Available online at www.sciencedirect.com
SCIENCE DIRECT
International Journal of Pharmaceutics 307 (2006) 77–82
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

The preparation of silybin–phospholipid complex and the study on its pharmacokinetics in rats

Xiao Yanyu, Song Yunmei, Chen Zhipeng, Ping Qineng *

China Pharmaceutical University, Nanjing, Jiangsu, China

Received 27 August 2005; accepted 1 October 2005
Available online 18 November 2005

Abstract

The aim of the present study was to find a way of prepare silybin–phospholipid complex to make oral bioavailability of silybin increase and to study its physicochemical properties and to compare the pharmacokinetic characteristics and bioavailability after oral administration of silybin–phospholipid complex and silybin-*N*-methylglucamine in rats. Using ethanol as a reaction medium, silybin and phospholipids were resolved into the medium, after the organic solvent was removed under vacuum condition, silybin–phospholipid complex was formed. The new complex's physicochemical properties including scanning electron microscopy (SEM), transmission electron microscopy (TEM), differential scanning calorimetry (DSC), solubility, dissolution, etc., were tested. The concentrations of silybin after oral administration of silybin–phospholipid complex and silybin-*N*-methylglucamine at different time in rats were determined by RP-HPLC. The pharmacokinetic parameters were computed by software program 3p97. Our data showed that silybin and phospholipids in the silybin–phospholipid complex were combined by non-covalent-bond, not forming a new compound and the solubility of silybin–phospholipid complex in water and in *n*-octanol was effectively enhanced. We found that mean plasma concentration–time curve of silybin after oral administration of silybin–phospholipid complex and silybin-*N*-methylglucamine in rats was both in accordance with open single-compartment model with first-order absorption. Pharmacokinetic parameters of silybin in rats were T_{max} 10 and 5 min; C_{max} 126.72 and 104.29 ng ml^{-1}; $AUC_{0-\infty}$ 1020.33 and 235.81 ng ml^{-1} h, respectively. The bioavailability of silybin in rats was increased remarkably after oral administration of silybin–phospholipid complex comparing to silybin-*N*-methylglucamine. This was mainly due to an impressive improvement of the lipophilic property of silybin–phospholipid complex and improvement of the biological effect of silybin.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Silybin–phospholipid complex; Silybin-*N*-methylglucamine; Physicochemical properties; Oral bioavailability; Pharmacokinetics; Silybin

1. Introduction

Silybin, the seeds extract of the milk thistle (*Silibum marianum* Gaertn), has been widely used to maintain liver health and treat liver disorders, reported by Kvasnicka et al. (2003) and Tedesco et al. (2004). Silybin is slightly soluble in water and in oil, the poor permeation across the intestinal epithelial cells and minor the gastrointestinal (GI) tract absorption in rats has been reported by Barzaghi et al. (1990) and Morazzoni et al. (1992). Phospholipids are an important component of cell membrane, having the actions of keeping cell membrane fluidity and treating hepatic disorder. It is expected that silybin combined with phospholipids might result in increase of oral bioavailability and improvement of the biological effect of silybin.

To take the advantages of phospholipids complexes, we studied silybin–phospholipid complexes to improve oral bioavailability of silybin.

2. Materials and methods

2.1. Materials

Both silybin and silybin-*N*-methylglucamine were purchased from Pan-jing-ge-ling-en Biology Technique Ltd., purity 98.6 and 100%, respectively, and phospholipid was purchased from Tai-wei-yao-ye Ltd., and the phosphatidyl content was approximately 82% (w/w). The other chemical reagents were of analytical grade or better.

* Corresponding author.
E-mail addresses: czpcpu@hotmail.com (X. Yanyu), pingqn@cpu.edu.cn (P. Qineng).

0378-5173/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.10.001

2.2. Preparation of silybin–phospholipid complexes

The required amounts of silybin and phospholipids were placed in a 100 ml round-bottom flask and dissolved in anhydrous ethanol. After ethanol was evaporated off under vacuum at 40 °C, the dried residues were gathered and placed in desiccators overnight, then crushed in the mortar and sieved with an 100 mesh. The resultant silybin–phospholipid complex was transferred into a glass bottle, flushed with nitrogen and stored in the room temperature.

