Sol and VANH-Lips were corresponded to the

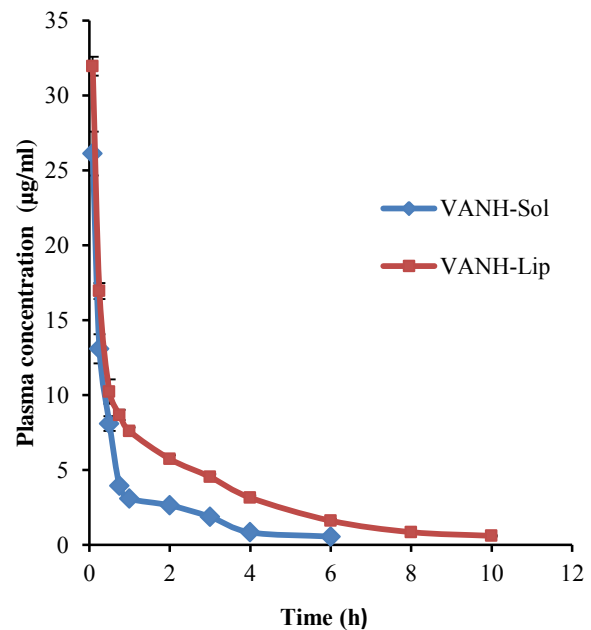


Fig. 5 – Mean plasma concentration–time curves of VANH in mice after intravenous administration of VANH-Sol and VANH-Lips at a dose of 15 mg/kg ($n = 5$).

Table 4 – Main pharmacokinetic parameters of VANH in mice after intravenous administration of VANH-Sol and VANH-Lips at a single dose of 15 mg/kg.

Parameter	VANH-Sol	VANH-Lips
$t_{1/2\alpha}$ (h)	0.145 ± 0.005*	0.101 ± 0.013*
$t_{1/2\beta}$ (h)	1.888 ± 0.067*	2.240 ± 0.066*
V1 (l/kg)	0.410 ± 0.032*	0.305 ± 0.024*
CL (l/h/kg)	0.667 ± 0.011*	0.336 ± 0.002*
AUC _{0–6} (mg/l·h)	18.918 ± 0.339*	36.190 ± 0.337*
AUC _{0–∞} (mg/l·h)	22.490 ± 0.366*	44.710 ± 0.306*
MRT _{0–6} (h)	1.285 ± 0.042*	1.776 ± 0.003*
MRT _{0–∞} (h)	1.699 ± 0.198*	2.768 ± 0.021*

Symbols * denote $P < 0.05$.

two-compartment model following intravenous administration, the weight coefficient was expressed as 1/cc.

After i.v. administration, VANH-Sol was removed from the blood circulation of mice with a half-life of about 1.888 h. On

the contrary, the half-life of VANH-Lips increased to 2.240 h compared with VANH-Sol. Furthermore, compared with VANH-Sol, MRT_{0–t} and MRT_{0–∞} of VANH-Lip increased by 0.83 and 0.61 fold, respectively. The extended MRT may due to the sustained release of VANH from VANH-Lips and the fact that the reticuloendothelial system (RES) removal could be avoided [28]. What's more, AUC_{0–t} and AUC_{0–∞} of VANH-Lips increased about 1.13-fold and 0.99-times compared with those of VANH-Sol, respectively. Therefore, the prolonged blood circulation and higher AUC value of VANH-Lips may contribute to higher therapeutic efficacy on long-periodic treatment of osteomyelitis.

