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Pharmacokinetics and Metabolic Profile of Free, Conjugated and Total Silymarin Flavonolignans in Human Plasma after Oral Administration of Milk Thistle Extract

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Pharmacokinetics and metabolism of silymarin flavonolignans

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Abbreviations used in this paper:

SC, silychristin; SD, silydianin; SB_A, silybin A; SB_B, silybin B; ISB_A, isosilybin A; ISB_B, isosilybin B; NG, naringenin; UGT, UDP-glucuronosyltransferase; D-SL, D-saccharic acid 1,4-lactone; ACN, acetonitrile; MeOH, methanol; HAc, glacial acetic acid; HPLC, high-performance liquid chromatography; LC, liquid chromatography; ESI, electrospray ionization; MS, mass spectrometry; SIM, selective ion monitoring; IS,

internal standard; C_{max}, maximum plasma concentration; T_{max}, peak time at C_{max}; $t_{1/2}$, terminal elimination half-life; $AUC_{0\rightarrow\infty}$, area under the plasma concentration-time curve from time 0 to infinity.

ABSTRACT

Silymarin, a mixture of polyphenolic flavonoids extracted from milk thistle (*Silybum marianum*), is mainly composed of silychristin (SC), silydianin (SD), silybin A (SB_A), silybin B (SB_B), isosilybin A (ISB_A), and isosilybin B (ISB_B). In this study, the plasma concentrations of free (unconjugated), conjugated (sulfated and glucuronidated), and total (free and conjugated) silymarin flavonolignans were measured using a liquid chromatography-electrospray ionization-mass spectrometry, after a single oral dose of 600 mg standardized milk thistle extracts to three healthy volunteers. Pharmacokinetic analysis indicated that silymarin flavonolignans were rapidly eliminated with short half-lives (1 to 3 and 3 to 8 h for the free and conjugated, respectively). The $AUC_{0\rightarrow\infty}$ values of the conjugated silymarin flavonolignans were 4- to 30-fold higher than those of their free fractions, with SB_B (mean $AUC_{0\rightarrow\infty}$ = 51 and 597 μg·h/l for the free and conjugated, respectively) and ISB_A (mean $AUC_{0\rightarrow\infty}$ = 30 and 734 μg·h/l for the free and conjugated, respectively) exhibiting higher $AUC_{0\rightarrow\infty}$ values in comparison to other flavonolignans. Near the plasma peak times (1 to 3 h), the free, sulfated, and glucuronidated flavonolignans represented approximately 17, 28, and 55 % of the total silymarin, respectively. In addition, the individual silymarin flavonolignans exhibited quite different plasma profiles for both the free and conjugated fractions. These data suggest that, after oral administration, silymarin flavonolignans are quickly metabolized to their conjugates, primarily forming glucuronides, and the conjugates are primary components present in human plasma.

Introduction

Silymarin, a mixed extract of polyphenolic flavonoids isolated from milk thistle (*Silybum marianum*), is mainly composed of six flavonolignans including silychristin (SC), silydianin (SD), silybin A (SB_A), silybin B (SB_B), isosilybin A (ISB_A), and isosilybin B (ISB_B) (Fig. 1). As an herbal remedy, silymarin is widely used for the self-treatment of liver disease and cancer (Flora, et al., 1998; Jacobs, et al., 2002; Fraschini, et al., 2002; Ladas and Kelly, 2003). Silymarin is also used for the treatment of *Amanita phalloides* mushroom poisoning (Desplaces, et al., 1975; Hruby, et al., 1983; Vogel, et al., 1984). In vitro and animal studies have demonstrated the hepatoprotective properties of silymarin or silybin (a mixture of SB_A and SB_B) (Fraschini, et al., 2002; Hoofnagle, 2005; Crocenzi and Roma, 2006). Several clinical trials have shown an excellent safety profile for silymarin in humans (Fraschini, et al., 2002; Ball and Kowdley, 2005; Dryden, et al., 2006). However, the clinical efficacy and dose-exposure relationships in humans remain unclear, because of the small number of participants and the lack of information on the exposure levels of the major silymarin flavonolignans with administration of standardized dosage regimens (Jacobs, et al., 2002; Ball and Kowdley, 2005; Mayer, et al., 2005; Rambaldi, et al., 2005).

Silymarin is believed to be primarily metabolized to conjugates (e.g., sulfates and glucuronides) both in vitro and in vivo (Rickling, et al., 1995; Kren, et al., 2000; Han, et al., 2004; D'Andrea, et al., 2005). Some previous studies have reported the plasma concentrations of the free and total silybin isomers in animals and humans after oral dose of silybin or silymarin (Barzaghi, et al., 1990; Mascher, et al., 1993; Morazzoni, et al., 1993; Gatti and Perucca, 1994; Schandalik and Perucca, 1994; Rickling, et al., 1995). However, the

pharmacokinetics and metabolism of individual silymarin flavonolignans in humans have not preciously been reported. In order to better understand the elimination and metabolic profile of the six major active isomers of silymarin, we investigated the pharmacokinetics and metabolic profile of the free (unconjugated), conjugated (sulfated and glucuronidated) and total (free and conjugated) silymarin flavonolignans in plasma after a single oral administration of 600 mg standardized milk thistle extracts to healthy volunteers, using a rapid and sensitive liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). The contents of the six silymarin flavonolignans in several commercial milk thistle products were also estimated using a simple high-performance liquid chromatography (HPLC)-ultraviolet (UV) assay to provide a better understanding of the relationship between the composition of the major flavonolignans in the administered product and their relative exposures in blood.

Materials and Methods

Chemicals. Silybin (Silibinin) was purchased from Sigma-Aldrich (St. Louis, MO). The composition of silybin was confirmed to be a mixture of SB_A and SB_B by a LC-ESI-MS, and the contents of SB_A and SB_B were analyzed to be 48 and 52% by an HPLC-UV assay, respectively. The measured ratios of SB_A and SB_B in silybin were used for the qualitative and quantitative analysis in this study. SC was obtained from ChromaDex (Santa Ana, CA). SD and powdered milk thistle extracts were purchased from U. S. Pharmacopoeia (USP; Rockville, MD). Standardized silymarin, naringenin (NG; the internal standard for quantification), sulfatase (EC 3.1.6.1; type H-1 from *Helix pomatia*), β-glucuronidase (EC 3.3.1.31; type B-10 from bovine liver), D-saccharic acid 1,4-lactone (D-SL; a specific β-glucuronidase inhibitor) and

glacial acetic acid (HAc) were purchased from Sigma-Aldrich (St. Louis, MO). Silymarin Plus™ tablets (labeled to contain 237 mg of milk thistle extracts per tablet) were obtained from Source Naturals (Scotts Valley, CA). Capsule 1 (labeled to contain 300 mg of milk thistle extracts per capsule) was from Nutraceutical Sciences Institute (NSI; Boynton Beach, FL). Capsule 2 (labeled to contain 175 mg of milk thistle extracts per capsule) was from Nature's Way (Springville, UT). Pooled human plasma was obtained from Valley Biomedical (Winchester, VA). Acetonitrile (ACN; HPLC grade) and methanol (MeOH; HPLC grade) were obtained from Mallinckrodt (Phillipsburg, NJ). All other chemicals and reagents used were of analytical grade.

