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Preparation and properties of a silybin-phospholipid complex

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Due to its poor solubility in water and oil, silybin is hardly absorbed orally and cannot be dissolved directly for parenteral preparations. In this study, a silybin-phospholipid complex was prepared by single-factor design and orthogonal experimental design. Lipophilicity was improved to a large extent, which was determined by solubility experiments and oil/water partition studies. Various methods were used to confirm the formation and explore the properties of the silybin-phospholipid complex, such as UV, IR, ATR, CNMR, XRD,DSC, SEM and TEM. The structure of the phytosome when the complex was dispersed in aqueous solution is suggested.

1. Introduction

Bombardelli et al. (1991, 1994) discovered that some naturally occurring flavonoids have a special affinity to phospholipids and form complexes with them. These complexes of natural active agents with phospholipids form a type of nanoparticle called phytosomes when dispersed in water, whose physico-chemical properties and biological effects differ to some extent from the original compounds.

Drugs of obvious pharmacological activity but limited clinical use due to their physico-chemical properties, such as high polarity and low aqueous solubility or liposolubility, which cause poor oral absorption, irritation to gastrointestinal tract, or strong adverse effects, can be combined with phospholipids to form a drug-phospholipid complex (Bombardelli et al. 1985, 1991). After complexation, the drug's oral absorption or permeability through skin can be improved. Due to the significant increase in its lipophilicity, a silybin-phospholipid complex has been used to improve its bioavailability (Kidd et al. 2005; DiSario et al. 2005). Also, it is likely that phospholipids can combine with or enwrap the component which may contribute to irritation or adverse effects. For example, when amphotericin B, an anti-fungal drug with high toxicity, was combined with phospholipids, its adverse effect was reduced such that its LD50 increased from $3\ mg \cdot kg^{-1}$ to $40\ mg \cdot kg^{-1}$. Frankenburg and others (Frankenburg et al. 1998) concluded that formation of an amphotericin B-phospholipid complex can significantly promote the permeation of amphotericin B through skin and transport the drug deep into the body tissues, giving an absorptive effect ten times higher than that of amphotericin B alone when used transdermally.

Lipid substances with phosphate groups are generally referred to as phospholipids and the main members are PC, PE, PI and PA, all of which are basic ingredients of cell membranes, nuclear membranes and lipid membranes of both animal and plant cells. Their biological effects include: firstly, increasing the excitability of MP; secondly, increasing the amount of hemoglobin to a significant extent; thirdly, improving the irritability of erythrocyte to hemolysis caused by hypisotonic solution; and fourthly, facilitating the dissipation of atheromatous plaque by interfering with the transport and deposition of cholesterin and fat. As amphipathic molecules in which the oxygen atom of the hydroxyl group attached to the phosphorus atom has a strong tendency to gain electrons and the nitrogen atom to lose electrons, phospholipids can form complexes with drugs of suitable structures under certain condition. Phospholipids play a significant role in maintaining the normal structure of liver and in energy exchange among organs. People who lack phospholipids are vulnerable to diseases such as fatty liver, hepatitis, and hepatocirrhosis (Wu et al. 1998). Silybin, a stabilizer of liver cell membrane, has the effect of protecting the membrane of liver cells and enhancing its function, and can protect the liver function from deterioration resulting from the invasion by deleterious substances. In this study, a silibin-phosphlipid complex was prepared in a two step process and the form of the phytosomes was validated. A silybin-phospholipid complex may give full play to their synergistic effect of liver protection, favoring the enhancement of drug efficacy. Due to its poor solubility in water and oil, silybin has quite a low bioavailability. The preparation of a silybin-phospholipid complex can improve the drug's lipophilicity to a large extent, thus further improving its bioavailability.

2. Investigations, results and discussion

2.1. Factors affecting the formation of silybin-phospholipid complex

2.1.1. Reaction solvent

According to the complexation principle, the nitrogen atom in phospholipids has a strong tendency to lose electrons, while the oxygen atom of the phenolic hydroxyl group in silybin is inclined to gain electrons, making complexation possible. Therefore, a non-protonated transfer solvent is needed to provide a reaction environment which does not interfere with the exchange of electrons (protons) between drug and phospholipids (Bombardelli 1991, 1994). In this study, four solvents with low dielectric constant were explored including acetone (21.4), methylene dichloride (8.9), tetrahydrofuran (7.6), and ethyl acetate (6.1).

When methylene dichloride and ethyl acetate were used as reaction solvents, the reaction system was turbid the whole time. Because formation of the complex is primarily determined by the re-clarification of the solvent, it is concluded that when methylene dichloride and ethyl acetate were used as reaction solvents, a great amount of the silybin was not involved in complexation. The complexation efficiencies of complexes prepared with different solvents with the same starting molar ratio may be compared as follows: 64.53% with acetone as solvent, 99.73% with tetrahydrofuran as solvent and the experiment failed when using "ethyl acetate or methylene dichloride" as solvent.

