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Studies on the Encapsulation of Oxymatrine into Liposomes by Ethanol Injection and pH Gradient Method

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ABSTRACT Oxymatrine is the major active alkaloid constituent extracted from traditional Chinese herb medicine *Sophora flavescens* Ait (Kushen) and *Sophora alopecuroides* (Kudouzi). In recent years, oxymatrine had been found to posses remarkable anti-hepatic activity and has been used for treating hepatitis B in clinical therapy in China. In this study, we attempted to entrap oxymatrine into liposomes to facilitate the delivery of oxymatrine to the liver and enhance the therapeutic efficiency for hepatitis. Preformulation investigation was performed to obtain the drug physicochemical properties such as solubility, pKa, and logP for rational liposomes preparation design. Liposomes were prepared with soybean lecithin by ethanol injection and pH gradient loading methods. At the same time the factors affecting the entrapment efficiencies were investigated and compared. Ethanol injection method yielded liposomes with entrapment efficiency less than 20%. The lipid composition and aqueous medium had some effects on entrapment efficiency. However, liposomes could be produced with entrapment efficiency above 50% by pH gradient method. The internal pH buffer capacity, the lipid composition, and drug-to-lipid ratio greatly influenced the entrapment efficiency, while the incubation temperature had almost no effect on entrapment efficiency in the active loading procedure.

KEYWORDS Liposomes, Oxymatrine, Entrapment efficiency, Ethanol injection, pH gradient

INTRODUCTION

Hepatitis B caused by the hepatitis B virus (HBV) which can attack and injure the liver is one of the major diseases affecting mankind. It has been a serious global public health problem. Two billion people (one out of three people) around the world have been infected with the hepatitis B virus, and 400 million people have been chronically infected (which means they are unable to get rid of the virus) (www.hepb.org). In much of the developing countries (sub-Saharan Africa, most of Asia, and the Pacific), liver cancer caused by HBV is among the top three causes death by cancer in men (www.who.int).

Oxymatrine (Fig. 1) is a major active alkaloid constituent in the dried root of *Sophora flavescens* Ait (Kushen), and is also obtained from the *Sophora*

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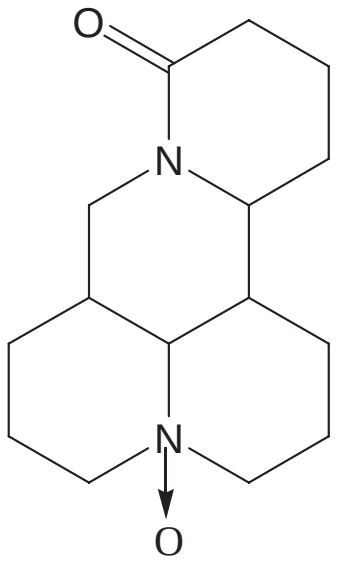

FIGURE 1 **Chemical Structure of Oxymatrine.**

alopecuroides (Kudouzi). Those herb medicines have been used in China for thousands of years. In recent years, oxymatrine has been found possess strong activities of inhibiting HBV (Chen et al. 2001; Cai et al. 1999). Clinical trial researchers concluded that oxymatrine was an effective and safe agent for the treatment of chronic hepatitis B (Lu et al. 2003). Injection and capsule formulation of oxymatrine have been approved on the market in China for treating HBV infection.

The pharmacokinetic study of oxymatrine showed that oxymatrine had a half-life time of about 2 hours (Wang et al, 2003). The drug administrated by injection will be distributed to all over the body and eliminated rapidly. To enhance the therapy efficacy of oxymatrine, it is necessary to increase the accumulation of drug in the liver and prolong the half-time in plasma. As drug carriers, liposomes could change the biodistribution of drugs and preferably distribute in reticular endothelial system (RES)-rich areas, especially in liver. At the same time, small-size liposomes (<200 nm) are preferentially taken up by the hepatocytes in the liver (Scherpphof & Kamps, 2001). Anti-hepatic virus drug entrapped in liposomes could be targeted to the site of action to increase the therapeutic efficiency. On the other hand, liposomes can control the release rate of encapsulated drugs. For these reasons, liposomes would be a favorable carrier for oxymatrine to maintain effective concentration level in the liver over longer time periods. Liposomal oxymatrine is expected to be more efficacious than free drug.