Analysis of Silymarin Flavonolignans in Commercial Milk Thistle Products. Milk Thistle extracts are currently marketed as dietary supplements in the United States, and are not regulated by the Food and Drug Administration (FDA) as drugs. Because the FDA has little control over the quality of herbal products like silymarin, it is necessary to quantitatively estimate the contents of the potentially active ingredients in botanically derived therapies before use. Determination of the six silymarin flavonolignans in various commercial milk thistle extracts was performed with a simple HPLC system using NG as the internal standard (IS). Chromatographic separation was carried out using an Agilent 1100 LC system (Palo Alto, CA) with a BrownLee RP-C18 guard column (15 mm × 3.2 mm, i.d., 7 μm; PerkinElmer, Shelton, CT) and an Axxiom ODS analytical column (150 mm × 4.6 mm, i.d., 5 μm; Thomson Instrument, Clear Brook, VA). HPLC conditions were as follows: mobile phase, MeOH:0.1% HAc (pH 3) (46:54, v/v) with isocratic elution; detection wavelength, 288 nm; flow rate, 1.5 ml/min; injection volume, 20 μl; run time,

20 min. Typical retention times of SC, SD, NG, SB_A, SB_B, ISB_A and ISB_B under the experimental conditions used were 4.1, 4.8, 9.1, 10.8, 12.1, 15.0, and 16.0 min, respectively (Fig. 2). Stock standard solutions of SC, SD, SB_A, and SB_B were separately prepared in MeOH, and diluted with 50% MeOH. Calibration curves were set up using mixed standard solutions containing SC, SD, SB_A, and SB_B. Concentrations of silymarin flavonolignans in the samples were estimated with $1/x^2$ weighted least-squares regression equations derived from the peak area ratios of individual silymarin flavonolignans to that of NG. Because ISB_A and ISB_B were not commercially available during this study, their concentrations were initially calculated using the calibration curves of SB_A and SB_B, assuming that ISB_A and ISB_B have the similar quantitative responses to those of SB_A and SB_B, respectively. These analytical responses were later evaluated and corrected using purified standards of ISB_A and ISB_B obtained from Madaus (Köln, Germany).

Sample Preparation for Commercial Milk Thistle Products. Ten tablets or capsules were weighed, and finely pulverized. Appropriate amounts of the powder corresponding to one tablet or capsule were separately weighed, and transferred to a 25-ml volumetric flask and then mixed with 20 ml of MeOH. The mixture was sonicated for 15 min at room temperature, and diluted to 25 ml with MeOH. The mixture was filtered by a Millex®-HX Nylon syringe filter (0.45 μm, 25 mm; Millipore, Bedford, MA) to remove any particles. The first 5 ml of the filtrates were discarded, and the following filtrates were collected. Appropriate aliquots of the filtrates were diluted with 50% MeOH, as well as the addition of NG (final concentration 5 μg/ml), and analyzed by the HPLC-UV assay as described above. Standardized silymarin

(Sigma) and powdered milk thistle extracts (USP) were directly dissolved in MeOH and then diluted with 50% MeOH, and determined by the HPLC-UV assay.

Analysis of Silymarin Flavonolignans in Human Plasma. Identification and quantification of the six silymarin flavonolignans in human plasma required more sensitivity than UV detection could provide; thus, it was carried out by a LC-ESI-MS. Separation of the six silymarin flavonolignans was performed using an Agilent HP 1050 LC system (Palo Alto, CA) with a C18 SecurityGuard cartridge (4 × 2.0 mm i.d.; Phenomenex, Torrance, CA) and a Luna C18 (2) analytical column (50 × 2.0 mm i.d., 3 μm; Phenomenex, Torrance, CA). HPLC conditions were as follows: mobile phase, MeOH:1% HAc (pH 2.8) (44:56, v/v) with isocratic elution; flow rate, 0.3 ml/min; injection volume, 25 μl; run time, 12 min. Typical retention times of SC, SD, SB_A, NG, SB_B, ISB_A, and ISB_B, under the experimental conditions used, were 1.9, 2.4, 5.2, 5.5, 5.9, 8.4, and 9.2 min, respectively (Fig. 3). MS analysis and detection were conducted by an API 100 LC/MS system (PerkinElmer Sciex, Toronto, Canada) with a TurboIonspray interface in the negative ESI ionization mode. MS parameters used for qualitative analysis were: ionspray voltage, -3100 V; ionspray temperature, 450ºC; orifice voltage, -30 V; focusing ring voltage, -200 V; nebulizer gas, 10 l/min; curtain gas, 8 l/min; dwell time, 1 ms; scan mode, full scan at the range of 100 to 800 *m/z*. MS parameters used for quantitative analysis were: ionspray voltage, -3100 V; ionspray temperature, 450 ºC; orifice voltage, -30 V; focusing ring voltage, -200 V; nebulizer gas, 10 l/ml; curtain gas, 8 l/ml; dwell time, 300 ms; scan mode, selective ion monitoring (SIM) with $[M-H]^-$ for silymarin flavonolignans (*m/z* 481), silymarin sulfates (*m/z* 561), silymarin glucuronides (*m/z* 657), and NG (*m/z*

271), respectively. Calibration curves were set up using standards of SC, SD, SB_A, and SB_B. Mixed standard solutions containing SC, SD, SB_A, and SB_B were spiked into pooled blank human plasma and then treated as described in the human plasma sample preparation (described below). Concentrations of silymarin flavonolignans in the samples were estimated with $1/x^2$ weighted least-squares regression equations derived from the peak area ratios of individual silymarin flavonolignans to that of NG (described above).

