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A sensitive LC–MS/MS assay for the simultaneous analysis of the major active components of silymarin in human plasma

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ABSTRACT

Silymarin, an extract of crushed achenes of the milk thistle plant *Silybum marianum* is a multi-constituent mixture, 70–80% of which consists of a complex assortment containing the flavonolignans silybin A and B, isosilybin A and B, silydianin, and silychristin, and the flavonoid taxifolin. To date, numerous pharmacological actions of the silymarin extract have been documented in the biomedical literature, including hepatoprotective, anti-inflammatory, anti-tumor, and anti-fibrotic activities. The present study describes a novel liquid chromatographic–tandem mass spectrometric method for simultaneous analysis of silychristin, silydianin, silybin A and silybin B, isosilybin A and isosilybin B, and taxifolin in human plasma employing liquid–liquid extraction. This assay provides excellent resolution of the individual silymarin constituents via utilization of a 100 A 250 mm × 2 mm, 5 μm C_{18} column with the mobile phase consisting of 51% methanol, 0.1% formic acid, and 10 mM ammonium acetate. The lower limit of quantification was 2 ng/ml for each constituent. Calibration curves were linear over the range from 2 ng/ml to 100 ng/ml for all analytes ($r^2 > 0.99$). The intra- and inter-day accuracies were 91–106.5% and 95.1–111.9%, respectively. The intra- and inter-day precision was within 10.5%. Additionally, recovery, stability, and matrix effects were fully validated as well. This method was successfully applied to human plasma samples from subjects treated with the milk thistle extract Legalon®.

1. Introduction

Silymarin is a complex mixture of at least 7 flavonolignans and 1 flavonoid that account for 65–80% of milk thistle extract. Obtained from the crushed seeds of the plant *Silybum marianum*, it has been used for centuries as adjunct treatment for a variety of liver ailments including cirrhosis, alcoholic liver disease, and hepatitis [1–4]. Silymarin extract has also been has also been employed for the treatment of mushroom poisonings secondary to ingestion of *Amanita phalloides* [2]. Additionally, there is ongoing interest in potential anti-tumor, anti-inflammatory, and anti-fibrotic actions of silymarin extracts that have been documented in the biomedical literature [3]. Silymarin is comprised of a mixture of flavonolignans including diastereomers silybin A and B, isosilybin A and B, silychristin A and B, and silydianin, all of which are thought to confer purported pharmacological activity to milk thistle extracts [5,6]. Taxifolin, a flavanolol that is also present in silymarin extract, is thought to play a role in the anti-tumor activity of this mixture and has been reported to inhibit ovarian cancer cell growth in a dose-dependent fashion and as a preventative measure against skin carcinogenesis [7–9]. The remainder of the silymarin extract has yet to be fully characterized but is believed to be comprised of polymeric and oxidized polyphenols [10]. Significant variability of the concentrations of the major active constituents among the many different commercially available milk thistle products has been observed, meaning that a given dose of one manufacturers supplement may achieve varying concentrations in the blood and tissue and thus very different pharmacological effects when compared to the supplement of another manufacturer [11]. Therefore, an assay that can accurately quantify these bioactive silymarin components in both commercially available supplements and human plasma is necessary for proper pharmacokinetic analysis.

To date, several analytical methods have been developed with the goal of quantifying the bioactive components of silymarin extracts (excluding taxifolin) including high-performance liquid chromatography (HPLC) assay coupled with various forms of detection including ultraviolet (UV), mass spectrometry (MS) or tandem mass spectrometry (MS/MS) [12–18]. In a previous assay published by Lee and coworkers in 2007 and in a separate assay published

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by Wen and associates in 2007, two analytical methods based on LC–MS/MS and LS-MS, respectively, were developed and applied to human plasma samples but neither assay was fully validated [19,20]. Additionally, the aforementioned assays were not able to individually quantify the discrete diastereomers within silymarin extracts (i.e. silybin A and B and isosilybin A and B), but rather the mixture diastereomers that was reported as a single value. This means that absolute quantification of each constituent, specifically the diestereomers silybin A and silybin B, and isosilybin A and isosilybin B, is improbable. Another shortcoming of the previously published assays is that none of them have the ability to quantitatively analyze taxifolin. Taxifolin is a vital bioactive component of the silymarin mixture and should be included in an analytical method that is created for the purpose of quantifying all known pharmacologically active constituents of silymarin extract.

Consequently, an assay combining HPLC-electrospray ionization (ESI)-MS/MS was developed to simultaneously detect and quantify silymarin components believed to be significant, including silybin A and B, isosilybin A and B, silychristin, silydianin, and taxifolin in human plasma. This method was then applied to samples collected during a pharmacokinetic study conducted in healthy human volunteers receiving escalating doses of milk thistle extract (administered as Legalon®) in order to assess the bioavailability of each silymarin constituent under different dosing regimens.

2. Materials and methods

2.1. Chemicals and reagents

Milk thistle capsules (175 mg dried extract equivalent to 140 mg silymarin: Legalon®) were generously donated by MADAUS GmbH (Cologne, Germany). Each Legalon® capsule contains 52.1 mg silybin A and B, 17.9 mg isosilybin A and B, and 40.4 mg silydianin and silychristin based on the certificate of analysis provided by MADAUS GmbH. Authentic analytical reference standards of taxifolin, silychristin, and silydianin, were obtained from ChromaDex™ (Santa Ana, CA), and silybin A, silybin B, isosilybin A, and isosilybin B were sourced from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany) (Fig. 1). The internal standard (IS) naringenin was purchased from SAFC Supply Solutions (St. Louis, MO). LC–MS grade methanol, ammonium acetate, formic acid, and ethyl acetate were all purchased from Sigma–Aldrich (St. Louis, MO). The blank plasma utilized in this study was obtained from healthy volunteers at both Shands at the University of Florida (Gainesville, FL) and BioChemed Services (Winchester, VA). All other chemicals were of analytical grade and commercially available.