The aim of the present study is to select a suitable preparation method for encapsulating oxymatrine into liposomes with high entrapment efficiency. Although oxymatrine has been frequently used in clinical therapy, its physicochemical proprieties are still not well reported. Preformulation investigation was performed to obtain the properties parameters of oxymatrine such as solubility, pKa, and octanol/buffer partition coefficients for rational experiment design. Liposomes were prepared by ethanol injection methods and pH gradient method, and the factors influencing encapsulation were investigated. The preparation method with higher entrapment efficiency was chosen to develop a suitable liposomal preparation in the further investigation.

MATERIALS AND METHODS

Soybean phosphatidylcholine (SPC, Epikuron200, PC >92%) and soybean phosphatidylserine (SPS, Leci-PS90PN, PS >85%) were kindly gifted from Degussa, Freising, Germany. Soybean phosphatidylcholine for injection (SPC for injection, PC>70%) was obtained from Jin Ban Pharmaceuticals, Shanghai, China. Cholesterol (Chol) was purchased from Shenyang Medicines Company, Shenyang, China. Oxymatrine was obtained from Chia Tai Tianqing Pharmaceuticals, Lianyungang, China. All other reagents were analytical grade.

Preformulation Investigation

Determination of Solubility

The approximate solubility of oxymatrine in three different aqueous media including distilled water, sodium carbonate solution (150mM/L), and citric acid solution (150mM/L) was determined according to the procedure in Pharmacopeia of the People's Republic of China (2000) with some modification. Briefly, three samples of 100 mg finely powdered oxymatrine were weighed out and put in three vials at temperature of 25°C, 100 μl three solvents mentioned above were added respectively, the vials were shaken vigorously for 30 sec at a interval of 5 min, and the solubility behavior was observed for 60 min. It was considered to be completely soluble if no particles of oxymatrine were observed.

Determination of Ionization Constant

The ionization constant (pK_a) of oxymatrine was determined according to both potential titration and

spectrophotometric indicator method as described in literature (Albert & Serjeant, 1984).

Determination of Partition Coefficient

The partition coefficient of oxymatrine was measured by shake-flask method. Oxymatrine was dissolved in six different pH phosphate buffer solutions (50 mM) with the pH ranging from 4.0 to 10.0. All buffers were adjusted to isoosmotric with sodium chloride. Oxymatrine solution was mixed with n-octanol (presaturated with water) in equal volume, shaken, and incubated in a water bath at 25°C for 2 h. After centrifugation, the concentrations of oxymatrine in the aqueous phase and in the original solution were determined by HPLC, and the octanol phase concentration was calculated by subtraction. Then the apparent partition coefficient was obtained by the ratio of the oxymatrine concentration in octanol phase to aqueous phase.

Liposome Preparation

For all preparations, the lipid mixtures were composed of SPC and Chol at a mass ratio of 4:1. The mass ratio of oxymatrine to total lipid was from 1:2 to 1:6. Liposomes were prepared by ethanol injection and pH gradient loading method. To get homogenous liposomes suspensions, liposomes were extruded in turn through 0.8, 0.45, 0.22 μm micropore filter by Extruder five times, respectively (Lipex Biomembranes Inc., Vancouver, Canada). Brief descriptions of the procedures follow.

Ethanol injection method

Ethanol injection method was performed with some modification (Pons et al., 1993). Oxymatrine and lipids were co-dissolved in 3 mL absolute ethanol at 60°C and injected into 10 mL empty aqueous medium under magnetically stirring at 60°C. The ethanol was evaporated to no odor, then water was added to adjust the volume of final liposomes suspension to 10 mL.

pH gradient loading method

pH gradient loading method was performed as Mayer did (1993). Empty liposomes were prepared by a combination of standard thin-film hydration method and repeated extrusion. The total lipids were co-dissolved in chloroform and put into in a round bottom flask, and the solvent was removed by rotary evaporation under reduced pressure to form thin film. Trace of residue solvent was removed by putting the flask into a lyophilizer under high vacuum condition for 2 h. The dried lipid films were hydrated at 40°C with 10 mL citric acid solution and dispersed by hand shaking with glass beads to yield empty liposomes.

