BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e20160

Article

Bioavailability augmentation of silymarin using natural bioenhancers: An *in vivo* pharmacokinetic study

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Pharmacokinetic studies were carried out in male and female rats to quantify silymarin as silybin (A+B) after the oral administration of various silymarin formulations combined with three bioenhancers, namely, lysergol, piperine, and fulvic acid, and compared with plain silymarin formulation (control). A non-compartmental analysis, model independent analysis, was utilized, and various pharmacokinetic parameters (C_{max}, T_{max}, and AUC_{0-t}) were calculated individually for each treatment group, and the values were expressed as mean ± SEM (n = 6). Plasma samples obtained from the rats were analyzed for the concentration of silymarin through a validated RP-HPLC method and on the basis of data generated from the pharmacokinetic studies. Results indicated that the bioenhancers augmented pharmacokinetic parameters and bioavailability increased 2.4–14.5-fold in all the formulations compared with the control. The current work envisages the development of an industrially viable product that can be further subjected to clinical trials and scientifically supports the development of silymarin as a contemporary therapeutic agent with enhanced bioavailability and medicinal values.

Keywords: Silymarin. Bioavailability. Piperine. Fulvic acid. Lysergol.

INTRODUCTION

Silymarin, extracted from *Silybum marianum* (milk thistle), has been used in traditional medicine to treat liver and biliary tract diseases, particularly acute and chronic, viral, and drug and alcohol-induced hepatitis (Radko, Cybulski, 2007; Kren, Walterova, 2005). Silymarin is a standardized extract consisting of seven flavonolignans and flavonoids, and silybin A and B are the primary and most active components. All the pharmacokinetic parameters of silymarin are referred to and standardized as silybin. Orally administered silymarin (silybin) is rapidly absorbed, with a T_{max} of 2–4 h and $t_{1/2}$ of 6 h (Pradhan, Girish, 2006). Only 20%–50% of oral silymarin is absorbed in the gastrointestinal tract, where it undergoes extensive enterohepatic circulation. Therefore, the absorption of silymarin in the gastrointestinal tract is low, rendering bioavailability poor (Wu *et al*., 2007). *In vivo* studies that investigated the absorption of silymarin or silybin after oral administration indicated extremely high variability in the values of C_{max} and T_{max}. This result may be due to differences in the concentrations of the isomers (silybin A and B) between the extract and dose administered. The oral bioavailability of silymarin (silybin) in rat plasma was 0.73%, indicating the low absorption of silymarin isomers after the oral administration of the plain extract (Wu *et al*., 2007).

Despite the potential medicinal benefits of silymarin, it has low oral bioavailability, which continues to be a major challenge in the development of formulations with clinical efficacy (Wu *et al*., 2007). Through the systematic application of modern formulation approaches, these biopharmaceutical barriers can be successfully overcome, and the true potential of silymarin can be fully exploited

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for the alleviation and prevention of complications of diseases. To offset the poor bioavailability of silymarin, various approaches have been made, but none of them proved to be potentially fruitful (Javed, Kohli, Ali, 2010a; Javed, Kohli, Ali, 2010b). The concept of using natural bioenhancers is a recent approach for increasing drug bioavailability and offers many advantages, including reduction in drug cost, toxicity, and adverse effects, and has a beneficial influence on the national economy (Javed, Kohli, Ahsan, 2016). This study was conducted to explore the use of three natural bioenhancers, namely lysergol, piperine, and fulvic acid, which are used in a variety of fixed dose combinations with silymarin for the preparation of oral formulations. These formulations were used in the *in vivo* pharmacokinetic studies on rats and compared with a plain silymarin tablet as control. Lysergol, piperine, and fulvic acid were used as bioenhancers owing to their promising bioavailabilty-enhancing activities. Lysergol, obtained from *Ipomoea* spp. has shown bioenhancing potential in several *in vitro* studies; it facilitates the transport of antibiotics across membranes, thereby increasing drug efficacy on target sites (Khanuja, Arya, Srivastava, 2007). It increases the bioavailability of berberine when administered together in rats (Patil *et al*., 2012) and increases the membrane permeability of drugs inside cells, thus inhibiting P-glycoprotein (P-gp)-mediated efflux. Piperine, an active constituent of *Piper longum*, inhibits the metabolism of various drugs by inhibiting CYP450 enzymes and increasing their blood levels. The third bioenhancer selected was Fulvic acid, a water soluble carrier which increases the water solubility of a drug by complexation. Given that the low bioavailability of silymarin is attributed to extensive first pass metabolism, increased P-gp efflux, and low permeability across membranes, the bioenhancers are expected to augment the bioavailabilty of silymarin.

