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# A lipid microsphere vehicle for vinorelbine: Stability, safety and pharmacokinetics

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**Abstract**

A lipid microsphere vehicle for vinorelbine (VRL) was designed to reduce the severe venous irritation caused by the aqueous intravenous formulation of VRL. Lipid microspheres (LMs) were prepared by high pressure homogenization. The physical stability was monitored by the appearance, particle size and zeta potential changes while the chemical stability was achieved by using effective antioxidants and monitored by long-term investigations. Safety tests were performed by testing rabbit ear vein irritation and a guinea pig hypersensitivity reaction. A pharmacokinetic study was performed by determining the drug levels in plasma up to 24 h after intravenous administration of VRL-loaded LMs and conventional VRL aqueous injection separately. The VRL-loaded LMs had a particle size of $180.5 \pm 35.2$ nm with a 90% cumulative distribution less than 244.1 nm, while the drug entrapment efficiency was 96.8%, and it remained stable for 12 months at $6 \pm 2\,^{\circ}C$. The VRL-loaded LMs were less irritating and toxic than the conventional VRL aqueous injection. The pharmacokinetic profiles were similar and the values of $AUC_{0-t}$ were very close for the two formulations. A stable and easily mass-produced VRL-loaded LM preparation has been developed. It produces less venous irritation and is less toxic but has similar pharmacokinetics *in vivo* to the VRL aqueous injection currently commercially available.


*Keywords:* Vinorelbine; Lipid microspheres; Stability; Safety; Pharmacokinetics; Drug delivery

## 1. Introduction

Vinorelbine is a semi-synthetic vinca alkaloid (Fig. 1) which has a wide anti-tumor spectrum of activity, and is especially active in advanced breast cancer (ABC) and advanced/metastatic non small cell lung cancer (NSCLC). Compared with other vinca alkaloids, vinorelbine has been shown to have a clearly higher activity and lower neurotoxicity (Sabot et al., 1998; Bonneterre et al., 2001). An injectable formulation of vinorelbine (Navelbine® IV) developed by Pierre Fabre Medicament France is now widely marketed for the treatment of NSCLC and ABC in many countries around the world. However, Navelbine® IV is not an optimal drug delivery system for vinorelbine, because vinorelbine has a vesicant action and is well known to cause venous irritation and phlebitis when directly administered intravenously as an aqueous solution (Yoh et al., 2004). Venous irritation has been reported, such as injection site reactions, local reactions, and superficial phlebitis. The symptoms erythema, pain at the injection site, vein discoloration and tenderness along the vein are often observed clinically (Mare et al., 2003). Also, the venous tolerability is dependent on the duration of the infusion (Rittenberg et al., 1995): the longer the infusion, the poorer the tolerability. In order to reduce patient discomfort, the injection must be infused rapidly, and then a large volume of 5% glucose or saline solution should be used to rinse out the blood vessels for over 15 min (Marquet et al., 1992). The latest report from Yoh et al. (2007) indicated that vinorelbine induced-local venous toxicity was observed in 33% of patients after a 6 min infusion and in 24% after a 1 min bolus, and the incidence of local venous toxicity per infusion was 16% for the 6 min infusion and 11% for the 1 min bolus. Thus, a new strategy is needed to reduce the venous irritation produced by aqueous injections of vinorelbine.

A stable oral dosage form of vinorelbine (Navelbine® Oral) developed in 1994 by Pierre Fabre Medicament is also available

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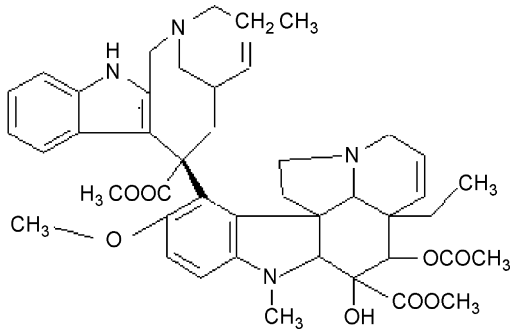

Fig. 1. Structure of vinorelbine.

on the market. It is a soft gelatin capsule filled with vinorelbine ditartrate/ethanol/water/glycerol/macrogol 400 solution (Bugat et al., 2002). The development of the oral formulation is due to the fact that i.v. administration is a major source of discomfort as well as patient pharmaco-economic issues. Patient preference and cost savings are additional advantages of oral chemotherapy (Liu et al., 1997; Bonneterre et al., 2001). An initial study published by Marty et al. (2001) described a bioavailability of oral vinorelbine which was close to 40%, although different results were subsequently published by Lush et al. (2005): 33% oral bioavailability of this oral formulation, and Puozzo (2006) gave a further explanation about the discrepancies between these two studies. According to a phase I oral dose-finding study, the recommended phase II oral dose was 80 mg/m$^2$/week (Chevallier et al., 1997). However, Depierre et al. (2001) found a high rate of early deaths (10%) due to complicated neutropenia toxicity when patients were given a weekly dose of 80 mg/m$^2$ by mouth; accordingly, the dose had to be reduced to 60 mg/m$^2$/week to continue the study. A study carried out by Marty et al. (2001) showed that a 60 mg/m$^2$ oral dose was comparable with a 25 mg/m$^2$ i.v. dose, and both formulations induced mainly haematological and gastrointestinal adverse effects. Nevertheless, the severity of these side effects was greater following oral administration than with the i.v. form. On account of the disadvantages of both Navelbine® IV and Navelbine® Oral, the present work was carried out to discover an ideal carrier for vinorelbine in order to reduce the severe venous irritation, enhance the anti-tumor activity and improve patient tolerance.