2.2. Preparation of stock solutions, calibrator solutions, and quality controls

Stock solutions of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, isosilybin B, and the IS naringenin were prepared in 100% methanol. The concentration of all stock solutions were 1 mg/ml with the exception of the naringenin working solution which was 10 mg/ml. Working solutions were prepared by diluting each silymarin component in water at the concentration of 1 ng/ml as well as the internal standard, naringenin, at the concentration of 0.4 μg/ml. All stock and working solutions were stored at −20 °C until use. Calibrator solutions were prepared by adding the desired amount of working solutions to 1 ml of blank human plasma. The calibrator concentrations were 100, 50, 20, 10, 5, and 2 ng/ml plasma for each analyte while the final concentration of the internal standard, naringenin, was 20 ng/ml. Quality control (QC) samples were used at the concentrations of 75, 15, and 4 ng/ml plasma.

2.3. Instrumentation

The LC–MS/MS analysis was performed on a Shimadzu HPLC system (Shimadzu, Tokyo, Japan) including a degasser (DGU-14A), two pumps (LC-10ATvp), an autosampler (SIL-10ADvp) and a system control (SCL-10Avp), coupled to an Applied Biosystems-Sciex API 3000 triple quadrupole mass spectrometer (Foster City, CA, USA). Samples were run through a C_{18} guard column (4 mm × 20 mm, SecurityGuard™, part no. AJO-4286, Torrence, CA) before separation on a Phenomenex™ Luna 5u C_{18} column (100 A 250 × 2 mm, 5 μm, Torrence, CA). The mobile phase consisted of 51% methanol and 49% water containing 0.1% formic acid and 10 mM ammonium acetate, and was delivered at a flow rate of 0.25 ml/min. The mass spectrometer was operated in negative ion mode using turbo electrospray ionization. The MS tuning parameters were optimized for taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B by infusing 0.1 μg/ml of each analyte dissolved in mobile phase at a flow rate of 20 μl/min. The following parameters were utilized for the MS analysis: curtain gas, 8 psi; nebulizer gas (gas 1), 12 psi; CAD gas, 6 psi; TurboIonSpray (IS) voltage, −4500 V; entrance potential (EP), −10 V; collision cell exit potential (CXP), −7 V; declustering potential (DP), −71 V; collision energy (CE), 40 eV for m/z: 481 > 125, 26 eV for m/z: 271 > 151, 30 eV for m/z: 303 > 125; source temperature, 400 °C; and dwell time, 250 ms. The following transitions were monitored in the Multiple Reaction Monitoring (MRM) mode: taxifolin, m/z 303 > 125; silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B, m/z 481 > 125; naringenin IS, m/z 271 > 151 Data was acquired and analyzed by AB Sciex Analyst software, version 1.4.2 (AB Sciex, Toronto, Canada).

2.4. Clinical study

All clinical samples were collected at the Medical University of South Carolina (MUSC) General Clinical Research Center (GCRC) in Charleston, SC. Each subject provided written informed consent approved by the MUSC Office of Research Integrity. Subjects were determined to be healthy by medical history and physical examination performed by the study physician. All participants were nonsmokers, not taking prescription or over-the-counter medications or dietary supplements. Additionally, participants were requested to abstain from grapefruit juice, caffeine-containing beverages, and ethanol use two weeks prior to, and during the study period. Lastly, subjects were asked to refrain from consumption of artichokes, or artichoke-containing foods that are also known to contain taxifolin.

Thirteen subjects participated in this study (8 men and 5 women). Following an overnight fast, subjects arrived at the MUSC GCRC the morning of each of 4 separate blood drawing phases of the study. An indwelling venous catheter was placed in each subject's arm to facilitate serial blood sampling. At approximately 8:00 AM subjects were provided one, two, or three 175 mg of milk thistle capsules (Legalon®). Subjects remained in a fasted state for 4 h following drug administration to reduce any effect of food on absorption. Standard meals were later provided by a registered dietician on the GCRC and did not include any were free of other sources of potentially interfering flavonoids. A total of 12 blood samples (10 ml each) were collected over the active study period at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 24 h. All samples were drawn in heparinized blood collection tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) and immediately placed on ice until centrifugation at 4 °C, which was timed at no longer than 15 min for any sample. Following centrifugation, plasma was transferred into

