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## Title Page

**Title:** Differences in the Disposition of Silymarin Between Patients with Non-Alcoholic Fatty Liver Disease and Chronic Hepatitis C

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## Running Title Page

**Running Title:** Pharmacokinetics of silymarin in chronic HCV and NAFLD patients

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## ABBREVIATIONS

NAFLD, nonalcoholic fatty liver disease; HCV, hepatitis C virus; LC-MS, liquid chromatography-mass spectrometry; HPLC, high performance liquid chromatography;  $C_{\max}$ , maximum plasma concentration;  $T_{\max}$ , peak time at  $C_{\max}$ ;  $t_{1/2}$ , terminal elimination half-life;  $CL/F$ , apparent clearance;  $AUC_{0 \rightarrow 24h}$ , area under the plasma concentration-time curve from time 0 to 24 hours; ALT, alanine aminotransaminase

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## ABSTRACT

Silymarin, derived from the milk thistle plant *Silybum marianum* and widely used for self-treatment of liver diseases, is comprised of six major flavonolignans including silybin A and silybin B which are the predominant flavonolignans quantified in human plasma. The single and multiple dose pharmacokinetics of silymarin flavonolignans were examined in patients with nonalcoholic fatty liver disease (NAFLD) or hepatitis C virus (HCV) to determine if silymarin's disposition, and therefore its potential efficacy, varies between liver disease populations. Cohorts of eight subjects with non-cirrhotic liver disease were randomized 3:1 to oral silymarin or placebo (280 or 560 mg) every 8 hours for 7 days. 48-Hour blood sampling was conducted following the first and final doses. In general, plasma concentrations of silybin A and silybin B were higher while concentrations of conjugates were lower in NAFLD compared to HCV. After adjusting  $AUC_{0-8h}$  for weight and dose, only silybin B and silybin B conjugates differed significantly between disease types. For NAFLD, the adjusted mean  $AUC_{0-8h}$  was higher for silybin B ( $p<0.05$ ) but lower for silybin B conjugates ( $p<0.05$ ) compared to HCV. At the 280 mg dose, steady-state plasma concentrations of silybin B conjugates for NAFLD subjects were characterized by 46% lower  $AUC_{0-8h}$  ( $p<0.05$ ) and 42% lower  $C_{max}$  ( $p<0.05$ ) compared to HCV subjects. Evidence of enterohepatic cycling of flavonolignans was only observed in NAFLD subjects. In summary, silymarin's efficacy may be more readily observed in NAFLD patients due to higher flavonolignan plasma concentrations and more extensive enterohepatic cycling compared to patients with HCV.

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## INTRODUCTION

Silymarin is an herbal product that has been used for centuries for diseases of the liver (Flora et al., 1998), and approximately one-third of patients seen in US liver clinics report the use of some CAM to self-treat their liver disease (Strader et al., 2002). Derived from the milk thistle plant, *Silybum marianum*, silymarin is a complex mixture of six major flavonolignans (silybins A and B, isosilybins A and B, silychristin, and silydianin), as well as other minor polyphenolic compounds (Kim et al., 2003). Silymarin has been shown to have antioxidant, anti-inflammatory/immunomodulatory, and anti-fibrotic properties in various *in vitro* and animal models (Abenavoli et al., 2010). However, it is the antioxidant activity of silymarin that is most likely to attenuate the pathologic effects initiated by oxidative stress in the liver which influence pathways of inflammation, necrosis, and fibrosis in chronic liver disease (Galli et al., 2005; Medina and Moreno-Otero, 2005).

Silymarin may be the most potent antioxidant in nature by virtue of its free radical scavenger reactivity and favorable membrane-lipid/water partitioning (György et al., 1992). Oxidative stress is thought to play a central role in the etiology of nonalcoholic steatohepatitis (NASH), a specific subset of nonalcoholic fatty liver disease (NAFLD), and is hypothesized to represent a 'second hit' triggering the necroinflammatory response characteristic of NASH (Day and James, 1998). Therefore, the antioxidant properties of silymarin may be particularly beneficial as a treatment for NASH since patients have significantly increased levels of serum lipid peroxidation products (Chalasani et al., 2004) as well as other oxidative stress markers and decreased levels of antioxidant enzymes (Koruk et al., 2004). In addition, oxidative stress is a key feature of disease activity in HCV infection. Elevated levels of oxidative stress markers have been associated with the grade and stage of liver disease in patients with HCV (Jain et al., 2002) which suggests that antioxidant therapy may be effective in slowing disease

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progression in the absence of antiviral effects. These observations provide the rationale for current Phase 2 trials on the effects of silymarin in HCV and NASH populations.

The type and stage of liver disease has been recently shown to influence the single dose pharmacokinetics of the major silymarin flavonolignans (Schrieber et al., 2008). An unexpected finding was that total silymarin flavonolignan exposures were 3- to 5- fold higher for patient cohorts compared to healthy controls (Schrieber et al., 2008). While this study demonstrated that the pharmacokinetics of silymarin depend upon the type and grade/stage of liver disease, pharmacokinetic differences between patients with chronic HCV infection and NAFLD were not fully elucidated due to the low plasma concentrations.

Silymarin flavonolignans are metabolized via phase 2 conjugation pathways (Jancova et al., 2011; Sridar et al., 2004) and the majority of glucuronide and sulfate conjugates undergo hepatobiliary excretion via multi-drug resistance protein-2 (Mrp2) (Miranda et al., 2008). In obesity and NAFLD animal models, Mrp2 has been shown to have altered hepatic expression and function (Cheng et al., 2008; Geier et al., 2005). In addition, functional genetic polymorphisms in *MRP2* have been associated with susceptibility to NAFLD and disease severity (Sookoian et al., 2009). Therefore, disease-specific modulation of silymarin metabolizing enzymes or hepatic transporters may account for alterations in silymarin pharmacokinetics that have been previously observed in different types of liver diseases and therefore may have a profound effect on the efficacy in different patient populations.

We have previously reported on the ascending multiple dose pharmacokinetics of silymarin in noncirrhotic patients with chronic HCV infection (Hawke et al., 2010) obtained from a double-blind, placebo-controlled Phase 1 trial that was conducted in patients with either HCV or NAFLD. Unexpectedly, dose proportionality in the pharmacokinetics of parent silymarin flavonolignans was not

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observed in HCV patients with well-compensated liver disease at silymarin doses above 560 mg when administered orally every eight hours (Hawke et al., 2010). Since the steady-state pharmacokinetics of silymarin in patients with NALFD has not been previously described, and because silymarin's pharmacokinetics may be different in different types of liver diseases (Schrieber et al., 2008), we now report on the pharmacokinetics of silymarin in NAFLD subjects enrolled in the Phase 1 trial. To determine if the disposition of silymarin differs between patients with NAFLD or HCV liver disease, we also compare the single and multiple dose pharmacokinetics of silybin A and silybin B and their conjugates between patients with NAFLD or HCV. Finally, since silymarin's pharmacokinetics appears to be nonlinear in patients with HCV, the pharmacokinetics of silymarin was evaluated at silymarin doses of 280 mg and 560 mg to assess the interaction between dose and disease type. These trials were conducted to optimize oral silymarin dosing for Phase 2 efficacy trials in patients with either HCV or NASH (Lang, 2006). In these Phase 2 trials, which are now ongoing, oral doses higher than the customary dose of 140 mg every 8 hours are utilized in an attempt to overcome silymarin's high first-pass metabolism and achieve therapeutic, steady-state plasma concentrations.