The plasma concentration–time profile after intravenous administration of VANH-Sol and VANH-Lips at a dose of 15 mg/kg VANH was shown in Fig. 5. The measured VANH plasma concentration achieved from VANH-Lips group was higher than that of the control VANH-Sol at each time point. Then the plasma concentration of VANH-Sol declined significantly more rapid than that of VANH-Lips. This is

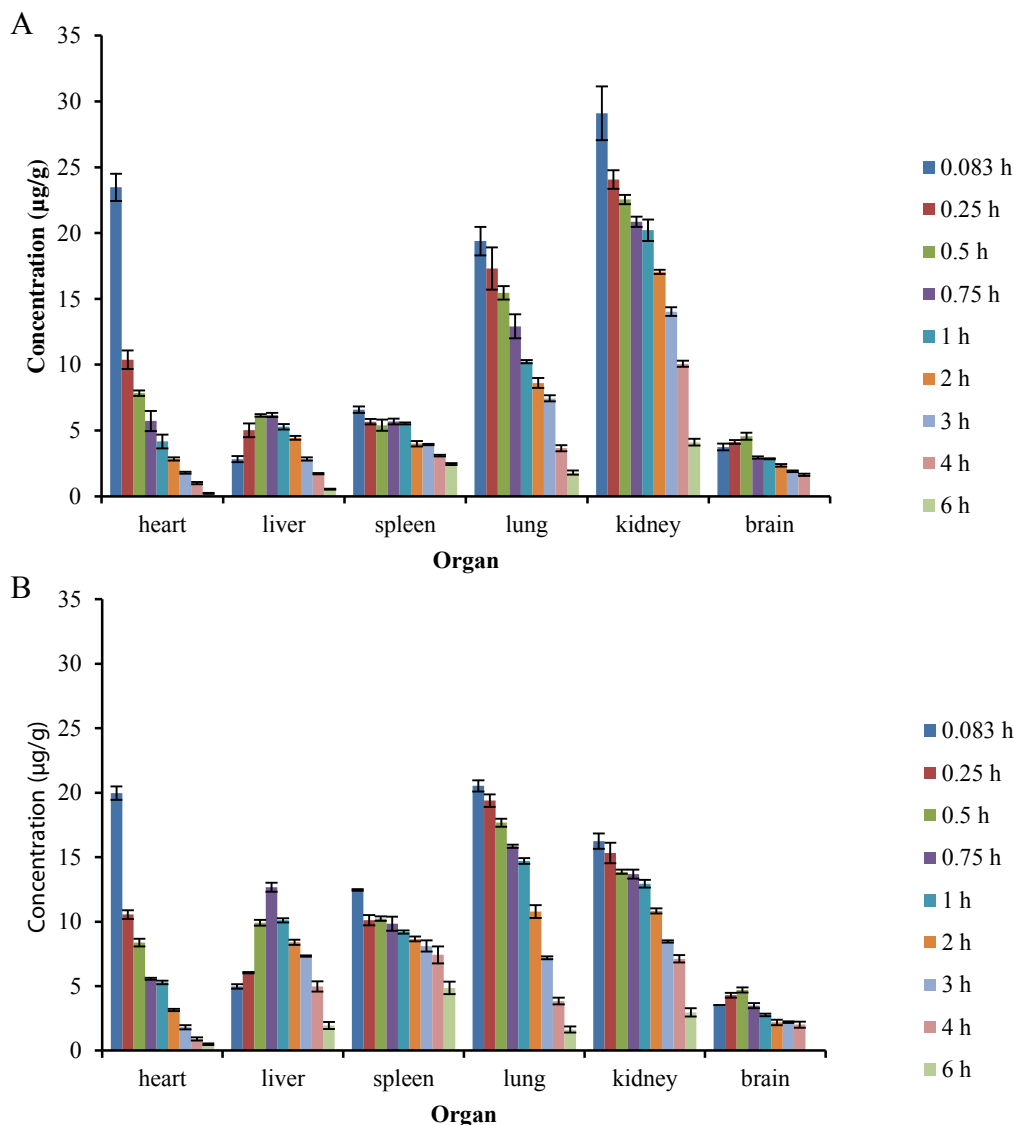


Fig. 6 – VANH concentration in tissue at different time points after intravenous administration of (A) VANH-Sol and (B) VANH-Lips (n = 5).

