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Bioavailability of a silybin–phosphatidylcholine complex in dogs

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Liver dysfunction often is associated with an imbalance in the production and removal of free radicals derived from oxygen and nitrogen and has been managed clinically with antioxidant supplements, including silymarin extract derived from milk thistle. The potential for enhanced bioavailability of a phytosome complex containing phosphatidylcholine and silybin, the primary active flavonolignan in silymarin extract, was tested in dogs. A group of eight beagles (four males, four females) were dosed orally with a silybin–phosphatidylcholine complex (SPC) and a commercially available standardized silymarin extract containing equivalent levels of silybin. Dosing with the SPC resulted in C_{max}, T_{max}, and AUC_{0-24h} values (mean ± SD) for total silybin of 1310 ± 880 ng/mL, 2.87 ± 2.23 h, and 11 200 ± 6520 ng·h/mL, respectively; corresponding values for a standardized silymarin extract were 472 ± 383 ng/mL, 4.75 ± 2.82 h, and 3720 ± 4970 ng·h/mL. A second, separate group of beagles were also dosed with the extract alone, yielding values of 449 ± 402 ng/mL, 6.87 ± 7.43 h, and 2520 ± 2976 ng·h/mL. These data show that a phytosome complex of phosphatidylcholine and silybin markedly enhances bioavailability in dogs.

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INTRODUCTION

Acute and chronic liver disorders frequently involve damage to hepatocytes by reactive oxygen and reactive nitrogen species (ROS/RNS) resulting from an imbalance in the production and removal of free radicals (Center, 2004; Medina & Moreno-Otero, 2005). Oxidative and nitrosative stress may occur within hepatocytes caused by conditions, such as xenobiotic toxicity, metal overload, or biological toxins or may be caused by free radicals derived from activated Kupffer cells or invading neutrophils. Recommended management options include antioxidant therapy with various antioxidant supplements, including intravenous *N*-acetylcysteine, and oral *S*-adenosylmethionine, vitamins C and E and milk thistle (Center, 2004).

Plant-derived flavonoids with potent antioxidant activity have a long history of use in counteracting human liver dysfunction. Flavonoids, commonly referred to as silymarin, extracted from the milk thistle *Silybum marianum* have received particular attention, with considerable clinical and basic research assessing effects and mechanisms (Wellington & Jarvis, 2001). Silymarin is composed of a mixture of the flavonolignans, such as silybin, isosilybin, silydianin, and silychristin, with silybin the major active component (Quaglia *et al.*, 1999; Skottova *et al.*, 1999; Kvasnicka *et al.*, 2003). Studies with silymarin or silybin have demonstrated hepatoprotection in conditions, such as iron overload (Pietrangelo *et al.*, 2002), xenobiotic toxicity (Mourelle *et al.*, 1989; Muriel *et al.*, 1992), and in high dosages for amanitoxin as well as tetrachloromethane poisoning in dogs (Vogel *et al.*, 1984; Paulova *et al.*, 1990). While the polyphenolic nature of silymarin components suggest that its activity is linked to its antioxidant properties, both *in vivo* and *in vitro* studies have shown a range of effects, including inhibition of stellate cell activation, collagen type I synthesis, and ensuing fibrogenesis (Fuchs *et al.*, 1997; Jia *et al.*, 2001; Di Sario *et al.*, 2005); inhibition of Kupffer cell function (Dehmlow *et al.*, 1996); stimulation of nucleic acid metabolism and hepatocyte regeneration (Sonnenbichler & Zetl, 1984; Sonnenbichler *et al.*, 1986; Sonnenbichler & Zetl, 1986); and stimulation of bile acid production (Crocenzi *et al.*, 2000) that cumulatively make its use an attractive treatment strategy for liver dysfunction.

