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Preparative chromatographic purification of diastereomers of silybin and their quantification in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

A novel preparative HPLC method separating silybin has been developed to meet the need for both silybin A and silybin B standard. After the preparation of silybin A and silybin B standard, a simple, sensitive, selective and reproducible liquid chromatography–tandem mass spectrometry (LC–MS–MS) method with negative electrospray ionization (ESI) was developed for the quantification of silybin A and silybin B in human plasma. Following rapid sample preparation, silybin A, silybin B and naringin (internal standard, ISTD) were separated on a Zorbax Eclipse XDB-C_{18} column, using methanol–water containing 0.1% formic acid (48:52, v/v) as the mobile phase. The mass spectrometer was operated in selected reaction monitoring (SRM) mode using the transition *m/z* 481.1 → 300.9 for both silybin A and silybin B and *m/z* 579.2 → 271.1 for naringin, respectively. Linear calibration curves were obtained in the concentration range of 2–5000 ng/ml with a lower limit of quantitation (LLOQ) of 2 ng/ml for both silybin A and silybin B, respectively. The intra- and inter-day precision values were below 7.5% and accuracy was within ±4.9% at all three quality control (QC) levels, for both silybin A and silybin B, respectively. This method was successfully applied to the stereospecific analysis of silybin in plasma samples from a pharmacokinetic study of silybin A and silybin B in 22 healthy male Chinese volunteers after a single oral dose of silybin–phosphatidylcholine complex (equivalent to 280 mg silybin, including 133 mg silybin A and 147 mg silybin B).

Keywords: Pre-HPLC; LC–MS–MS; Silybin; Pharmacokinetic; Silybin A; Silybin B; Plasma

1. Introduction

Silybin is the principal component and main active substance of silymarin, a standardized extract of flavanolignans from the fruit of *Silybum marianum*, which has been used for centuries as a natural remedy in the treatment of hepatitis and cirrhosis, as well as in the protection of the liver from toxic substances [1–3]. Recent reports have demonstrated that silybin also has exceptionally high anti-tumor activity and many other pharmacological activities such as anti-inflammatory and anti-fibrotic effects [3–5].

Silybin is slightly soluble in water and in oil, the poor permeation across the intestinal epithelial cells and the minor gastrointestinal tract absorption in rats has been reported [6,7]. Phospholipid is an important component of cell membrane, having the actions of keeping cell membrane fluidity and treating hepatic disorder, the structure and biochemical characters of phospholipid are similar to phosphatidylcholine. It is reported that silybin combined with phosphatidylcholine result in great increase of oral bioavailability [8–10] and improvement of the biological effect of silybin [1,11].

Silybin is mixture of diastereoisomers, and is consisted of silybin A and silybin B. It is difficult to separate and purify sily-

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bin A and silybin B as standards for their respective quantitative analysis and up to now the preparation and purification of silybin A and silybin B standard has not been reported [3,5,12,13], so pharmacokinetics of silybin usually were researched by taking silybin as a single entity [6–10,14–16]. But the pharmacokinetics of diastereomers could be different in view of the difference of stereoselectivity of silybin A and silybin B, then it is essential and important to research the pharmacokinetics of silybin A and silybin B, respectively. A method using high-performance liquid chromatography (HPLC) equipped with electrochemical detector have been developed to determine silybin A and silybin B in human plasma, but the accuracy of the method had suffered from the absence of standards of silybin A and silybin B [17].

Previous investigations have shown that silybin in biological fluids can be detected using radiolabel method [14] and high-performance liquid chromatography (HPLC) equipped with ultraviolet (UV) [6–10,15–17] or electrochemical (EC) detector [17]. The radiolabel method was applied to a pharmacokinetic study in rat using ^{125}iodine-labeled silybin, but the method could not be used in human and it was unknown if the radiolabel of silybin would change the pharmacokinetics of silybin. And these HPLC methods usually suffered from bad sensitivity or complicated sample preparation.

The aim of this research was to separate and purify silybin A and silybin B for their respective quantitative analysis using preparative high-performance liquid chromatography (pre-HPLC) and to develop a fast and sensitive LC–MS–MS method for the determination of both silybin A and silybin B separately in human plasma with negative electrospray ionization (ESI) in selective reaction monitoring (SRM) mode. Following validation, this method was successfully applied to pharmacokinetic studies of silybin A and silybin B performed in 22 healthy male Chinese volunteers after a single oral dose of silybin–phosphatidylcholine complex (equivalent to 280 mg silybin, including 133 mg silybin A and 147 mg silybin B).