The complex prepared with tetrahydrofuran had a higher complexation efficiency than those prepared with the other three solvents. Therefore, tetrahydrofuran was selected in preference as the optimum reaction solvent.

2.1.2. Starting molar ratio

Complexation efficiencies of complexes prepared at three molar ratios (drug: phospholipids) were found as follows: when prepared at molar ratios of 1 : 0.3, 1 : 0.5 and 1 : 1 (drug: phospholipids), the corresponding complexation efficiencies were 18.45%, 47.31% and 97.65%, respectively.

The complexation efficiency increased with an increase in phospholipids. Therefore, it is concluded that single-bond combination of drug and phospholipid molecules was involved in the complexation process.

2.1.3. Drying technique

During freeze drying, tetrahydrofuran was found to be lost from the container because of its low freezing point. Therefore, products prepared by freeze drying had a low yield of about $50 \pm 10\%$. Products prepared by spray-drying possess the best appearance, light yellow dry powders, but a slightly lower yield than that of rotary evaporation under reduced pressure. To protect phospholipids from oxidation during the drying process, products were dried at a relatively low temperature using nitrogen as the atomizing gas. The amount of residual organic solvent in products prepared by rotary evaporation under reduced pressure was higher than that obtained by spray-drying with a yield of about $68 \pm 12\%$. Yields were mostly above 75% when prepared under vacuum rotary evaporation. However, this can be overcome by placing the products in a vacuum drying oven at 40 °C overnight.

2.2. Evaluation of complex formation optimized by orthogonal experimental design

It has been proved by many experiments that a complex with high complexation efficiency remains stable after dissolving in chloroform for 72 h. On the other hand, a white sediment appeared to different extents after dissolving in chloroform for 24, 48, and 72 h. The amount of sediment and time needed for its appearance depend on the complexation efficiency and stability of the complex. Higher complexation efficiency and better stability result in both a smaller amount of sediments and a longer time for its appearance. Generally speaking, the solutions all reached a stable state after dissolving in chloroform for 72 h. Complexation efficiencies of nine complexes prepared by orthogonal experimental design were evaluated at 24, 48, 72 and 96 h using the same method as described above. The results are given in Table 1.

It can be seen from Table 1 that when prepared with an appropriate starting ratio of silybin and phospholipids, the complex can remain stable in chloroform for quite a long time. Based on literature reports (Bombardelli et al. 1985) and the structures of silybin and phospholipids, it is suggested that complexation is most likely to happen when silybin and phospholipids react in a molar ratio of 1 : 1, that is, a single molecule of silybin can form a relatively stable weak bond with a single phospholipid molecule, thus achieving complexation. Therefore, when silybin and phospholipids react in a molar ratio of less than 1, the complex is stable, and a chloroform solution can remain clear for a long time. When silybin and phospholipids react in a molar ratio of 1 : 0.5, half the silybin molecules can form only a weak inter-molecular force, instead of a strong one, with the polar groups of phospholipids, thus resulting in the complex remaining stable in chloroform and the chloroform being clear for only 24 h. However, with the progress of time, free silybin will be released and precipitate because of the dissociation of unstable intermolecular forces.

Table 1: Orthogonal test results

No.	Ratio (silybin : phospholipids) (mol)	24 hours complexing rate	48 hours complexing rate	72 hours complexing rate	96 hours complexing rate
1		80.67%	69.14%	47.65%	45.32%
2	1 : 0.5	97.52%	80.63%	36.40%	35.89%
3		101.93%	75.20%	44.04%	43.21%
4		106.31%	98.32%	103.87%	101.26%
5	1 : 1	102.13%	96.14%	97.62%	99.52%
6		96.18%	105.09%	101.53%	99.86%
7		98.01%	95.70%	97.36%	98.18%
8	1 : 2	94.64%	98.76%	101.64%	97.83%
9		91.57%	93.44%	104.03%	101.73%

Table 2: The solubility of silybin-phospholipid complex

Groups	Water	Solubility in oil (mg/ml)			
		MCT	Soybean oil	Cottonseed oil	Castor oil
Silybin	0.016	0.256	0.188	0.121	0.169
Silybin-phosphatidylcholine mixture (1 : 1)	0.022	0.449	0.240	0.198	0.297
Silybin-phosphatidylcholine complex (1 : 1)	0.056	15.493	9.124	4.391	6.692

The experimental data were subjected to ANOVA. The results indicate that the starting ratio has the most significant effect on complexation efficiency, with reaction time and temperature in between and reaction concentration the least important.

The optimum preparation is achieved when silybin and phospholipids are dissolved in tetrahydrofuran at a molar ratio of 1 : 1 (equivalent to 25 mg/ml of silybin). The reaction is carried out under room temperature (25 °C ± 2 °C) for 2 h. The resulting solution is evaporated by rotary evaporation at 40 °C under reduced pressure or by spray-drying, and then put in a vacuum desiccator at 40 °C for 12 h to assure complete removal of traces of organic solvent. The ultimate product is light yellow crystals.