Because of the pervasive role that oxidative/nitrosative stress is now known to play in pathophysiology, the flavonolignans in silymarin have received increasing attention in treatment and prevention of various forms of cancer (Kren & Walterova, 2005). Inhibitory effects on multi-drug resistance proteins (Chung *et al.*, 2005), anti-angiogenic activity (Jiang *et al.*, 2000), antagonism of UV induction of skin cancer (Singh & Agarwal, 2002, 2005), potentating effects with chemotherapeutic agents (Giacomelli

et al., 2002; Tyagi *et al.*, 2002; Chon & Kim, 2005) and attenuating effects in animal models of diabetes (Soto *et al.*, 2004) have widened the range of potential applications of the unique polyphenols in silymarin.

The *in vivo* effectiveness of silymarin flavonolignans, as with many polyphenols, depends on bioavailability and achieving therapeutic concentrations in the organs of interest (Manach *et al.*, 2004, 2005; Silberberg *et al.*, 2006). The components of silymarin are poorly soluble in water, and studies in both laboratory animals and humans have shown only nanogram per milliliter concentrations in plasma following oral administration of powdered extracts (Barzaghi *et al.*, 1990; Schandalik *et al.*, 1992; Weyhenmeyer *et al.*, 1992; Morazzoni *et al.*, 1993; Gatti & Perucca, 1994). However, pharmacokinetic studies in rats, dogs, and human subjects have shown substantial increases in bioavailability by combining silybin with phosphatidylcholine enriched in linoleic acid by a process that yields a 'phytosome', an entity structurally distinct from a liposome (Orlando *et al.*, 1991; Schandalik *et al.*, 1992; Morazzoni *et al.*, 1993; Gatti & Perucca, 1994; Kidd & Head, 2005). This complex has been used extensively in human clinical studies of hepatic disorders and with laboratory models of hepatotoxicity (Kidd & Head, 2005). While silymarin has been shown to alleviate amanitoxin poisoning in dogs (Floersheim *et al.*, 1978; Vogel *et al.*, 1984), no information is available on the relative availability of this complex or of silymarin extracts in companion animals. This study was conducted to analyze the pharmacokinetics of silybin in beagles administered equivalent amounts of silybin derived from a silybin–phosphatidylcholine complex (SPC) or a standardized extract of silymarin orally.

MATERIALS AND METHODS

Study protocol

The study protocol was reviewed and approved before study initiation by an Institutional Animal Care and Use Committee (IACUC) and complied with the Animal Welfare Act. Sixteen healthy adult purpose-bred beagles were used (eight males and eight females), weighing between 10.14 and 12.49 kg and routinely fed once per day. Dogs were fed a standard colony diet (Joy Special Meal; Joy Pet Foods, St. Marys, OH, USA) that contained not <26% protein and 12% fat and not more than 4.0% crude fiber and 10% water.

This study was designed as a parallel design to evaluate the acute pharmacokinetics (24 h) of a silymarin extract and SPC (Marin®; Nutramax Laboratories, Inc., Edgewood, MD, USA) and chronic administration of SPC in dogs. The dogs were randomized into two groups of eight dogs each. Each group of dogs received either SPC or a silymarin extract, each containing equivalent amounts of silybin. After a 23-h fast (day 0), administration by direct insertion into the dog's mouth was followed by emesis after SPC (group A) but not the silymarin extract (group B, silymarin). Gastric irritation following zinc administration was not anticipated, but is known to occur in dogs. Therefore, after a 6-day washout period the test articles were administered after a shorter, 2 h fast (day 7) with complete acceptance of the SPC. On day 7, the test article administrations were switched (Fig. 1). This allowed collection of data on both silymarin extract (group B, silymarin) and SPC (group B, SPC) on the same set of dogs, in addition to a second silymarin extract-treated group of dogs (group A, silymarin).

Dogs received either (i) a tablet containing 178 mg silybin (A + B) equivalents (mean 16.2, range 14.6–17.3 mg/kg) complexed with phosphatidylcholine (Marin®; Nutramax Laboratories, Inc.), or (ii) a capsule containing 178 mg silybin equivalents (mean 15.74, range 14.2–17.6 mg/kg) of a silymarin extract standardized to 80% silymarin (Indena USA, Inc., Seattle, WA, USA), along with 10 mL of water to assure timely entry into the stomach. Marin® is a flavored chewable tablet that contains active ingredients a stabilized combination of silybin in a phosphatidylcholine complex, elemental zinc as zinc gluconate, and Vitamin E (RRR-α tocopheryl acetate). The lot used in this study contained 89 mg of silybin in a phosphatidylcholine complex, 59 mg of elemental zinc as zinc gluconate, and 308 IU of Vitamin E (RRR-α tocopheryl acetate). The silymarin used was a powdered extract and was analyzed by HPLC to determine silybin content.