MATERIAL AND METHODS

Drug and bioenhancers

Milk thistle seed extract containing 70% silymarin was obtained as a gift sample from Maneesh Pharmaceuticals Ltd. Mumbai, India and was used as received. Lysergol (97%) was obtained as a gift sample from Chemical Resources Pvt. Ltd. Panchkula, Haryana, India and was used as received. Piperine (97%) was purchased from Sigma Aldrich, New Delhi, India and was used as received. Fulvic acid was extracted from raw shilajit extract obtained from marketed shilajit capsules through a previously described procedure (Javed, Kohli, Ali, 2013).

Chemicals and Instruments

Silybin standard (98%) and naringenin (98%, HPLC grade) internal standard were purchased from Sigma Aldrich, New Delhi, India. Acetonitrile and water (HPLC grade) were obtained from S.D. Fine-chem Ltd. India, and methanol (HPLC grade) was obtained from Spectrochem Pvt. Ltd. India. Chromatographic analyses were performed on HPLC (HPLC 1120 series, Agilent Technologies with TC- C18 column) instrument coupled with a UV-visible detector.

Preparation of solutions

Preparation of drug standard stock solution

All stock solutions were prepared fresh for use. A 100 µg/mL drug standard solution was produced by dissolving 10.0 mg of silybin (98%) in 100 mL of methanol.

Preparation of internal standard stock solution (w/v)

A 100 µg/mL internal stock (IS) solution was prepared by dissolving 10.0 mg of naringenin (98%) in 100 mL of acetonitrile.

Preparation of working solutions for calibration curve construction

Serial dilutions of each stock solution (2.5, 5.0, 10, 25, 50, and 100 µg/mL) were prepared by taking aliquots of 25, 50, 100, 250, 500, and 1000 µL of the stock solutions and adding methanol to a final volume to 10 mL.

Spiking of plasma for calibration curve

Curves were prepared by spiking 300 µL plasma with 700 µL of IS solution and 20 µL of a silybin working solution. The calibration points represented 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 µg/mL of standard silybin. The spiked plasma samples were then centrifuged at 5000 rpm for 10 min, and the supernatant fluid (20 µL) was injected into RP-HPLC-UV for analysis. After HPLC analysis, the peak areas of silybin (A + B) and the peak areas of IS were obtained, and the ratio of the peak areas of silybin A + B/peak area of IS were calculated.

Chromatographic conditions

An Agilent TC C18 reverse phase column (250 mm × 4.6 mm, 5 µm) with a column temperature of 37.5 °C and isocratic mobile phase consisting of methanol and water (1% *v/v* acetic acid, adjusted to pH 3.0) in the ratio 55:45 *v/v* was used. The eluent was monitored at 288 nm at a flow rate of 1.0 mL/min and injection volume of 20 µL.

Pharmacokinetic study

The pharmacokinetic study protocol was submitted to and approved by Institutional Animal Ethics Committee, and the animals were provided by the Central Animal House Facility, Jamia Hamdard (CPCSEA registration no. 173/CPCSEA). All the studies were performed at the Department of Pharmaceutics, School of Pharmaceutical Education and Research, Jamia Hamdard. The animals were sacrificed at the end of the study, and the carcasses were disposed through a central incineration facility.

Animal dosing and sampling

Male and female Wistar rats with an average weight of 250 g were fasted overnight before the drug was administered but had free access to water. Silymarin was administered as a single oral dose (140 mg/kg) to five groups of rats (n = 6). Group I received plain silymarin tablet formulation and was marked as control (No.1), Group II received silymarin (140 mg/kg) + lysergol (1.4 mg/kg) formulation (No. 2), Group III received silymarin (140 mg/kg) + piperine (1.4 mg/kg) formulation (No. 3), Group IV received silymarin (140 mg/kg) + fulvic acid (140 mg/kg) formulation (No. 4), and Group V received silymarin (140 mg/kg) + fulvic acid (140 mg/kg) + piperine (1.4 mg/kg) formulation (No. 5). Blood samples (300 µL) were collected from the tail vein under anesthesia at 0 (predose), 0.5, 1, 1.5, 2, 4, 6, and 8 h after oral administration, and plasma was separated by centrifugation at 5000 rpm for 15 min and stored at −80 °C until further analysis.

Preparation of samples for analysis

Plasma samples were thawed, and an aliquot of 25 µL was vortex-mixed with 50 µL of internal standard solution (10 µg/mL) in acetonitrile. Protein precipitation was achieved, and an aliquot (20 µL) of the supernatant was directly injected onto the HPLC system for analysis. The quantification was based on $1/x^2$ weighted least-squares regression equations derived from the peak area ratios of silybin to that of naringenin, and the area of silybin isomers was calculated as a whole.