In recent years, much attention has been focused on drug delivery systems (DDS) for cancer chemotherapy which aim at the specific targeting of tumor cells or tumor tissues, thus enhancing the efficacy and reducing the toxicity of antitumor agents. Moreover, lipid microspheres (LMs), have been developed as a very useful approach for drug delivery (Mitsuko, 1996). LMs are prepared by dispersing biocompatible oil or triglyceride containing lipid soluble drugs using a homogenizer and/or ultrasonicator, with lecithin or phospholipids as the emulsifying agents. Drug-free LMs (known as lipid emulsions) have been marketed for parenteral nutrition (Intralipid®, Lipofundin®) for many years and are administrated in doses of 300–500 ml (Venkateswarlu et al., 2001). Several antitumor drug-loaded LMs have been reported to be less toxic, with enhanced activity and better stability, such as antitumor prostaglandins (PGs) LMs (Fukushima et al., 1997) and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) LMs (Mitsuko, 1996). Therefore, lipid microspheres are an ideal drug carrier. Schwarz et al. (1994) introduced the advantages and disadvantages of polymeric nanoparticles, fat emulsions (similar to LMs) and liposomes, and claimed that the advantages of polymeric nanoparticles and fat emulsions can be combined by producing particles from solid lipids. Solid lipid nanoparticles (SLNs) possess a solid matrix allowing controlled drug release, can be sterilized by autoclaving and can be produced by large scale. However, considering the drug loading capacity, storage stability and biocompatibility, we still choose LMs as a drug delivery system for vinorelbine. When drugs are incorporated into the core of the LM, this effectively protects them from degradation and, above all, the encapsulated drugs cause less irritation and fewer toxic effects and also exhibit sustained release and targeted delivery to various organs. Furthermore, LMs have many other advantages, such as being easy to mass-produce, safe for intravenous use, stable for long periods, with a high drug loading capacity and excellent cost-effectiveness compared with other drug carriers (L. Wang et al., 2006). Semple et al. (2005) designed an optimized liposomal system composed of Sphingomyelin/cholesterol to maximize *in vivo* drug retention, plasma circulation time, and therapeutic activity of vinorelbine. The report indicated that SM/Chol liposomal formulation of VRL had long plasma circulation times, a promising antitumor activity and an excellent pharmaceutical stability. The superiority of liposomes as drug carriers is now widely recognized and great advances have been made in this field. However, it is still not easy to prepare acceptable aseptic liposomal drugs with desirable properties, high encapsulation efficiency, and long-term stability without drug leakage and loss (T. Wang et al., 2006). Firstly, liposomal formulations cannot bear autoclaving, and other methods of sterilization all have disadvantages when used for liposomes (Gulati et al., 1998). The leakage of various types of drugs on autoclaving was studied by Zuidam et al. (1993). Thus, sterile operations have to be involved in preparing liposomes though the aseptic conditions were difficult to control; secondly, their difficulties in manufacturing, poor particle size reproducibility and questionable stability hindered more widespread applications of liposomes (Edwards and Baeumner, 2006); finally, liposomal formulations need higher cost than other drug delivery systems. Semple et al. prepared a vinorelbine liposomal system composed of Sphingomyelin/cholesterol for parenteral use (Semple et al., 2005), but did not mention the method of sterilization, and what is more, ionophore and solvent were not easy to remove completely, the process of liposome preparation was complicated and the cost of manufacture was high. Therefore, the development of lipid microsphere vehicle for vinorelbine seems to be another more feasible strategy.

In this study, vinorelbine ditartrate loaded lipid microspheres with a high drug entrapment efficiency were initially designed and prepared. Then their physicochemical stability, safety and pharmacokinetics were investigated to evaluate the formulation in detail.

## 2. Materials and methods

### 2.1. Materials and animals

Egg lecithin (Lipoid E80®) and medium-chain triglyceride (MCT) were obtained from Lipoid KG (Ludwigshafen, Germany); Poloxamer 188 (Pluronic F68®) was purchased from BASF AG (Ludwigshafen, Germany); Tween80 for parenteral use was obtained from Shenyu Medicine and Chemical Industry Limited Co. (Shanghai, China); Soybean oil for parenteral use was obtained from Tieling BeiYa Medicated Oil Co. (Tieling, China); Vinorelbine ditartrate and Navelbine® IV were purchased from Hangzhou Minsheng Pharmaceutical Co.(Hangzhou, China), and ketoconazole, the internal standard, was kindly provided by Nanjing Baijingyu Pharmaceutical Co. (Nanjing, China). All other chemicals and reagents were of analytical or chromatographic grade.

All the laboratory animals in this study were purchased form the Animal Center of Shenyang Pharmaceutical University (Shenyang, China).

### 2.2. Formulation and preparation of VRL-loaded LMs

Soybean oil, MCT and α-tocopherol were mixed under stirring at 70 °C to make the oil phase while the egg lecithin, F68, Tween80, oleate sodium, glycerol, and some of the antioxidants were dispersed in water for injection stirring at 75 °C to obtain a water phase. The oil phase was added to the water phase and mixed using a high-shear mixer at 8000 rpm to prepare a coarse emulsion, then vinorelbine ditartrate was added, with further stirring until the primary emulsion was obtained. After adjusting the pH to about 8.0 with 0.1 mol/l HCl or NaOH solution, the primary emulsion was passed through a high pressure homogenizer (Niro Soavi NS10012k, Niro Soavi S.p.A., Via M. Da Erba, Italy). Finally, the preparation was gassed with $N_2$ and sealed in 50 ml glass bottles followed by autoclaving for 15 min at 121 °C. The resulting formulation contained (%, w/w): Soybean oil 2%, MCT 8%, egg lecithin 1.2%, F68 0.2%, Tween80 0.2%, sodium oleate 0.05%, glycerol 2.5%, total antioxidants 0.32%, and water for injection to 100%.

### 2.3. Physical stability of VRL-loaded LMs

The physical stability of VRL-loaded LMs was evaluated by measuring the particle size, zeta potential, and examining the physical appearance. The particle size and zeta potential were measured using a NICOMP™ 380 Zeta Potential/Particle Sizer (Particle Sizing Systems, Santa Barbara, USA). The mean particle size and distribution were measured based on photon correlation spectroscopy (PCS, dynamic light scattering, DLS) technique, which is a powerful and versatile tool for estimating the particle size distribution of fine-particle materials ranging from a few nanometers to several micrometers (Komatsu et al., 1995). The zeta potential is a very useful way of evaluating the stability of any colloidal system, and it was determined based on an electrophoretic light scattering (ELS) technique. The physical appearance was examined by macroscopic observation or using a XSZ-G type light microscope (Chongqing Optical Instrument Co., Chongqing, China). The products were gassed with $N_2$, sealed in glass bottles and stored in darkness at 6 ± 2 °C unless otherwise indicated.

