Formulation, Characterization and Hypersensitivity Evaluation of an Intravenous Emulsion Loaded with a Paclitaxel–Cholesterol Complex

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The objective of this paper was to develop a novel Cremophor-free, autoclave stable, intravenous emulsion for paclitaxel (PACE). A paclitaxel–cholesterol complex was used as the drug carrier to improve the solubility of paclitaxel in the oil phase of emulsions. The complex and PACE were prepared by rotary evaporation and high-pressure homogenization, respectively. Effects of oil phases, emulsifiers and pH values on the characteristics of PACE were investigated. PACE was characterized with regard to its appearance, morphology, osmolality, pH value, particle size, zeta potential, encapsulation efficiency and stability. Hypersensitivity was evaluated by guinea pig hypersensitivity reaction. The final formulation was composed of the complex, soybean oil, medium-chain triglyceride, soybean lecithin, poloxamer 188 and glycerol. The resulting PACE had an encapsulation efficiency of 97.3% with a particle size of 135 nm and a zeta potential of 46 mV. Osmolality and pH of the formulation were 383 mOsmol/kg and 4.5, respectively. The formulation survived autoclaving at 115 °C for 30 min and remained stable for at least 12 months at 6 °C. PACE also exhibited a better tolerance than an equal dose of Cremophor-based paclitaxel injection in guinea pigs, as no obvious hypersensitivity reaction was observed. These results suggested that PACE has a great potential for industrial-scale production and clinical applications.

Key words paclitaxel; emulsion; complex; characterization; stability; hypersensitivity

Paclitaxel (PAC) is one of the most effective antineoplastic agents used to treat a wide range of tumors, including refractory ovarian cancer, metastatic breast cancer, non-small cell lung cancer, head and neck cancer and AIDS-related Kaposi’s sarcoma.1,2 It is a taxane that interferes with microtubule depolymerization in tumor cells resulting in an arrest of the cell cycle in mitosis followed by the induction of apoptosis. PAC has brought much hope to people with cancer, but its poor solubility has limited its clinical application.3

At present, many generic PAC-based products that are suitable for formulation as an intravenous infusion, besides the first patented PAC named Taxol, are available on the market. However, all these products contain Cremophor as a solubilizer. Cremophor is allegedly responsible for many paclitaxel-associated hypersensitivity reactions,4–6 which may prove to be fatal to patients. In addition, this formulation has been associated with a number of issues, such as sterilization by filtration, the possibility for drug precipitation upon dilution, filtering requirements and the use of non-plasticized containers and administration sets.7 Therefore, it is very important to develop a new intravenous dosage form of PAC with improved solubility and in formulations devoid of Cremophor.

An intravenous emulsion stabilized with amphipathic lipids is an appealing alternative as a drug carrier for anticancer drug delivery. The potential pharmaceutical applications include use as a carrier for lipophilic drugs7–11 and for site-specific drug delivery by attaching ligands for various cell surface receptors to the particle surface.12 Generally, intravenous emulsions are biodegradable, biocompatible, physically stable, easy to scale up and cost effective when compared to other drug carriers. In addition, lipophilic drugs can be incorporated into the interior oil phase to sequester them from direct contact with exterior water and oxygen. Thus, for some drugs, emulsions could afford a better chemical stability. However, emulsions based largely on vegetable oils are unsuitable for PAC, because PAC does not show a sufficiently high solubility in registered oils (e.g., long-chain triglyceride (LCT) in the form of soybean oil, medium-chain triglyceride (MCT) or a mixture of LCT and MCT).

As reported in the literature, several emulsion formulations, such as using surfactants which were not clinically accepted in intravenous applications,13,14 adding excess amount of oils15 or adopting organic solvents as co-solvents,16,17 have successfully resolved the problem of the solubility of PAC in the oil phase. However, none of these emulsion formulations have been introduced into clinical practice because of the lack of sufficient biocompatibility and stability14,15,18,19 to meet the requirements of clinical applications and industrial-scale production.

The objective of this paper was to prepare a novel Cremophor-free, autoclave stable, intravenous emulsion for paclitaxel (PACE). A paclitaxel–cholesterol complex was adopted as the drug carrier to improve the solubility and entrapment efficiency of paclitaxel in the oil phase of the emulsions. All the excipients used in the PACE formulation were clinically acceptable and approved for use in intravenous infusion by the U.S. Food and Drug Administration (FDA). The complex and PACE used in this study were prepared by conventional rotary evaporation and high-pressure homogenization, respectively. The formulation, its characterization and the hypersensitivity evaluation were investigated to describe the formulation in detail.