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## MATERIALS AND METHODS

### Subjects

Forty male and female subjects  $\geq 18$  years of age with chronic noncirrhotic NAFLD and HCV were enrolled into the study within 28 days of screening (N=8 per cohort). Subjects were required to have elevated alanine aminotransferase levels  $\geq 65$  IU/L within 1 year prior to screening, and a creatinine clearance (calculated according to Cockcroft-Gault equation)  $> 60$  ml/min at screening as well as a negative urine pregnancy screen for females of child-bearing potential who were also required to use barrier methods of contraception during the study. .

Subjects were excluded if they had either a history of or, in the clinical opinion of the investigator's, evidence of decompensated liver disease defined by: serum albumin  $< 3.2$  g/dl, total bilirubin  $> 1.5$  mg/dl, or PT/INR  $> 1.3$  times normal, history or presence of ascites, encephalopathy, portal hypertension, or bleeding from esophageal varices. Subjects were also excluded if they had evidence of other chronic liver disease or serologic evidence of infection with human immunodeficiency virus. Other exclusion criteria included: an allergy to milk thistle or its preparations; use of silymarin or other milk thistle preparations, or use of high doses of other antioxidants such as vitamin E, vitamin C, glutathione, alpha-tocopherol, within 30 days of randomization through study completion. However, use of standard doses of over-the-counter multivitamins or cough/cold preparations was allowed. Also excluded was the chronic use of acetaminophen  $> 2$  grams/day; use of oral contraceptive, warfarin, metronidazole, or concurrent use of the following cytochrome CYP3A4 inducers: aminoglutethimide, aprepitant, carbamazepine, dexamethasone, efavirenz, ethosuximide, garlic supplements, glucocorticoids, glutethimide, griseofulvin, modafinil, nafcillin, nevirapine, oxcarbazepine, phenobarbital, phenytoin, primidone, rifabutin, rifampin, rifapentine, and St. John's Wort; historical liver

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biopsy demonstrating the presence of cirrhosis (Ishak stage 5 or 6), or  $\geq 15\%$  steatosis, or evidence of steatohepatitis; positive urine screen for drugs of abuse; alcohol consumption of  $> 12$  grams/day for  $\geq 6$  months prior to screening; or other evidence of alcohol or drug abuse within 6 months of screening. Women who were pregnant or breast-feeding were also excluded. All subjects agreed not to consume alcohol 48 hours prior to study randomization through study completion.

## Trial Design

Specific details on the design of this Phase 1 study have been previously described (Hawke et al., 2010). Briefly, dose cohorts of eight subjects each were randomized 3:1, via a web-based randomization system used by each site's pharmacist, to receive oral silymarin or placebo every 8 hours for 7 days. 48-hour pharmacokinetic samples were collected following an initial single dose administration before the 7 day treatment and a final dose following the 7 day treatment for a total of 23 doses. Only pharmacists were unblinded to treatment assignments until trial completion. The sample size was selected to provide information on safety, tolerability, and pharmacokinetics of silymarin and based on historical experience for Phase 1 trials and not on statistical considerations. Cohorts were enrolled sequentially at doses of 280 mg or 560 mg Legalon<sup>®</sup>. Legalon<sup>®</sup> (Madaus, Germany now Rottapharm|Madaus, Italy) brand of silymarin was selected as the clinical trial material for the Silymarin Product Development Program for use in NIH-sponsored clinical trials for liver diseases from competing bids in response to a Notice of Opportunity by the National Center for Complementary and Alternative Medicine and the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health.

The first and last doses for the pharmacokinetic studies were administered on days 1 and 10, respectively. To control for potential variability induced by fed versus fasted states, doses were



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administered with 240 ml of water 30 minutes after breakfast to subjects who were fasted overnight. Subjects were allowed to choose from a fixed list of items on the clinical research breakfast menu. Grapefruit juice was not allowed. Subjects remained in the research unit for 48 hours for collection of blood. Fourteen serial blood samples were collected at 0 (pre-dose), 0.5, 1, 1.5, 2, 4, 6, 8, 12, 15, 18, 24, 32, and 48 hours post-dose. Twenty-one doses were dispensed to subjects upon discharge after collection of the 48 hour post-dose sample on day 3. The first of these 21 doses was self-administered under direct supervision in the clinical research center. 8-Hour post-dose trough plasma samples were collected during safety visits on days 6 and 8. Patient adherence was assessed by patient drug diary, pill counts, and by maintaining records of drugs dispensed and returned.

Subjects were enrolled from December 2006 to July 2008 at 4 clinical centers, which included University of North Carolina at Chapel Hill, Beth Israel Deaconess Medical Center, University of Pennsylvania, and Thomas Jefferson University. Institutional review boards of participating centers approved the protocol; all subjects provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and guidelines on Good Clinical Practice.

## Safety Assessment

Safety was assessed before dosing on study days 1 (baseline), 6, 8, and 10, which consisted of clinical laboratory tests and reports of clinical adverse events using a symptom assessment questionnaire. Additionally, on days 1 and 10, the questionnaire was also completed at approximately 24 and 48 hours postdose. Common Terminology Criteria for Adverse Events (CTCAE v3.0) was utilized to grade the severity of adverse events. Physical examinations and electrocardiograms were completed at baseline and at end-of-study. Decisions to dose escalate were made after a safety

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evaluation by a designated safety committee masked to treatment. The safety committee consisted of the principal investigators from the four clinical centers and an external safety monitor.

## Study Drug

Silymarin (Legalon<sup>®</sup>) and matching placebo were manufactured in hard capsules by Madaus Rottapharm Group (Cologne, Germany); all study doses were administered from Lot No. 0418901. Each dose consisted of five silymarin and/or placebo capsules packaged in single use medicine dose cups. The flavonolignan content of each capsule was determined according to previously published LC-MS methods as follows: 23.2 mg, silybin A; 32.0 mg, silybin B; 11.8 mg, isosilybin A; 6.6 mg, isosilybin B; 24.9 mg, silychristin; and 29.0 mg, silydianin (Wen et al., 2008). These six flavonolignans account for 70.8% (127.5 mg silymarin equivalent to 140 mg of silymarin as determined by the manufacturer's DNPH method) of the 180 mg milk thistle extract contained in each capsule. Based on interim stability testing results performed by the manufacturer, Legalon<sup>®</sup> capsules are stable under normal conditions (25° C, 60% relative humidity) for at least 9 months. For the purpose of the pharmacokinetic analyses described in this report, one Legalon<sup>®</sup> capsule was considered equal to 140 mg of silymarin in accordance with the manufacturer's specifications.