Blood samples for determination of silybin levels were collected 24 h and immediately prior to dosing, then at 0.5, 1, 2, 4, 6, 8 and 24 h for each group on days 0 and 7. Dosing was continued for seven additional days at 89 mg silybin daily in both groups of dogs and blood samples collected again prior to dosing on day 14. Dosing with the SPC (group B, SPC) continued to 35 days with blood samples taken after 21 and 35 days. Blood samples drawn for plasma analysis were collected in lithium–heparin tubes, centrifuged, and plasma samples stored at −70 or −80 °C until analyzed. Additional blood samples were collected for complete blood count (CBC) and serum chemistry on the SPC treated dogs on days -1, 21, and 35 (Fig. 1). In a separate study, dogs were orally dosed with SPC at 8.8, 12.9, and 17.7 mg/kg and blood samples collected as described above over a 24-h period, plasma prepared, and the samples analyzed for total silybin A and B.

Assay method

The total plasma concentrations of silybin were analyzed by an HPLC method (B.T. Biotecnica, Varese, Italy) after overnight digestion of 50 μL samples in 0.5 mL 0.45 M sodium acetate, pH

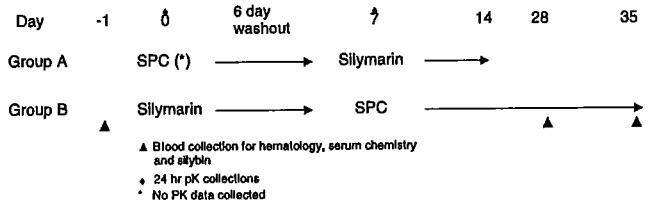

Fig. 1. Time course for study showing test article administration and blood collection points.

5.0 with type H-1 glucuronidase (Sigma-Aldrich, St Louis, MO, USA). Following digestion, eriodictyol, an aglycon flavanone (Fluka), was added as the internal standard, followed by 1 mL of 50 mM sodium citrate and by 6 mL of *tert*-butylmethylether. The samples were then mixed for 20 min, centrifuged at 1000 *g* for 5 min, and frozen at −80 °C for at least 10 min. The liquid upper organic layer was decanted and evaporated under nitrogen and the residue reconstituted with 100 μL of water: methanol (70:30 v/v). Samples of 50 μL were analyzed using a Luna C18 column, 250 × 4.6 mm (Phenomenex, Torrance, CA, USA) on a Shimadzu (Manchester, UK) 10 HPLC system at 26 °C with monitoring at 288 nm. Separations were achieved using a mobile phase of 5% acetic acid: methanol (70:30) over a period of 30 min. Data were expressed as total silybin in nanogram (silybin A + B isomers) per milliliter of plasma. Standard curves for silybin were generated by analyzing pure silybin prepared and diluted in methanol, spiked with the internal standard, then extracted and reconstituted as described above for plasma samples, and analyzed. Nominal values for each isomer were calculated based on the 49% silybin A, 51% silybin B composition of silybin standard.

Pharmacokinetics and statistical analysis

The observed values for maximal plasma concentration (C_{max}), time to reach maximal plasma concentration (T_{max}) and half-life ($t_{1/2}$) were determined on individual animals using PK Solutions 2.0 (Summit Research Services, Montrose, CO, USA; http://www.SummitPK.com) and the results used to calculate mean values. The area under the concentration curve (AUC_{0-24}) was calculated using the trapezoidal method.

Statistical analysis for C_{max}, T_{max}, AUC_{0-24} and $t_{1/2}$ were evaluated using ANOVA with group or phase as factors at an alpha of 0.5. The data for C_{max} and $t_{1/2}$ were log-transformed for analysis to meet the assumptions of the ANOVA. Statistical analysis was performed with NCSS statistical and power analysis software (Kaysville, UT, USA).

