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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