JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Quantitation of Silibinin, a Putative Cancer Chemopreventive Agent Derived from Milk Thistle (*Silybum marianum*), in Human Plasma by High-Performance Liquid Chromatography and Identification of Possible Metabolites

CARMEN S. L. HOH,[†,‡] DAVID J. BOOCOCK,*[,†] TIMOTHY H. MARCZYLO,[†] V. A. BROWN,[†] HONG CAI,[†] WILLIAM P. STEWARD,[†] DAVID P. BERRY,[‡] AND ANDREAS J. GESCHER[†]

Cancer Biomarkers and Chemoprevention Group, Department of Cancer Studies and Molecular Medicine, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, Leicester, LE2 7LX, United Kingdom; and Department of Hepatobiliary and Pancreatic Surgery, The Leicester General Hospital, Gwendolen Road, Leicester, LE5 4PW, United Kingdom

Silibinin has recently received attention as a potential cancer chemopreventive agent because of its antiproliferative and anticarcinogenic effects. A simple and specific reversed-phase high-performance liquid chromatography method was developed and validated for the quantitation of silibinin in human plasma. Sample preparation involved simple protein precipitation, and separation was achieved on a Waters Atlantis C_{18} column with flow rate of 1.0 mL/min at 40 °C and UV detection at 290 nm. Silibinin was detected as two peaks corresponding to trans-diastereoisomers. The peak area was linear over the investigated concentration range (0−5000 ng/mL). The limits of detection were 2 and 1 ng/mL for the two diastereoisomers (d1 and d2), with a recovery of 53−58%. This method was utilized to detect silibinin in plasma of colorectal patients after 7 days of treatment with silipide (silibinin formulated with phosphatidyl choline).

KEYWORDS: Silibinin; silipide; silybin; IDB 1016; HPLC; human plasma; milk thistle; *Silybum marianum*

INTRODUCTION

Silymarin, a mixture of flavonolignans extracted from the seeds, fruits, and leaves of milk thistle (*Silybum marianum*), has been used traditionally for the treatment of hepatic disorders such as acute viral hepatitis (*1*), alcoholic liver disease (*2*, *3*), and death cap mushroom poisoning (*4*). Silymarin consists of silibinin (34%), isosilibinin (26%), silydianin (20%), silycristin (20%), and traces of taxifolin (*5*). Silibinin exists as two trans-diastereoisomers (*6*) (**Figure 1**). The names silybinin, silibinin, silibin, or silybin have been used interchangeably; here, the term "silibinin" will be used throughout.

In recent years, silibinin has received much attention regarding its antiproliferative and anticarcinogenic effects in a variety of neoplasias as reflected in experiments with cancer cell lines (*7*, *8*) and in rodents (*9*, *10*). Silibinin has previously been shown to have low oral bioavailability (*11*). Therefore, a formulation (Silipide) combining silibinin with phosphatidyl choline at a molar ratio has been developed to increase its absorption (*12*). Silipide is well-tolerated in both animal models and healthy human volunteers (*12*, *13*). Clinical development of silibinin requires the development of a robust analytical technique for its identification and quantitation in biological matrices. Previously, Martinelli et al. (*14*) described a high-performance liquid chromatography (HPLC) method for silibinin using normal-phase solid extraction columns, *tert*-butylmethylether as the eluent, and *n*-hexane/ethanol as the mobile phase. This method did not separate silibinin diastereoisomers, and *n*-hexane is known to be toxic (*15*, *16*). In 1993, Mascher et al. (*17*)

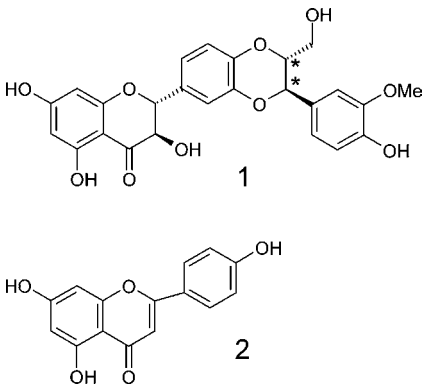

Figure 1. Molecular structure of the *trans*-diastereoisomers of silibinin (**1**) showing the chiral centers (*) and apigenin (internal standard) (**2**).