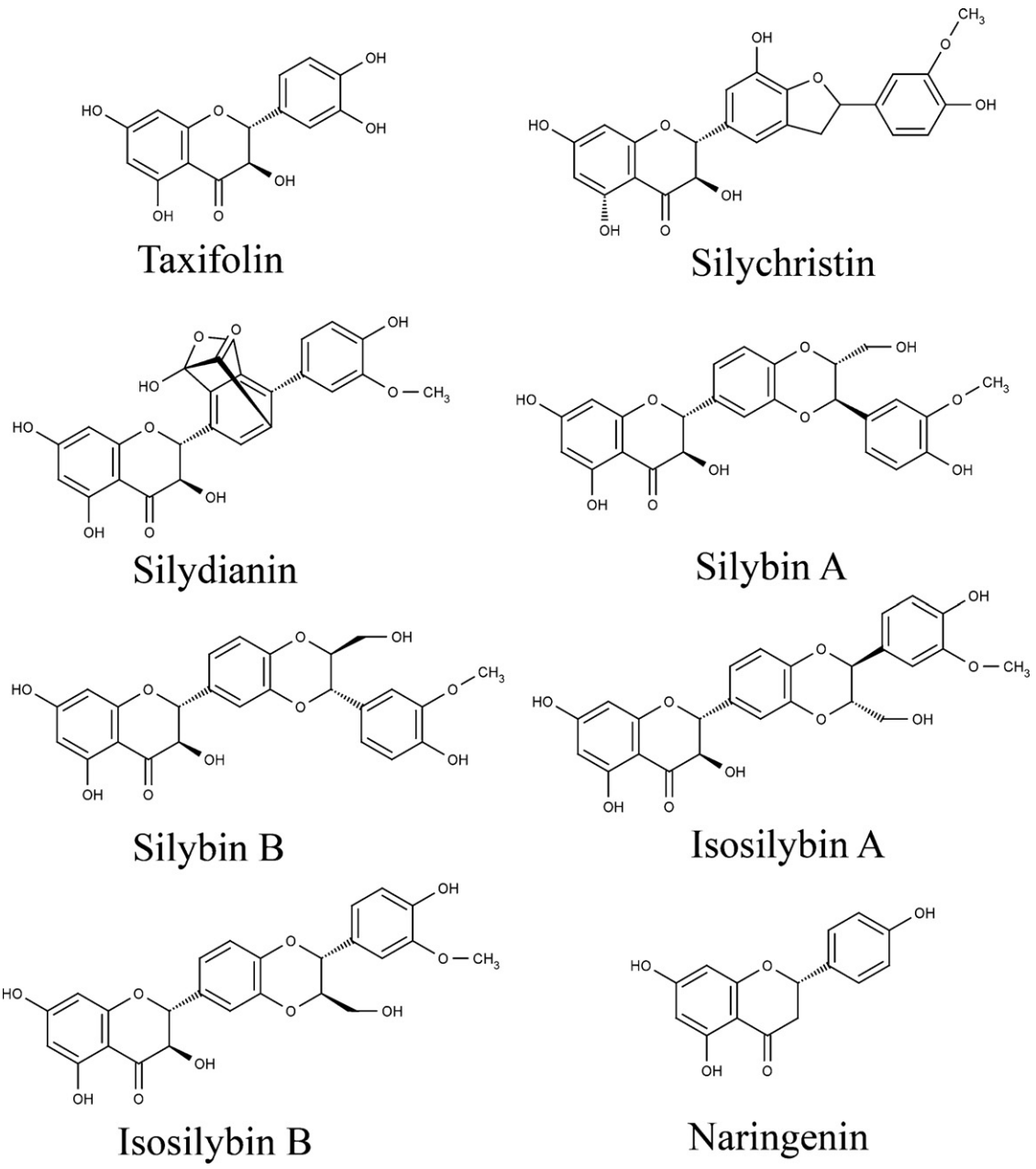

Fig. 1. Structural representation of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, isosilybin B, and the internal standard naringenin.

plastic vials, acidified with 10 μl of 1 M acetic acid/ml plasma and frozen immediately at −70 °C and stored until analysis.

2.5. Plasma sample preparation

A liquid–liquid extraction method was utilized to extract all silymarin components from human plasma. One milliliter of plasma samples, calibrators, and QCs were added with 20 ng/ml internal standard and 2 ml of ethyl acetate containing 0.1% formic acid. The samples were then shaken at 200 cycles/min for 10 min, followed by centrifugation at 3000 rpm for 10 min at room temperature. The organic phase was then transferred to clean glass tubes. This process was then repeated and produced approximately 4 ml of organic solvent per sample that was then evaporated to dryness under a gentle stream of nitrogen gas at 40 °C. The remaining residue was then reconstituted with 100 μl of mobile phase and 40 μl of each were injected for analysis.

3. Results and discussion

3.1. Mass spectrometry

Product ion mass spectra of each analyte and the IS naringenin were obtained in the negative ion mode and presented in Fig. 2, along with their respective daughter ion chemical structures. The patterns of fragmentations were consistent with the previously published reports [11,19,21,22]. Based on the observed ion fragmentation, the ion transition of m/z 481 > 125 was utilized for monitoring the flavonolignans including silybin A and B, isosilybin A and B, silychristin, and silydianin, while the m/z transitions