2. Experimental

2.1. Materials and reagents

Silybin A and Silybin B (Fig. 1) were separated and purified by Tasly R&D institute (Tianjin, China). Naringin (Fig. 1) was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Silybin and Silybin–phosphatidylcholine complex were obtained from Tianjin Tasly Pharmaceutical Co., Ltd. (Tianjin, China). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were of analytical grade and used without further purification.

2.2. Instrumentation and conditions

2.2.1. Preparative HPLC

The preparative HPLC was performed with a Waters 600E HPLC system equipped with a Waters HPLC autosampler and a Waters 440 variable wavelength UV detector, using a Agilent Zorbax Eclipse XDB-C_{18} preparative column (5 μm particle size, 250 mm × 21.2 mm i.d.). The mobile phase of methanol–water containing 0.1% formic acid (45:55, v/v) was delivered isocratically at 18 ml/min. The chromatograms were monitored at 288 nm. The HPLC system was controlled by Waters Empower software (Waters, Milford, MA, USA).

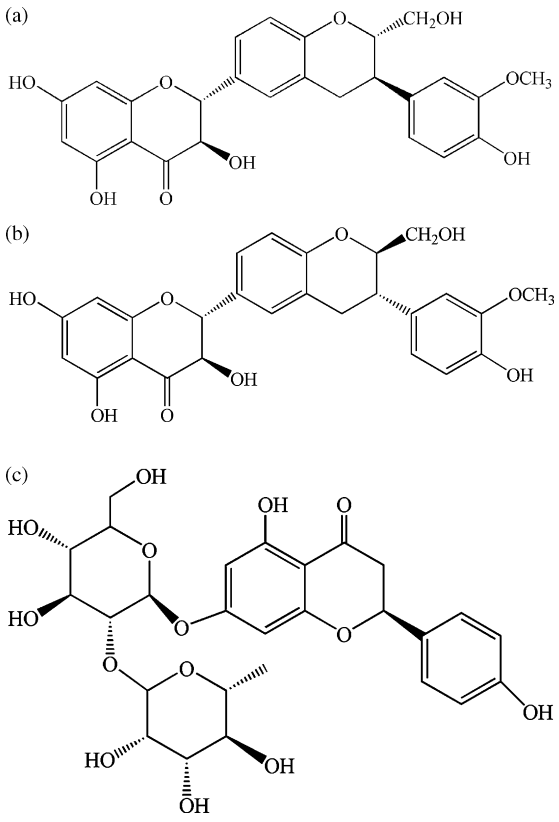

Fig. 1. Chemical structures of silybin A (a), silybin B (b) and Naringin (ISTD) (c).

Silybin solution was prepared by dissolving the accurately weighed compound in methanol to give final concentration of 1.5 mg/ml. A 400 μl volume of silybin solution was applied to the chromatographic system. The silybin A and silybin B peaks were collected manually, respectively.

2.2.2. Analytical HPLC

The analysis was performed with an Agilent series 1100 HPLC equipped with a quaternary gradient pump, autosampler and diode array detector using a Agilent Zorbax Eclipse XDB-C_{18} analytical column (5 μm particle size, 150 mm × 4.6 mm i.d.). The mobile phase of methanol–water containing 0.1% formic acid (50:50, v/v) was delivered isocratically at 1.0 ml/min. The chromatograms were monitored at 288 nm.

Standard solutions of silybin A and silybin B were prepared by dissolving the accurately weighed standard compounds in mobile phase to give final concentration of 0.2 mg/ml, respectively. A 10 μl volume of silybin A solution and a 10 μl volume of silybin B solution were applied to the chromatographic system for the determination of purity, respectively.

2.2.3. LC–MS–MS

The LC–MS–MS system consisted of a Surveyor MS pump, a Surveyor autosampler (ThermoFinnigan, USA) and a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. Data acquisition was performed with *X*calibur 1.3 software (ThermoFinnigan, USA). Chromatographic analysis was performed on a Agilent Zorbax Eclipse XDB-C_{18} analytical column (5 μm particle size, 150 mm × 2.1 mm i.d.). The mobile phase of methanol–water containing 0.1% formic acid (48:52, v/v) was delivered isocratically at 0.25 ml/min. Mass spectrometer with the ESI source was performed in negative ion mode, using selected reaction monitoring (SRM). Quantitation was performed using the transition *m/z* 481.1 → 300.9 for silybin A and silybin B and *m/z* 579.2 → 271.1 for ISTD, respectively. For analytes and ISTD, the following optimized parameters were obtained: capillary temperature of 390 °C, electrospay voltage of 3.8 kV, nitrogen was used as sheath gas and auxiliary gas at the pressures of 25 psi and 3 psi, respectively. At the same time, collision-induced dissociation (CID) was performed using argon at a collision gas pressure of 1.4 mTorr and the collision energy of 20 ev was used for the analytes and 34 ev for ISTD. The peak width settings for both Q_1 and Q_3 were 0.7 u.