Sample Preparation for Human Plasma. Plasma samples were treated with and without enzyme hydrolysis. The free (unconjugated) silymarin flavonolignans were directly determined without enzyme hydrolysis. The total (free and conjugated) silymarin flavonolignans were measured after hydrolysis using a mixed enzyme solution containing sulfatase and *β*-glucuronidase. Concentrations of the conjugated (sulfated and glucuronidated) silymarin flavonolignans were calculated from the differences between the total and free. Sulfated or glucuronidated silymarin flavonolignans were calculated from the differences between the measured concentrations of the free and those of after hydrolysis with sulfatase (containing D-SL, a specific *β*-glucuronidase inhibitor) or *β*-glucuronidase. In brief, 100-μl aliquots of plasma were treated with sulfatase (80 U/ml in the final incubation) containing D-SL (10 mM in the final incubation), *β*-glucuronidase (8000 U/ml in the final incubation), and a mixture of sulfatase (80 U/ml in the final incubation) and *β*-glucuronidase (8000 U/ml in the final incubation), respectively. Plasma samples with different hydrolytic enzymes were buffered using sodium acetate (pH 5.0, 0.125 M in the final incubation), and incubated (final volume 120 μl) at 37ºC with gentle shaking for 4 h. Preliminary experiments

demonstrated that, after each hydrolysis, no peaks corresponding to the conjugated (sulfated or glucuronidated) silymarin flavonolignans were found by the LC-ESI-MS, indicating the enzyme hydrolyses were complete. After the addition of 0.6 ml of ice-cold ACN containing 1% HAc and NG (20 ng) into the incubations or plasma (for free silymarin flavonolignans), the mixture was centrifuged at 15,000g for 15 min at 4ºC. The supernatants were transferred and then evaporated with a gentle stream of nitrogen at 45ºC in a water bath. The residue was reconstituted in 100 μl of HPLC mobile phase (MeOH:1% HAc, 44:56, v/v) and then centrifuged at 10,000g for 10 min at 4ºC, and 25 μl of the reconstituted supernatants were introduced for LC-ESI-MS analysis.

Pharmacokinetics of Silymarin Flavonolignans in Human Plasma. Plasma samples were obtained from three healthy volunteers following a single oral dose of 600 mg standardized milk thistle extracts (Capsule 1, labeled to contain 300 mg of milk thistle extracts per capsule) at 0 (pre-dose) and 0.25-24 h. 100-μl Aliquots of plasma at each time points were prepared with and without a mixed enzyme hydrolysis (80 U/ml sulfatase and 8000 U/ml β-glucuronidase), and measured by the LC-ESI-MS as described above. Pharmacokinetic parameters of individual silymarin flavonolignans were estimated by a noncompartmental analysis using WinNonlin (Pharsight, Mountain View, CA). The maximum plasma concentration (C_{max}) and time to maximum plasma concentration (T_{max}) were obtained directly from the plasma concentration-time data. The terminal elimination rate constant (λ_z) was estimated by linear least-squares regression of the terminal portion of the plasma concentration-time curve, and the corresponding elimination half-live ($t_{1/2}$) was then obtained by $t_{1/2} = 0.693/ \lambda_z$. The area under the plasma

concentration-time curve from time 0 to infinity ($AUC_{0\rightarrow\infty}$) was calculated according to the linear trapezoidal rule.

Results

Estimation of Silymarin Flavonolignans in Milk Thistle Extracts. The limit of detection (signal-to-noise ratio >3:1) and linear quantitative range for the determination of each silymarin flavonolignans in milk thistle extracts using the HPLC-UV assay were 20 ng/ml, and 0.05-200 μg/ml, respectively. The intra-day and inter-day precisions, expressed as the relative standard deviation (RSD, n = 5), were 0.74 to 10%, and 1.9 to 12%, respectively. The contents of the six silymarin flavonolignans in various commercial milk thistle extracts are summarized in Table 1. This composition allowed us to select a marketed silymarin formulation for the clinical study with reasonably high purity and known content. The relative percentages of individual silymarin flavonolignans in the standardized milk thistle extracts measured were very similar, with SC at 22 to 24%, SD at 9 to 15%, SB_A at 18 to 22%, SB_B at 30 to 35%, ISB_A at 8 to 9%, and ISB_B at 3 to 4%, respectively. These results indicated that SC, SB_A, and SB_B were the three predominant constituents (total 70 to 80%), whereas SD, ISB_A, and ISB_B were the minor components. Using the sum of six flavonolignans as the total content of silymarin, the measured contents of silymarin ranged from 57 to 71%. Standardized milk thistle extracts are generally considered to contain about 70 to 80% as silymarin (Flora, et al., 1998; Jacobs, et al., 2002; Simanek, et al., 2000; Venkataramanan, et al., 2006), whereas the content of silybin (SB_A + SB_B) represents about 40 to 70% of the total amounts of silymarin (Jacobs, et al., 2002; Venkataramanan, et al., 2006; USP, 2006). As shown

in Table 1, the relative percentages of silybin (SB_A + SB_B) were found to be 48 to 56% in standardized milk thistle extracts (Sigma and USP), and 54 to 57% in commercial milk thistle tablets and capsules. However, there were substantial differences in silymarin content between the various commercial milk thistle products measured. The total silymarin contents of USP and Sigma standardized milk thistle extracts were found to be 69 and 54%, respectively, whereas the three marketed milk thistle products were actually 63% (tablet), 57% (Capsule 1), and 71 % (Capsule 2), well below their labeled values (80%). This apparent discrepancy may be due to the use of nonspecific or colorimetric assays for determining silymarin content by the manufacturers.