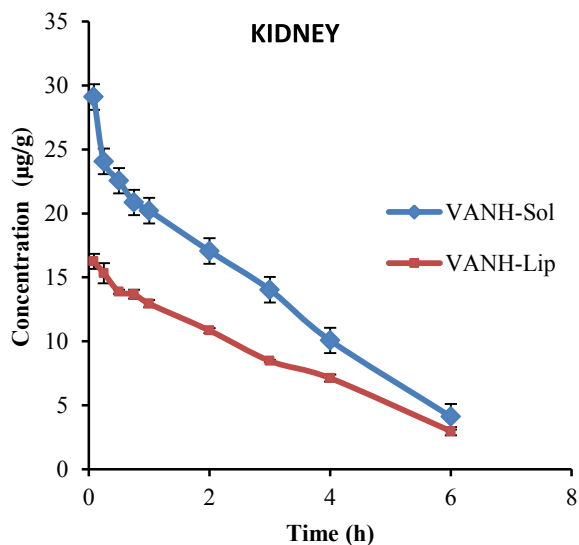


Fig. 7 – Kidney concentration–time curves of VANH after the intravenous administration of VANH-Sol and VANH-Lips in mice (n = 5). *Unit of blood drug level (µg/g).

possibly because that VANH was encapsulated into phospholipid bilayer and released into the plasma for a prolonged period of time. Moreover, it was reported that nanoparticles smaller than 220 nm could escape macrophage clearance to some extent. So the VANH-Lips can reduce clearance by macrophage as well, which will contribute to long circulation in blood [28]. Pharmacokinetic results in this study indicated VANH encapsulated in liposomes retains in the blood stream much longer than the VANH-Sol. So the VANH-Lip has more time to interact with the APL cell, which may benefit to the long-period clinical application of VANH.

3.4.2. Drug distribution studies

Within 0.2–30 µg/ml VANH concentration, the standard curves of all measured organs were established and the correlation coefficients range from 0.9990 to 0.9997. The optimum liposome-loaded VANH formulation by modified reverse phase evaporation method was used to investigate the bio-distribution of liposomal VANH in mice and evaluate its relative retaining activities to the certain organs such as heart, liver, spleen, lung, kidney and brain by determining the concentration of vancomycin hydrochloride. The tissue distributions of VANH after intravenous injection of a single 15 mg/kg dose of VANH-Sol and VANH-Lips were shown in Fig. 6, respectively. Remarkably, the drug distribution was different in the VANH-Lips group and the VANH-Sol group. Briefly, the concentration of VANH in kidney, an important excretory organ for i.v. delivery of VANH, was much lower for the VANH-Sol group, which may be expected to reduce or avoid the potential side effects to kidney. Furthermore, VANH-Lips resulted in a higher drug accumulation in liver and spleen compared with VANH-Sol ($P < 0.05$), which may be explained by the fact that liposome presented accumulative activity in RES sites such as lung, liver and spleen [29]. Conversely the biodistribution of VANH-Lips in non-RES sites such as in kidney decreased compared with VANH-Sol, which potentially resulted in the reduction of renal damages.

Notably, as is shown in Fig. 7, the drug concentrations in kidney were decreased tremendously after the administration of VANH-Lips compared with the VANH-Sol, which could improve the efficiency of VANH and decrease the side effects. This phenomenon may be explained by the fact that liposome encapsulation results in a decrease of the renal clearance [30].

The C_{max} , AUC and MRT values for the two groups in different tissue were calculated and are reported in Table 5. The tissue distribution results were evaluated according to Re ($Re = AUC_{VANH-Lip}/AUC_{VANH-Sol}$). If the value of Re exceeds 1, the tissue is exposed to drug to a greater extent by the

Table 5 – Biodistribution of vancomycin in liver, spleen and kidney tissue after intravenous administration of a dose (15 mg/kg) of solution and liposome formulations.