## Analysis of Silymarin Flavonolignans

Whole blood samples were collected in two 3 ml EDTA-lined tubes (K<sub>2</sub>-EDTA tubes; BD, Franklin Lakes, NJ, USA) and centrifuged at 1200 x g for 10 minutes at 4°C. Plasma was aspirated and transferred to polypropylene tubes. Plasma samples were temporarily stored at -70°C by each clinical

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site for < 30 days prior to shipment to the University of North Carolina where they were acidified by addition of glacial acetic acid (final concentration 1% acetic acid) and stored at -70°C until analysis.

For the determination of parent (i.e. nonconjugated) flavonolignan concentrations in plasma, a 125 µl aliquot of each patient sample was buffered using sodium acetate (pH 5.0, 0.125 M) and incubated for 6 hours at 37°C without hydrolytic enzymes. A second 125 µl aliquot was also buffered using sodium acetate (pH 5.0, 0.125 M) and incubated with a mixture of sulfatase (80 U/ml, S9626 Type H-1) and β-glucuronidase (8000 U/ml, G0501 Type B-10) (Sigma-Aldrich, St. Louis, MO) for the determination of total (i.e. parent + conjugates) flavonolignan concentrations which were expressed as “Parent Flavonolignan Equivalents”. After incubation, 50 ng of naringenin (internal standard) in 25 µl of 50% MeOH was added to the samples which were then deproteinized and processed using a high-throughput protein filtration procedure as previously described (Hawke et al., 2010). Following filtration, 75 µl of the plasma sample supernatants were transferred to glass HPLC vials and concentrations of silymarin flavonolignans were quantified by LC-ESI-MS as previously described using a Luna C<sub>18</sub> analytical column (50 × 2.0 mm i.d., 3 µm; Phenomenex, Torrance, CA); an isocratic mobile phase consisting of 43% methanol, 56% water, and 1% glacial acetic acid (pH 2.8); a flow rate, 0.3 ml/min; a 25 µl injection volume; and a 13 minute run time (Wen et al., 2008). For each silymarin flavonolignan, the limit of detection was 20 ng/ml and the quantitative ranges for parent and for total flavonolignan were 50 – 2,500 ng/ml and 100 – 20,000 ng/ml, respectively. The accuracy for each flavonolignan was within 95.4 – 107.4% and intra- and inter-day precisions were 1.7 – 11% and 4.5 – 14%, respectively.

## Data Analysis

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Pharmacokinetic parameters including: area under plasma concentration-time curve (AUC); maximum plasma concentration ( $C_{\max}$ ); time to  $C_{\max}$  ( $T_{\max}$ ); and terminal half-life ( $T_{1/2}$ ) were calculated using noncompartmental methods, WinNonlin-Pro (v5.2; Pharsight Corp, Mountain View, CA). A constant dosing interval ( $\tau$ ) of 8 hours was assumed for the calculation of steady-state  $AUC_{0-8h}$  using the linear up/log down trapezoidal method. To obtain pharmacokinetic parameters for the conjugate flavonolignan concentrations, the parent flavonolignan concentrations were subtracted from the total flavonolignan concentrations at each time point over the entire sampling period prior to performing a pharmacokinetic analysis. Pharmacokinetic parameters are reported as geometric means with 95% confidence intervals, except for  $T_{\max}$  which is reported as median with minimum and maximum values. For our primary analysis, differences in steady-state exposures (i.e.,  $AUC_{0-8h}$ ) between disease cohorts were compared, following log transformation, using a parametric two-sample t-test,  $p < 0.05$  was used for statistical significance. In addition, to eliminate weight as a potential confounder in the assessment of differences in flavonolignan exposures between cohorts, a linear regression model with log  $AUC_{0-8h}$  as outcome was used. The model included dose, disease, and weight as independent variables in order to adjust for variable weights across dose groups (280 mg vs. 560 mg) or disease type (HCV vs. NAFLD) while comparing  $AUC_{0-8h}$ . Least-square means (adjusted means) were reported with 95% confidence intervals and tested using t-tests. All statistical analyses were performed by using SAS 9.2 or SAS JMP 9 (SAS Institute Inc., Cary, NC).

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## RESULTS

### Subjects

Baseline demographics are presented in Table I. Study subjects in the HCV cohorts were predominantly males with ages ranging from 43 – 59 years, while males and females were more equally represented in the NAFLD cohorts with ages ranging from 28 – 58 years. Subjects were characterized by well-compensated, noncirrhotic liver disease as evidenced by total bilirubin (range: 0.3 – 2.6 mg/dl) and platelet counts (range: 150 – 327 cells/mm<sup>3</sup>).

### Efficacy and Safety Endpoints

When compared to their screening baseline values, no reductions in serum transaminases for either HCV or NAFLD subjects, or reductions in HCV RNA titer for HCV subjects were observed at the end of the 7 day treatment period (data not shown).

There were no abnormal deviations from baseline laboratory values reported with silymarin administration for any cohort. For the HCV cohorts, 3 subjects who received a single 280 mg dose of silymarin reported a total of 4 adverse events. Three of the adverse events were classified as neurological (e.g., headache) while the other was classified as gastrointestinal. Only one adverse event (dizziness) was considered possibly related Legalon® administration and resolved in less than 1 day.

For NAFLD cohorts, two out of 12 subjects (16.7%) receiving silymarin reported at least one adverse event compared to 1 out of 4 subjects (25%) receiving placebo. Adverse events reported with silymarin included upper respiratory infection and abdominal pain, both of which occurred in the 560 mg dose cohort. All adverse events reported with silymarin were determined to be mild to moderate, self-limiting, and were considered unrelated to treatment.

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### **Single dose Pharmacokinetics of Silybin A and Silybin B**

A comparison of the pharmacokinetics of silybin A and silybin B between HCV and NAFLD cohorts following single oral doses of either 280 or 560 mg silymarin are presented in Table II. Silybin A was the predominant flavonolignan in plasma for both HCV and NAFLD cohorts and was characterized by a 2.7- to 3.3-fold greater  $C_{\max}$  and a 2- to 4.5-fold greater  $AUC_{0-48h}$  compared to silybin B.

At the 280 mg dose, no differences were observed in the pharmacokinetics of silybin A or silybin B between HCV and NAFLD subjects. Short elimination half-lives were observed for both silybin A and silybin B (range 0.9 – 1.8 hours).

However, at the 560 mg dose, pharmacokinetic differences were observed between HCV and NAFLD subjects. Compared to HCV subjects,  $AUC_{0-48h}$  for silybin A and silybin B were 1.5-fold ( $p > 0.05$ ) and 2.1-fold ( $p < 0.05$ ) greater, respectively, for NAFLD subjects. A similar trend was observed in the  $C_{\max}$  for silybin A and silybin B, although the 1.4- to 1.6-fold differences between HCV and NAFLD subjects did not achieve statistical significance. Elimination half-lives were similar between the disease groups (range 1.1 – 1.5 hours), while  $T_{\max}$  was delayed by 1 hour in NAFLD subjects.