Determination of Silymarin Flavonolignans in Human Plasma. Identification of silymarin flavonolignans was based on their chromatographic retentions, MS in-source fragmentations, and confirmed by comparing the standardized milk thistle extracts (Sigma or UPS). The six flavonolignans of silymarin exhibited different chromatographic retentions (Fig. 3), but very similar MS fragmentations (Fig. 4), with the base peak at *m/z* 481 ($[M-H]^-$, deprotonated molecule ion of silymarin), and less abundant multiple sub-fragmentation ions (e.g., *m/z* 463, $[M-H-H_2O]^-$; *m/z* 453, $[M-H-CO]^-$; *m/z* 301, $[M-H-C_8O_5H_4]^-$) in the negative ion mode. The MS spectra of individual silymarin flavonolignans (Fig. 4) were in agreement with published literature (Kim, et al., 2003; Lee and Liu, 2003; Lee, et al., 2006). Quantification of the six silymarin flavonolignans in human plasma was conducted by a LC-ESI-MS with SIM detection at *m/z* 481 in the negative ion mode, which has higher sensitivity than in the positive ion mode. Although identification and quantification of the individual sulfated (*m/z* 561) and glucuronidated

(*m/z* 657) silymarin flavonolignans was attempted, it was not possible to separate the conjugated products from each other with current analytical conditions (data not shown), because each silymarin flavonoligan has multiple phenolic and alcoholic hydroxyl sites for conjugation (Fig. 1), resulting in the formation of a multitude of possible conjugates. Thus, the total sulfated and glucuronidated silymarin flavonolignans were indirectly determined with enzyme hydrolysis using sulfatase and *β*-glucuronidase, respectively. In order to avoid the potential for simultaneous cleavage of glucuronides during desulfation, the addition of D-saccharic acid 1,4-lactone (a specific *β*-glucuronidase inhibitor) was employed. The limit of detection and linear quantitative range of the six silymarin flavonolignans by the LC-ESI-MS assay were 2 ng/ml, and 5-1000 ng/ml, respectively. The intra-day and inter-day precisions (RSD, n = 4) were 1.7 to 11%, and 4.5 to 14%, respectively.

Pharmacokinetic Parameters of Silymarin Flavonolignans in Human Plasma. Pharmacokinetic analysis indicated that, after oral administration, silymarin flavonolignans were rapidly eliminated with short half-lives (1 to 3, 3 to 6, and 3 to 5 h for the free, conjugated and total silymarin flavonolignans, respectively) (Fig. 5 and Table 2). Conjugated SC exhibited relatively longer half-life (~8 h) than other flavonolignans. Free SC and SD were not detectable or at very low concentrations. The C_{max} values of free SB_A, SB_B, ISB_A, and ISB_B ranged from 9 to 23 ng/ml, whereas the C_{max} values of total silymarin flavonolignans were 2- to 12-fold higher than the free fractions, with total SB_B (C_{max} = 131 ng/ml), and ISB_A (C_{max} = 113 ng/ml) exhibiting the highest peak plasma concentrations. The C_{max} values of the conjugated silymarin flavonolignans were similar (SC and SD) or slightly lower (SB_A, SB_B, ISB_A, and

ISB_B) in comparison with those of total silymarin flavonolignans (Table 2). The $AUC_{0\rightarrow\infty}$ values of the conjugated and total silymarin flavonolignans were 4- to 30-fold higher than the free fractions, with SB_B ($AUC_{0\rightarrow\infty}$ = 51 and 597 μg·h/l for the free and conjugated, respectively), and ISB_A ($AUC_{0\rightarrow\infty}$ = 30 and 734 μg·h/l for the free and conjugated, respectively) exhibiting the highest *AUC* values, suggesting that conjugated silymarin flavonolignans, particularly with SB_B and ISB_A, were the major metabolites in human plasma.

Metabolic Profile of Free, Conjugated and Total Silymarin Flavonolignans in Human Plasma. Plasma samples near the peak times (1 to 3 h) for each flavonolignans were pooled from all three healthy volunteers. The relative proportions of the free (unconjugated), conjugated (sulfated and glucuronidated), and total (free and conjugated) silymarin flavonolignans in human plasma are shown in Figure 6 and Table 3. Near the peak times (1 to 3h), the fractions of the free, sulfated, and glucuronidated silymarin were 17, 28, and 55% of the total silymarin, respectively. These data suggested that, after oral administration, silymarin flavonolignans were metabolized to their conjugates (sulfates and glucuronides), which represented about 83% of the total silymarin measured at the plasma peak times in healthy volunteers. In addition, the individual flavonolignans of silymarin exhibited quite different plasma profiles for the parents and conjugates. The major isomeric flavonolignans found in human plasma were SB_B (~30% of the total silymarin) and ISB_A (~21% of the total silymarin). Free and sulfated SD were not detectable in this study. SB_A mainly remained in the free form (~60% of total), whereas SB_B and SD were predominantly in their glucuronides (~71 and 100% of total, respectively). Based on plasma exposure at the time of peak plasma

concentration, ISB_A preferred the formation of sulfates (~60% of total) to glucuronides (~35% of total), whereas SC and ISB_B preferred the formation of glucuronides (~49 and ~60% of total, respectively) to sulfates (~37 and ~21% of total, respectively).

Discussion

Silymarin is considered to be primarily conjugated and excreted into bile and urine, and appears to have minimal phase 1 metabolism (Flora, et al., 1998; Fraschini, et al., 2002). However, limited data exists for phase 2 metabolic pathways and the role of transporters in vivo (Venkataramanan, et al., 2006). Silymarin metabolism in vivo may have a role in herbal-drug interactions, especially as doses for silymarin are increased. Prior to systematically evaluating the safety, efficacy and tolerability of orally administrated silymarin in patients with liver disease and other disorders, it is necessary to estimate the actual contents of the six principal isomers in the standardized silymarin extracts and obtain some preliminarily information about the pharmacokinetics of silymarin in humans. The plasma pharmacokinetics and metabolism of silybin (a mixture of SB_A and SB_B) in humans (Barzaghi, et al., 1990; Mascher, et al., 1993; Gatti and Perucca, 1994; Schandalik and Perucca, 1994; Rickling, et al., 1995) and rats (Morazzoni, et al., 1993; Rickling, et al., 1995) have been reported previously, showing the fast elimination of both the free and conjugated silybin. In this study, we investigated the pharmacokinetics and metabolic profile of the free (unconjugated), conjugated (sulfated and glucuronidated) and total (free and conjugated) silymarin flavonolignans in human plasma after a single oral dose of 600 mg standardized milk thistle extracts to healthy volunteers. The results demonstrated that all six silymarin flavonolignans were rapidly eliminated,

and the conjugated silymarin flavonolignans had relatively longer half-lives and much higher $AUC_{0\rightarrow\infty}$ values than their free forms (Table 2). These results are in agreement with the previous observations after administration of silybin to humans ((Barzaghi, et al., 1990; Mascher, et al., 1993; Gatti and Perucca, 1994; Schandalik and Perucca, 1994; Rickling, et al., 1995), although the individual silymarin flavonolignans were not completely characterized in the earlier studies. Our data also demonstrate that, at the peak times (1 to 3 h), the fractions of the free, sulfated, and glucuronidated silymarin in human plasma were about 17, 28, and 55 % of the total (Table 3), respectively. These results confirmed that silymarin flavonolignans are rapidly metabolized to their conjugates (83% of the total silymarin at C_{max}), mainly present as glucuronides in plasma.