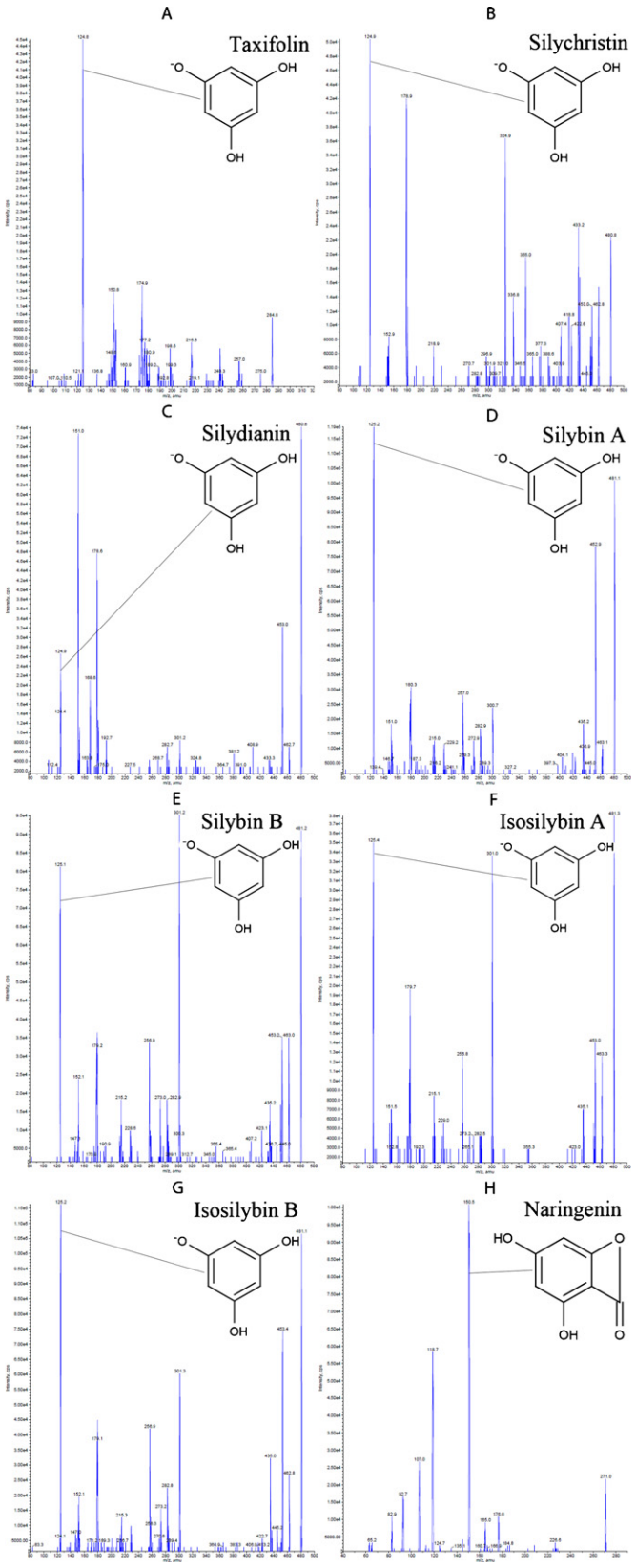

Fig. 2. Product ion spectra in negative ion mode of taxifolin (A), silychristin (B), silydianin (C), silybin A (D), silybin B (E), isosilybin A (F), isosilybin B (G), and naringenin (H), along with the chemical structures of the monitored product ions.

Table 1
Assessment of the recovery of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, isosilybin B, and the IS.

Analyte (ng/ml)	Mean peak area ($\times 10^4$, $n = 6$)		Recovery percentage[c] (%, mean ± SD)
	Unextracted[a]	Extracted Plasma[b]	
Taxifolin			
4	2.2	1.85	84.6 ± 0.8
15	8.41	7.11	86.4 ± 4.3
75	43.0	33.1	80.0 ± 3.1
Silychristin			
4	0.6	0.45	75.2 ± 6.2
15	2.35	1.65	69.0 ± 1.6
75	120.0	79.3	66.9 ± 1.1
Silydianin			
4	0.39	0.33	81.7 ± 3.9
15	1.50	1.21	82.7 ± 4.6
75	72.0	57.8	82.4 ± 3.6
Silybin A			
4	1.27	0.88	67.2 ± 2.4
15	4.90	3.24	66.0 ± 0.5
75	238.3	165.7	71.0 ± 3.3
Silybin B			
4	1.4	0.89	64.0 ± 0.8
15	5.12	3.40	66.7 ± 1.1
75	256.7	172.7	69.0 ± 3.3
Isosilybin A			
4	1.96	1.13	57.5 ± 1.0
15	72.1	4.47	62.7 ± 0.8
75	362.3	233.3	66.4 ± 2.6
Isosilybin B			
4	1.93	1.07	55.1 ± 2.0
15	7.20	4.20	59.2 ± 0.8
75	356.7	230.0	67.0 ± 2.7
Naringenin			
20	4.08	2.33	55.7 ± 3.1

[a] Peak area of analytes solution spiked in mobile phase.
[b] Peak area of analytes extracted from spiked plasma.
[c] Recovery was expressed as the percentage of the mean peak area of the analytes prepared in the mobile phase relative to that of analytes extracted from plasma.

for taxifolin and naringenin were 303 > 125 and 271 > 151, respectively.

3.2. Selectivity

Selectivity was evaluated by analyzing blank human plasma and plasma spiked with 2 ng/ml (for individual isomers) of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B or the IS naringenin. The blank plasma was obtained from 6 different sources and tested individually in the present selectivity study. As shown in Fig. 3, no endogenous interfering peaks were observed in blank plasma for all 7 analytes and the IS naringenin. Excellent separation was achieved for silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B, taxifolin, and the IS naringenin under the present chromatographic conditions.

3.3. Recovery

Recovery experiments were carried out by comparing the analytical results of extracted 3 QC samples with unextracted QCs using 3 replicates. The recovery of IS was determined at the concentration of 20 ng/ml. The results are depicted in Table 1. The recovery ranged from 55.1% to 86.4%.