### **Steady-State Pharmacokinetics of Silybin A and Silybin B**

The steady-state pharmacokinetics of silybin A and silybin B for the HCV and NAFLD cohorts following chronic oral administration of either 280 or 560 mg silymarin every 8 hours for 7 days are presented in Table III. Similar to the data obtained following single doses, silybin A was the predominant flavonolignan in plasma for both HCV and NAFLD cohorts and was characterized by a 2.1- to 3.6-fold greater  $C_{\max}$  and a 2.6- to 4.9-fold greater  $AUC_{0-8h}$  compared to silybin B. In addition,

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there was no evidence of accumulation for either flavonolignan following repeated dosing with elimination half-lives ranging between 0.7 to 1.3 hours. Also similar to the single dose data, pharmacokinetic differences between HCV and NAFLD cohorts were only observed at the 560 mg dose. The  $AUC_{0-8h}$  for silybin A and silybin B were 1.6-fold and 2.5-fold greater, respectively, in NAFLD subjects compared to HCV subjects at the 560 mg while differences in the  $C_{max}$  between cohorts ranged between 1.5- to 2.2-fold. After adjusting for weight and disease type, silybin A and silybin B  $AUC_{0-8h}$  differed significantly between the 280 and 560 mg dose groups ( $p \leq 0.004$ ), such that for either HCV or NAFLD or at any weight level, the 560 mg dose was associated with higher  $AUC_{0-8h}$ . When adjusted for weight and dose, only silybin B differed significantly across disease types such that adjusted mean  $AUC_{0-8h}$  for silybin B was higher for NAFLD compared to HCV ( $p = 0.004$ ). The higher silybin B exposures in NAFLD subjects suggest the metabolism or hepatic uptake of silybin B may be reduced in NAFLD compared to HCV.

#### Single dose and Steady-State Pharmacokinetics of Silybin A and Silybin B Conjugates

To further explore the effect of NAFLD on silymarin's metabolism, differences in the plasma concentrations of silybin A and silybin B conjugates between HCV and NAFLD subjects were examined. As defined in *Methods*, plasma concentrations of conjugates were estimated from the subtraction of parent flavonolignan concentrations from total (parent + conjugate) flavonolignan concentrations.

The single dose and steady-state pharmacokinetic data for total conjugates of silybin A and silybin B for both disease cohorts are presented in Tables IV and V, respectively. Whereas plasma concentrations were observed to be greater for silybin A than for silybin B, the converse was true for

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their conjugates. The  $C_{\max}$  and  $AUC_{0-8h}$  for silybin B conjugates were 3- to 4-fold greater than for silybin A conjugates across both dose levels and disease cohorts.

Differences between HCV and NAFLD subjects were observed in the pharmacokinetics for plasma conjugates of silybin A and silybin B at either dose level following single or chronic dosing. However, these differences only achieved significance between HCV and NAFLD cohorts dosed at 280 mg every 8 hours whereas conjugates of silybin B in plasma of NAFLD subjects were characterized by 46% lower  $AUC_{0-8h}$  ( $p < 0.05$ ) and 42% lower  $C_{\max}$  ( $p < 0.05$ ) compared to HCV subjects. Figure 1 depicts the mean steady-state plasma concentration versus time profiles for silybin B (inset) and silybin B conjugates for HCV and NAFLD subjects at the 280 mg dose. Plasma concentrations of silybin B conjugates were lower in NAFLD subjects compared to HCV subjects over the entire 8 hour dosing interval (Figure 1). In contrast, plasma concentrations of silybin B were higher in NAFLD subjects until peak concentrations were achieved and then declined similarly (Figure 1 inset). These data suggest that reduced silymarin metabolism may result in differences in silymarin exposures between NAFLD and HCV subjects, rather than differences in absorption.

After adjusting for weight and disease type, the  $AUC_{0-8h}$  for silybin A conjugates and for silybin B conjugates differed significantly between the 280 and 560 mg dose groups ( $p \leq 0.004$ ), such that for either HCV or NAFLD or at any weight level, the 560 mg dose was associated with higher  $AUC_{0-8h}$ . When adjusted for weight and dose, only silybin B conjugates differed significantly across disease types such that adjusted mean  $AUC_{0-8h}$  for silybin B conjugates was significantly lower for NAFLD subjects compared to HCV ( $p = 0.03$ ).

To further quantify differences in the extent of flavonolignan conjugation between HCV and NAFLD subjects, steady-state metabolic ratios were calculated as the ratio of  $AUC_{0-8h}$  for silybin B



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divided by  $AUC_{0-8h}$  for silybin B conjugates at the 560 mg dose. Metabolic ratios differed 4-fold ( $p < 0.05$ ) between HCV and NAFLD with means ( $\pm$  SD) of  $0.016 \pm 0.011$  and  $0.060 \pm 0.041$ , respectively. These data suggest that there is less conjugation of silybin B in NAFLD subjects compared to HCV at a silymarin dose of 560 mg. In summary, plasma concentrations of silybin A and silybin B were generally greater and the concentrations of their conjugates lower in NAFLD subjects compared to HCV subjects irrespective of the dose and frequency of oral silymarin administration.

### Flavonolignan Accumulation

The ratio of parent silybin A steady-state  $AUC_{0-8h}$  divided by single-dose  $AUC_{0-8h}$  was calculated as an indication of the extent of accumulation following chronic three times daily dosing. Silybin A ratios of 1.3 and 1.4 were calculated for HCV and NAFLD, respectively, at the 560 mg dose, which indicates no significant accumulation in either cohort with repeated dosing. Similar ratios were calculated for silybin B. This finding is consistent with the short half-life of the silymarin flavonolignans.

While no evidence for parent silybin A and silybin B accumulation was observed, the overall amount of parent flavonolignans in plasma was significantly higher in NAFLD subjects compared to HCV subjects at the 560 mg dose due to the appearance of additional parent flavonolignans. Figure 2 compares mean steady-state peak plasma concentrations of the six parent silymarin flavonolignans for HCV and NAFLD subjects at the 560 mg dose, as well as their sum concentration. As seen in Figure 2, plasma concentrations of isosilybin A, isosilybin B, silychristin, and silydianin were significantly greater in NAFLD subjects compared to HCV subjects. Interestingly, silychristin and silydianin were not detected in the plasma of HCV subjects. To gain insight into the mechanism(s) behind these observed

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differences, we evaluated the plasma concentration versus time profile for each flavonolignan over the 48 hour sampling period following administration of the last 560 mg dose (Figure 3). Significant enterohepatic cycling of the six flavonolignans were observed in NAFLD subjects as indicated by a prominent second peak at 4 hours following the absorption peak at 1 hour. Most flavonolignans also showed evidence of a third peak at 8 hours post dose. In contrast, there was less evidence of enterohepatic cycling in HCV subjects where no secondary peaks were observed for either silybin A or silybin B following the early absorption peak. Silychristin represented a major flavonolignan in the plasma of NAFLD subjects at the dose 560 mg dose. Silychristin's steady-state pharmacokinetics (geometric mean and 95% confident intervals) were characterized by a  $C_{\max}$  of 67 ng/ml (-2.5, 174), an  $AUC_{0-8h}$  of 325 ng•hr/ml (-145, 1100), and a  $T_{1/2}$  of 3.1 hr (1.2, 6.3). The steady-state pharmacokinetics of the conjugates of silychristin in NAFLD subjects were characterized by a  $C_{\max}$  of 663 ng/ml (367, 1394), an  $AUC_{0-8h}$  of 3800 ng•hr/ml (1628, 8462), and a  $T_{1/2}$  of 4.5 hr (2.2, 8.6).