2.3. Determination of the content of silybin in phospholipids complex

The content of silybin in phospholipids complex was determined as follows. Approximately 5 mg of phospholipids complex were dissolved in 50 ml of solvent A (methanol:water = 40:60, v/v), and a 20 μl aliquot of the resulting solution was injected into a HPLC system. The stationary phase, μBondapak C_{18} column (150 mm × 4.6 mm, 5 μm), was kept at 40 °C. The mobile phase was a mixture of methanol:double distilled water:0.05 M 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.

2.4. Scanning electron microscopy (SEM)

Phospholipids complex powders were coated with platinum in a sputter coater (JFC-1100, Jeol, Japan), and their surface morphology was viewed and photographed with a Jeol scanning electron microscope (JSM-5310-LV, Jeol).

2.5. Transmission electron microscopy (TEM)

Samples were prepared by dropping distilled water to phospholipids complex powders, then swirled for 3 min. A drop of the resultant phospholipids complex dispersions was placed onto a carbon-coated copper grid, leaving a thin liquid film. The films on the grid were negatively stained by immediately adding a drop of 2% (w/w) ammonium molybdate in 2% (w/v) ammonium acetate buffer (pH 6.8), removing the excess staining solution with a filter paper, and followed by through air-dry. The stained films were then viewed on a transmission electron microscope (Jeol-200 CX, Jeol, Japan) and photographed.

2.6. Differential scanning calorimetry (DSC)

The samples sealed in the aluminum crimp cell were heated at the speed of 5 °C/min from 0 to 300 °C in the atmosphere of nitrogen (DSC7, Perkin-Elmer, USA). Peak transition onset temperature was determined by means of an analyzer. The peak transition onset temperatures of phospholipids, pure silybin, the mixture of phospholipids and silybin and phospholipids complex were compared.

2.7. Solubility studies

Solubility determination of silybin of silybin material and phospholipids complex was carried out by adding excess of silybin material and phospholipids complex to 6 ml of water or *n*-octanol in sealed glass containers at 25 °C. Each experiment was performed in triplicate. The liquids were agitated for 24 h, then centrifuged to remove excessive silybin (15 min, 4000 rpm). The supernatant was filtrated through a 0.45 μm membrane. The 1 ml filtrate was mixed with 9 ml of solvent A and a 20 μl aliquot of the resulting solution was injected into a HPLC and detected at a wavelength of 288 nm, the concentration of silybin was measured.

2.8. Dissolution studies

The dissolution studies were carried out according to a dissolution test apparatus of China pharmacopoeia (2000 edition, paddle method). The dissolution flasks were immersed in a water bath at 37 °C. The dissolution medium (pH 1.2 HCl or pH 6.8 phosphate buffer saline, 900 ml) was continuously stirred at 100 rpm. Phospholipids complex which is equivalent to 162 mg of silybin was added on the surface of the stirred dissolution medium at the beginning of the study. At different time intervals, 10-ml samples were withdrawn and filtrated using 0.8 μm cellulose nitrate membrane, 10-ml fresh mediums were added into the flask. The 1 ml filtrate was mixed with 9 ml of solvent A and a 20 μl aliquot of the resulting solution was injected onto a HPLC and detected at a wavelength of 288 nm, the concentration of silybin was measured.

2.9. Rats bioavailability experiments

2.9.1. Chromatography

The plasma concentrations of silybin were determined by a HPLC. The stationary phase, μBondapak C_{18} column (150 mm × 4.6 mm, 5 μm), was kept at 40 °C. The mobile phase was a mixture of methanol:double distilled water:0.05 M KH_2PO_4 = 50:50:5, adjusted to pH 4.0 with phosphoric acid. The flow rate was l.0 ml/min. Effluent was monitored at 288 nm. Two peaks were detected at about 12 and 13 min, because silybin is an isomeric compound.

2.9.2. Plasma sample preparation and validity

The rats were anaesthetized with aether, and 500 μl blood was taken from the eyeground veins. The plasma was obtained after centrifugation (15 min, 4000 rpm) was stored at −20 °C until analyzed.

When the plasma sample was thawed, 100 μl of 1 M Na_2CO_3 solution and 500 μl of borate buffer saline (pH 8.0) were added, and agitated for 30 s. After 4 ml aether was added to the solution above, this mixture was shaken for 3 min and then centrifuged (15 min, 4000 rpm). The organic phase was quantitatively decanted into a clear tapered centrifuging tube and the eluate were evaporated under nitrogen at 37 °C. The residues were resuspended in 100 μl of mobile phase and centrifuged (15 min, 4000 rpm). Aliquots (20 μl) of the supernatant were injected for HPLC analysis.