RESULTS

Assay validation

Chromatograms of the internal standard eriodictyol peak and both silybin A (1) and silybin B (2) isomers at 200 ng/mL silybin and at 5000 ng/mL silybin are presented in Fig. 1a–c. Standard curves for three assays of pure silybin were linear from 100 ng/mL to 25 μg/mL for each isomer with $R^2 > 0.995$ for silybin A (1) and silybin B (2). Intra-assay precision was <7.3% and intra-assay accuracy was 97.7–112.9%. The lower limit of quantification was 100 ng/mL for each isomer. Recovery of 0.5–5.0 μg/mL standards added to plasma was 43–61% of the same standard evaporated and directly reconstituted in mobile phase and recovery of standards from water was 104 ± 6% that from plasma, indicating no loss due to plasma protein binding.

Representative chromatograms of one SPC dog are presented in Fig. 2 showing the internal standard and both isomers of silybin at 0, 2, 6 and 24 h post dosing (A, B, C, & D, respectively). The 0 and 24 h chromatograms show the presence of a small silybin B peak at baseline. The absorption of silybin into the plasma is observed in the substantial increase in peak height/area at 2 h (C_{max}) and 6 h samples. The 24 h post sample shows a small peak similar to the 0 h 'background' level. Analysis of the data has been performed by subtracting the averaged day minus 1 and 0 times for silybin from the post administration samples.

Pharmacokinetics

The plasma concentration vs. time curve following oral administration of SPC or silymarin extract is shown in Fig. 3. A single oral dose of SPC resulted in a rapid increase during the first 2 h, then dropped by over 50% at 8 h but remained at a detectable level out to 24 h (Fig. 4). Both groups of silymarin extract dogs exhibited similar plasma concentration vs. time curves with minimal and variable change in levels over time. When dogs were dosed with equal amount of silybin in SPC and silymarin extract, the mean C_{max} plasma levels observed were 270% that of the extract, with a relative bioavailability of 3.03 and 4.44 that of the extract (Table 1). There was no statistically significant difference in the T_{max} values between the SPC and silymarin extract group of dogs. The $t_{1/2}$ for both the SPC and silymarin extract dogs was very similar. The pharmacokinetic parameters are summarized in Table 1. The high animal to animal variability typically seen in all pharmacokinetic studies of silymarin or silybin was observed in the current study. However, the data clearly demonstrate dosing the dogs with silybin with the phytosome form of a SPC prepared by the 'phytosome' method provides a marked enhancement in bioavailability over an extract alone (Fig. 3).

In an effort to establish whether continued dosing results in increased residual levels of silybin in plasma, dogs receiving the SPC were dosed with 89 mg of silybin for days 8–35. The plasma levels at 24 h after dosing on days 21 and 35 were 0.234 ± 0.104 ng/mL and 0.090 ± 0.101 ng/mL, respectively, indicating minimal concentration of silybin in the plasma.

DISCUSSION

Although polyphenolic flavonoids have been shown to have antioxidant activity and other properties that add to their potential to positively impact a range of pathophysiological conditions, their effectiveness may be limited by their relatively low bioavailability (Manach & Donovan, 2004; Manach *et al.*, 2004, 2005). Various approaches have been tested to enhance water solubility and bioavailability of silymarin, including complexing with *N*-methylglucamine (Yanyu *et al.*, 2006), succinate (Campos *et al.*, 1989), B-cyclodextrin (Arcari *et al.*, 1992), and phosphatidylcholine (Barzaghi *et al.*, 1990; Orlando *et al.*, 1991; Schandalik *et al.*, 1992; Weyhenmeyer *et al.*, 1992;