Experimental

Materials Paclitaxel and its Cremophor-based paclitaxel injection (paclitaxel injection) were purchased from Beijing Union Pharmaceutical Factory (Beijing, China). Soybean oil and MCT were purchased from Tieling Beiya Pharmaceutical Co. (Liaoning, China). Soybean lecithin (S75),
eggoyolk lecithin (E30), cholesterol, dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl glycerol (DMPG), hydroxyl soybean phosphatidyl choline (SPC-3) and distearoyl phosphatidyl glycerol (DSPG) were obtained from Lipoid (Ludwigshafen, Germany), and glycerol from Zhejiang Suichang Glycol Plant (Zhejiang, China). Poloxamer 188 (Pluronic F68) was purchased from BASF (Ludwigshaven, Germany). Sodium chloride injection was purchased from Shijiazhuang No. 4 Pharmaceutical Co., Ltd. (Hebei, China). Osmolalimeter (Tianjin Tianhe Medical Instrument Co., Ltd., China) with refer-
tility was based on the freezing-point method. After calibration of a SMC 30B Osmometer (Tianjin Tianhe Medical Instrument Co., Ltd., China). Double distilled de-ionized water was used for all experiments. All other chemicals and reagents were of analytical grade.

Guinea pigs used in this paper were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Animal experiments were approved by the Animal Care & Welfare Commit-
tee of the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College.

Solubility of PAC in Oils The solubility of PAC was determined in soybean oil and LCT/MCT. Excess PAC was added into 5 mL of oils in a centrifuge tube and mixed (100 rpm) in a shaking incubator at 60 °C for 5 h. The solutions were then centrifuged at 13000 rpm for 5 min to remove the excess PAC, and the concentrations of PAC in the supernatant were measured by HPLC after appropriate dilution with ethanol.

Preparation of Paclitaxel–Cholesterol Complex The paclitaxel–cholesterol complex was prepared by a rotary evaporation method. The required amount of paclitaxel and cholesterol were put in a round bottom flask and reaction solvent was added. The mixture was refluxed at 40 °C for 1.5 h. Then, the settled solution was evaporated to obtain the dried pacli-
taxel–cholesterol complex. Paclitaxel, cholesterol and the dried paclitaxel–cholesterol complex were weighed on an electronic balance before and after reaction to make sure the reaction solvent had been completely evaporated.

Preparation of Paclitaxel Emulsion (PAC) and Blank PAC The drug-loading of PAC was initially set at 1.0 mg/ml. PAC was prepared by high-pressure homogenization. At first, the PAC–cholesterol complex was dissolved in 20% MCT/LCT at 60 °C. Next, the aqueous phase consisting of soybean lecithin, poloxamer 188 and glycerol was uniformly dispersed at 60 °C in a water bath. Then, the coarse emulsion was prepared at 60 °C with high shear mixing using a Fluko homogenizer FA25 model (Fluko Equip-
ment Shanghai Co., Ltd., China) by rapidly adding the oil phase to the aque-
ous phase at 10000 rpm. The high shear mixing process was carried out for 10 min at 19000 rpm and the final emulsion was obtained by high-pressure homogenization using Amo No Soavi NS 1001LK homogenization equipment (Niro Soavi S.p.A., Via Mda Erba Edoari, 29A-A3100 Parma, Italy) at 80MPa for 7 cycles. The temperature of the whole homogenization process was maintained below 40 °C in a cycle ice-water bath. The pH value was adjusted to 4.5. Measurement of the PAC osmolal-
ty was performed at 115 °C for 30 min. Osmolality and pH Measurement

Morphology

Results and Discussion

Solubility of PAC in Oils The generally accepted dose of paclitaxel for clinical applications has been 135—250 mg/m^2.\textsuperscript{11} Based on this dose, a drug-loading of 0.8 mg/ml or above was required for PAC to have a total volume below 500 mL. According to the solubility of paclitaxel in a 20% oil phase formulation, however, its concentration should reach at least 4 mg/ml. Despite this, the low solubility of paclitaxel, 0.3 mg/ml in the soybean oil and 1.0 mg/ml in LCT/MCT,
made it impossible for a direct preparation of these emulsions (Table 1). Therefore, it was essential to enhance the solubility of paclitaxel in the oil phase.