3.4. Linearity

Calibration curves were determined by plotting the concentration versus analyte-to-IS peak area ratio and were found to be linear within the range of 2–100 ng/ml for each analyte. This range of concentrations was used to bracket all anticipated concentrations of

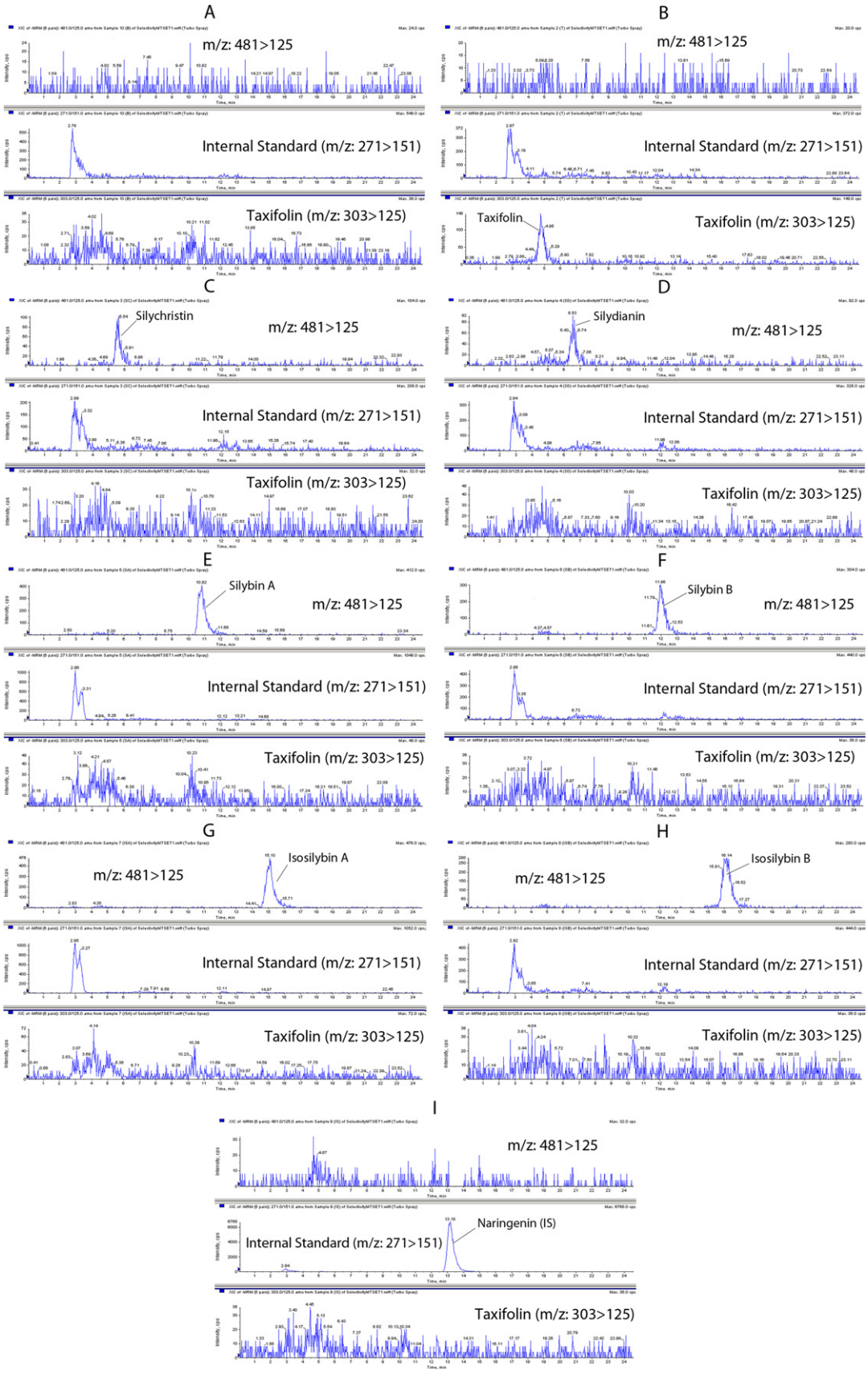

Fig. 3. Representative LC–MS/MS chromatograms of blank human plasma (A) and of plasma spiked with 2 ng/ml of taxifolin (B), silychristin (C), silydianin (D), silybin A (E), silybin B (F), isosilybin A (G), isosilybin B (H), and naringenin (I).