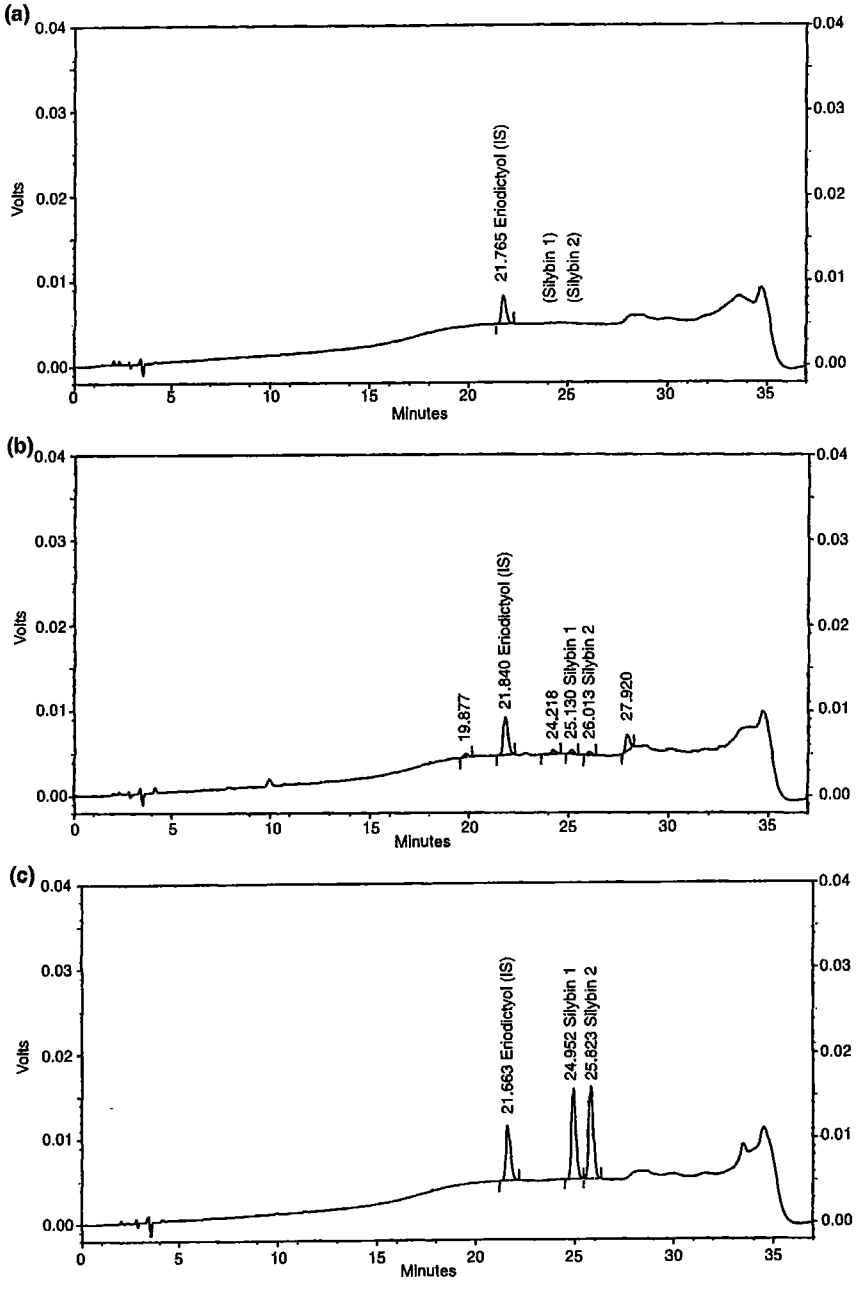

Fig. 2. Chromatograms of silybin A [1] and B [2] and the internal standard eriodyctyol. (a), internal standard; (b), plasma with added eriodictyol and 200 ng/mL total silybin; (c), plasma with added eriodictyol and 5000 ng/mL total silybin.