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## DISCUSSION

The expression of drug disposition genes and their protein products have been shown to be altered in liver disease (Congiu et al., 2009; Fisher et al., 2009; Congiu et al., 2002), and effects of liver disease on the disposition of drugs have been demonstrated and tend to be more severe in patients with more advanced cirrhotic disease (Chalon et al., 2003). In contrast, significant differences in the disposition of drugs between different types of liver disease have not been demonstrated. We have shown that the disposition of silymarin, an herbal medicine widely used by patients with liver disease, is significantly altered in patients with liver disease (Schrieber et al., 2008). Concentrations of total silymarin species found in plasma, which consist primarily of flavonolignan conjugates, were found to be approximately 5-fold higher in patients with chronic HCV infection or NAFLD when compared to healthy controls. Pharmacokinetic differences were also observed between healthy subjects and patients with NAFLD or patients with HCV cirrhosis. In contrast, differences were not observed between healthy subjects and patients with noncirrhotic HCV disease possibly due to wide disease heterogeneity in patient cohorts or reduced sensitivity as a result of low plasma concentrations of flavonolignans associated with the low oral dose of a generic brand of silymarin that was used in this study (Schrieber et al., 2008). These results raised the possibility that the disposition of silymarin, and its potential beneficial effects, may be different in various liver disease populations with early stage disease. To determine if the disposition of silymarin is different between patients with different types of the liver disease, this study examined the pharmacokinetics of higher than customary oral doses of silymarin in noncirrhotic patients with either chronic HCV infection or NAFLD. The results of our study show that NAFLD patients are characterized by higher plasma concentrations of certain silymarin flavonolignans and lower concentrations of flavonolignan conjugates compared to HCV patients administered the same

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dose. While silymarin flavonolignans appear to share common pathways of metabolism and transport, differences in their affinity for these processes have been noted (Miranda et al., 2008; Sridar et al., 2004) which likely account for the different relationships between AUC exposure and dose for silybin A and silybin B observed in our study.

*In vitro* and *in vivo* studies suggest silymarin flavonolignans are primarily metabolized through glucuronidation and sulfation pathways with various UDP-glucuronosyltransferases (UGTs) sharing overlapping specificity (Sridar et al., 2004; Jancova et al., 2011). In addition, the extent in which various flavonolignans undergo glucuronidation or sulfation appears to vary (Wen et al., 2008). There are several possibilities that could explain why the ratio of parent flavonolignan (e.g., silybin B) to flavonolignan conjugates was higher in patients with NAFLD compared to HCV in our study. The simplest explanation is that the expression or activity of UGTs is decreased in NAFLD subjects. Nonalcoholic steatohepatitis, a specific subset of NAFLD, is characterized by hepatic steatosis, and varying degrees of inflammation which can lead to decreased UGT expression which has been observed in rodents (Richardson et al., 2006) and in human liver tissue (Congiu et al., 2002). Therefore it is plausible that the major UGT isoforms involved in metabolism of silymarin may be lower in NAFLD subjects resulting in higher plasma levels of parent flavonolignans and lower concentrations of conjugates. Since silybin B conjugates represents 99% of the total (parent + conjugates) silybin B species in HCV patient plasma, metabolism stoichiometry predicts that the 40% reduction in silymarin conjugates observed in our NAFLD cohort should result in an ~30-fold increase in silybin B plasma concentrations. However, plasma concentrations of silybin B were comparable between HCV and NAFLD patients. Therefore, reduced UGT activity does not appear to be a viable explanation for the differences in silymarin pharmacokinetics between HCV and NAFLD in our study. In addition, the

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lower plasma concentration of flavonolignan conjugates in NAFLD compared to HCV does not appear to be related to reduced intestinal absorption since parent flavonolignans would also be expected to be lower in plasma.

Alterations in the expression and function of hepatobiliary transporters may be a more plausible explanation for the decrease in flavonolignan conjugates and the higher plasma concentrations of parent flavonolignans observed in the NAFLD cohorts. Evidence for extensive enterohepatic cycling of silymarin and their conjugates has been observed at high doses of silymarin (Hawke et al., 2010; Schrieber et al., 2008). Enterohepatic cycling is regulated by hepatobiliary transporters involved in the active uptake of anionic and cationic compounds from the blood such as the organic anion transporting polypeptides, OATP1B1 and OATP2B1, located on the basolateral membrane of the hepatocyte (Chandra et al., 2004). In many instances, these compounds undergo metabolism to more polar conjugates followed by transport and biliary excretion by ATP-binding cassette transporters such as P-glycoprotein, multidrug resistance associated protein 2 (MRP2), and breast cancer resistance protein, located at the canalicular membrane of the hepatocyte (Leslie et al., 2005; Schinkel and Jonker, 2003). Once delivered to the small intestine, parent compounds can be reformed by bacterial deconjugation and returned to portal blood for delivery to the liver for reuptake. In competition with biliary efflux, is the efflux of substrates from the hepatocyte to blood by other members of the MRP family, such as MRP3 and MRP4 (MRPs 3/4), which are located on the basolateral (sinusoidal) membrane. It is generally thought that MRP2 and MRP3 work in concert in liver disease to promote hepatic efflux and protect the hepatocyte from the effects of cholestasis (Van de Steeg et al., 2010; Wagner et al., 2005).

The most intriguing observation in the current study was the suggestion of significant enterohepatic recycling of silymarin flavonolignans in NAFLD subjects in contrast to HCV subjects

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where there was no evidence of enterohepatic cycling (see Figure 3). Silymarin flavonolignans demonstrate high affinity for MRP4 (Wu et al., 2005) while silymarin conjugates, but not parent flavonolignans, appear to be better substrates for MRP2 (Miranda et al., 2008). Glucuronides that are substrates for MRP2, such as conjugated bilirubin, can also be substrates for MRPs 3/4 (Borst et al., 2006; Zelcer et al., 2006). Therefore, differences in the disposition and enterohepatic cycling of silymarin flavonolignans may reflect alterations in the function of hepatobiliary transporters as a result of liver disease.

In obesity and NAFLD animal models, Mrp2 has been shown to have altered hepatic expression and function (Cheng et al., 2008; Geier et al., 2005). In addition, Mrp2, Mrp3, and Mrp4 protein expression were significantly increased in a rodent model of NAFLD (Lickteig et al., 2007). The biliary excretion of glucuronide and sulfate conjugates of silymarin flavonolignans was shown to be dependent on Mrp2 using isolated perfused livers, and some flavonolignans such as silychristin and silydianin were almost quantitatively secreted into bile (Miranda et al., 2008). Therefore, enterohepatic cycling of silymarin flavonolignans may be increased in NAFLD due to increased MRP2-dependent biliary efflux and diversion of silymarin conjugates away from sinusoidal efflux to blood. An increase in MRP4 would also contribute to greater sinusoidal efflux of parent flavonolignans. These changes would result in lower plasma concentrations of silymarin conjugates with higher concentrations of recycling silymarin flavonolignans in NAFLD patients compared to HCV.