### 2.4. High-performance liquid chromatographic analysis

The vinorelbine content of the lipid microspheres was measured by high-performance liquid chromatographic (HPLC) analysis as described in the ChP 2005 edition (Chinese Pharmacopeia 2005), and it is similar to the method described in USP 26 (United States Pharmacopeia 26, pp. 1932–1933). The equipment consisted of a Jasco PU-980 pump (Japan), a Jasco UV-975 detector and a C18 analytical column (Phenomenex, 5 μm, 250 mm × 4.6 mm i.d.). The mobile phase consisted of a mixture of methanol (with 0.2% sodium 1-decanesulfonate added) and a 0.05 mol/l phosphate buffer solution (pH 4.2) (67:33, v/v). The phosphate buffer solution was prepared by dissolving monobasic sodium phosphate in water and the pH was adjusted to 4.2 with phosphoric acid. The flow rate was 1.0 ml/min and peak detection was performed at 267 nm. The column temperature was maintained at 40 °C. VRL-loaded LMs were directly dissolved in methanol prior to injection.

Related compounds were also determined by HPLC analysis according to the above chromatographic conditions (Chinese Pharmacopeia 2005). The *Test solution* was prepared by dissolving VRL-loaded LMs in methanol, and the *Contrast solution* was prepared by diluting the corresponding *Test solution* 100-fold with methanol. The same volume (20 μl) of both solutions was injected into the HPLC system, and ratios of each peak response for impurities obtained from the *Test solution* to the peak response for vinorelbine from the *Contrast solution* were calculated. The relative retention times were about 0.8 for the photodegradation product, 1.0 for vinorelbine, and 1.2 for vinorelbine-related compound A (4-*O* deacetyl vinorelbine).

### 2.5. Determination of entrapment efficiency of VRL-loaded LMs

The entrapment efficiency of the system was determined by measuring the concentration of free vinorelbine in the aqueous phase. Ultrafiltration was performed using VIVASPIN 4 filters (VIVASCIENCE Ltd. Co., Germany) at 3000 rpm for 30 min. The equipment consisted of a filter membrane with molecular weight cut-off of approximately 10 kDa and a sample recovery chamber at the base. The amount of vinorelbine in the separated aqueous phase was measured by high-performance liquid chromatography (HPLC) in accordance with the above vinorelbine assay. Then, drug concentrations in the aqueous phase and in the whole LMs were compared to calculate the drug entrapment efficiency (Zurowska-Pryczkowska et al., 1999).

### 2.6. Safety test of VRL-loaded LMs

#### 2.6.1. Rabbit ear vein irritation test ( L. Wang et al., 2006)

Rabbits (New Zealand white, 2.5–3.0 kg) were divided into two groups (Group A and Group B) with three in each group. The

dosage for rabbits calculated by the skin surface area conversion table based on a human dose (30 mg/m$^2$) was 1.4 mg/kg. Every rabbit in group A was given an injection of VRL-loaded LMs (1 mg/ml) into their right ear marginal vein, and an injection of normal saline into their left ear marginal vein while every rabbit in group B was given an injection of VRL aqueous injection (1 mg/ml), likewise, normal saline into their left ear marginal vein. All injections were made at a rate of 2.8 ml/min, and injections were performed once a day for three consecutive days. The rabbits were killed 24 h after the last injection and then the appearance of the veins was examined by an experienced unbiased observer and they were subsequently dissected from the surrounding tissues and pathological sections prepared.

#### 2.6.2. Hypersensitivity reaction ( He et al., 2003)

Eight guinea pigs (weight 250–280 g) were divided into two groups (Group A and Group B). Each group consisted of four guinea pigs, two of which were male and the others were female. The dose for the guinea pigs was calculated from the skin surface area conversion table based on a human dose (30 mg/m$^2$) and was 2.3 mg/kg. Group A was given VRL-loaded LMs (1 mg/ml) and Group B was given a VRL aqueous injection (1 mg/ml). Every other day 0.5 ml of the formulations was intraperitoneally injected, and this was repeated three times. On the 14th and 21st day after the first injection, every guinea pig was given a 1 ml i.v. dose of the corresponding formulation. The animals were monitored for 2 h in order to see if there was any nose scratching, sneezing, erect hair, twitching, dyspnea, gatism, shock or death.

### 2.7. Pharmacokinetic studies of VRL-loaded LMs

A pharmacokinetic study was designed to compare and evaluate the VRL-loaded LMs (1 mg/ml) and VRL aqueous injection (dilute to 1 mg/ml). Male Wistar rats (weight 250 ± 10 g) were divided into two groups with six animals in each group. The dosage of the VRL aqueous injection for humans is 25–30 mg/m$^2$ and the dosage for rats was calculated from the skin surface area conversion table. As far as the injection volume was concerned, a dose of 10 mg/kg via the femoral vein was selected. Then blood samples of about 0.3 ml were collected by retro-orbital puncture at predetermined time points (predosing, 5 min, 15 min, 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h) and transferred to heparinized Eppendorf tubes, and centrifuged immediately at 4000 rpm for 15 min to obtain plasma. The plasma samples were stored at −20 °C in a refrigerator until further analysis.

A 100 μl aliquot of a plasma sample was placed in a centrifuge tube and 10 μl internal standard solution (125 μg/ml ketoconazole methanol solution) was added, vortexed for 3 min, then 400 μl methanol was added. After vortexing for 3 min, the mixture was centrifuged at 8000 rpm for 15 min. The supernatant liquid was transferred to a clean tube and dried under nitrogen at 50 °C. The residue was reconstituted in 100 μl mobile phase and 20 μl was injected into the HPLC system. The HPLC conditions were the same as described above. Standard curves were obtained by plotting the vinorelbine:internal standard peak area ratio as a function of the nominal vinorelbine concentration of the plasma standards. Linear least square regression with a weighting factor of 1/$C$ was carried out to calculate the best-fit line and regression coefficient. Vinorelbine concentrations in plasma samples were obtained by applying the resulting linear function to the peak area ratios (vinorelbine:internal standard) for each sample. Other method validations including accuracy, precision, recovery, and limits of quantitation were also carried out.