After extrusion, the extravesicular pH of empty liposomes was adjusted to 8.0 by sodium carbonate solution (150 mM/L). Oxymatrine solution of 10 mg/mL was added into the alkalinized empty liposomes and incubated in a water bath at 50°C for 10 min, then cooled with cold water immediately.

Entrapment Efficiency

The entrapment efficiency was calculated by the percentage of oxymatrine incorporated into liposomes relative to the amount of oxymatrine in liposomes suspension. To determine the entrapment efficiency, the oxymatrine concentration of final liposome preparation was adjusted to 1 mg/mL with 5% glucose solution and then divided into two portions. In one portion, unentrapped free drug was separated from the liposomes by ultrafiltration (10 mL Omegacell™ stirred cell devices, membrane: polyethersulfone, cutoff: 100 kDa, Pall life sciences, Ann Arbor). In the other portion, a mixture of entrapped and unentrapped oxymatrine was disrupted by the addition of 10% Triton X-100 to form a clear solution. The amount of unentrapped and originally total oxymatrine was determined by HPLC. The entrapment efficiency (EE) could be calculated according to the following equation:

$$EE\% = 1 - C_{\text{free drugs}} / C_{\text{total drugs}}$$

(C is the concentration of oxymatrine)

Determination of Oxymatrine

A Shimadzu HPLC system (Kyoto, Japan) consisting of an LC-10AT pump, an SPD-10A UV-VIS detector was used for the assay of oxymatrine. A 150 × 4.6 mm Kromasil ODS (5 μm) column (Dalian institute of chemical physics, China) were used. The mobile phase was a mixture of 92% phosphate buffer (0.1% phosphoric acid solution, adjusted pH to 3.0 with triethylamine) and 8% acetonitrile. The flow rate was 0.8 mL/min. A sample volume of 20 μl was injected. The drug was detected at a wavelength of 220 nm.

RESULTS

Preformulation Investigation

Three samples of 100 mg oxymatrine completely dissolved in 0.1 mL citric acid solution (pH1.65), distilled water, and sodium carbonate solution (pH 11.34), respectively. It suggested that oxymatrine is a very soluble drug (approximate water solubility>1:1)

The pKa of oxymatrine determined by both potential titration and spectrophotometric indicator methods results in a similar value of 6.7, which is higher than pKa 6.0 determined by CE method (Gong et al., 2003). The difference of pKa may be due to the different determinatation method used. The apparent partition coefficient of oxymatrine at different pH buffer is shown in the Fig. 2. The true partition coefficient of oxymatrine was 0.2, which was obtained at pH 10 because all oxymatrine is unprotonated at this pH value. The logP of oxymatrine was log0.2, which is –0.7.

The Effecting Factors on Entrapment Efficiency of Liposomes Prepared by Ethanol Injection Method

The Effect of Aqueous Medium

In ethanol injection method, when pure water was used as the aqueous medium, entrapment efficiency of liposomes prepared by epikuron 200 was less than 10% before extrusion. When the lipid was replaced by SPC for injection, the entrapment efficiency could reach 16%. While in the aqueous medium of PBS, liposomes prepared by both lipids result in low entrapment efficiency less than 5% before extrusion. The result was shown in Fig. 3.