2.3. Solubility of silybin and its phospholipid complex

2.3.1. Solubility of silybin-phospholipid complex and physical mixture in water and oil

As shown in Table 2, compared with silybin and a silybin-phosphatidylcholine mixture, the solubility of complex in various oils improves significantly due to the increase in lipophilicity. The phenomenon that the solubility of the complex in oil increases significantly in comparison to either silybin or phospholipids before complexation can also indicate the formation of the complex.

2.3.2. Solubility of silybin-phospholipid complex prepared at different starting ratios in medium chain triglycerides (MCT)

The solubilities of the silybin-phospholipid complex in medium chain triglycerides were 11.1 mg/ml, 15.5 mg/ml and 16.3 mg/ml when prepared at molar ratios (silybin : PC) of 1 : 0.5, 1 : 1 and 1 : 2, respectively.

After complexation with phospholipids, the solubility of silybin in MCT improved significantly from the original 0.256 mg/ml to15.493 mg/m, about a 60 fold increase. When the ratio of silybin to PC changed from 2 to 0.5, the solubility of silybin in MCT increased from 11.1 to 16.3 mg/ml. It appears that complexation efficiency is the dominant factor in determining the solubility of the complex in MCT. After complexation, excess phospholipids can serve as surfactant to facilitate the solubilization of silybin in MCT, which has little effect on the improvement of the aqueous solubility of silybin.

2.3.3. Physical characteristics of phospholipid complex

Oil/water apparent partition obtained from experiments was 22.6 ± 6.8 silybin, 33.1 ± 7.1 silybin-PC mixture, and 332.8 ± 14.6 silybin-PC complex. It can be concluded from the fact that the oil/water apparent partition coefficient of the silybin-phospholipid complex increased almost 10 fold, that the lipophilicity of the complex prepared under certain conditions is significantly improved.

2.4. Results of studies of silybin, phospholipids and phospholipid complex

2.4.1. UV-spectra

The result shows there is no difference between the UV absorption spectra of silybin before and after complexation, which is a strong indication that the bonding of the drug with phospholipids does not affect the conjugation system of silybin and the characteristics of its chromophore are not altered.

2.4.2. TR and Attenuated Total Reflection (ATR)

The stretching vibration peak at 1247 cm^{-1} associated with P=O in phospholipid molecules has disappeared in the spectrum of the complex, which indicates that there are some interactions between P=O and silybin, affecting peak formation. The pointed peak at 3455 cm^{-1} attributed to the stretching vibration of free −O−H (with no hydrogen bonding) in silybin has also disappeared in the spectrum of the complex, which indicates the formation of a hydrogen bond between the free −O−H in silybin and phospholipids, weakening the intensity of the bonds concerned of proton donors and acceptors, thus altering the frequency of stretching and bending vibrations correspondingly. The stretching vibration band of X−H has moved to the lower wave number region coupled with increased absorption intensity and spectral band broadening, resulting in a wide peak at 3500–3200 cm^{-1} in the spectrum of the complex.

No new conjugation bond was formed between silybin and phospholipids. However, as can be seen from the

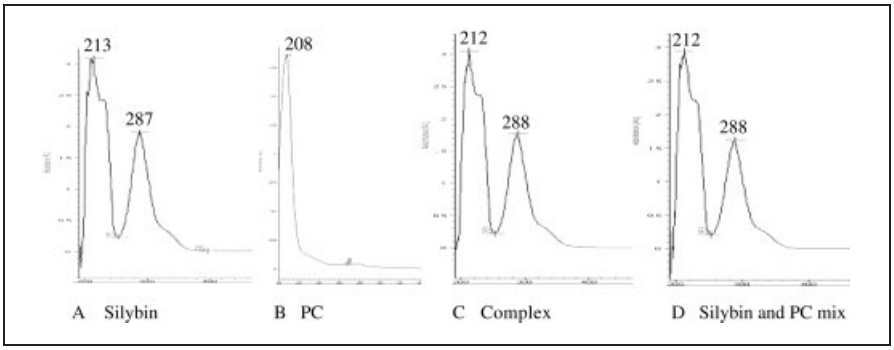


Fig. 1: UV-Spectra

slight difference in the fingerprint peak regions, there might be some weak bonds forming between silybin and phospholipids.

The absorption peak positions of functional groups in TR basically coincide with those of ATR. The ATR spectra of the complex and phospholipids are almost the same with all the characteristic groups of phospholipids visible in the ATR spectrum of the complex, whereas many of the characteristic groups of silybin have disappeared in the spectrum of the complex. It can be concluded that it is the phospholipids enclosing some of the groups of silybin that results in the disappearance of the characteristic groups of silybin in the ATR spectrum of the complex. It can be further concluded that the complex is distinguished from solid dispersions containing molecules highly dispersed in the matrix with regard to structure.