Morazzoni *et al.*, 1993; Gatti & Perucca, 1994; Yan-Yu *et al.*, 2006; Yanyu *et al.*, 2006). The SPC used in the present study was produced with phosphatidylcholine by a method that forms a 'phytosome' that has properties distinct from liposomes or proliposomes (Yan-Yu *et al.*, 2006; Yanyu *et al.*, 2006). This formulation has been used in earlier studies on humans (Barzaghi *et al.*, 1990; Orlando *et al.*, 1991; Schandalik *et al.*, 1992; Weyhenmeyer *et al.*, 1992; Gatti & Perucca, 1994) and rats (Morazzoni *et al.*, 1993). Pharmacokinetic studies have shown the 'phytosome' complex to provide a fivefold improvement in bioavailability in human subjects (Barzaghi *et al.*, 1990; Schandalik *et al.*, 1992; Gatti & Perucca, 1994) and an even greater increase in rodents (Morazzoni *et al.*, 1993). The present study indicates that a substantial advantage over a standardized extract also occurs in the dog, with a 3–4.4 fold gain.

Previous studies on amanitin toxicity in beagles showed that 50–150 mg/kg silymarin extract provided protection if given 5 and 24 h after intoxication. If given 10 min after intoxication, a 15 mg/kg dose provided partial protection, while a 100 mg/kg dose gave total protection (Vogel *et al.*, 1984). As it is likely that these extracts suffered from the limitations on bioavailability mentioned above, it is clear that a 'phytosome' preparation at substantially lower doses should be hepatoprotective against this extreme form of toxicity as well as other sources of toxicity. It is noteworthy that a comparable pharmacokinetic study on beagles using a silybin in a proliposome containing phosphol-

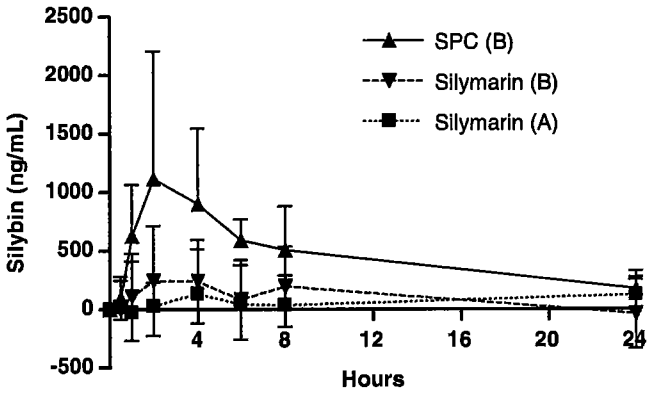

Fig. 3. Silybin plasma concentration time course after dosing with silybin–phosphatidylcholine complex (SPC) or silymarin extract. Values represent the mean values ± standard deviation of measured levels after subtraction of background peaks averaged for days -1 and 0. The group B dogs were switched, while a separate group A received only the silymarin extract.

ipids at a dose of 7.7 mg/kg (Yan-Yu *et al.*, 2006) compared with 14–15 mg/kg in this study, showed C_{max} and *AUC* values 20–25% of those shown here. In addition the more rapid uptake and disappearance of silybin given as a proliposome produced a much shorter mean residence time, thereby decreasing bioavailability and any bioactivity (Yan-Yu *et al.*, 2006).

Many *in vitro* studies of the effects of silybin, as well as many other polyphenols, have shown effects at μM levels that are frequently somewhat higher than plasma levels typically observed in pharmacokinetic studies (Manach & Donovan, 2004; Manach *et al.*, 2004, 2005; Williamson & Manach, 2005). While this disparity may raise valid questions as to the significance of the *in vitro* studies for effects on specific organs or conditions, in the case of silymarin and its main component silybin, there is clear evidence that levels achieved in the liver are sufficient to be clinically effective (Lorenz *et al.*, 1984; Schandalik *et al.*, 1992; Weyhenmeyer *et al.*, 1992; Saller *et al.*, 2001). Silybin in a phosphatidylcholine complex given to rats resulted in increased plasma levels and was detected in liver microsomes, which then exhibited antioxidant properties when compared with microsomes from control or silymarin extract groups (Comoglio *et al.*, 1990). The fact that hepatoprotection has been

Table 1. Main pharmacokinetic parameters for total plasma silybin in dogs dosed with equivalent amounts of silybin in silybin–phosphatidylcholine complex (SPC) or a silymarin extract

	Group A	Group B	
	Silymarin (mean ± SD)	Silymarin (mean ± SD)	SPC (mean ± SD)
C_{max} (ng/mL)	449 ± 402*	472 ± 383*	1310 ± 880*
T_{max} (h)	6.87 ± 7.43	4.75 ± 2.82	2.87 ± 2.23
AUC_{0-24} (ng·h/mL)	2520 ± 2976*	3720 ± 4970*	11 200 ± 6520*
$t_{1/2}$ (h)	n/d	17.4 ± 9.26	15.3 ± 13

*$P < 0.05$ between silymarin extract groups and SPC group.