* To whom correspondence should be addressed. Tel: + 44(0)116 223 1856. Fax: + 44(0)116 223 1855. E-mail: djb27@leicester.ac.uk.
† Leicester Royal Infirmary.
‡ The Leicester General Hospital.

10.1021/jf063156c CCC: $37.00
Published on Web 03/14/2007

published a semivalidated HPLC method that, for the first time, separated the two diastereoisomers of silibinin, but this method employed a mobile phase composed of 0.02 M perchloric acid, which is a powerful oxidizing agent and as such raises safety concerns for large-scale use in the laboratory. The purpose of this study was to develop a safe, simple, and robust reversed-phase HPLC method validated for detection in human plasma and suitable for use in the further clinical development of silibinin.

MATERIALS AND METHODS

Compounds and Biomatrices. Silibinin (CAS 22888-70-6) and apigenin (CAS 520-36-5) were purchased from Sigma-Aldrich (Poole, United Kingdom). Silibinin was >98% pure and consisted of a racemic mixture of two diastereoisomers. Silipide (IdB 1016: a 1:1 molar ratio of silibinin and phosphatidylcholine, i.e., 40% of silibinin and 60% of phosphatidylcholine by weight) was supplied by Indena s.p.a (Milan, Italy). Human plasma was obtained from the National Blood Transfusion Centre (Sheffield, United Kingdom). Plasma samples were also obtained from colorectal cancer patients receiving silipide capsules (containing 480 mg of silibinin) three times a day for 7 days prior to surgery. The Leicestershire Local Research Ethics Committee authorized the investigation of silibinin bioavailability in colorectal cancer patients. Patients gave informed consent prior to the commencement of the study.

Extraction of Silibinin from Human Plasma. Pooled human plasma was stored at −80 °C and thawed at room temperature prior to use. Silibinin stock solutions were prepared in methanol. Dilutions were made using 70% aqueous methanol containing 5% acetic acid. Human plasma samples spiked with silibinin (0−5 μg/mL) were analyzed in triplicate and incorporated an internal standard (apigenin) added at a final concentration of 500 ng/mL. The samples were vortexed at high-speed setting for at least 20 s prior to adding 3 mL of ice-cold methanol to precipitate the proteins. Efficient protein precipitation was achieved by keeping samples at −20 °C for 30 min. Samples were centrifuged (6000*g* for 20 min), and the supernatant was removed and transferred to fresh plastic tubes and dried under a constant stream of nitrogen. Residues were reconstituted in 100 μL of mobile phase B followed by a final 13000*g* centrifugation at 4 °C, and the supernatant was transferred to vials prior to HPLC analysis (50 μL injection volume).

Blood from colorectal cancer patients was collected into heparinized tubes before treatment and 2 h after the final dose. Plasma was isolated immediately by centrifugation and stored at −80 °C until analysis. Samples were thawed to room temperature, and 5 μL (100 μg/mL) of apigenin (internal standard) was added to patient plasma (995 μL). Silibinin was extracted as described above.

The extraction method for plasma was validated for various parameters. Linearity was assessed for each diastereoisomer by r^2 regression using Microsoft Excel 2002 software. Recovery was assessed by comparison of silibinin isomers extracted from spiked plasma ($n = 6$) with nonextracted standard solutions in methanol ($n = 6$). Precision and accuracy for silibinin diastereoisomers (d1 and d2) were determined at four concentrations (2500, 250, 50, and 25 ng/mL). The limits of detection (LOD) and quantification (LOQ) were estimated as the concentrations of silibinin diastereoisomers that generate a signal-to-noise ratio of three and seven, respectively.