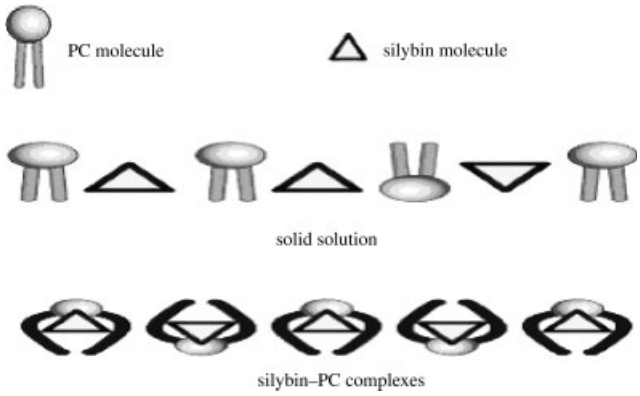


Fig. 2: Solid state of solid solution and phytosomes

2.4.3. NMR

In the ^{1}H NMR spectrum of the silybin-phospholipid complex (Bombardelli et al. 1985; Zhai et al. 2001; Wu et al. 2001), signals of the protons in silybin with high activity and some of the phospholipid protons have weakened remarkably to the extent that they cannot be observed, which indicates these protons are involved in the formation of the complex, whereas the proton signals of the fatty acid chains in phospholipids are still clear with no change, indicating that they are not involved in complexation. Information obtained from ^{13}C NMR spectra is similar to that from ^{1}H NMR. The relaxation time of the drug's carbon cores reacting with phospholipids has decreased significantly, making the corresponding signals of the carbon spectrum decrease or disappear. Meanwhile, signals of $-N(CH_3)_3^+$ in phospholipids have broadened, whereas conjugant signals of fatty acid chains retain their original pointed peaks.

In the ^{31}P NMR spectrum, the peak position of P in the complex has moved to high ppm with the peak shape broadening compared with that in free silybin, which, coupled with the disappearance of P=O absorption peak in the IR spectra, indicates the involvement of P=O in complexation.

It has been reported (Wu et al. 1998) that the relatively strong interaction between natural active components and the polar parts of phospholipids inhibits free rotation of the single bonds in the phospholipid molecule, whereas the two long fatty chains of phospholipid are not involved in complexation and can still turn freely, enwrapping the polar parts of the phospholipid and forming a lipophilic

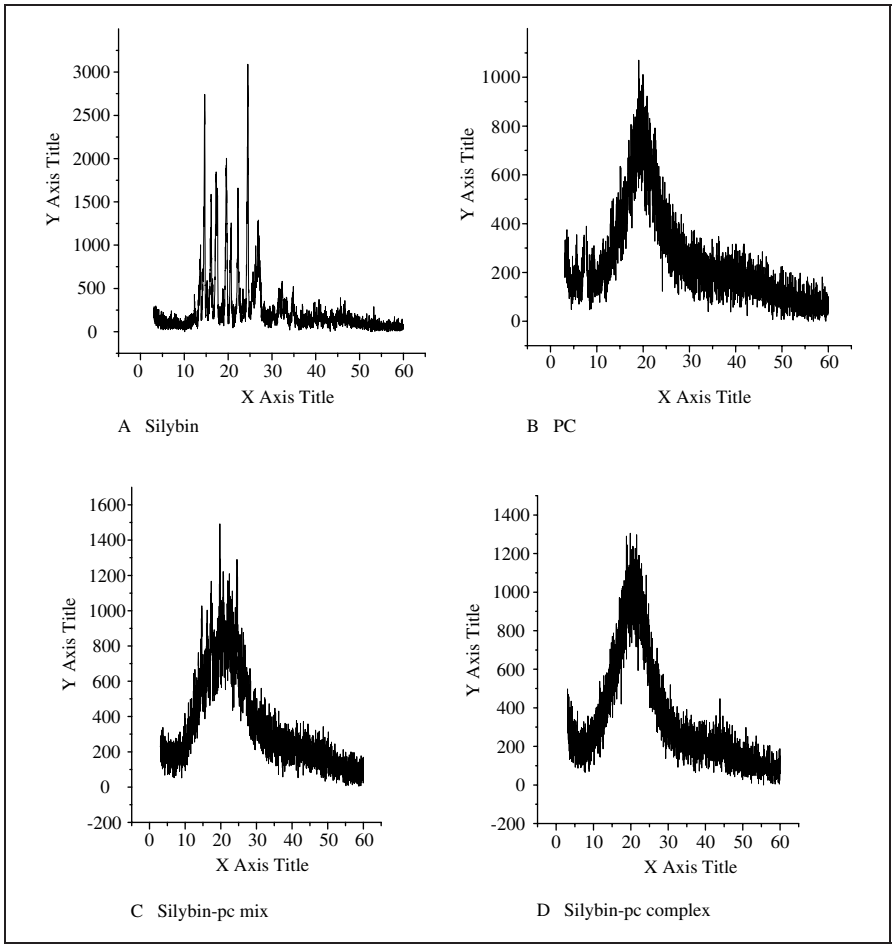


Fig. 3:
X-ray diffractogram

appearance, which apparently improves the lipophilicity of the complex.