2.3. Preparation of standard and quality control solutions

Stock solutions of silybin A, silybin B and ISTD were prepared by dissolving the accurately weighed standard compounds in acetonitrile to give final concentrations of 1 mg/ml. Successive dilutions from stock solutions of silybin A and silybin B with purified water gave working standard solutions at concentrations of 2 ng, 5 ng, 20 ng, 100 ng, 500 ng, 1000 ng, and 5000 ng, respectively. The quality control working solutions were prepared at three different concentration levels, low level (5 ng/ml), middle level (100 ng/ml), and high level (1 μg/ml). The ISTD working solution (100 ng/ml) was prepared by diluting stock solution of internal standard with acetonitrile.

The analyte working solutions (100 μl) were used to spike blank plasma (100 μl) either for calibration curves or for QC samples in method validation and during the pharmacokinetic study.

All the solutions were stored at 4 °C and were brought to room temperature prior to use.

2.4. Sample preparation

A 100 μl aliquot of plasma was mixed with 200 μl of the ISTD solution. After vortex mixing for 20 s and centrifugation at 15,000 × *g* for 3 min, 10 μl of the clear supernatant was directly injected onto the LC–MS–MS system for analysis.

2.5. Method validation

The method was validated for selectivity, linearity, precision, accuracy, recovery and stability. The selectivity of the method was measured by analysis of six blank plasma samples of different origin for interference at the retention times of the analytes and ISTD. The selective determination of silybin A and silybin B were illustrated by analysis of SRM transitions characteristic of the analytes and ISTD. Linearity was assessed by preparing and analyzing silybin A and silybin B samples over 2–5000 ng/ml in human plasma, respectively. Calibration curves were analyzed by weighted linear regression ($1/x^2$) of the peak area of analytes over that of ISTD.

In order to assess the intra- and inter-day precision and accuracy, complete analytical runs were performed on the same day and on four consecutive days. Each analytical run consisted of a matrix blank, a set of calibration standards, six replicate LLOQ samples, and a set of low, medium and high concentration QC samples. Concentrations for the QC samples were calculated by reference to the calibration curve generated from the working standards. The LLOQ was defined as the concentration of the lowest concentration standard in the calibration curve that was analyzed with both accuracy and precision ≤15%. During routine analysis each analytical run included a matrix blank, a set of calibration samples, a set of QC samples in duplicate and unknowns.

The recoveries of silybin A and silybin B were evaluated by comparing the peak areas of five extracted low, medium and high quality control samples to mean peak areas of five neat reference solutions (unprocessed). Recovery of IS was evaluated by comparing the peak areas of five extracted quality control samples to mean peak areas of five neat reference solutions (unprocessed) of the same concentration.

Stability tests were performed for analyte-spiked plasma samples under various conditions (four freeze–thaw cycles; storage at room temperature for 24 h) by analyzing six replicates at low, medium and high QC concentrations.

2.6. Application to clinical study

Twenty-two healthy male Chinese volunteers were enrolled in this study. Their ages, weights and heights were 20–32 years (25.4 ± 5.2), 53–78 kg (69 ± 7.2) and 165–184 cm (172.8 ± 7.7), respectively. All were in good health on the basis of their medical histories, physical examinations, blood chemistries and urinalyses. Subjects, after an overnight fast, received silybin–phosphatidylcholine complex (equivalent to 280 mg silybin, including 133 mg silybin A and 147 mg silybin B). All subjects remained fasting for 4 h after dosing. Plasma samples were collected at times 0 h (before dosing), 0.33 h, 0.67 h, 1.0 h, 1.5 h, 2.0 h, 2.5 h, 3.5 h, 4.5 h, 6.5 h, and 11.0 h after administration and stored at −20 °C until assay.