**Investigations of Paclitaxel–Cholesterol Complex** As reported in the literature, the formation of a complex between a drug and lipids could improve the physicochemical properties of the drug, especially its liposolubility. 21,22 In this paper, paclitaxel–lipid complexes were prepared to improve the solubility of paclitaxel in the oil phase of emulsions. S75, E80, cholesterol, DMPC, DMPG, SPC-3 and DSPG were adopted as lipid materials to optimize paclitaxel–lipid complexes. According to the solubility results of paclitaxel in oils (Table 1), LCT/MCT showed increased solubility for PAC, which might be due to the higher polarity of MCT compared with LCT and resulting in an increased drug uptake capability for MCT. Thus, the selection of the complexes was based on a comparison of their solubility in LCT/MCT. The results showed that the solubility of the complexes with S75, E80, cholesterol, DMPC, DMPG, SPC-3 and DSPG were 0.5, 0.3, 19.8, 0.2, 8.5, 1.0 and 10.2 mg/ml, respectively. Paclitaxel–cholesterol complex exhibited a higher solubility and a lower price, and thus the latter was selected as lipid material for preparation of PACE and its solubility and stability in oils were investigated in detail. From Table 1, it can be seen that the physical mixture of drug and cholesterol at the same ratio as the complex exhibited a solubility of 0.3 mg/ml in soybean oil and 1.2 mg/ml in LCT/MCT, respectively, which was similar to the paclitaxel. Meanwhile, the complex exhibited a solubility of 8.0 mg/ml in soybean oil and 19.8 mg/ml in LCT/MCT, respectively. In addition, when the complex was dissolved in LCT/MCT, it remained stable after autoclaving at 115 °C for 30 min with no degradation product detected, whereas degradation products of paclitaxel injection was 23%. Furthermore, the LCT/MCT oil solution of the complex did not precipitation after 4 weeks at 25 °C. These results showed that the paclitaxel–cholesterol complex formulation significantly increased drug solubility and stability in the oil phase. Therefore, the paclitaxel–cholesterol complex could be a suitable drug carrier for the paclitaxel emulsion preparation.

**Differential Scanning Calorimetry (DSC) of Paclitaxel–Cholesterol Complex** DSC is a rapid and reliable method to analyze the possible interactions between drug and excipient. Figure 1 shows the DSC curves of paclitaxel (A), cholesterol (B), a physical mixture of paclitaxel and cholesterol (C) and the paclitaxel–cholesterol complex (D). PAC and cholesterol exhibited an endothermic peak at 225.7 °C and 150.9 °C, respectively, which corresponded to the melting point of PAC and cholesterol. In the DSC curve of the physical mixture of PAC and cholesterol, there are two endothermic peaks. The first endothermic peak was at 148.1 °C, which were almost at the same temperature as the one in the cholesterol thermogram of 150.9 °C. The second endothermic peak was at 205.1 °C, which was lower than the melting peak of 225.7 °C. This might be because of the fact that when the temperature rises, PAC and cholesterol partly interact to form a complex. The DSC curve of the PAC–cholesterol complex shows that the original peaks of PAC and cholesterol have disappeared and a broad endothermic peak at 137.0 °C appeared. This indicated that the complex of PAC and cholesterol has a lower phase transition temperature than the individual components. It is evident by comparison of the four DSC curves that PAC and cholesterol exhibit some interactions, such as the formation of hydrogen bonds and van der Waals forces.

**Formulation Development of PACE** An initial formulation of PACE was composed of 20% LCT/MCT as the oil phase, soybean lecithin as the emulsifier and poloxamer 188 as the co-emulsifier. The species of oil phase (compared to soybean oil), the amount of oil phase (compared to 10%) and the species of emulsifier (compared to egg yolk lecithin) best suited to optimization were then investigated. The appearance, pH value, particle size, related substances and centrifugal stability test were used as parameters for the optimization of the formulation. The main optimization results are shown in Table 2.