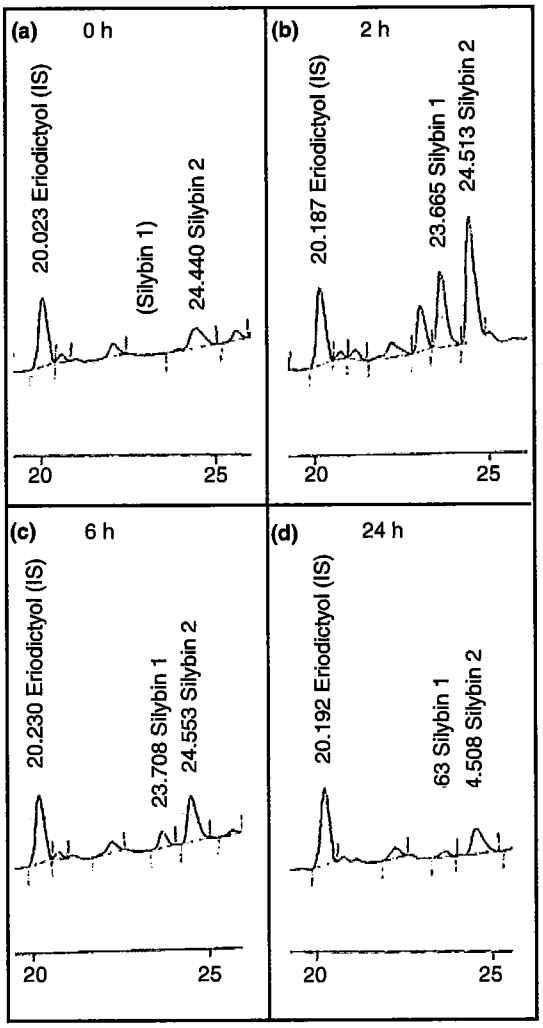

Fig. 4. Chromatograms of internal standard, silybin A [1] and silybin B [2] in a representative silybin–phosphatidylcholine complex (SPC) dog at 0 h (a), 2 h (b), 6 h (c) and 24 h (d).

shown in various animal models of toxicity, along with some attenuation of markers of hepatitis in humans (Flora *et al.*, 1998; Giese, 2001; Saller *et al.*, 2001; Wellington & Jarvis, 2001) may be due to relatively high intrahepatic concentrations following intake. Plasma silymarin is largely protein-bound, with clearance occurring largely through bile secretion (Flory *et al.*, 1980; Lorenz *et al.*, 1984; Barzaghi *et al.*, 1990), which continues for over 24 h. Bile levels greatly exceed serum levels (Lorenz *et al.*, 1984; Schandalik *et al.*, 1992; Weyhenmeyer *et al.*, 1992; Saller *et al.*, 2001), indicating that substantial levels are achieved in the liver and may remain high due to enterohepatic recirculation. Thus the liver is particularly suited to benefit from exposure to the silymarin flavonolignan. In addition, the increasing numbers of studies, both *in vitro* and *in vivo*, suggest that it may have wide ranging benefits, from attenuating various forms of toxicity to acting synergistically with various agents that treat cancer. The availability of a well-characterized form of its major component, silybin, in a

bioavailable form provides for its use in a wider range of conditions found in companion animals. This study clearly shows that oral administration of silybin in a phosphatidylcholine complex (phytosome) results in greater bioavailability of silybin compared with standard silymarin extract. Zinc and vitamin E have been recommended to clinically manage liver dysfunction in dogs (Center, 2004) and do not appear to attenuate the enhanced bioavailability derived from complexing the silybin with phosphatidylcholine.

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