The elimination rate constant (λ_z) was obtained as the slope of the linear regression of the log-transformed concentration values versus time date in the terminal phase. The elimination half-life ($t_{1/2\beta}$) was calculated as $0.693/\lambda_z$. The peak plasma concentration (C_{max}) and the corresponding time (T_{max}) were directly obtained from the raw data. The area under the curve to the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The area under the curve to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-\infty} = AUC_{0-t} + C_t/\lambda_z$, where C_t is the last measurable concentration.

3. Results and discussion

3.1. Preparative and analytical HPLC

Different type of HPLC columns and various HPLC conditions were evaluated in this study and the best chromatographic conditions for the separation of silybin A and silybin B with shortest run time was a Agilent Zorbax Eclipse XDB-C_{18} column with mobile phase consisting of methanol/water/formic acid.

The solubility of silybin in water and methanol at 25 °C was evaluated. The values in water was just 0.05 mg/ml when the values in methanol was 1.5 mg/ml, so methanol was selected as the solvent of silybin. For attaining most quantity of pure silybin A and silybin B and avoiding the expansion of their chromatographic peaks that would cause bad resolution, 400 μl was used as the volume of injections.

The application of a larger column (21.2 mm i.d.) allowed the separation and purification of silybin A and silybin B on a larger scale. As shown in Fig. 2, a 400 μl aliquot of 1.5 mg/ml silybin solution equivalent to 0.6 mg silybin was injected into the pre-HPLC system, the resolution of silybin A and silybin B on the preparative column was high enough to obtain a pool of fractions containing practically pure single isomers.

When fractions are collected from the preparative HPLC, it is then necessary to remove the mobile phase in order to purify the silybin A and silybin B. With a view to the comparative difficulty of removing the mobile phase salt, a mobile phase without salts was used. Formic acid was chosen as the pH modifier as it is reasonably volatile and could be readily removed with the other components of the mobile phase.

Fractions of silybin A and silybin B from 80 times injections are collected, respectively. And then amorphous colourless powder of silybin A (17.2 mg) and silybin B (20.7 mg) were attained by drying under vacuum after removal of the mobile phase under reduced pressure at 60 °C.

Fig. 3 shows the purities of prepared silybin A and silybin B standard by the use of analytical HPLC method. It was proved that purities of silybin A and silybin B were more than 98% and 97%, respectively.

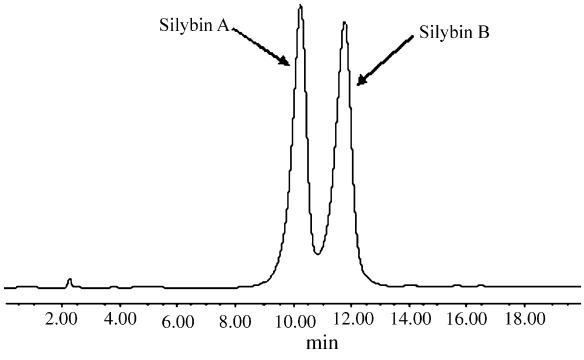

Fig. 2. Preparative chromatographic separation of silybin, Agilent Zorbax Eclipse XDB-C_{18} preparative column (5 μm particle size, 250 mm × 21.2 mm i.d.), methanol–water containing 0.1% formic acid (45:55, v/v), 288 nm, 18 ml/min.

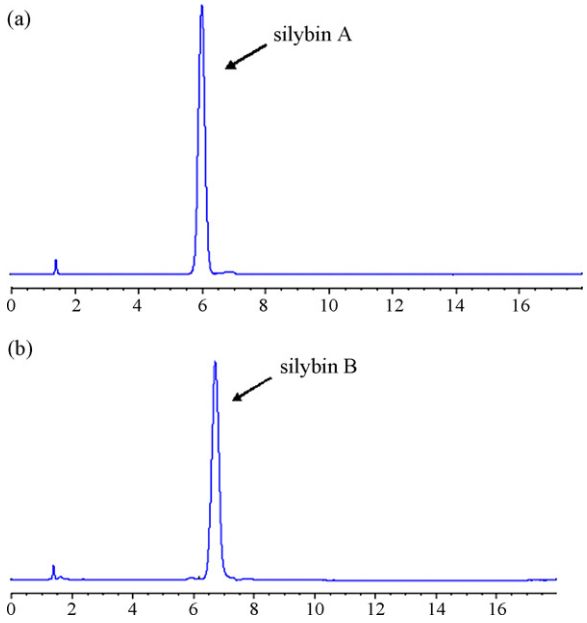

Fig. 3. Analysis of silybin A (a) and silybin B (b) prepared using pre-HPLC, Agilent Zorbax Eclipse XDB-C_{18} analytical column (5 μm particle size, 150 mm × 4.6 mm i.d.), methanol–water containing 0.1% formic acid (50:50, v/v), 288 nm, 1.0 ml/min.