HPLC Analysis of Silibininin in Human Plasma. Chromatographic separation was accomplished using 150 mm × 4.6 mm i.d., 3 μm, C_{18} Atlantis column (Waters, Elstree, United Kingdom) in combination with a 20 mm × 4.6 mm i.d., 5 μm guard column at 40 °C. The Atlantis stationary phase was chosen for this analysis due to its ability to run at high aqueous concentrations and to separate the diastereoisomers of silibinin. The HPLC system consisted of a Varian ProStar 230 Pump, ProStar 410 autosampler, and a 310 UV/vis detector at 290 nm (Varian Analytical Instruments, Oxford, United Kingdom). The collected data were analyzed using Star LC Workstation software version 5.5. Mobile phase A consisted of glacial acetic acid and distilled water at a ratio of 1:19 (pH unaltered ∼2.4). Mobile phase B contained glacial acetic acid and methanol (1:19 v/v). The flow rate was 1.0 mL/min, the total run time was 35 min, and the gradient employed was as follows (mobile phase B): 5% at 0 min, 30% at 5 min, 50% at 20 min, 60% at 25 min, and 95% at 30 min and held at 95% for 5 min. Quantitation of silibinin was accomplished by reference to a calibration curve of silibinin peak area ratio (silibinin d1 or d2 to internal standard apigenin) plotted against silibinin concentration.

Identification of Silibinin and Metabolites by Liquid Chromatography/Mass Spectrometry (LC/MS). LC/MS analysis was performed using an API2000 mass spectrometer (Applied Biosystems, Warrington, United Kingdom) with sample delivery via an 1100 series HPLC instrument (Agilent Technologies UK Ltd., South Queensferry, United Kingdom). The HPLC separation used was essentially as described above with the following modifications: 150 mm × 2.1 mm i.d., 3 μm Atlantis column with 20 mm × 2.1 mm i.d. guard column and the flow rate reduced to 300 μL/min to allow direct injection in the mass spectrometer without the need for eluant splitting. Mass spectrometric analyses were performed in negative mode under the following conditions: declustering potential, −121 V; focusing potential, −300 V; entrance potential, −10 V; collision energy exit potential, −20 V; ion spray voltage, −4500 V; and temperature, 500 °C. Silibinin was identified by a Q1 multiple ion scan looking at *m*/*z* 481 ($M - H^+$).

RESULTS AND DISCUSSION

Validation of Silibinin Extraction. The method described here resolves the two diastereoisomers of silibinin into two baseline-separated chromatographic peaks at retention times 21.2 and 21.7 min, which were arbitrarily assigned as silibinin d1 and silibinin d2, respectively (**Figure 2**). Plasma spiked with silibinin yielded calibration curves that were linear over the observed range (0−5 μg/mL). Silibinin d1 was characterized by the calibration equation [$y = (0.0005 \pm 0.0001)x - (0.0166 \pm 0.0196)$, silibinin d2 by $y = (0.0005 \pm 0.0001)x - (0.0271 \pm 0.0232)$] (mean ± SD of $n = 6$). The mean correlation coefficient (r^2) for the interday analysis was consistently above 0.995 ($n = 6$).

Recovery of silibinin d1 and d2 from pooled plasma samples and precision and accuracy was estimated at four concentrations (2500, 250, 50, and 25 ng/mL). Recovery was 53−58% (**Table 1**). The precision determined at each concentration level as reflected by the coefficient of variation (CV) did not exceed 15%. Accuracy was within 15% for silibinin d1 at all concentrations and silibinin d2 for concentrations 50−2500 ng/mL (**Table 1**).

The LODs of silibinin d1 and d2 were determined by extraction from spiked plasma. The LOD for silibinin d1 was 3 ng/mL, and for silibinin d2, the LOD was 2 ng/mL. LOQs for silibinin d1 and d2 in human plasma were found to be 7 and 5 ng/mL, respectively. The CV for LOQ is within the acceptable limits of the FDA method validation guidelines (*18*).