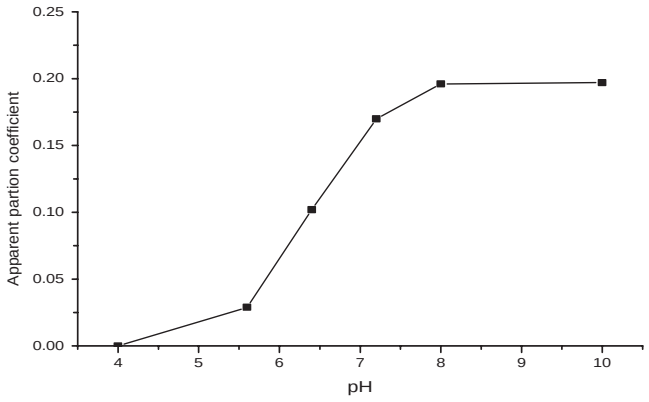

FIGURE 2 **The Partition Coefficient of Oxymatrine in Different pH.**

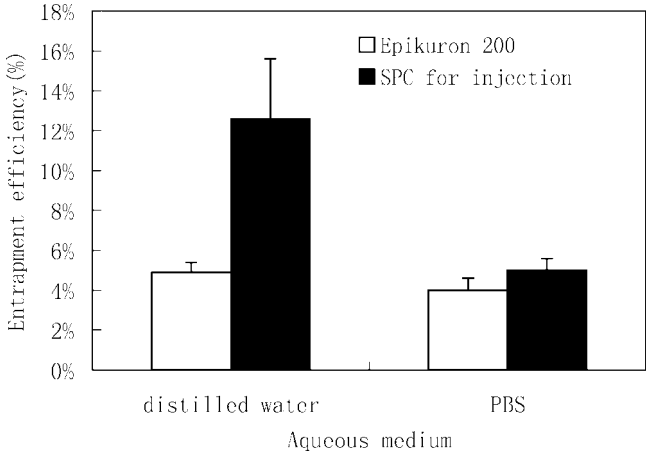

FIGURE 3 **The Effect of Aqueous Medium on Entrapment Efficiency of Liposomes Prepared by Ethanol Injection Method (Drug to Lipid Ratio 1:6(w/w), Liposomes Unextruded).**

The Effect of Lipid Composition

Liposomes consisting of different composition of lipids were prepared in the aqueous medium of pure water. As were shown in the Fig. 4, before extrusion, liposomes with various entrapment efficiencies ranging from 8% to 35% could be obtained. The neutrally charged lipid Epikuron 200 yielded the lowest entrapment efficiency, about 10%. When negatively charged lipid PS was introduced, the entrapment efficiency of liposomes increased to 18%. Liposomes composed of all negative-charged lipid PS resulted in the highest entrapment, about 35%. After extrusion, entrapment

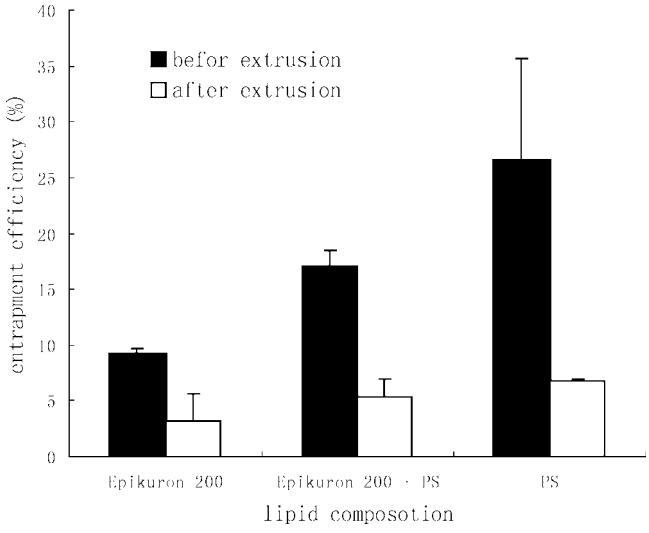

FIGURE 4 **The Entrapment of Oxymatrine Liposomes Prepared by Ethanol Injection Method with Different Lipid (The Drug Concentration is 10 mg/mL, the Lipid Concentration is 60 mg/mL).**

efficiency of all liposomes decreased but still remained the increasing trends with the adding of PS.