3.2. LC–MS–MS

A better resolution for silybin A and silybin B and a shorter run time were achieved by the combination of Agilent Zorbax Eclipse XDB-C_{18} analytical column (5 μm particle size, 150 mm × 2.1 mm i.d.) and the mobile phase of methanol–water containing 0.1% formic acid (48:52, v/v).

Atmosphere pressure chemical ionization (APCI) was also investigated in the analysis but provided no sensitivity advantages over electrospray. The negative ion mode was selected because it was far more sensitive than the positive ion mode for silybin and ISTD. Following the selection of the negative ESI mode, silybin and ISTD, respectively, were found to form deprotonated ion $[M-H]^-$ at m/z 481.1 and 579.2 that could be used as precursor ions for the analysis. Fig. 4 shows the production ion mass spectra of the $[M-H]^-$ ion of silybin A, silybin B and ISTD, fragment ions at m/z 300.9 and 271.1 were observed to be the stronger for silybin and ISTD, respectively. Based on the results of these studies, the transitions of m/z 481.1 → 300.9 for silybin and m/z 579.2 → 271.1 for ISTD were used. The collision energy was set at 20 eV for silybin and 34 eV for ISTD because the dissociation of $[M-H]^-$ ions of silybin and ISTD was found to depend strongly on this parameter. Other LC–MS–MS parameters were optimized to obtain the maximum sensitivity for the m/z 481.1 → 300.9 of silybin.

3.3. Sample preparation

HPLC–UV methods [6–10,15–17] for the determination of silybin usually suffered from bad sensitivity, so laborious solid phase or liquid–liquid extraction methods were used because the