Silibinins d1 and d2 were stable for at least 1 month at 4 °C in the dark when kept in methanol. The stability of silibinin and apigenin in human plasma was evaluated following sample storage at −20 and −80 °C for 24 h. Both silibinins d1 and d2 were stable when kept at −20 and −80 °C after three freeze−thaw cycles (**Table 2**).

Determination of Silibinin in Human Plasma from Patients Receiving Oral Silipide. Silibinin was successfully detected at quantifiable levels in the plasma of colorectal cancer patients receiving oral silipide. **Figure 2** shows typical HPLC chromatograms of plasma obtained from a colorectal cancer patient before and after consumption of silipide capsules, at 1440 mg silibinin per day in three divided doses for 7 days. Peaks at 21.1 and 21.7 min were identified as silibinin diastereoisomers by cochromatography with authentic standard. These peaks were absent from plasma samples taken from the patient before silipide ingestion (**Figure 2D**). Several peaks appeared in

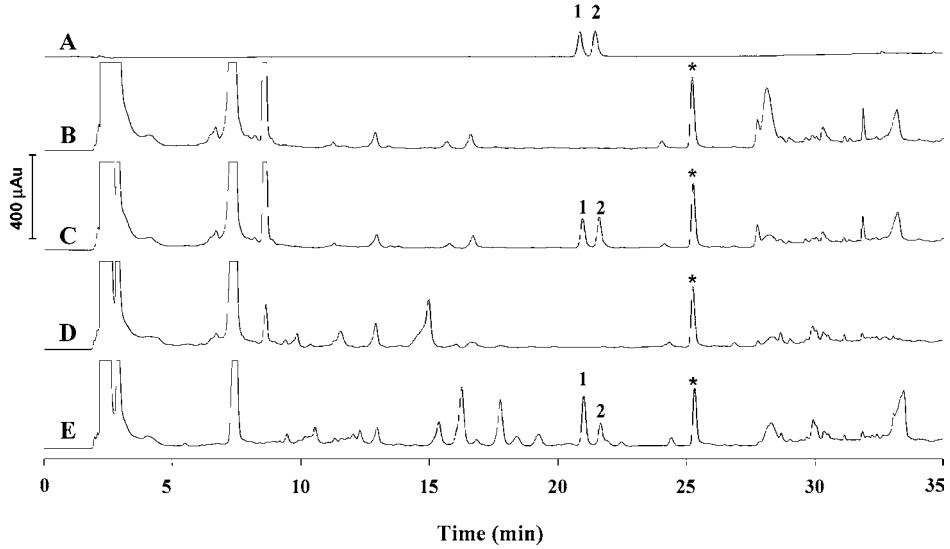

Figure 2. Separation and identification of silibinin diastereoisomers in human plasma. Silibinin d1 and d2 (peaks 1 and 2, respectively) in methanol (**A**) spiked in human plasma (**C**) and in plasma obtained from a colorectal cancer patient after a 7 day treatment of silipide capsules (containing 480 mg of silibinin three times daily) (**E**). Blank pooled human plasma (**B**) and plasma from patient prior to initiating silipide treatment (**D**) demonstrate the absence of any coeluting peaks (* indicates the internal standard, apigenin).

Table 1. Summary of Recovery, Precision, and Accuracy ($n = 6$) for Extraction and Measurement of Silibinin Diastereoisomers from Human Plasma

silibinin diastereoisomer	concn (ng/mL)	recovery (%)	recovered concn (mean ± SD)	accuracy[a] (% ± SD)	coefficient of variation RSD (%)
d1	25	58 ± 8	29.04 ± 4.08	93.6 ± 6.2	14.04
d2	25	55 ± 7	27.44 ± 3.29	75.3 ± 6.3	11.98
d1	50	56 ± 4	55.61 ± 3.83	97.4 ± 6.9	6.89
d2	50	53 ± 7	54.41 ± 2.15	85.8 ± 4.1	4.02
d1	250	54 ± 6	270.6 ± 32.2	106.7 ± 12.1	11.92
d2	250	55 ± 6	275.6 ± 28.7	106.3 ± 11.4	11.40
d1	2500	55 ± 6	2598 ± 272	100.0 ± 10.5	10.48
d2	2500	55 ± 5	2732 ± 255	113.5 ± 10.6	9.32

[a] $n = 6$.