The experimental procedures complied with the University Animal Ethics Committee Guidelines. The study was approved by the University Animal Ethics Committee.

### 2.8. Pharmacokinetic data analysis

The 3p87 computer program was employed to analyze the plasma concentration-time data. It was produced by the Mathematics Pharmacological Committee of the Chinese Academy of Pharmacology, and it provided the most appropriate pharmacokinetic model to describe the experimental data. The model was selected based on the residual sum of squares and the minimum Akaike's information criterion (AIC) value. The area under the concentration–time curve ($AUC_{0-t}$) from zero to the last time point, the area under the cross product of the time and plasma concentration–time curve ($AUMC_{0-t}$), mean residence time (MRT), clearance (CL), steady-state apparent volume of distribution (Vss), half-life ($T_{1/2}$) and the elimination rate constant ($K_e$) of the drug were all obtained using the statistical moment method (Yu et al., 2006).

The effect of the formulation on the various pharmacokinetic parameters was statistically compared using an independent $t$-test. Significance was assumed at the 0.05 level of probability. Values are reported as mean ± standard deviation. The statistical data were obtained using SPSS version 13.0 for Windows statistical software.

## 3. Results and discussion

### 3.1. Preparation of VRL-loaded LMs

According to literatures on lipid emulsion preparation and our own experimental experience, the three main factors that influence the physical stability of LMs are: (a) suitable oil phase; (b) proper emulsifiers; (c) better controlled homogenization process. In the following, the formulations were prepared with only one factor being altered at a time and then the three main factors that influence the physical stability of LMs were investigated.

Soybean oil (long-chain triglycerides, LCTs) and a medium-chain triglycerides (MCTs) mixture is currently used to prepare commercial parenteral emulsions. Long-chain triglycerides have some toxic effects, such as causing immune dysfunction, accumulation in reticuloendothelial cells and deposition of adipochrome in liver or lung after long-term use. Including MCTs in alternative lipid emulsions allows the large amount of linoleic acid to be reduced, giving a better balance of fatty acids (Carpentier and Hacquebard, 2006), which may reduce the toxicity associated with pure LCT-based lipid emulsions (Smyrniotis et al., 2001) and may also provide more stable

Table 1
Effects of different emulsifier compositions on VRL-loaded LMs

| Formulation | Emulsifiers | Particle size (nm) | Visual appearance and physical stability |
|---|---|---|---|
| A1 | Egg lecithin 1.2% | 227.5 ± 67.0 | Visible supernatant oil drops; creaming after a few days |
| A2 | Egg lecithin 1.8% | 203.1 ± 56.2 | Visible supernatant oil drops; creaming after a few days |
| A3 | Egg lecithin 1.2% + Tween80 0.2% | 202.8 ± 52.3 | Visible supernatant oil drops after autoclaving; creaming after a few months |
| A4 | Egg lecithin 1.2% + Tween80 0.2% + F68 0.2% | 180.5 ± 35.2 | Homogenous; stable for 12 months |
| A5 | Egg lecithin 1.8% + Tween80 0.2% | 195.2 ± 50.2 | Visible supernatant oil drops after autoclaving; creaming after a few months |
| A6 | Egg lecithin 1.8% + Tween80 0.2% + F68 0.2% | 182.2 ± 52.2 | Homogenous; stable for 12 months |

Formulations in the table contained (%, w/w): soybean oil 2%, MCT 8%, oleate sodium 0.05%, glycerol 2.5%, total antioxidants 0.32%, and water for injection to 100%, with emulsifier as the only difference. The particle size data are given directly as mean ± S.D. by the NICOMP™ 380 Particle sizer. The S.D. value indicates the particle size distribution.

all-in-one admixtures (Driscoll et al., 2000). Three ratios of MCT:LCT were chosen to evaluate the entrapment efficiency of the corresponding formulations. Three groups of formulations were prepared with three different ratios of MCT:LCT (2:8; 5:5; 8:2) while maintaining the other conditions constant (the pH value of primary emulsion in each formulation was adjusted to 8.0). The drug entrapment efficiencies were 35.5 ± 0.4%, 58.4 ± 0.3% and 96.6 ± 0.3% (mean ± S.D., $n = 3$), respectively. These results indicate that when the ratio of MCT:LCT is 8:2, the highest drug entrapment efficiency is obtained, so, 8:2 was the optimal choice.

Because of their biocompatibility and application in commercial intravenous lipid emulsions, phospholipids are usually the first candidates selected as emulsifiers (Han et al., 2004). Lecithin can be totally biodegraded and metabolized, moreover it is an integral part of biological membranes and is virtually non-toxic. Thus, egg lecithin was chosen as the main emulsifier. Table 1 shows that samples were not homogeneous or stable when egg lecithin acted as the only emulsifier, because the egg lecithin (HLB about 8) had a relatively good solubility in the oil phase, so the phospholipid monolayer interfacial film was not stable enough. When Tween80 (HLB 15) was added, the formulations became more stable because the Tween80 reduced the oil–water-interfacial tension and consequently strengthened the viscoelasticity of the interfacial film to some degree. However, that was still not enough. An even stronger effect was observed when Pluronic F68 (dissolved in water) was used as the water-soluble emulsifier. As reported, Pluronic F68 (HLB 29) stabilized the newly created interface immediately. Within only one homogenization cycle maximum dispersity had already been achieved when Pluronic F68 was present. Pluronic F68 is a non-ionic emulsifier and produces sterically stabilized emulsions (Eccleston, 1992). In addition, it was found that when only 0.05% (weight percent) sodium oleate was added, the absolute value of the zeta potential increased markedly. Obviously, the increased surface charge enhances the force of repulsion among LM particles and helps to stabilize the formulation. Sodium oleate, as a water-soluble co-emulsifier, was incorporated into the oil–water interface forming an integral part of the emulsifier film so as to increase the repulsive surface charge and correspondingly prevent LM recoalescence. However, if there is too much egg lecithin, small particles could be formed by the surplus lecithin and the particle size exhibits a bimodal Nicomp distribution when measured by the NICOMP™ 380 Particle sizer and the S.D. value is increased. Accordingly, the appropriate amount of egg lecithin was 1.2%.