As shown in Table 1, using an MCT/LCT mixture promoted the solubility of drug, increasing the entrapment efficiency and reducing the interfacial tension, which in turn had an effect on the physical and chemical stability of the emulsions. It has been reported that the LCT/MCT mixture could reduce the viscosity of LCT and the particle size distribution of the emulsion. 23 In addition, excessive oils were not conducive to the stability of the emulsion, leading to a larger mean particle size. If the amount of oil was too low, however, it was difficult to meet the drug solubility requirements and

<table>
<thead>
<tr>
<th>Component</th>
<th>Solubility (mg/ml)</th>
<th>Soybean oil</th>
<th>LCT/MCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel</td>
<td>0.3</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Physical mixture</td>
<td>0.3</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Complex</td>
<td>8.0</td>
<td>19.8</td>
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</tr>
</tbody>
</table>

Table 1. The Solubility of Paclitaxel, the Physical Mixture and the Complex of Paclitaxel and Cholesterol in Soybean Oil and LCT/MCT
the drug easily leak into the water phase, thereby affecting the chemical stability of the drugs.24,25) Similar results were observed in our experiments as the particle size of the formulation with 20% LCT/MCT was smaller than that of 20% LCT, but larger than that of 10% LCT/MCT. The amounts of autoclave degradation products of the formulation with 20% LCT/MCT, 10% LCT/MCT and 20% LCT were 3.4%, 5.2% and 14.4%, respectively.

Lecithin is regarded as a well-tolerated and non-toxic compound, making it suitable for long-term use and high-dose infusion. The screening results for lecithin species proved that soybean lecithin exhibited better emulsifying properties. Additionally, after a regular test of centrifugal stability, a separation of the oil and water phases occurred and visible oil droplets were observed in the formulation with the egg yolk lecithin, but none for the formulation with the soybean lecithin. Moreover, the formulation with soybean lecithin had significantly less degradation products after autoclaving. This could be because soybean lecithin contains more phosphatidic acid (PA) and phosphatidylinositol (PI) than egg lecithin, as these charged phospholipid components contributed to more negative potentials and, thus increased the stability of the emulsions to stress.26,27) Thus, the optimized PACE formulation consisted of 20% LCT/MCT as the oil core, soybean lecithin as the emulsifier and poloxamer 188 as the co-emulsifier. Glycerol was also added to maintain the osmotic pressure of the formulation for intravenous administration.

In a series of experiments mentioned above, emulsions were prepared by a conventional high-pressure homogenization method. Based on the optimized formulation, the preparation process of PACE was further investigated. Results indicated that an 80 MPa homogenization pressure for 7 continuous cycles resulted in a PACE with particle sizes less than 200 nm. Increased pressure and cycle times did not improve this result.

Most importantly, although a neutral pH value was important to ensure emulsions a high absolute zeta potential28) and a low phospholipids and triglycerides hydrolysis,29—31) PAC was instable at this pH. Further study showed that, under acidic pH conditions, the degradation products decreased with pH values (Table 2). However, when the pH value was reduced to below 4.0, a precipitate of soybean lecithin appeared. This observation was consistent with the fact that the acidic pH was not conducive to the physical stability of the emulsion. Hence, a satisfactory pH value (pH 4.0—5.5) was important for PACE to survive autoclaving and remain stable during long-term storage.

**Characterization of PACE** For the characterization of PACE, the pH was 4.5 and the osmolality was 383 mOsmol/kg. Transmission electron micrographs of PACE showed that the particles were spherical in shape (Fig. 2).

The surface charge (zeta potential) is relevant to the stability of the emulsions. PACE has a high negative zeta potential of $-38.3 \text{ mV}$, which reflects the physical stability by preventing coalescence of droplets upon random collisions.29) The mean particle size of PACE was 135 nm with a size distribution of approximately 50—300 nm (Fig. 3), and it has been reported that particles in the 60—400 nm size range can slowly leak out and accumulate in tumors32—36) via a passive mechanism referred to as the ‘enhanced permeability and retention’ (EPR) effect,37,38) which might be conducive to a better cancer treatment.

Drug encapsulation efficiency is an important parameter in colloidal drug delivery systems. High solubility and partitioning of the drug into the oil phase are essential for good encapsulation efficiency of emulsions, which is conducive to the stability of the drugs.39) PACE exhibited an encapsulation efficiency of 97.3%, which proved that the majority of the PAC remained in the oil phase and oil–water interface of PACE.