Table 2. Stability of Silibinin Diastereoisomers in Spiked Plasma after Three Freeze–Thaw Cycles

		silibinin diastereoisomer concentration (ng/mL)					
		25		50		2500	
diasterioisomer	storage	peak area ratio[a]	RSD (%)	peak area ratio[a]	RSD (%)	peak area ratio[a]	RSD (%)
d1	immediate	0.056	5.669	0.125	16.224	7.206	1.988
d2	($t = 0$)	0.075	2.365	0.140	8.592	8.013	1.947
d1	−20 °C	0.035	5.612	0.118	6.067	6.960	0.582
d2		0.060	11.191	0.114	8.613	7.637	0.367
d1	−80 °C	0.062	2.212	0.111	4.693	6.990	3.380
d2		0.069	7.270	0.116	7.791	7.863	2.391

[a] Peak area ratio of silibinin diastereoisomer to the internal standard apigenin.

postdose plasma samples that may be attributable to silibinin metabolites (e.g., at retention times 15.3, 16.2, 17.7, 18.3, and 19.2 min).

Identification of Silibinin and Possible Metabolites in Human Plasma from Patients Receiving Oral Silipide. Plasma obtained from patients was investigated by LC/MS in Q1 scan mode (*m*/*z* 100−900) as described previously (*19*). Peaks in the mass chromatogram at 20.8 and 21.5 min (*m*/*z* 481) were identified as the two diastereoisomers of silibinin, corroborated by coelution with authentic standard, and these are the same peaks as seen by HPLC. Various *m*/*z* values were scanned for in multiple ion scan mode corresponding to probable phase I and phase II metabolites. Of these, only the following showed peaks of significant intensity: *m*/*z* 657 (monoglucuronide) 13.1, 15.6, 16.4, and 17.8 min; *m*/*z* 833 (diglucuronide) 10.8 min; *m*/*z* 561 (monosulfate) 17.0, 17.8, and 18.8 min; and *m*/*z* 737 (glucuronide sulfate) 9.1 and 11.0 min. There was also some evidence for the presence of *O*-desmethyl silibinin glucuronide (*m*/*z* 643) and silibinin triglucuronide (*m*/*z* 1008).

Although several methods have been developed for the analysis of silibinin in human plasma, to the best of our knowledge, no simple, fully validated method suitable for clinical evaluation of silibinin and its metabolites has yet been published. Here, we describe a simple method utilizing plasma protein precipitation and robust, precise, and reproducible reverse-phase HPLC to separate silibinin diastereoisomers with LOD and LOQ comparable with, or better than, previously published HPLC-UV detection methods (*14*, *17*, *20*). Rickling et al. (*21*) published a LOD for silibinin diastereoisomers of 0.25 ng/mL, which are superior to those quoted here; however, this level of sensitivity was achieved by using electrochemical detection and column switching, whereas our method has the advantage of employing simple UV/vis detection at 290 nm and without the need for column switching. Other techniques have required the use of less common constituents of mobile phases such as dioxane and perchlorate for separation (*14*, *17*). A published method looking at the glucuronidation of silibinin

using bovine liver microsomes (*22*) was not optimized for human plasma nor was it optimized for the various phase I and II metabolites likely to be present in humans taking silibinin.