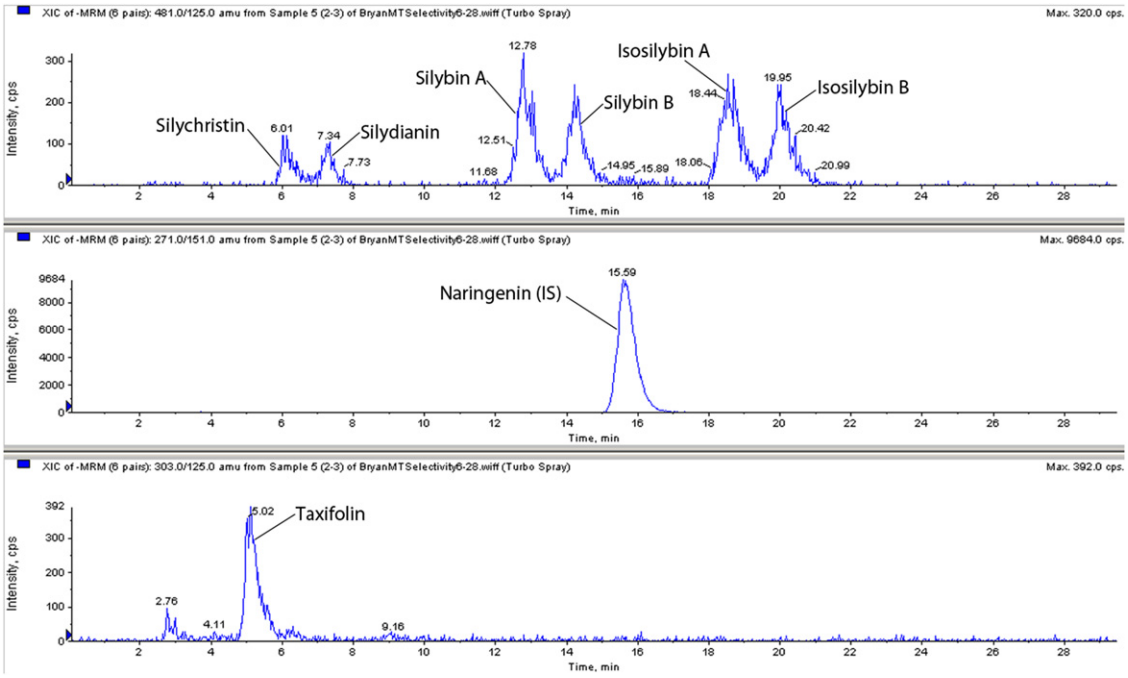

Fig. 4. Representative chromatogram of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B at the concentration of 2 ng/ml (LLOQ).

analytes in human plasma within the first 24 h of administration of the clinically relevant doses of milk thistle extract. The correlation coefficients from three independent experiments were ≥0.99 for all 7 compounds.

Table 2
Intra-day and inter-day accuracy and precision of enantioselective LC–MS/MS method for determination of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, isosilybin B, and IS in human plasma.

Analyte (ng/ml)	Intra-day ($n=5$)		Inter-day ($n=15$)	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Taxifolin				
4	101.4	7.7	105.0	7.5
15	105.7	4.4	102.6	6.9
75	91.0	4.4	95.1	9.8
Silychristin				
4	102.9	4.4	107.4	8.83
15	104.0	4.6	101.0	9.2
75	94.6	7.0	100.3	10.0
Silydianin				
4	105.7	8.2	111.9	10.5
15	102.1	5.8	103.1	8.8
75	96.8	5.5	101.8	6.8
Silybin A				
4	104.0	4.15	109.3	6.3
15	102.1	4.8	104.7	9.8
75	106.5	7.45	107.2	5.8
Silybin B				
4	101.2	6.2	105.5	9.5
15	101.2	7.2	102.5	8.1
75	105.6	5.6	107.0	5.8
Isosilybin A				
4	100.0	3.5	104.7	5.6
15	98.7	7.5	103.2	7.7
75	105.6	6.6	105.4	7.5
Isosilybin B				
4	104.6	6.2	103.4	5.7
15	98.7	8.0	100.3	7.6
75	105.7	6.2	106.0	6.4
Naringenin				
20	95.4	10.1	97.1	9.7

3.5. Lower limit of quantification

Lower limit of quantification (LLOQ) of each analyte was determined to be 2 ng/ml using the criteria in the Guidance for Industry

Table 3A
Assessment of the bench top stability of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, isosilybin B, and the IS.

Analyte (ng/ml)	Measured concentration ($n=3$)		Ratio[c] (%, mean ± SD)
	Control[a]	After storage[b]	
Taxifolin			
4	4.18	3.76	111.1 ± 13.1
15	15.2	15.2	100.6 ± 10.9
75	72.4	74.2	102.6 ± 8.2
Silychristin			
4	4.6	4.3	94.8 ± 12.6
15	13.6	13.8	101.7 ± 3.2
75	70.8	71.4	100.6 ± 8.0
Silydianin			
4	4.9	4.4	90.4 ± 14.1
15	13.3	13.5	102 ± 6.1
75	70.0	68.7	98.2 ± 5.0
Silybin A			
4	4.4	4.36	98.4 ± 7.7
15	13.9	14.9	106.7 ± 8.4
75	75.1	74.5	99.6 ± 10.5
Silybin B			
4	4.8	4.22	88.1 ± 6.8
15	14.0	14.4	102.9 ± 8.7
75	76.3	76.8	100.8 ± 8.6
Isosilybin A			
4	4.9	4.71	95.9 ± 10.5
15	14.0	14.5	104.1 ± 4.0
75	73.7	75.3	102.4 ± 10.6
Isosilybin B			
4	5.0	5.25	106 ± 15.2
15	14.1	14.7	104.6 ± 9.6
75	74.4	77.1	103.9 ± 7.7

[a] Measured concentration of analyte after immediate extraction.
[b] Measured concentration of spiked plasma left on bench top for 4 h followed by extraction.
[c] Ratio was expressed as the percentage of the measured concentrations of the analytes left at room temperature for 4 h relative to that of analytes extracted from plasma immediately.

Table 3B
Assessment of the freeze–thaw stability of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, isosilybin B, and the IS.