It was noted that the C_{max} and $AUC_{0\rightarrow\infty}$ values of total SB_B and ISB_A were 2- to 6-fold higher than those of other flavonolignans (Table 2). These data suggest that the conjugated SB_B and ISB_A are the major metabolites in plasma of healthy volunteers. According to the analysis of silymarin flavonolignans in the standardized milk thistle extracts (600 mg) orally dosed in this study, the estimated contents of SC, SD, SB_A, SB_B, ISB_A, and ISB_B were 78, 31, 75, 117, 30, and 13 mg (Table 1), respectively. Obviously, ISB_A exhibited a relatively higher accumulation of conjugates in plasma in spite of a lower dose than other flavonolignans, with 60% of sulfates and 35% of glucuronides (Table 3). This relatively high metabolite content of ISB_A in human plasma is consistent with a previous finding in rats (Morazzoni, et al., 1993), although the underlying accumulation mechanism for this isomer is still unknown.

In vitro studies showed that the glucuronidation of silybin (a mixture of SB_A and SB_B) was stereoselective,

and SB_B was more efficient and faster than its diastereoisomer, SB_A (Han, et al, 2004; Kren, et al., 2000). In this study, higher plasma percentage of glucuronidated SB_B (71%) was observed, in comparison with that of glucuronidated SB_A (25%). Thus, this in vivo data is supported by the in vitro results regarding the stereoselective glucuronidation of SB_A and SB_B. Similarly, the in vivo glucuronidation of ISB_A and ISB_B also showed different steroselectivities of metabolite present in plasma at the peak time (Table 3). Sulfated SB_A and SB_B had very similar plasma percentages (16 and 14%), whereas the plasma percentage of sulfated ISB_A (60%) was higher than that of ISB_B (21%), this may suggest that the sulfation of ISB_A and ISB_B is also stereoselective.

The profile of the conjugates in plasma exhibited secondary peaks and irregular profiles for some of the silymarin isomers which varied between individual subjects (Fig. 5). Within an individual the secondary peaks were not consistent across isomers, which would suggest that irregular absorption from the dosage form was not a factor. Secondary peaks were also apparent in some of the free profiles, indicative of enterohepatic recycling. The fairly rapid absorption of silymarin, early peak times of the conjugates, and much higher maximum concentrations of the metabolites, supports rapid metabolism and low bioavailability. However, the expected lower volume of distribution of the conjugates relative to the parent silymarin isomers makes it difficult to provide any estimate of the fraction of dose reaching the systemic circulation as conjugates relative to parent silymarin isomer.

In conclusion, after oral administration of standardized milk thistle extracts, silymarin flavonolignans, the major biologically active components in milk thistle, are rapidly metabolized and measurable in plasma,

mainly in the form of glucuronides. The individual silymarin flavonolignans exhibited quite different plasma profiles for the parents and metabolites, with longer half-lives for the metabolites and conjugated SB_B and ISB_A as the major metabolites in the plasma of healthy volunteers. The role of silymarin or flavonoid metabolism on potential herbal-drug interactions, as well as that of modulating flavonoid disposition and pharmacological activity, is still poorly understood. This study provides a basic understanding of silymarin disposition and metabolism in healthy individuals that should help clarify and understand these complex relationships and provide a basis for comparison with studies of silymarin in patients that are ongoing.

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Footnotes

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Legends for figures

FIG. 1. Chemical structures of the silymarin flavonolignans and naringenin (IS).

FIG. 2. Representative HPLC-UV chromatogram for the determination of silymarin flavonolignans in milk thistle extracts. HPLC-UV (288 nm) analysis was conducted as described under *Materials and Methods*. Typical retention times of SC, SD, NG, SB_A, SB_B, ISB_A, and ISB_B under the experimental conditions used were 4.1, 4.8, 9.1, 10.8, 12.1, 15.0, and 16.0 min, respectively.

FIG. 3. Representative LC-ESI-MS chromatograms for the determination of silymarin flavonolignans in human plasma. Standardized milk thistle extracts (2.5 µg extract/ml in final) from Sigma-Aldrich were spiked into 100 µl of pooled blank human plasma, and analyzed by the LC-ESI-MS assay as described under *Materials and Methods*. Typical retention times of SC, SD, SB_A, NG, SB_B, ISB_A, and ISB_B under the experimental conditions used were 1.9, 2.4, 5.2, 5.5, 5.9, 8.4, and 9.2 min, respectively. **A**-SIM chromatogram for the six silymarin flavonolignans with $[M-H]^-$ (*m/z* 481); **B**-SIM chromatogram for NG (IS) with $[M-H]^-$ (*m/z* 271).

FIG. 4. MS spectra of the silymarin flavonolignans in the negative ion mode. LC-ESI-MS conditions were described under *Materials and Methods*.

FIG. 5. Plasma concentration-time profiles of the free (unconjugated) and total (free and conjugated) silymarin flavonolignans after a single oral administration of 600 mg standardized milk thistle extracts to three healthy volunteers. Quantification of silymarin flavonolignans was carried out by the LC-ESI-MS assay as described under *Materials and Methods*. Free-1~3; the free silymarin flavonolignans of the individual healthy volunteers; Total-1~3; the total silymarin flavonolignans of the individual healthy volunteers.

FIG. 6. Representative LC-ESI-MS chromatograms (SIM) of the free (unconjugated), conjugated (sulfated and glucuronidated), and total (free and conjugated) silymarin flavonolignans in plasma pooled from three healthy volunteers near the peak times (1 to 3 h) after a single oral administration of 600 mg standardized milk thistle extracts. Enzyme hydrolysis and LC-ESI-MS analysis were conducted as described under *Materials and Methods*. **A**-free silymarin (without enzyme hydrolysis); **B**-after sulfatase hydrolysis (containing D-saccharic acid 1,4-lactone); **C**-after β-glucuronidase hydrolysis; **D**-after mixed enzyme hydrolysis (sulfatase + β-glucuronidase).