The method described in this paper was used to separate silibinin and its metabolites in a patient who had received silibinin at a dose of 480 mg, three times daily oral dose for 7 days in a clinical pilot study (**Figure 2**). The results of the clinical study (*19*) suggests that silibinin is orally bioavailable and present in the systemic circulation at clearly quantifiable concentrations. The peaks eluting before silibinin in **Figure 2** suggest the presence of silibinin conjugates. Consistent with this observation, silibinin conjugates have been putatively identified (*19*) using this method, the validation for which is described here. This method seems highly suitable for the development of silibinin as a cancer chemopreventive agent in humans.

ACKNOWLEDGMENT

We thank Indena S.p.a. for the gift of silipide capsules and for the kind assistance of Dr. P. Morrazzoni.

LITERATURE CITED

(1) Magliulo, E.; Gagliardi, B.; Fiori, G. P. Results of a double blind study on the effect of silymarin in the treatment of acute viral hepatitis, carried out at two medical centres. *Med. Klin.* **1978**, *73*, 1060−1065.

(2) Ferenci, P.; Dragosics, B.; Dittrich, H.; Frank, H.; Benda, L.; Lochs, H.; Meryn, S.; Base, W.; Schneider, B. Randomized controlled trial of silymarin treatment in patients with cirrhosis of the liver. *J. Hepatol.* **1989**, *9*, 105−113.

(3) Pares, A.; Planas, R.; Torres, M.; Caballeria, J.; Viver, J. M.; Acero, D.; Panes, J.; Rigau, J.; Santos, J.; Rodes, J. Effects of silymarin in alcoholic patients with cirrhosis of the liver: Results of a controlled, double-blind, randomized and multicenter trial. *J. Hepatol.* **1998**, *28*, 615−621.

(4) Hruby, K.; Csomos, G.; Fuhrmann, M.; Thaler, H. Chemotherapy of *Amanita phalloides* poisoning with intravenous silibinin. *Hum. Toxicol.* **1983**, *2*, 183−195.

(5) Kvasnicka, F.; Biba, B.; Sevcik, R.; Voldrich, M.; Kratka, J. Analysis of the active components of silymarin. *J. Chromatogr. A* **2003**, *990*, 239−245.

(6) Lee, D. Y. W.; Liu, Y. Z. Molecular structure and stereochemistry of silybin A, silybin B, isosilybin A, and isosilybin B, isolated from *Silybum marianum* (milk thistle). *J. Nat. Prod.* **2003**, *66*, 1171−1174.

(7) Zi, X.; Grasso, A. W.; Kung, H. J.; Agarwal, R. A flavonoid antioxidant, silymarin, inhibits activation of erbB1 signaling and induces cyclin-dependent kinase inhibitors, G1 arrest, and anticarcinogenic effects in human prostate carcinoma DU145 cells. *Cancer Res.* **1998**, *58*, 1920−1929.

(8) Agarwal, C.; Singh, R. P.; Dhanalakshmi, S.; Tyagi, A. K.; Tecklenburg, M.; Sclafani, R. A.; Agarwal, R. Silibinin upregulates the expression of cyclin-dependent kinase inhibitors and causes cell cycle arrest and apoptosis in human colon carcinoma HT-29 cells. *Oncogene* **2003**, *22*, 8271−8282.

(9) Kohno, H.; Tanaka, T.; Kawabata, K.; Hirose, Y.; Sugie, S.; Tsuda, H.; Mori, H. Silymarin, a naturally occurring polyphenolic antioxidant flavonoid, inhibits azoxymethane-induced colon carcinogenesis in male F344 rats. *Int. J. Cancer* **2002**, *101*, 461−468.

(10) Singh, R. P.; Tyagi, A. K.; Zhao, J. F.; Agarwal, R. Silymarin inhibits growth and causes regression of established skin tumors in SENCAR mice via modulation of mitogen-activated protein kinases and induction of apoptosis. *Carcinogenesis* **2002**, *23*, 499−510.