**Stability of PACE** As mentioned above, the stability of PAC during autoclaving was a key for further PACE applications. It has been reported that the autoclaving process might have caused re-emulsification or irreversible redistribution of
emulsifier compounds within the oil and aqueous compartments. In addition, environmental stress during the storage process of emulsions, such as temperature, freezing, light and intense shaking, could affect the physicochemical stability of emulsions.

The effect of autoclaving and long-term storage on PACE are shown in Table 3. PACE prepared according to the final formulation had a uniform milky appearance. After autoclaving at 115 °C for 30 min, the appearance, pH value, particle size and content were nearly unchanged. As far as the related substances were concerned, an increase of 0.4% in degradation impurities after autoclaving compared with the data obtained before autoclaving was acceptable based on major pharmacopoeias, which require related substances below 2%.\(^{41,42}\)

The results of long-term storage revealed that the samples stored at 6 °C were stable for 12 months. Variations in the appearance, pH value, particle size and content were very small, whereas an acceptable increase of 1.0% in degradation impurities was observed. In addition, all other parameters of PACE after 12 months were also still within the range required for safe intravenous administration. However, the samples stored at 30 °C for 3 months showed a significantly increase in particle size and related substances, indicating that room temperature was unsuitable for the storage of PACE.

### Hypersensitivity Evaluation

One of the most important objectives of this paper was to reduce the hypersensitivity reaction caused by Cremophor-based paclitaxel solution. In a series of pre-experiments, hypersensitivity tests were performed at an allergen dose of 4 mg/kg. From these investigations, PACE exhibited a lower toxicity than an equal dose of Cremophor-based paclitaxel injection. Five guinea pigs out of six in the PACS group died during the experimental period, whereas only two guinea pigs out of six in the PACE group died after the experiments. Based on these pre-experiments, the allergen dose of paclitaxel was reduced to 2 mg/kg and the challenge dose was set at 6 mg/kg in the formal hypersensitivity studies. After challenge, in positive control group, strong positive allergic reaction symptoms, such as dyspnea, unsteady gait, gasp, spasm and rotation, were observed. Additionally, the PACS group and the blank PACS group caused equally strong positive hypersensitivity reactions. Thus, it could be concluded that the hypersensitivity reaction of the paclitaxel injection was mainly caused by Cremophor, which was in accordance with previous reports already discussed. However, the PACE group and blank PACE group did not respond to the last challenge as the hypersensitivity reaction of PACE and blank PACE was negative. Therefore, it could be concluded that PACE used intravenously at a dose of 6 mg/kg did not cause hypersensitivity.

This observation could be explained by the fact that PACE and blank PACE formulations devoid of Cremophor had no hypersensitivity reactions.

### Conclusion

The formation of a paclitaxel–cholesterol complex significantly increased the solubility of paclitaxel in the oil phase. With the complex as drug carrier, a new kind of paclitaxel intravenous emulsion was developed. This emulsion was composed of the complex, LCT/MCT, soybean lecithin, poloxamer 188 and glycerol. PACE with high encapsulation efficiency could survive autoclaving at 115 °C for 30 min and was stable for at least 12 months stored at 6 °C. PACE also exhibited a lower toxicity than equal doses of PACS in guinea pigs, as no hypersensitivity reactions were observed for guinea pigs treated with PACE. Undoubtedly, PACE has a great potential for industrial-scale production and clinical applications. Further studies are ongoing to determine the pharmacokinetic, anticancer activity and safety of PACE.

### Acknowledgement

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### References


<table>
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<tr>
<th>Sample</th>
<th>Appearance</th>
<th>pH</th>
<th>Particle size (nm)</th>
<th>Content (%)</th>
<th>Related substance (%)</th>
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<tbody>
<tr>
<td>Initial</td>
<td>Before autoclaving</td>
<td>Homogenous</td>
<td>4.48</td>
<td>141</td>
<td>101.2</td>
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<tr>
<td>After autoclaving</td>
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<td>101.1</td>
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<td>Long-term storage</td>
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<td>Homogenous</td>
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<td>101.2</td>
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<tr>
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<td>4.39</td>
<td>209</td>
<td>98.2</td>
<td>2.7</td>
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</tbody>
</table>
41) USP32-NF27, United States Pharmacopoeial Convention, Maryland, 2009, p. 3189.