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TABLE 1

Estimation of the silymarin flavonolignans in various commercial sources of milk thistle extracts

Milk Thistle Extract		SC	SD	SB_A	SB_B	ISB_A	ISB_B	Total	% of labeled silymarin (milk thistle extracts)
Sigma	mg/g[a]	122 ± 2.1	48.9 ± 1.0	115 ± 2.0	188 ± 3.2	44.6 ± 0.8	21.1 ± 0.5	540 ± 9.3	
	% (w/w)[b]	22.6	9.06	21.3	34.9	8.26	3.91	100	
USP	mg/g[a]	161 ± 2.0	102 ± 3.3	127 ± 1.5	205 ± 2.8	63.7 ± 1.2	27.5 ± 0.6	686 ± 11	
	% (w/w)[b]	23.5	14.9	18.4	29.9	9.28	4.01	100	
Tablet[c]	mg/tablet[a]	32.9 ± 1.4	13.6 ± 0.6	33.1 ± 1.5	52.8 ± 2.4	12.6 ± 0.6	5.50 ± 0.3	150 ± 6.7	79 (63)
	% (w/w)[b]	21.9	9.02	22.0	35.1	8.37	3.66	100	
Capsule 1[c]	mg/capsule[a]	39.2 ± 1.7	15.3 ± 0.6	37.7 ± 1.6	58.5 ± 2.5	14.8 ± 0.6	6.31 ± 0.23	172 ± 7.2	72 (57)
	% (w/w)[b]	22.8	8.93	22.0	34.0	8.59	3.67	100	
Capsule 2[c]	mg/capsule[a]	29.7 ± 1.1	10.9 ± 0.4	26.0 ± 0.9	41.6 ± 1.4	11.4 ± 0.4	5.11 ± 0.2	125 ± 3.9	89 (71)
	% (w/w)[b]	23.8	8.75	20.8	33.3	9.17	4.10	100	

[a] mean ± SD (n = 6).

[b] percentage of individual component relative to the total content of six silymarin flavonolignans

[c] labelled total silymarin contents (80% of milk thistle extracts): tablet, 190 mg; capsule 1, 240 mg; capsule 2, 140 mg.

TABLE 2

Pharmacokinetic parameters of the free (unconjugated), conjugated (sulfated and glucuronidated), and total (free and conjugated) silymarin flavonolignans in human plasma

Compound	Fraction	C_{max} *ng/ml*	T_{max} *h*	$t_{1/2}$ *h*	$AUC_{0\rightarrow\infty}$ *μg·h/l*
SC	Free	ND	ND	ND	ND
	Total	37 ± 7.8	3.7 ± 0.6	7.9 ± 1.7	401 ± 93
	Conjugated	37 ± 7.8	3.7 ± 0.6	7.9 ± 1.7	401 ± 93
SD	Free	ND	ND	ND	ND
	Total	21 ± 3.1	2.8 ± 1.3	3.3 ± 0.6	140 ± 47
	Conjugated	21 ± 3.1	2.8 ± 1.3	3.3 ± 0.6	140 ± 47
SB_A	Free	23 ± 7.9	1.0 ± 0.4	1.3 ± 0.5	63 ± 38
	Total	45 ± 16	1.3 ± 0.3	5.3 ± 2.3	260 ± 95
	Conjugated	26 ± 6.8	2.2 ± 0.8	6.0 ± 1.9	208 ± 65
SB_B	Free	15 ± 7.4	0.83 ± 0.1	2.3 ± 0.7	51 ± 19
	Total	131 ± 28	1.3 ± 0.3	3.4 ± 0.8	647 ± 234
	Conjugated	118 ± 21	1.5 ± 0.5	3.5 ± 0.8	597 ± 204
ISB_A	Free	9.7 ± 4.8	0.75 ± 0.3	3.2 ± 0.7	30 ± 8
	Total	113 ± 30	1.7 ± 0.3	4.1 ± 1.3	799 ± 403
	Conjugated	108 ± 30	2.5 ± 1.3	4.1 ± 0.1	734 ± 346
ISB_B	Free	8.5 ± 4.4	0.75 ± 0.3	2.4 ± 0.8	26 ± 12
	Total	59 ± 16	1.5 ± 0.0	4.0 ± 1.1	383 ± 195
	Conjugated	53 ± 13	2.5 ± 0.9	4.7 ± 2.3	355 ± 170

Plasma samples were obtained from three healthy volunteers following a single oral administration of 600 mg standardized milk thistle extracts at 0 (pre-dose) and 0.25-24 h. Concentrations of the free (without enzyme hydrolysis), total (mixed enzyme hydrolysis with sulfatase and *β*-glucuronidase) and conjugated (sulfated and glucuronidated) silymarin flavonolignans were measured or calculated as described under *Materials and Methods*. Data were expressed as mean ± SD (n = 3). ND, not detected.

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TABLE 3

Metabolic profiles of the free (unconjugated), conjugated (sulfated and glucuronidated), and total (free and conjugated) silymarin flavonolignans in human plasma near T_{max}

Plasma Concentration, *ng/ml* (Percentage of total, %)	SC	SD	SB_A	SB_B	ISB_A	ISB_B	Silymarin
Free	7.52	ND	21.7	13.9	3.72	6.30	53.1
	(14.0)	ND	(60.0)	(15.2)	(5.73)	(18.5)	(17.4)
Sulfated	19.8	ND	5.83	12.8	38.6	7.26	84.3
	(36.8)	ND	(15.8)	(14.1)	(59.5)	(21.4)	(27.6)
Glucuronidated	26.5	24.4	9.26	64.4	22.6	20.5	168
	(49.2)	(100)	(25.2)	(70.7)	(34.8)	(60.1)	(55.0)
Total	53.8	24.4	36.8	91.1	64.9	34.1	305

Plasma samples were pooled from three healthy volunteers near the peak times (1 to 3 h) for each flavonolignans after a single oral administration of 600 mg standardized milk thistle extracts. Enzyme hydrolysis and quantification of the free, sulfated, glucuronidated and total silymarin flavonolignans were conducted as described under *Materials and Methods*. Data were expressed as the mean of duplicates. Silymarin, sum of six flavonolignans. ND, not detected.