The method was validated by adding various quantities of silybin to blank rat plasmas. The resulting concentrations of silybin were 0.05, 0.1, 0.2, 0.6, 1.0, 1.8 and 2.6 μg ml^{-1}. These

calibrations were subjected to the entire analytical procedure, so as to test the linearity, precision and accuracy of the method.

2.9.3. Pharmacokinetic study of silybin–phospholipid complex and silybin-N-methylglucamine in rats

Twelve male rats (body weight 200–250 g) divided randomly into two groups were fasted for 12 h, but allowed to take water freely. A sample equivalent to 9.1 mg/kg of silybin of phospholipids complex suspended in 2 ml of water was orally administered to one group of rats. The suspensions of silybin-*N*-methylglucamine equivalent to 9.1 mg/kg of silybin were orally administered to another group of rats.

Peak concentration (C_{max}) and peak times (T_{max}) were derived directly from the experimental points. The other pharmacokinetical parameters were computed by software program 3p97.

3. Results and discussions

3.1. Preparation of silybin–phospholipid complex

We prepared silybin–phospholipid complex according to different quantity ratio of phospholipids and drugs, such as 0.1, 0.5, 1 and 2. The results showed that when the ratio was more than 1, the appearance of resultant materials appeared viscous and it was not easy that resultant materials were prepared to other preparations, but when the ratio was lower than 1, the stability of phospholipid complexes was worse. For the purpose to get the best quality and use the fewest quantity of phospholipids, at last we prepared silybin–phospholipid complex in term of the quantity ratio 1. The content of silybin in the phospholipids complex was 49.73% (w/w).

3.2. Scanning electron microscopy

The surface morphology of phospholipids complex as examined by SEM is Fig. 1. Phospholipids complex were made up of phospholipids and drugs and appeared column shape. When at ×1000 magnification, we could see that phospholipids did not exit on the appearance of drugs but drugs uniformly dispersed in phospholipids and formed the structure of column shape.

3.3. Transmission electron microscopy

The TEM of phospholipids complex after slightly shaking in distilled water are shown in Fig. 2. After slightly shaking in distilled water, we could see that there were many particles suspended in the water and infusible particles still exited in the solution. For phospholipids complex, the drugs were combined with phospholipids by the polar part of phospholipids, when swirled in distilled water many complex molecules arranged in order and formed the structure of vesicles different from liposomes.

3.4. Differential scanning calorimetry

Fig. 3 shows the DSC curves of phospholipids, silybin physical mixture and phospholipids complex. Phospholipids show two different kinds of endothermal peaks, and the first endothermal peak appears mild, it was considered that the for-

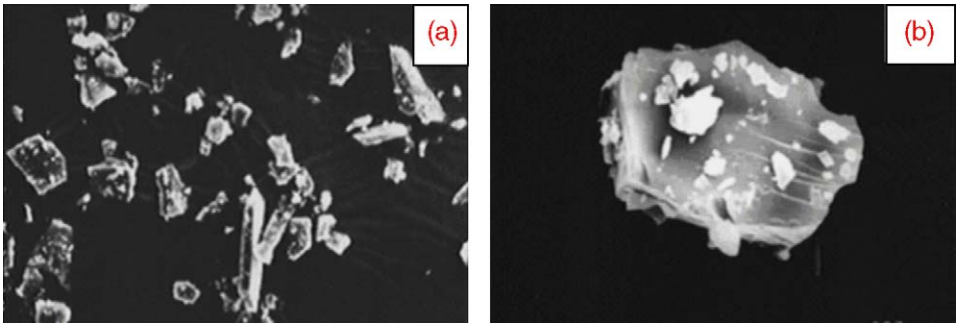

Fig. 1. Scanning electron micrographs of phospholipids complex (a) at ×200 magnification and (b) at ×1000 magnification.