Combining with the Attenuated Total Reflection Spectra (ATR) of the silybin-phospholipid complex, it can be concluded that the structures of the silybin-phospholipid complex and solid solution (a kind of solid dispersion) in the solid state are as follows. As we can see (Fig. 2), silybin-phospholipid complex is a special kind of solid dispersion which has mono-molecule bonding within the complex, while the solid solution is actually a mixture which contains molecules highly dispersed in the matrix.

2.4.4. x-ray diffraction (XRD) analysis

XRD (Fig. 3) reveals the amorphism characteristic of the silybin-phospholipid complex with all the diffraction peaks of the silybin crystal being basically invisible, indicating that, after complexation, the orientated combination of the polar ends of phospholipids with silybin actually led to their being highly dispersed, thus inhibiting their individual crystalline characteristics. Generally, amorphic molecules display an irregular arrangement with relatively higher free energy and solubility and a faster dissolution rate. Moreover, the higher affinity with water relative to crystals might be one of the reasons leading to the improved solubility of the complex (Wu et al. 2001).

2.4.5. Differential scanning calorimetry (DSC)

DSCs (Fig. 4) of silybin, the phospholipid, phospholipid complex, and physical mixture indicate a decrease of the phase transition temperature of the silybin-phospholipid complex. Two different endothermal peaks were observed in the DSC of PC. The former peak had lower intensity and a smaller peak area, and was due to thermal movement of the polar end of phospholipid molecules, while the latter associated with the transition from gel state to liquid crystalline state obviously appeared much sharper, and probably resulted from the melting, isomerization or change of crystal form of the carbon-hydrogen chains in phospholipids (Bombardelli et al. 1985). Therefore, the phase transition temperature of phospholipid was 229.6 °C. SB was not pure, so the DSC of SB shows a broad endothermal peak with an onset melting point of 136.5 °C. Both drug and phospholipid peaks were replaced by a relatively less intense exothermal peak at 28.8 °C in the DSC curve of SLC. The phase transition temperature of SLC was lower than that of phospholipids and this was probably due to the fact that after combination of silybin with the polar end of PC, the carbon-hydrogen chains of PC can still move freely, enwrapping the polar parts of phospholipids, making the sequence of aliphatic carbon-hydrogen chains of phospholipids decrease and the second endothermal peak of PC disappear. (Wu et al. 2001; Dobretsov et al. 1998). The typical peak of silybin can be seen clearly in the DSC curve of the silybin-PC physical mixture, but the typical peak of PC was replaced by a peak at 28.8 °C identical to the exothermal peak of the silybin-PC complex. This phenomenon was caused by the complexation of part of the mixture during the melting process while heated.

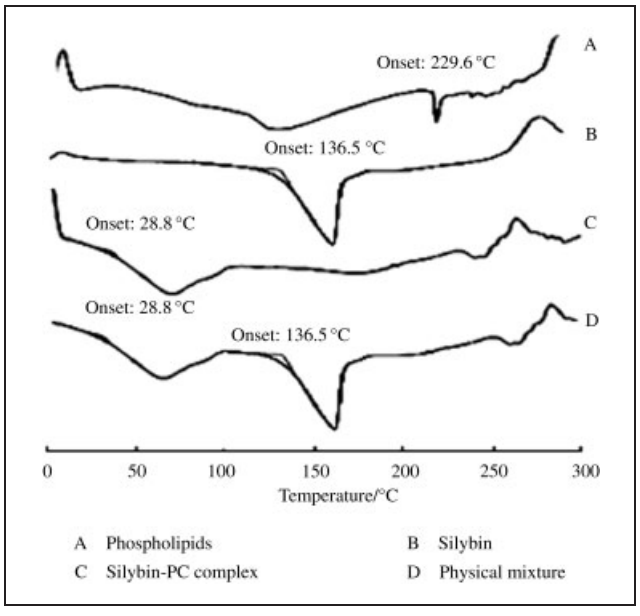


Fig. 4: DSC spectra

2.4.6. SEM and TEM

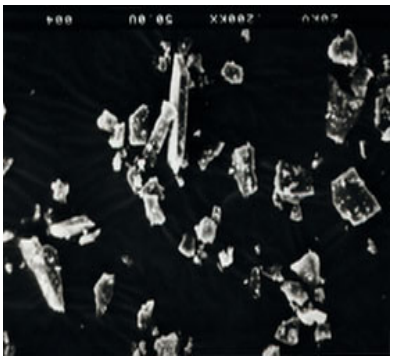
As shown in Fig. 5, the appearance of the silybin-phospholipid complex is light yellow transparent agglomerates, resembling the phospholipids in appearance, but lacking the regularity of the common crystalline appearance.