High pressure homogenization has the advantages of being simple to carry out and easy to use on an industrial scale and, consequently, it has been developed and used extensively to

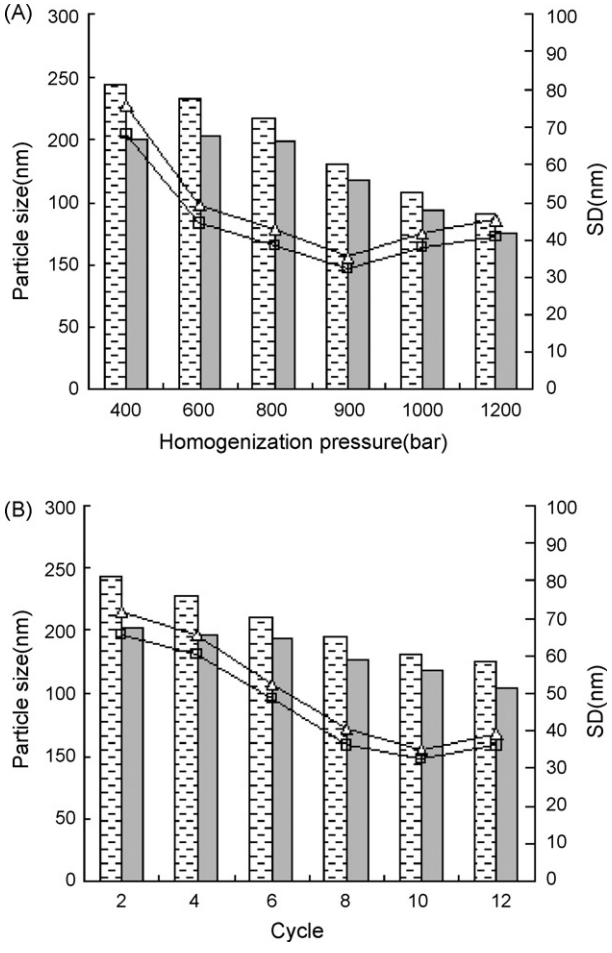


Fig. 2. (A) Influence of different homogenization pressures on the particle size of VRL-loaded LMs (cycle number was 10); (B) influence of different cycle numbers on the particle size of VRL-loaded LMs (homogenization pressure was 900 bar). The data involved the photon correlation spectroscopy (PCS) diameter Gaussian distribution obtained using a NICOMP™ 380 Zeta Potential/Particle Sizer (10 bar equals 1 Mpa); (▭) intensity-weighting particle size; (▭) volume-weighting particle size; (△) intensity-weighting standard deviation of particle size; (□) volume-weighting standard deviation of particle size.

prepare emulsions, lipid microspheres, and liposomes. According to Washington and Davis (1988) and Bock et al. (1994), the most important homogenization parameters for controlling droplet size are homogenization pressure, temperature and duration (number of cycles). We chose 40 °C as the homogenization temperature in order to avoid disrupting droplets overprocessed according to Bock's study. A circulator bath was used to keep the formulation around 40 °C during the homogenizing process. In Fig. 2(A), six different homogenization pressures were involved to prepare VRL-loaded LMs while maintaining the other conditions constant (cycle number 10). The figure shows that the particle size and S.D. value (indicates the particle size distribution) were reduced as the homogenization pressure increased from 400 to 900 bar, but after 900 bar, both the particle size and S.D. value increased. This phenomenon has been examined by Davis and Bock (Davis et al., 1985; Bock et al., 1994). Slightly broader particle size distributions and larger diameters for the largest droplets result when a high temperature is used together with a high homogenization pressure for more than three cycles. This can be regarded as a typical case of overprocessed emulsions, where dispersion efficacy exceeds the stabilization capability of the emulsifier. At an excessively high homogenization pressure, temperature increases dramatically with the number of homogenization cycles, and the kinetic energy of the oily drops also increases dramatically. Then, collisions are accelerated leading to coalescence and the production of larger particles. In Fig. 2(B), six different homogenization cycles were used to prepare VRL-loaded LMs while the other conditions were kept constant (homogenization pressure 900 bar). Likewise, too many cycles resulted in a larger particle size and broader particle size distributions, and 10 times was the final choice.

Other conditions involved in the process of preparing VRL-loaded LMs, such as the controlled factors in preparing primary emulsions (temperature, stirring speed and time, oil added to water or water to oil) and the sterilizing methods were also investigated to obtain the most satisfactory formulations. The optimal VRL-loaded LM formulation had a particle size of $180.5 \pm 35.2$ nm with a 90% cumulative distribution less than 244.1 nm, zeta potential −26.58 mV and no phenomena such as creaming, or precipitation were observed. Then, these physical characteristics (appearance, particle size distribution and zeta potential) of the optimal VRL-loaded LM formulation were investigated for 12 months at $6 \pm 2$ °C to examine the stability.

### 3.2. Entrapment efficiency of vinorelbine

Vinorelbine ditartrate is a water-soluble drug with an aqueous solution pH of about 3.5 while vinorelbine ($\log P$1.32), a vinca alkaloid, is lipid-soluble (Zhigaltsev et al., 2005). Thus, it is possible to incorporate vinorelbine into the lipid core of lipid microspheres by adjusting the pH during the preparation process. The primary emulsion was adjusted to a series of pH values, and the entrapment efficiency increased with the pH of the primary emulsions (Table 2). When the pH value exceeds 8.0, the drug entrapment efficiency does not increase any more,

Table 2
Influence of pH on the efficiency and particle size of VRL-loaded LMs

| pH of the primary emulsions | pH of the formulations | Particle size (nm) | EE (%) |
|---|---|---|---|
| 6.00 | 5.81 | 210.0 ± 50.2 | 60.5 ± 0.4 |
| 7.01 | 6.73 | 170.4 ± 42.1 | 74.3 ± 0.3 |
| 8.02 | 7.86 | 163.4 ± 34.5 | 96.8 ± 0.4 |
| 8.99 | 8.78 | 169.9 ± 40.3 | 96.5 ± 0.5 |

Formulations in the table contained (%, w/w): soybean oil 2%, MCT 8%, egg lecithin 1.2%, F68 0.2%, Tween80 0.2%, oleate sodium 0.05%, glycerol 2.5%, total antioxidants 0.32% and water for injection to 100%, with the pH value of the primary emulsions as the only difference. EE (%), entrapment efficiency (mean ± S.D., $n = 3$).