The Effecting Factors on Entrapment Efficiency of Liposomes Prepared by pH Gradient Method

The Effect of Lipid Composition

Liposomes prepared with epikuron 200 by pH gradient method could entrap oxymatrine more than 50% at the mass ratio of drug-to-lipid range below 0.5. Liposomes prepared with SPC for injection entrapped oxymatrine less than 40%. (Fig. 5).

In order to evaluate the effect of complex phospholipids, Epikuron 200 and PS were mixed with mass ratio of 3:1, and liposomes composed of that complex phospholipids and chol with mass ratio 4:1 were prepared. The liposomes aggregated seriously in citric acid solution, and the entrapment efficiency obtained was 20%.

Effect of Citric Acid Concentration

The ΔpH between vesicular and internal-vesicular buffer capacity greatly influences the entrapment efficiency. As shown in Fig. 6, higher concentration citric acid provided higher ΔpH and internal-vesicular buffer capacity, thus leading to higher entrapment efficiency.

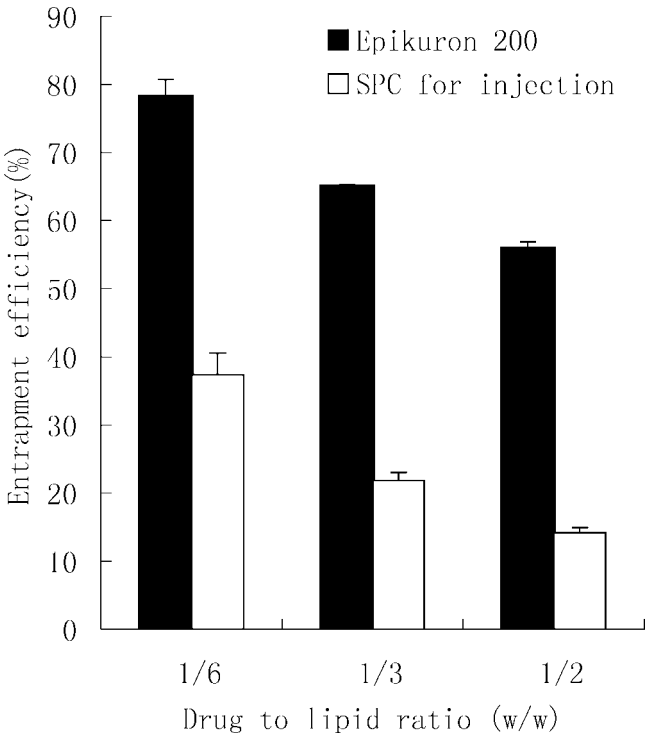

FIGURE 5 **The Effect of Lipid Composition on Entrapment Efficiency of Oxymatrine Liposomes Prepared by pH Gradient Method at Different Drug to Lipid Ratio (Citric Acid Concentration is 150 mM/L).**

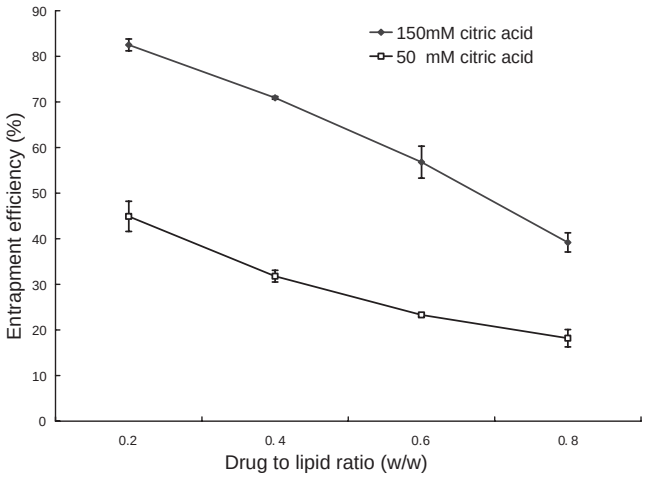

FIGURE 6 **The Entrapment Efficiency of Oxymatrine by pH Gradient Method of Different Citric Acid Concentration at Different Drug to Lipid Ratio.**

Effect of Incubation Temperature on Entrapment Efficiency

The pH gradient method was performed at both room temperature (25°C) and 50°C. Similar entrapment efficiency was obtained, which was seen in Fig. 7.