Silybin-phospholipid was redissolved in double distilled water, vortexed, and observed by transmission electron microscopy. The dispersion of the complex in water resembles liposome micelles (Fig. 6).

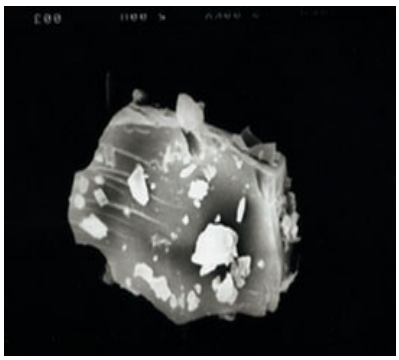
Silybin-phospholipid complexes can form agglomerates in water, resembling in appearance liposomes. However, the formation mechanism of the aqueous solution of the complex is distinguished, both theoretically and practically, from that of liposome solutions, resulting in different internal structures. Liposomes are closed vesicles formed by lipid bilayers, encapsulating drugs within an aqueous compartment or the multiple lipid bilayers. On the other hand, the silybin-phospholipid complex is formed by the combination of silybin with the polar end of the phospholipids and its aqueous dispersion is characteristic of phospholipids. When the drug-loaded complex is dissolved in water, the regular arrangement of multiple complex molecules forms spheroids with an appearance similar to multilamellar vesicles, but which are actually distinguished from them internally (Bombardelli 1985). Combined with their experimental conclusions and the results of this study, we conclude that the structure of the silybin-PC complex is as shown in Fig. 7.

Silybin-phospholipid complexes with high solubility and stability were prepared after evaluation of the preparation techniques by single-factor and orthogonal experimental design. Experimental data show that the complexation of silybin with phospholipids improves drug lipophilicity significantly. The improvement of drug lipophilicity favors the drug permeating cell membranes and entering into systemic circulation or the interior of tissue cells to exert pharmacological effects (Kidd et al. 2005).

It can be inferred from the spectral characteristics of the complex that a strong interaction exists between silybin and the polar groups of phospholipids, while the two long fatty acid chains of the phospholipids are not involved in complexation and can still rotate freely, enwrapping the polar parts of phospholipids to form a lipophilic appearance, thus resulting in higher lipophilicity of the complex. Various spectra of the complex reveal that silybin and phospholipids form a molecule-based complex with no generation of new compounds; therefore the individual

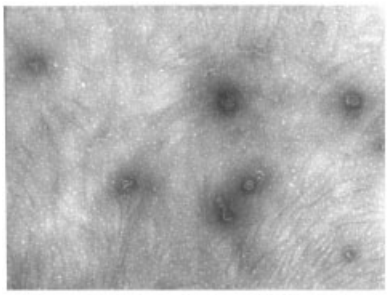


A ×200 magnification while



B ×1000 magnification

Fig 5:
Scanning electron micrographs of phospholipid complex



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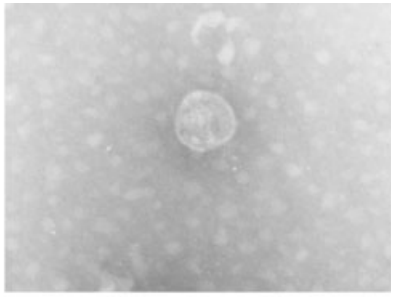

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Fig. 6:
Transmission electron micrographs of complex after slightly shaking in distilled water