and formulations with a pH of 8.02 exhibited the highest drug entrapment efficiency. Dissolving vinorelbine ditartrate in water and then adjusting the pH value with sodium hydroxide solution, gives a pH value of over 7.0, resulting in the precipitation of white vinorelbine. However, precipitation was never observed when preparing VRL-loaded lipid microspheres, although the pH value of the coarse emulsion was over 8.0 (with sodium oleate being present before vinorelbine ditartrate was added). Owing to the p$K_a$ value (5.4) of vinorelbine (Mouchard-Delmas et al., 1995), this drug is protonated when in a solution of lower pH and is in the molecular form when in a higher pH solution. When vinorelbine ditartrate was added to the alkaline coarse emulsions, the molecular type vinorelbine formed and dissolved in the oil phase simultaneously with the help of high speed stirring and sufficient surfactant. Then, because the pH value would fall when vinorelbine ditartrate was added, it was necessary to adjust the pH value of the drug-loaded primary emulsion to about 8.0 before starting the high pressure homogenization process to obtain a high drug entrapment efficiency.

### 3.3. Selection of effective antioxidants

During the 12-month investigation, VRL related compounds complied with the limits of the Chinese Pharmacopeia 2005 edition except for compound A (4-*O* deacetyl vinorelbine). Then, a series of formulations were designed and investigated to obtain a chemically stable VRL-loaded LM preparation. The content of VRL-loaded LMs with no antioxidants degraded about 10% over one month and 50% over six months at $6 \pm 2$ °C protected from light. On the other hand, compound A increased from 1.0% to 10.8% (first month) then 50.6% (6 months) (Table 3). As Table 3 shows, with the addition of different antioxidants, degradation of vinorelbine was generally inhibited. Disodium ethylenediamine tetraacetic acid (EDTA), a metal complexing agent, was used to complex metal ions and consequently block the oxidizing reaction. Lipid-soluble α-tocopherol was added to the oil phase to protect oil, egg lecithin, and vinorelbine from being oxidized. However, that was still not enough. Formulations with EDTA and added α-tocopherol degrade about 20% over three months at $6 \pm 2$ °C protected from light. Then, ascorbic acid (Vitamin C), L-cysteine and $Na_2SO_3$ were employed to investigate their effectiveness. Vitamin C was so instable that it was oxidized during the preparation of the lipid microspheres, and after three months,

Table 3
Chemical stability of VRL-loaded LMs with different antioxidants

| | Formulations | pH value | Content (%)* | Compound A (%)** | EE (%) |
|---|---|---|---|---|---|
| 0 time | F1 | 7.90 | 100.1 ± 0.10 | 1.0 ± 0.01 | 96.8 ± 0.3 |
| | F2 | 7.89 | 99.6 ± 0.09 | 0.6 ± 0.005 | 95.7 ± 0.5 |
| | F3 | 7.48 | 98.7 ± 0.12 | 0.5 ± 0.004 | 92.2 ± 0.3 |
| | F4 | 7.72 | 99.3 ± 0.14 | 0 | 94.5 ± 0.7 |
| | F5 | 7.86 | 100.8 ± 0.16 | 0 | 96.4 ± 0.3 |
| First month | F1 | 7.91 | 90.2 ± 0.09 | 10.8 ± 0.06 | 96.5 ± 0.5 |
| | F2 | 7.87 | 92.3 ± 0.12 | 7.9 ± 0.05 | 95.4 ± 0.4 |
| | F3 | 7.41 | 97.9 ± 0.15 | 1.2 ± 0.01 | 92.3 ± 0.4 |
| | F4 | 7.70 | 97.8 ± 0.07 | 1.3 ± 0.006 | 93.8 ± 0.6 |
| | F5 | 7.87 | 100.6 ± 0.08 | 0 | 96.5 ± 0.4 |
| 3 months | F1 | 7.88 | 71.2 ± 0.13 | 29.9 ± 0.05 | 95.3 ± 0.3 |
| | F2 | 7.86 | 78.0 ± 0.08 | 22.3 ± 0.04 | 94.1 ± 0.2 |
| | F3 | 7.33 | 87.4 ± 0.06 | 11.6 ± 0.02 | 91.6 ± 0.4 |
| | F4 | 7.58 | 90.2 ± 0.05 | 9.0 ± 0.06 | 93.5 ± 0.5 |
| | F5 | 7.85 | 100.3 ± 0.07 | 0.5 ± 0.002 | 96.2 ± 0.4 |
| 6 months | F1 | 7.85 | 50.2 ± 0.09 | 50.6 ± 0.09 | 95.9 ± 0.6 |
| | F2 | 7.84 | 57.4 ± 0.07 | 42.8 ± 0.07 | 93.7 ± 0.4 |
| | F3 | 7.14 | 68.8 ± 0.08 | 30.2 ± 0.05 | 92.1 ± 0.7 |
| | F4 | 7.55 | 75.3 ± 0.11 | 23.7 ± 0.06 | 94.1 ± 0.5 |
| | F5 | 7.87 | 100.1 ± 0.06 | 0.6 ± 0.005 | 96.0 ± 0.3 |
| 12 months | F1 | 7.84 | 26.3 ± 0.14 | ND | ND |
| | F2 | 7.85 | 33.6 ± 0.10 | ND | ND |
| | F3 | 7.10 | 44.1 ± 0.09 | ND | ND |
| | F4 | 7.52 | 52.1 ± 0.12 | ND | ND |
| | F5 | 7.84 | 99.8 ± 0.08 | 0.8 ± 0.003 | 96.2 ± 0.3 |