DISCUSSION

Passive loading mainly depends on the entrapped volume and the interaction between drug and liposomes bilayer (Barenholz, 1998). In ethanol injection

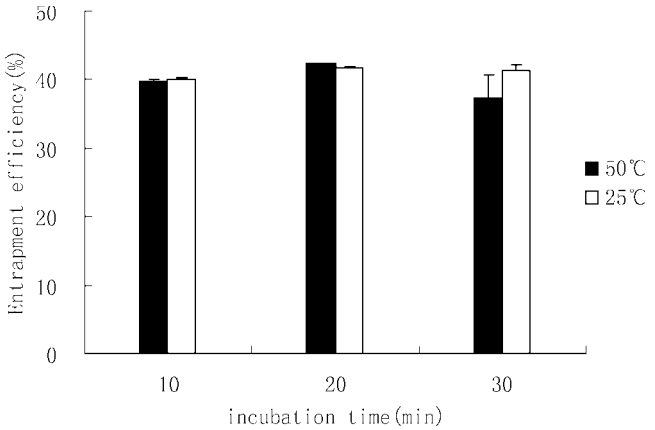

FIGURE 7 **The Entrapment Efficiency of Oxymatrine Liposomes in pH Gradient Method at Different Temperature (Drug to Lipid Mass Ratio 1:4, Citric Acid Concentration 50 mM/L).**

method, the effect of both lipid composition and aqueous medium on entrapment efficiency, may be due to the electrostatic interaction between lipid and oxymatrine. As SPC for injection was a mixture, the PC content of SPC for injection was about 70%, and there was some other negatively charged lipid such as PS and PG in the mixture. When liposomes composed of SPC for injection were prepared in pure water, these negatively charged lipid may adsorb positively charged oxymatrine by electrostatic interaction. When liposomes were prepared in the PBS medium, the electrolyte in PBS would decrease the electrostatic interaction between drug and negative-charged lipid, thus leading to low entrapment efficiency. Liposomes composed of high-purity PC of Epikuton 200 (PC content above 92%) consisted of almost all neutrally charged lipid. There was little interaction between neutrally charged lipid and oxymatrine, which did little effect on the entrapment efficiency, thus yielding liposomes with low entrapment efficiency in both aqueous media.

Although there was some weak interaction between oxymatrine and negative-charged lipid, most of the drug leaked from the liposomes after extrusion. The less-entrapped volume of liposomes by passive loading method results in lower entrapment efficiency.

To deliver a sufficient amount of drug for providing a therapeutic effect, a high entrapment efficiency of drug in liposomes is required. The pH gradient method provides a feasible loading method for weak base and weak acid drug with high entrapment efficiency. Although oxymatrine is a very hydrophilic drug with low logP, as a weakly alkaline drug, sufficient amount of oxymatrine could be entrapped into liposomes by pH gradient loading method, which is similar to some weak base drug reported in literature (Madden et al., 1990).

In pH gradient method, liposomes composed of high-purity PC are superior to low-purity lipid for higher entrapment efficiency. The adding of negatively charged lipid PS greatly reduced the entrapment efficiency, which may be due to the aggregated lipid lost during the extrusion process. Although liposomes composed of the SPC for injection aggregate slightly in citric acid solution, the entrapment efficiency is still low. It seems that complex phospholipids containing negatively charged lipids may not reduce the leakage of proton and maintain the pH gradient across the bilayer. The incubation temperature and time did little effect on oxymatrine entrapment efficiency. That is not the same as adriamycin, which prefers higher temperature in active loading procedure (Mayer, 1995). The difference may relate to the difference of physicochemical propriety of drug entrapped. As a small molecule drug, oxymatrine need to overcome lower active energy than adriamycin in the active loading procedure.