Alternatively, the differences observed in the disposition of silymarin between NAFLD and HCV patients may reflect HCV-specific alterations in hepatobiliary function. HCV infection was shown to be associated with increased hepatic expression of MRP4, decreased expression of MRP2, and decreased expression of OATP1B1 in cirrhotic and noncirrhotic liver while the expression of MRP3 and

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OATP2B1 were similar to that in normal human liver (Ogasawara et al., 2010). Therefore, the differences in the disposition of silymarin between HCV and NAFLD subjects observed in our study may reflect a diversion of silymarin conjugates to sinusoidal efflux in HCV patients due to reduced biliary efflux by MRP2 or reduced uptake by OATP1B1, which would also result in higher plasma concentrations of silymarin conjugates and decreased enterohepatic cycling of silymarin flavonolignans compared to patients with NAFLD. While the results of our study cannot delineate between these various potential mechanisms, it is possible that silymarin's disposition is altered by different, disease-specific mechanisms in NAFLD and HCV populations. This conclusion is supported by our previous observation that plasma concentrations of silymarin conjugates are significantly higher in both NAFLD and HCV patients compared to concentrations found in healthy volunteers (Schrieber et al., 2008).

In summary, differences in the disposition of silymarin between NAFLD and HCV patients may reflect different disease-specific alterations in the function of hepatobiliary transport proteins. These observations are significant because differences in the disposition of drugs between different types of liver disease have not been demonstrated, perhaps because of their more restrictive use indications. Importantly, the antioxidant activity and potential antiinflammatory and antifibrotic effects of silymarin on disease progression will be dependent on its hepatic disposition. Oxidative stress has been associated with all stages of chronic HCV liver disease (Jain et al., 2002) and recent data from the HALT-C trial suggest that silymarin use among patients with advanced HCV liver disease may be associated with reduced progression to cirrhosis (Freedman et al., 2011). Compared to HCV infection, silymarin may demonstrate greater benefits in patients with NAFLD since oxidative stress is thought to play a central role in the etiology of NASH (Day et al., 1998) and there are no approved therapies. In addition, the results of this study suggest silymarin's effects on liver disease progression may also be greater in

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NAFLD patients due to higher flavonolignan plasma concentrations and more extensive enterohepatic cycling compared to patients with HCV. These observations were critical in the design of a Phase 2 silymarin trial in NASH which is currently ongoing (Lang, 2006).



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### **Authorship Contributions**

*Participated in research design:* Hawke, Reddy, Belle, Afdhal, Navarro, Meyers, Doo, Fried

*Conducted experiments:* Wen, Schrieber

*Contributed new reagents or analytic tools:* Hawke, Smith

*Performed data analysis:* Schrieber, Wahed

*Wrote or contributed to the writing of the manuscript:* Schrieber, Hawke

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## Footnote

This was an investigator-initiated trial, and Rottapharm|Madaus had no direct or indirect involvement in the design of the trial, data collection, preparation, or submission of the manuscript for this registered (<http://clinicaltrials.gov/ct2/show/NCT00389376>) investigator-initiated trial. None of the authors have a personal conflict of interest with the manufacturer of any of the marketed silymarin formulations.

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## Figure Legends

**Figure 1. Steady-state plasma concentration vs. time profiles for silybin B conjugates and parent silybin B (inset) at 280 mg silymarin in HCV (●) and NAFLD (□) subjects.**

48-hour plasma samples were obtained after a final single dose administration following an every 8 hour x 7 day dose regimen.  $AUC_{0-8h}$  and  $C_{max}$  for silybin B conjugates were 46% and 42% lower, respectively, in NAFLD subjects compared to HCV subjects,  $p < 0.05$ .

**Figure 2. Maximum steady-state plasma concentrations for silymarin flavonolignans at 560 mg silymarin in HCV (■) and NAFLD (□) subjects.**

Plasma concentrations of isosilybin A, isosilybin B, silychristin, and silydianin were significantly greater in NAFLD subjects compared to HCV subjects. Silychristin and silydianin were not detected in the plasma of HCV subjects.

**Figure 3. Steady-state plasma concentration vs. time profiles for silymarin flavonolignans at 560 mg silymarin in HCV and NAFLD subjects.**

48-hour plasma samples were obtained after a final single dose administration following an every 8 hour x 7 day dose regimen. Evidence of enterohepatic recycling of flavonolignans by the appearance of secondary peaks was observed in NAFLD subjects (–), while no evidence of enterohepatic recycling for silybin A or silybin B was observed in HCV subjects (--). In addition to silybin A and silybin B, silychristin (▲) represented a major flavonolignan in NAFLD subjects. For presentation clarity, error bars were not included.

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**Table I. Subject baseline demographics.**

	Group and Cohort			
	HCV		NAFLD	
	280 mg*	560 mg	280 mg	560 mg
Male : Female, n	8:4	5:1	3:3	2:4
White : Black, n	10:2	4:2	6:0	6:0
Age, years	50 (44, 59)	51 (43, 54)	51 (28, 58)	48 (28, 52)
Weight, kg	92 (67, 99)	104 (86, 123)	104 (79.0, 150)	101 (78.2, 143)
BMI, kg/m <sup>2</sup>	28 (25, 42)	33 (26, 41)	38 (27, 42)	38 (27, 43)
Total bilirubin, mg/dl	0.8 (0.3, 1.0)	0.6 (0.3, 1.4)	0.6 (0.3, 1.1)	0.5 (0.3, 2.6)
ALT, U/L	95 (58, 288)	113 (81, 214)	99 (52, 322)	104 (77, 115)
AST, U/L	64 (43, 271)	89 (46, 110)	60 (38, 325)	66 (54, 104)
Platelets, cells/mm <sup>3</sup>	208 (177, 327)	192 (150, 225)	275 (157, 341)	282 (162, 319)

\*Two cohorts of 6 subjects were used to study single and multiple dose pharmacokinetics. Data are presented as medians (minimum, maximum). BMI, body mass index.

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**Table II. Single dose pharmacokinetics of parent silybin A and silybin B.**

Single Dose Pharmacokinetics <sup>a</sup>					
	Flavonolignan	SA		SB	
Cohort	PK Parameter	HCV	NAFLD	HCV	NAFLD
280 mg	AUC <sub>0-48h</sub> (ng•hr/ml)	201 (115, 338)	228 (75, 469) <sup>b</sup>	93 (15, 188) <sup>c</sup>	80 (70, 91) <sup>b</sup>
	C <sub>max</sub> (ng/ml)	78 (32, 147)	82 (35, 153) <sup>b</sup>	27 (8, 50) <sup>c</sup>	30 (16, 53) <sup>b</sup>
	T <sub>max</sub> (hr)	2.0 (1.0, 4.0) <sup>c</sup>	2.0 (1.0, 6.0) <sup>b</sup>	3.0 (1.0, 4.0) <sup>c</sup>	2.0 (2.0, 6.0) <sup>b</sup>
	T <sub>1/2</sub> (hr)	1.3 (0.7, 2.0)	1.8 (-2.2, 7.6)	0.9 (-0.9, 6.3) <sup>c</sup>	1.8 (-1.6, 6.6)
560 mg	AUC <sub>0-48h</sub> (ng•hr/ml)	557 (470, 657)	859 (508, 1397)	125 (94, 160) <sup>b</sup>	261 (164, 395)
	C <sub>max</sub> (ng/ml)	192 (147, 250)	275 (127, 491)	58 (31, 92) <sup>b</sup>	93 (55, 145)
	T <sub>max</sub> (hr)	1.5 (1.0, 4.0)	2.7 (1.5, 4.0)	1.5 (0.5, 4.0) <sup>b</sup>	2.7 (1.0, 4.0)
	T <sub>1/2</sub> (hr)	1.4 (0.9, 2.1)	1.4 (0.8, 2.3)	1.1 (0.7, 1.7) <sup>b</sup>	1.5 (0.6, 2.9)

<sup>a</sup> Results are shown as geometric mean (95% confidence interval), except for T<sub>max</sub> which is shown as median (minimum, maximum). Data are for N=6 subjects.