(11) Skottova, N.; Svagera, Z.; Vecera, R.; Urbanek, K.; Jegorov, A.; Simanek, V. Pharmacokinetic study of iodine labeled silibinins in rat. *Pharmacol. Res.* **2001**, *44*, 247−253.

(12) Morazzoni, P.; Magistretti, M. J.; Giachetti, C.; Zanolo G. Comparative bioavailability of Silipide, a new flavanolignan complex, in rats. *Eur. J. Drug Metab. Pharmacokinet.* **1992**, *17*, 39−44.

(13) Barzaghi, N.; Crema, F.; Gatti, G.; Pifferi, G.; Perucca, E. Pharmacokinetic studies on IdB 1016, a silybin-phosphatidylcholine complex in healthy human subjects. *Eur. J. Drug Metab. Pharmacokinet.* **1990**, *15*, 333−338.

(14) Martinelli, E. M.; Morazzoni, P.; Livio, S.; Uberti, E. Liquid Chromatograhic assay of silybin in human plasma. *J. Liq. Chromatogr.* **1991**, *14*, 1285−1296.

(15) Burgaz, S.; Erdem, O.; Cakmak, G.; Erdem, N.; Karakaya, A.; Karakaya, A. E. Cytogenetic analysis of buccal cells from shoerorkers and pathology and anatomy laboratory workers exposed to n-hexane, toluene, methyl ethyl ketone and formaldehyde. *Biomarkers* **2002**, *7*, 151−161.

(16) Spencer, P. S.; Kim, M. S.; Sabri, M. I. Aromatic as well as aliphatic hydrocarbon solvent axonopathy. *Int. J. Hyg. Environ. Health* **2002**, *205*, 131−136.

(17) Mascher, H.; Kikuta, C.; Weyhenmeyer, R. Diastereomeric separation of free and conjugated silibinin in plasma by reversed phase HPLC after specific extraction. *J. Liq. Chromatogr.* **1993**, *16*, 2777−2789.

(18) U.S. Department of Health and Human services guidelines, Food and Drug Administration, Center for drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM) May 2001; http://www.fda.gov/cder/guidance/index.htm.

(19) Hoh, C.; Boocock, D.; Marczylo, T.; Singh, R.; Berry, D. P.; Dennison, A. R.; Hemmingway, D.; Miller, A.; West, K.; Euden, S.; Garcea, G.; Farmer, P. B.; Steward, W. P.; Gescher, A. J. Pilot study of silibinin, a putative chemopreventive agent, in colorectal cancer patients: Silibinin levels in plasma, colorectum, and liver and their pharmacodynamic consequences. *Clin. Cancer Res.* **2006**, *12*, 2944−2950.

(20) Ding, T.; Tian, S.; Zhang, Z.; Gu, D.; Chen, Y.; Shi, Y.; Sun, Z. Determination of active components in silymarin by RP-LC and LC-MS. *J. Pharm. Biomed. Anal.* **2001**, *26*, 155−161.

(21) Rickling, B.; Hans, B.; Kramarczyk, R.; Krumbiegel, G.; Weyhenmeyer, R. Two HPLC assays for the determination of free and total silibinin diastereomers in plasma using column switching with ECD and reversed-phase chromatography with ultraviolet detection. *J. Chromatogr.* B **1995**, *670*, 267−277.

(22) Gunaratna, C.; Zhang, T. Application of liquid chromatography−electrospray ionization-ion trap mass spectrometry to investigate the metabolism of silibinin in human liver microsomes. *J. Chromatogr. B* **2003**, *794*, 303−310.

(23) Han, Y. H.; Lou, H. X.; Ren, D. M.; Sun, R. L.; Ma, B.; Ji, M. Stereoselective metabolism of silybin diastereoisomers in the glucuronidation process. *J. Pharm. Biomed. Anal.* **2003**, *34*, 1071−1078.

Received for review November 2, 2006. Revised manuscript received December 12, 2006. Accepted December 20, 2006.

JF063156C