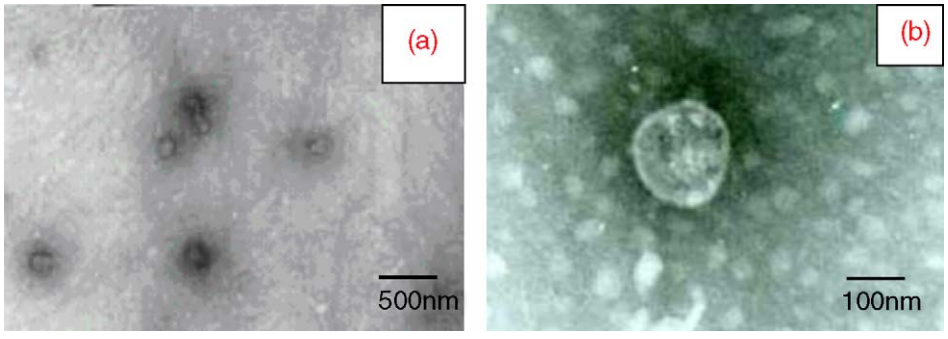

Fig. 2. Transmission electron micrographs of phospholipids complex after slightly shaking in distilled water at ×4000 magnification (a) and at ×20,000 magnification (b), respectively.

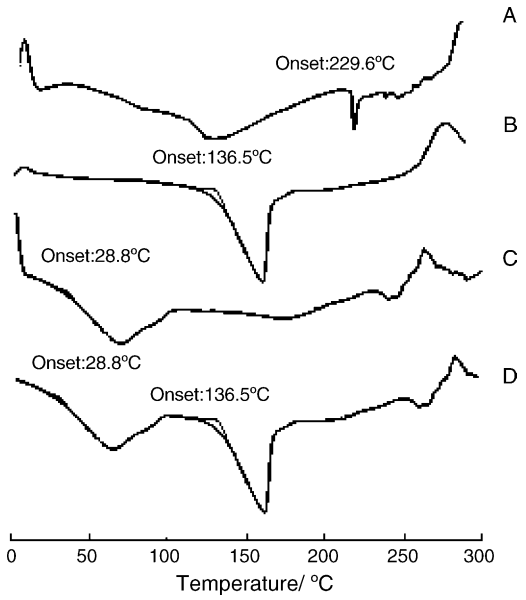

Fig. 3. DSC thermograms of phospholipids (A), silybin (B), phospholipids complex (C) and physical mixture (D).

mation of this peak was duo to hot movements of phospholipids molecule polarity parts. However, the second endothermal peak at 229.6 °C appears sharp-pointed, it was considered that owing to the transition from gel state to liquid crystal state, the carbon–hydrogen chain in phospholipids perhaps happened to be melt, isomerous or the crystal changes. Silybin is not pure, so it shows abroad endothermal peak, and its beginning melting point at 136.5 °C. Physical mixture of silybin and phospholipids shows that there are two endothermal peaks, and the former is 28.8 °C, the same with the onset temperature of phospholipids complex; another is 136.5 °C, the same with the onset temperature of silybin. It was considered that when the temperature was increased, phospholipids were melt and drugs were dissolved in the phospholipids and partly formed phospholipids complex, which could be explained through the theory of preparation by melt-out method. DSC of phospholipids complex shows the endothermal peaks of drug and phospholipid are disappeared and the phase transition temperature is lower than the phase transition temperature of phospholipids, it was considered by Hwang and Shen (1981), Venema and Weringa (1988) and Lasonder and Weringa (1990) that silybin and phospholipids should have some interaction, such as the combination of hydrogen bonds or van der Waals force. After the combination of silybin and the phospholipids molecule polarity parts, the carbon–hydrogen chain in phospholipids could turn freely and enwrap the phospholipids molecule polarity parts, which made the sequence decrease between phospholipids aliphatic hydrocarbon chains, made the second endothermal peak of phospholipids disappear and depressed the phase transition temperature.

3.5. Solubility studies

Tables 1 and 2 shows the solubility of silybin, physical mixture and phospholipids complex in water or *n*-octanol. The data show that solubility of silybin–phospholipid complex in *n*-octanol was about 1000 multiples than that in water.

Table 1
Apparent solubility of silybin and silybin–phospholipid complex in water at 25 °C

Sample	Apparent solubility ($\mu g\,ml^{-1}$)			$\bar{x} \pm s$
	1	2	3	
Silybin	39.56	41.90	41.02	40.83 ± 1.18
Physical mixture	48.72	46.53	50.24	48.50 ± 1.87
Silybin–phospholipid complex	75.47	80.82	78.45	78.25 ± 2.68

Table 2
Apparent solubility of silybin and silybin–phospholipid complex in *n*-octanol at 25 °C

Sample	Apparent solubility ($mg\,ml^{-1}$)			$\bar{x} \pm s$
	1	2	3	
Silybin	0.682	0.761	0.717	0.72 ± 0.04
Physical mixture	5.52	6.16	8.84	6.84 ± 1.76
Silybin–phospholipid complex	64.79	61.31	61.90	62.67 ± 1.86