<sup>b</sup> N=5

<sup>c</sup> N=4

† p < 0.05

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**Table III. Steady-state pharmacokinetics of silybin A and silybin B.**

<b>Steady-State Pharmacokinetics<sup>a</sup></b>					
	Group	SA		SB	
Cohort	PK Parameter	HCV	NAFLD	HCV	NAFLD
280 mg	AUC <sub>0-8h</sub> (ng•hr/ml)	370 (279, 480)	317 (191, 499)	86 (66, 109)	123 (53, 219) <sup>b</sup>
	C <sub>max</sub> (ng/ml)	143 (78, 242)	133 (60, 262)	45 (28, 72)	64 (30, 111) <sup>b</sup>
	T <sub>max</sub> (hr)	1.8 (1.0, 4.0)	1.3 (0.5, 4.4)	1.5 (0.5, 4.0)	1.2 (0.5, 2.0) <sup>b</sup>
	T <sub>1/2</sub> (hr)	1.1 (0.7, 1.6)	1.1 (0.3, 2.6)	0.7 (0.4, 1.2) <sup>b</sup>	0.9 (0.2, 2.1) <sup>b</sup>
560 mg	AUC <sub>0-8h</sub> (ng•hr/ml)	729 (371, 1195)	1166 (589, 2128)	149 (40, 310)	376 (161, 759)
	C <sub>max</sub> (ng/ml)	308 (104, 620)	448 (255, 724)	86 (26, 186)	187 (93, 332)
	T <sub>max</sub> (hr)	1.5 (1.5, 2.0)	3.0 (0.5, 4.0)	1.5 (1.5, 2.0)	3 (0.5, 4.0)
	T <sub>1/2</sub> (hr)	1.3 (0.9, 1.9)	1.0 (0.6, 1.6)	1.1 (0.3, 2.2)	0.7 (0.4, 1.0)

<sup>a</sup> Results are shown as geometric mean (95% confidence interval), except for T<sub>max</sub> which is shown as median (minimum, maximum). Data are for N=6 subjects, except for the HCV 560 mg steady-state cohort where N=5, one subject was dropped from the pharmacokinetic analysis due to incorrect dosing for pharmacokinetic sampling at steady-state on day 8.

<sup>b</sup> N=5

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**Table IV. Single dose pharmacokinetics of silybin A conjugates and silybin B conjugates.**

Single Dose Pharmacokinetics <sup>a</sup>					
	Group	SA <sub>conjugates</sub>		SB <sub>conjugates</sub>	
Cohort	PK Parameter	HCV	NAFLD	HCV	NAFLD
280 mg	AUC <sub>0-48h</sub>	1327 (860,	1003 (672,	4094(2465,	3120 (2247,
	(ng•hr/ml)	1925)	1456)	6308)	4233)
	C <sub>max</sub> (ng/ml)	144 (96, 205)	80 (45, 125)	586 (441,	388 (266,
				762)	544)
	T <sub>max</sub> (hr)	2 (1.5, 4.0)	4.0 (2.0, 12.0)	2.0 (1.5, 4.0)	3.0 (2.0, 8.0)
560 mg	T <sub>1/2</sub> (hr)	5.7 (4.0, 7.7)	6.4 (4.5, 9.0)	4.3 (3.1, 5.6)	4.5 (2.5, 7.2)
	AUC <sub>0-48h</sub>	3468 (1747,	2844 (1493,	12760 (6505,	7850 (4501,
	(ng•hr/ml)	6024)	4779)	22432)	12664)
	C <sub>max</sub> (ng/ml)	339 (169,	278 (126,	1691 (1003,	1125 (805,
		621)	488)	2774)	1570)
	T <sub>max</sub> (hr)	3.0 (2.0, 4.0)	4.0 (4.0, 6.2)	4.0 (1.0, 4.0)	3.0 (1.5, 6.0)
	T <sub>1/2</sub> (hr)	6.6 (4.5, 9.2)	7.0 (5.0, 9.7)	4.7 (3.7, 6.0)	6.2 (3.3, 10.3)

<sup>a</sup> Results are shown as geometric means (95% confidence interval), except for T<sub>max</sub> which is shown as median (minimum, maximum). Data are for N=6 subjects.

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**Table V. Steady-state pharmacokinetics of silybin A conjugates and silybin B conjugates.**

Steady-State Pharmacokinetics <sup>a</sup>					
	Group	SA <sub>conjugates</sub>		SB <sub>conjugates</sub>	
Cohort	PK Parameter	HCV	NAFLD	HCV	NAFLD
280 mg	AUC <sub>0-8h</sub> (ng•hr/ml)	2048 (1465, 2815)	1297 (652, 2271)	7278 (5633, 9287) †	3962 (2338, 6292)
	C <sub>max</sub> (ng/ml)	369 (294, 459) †	233 (146, 352)	1294 (1040, 1589) †	750 (563, 981)
	T <sub>max</sub> (hr)	2 (1, 4)	2 (0, 4)	2 (1, 4)	1.8 (0, 2)
	T <sub>1/2</sub> (hr)	5 (3, 8.3)	6.8 (2.5, 15.5)	4.3 (2.8, 6.3)	5.1 (1.2, 12.7)
560 mg	AUC <sub>0-8h</sub> (ng•hr/ml)	3229 (1618, 5348)	2902 (1403, 5163)	11003 (3244, 21930)	7745 (4312, 12547)
	C <sub>max</sub> (ng/ml)	543 (182, 1050)	529 (287, 894)	2074 (700, 4103)	1592 (927, 2600)
	T <sub>max</sub> (hr)	2 (0, 4)	4 (0, 6)	2 (2, 4)	3 (0, 4)
	T <sub>1/2</sub> (hr)	6.2 (3.6, 9.3)	3.6 (2.7, 4.7)	4.1 (2.9, 5.6)	2.8 (1.9, 3.9)

<sup>a</sup> Results are shown as geometric means (95% confidence interval), except for T<sub>max</sub> which is shown as median (minimum, maximum). Data are for N=6 subjects, except for the HCV 560 mg steady-state cohort where N=5; one subject was dropped from the pharmacokinetic analysis due to incorrect dosing for pharmacokinetic sampling at steady-state on day 8.

† p < 0.05



Figure 1.