Analytical method validation

To validate assay specificity, the chromatogram of blank rat plasma was compared with that of a rat plasma sample spiked with silymarin. Linearity was assessed using a calibration curve for silymarin over a concentration range of 0.05–2.0 µg/mL. The inter-day and intra-day accuracy and precision of the method were evaluated by analyzing three silymarin quality control samples with concentrations of 0.05, 0.5, and 2.0 µg/mL on the same day and in three consecutive days. Accuracy (% bias) was calculated by dividing the measured mean drug concentration by the theoretical drug concentration. Precision (% R.S.D.) was the ratio of the standard deviation to measured mean drug concentration. Lastly, percent recovery, limit of detection (LOD), and limit of quantification (LOQ) were determined for the assessment of the sensitivity of detection.

RESULTS

Optimization of chromatographic conditions

The HPLC method used was based on the method in the literature with modifications (Wu *et al*., 2007; Yanyu *et al*., 2006). A variety of solvents were tried as mobile phase (acetonitrile, methanol, distilled water, KH_2PO_4 and NaH_2PO_4 buffers) and after optimization, methanol and double distilled water was found to be most optimal. Secondly, various ratios of the mobile phase (60:40, 50:50, 45:55, 55:45) were tested, and the best resolution was obtained at a ratio of 55:45. In the ratio of 60:40, a methanol concentration resulted in the inadequate separations of silybin diastereomeric peaks, and when organic content was reduced to ratios of 50:50 and 45:55, separation occurred but showed excessive tailing and long retention time (RT) increased. Optimum separation with reasonable RT was achieved by using a methanol-to-water ratio of 55:45. For improved peak symmetry, the pH value of the mobile phase was adjusted to 2.0, 2.5, 3.0, and 4.0, and pH 3.0 improved the resolution of the isomeric peaks of silybin without any change in RT. Different flow rates were tested (0.5, 0.8, 1.0, and 1.5 mL/min), and the best resolution and retention were obtained at 1.0 mL/min flow rate.

Thus, the applicability of an Agilent TC C18 reverse phase column (250 mm × 4.6 mm, 5 µm) with a column temperature at 37.5 °C was investigated, and satisfactory separation was obtained using an isocratic mobile phase consisting of methanol and water (pH adjusted to 3.0 with 1% *v/v* acetic acid) with a ratio of 55:45 *v/v* at 288 nm, flow rate of 1 mL/min, and injection volume of 20 µL on standard silybin and silymarin sample, as shown in Figure 1. In a standard silybin powder, one of the silybin diastereomeric peaks appeared at an RT of 10.32 min, and the other at an RT of 11.38 min. The sum of the areas of both peaks were calculated. Silymarin (500 ng/mL) was then spiked with blank rat plasma and allowed to run under the same chromatographic conditions in order to access any interference by plasma. Silybin A and B peaks appeared at 10.29 and 11.30 min, respectively showing no shift in peaks in the plasma-spiked samples. This method enabled good separation and quantification of silybin peaks within 12 min.