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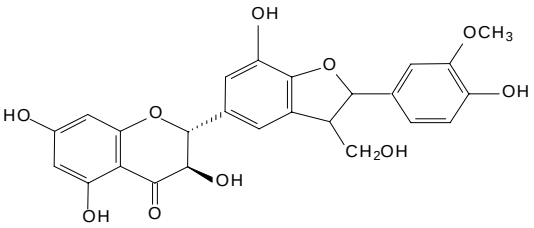
Fig. 1



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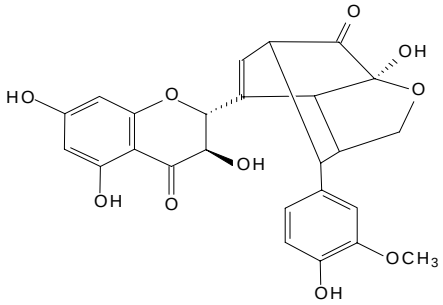
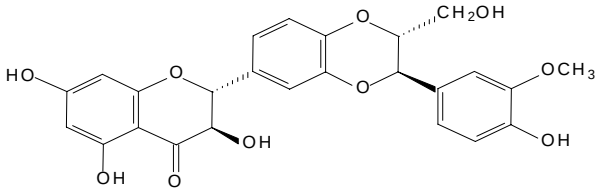
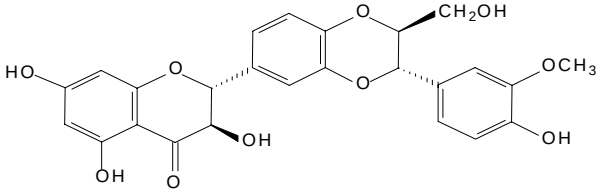


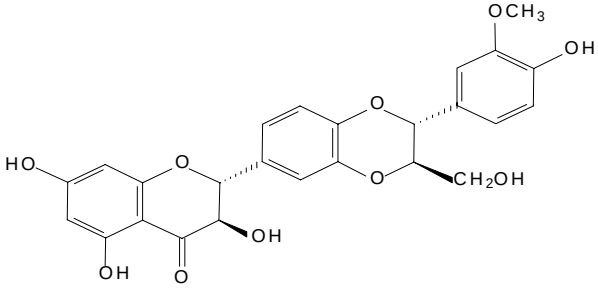
Silydianin (SD)



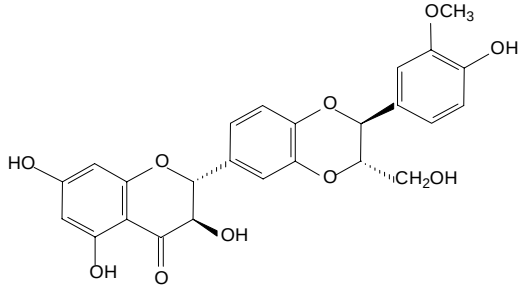
Silybin A (SB_A)



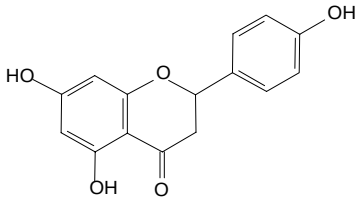
Silybin B (SB_B)



Isosilybin A (ISB_A)



Isosilybin B (ISB_B)



Naringenin (NG, internal standard)