Organs	Parameter	VANH-Sol	VANH-Lip	Re
Heart	AUC _{0–6} (mg/l h)	18.549 ± 0.726	19.191 ± 0.154	1.040
	C _{max} (µg/g)	23.474 ± 1.040	19.975 ± 0.523	
	MRT (h)	1.288 ± 0.027	1.372 ± 2.283	
Liver	AUC _{0–6} (mg/l h)	12.804 ± 0.375*	27.750 ± 1.039*	2.167
	C _{max} (µg/g)	6.245 ± 0.078	12.669 ± 0.358	
	MRT (h)	2.285 ± 0.066	3.054 ± 0.156	
Spleen	AUC _{0–6} (mg/l·h)	23.851 ± 0.346*	47.266 ± 1.559*	1.982
	C _{max} (µg/g)	6.531 ± 0.277	12.469 ± 0.064	
	MRT (h)	6.405 ± 0.053	11.044 ± 5.778	
Lung	AUC _{0–6} (mg/l h)	43.679 ± 0.596*	50.435 ± 0.663*	1.155
	C _{max} (µg/g)	19.391 ± 1.080	20.530 ± 0.440	
	MRT (h)	1.825 ± 0.014	1.816 ± 0.026	
Kidney	AUC _{0–6} (mg/l·h)	80.085 ± 1.351*	51.631 ± 1.482*	0.645
	C _{max} (µg/g)	29.102 ± 2.047	16.241 ± 0.588	
	MRT (h)	3.057 ± 0.125	3.672 ± 0.313	
Brain	AUC _{0–6} (mg/l h)	10.197 ± 0.164	7.647 ± 5.022	0.750
	C _{max} (µg/g)	3.557 ± 1.482	4.706 ± 0.203	
	MRT (h)	1.649 ± 0.004	1.711 ± 0.534	

Re = AUC_{VANH-Lip}/AUC_{VANH-Sol}, Symbols * denote $P < 0.05$.

liposomes [31]. The Re of heart, liver, spleen, lung and kidney were 1.040, 2.167, 1.982, 1.155, 0.645 and 0.750, respectively, shown in Table 5. It is in accordance with Fig. 6. It demonstrated that liposomes presented accumulative activity in reticuloendothelial system (RES) sites such as spleen and liver. In addition, the value of Re for lung were also higher than 1, which showed that VANH-Lips were also inclined to distribute to lung though less preferable to liver and spleen. Therefore VANH-Lips performed their potential as a delivery system targeting to lung for the treatment of pulmonary inflammation. The AUC of heart and brain were not significantly changed. Conversely, the biodistribution of liposomes in non-RES sites such as in kidney decreased with descending Re compared with VANH-Sol, which potentially resulted in the reduction of nephrotoxicity or renal damages. This prompted the prepared VANH-Lips had the advantage in the long-periodic treatment of osteomyelitis.

4. Conclusions and outlook

In this work, VANH-Lips was successfully developed. The EE of VANH-Lips were significantly increased. The in vitro and in vivo physicochemical properties of VANH-Lips were characterized and evaluated in detail. The in vitro drug release experiments exhibited biphasic drug release characteristic and slower release than VANH-Sol. The pharmacokinetics studies confirmed that the circulation time of VANH in blood can be extended by liposome encapsulation. The tissue distribution study indicated that VANH-Lips decreased the drug accumulation in kidney following intravenous injection in mice. We, therefore, conclude that VANH-Lips may serve as a promising carrier for systematic delivery of VANH in the treatment of osteomyelitis.

Liposome as drug carriers significantly influence on drug distribution and reduce toxic side effects during antibiotic therapy [32–34]. In this study, VANH-Lips were found to reduce the distribution of VANH in kidney, which might help to lower its renal toxicity and eliminate its potential toxicity of kidney tubules. These findings suggested that VANH-Lips might have a potential to be developed as a promising pharmaceutical preparation for the treatment of chronic osteomyelitis.

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