EE, entrapment efficiency of VRL-loaded LMs determined by ultrafiltration; formulations in this table contained (%, w/w): soybean oil 2%, MCT 8%, egg lecithin 1.2%, F68 0.2%, Tween80 0.2%, oleate sodium 0.05%, glycerol 2.5%, and water for injection to 100%, with antioxidants as the only difference. The pH value of the primary emulsion in each formulation was adjusted to 8.0. F1, no antioxidants; F2, α-tocopherol 0.05% and EDTA 0.02%; F3, α-tocopherol 0.05%, EDTA 0.02% and antiscorbic acid 0.2%; F4, α-tocopherol 0.05%, EDTA 0.02% and L-cysteine 0.05%; F5, α-tocopherol 0.05%, EDTA 0.02%, L-cysteine 0.05% and $Na_2SO_3$ 0.2%; *Content of vinorelbine in the formulations; **Counted as a ratio of the peak response for VRL related compound A to the peak corresponding to vinorelbine as described in item 2.4; ND, not determined. All the formulations were stored at 6 ± 2 °C protected from light.

formulations with Vitamin C added appeared to be yellow and the degradation of vinorelbine accelerated. The long-term stability investigation indicated that α-tocopherol, EDTA, L-cysteine and $Na_2SO_3$ were the most effective antioxidants. Following the addition of α-tocopherol 0.05%, EDTA 0.02%, L-cysteine 0.05% and $Na_2SO_3$ 0.2%, vinorelbine loaded in the lipid microspheres remains chemically stable for 12 months at 6 ± 2 °C protected from light. In addition, the drug entrapment efficiencies of VRL-loaded LMs were also investigated. Because of the ratio of the native vinorelbine concentration in the oil phase to that in the whole LMs, the drug entrapment efficiency of VRL-loaded LMs did not vary significantly with the degradation.

### 3.4. Satety test

#### 3.4.1. Rabbit ear vein irritation test

One of the most important objectives of this study was to reduce the venous irritation caused by VRL aqueous injection, and avoid clinical injection site reactions and phlebitis. Macroscopic observation indicated that vascular engorgement and dropsy were seen at the injection site following VRL aqueous injection. Furthermore, light microscopy showed that angiectasia and erythrocyte aggregation were observed at or away from the site of injection after VRL aqueous injection was administered as shown in Fig. 3 while similar phenomena were not observed in the case of normal saline and LM injections. Therefore, VRL-loaded LMs produced less irritation than VRL aqueous injections.

#### 3.4.2. Hypersensitivity reaction

Neither the VRL-loaded LMs nor VRL aqueous injection group exhibited conventional hypersensitivity reactions, such as nose scratching, sneezing, erect hair, twitching, dyspnea, gatism and shock. However, three guinea pigs out of four in Group B (VRL aqueous injection group) died, respectively, 4, 6 and 9 days during the experimental period. These results were not due to a hypersensitivity reaction, but were due to the fact that the toxicity of VRL aqueous injections is higher than that of VRL-loaded LMs.

With more than 95% vinorelbine encapsulated in VRL-loaded LM, VRL are not in direct contact with the vessel walls, and might act differently *in vivo*, thus the venous irritation and toxicity were reduced dramatically. Undoubtedly, lipid microspheres are an ideal drug carrier which will increase the beneficial action of the drug and reduce local and systemic adverse effects.

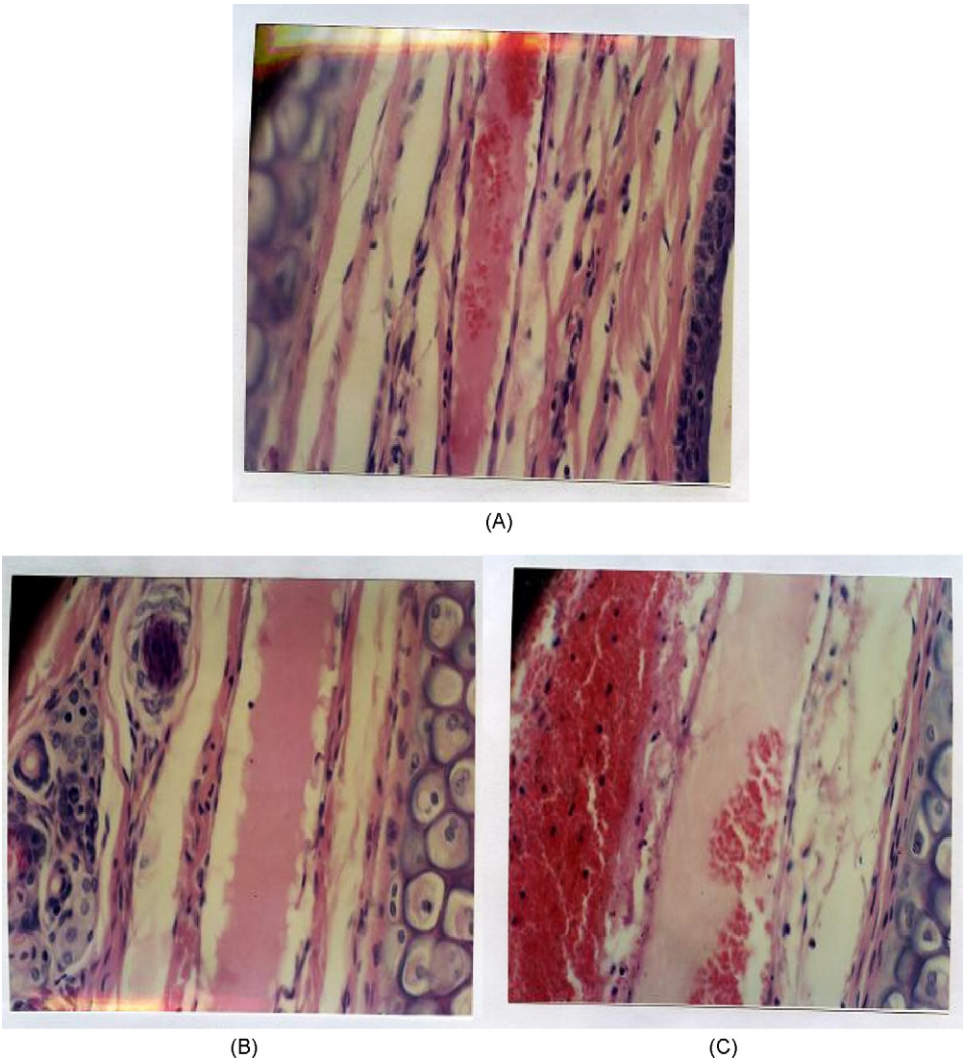


Fig. 3. Pathological section photos of a rabbit ear-rim auricular vein following different injections. (A) Normal saline; (B) VRL-loaded LM; (C) VRL aqueous injection.