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Fig. 2

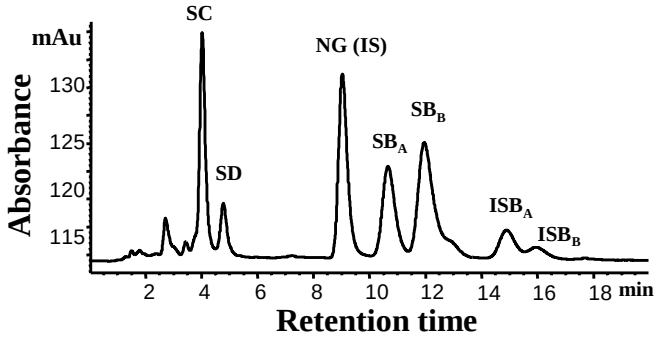

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Fig. 3

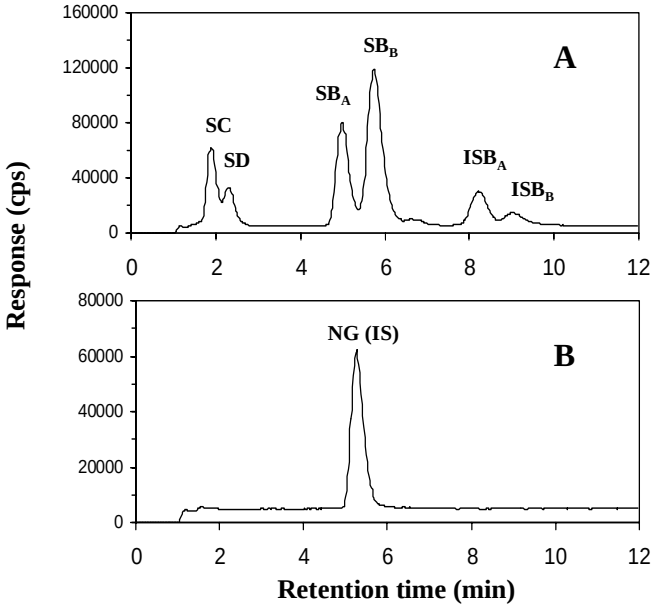

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Fig. 4

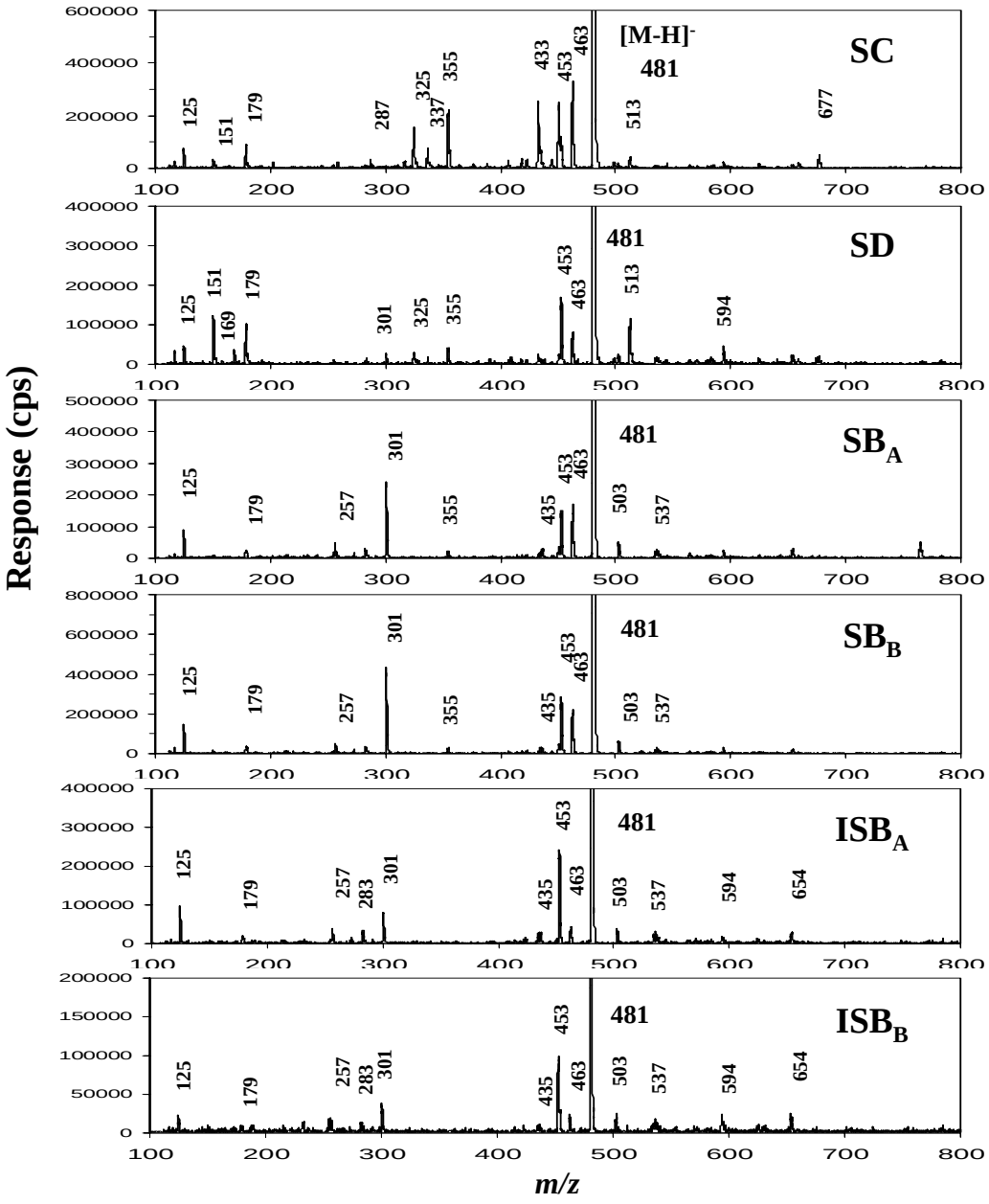

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Fig. 5

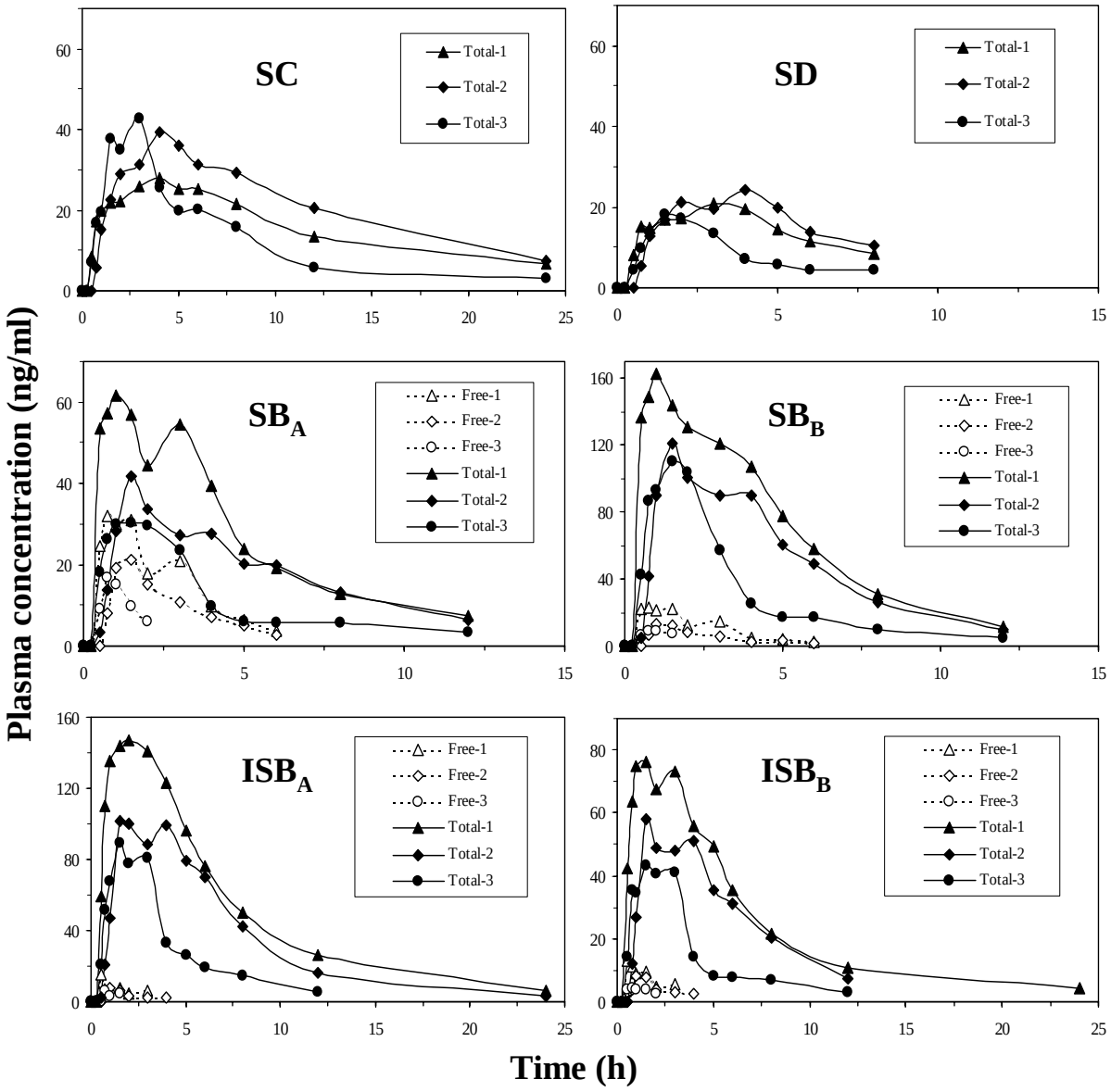

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Fig. 6