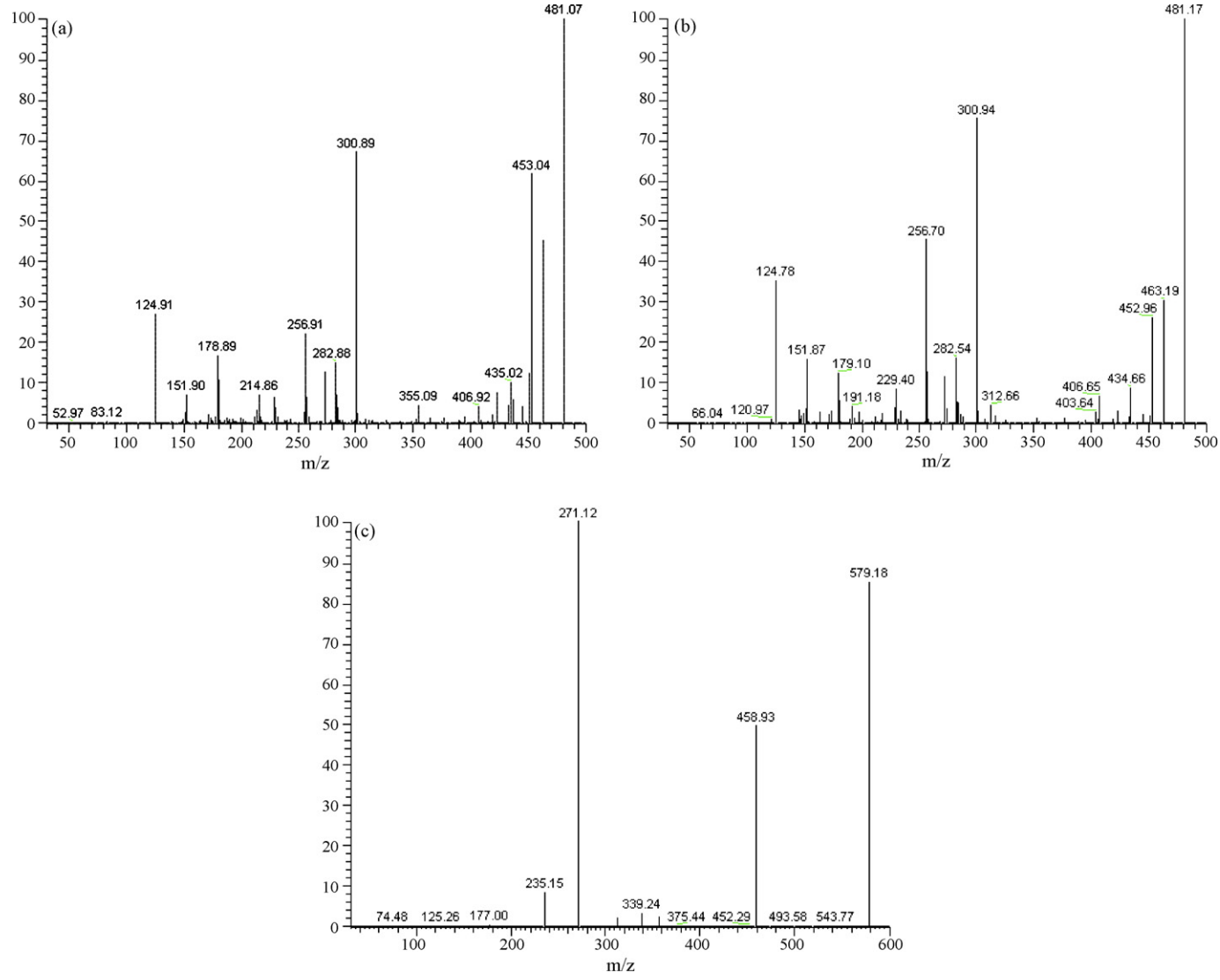

Fig. 4. Product ion mass spectra of $[M-H]^-$ ions of silybin A (a), silybin B (b) and Naringin (c).

steps of evaporation and reconstitution could condense samples, and protein precipitation extraction could not be used because it causes worse sensitivity by diluting samples.

In the present study, simpler sample preparation is better since HPLC/MS/MS method has better sensitivity and LLOQ is not the first consideration. And then the optimized protein precipitation extraction procedure have been developed through the use of acetonitrile as the solvent of ISTD, the proportion of acetonitrile (acetonitrile/plasma = 2:1) is enough to precipitate more than 98% protein. The optimized protein precipitation extraction is very simple and time-saving.

3.4. Method validation

3.4.1. Selectivity and matrix effects

Interference from endogenous substances was investigated by measurement of six blank plasma of different origin, and assay selectivity was confirmed by the absence of interfering peaks at the retention times of silybin and ISTD (Fig. 5).

The matrix effects were revealed by the recoveries of silybin and ISTD, since the method of protein precipitation was used during sample preparation. No significant matrix effects were observed for silybin and ISTD.

3.4.2. Linearity of calibration curve and lower limit of quantitation

The squared correlation coefficient (r^2) for the daily calibration curves were all $\geq$0.996 ($n = 5$) over the concentration range of 2–5000 ng/ml for both silybin A and silybin B. For each point on the calibration curves for the two analytes, the concentrations back-calculated from the equation of the regression analysis were within acceptable limits for accuracy and precision of $\pm$ 10%. The current assay had a lower limit of quantitation of 2 ng/ml ($n = 6$) with signal-to-noise ratio above 50.

3.4.3. Precision, accuracy and recovery

Data for intra- and inter-day precision and accuracy of the assay are summarized in Tables 1 and 2, respectively. The intra-