### 3.5. Evaluation of VRL-loaded LMs in vivo

In this experiment, plasma treatment was performed by methanol protein precipitation instead of the conventional two step liquid–liquid extraction (Robleux et al., 1996). Then, methanol was dried under a nitrogen stream at 50 °C and reconstituted in 100 μl mobile phase. This method had an acceptable recovery rate and was easy to perform. The linear range of VRL in plasma was 50–5000 ng/ml, the inter- and intra-day variations were less than 6%, and the relative recovery of VRL from plasma was more than 90%.

A comparative pharmacokinetic study was performed by determining drug levels in plasma up to 24 h after administration. Analysis was carried out using a pharmacokinetic program 3p87, and data on both the preparations fitted a two-compartment model. The main pharmacokinetic parameters were calculated by the statistical moment method shown in Table 4 and most of them exhibited no statistically significant differences ($n = 6$, $P > 0.05$) except Vss ($n = 6$, $P < 0.05$) calculated by SPSS. Vss was 1.5 times higher for the aqueous injection than for the lipid microspheres. Similar results have also been reported for propofol (Sandeep and Ebling, 1998) and tirilazad (Wang et al., 1999). In the propofol study, a lipid-free formulation resulted in a three-fold increase in Vss and a two-fold increase in CL as

Table 4
Pharmacokinetic parameters after i.v. administration of VRL-loaded LM and VRL aqueous injection to rats

| Parameters | VRL-loaded LMs | VRL aqueous injection |
|---|---|---|
| Ke (l/h) | 0.021 ± 0.003 | 0.022 ± 0.007 |
| CL (l/h) | 0.27 ± 0.04 | 0.37 ± 0.11 |
| $AUC_{0-t}$ (μg h/ml) | 7.47 ± 1.13 | 6.01 ± 2.32 |
| Vss (l) | 10.73 ± 1.09 | 15.00 ± 2.18 |
| $T_{1/2}$ (h) | 32.97 ± 4.47 | 35.37 ± 14.54 |
| MRT (h) | 39.75 ± 4.68 | 45.56 ± 21.04 |
| $AUMC_{0-t}$ (μg $h^2$/ml) | 300.10 ± 74.85 | 312.74 ± 279.88 |

The data were mean ± S.D. ($n = 6$). $K_e$, elimination rate constant; $T_{1/2}$, half-life; $AUC_{0-t}$, area under the concentration–time curve; $AUMC_{0-t}$, area under the cross product of the time and plasma concentration–time curve; MRT, mean residence time; CL, clearance; Vss, steady-state apparent volume of distribution.

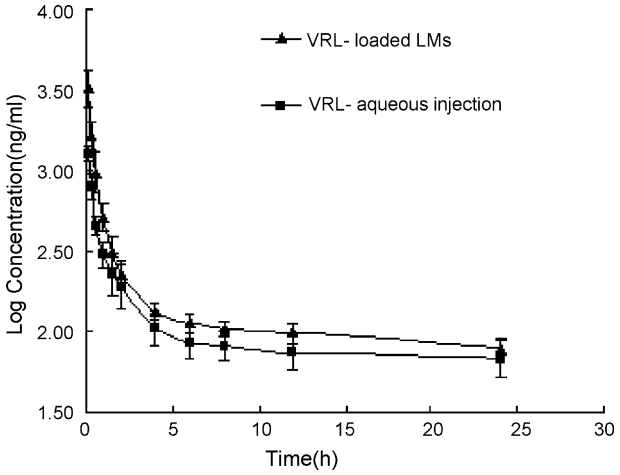


Fig. 4. Mean plasma concentration–time profiles after i.v. VRL-loaded LMs and VRL aqueous injection to rats ($n = 6$) at a dose of 10 mg/kg.

compared with the lipid emulsion formulation, and in the tirilazad study, Vss was 20 times higher for the solution than for the emulsion. These results could be attributable to the vehicle since when the drug is incorporated in the lipid core of lipid microspheres or the emulsions, it could reduce the penetration of drug into the tissues, and produce a higher plasma concentration, consequently resulting in a lower Vss of the drug since the ratio of the concentrations of drug in the tissues to that in plasma was reduced. In addition, the distribution of vinorelbine, *in vitro*, demonstrated that 84% is bound to blood cells, mainly to platelets (78%) (Gauvin et al., 2000), so vinorelbine loaded in LMs distributes mostly in plasma while free vinorelbine distributes mostly in platelets removed during centrifugation, and this, in some degree, also caused the plasma concentrations of VRL-loaded LM formulation to be higher than those following VRL aqueous injection (Fig. 4). Nevertheless, from Fig. 4, the curves of the two preparations are similar and no significantly delayed release was found for VRL-loaded LMs. So, lipid microspheres do not significantly change the pharmacokinetics of vinorelbine *in vivo*. The published paper by Semple et al. (2005) indicated that SM/Chol liposomal formulation of VRL had longer circulation times, an altered tissue distribution, and a larger increase in plasma AUC (>330-fold) compared with VRL aqueous injection. So, the liposomal formulation may offer an improved therapeutic index compared with VRL-loaded LMs, however, the difficulty of sterilization, high cost of manufacture and complicated process of preparation might hinder the mass-produce and widespread applications of VRL liposomes.

## 4. Conclusions

The VRL-loaded lipid microspheres had a particle size of $180.5 \pm 35.2$ nm, a drug entrapment efficiency of 96.8%, and remained stable for 12 months at $6 \pm 2\,^{\circ}C$ protected from light when α-tocopherol 0.05%, EDTA 0.02%, L-cysteine 0.05% and $Na_2SO_3$ 0.2% were added as antioxidants. VRL-loaded LMs caused less irritation and were less toxic than conventional VRL aqueous injections. The pharmacokinetic profiles were similar for the two preparations. Accordingly, stable and easily mass-produced vinorelbine lipid microspheres have been developed. These cause less venous irritation and toxicity but have similar pharmacokinetics *in vivo* compared with the vinorelbine aqueous injection currently commercially available. This lipid microsphere system represents a valuable and attractive option for the mass production of a non-irritant vinorelbine intravenous formulation.

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