3.6. Dissolution in HCl (pH 1.2) and phosphate buffer saline (pH 6.8)

Fig. 4 shows the dissolution profile of silybin from phospholipids complex and silybin material in HCl (pH 1.2) and phosphate buffer saline (pH 6.8), respectively. The dissolution of silybin from phospholipids complex in pH 6.8 phosphate buffer saline was not complete until 60 min, the amount about 158.4 mg; however, at about 30 min, the dissolution in 0.1N HCl was complete, and the amount only 6.3 mg. From Fig. 1, we can know that the curves of phospholipids complex dissolution procedure are greatly influenced by the pH of media, and with the increase of the pH of media the dissolution amount of silybin is increased.

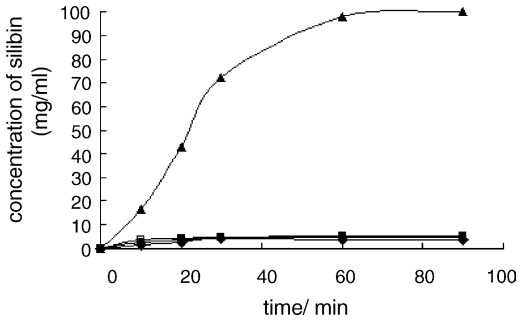

Fig. 4. Dissolution behaviors of phospholipids complex and silybin material in HCl (pH 1.2) and phosphate buffer saline (pH 6.8), respectively. (–▲–) Phospholipids complex in phosphate buffer saline (pH 6.8); (–■–) phospholipids complex in HCl (pH 1.2); (–♦–) silybin material in phosphate buffer saline (pH 6.8); (–□–) silybin material in HCl (pH 1.2).

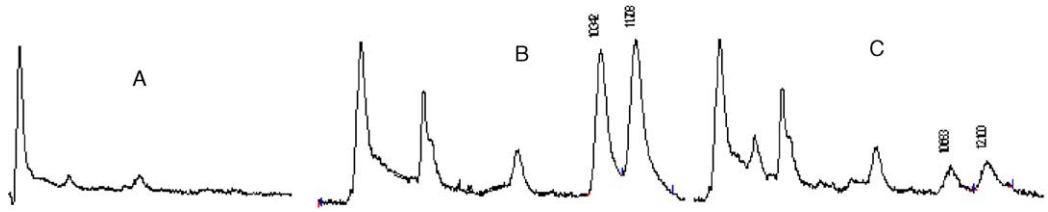

Fig. 5. Typical chromatograms of silybin. (A) Blank rat plasma; (B) blank rat plasma spiked with silybin; (C) a sample after oral administration of silybin–phospholipid complex.

3.7. Rats bioavailability experiments

Silybin in plasma was completely separated under analytical conditions, and standard curves ranging from 0.05 to 2.6 μg ml^{-1} were linear ($r = 0.9932$) (Fig. 5). The results attained from the method recoveries of high, middle and low concentrations were 81.24, 83.49 and 82.40%, respectively. The R.S.D. in days were 3.12, 2.56 and 3.74%, respectively, the R.S.D. intra-days were 4.83, 4.07 and 5.27%, respectively, which showed recoveries and R.S.D. in days or intra-days were satisfying, and the lowest detection limit was 25 ng ml^{-1}.

Fig. 6 shows the sample equivalent to 9.1 mg/kg of silybin of phospholipids complex and silybin-*N*-methylglucamine was respectively orally administered to rats ($n = 6$). From the above profile, we could know the average values of C_{max} is 126.72 ng ml^{-1} after oral administration of phospholipids complex with a T_{max} of about 10 min. However, the average value of C_{max} was 104.29 ng ml^{-1} after oral administration of silybin-*N*-methylglucamine solution with a T_{max} of about 5 min. The other parameters were gotten by 3p97 procedure (Tables 3 and 4). The parameters shows that silybin-*N*-methylglucamine is metabolized more rapidly than phospholipids complex with the approximate same C_{max}. The complex stayed longer than silybin-*N*-methylglucamine, which make the complex achieved higher bioavailability of silybin.

The pharmacokinetical data were simulated by non-linear least squares. The results showed that open single-compartment model and one-order absorption were fitted to both phospholipids complex and silybin-*N*-methylglucamine plasma concentration–time course in vivo of rats.