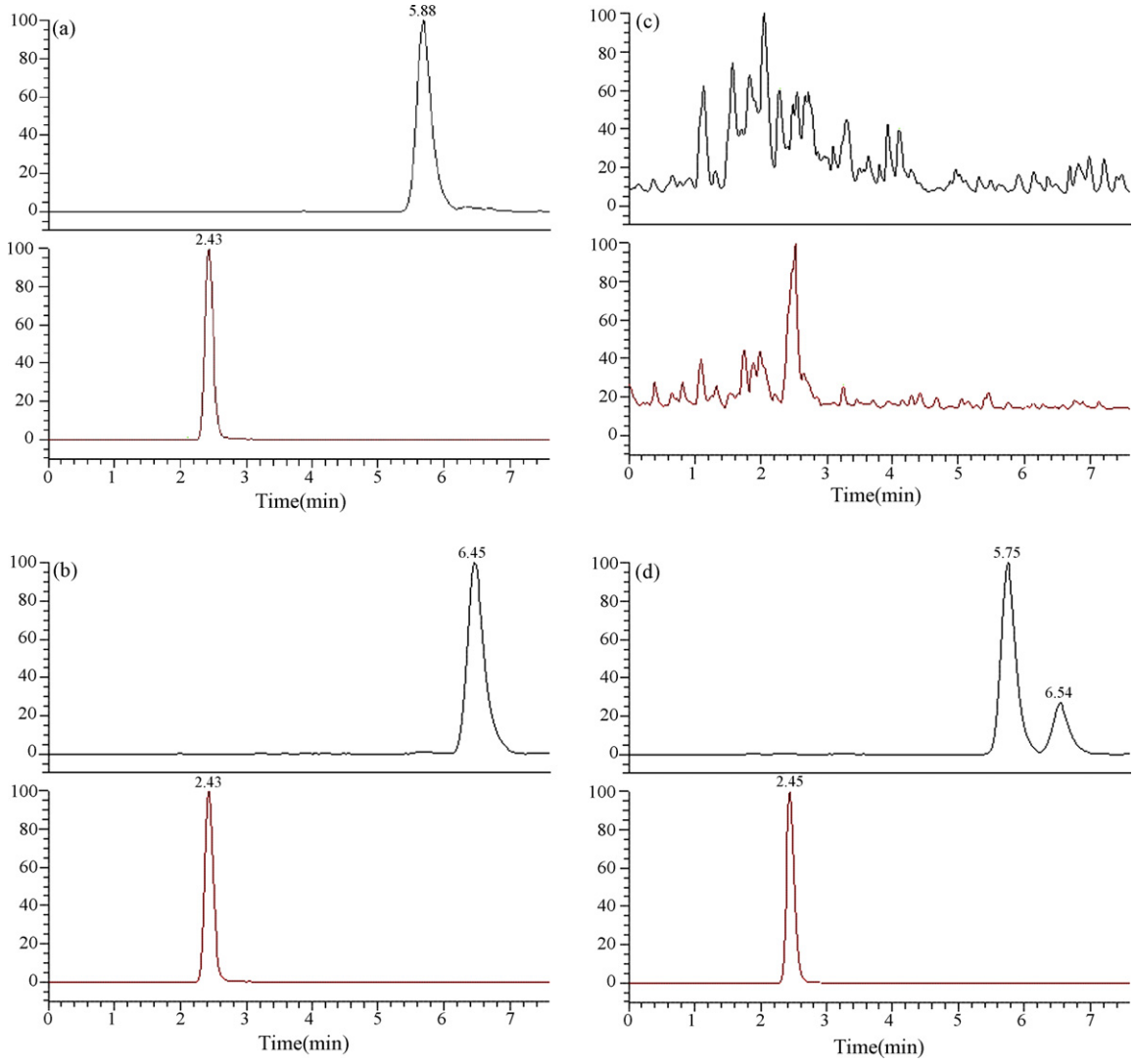

Fig. 5. SRM chromatograms for silybin A (5.7 min), silybin B (6.5 min) and ISTD (2.4 min) in human plasma: (a) blank plasma spiked with silybin A (100 ng/ml) and ISTD (100 ng/ml); (b) blank plasma spiked with silybin B (100 ng/ml) and ISTD (100 ng/ml); (c) blank plasma; (d) a plasma sample 1 h after a single oral dose of silybin–phosphatidylcholine complex (equivalent to 280 mg silybin, including 133 mg silybin A and 147 mg silybin B).

Table 1
Intra-day precision, accuracy and LLOQ results for silybin isomers (six replicates)

Silybin A (ng/ml)	Intra-day R.S.D. (%)	Relative error (%)	Silybin B (ng/ml)	Intra-day R.S.D. (%)	Relative error (%)
5.0	6.8	−3.3	5.0	5.9	1.9
100.0	2.5	2.9	100.0	1.5	3.0
1000.0	1.5	1.2	1000.0	0.9	1.1
2.0	7.2	5.3	2.0	6.9	−3.7

Table 2
Inter-day precision, accuracy and LLOQ results for silybin isomers ($n = 5$ day, six replicates per day)

Silybin A (ng/ml)	Inter-day R.S.D. (%)	Relative error (%)	Silybin B (ng/ml)	Inter-day R.S.D. (%)	Relative error (%)
5.0	7.5	−3.6	5.0	6.1	3.4
100.0	2.8	2.7	100.0	3.0	4.9
1000.0	1.1	3.1	1000.0	1.2	1.8
2.0	7.8	5.5	2.0	8.1	−5.5