The low pH of citric acid solution in active loading procedure may not be suitable for liposomes suspension for long-time stability due to the phospholipid hydrolysis. A further lyophilized liposome formulation, which may solve stability problems for liposomes suspension mentioned above, will be developed in the future. This investigation is undertaken in our lab.

CONCLUSION

Preformulation investigation suggests oxymatrine is a very soluble weak alkaline drug with low logP. The liposomes prepared by ethanol injection method yield low entrapment efficiency due to the high water solubility of oxymatrine and its weak interaction with lipid. While as a weak base drug, oxymatrine could be successfully encapsulated into liposomes with high entrapment efficiency by pH gradient method.

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REFERENCES

Albert, A, & Serjeant, E. P. (1984) *The Determination of Ionization Constant*. 3rd Ed. London: Chapman and Hall, 14–35, 70–95.

Barenholz, Y. (1998) Design of liposome-based drug carriers: From basic research to application as approved drugs. In *Medical Application of Liposomes*; Lasic D.D and Papahadjopoulos D., Eds. Amsterdam: Elsevier Science, 545–565.

Cai, X., Wang, G. J., Qu, Y., Fan, C. H., Zhang, R. Q., & Xu, W. S. (1997) Clinical efficacy of kurorinone in the treatment of chronic hepatitis B. *Dier Junyi Daxue Xuebao. 18*, 47–49.

Chen, X. S., Wang, G. J., Cai, X., Yu, H. Y., & Hu, Y. P. (2001) Inhibition of hepatitis B virus by oxymatrine in vivo. *World J Gastroenterol. 7*(1), 49–52.

Gong, S. X., Su, X. D., Bo, T., Zhang, X., Liu, H. W., & Li, K. A. (2003). Determination of dissociation constants of ten alkaloids by capillary zone electrophoresis. *J. Sep. Sci*. 26, 549–554.

http://www.hepb.org/02-0592.hepb (accessed Oct 2004)

Lu, L. G., Zeng, M. D., Mao, Y. M., Li, J. Q., Wan, M. B., Li, C. Z., Chen, C. W., Fu, Q. C., Wang, J. Y., She, W. M., Cai, X., Ye, J., Zhou, X. Q., Wang, H., Wu, S. M., Tang, M. F., Zhu, J. S., Chen, W. X., & Zhang, H. Q. (2003) Oxymatrine therapy for chronic hepatitis B: a randomized double-blind and placebo-controlled multi-center trial. *World J Gastroenterol. 9*(11), 2480–3.

Madden, T. D., Harrigan, P. R., Tai, L. C., Bally, M. B., Mayer, L. D., Redelmeier, T. E., Loughrey, H. C., Tilcock, C. P., Reinish, L. W., & Cullis, P. R. (1990). The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. *Chem. Phys. Lipids*, 53, 37–46.

Mayer, L. D., Madden, T. D., Bally, M. B., & Cullis, P. R. (1993) pH Gradient-Mediated Drug Entrapment in Liposomes. In *Liposome Technology*. 2nd Ed. Gregoriadis G.; Ed. London, CRC Press, Vol. 2, 27–44.

Pons, M., Foradada, M., & Estelrich, J. (1993) Liposomes obtained by the ethanol injection method. *Int. J. Pharm. 95*(1–3), 51–56.

Scherpphof, G. L., & Kamps, J. A. A. M. (2001) The role of hepatocytes in the clearance of liposomes from the blood circulation. *Pro Lipid Res*. 40, 149–166.

State Pharmacopeia Commission of P.R. China. (2000) *Pharmacopeia of the People's Republic of China* (English editon 2000). Beijing: Chemical Industry Press, Vol.2, XX.

Wang, Y., Zheng, W. Y., Menggen, D. L., & Liu, S. D. (2003) Pharmacokinetic study of oxymatrine injection in chinese healthy volunteers. *The chinese journal of clinical pharmacology 19*(4), 301–305.

http://www.who.int/mediacentre/factsheets/fs204/en/ (accessed Oct 2004).