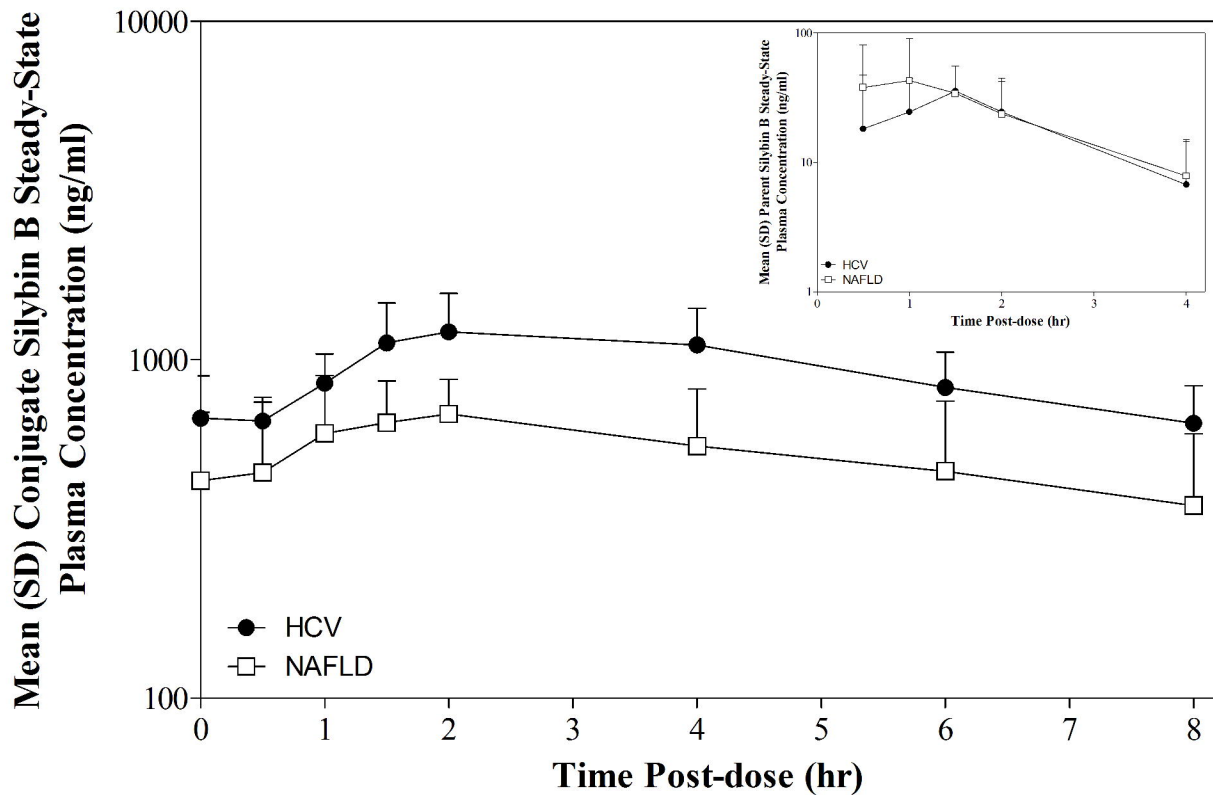


Figure 2.

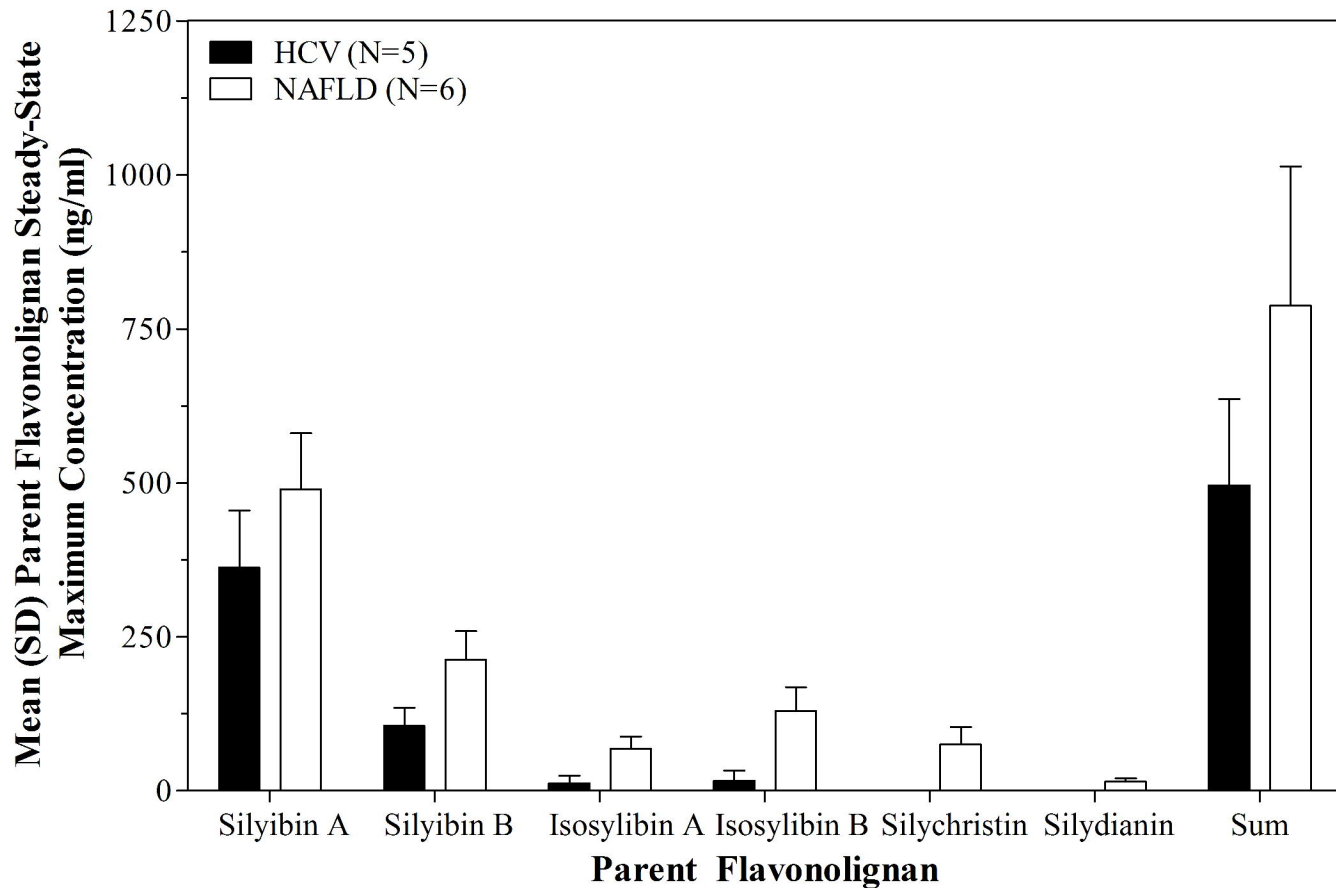


Figure 3.

Mean Parent Flavonolignan Steady-State  
Plasma Concentration (ng/ml)