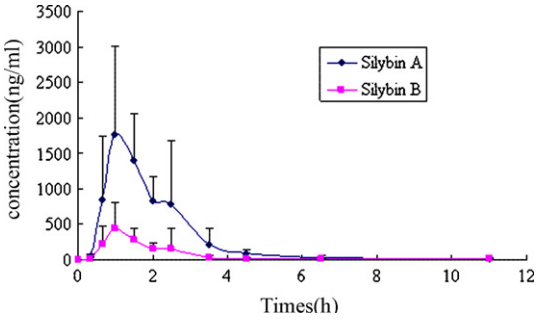

Fig. 6. Mean plasma concentration–time profiles of silybin A and silybin B in 22 subjects after a single oral dose of silybin–phosphatidylcholine complex (equivalent to 280 mg silybin, including 133 mg silybin A and 147 mg silybin B).

and inter-day precision were below 7.5% and accuracy was within ± 4.9% at all three quality control (QC) levels, for both silybin A and silybin B, respectively.

The mean recoveries ($n = 5$) of silybin A were 104.3 ± 4.2%, 98.6 ± 2.8%, and 94.6 ± 2.9% and that of silybin B were 102.3 ± 4.8%, 98.1 ± 3.2%, and 97.9 ± 2.1% at concentrations of 5 ng/ml, 100 ng/ml, and 1000 ng/ml, respectively. The mean recovery ($n = 5$) of ISTD was 95.2 ± 3.4%.

3.4.4. Stability

Analyte-spiked plasma samples stored at room temperature for 24 h showed no sign of degradation when compared with freshly prepared samples. The analytes were also shown to be stable after four freeze–thaw cycles. The REs of the three QC levels for silybin A and silybin B were ranged from −2.2% to 3.5% and from −3.2% to 2.9%, respectively.

3.5. Application to clinical study

The mean plasma concentration versus time profiles of silybin A and silybin B in 22 subjects after a single oral dose of silybin–phosphatidylcholine complex are presented in Fig. 6. Pharmacokinetic parameters of silybin A and silybin B in human after single oral administration of silybin–phosphatidylcholine complex are summarized in Table 3.

In literature there were no reports on the pharmacokinetic parameters of silybin A and silybin B in human plasma except the AUC_{0-t} of silybin A and silybin B [17]. In the reported literature, the AUC_{0-t} of both silybin A and silybin B was much smaller compared to the AUC_{0-t} of both silybin A and silybin B reported by the present research. The main reasons for the difference of AUC_{0-t} may be that subjects were administered a higher dose in the present research and compared to silybin as a part of silymarin, the better bioavailability of silybin A and silybin B was achieved by the complex of silybin and phosphatidylcholine used in the present research [1,6–10].

From these data of Table 3, it is found that the C_{max} and AUC of silybin A are about four times higher than those of silybin B; elimination half-life ($t_{1/2\beta}$) was about three times shorter for silybin A than for silybin B; and T_{max} is similar between silybin A and silybin B. So it is concluded that silybin A has better absorption and slower elimination than silybin B, and the velocity of absorption is similar between the two isomers. The pharmacokinetic difference between silybin A and silybin B may be explained by stereoselective absorption, distribution and metabolism.

Table 3
Pharmacokinetic parameters of silybin A and silybin B in 22 subjects after a single oral dose of silybin–phosphatidylcholine complex (equivalent to 280 mg silybin isomers, including 133 mg silybin A and 147 mg silybin B)

Parameter	Silybin A	Silybin B
C_{max} (ng/ml)	2333.7 ± 1037.1	582.4 ± 350.3
T_{max} (h)	1.46 ± 0.60	1.36 ± 0.58
$T_{1/2\beta}$ (h)	2.73 ± 0.63	7.74 ± 5.75
AUC_{0-t} (ng/ml h)	3495.7 ± 1007.5	780.4 ± 301.0
$AUC_{0-\infty}$ (ng/ml h)	3580.8 ± 1007.2	886.3 ± 288.7

4. Conclusions

Pure silybin A (17.2 mg) and silybin B (20.7 mg) were obtained from commercial silybin using preparative HPLC. The purities of silybin A and silybin B were more than 98% and 97%, respectively which had been assessed by analytical HPLC. The described preparative HPLC method is effective to attain the standards of silybin A and silybin B for the study of quantitative analysis though it is laborious. This research also outlines a simple, sensitive, selective and reproducible LC–MS–MS method that has been validated for the determination of both silybin A and silybin B in human plasma with a lower limit of quantitation of 2 ng/ml. The LC–MS–MS method described has been successfully applied to pharmacokinetic studies in healthy subjects.

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