Analyte (ng/ml)	Measured concentration ($n = 3$)		Ratio[c] (%, mean ± SD)
	Control[a]	After storage[b]	
Taxifolin			
4	4.14	3.81	93.1 ± 8.8
15	15.0	13.4	89.4 ± 3.5
75	78.9	84.3	106.6 ± 13.9
Silychristin			
4	4.7	4.8	102.5 ± 9.3
15	13.6	14.9	109.8 ± 6.9
75	81.2	81.7	99.5 ± 5.6
Silydianin			
4	5.1	4.45	86.7 ± 4.6
15	13.8	13.5	98.2 ± 7.8
75	77.8	77.8	82.4 ± 4.5
Silybin A			
4	4.4	4.48	101.8 ± 5.2
15	15.3	14.4	94.2 ± 4.0
75	79.6	75.3	98.0 ± 3.3
Silybin B			
4	4.6	4.26	94.8 ± 8.8
15	14.8	14.1	95.3 ± 4.8
75	76.7	77.5	101.0 ± 2.6
Isosilybin A			
4	4.7	4.1	87.5 ± 4.5
15	15.7	14.8	94.4 ± 1.3
75	81.2	76.0	93.6 ± 3.9
Isosilybin B			
4	4.2	4.0	96.8 ± 8.0
15	15.3	14.6	95.3 ± 2.6
75	77.8	78.3	100.9 ± 6.8

[a] Measured concentration of analyte after immediate extraction.
[b] Measured concentration of analytes after three freeze–thaw cycles.
[c] Ratio was expressed as the percentage of the measured concentrations of the analytes that went through three freeze–thaw cycles relative to that of analytes extracted from plasma immediately.

Table 3C
Assessment of the autosampler stability of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, isosilybin B, and the IS.

Analyte (ng/ml)	Mean peak area ($\times 10^4$, $n = 6$)		Ratio[c] (%, mean ± SD)
	Control[a]	After storage[b]	
Taxifolin			
4	0.45	0.403	89.5 ± 4.3
15	3.28	3.64	107.5 ± 10.0
75	6.6	6.4	97.3 ± 8.5
Silychristin			
4	0.126	0.107	84.5 ± 4.0
15	1.0	0.99	98.4 ± 7.5
75	3.06	3.0	105.6 ± 14.6
Silydianin			
4	0.228	0.195	85.6 ± 5.0
15	1.24	1.27	102.2 ± 6.9
75	2.23	2.4	109.3 ± 10.2
Silybin A			
4	0.754	0.684	88.7 ± 9.8
15	5.17	5.08	97.9 ± 2.6
75	6.83	7.3	107.8 ± 1.6
Silybin B			
4	0.815	0.791	97.3 ± 9.7
15	5.2	5.13	98.6 ± 3.1
75	6.87	7.2	107.6 ± 4.3
Isosilybin A			
4	1.35	1.18	89.4 ± 7.8
15	7.0	6.97	99.7 ± 7.6
75	14.0	15.6	110.8 ± 7.8
Isosilybin B			
4	1.17	1.13	96.3 ± 1.3
15	7.26	7.14	98.9 ± 6.5
75	14.6	16.3	111.6 ± 9.4

[a] Peak area of analytes extracted from plasma.
[b] Peak area of analytes extracted from spiked plasma that were left in autosampler for 10 h.
[c] Ratio was expressed as the percentage of the measured concentrations of the analytes left in the autosampler for 10 h to that of analytes injected immediately.

Bioanalytical Method Evaluation presented by the Center for Drug Evaluation and Research [23]. The accuracy of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B were within 91.2–112.8%, and the %RSDs of precision were below 10.6% from using five replicates at the LLOQ concentration. The signal (peak height)-to-noise ratios were higher than 5 for all 7 analytes (Fig. 4).

3.6. Accuracy and precision

The three concentrations of QC samples in five replicates were utilized to validate the accuracy and precision of the developed method. The results showed that the intra and inter-day accuracy ranged between 91.0% and 111.9%. All accuracy and precision results were within acceptable limits (see Table 2). The %RSD of intra- and inter-day precision was less than 10.5% (Table 2). The present LC–MS/MS method was thus found to meet the accepted requirements of accuracy and precision.

3.7. Stability

The bench top, freeze–thaw, and autosampler stability of each analyte was evaluated using three QC samples with three replicates at each concentration. The bench top stability was assessed by measuring the concentration of each analyte after the samples remained at room temperature for 4 h. This time was selected based on the expected duration that the samples could be maintained at room temperature during the sample preparation. The ratios of the concentrations of each analyte determined after 4 h exposure to room temperature to that measured immediately before the preparation ranged from 0.82 to 1.11 (Table 3A). Freeze and thaw stabilities were determined after three freeze–thaw cycles. The QC samples (1 ml) were allowed to thaw unassisted at room temperature, and left on the bench for 1 h before being refrozen at −70 °C for 24 h. After three freeze–thaw cycles, the remaining concentrations of each analyte were determined to be within 82.4% and 109.8% (Table 3B). To study the autosampler stability, the QC samples were prepared, transferred to autosampler vials, and kept in the autosampler (4 °C) for 10 h prior to analysis. The results demonstrated that the concentrations determined in the samples stored in the autosampler for 10 h ranged between 80% and 122% of that from the samples measured immediately after preparation (Table 3C).

3.8. Matrix effect

In order to assess the assay for any potential matrix-induced ion suppression/enhancement, quantitative matrix effect studies were carried out by comparing the absolute peak area of the analytes dissolved in the mobile phase to that of the same analyte solutions containing plasma extracts. Three QC concentrations of each tested analyte and 20 ng/ml IS were utilized in the test with six different sources of human plasma. As shown in Table 4, no significant ion suppression or enhancement was observed for all analytes and the IS under the present experimental conditions.