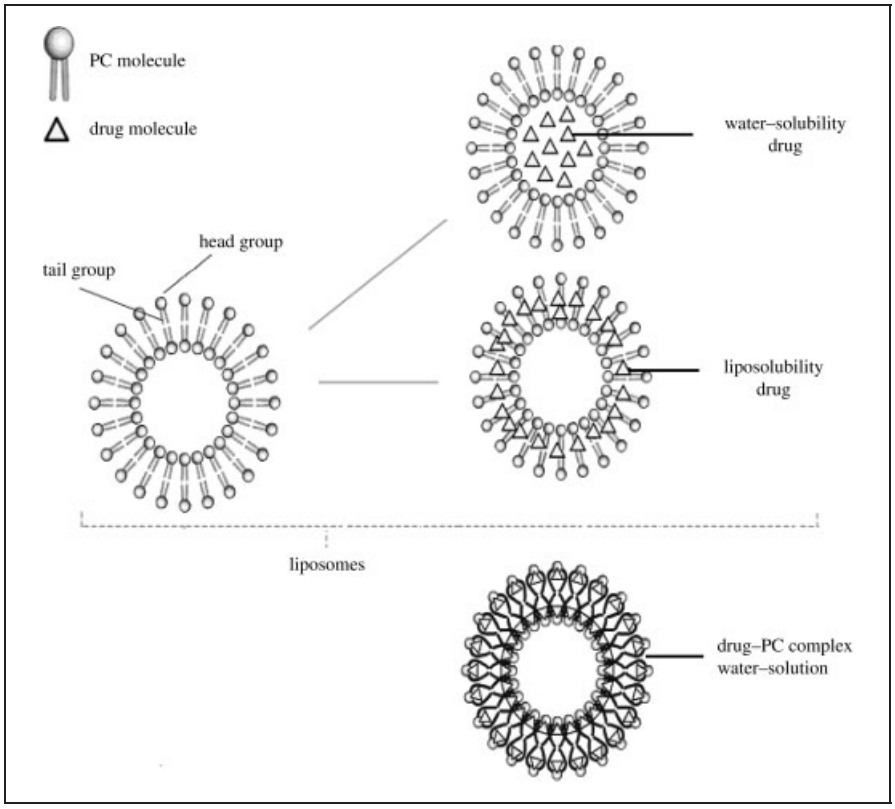

Fig. 7:
Difference between liposome and silybin-PC complex in water

chemical properties of silybin and phospholipids will not change *in vivo*.

3. Experimental

3.1. Materials

Silybin was purchased from Pan-jing-ge-ling-en Biology Technique Ltd, and phospholipid was purchased from Tai-wei-yao-ye Ltd. The phosphatidyl content of the phospholipid was approximately 82% (w/w). The other chemical reagents were of analytical grade or better.

3.2. Determination of the content of silybin in phospolipid complexes

Approximately 5 mg of the phospolipid complex was dissolved in 10 ml of a mixed solvent (methanol: water = 40 : 60, v/v), and a 20 µl aliquot of the mixture was injected into a HPLC system. The stationary phase, µBondapak C_{18} (150 mm × 4.6 mm, 5 µm), was maintained at 40 °C. The mobile phase was a mixture of methanol: double distilled water: 0.05 mol/L KH_2PO_4 = 60 : 40 : 5, adjusted to pH 4.0 with phosphoric acid. The flow rate was 1.0 ml/min. Effluent was monitored at 288 nm.

In order to construct a calibration curve, methanol was added to dissolve 5 mg standard silybin to make a stock solution. Silybin standards were prepared by removing 0.5, 1, 1.5, 2 and 2.5 ml of the stock solution to

five 10 ml volumetric flasks and diluting it with mobile phase. Concentrations of the silybin standards were analyzed by HPLC, and the peak areas were plotted against silybin concentrations to generate a calibration curve. Linear regression was used to calculate the equation of the line. Equation of the standard curve: $C = 8 \times 10^{-8} A + 0.0009$ ($r = 0.99994$). Standard curves generated acceptable data over the ranges 0.025–0.125 mg silybin/ml.

3.3. Evaluation of the complexation efficiency of silybin-phospholipid complex

The complexation efficiency was determined by dividing the content of drug combined with phospholipids over the original content of drug present in the loading solution.

Because both free silybin and its phospholipid complex dissolve well in methanol, the original content of the drug can be determined by dissolving a certain amount of the silybin-phospholipid complex in methanol, and measuring the content of the drug dissolved in methanol. The content of drug combined with phospholipids can be determined as follows: the same amount of silybin-phospholipid complex was dissolved in chloroform, and the uncombined silybin precipitated because free silybin is insoluble in chloroform. The precipitates were removed by filtration and the content of the drug dissolved in chloroform was measured. Therefore, the complexation efficiency was calculated as follows (1):

$$\text{complexation efficiency } \% = W_c / W_t{}^* \, 100\% \quad (1)$$

where W_c is the content of silybin dissolved in chloroform, and W_t is the content of silybin dissolved in methanol.

3.4. Determination of factors affecting the preparation of silybin-phospholipid complex by single-factor design

3.4.1. Reaction solvent selection

The effect of different solvents on the complexation efficiency of the complex was determined under preliminary experimental conditions: silybin and phospholipid were dissolved in different solvents in a molar ratio of 1 : 1 (equivalent to 25 mg/ml silybin). The reaction was carried out at 25 °C for 1 h. The product was dried by spray-drying. The candidate solvents were: acetone, methylene dichloride, tetrahydrofuran, and ethyl acetate.

3.4.2. Effect of feeding ratio

The effect of different starting ratios of silybin and phospholipids on the complexation efficiency of the complex was determined under preliminary experimental conditions: silybin and phospholipids were dissolved in tetrahydrofuran in different molar ratios (equivalent to 25 mg/ml silybin). The reaction was carried out at 25 °C for 1 h. The product was dried by spray-drying. Three candidate molar ratios of phospholipids and silybin were used: 1 : 0.3, 1 : 0.5, 1 : 1.

3.4.3. Effect of drying technique

During the preparation of the silybin-phospholipid complex, the end product was dissolved in reaction solvent. The organic solvent was removed by three conventional drying techniques to produce the complex: spray-drying, freeze-drying, and rotary evaporation under reduced pressure. The three methods were compared in terms of the appearance and yield of the product.