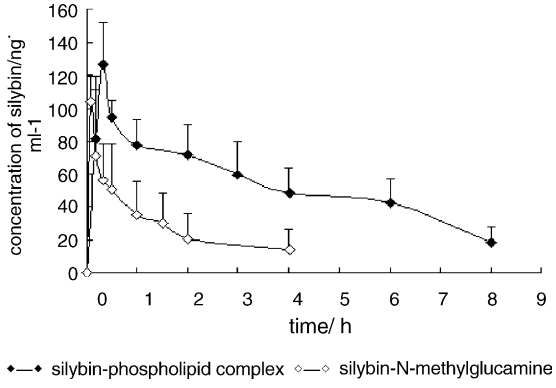

Fig. 6. Mean plasma concentration–time curve of silybin in rats after oral administration of silybin–phospholipid complex and silybin-*N*-methylglucamine equivalent to 9.1 mg/kg of silybin ($n = 6$), respectively. (–♦–) Silybin–phospholipid complex; (–◇–) silybin-*N*-methylglucamine.

Table 3
The main pharmacokinetic parameters of phospholipids complex and silybin-*N*-methylglucamine in rats ($n = 6$)

Parameters	Phospholipids complex	Silybin-*N*-methylglucamine
A (ng ml^{-1})	89.97	65.65
K_a (h^{-1})	39.38	61.05
K_e (h^{-1})	0.21	0.48
T_{max} (h)	0.17	0.08
C_{max} (ng ml^{-1})	126.72	104.29
AUC (ng ml^{-1} h)	1220.33	235.81
CL(s) (mg h^{-1} ng^{-1} ml)	0.006	0.02
V/F(c) (mg h^{-1} ng^{-1} ml)	0.03	0.05

Table 4
The main pharmacokinetical parameters of phospholipids complex and silybin-*N*-methylglucamine with non-model in rats ($n = 6$)

Parameters	Phospholipids complex		Silybin-*N*-methylglucamine	
	T (0–8 h)	T (0–∞)	T (0–8 h)	T (0–∞)
AUC(s0) (ng ml^{-1} h)	1059.02	1429.56	233.19	363.24
AUMC(s1)	3304.14	5375.15	293.82	759.17
MRT (h)	3.12	3.76	1.26	2.09

4. Conclusion

In this protocol, we successfully prepared silybin–phospholipid complex by a simple, novel method. DSC curves of phospholipids complex showed that drugs and phospholipids combined and formed some kind bond, such as hydrogen bonds or van der Waals force. Solubility studies showed there was a higher solubility in water or *n*-octanol for phospholipids complex than that of physical mixture. Drugs and phospholipids of phospholipids complex in vitro had not changed characteristics of themselves, but the combination changed greatly their characteristics in vivo, such as a remarkable enhancement of GI tract absorption. It would be further studied about the absorbed mechanism of silybin through small intestine.

References

Barzaghi, N., Crema, F., Gatti, G., Pifferi, G., Perucca, E., 1990. Pharmacokinetic studies on IdB 1016, a silybin–phosphatidylcholine complex, in healthy human subjects. Euro. J. Drug. Metab. Pharmacokinet. 15, 333–338.

Hwang, S.B., Shen, T.Y., 1981. Membrane effects of anti-inflammatory agents. 2. Interaction of nonsteroidal anti-inflammatory drugs with liposome and purple membranes. J. Med. Chem. 24, 1202–1212.

Kvasnicka, F., Biba, B., Sevcik, R., Voldrich, M., Kratka, J., 2003. Analysis of the active components of silymarin. J. Chromatogr. A 990, 239–245.

Lasonder, E., Weringa, W.D., 1990. An NMR and DSC study of the interaction of phospholipids vesicles with some anti-inflammatory agents. J. Colloid Interface Sci. 139, 469–478.

Morazzoni, P., Magistretti, M.J., Giachetti, C., Zanolo, G., 1992. Comparative bioavailability of silipide, a new flavanolignan complex, in rats. Euro. J. Drug. Metab. Pharmacokinet. 17, 39–44.

Tedesco, D., Tava, A., Galletti, S., Tameni, M., Varisco, G., Costa, A., Steidler, S., 2004. Effects of silymarin, a natural hepatoprotector, in periparturient dairy cows. J. Dairy. Sci. 87, 2239–2247.

Venema, F.R., Weringa, W.D., 1988. The interactions of phospholipid vesicles with some anti-inflammatory agents. J. Colloid Interface Sci. 125, 484–500.