3.9. Clinical application

This assay was successfully applied to a pharmacokinetic study of milk thistle capsule in healthy volunteers. Fig. 5 shows plasma concentration of the analytes including silybin A, silybin B, isosilybin A, isosilybin B, silychristin, silydianin, and taxifolin in a subject

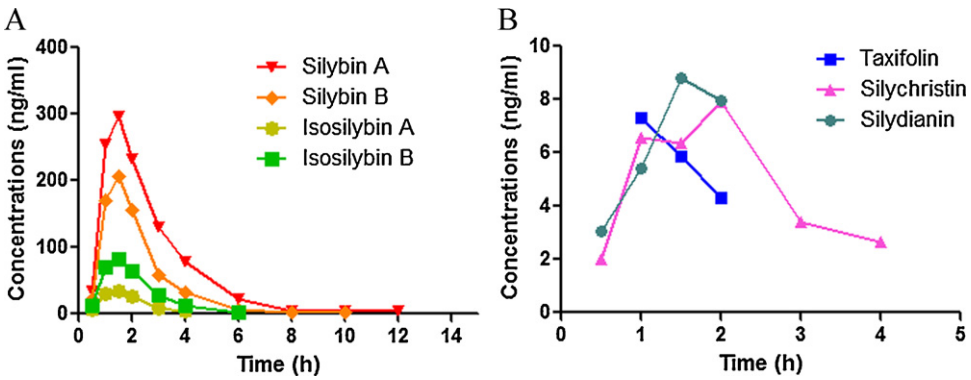

Fig. 5. Plasma concentration–time profile of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B from a healthy male volunteer administered 525 mg Legalon® milk thistle capsules.

Table 4
Assessment of matrix effect of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B, and the IS naringenin.

Analyte (ng/ml)	Mean peak area ($\times 10^4$, $n = 6$)		Matrix effect[c] (%, mean ± SD)
	Without extracts[a]	With extracts[b]	
Taxifolin			
4	1.19	1.14	95.1 ± 6.3
15	4.46	4.07	91.6 ± 5.1
75	24.3	21.4	88.9 ± 6.1
Silychristin			
4	0.575	0.584	100.9 ± 13.1
15	2.29	2.12	92.0 ± 4.2
75	13.7	12.5	90.9 ± 6.3
Silydianin			
4	0.835	0.835	100.2 ± 7.7
15	3.04	2.88	95.0 ± 6.7
75	16.6	16.2	97.6 ± 5.2
Silybin A			
4	3.89	3.6	90.6 ± 7.4
15	12.4	11.9	95.0 ± 9.8
75	72.0	67.7	93.9 ± 4.2
Silybin B			
4	3.69	3.49	92.7 ± 10.3
15	12.3	11.3	91.3 ± 4.9
75	70.7	66.4	93.6 ± 6.0
Isosilybin A			
4	5.48	5.16	93.4 ± 10.7
15	17.9	17.1	95.4 ± 9.8
75	106.0	99.6	93.6 ± 5.7
Isosilybin B			
4	5.82	5.26	90.3 ± 12.0
15	18.3	17.5	95.6 ± 4.6
75	105.0	103.0	98.3 ± 3.2
Naringenin			
20	51.4	50.7	98.2 ± 4.8

[a] Peak area of analytes solution without plasma extracts.
[b] Peak area of analytes spiked in plasma extracts.
[c] Matrix effect was expressed as the percentage of the mean peak area of the analytes prepared in the mobile phase relative to that of analytes spiked in plasma extracts.

Table 5
Pharmacokinetic parameters for taxifolin, silychristin, silydianin, silybin A and B, and isosilybin A and B from a healthy male volunteer administered 525 mg Legalon® milk thistle capsules.

Analyte	C_{max} (ng/ml)	T_{max} (h)	$T_{1/2}$ (h)	$AUC_{0-\infty}$ (ng h/ml)
Silybin A	296	1.5	1.6	786.4
Silybin B	206	1.5	1.2	433.1
Isosilybin A	34.4	1.5	1.3	79.3
Isosilybin B	82.1	1.5	1	185.8
Taxifolin	7.3	1	ND	ND
Silychristin	7.9	2	ND	ND
Silydianin	8.8	1.5	ND	ND

ND: not determined due to limited data available in the elimination phase.

receiving a single dose of 525 mg milk thistle capsules (Legalon®). The study demonstrated that silybin A, silybin B, isosilybin A, and isosilybin B are the major active substances found in the patient's plasma treated with milk thistle, whereas the concentrations of silychristin, silydianin, and taxifolin were relatively low. All compounds reached their maximum plasma concentrations 1–2 h after drug administration. The pharmacokinetic parameters C_{max}, T_{max}, half-life, and area under the curve for taxifolin, silychristin, silydianin, silybin A and B, and isosilybin A and B are depicted in Table 5.

4. Conclusions

A sensitive ESI–MS/MS assay for the simultaneous analysis of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B in human plasma has been developed and validated in the present study. To the best of our knowledge, this presents the first analytical method with the capacity of quantifying all 7 major bioactive components of milk thistle in any biological matrix. This method was found to be readily applicable to the clinical study of silymarin pharmacokinetics following a typical therapeutic dose of silymarin.

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