3.5. Optimization of complex preparation by orthogonal experimental design

Based on the results of the single-factor design investigations described above and related literature reports, the starting ratio of silybin and phospholipids, reaction temperature, reaction time, and drug concentration were defined as working factors affecting the complexation efficiency of the ultimate product. Therefore, a four-factor, three-level orthogonal experimental design (L9(34)) was used, with the complexation efficiency as the target, to optimize the preparation process as listed in Table 3. (where 9 = number of experiments; 3 = number of levels; and 4 = number of factors).

Table 3: Factors and levels in orthogonal design

Level	Factors			
	Ratio	Conc. (mg/ml)	Temperature (°C)	Time (h)
1	1 : 0.5	5	25	0.5
2	1 : 1	25	40	2
3	1 : 2	40	60	4

3.6. Solubility of silybin and its phospholipid complex

3.6.1. Solubility of silybin, phospholipid complex, and physical mixture in different oils

Appropriate amounts of silybin were placed in vials, and 5 ml of medium chain triglycerides (MCT), castor oil, soybean oil, and cottonseed oil were added to each vial, respectively. The mixture was magnetically stirred at 40 °C for 72 h followed by continued magnetic stirring at room temperature for 24 h, and then allowed to stand for a while. The supernatants were removed for centrifugation at 8000 r/min for 15 min. Afterwards, precisely 0.5 ml of the supernatant was removed to a 10 ml volumetric flask, and methanol was added up to the mark. 0.5 ml of the resulting solution was removed to a 5 ml volumetric flask, and mobile phase was added up to the mark. The content of silybin was determined by HPLC analysis.

The same approach was applied to the determination of the solubility of the phospholipid complex and physical mixture in double distilled water and different oils.

3.6.2. Oil/water apparent partition coefficient of silybin and phospholipid complex

5 ml of saturated aqueous solution (double-distilled water) of silybin, silybin-phospolipid complex and silybin-phospolipid physical mixture were removed to clean 20 ml test tubes, respectively, and then 5 ml n-octanol (pre-saturated by double-distilled water) was added to each tube. The tubes were stoppered, vortexed for 120 s, and allowed to stand overnight. The solubilities of silybin in water before and after the addition of n-octanol were determined by HPLC. The oil/water apparent partition coefficient (P) was calculated as follows (2, 3):

$$P = C_n / C_w \quad (2)$$

$$C_n = C_s - C_w \quad (3)$$

where C_s and C_w are the solubility of silybin in water before and after the addition of n-octanol, respectively.

3.7. Characteristics of silybin-phospholipid complex

The crystalline and phase transition properties of silybin and phospholipids before and after complexation were analyzed by x-ray diffraction (XRD) and differential scanning calorimetry (DSC). UV spectrophotometry, TR, Attenuated Total Reflection Spectra (ATR), and NMR were utilized to reveal the microstructure of the silybin-phospholipid complex for investigation of its formation mechanism.

Siliybin, phospholipids, phospholipid complex, and a physical mixture were subjected to the measurements described above.

3.7.1. UV

Appropriate amounts of the test samples were dissolved in methanol and their UV absorbances were determined from 200–500 nm. The differences of UV absorption characteristics of silybin and phospholipids before and after complexation were compared.

3.7.2. TR and Attenuated Total Reflection Spectra (ATR)

ATR can show the surface characteristics of the complex, thus giving information on the original "*in-situ*" structure of the complex in the solid state. TR can be used to determine the complexation mechanism by comparing the shift of spectral position of the typical functional groups in the complex. Test samples were tableted with KBr and detected by TR ranging from 400–4000 cm^{-1}, and ATR from 500–4000 cm^{-1}.

3.7.3. NMR

Test samples were studied by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, respectively.

3.7.4. XRD

Detection condition: the experiment was conducted using a graphite monochromator with CuK radiation with a voltage window of 40 KV and current density of 50 mA with a scanning rate of 10°/min ranging from 3 to 50 °C.

3.7.5. DSC

DSC measurement was performed at a heating rate of 10.00 K/min from 30 to 350 °C.

3.7.6. SEM and TEM

The morphologies of the silybin-phospholipid complex in solid and liquid state were observed by SEM and TEM, respectively.

Silybin-phospholipid complex powders were coated with platinum in a sputter coater, and their surface morphology was viewed and photographed with a scanning electron microscope under an acceleration voltage of 20 KV, beam current of 5×10^{8} mA, and working distance of 15 mm.

Samples of phospolipid complexes were prepared by dropping distilled water on to phospolipid complexes powders, and then swirling for 3 min. A drop of the resultant phospolipid complex dispersion was placed onto a carbon-coated copper grid, leaving a thin liquid film. The films on the grid were negatively stained by adding immediately a drop of 2% (w/w) ammonium molybdate in 2% (w/v) ammonium acetate buffer (pH 6.8). The excess staining solution was removed with filter paper and the films were exposed to thorough air-drying. The stained films were then viewed on a transmission electron microscope and photographed.

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