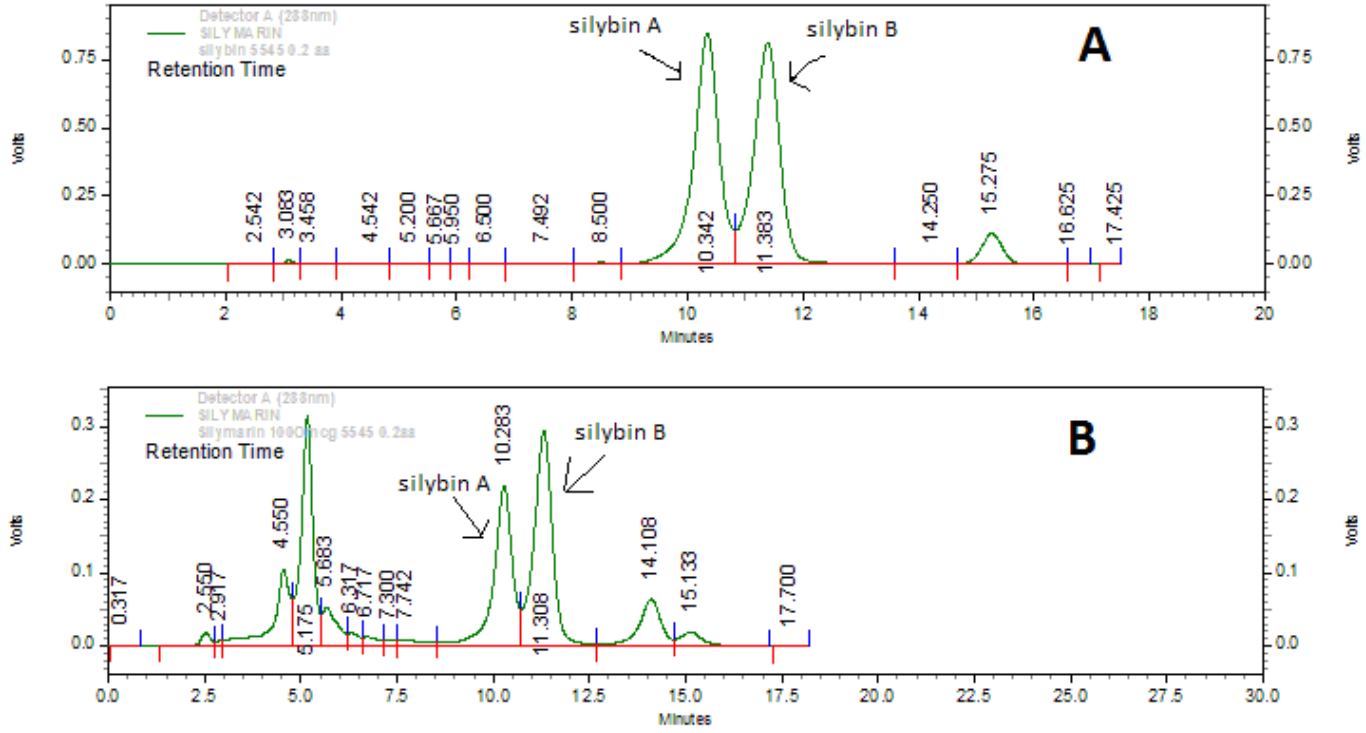

FIGURE 1 - Chromatograms of A) standard silybin and B) whole silymarin extract under optimized chromatographic conditions.

Calibration plot development

A calibration graph was plotted between concentration (µg/mL), and the ratio of the peaks and a straight line were obtained with $y = 0.0782x + 0.1085$ and $r^2 = 0.979$. From this straight line equation, the plasma concentrations of silybin (A+B) were calculated.

Validation of the HPLC method

The calibration curve of standard silybin demonstrated good linearity ($r^2 = 0.979$) between the response and the nominal concentration of silybin over the range of 0.05–2.0 µg/mL. LOD and LOQ values were 0.038 and 0.118 µg/mL, respectively. For the calculation of the concentrations of silybin in the plasma samples obtained from an animal study, the calibration curve based on the peak area ratios (silybin-to-internal standard) versus analyte concentration was used. The method was validated for linearity, selectivity, inter-day and intra-day precision and accuracy, limit of quantification, and percent recovery. The precision of the method was determined by measuring intra- and inter-day variations and was expressed as the percent relative standard deviation (% R.S.D.) of the mean measured concentration, as shown in Table I. Repeatability and reproducibility for the quality control samples at high, medium, and low concentrations were <2%, and measurements using the proposed method were found to be reliable. The method was found to be robust and stable when tested for robustness and stability issues. No major chromatographic changes were observed in both cases.

TABLE I - Method validation for the inter-day and intra-day assay precision (% R.S.D) and accuracy (% bias) of the HPLC method for the determination of silybin in plasma

Precision	Observed concentration (µg/mL)	Precision R.S.D (%)	Accuracy Bias (%)
Inter day			
0.05	0.061 ± 0.06	0.107	1.22
0.5	0.59 ± 0.05	0.092	1.19
2.0	2.56 ± 0.34	0.119	1.28
Intra day			
0.05	0.054 ± 0.07	0.128	1.09
0.5	0.6 ± 0.062	0.104	1.21
2.0	2.63 ± 0.35	0.116	1.32
Repeatability	Repeatability (intra-day % R.S.D, n = 3) = 0.10- 0.12%.		
Reproducibility	Reproducibility (inter-day % R.S.D, n=3) = 0.09 – 0.11%.		
Selectivity	Assessed by preparing various concentrations of standard silybin within the linear range and analyzing using this method.		
Recovery (%)	Good % recovery values at 0.05 (82.25%), 0.5 (83.45%) and 2.0% (84.40%) suggested accuracy of the method.		
Limit of Detection	The Limit of Detection was found to be 0.038 µg/mL		
Limit of Quantification	The Limit of Quantification was found to be 0.118 µg/mL.		

Pharmacokinetics of various silymarin formulations in rats

The pharmacokinetic parameters were evaluated using WinNonlin version 5.2, a computer program produced by Pharsight, St. Louis, Missouri. The AUC_{0-t} was the area under the plasma concentration time curve from time 0 to the observed final concentration time point upon oral administration and was calculated using the linear trapezoidal rule. The maximum serum concentration (C_{max}) and the time to reach the peak serum concentration (T_{max}) were obtained directly from experimental data.

Pharmacokinetic studies were carried out on male and female rats for the quantification of silymarin as silybin (A+B) after oral administration of 140 mg/kg of various silymarin formulations containing bioenhancers and were compared to the control formulation. The chromatograms obtained using different formulations are presented in Figure 2. Pharmacokinetic parameters were calculated by non-compartmental analysis, also called model independent analysis. All the pharmacokinetic parameters (C_{max}, $T_{max,}$ and AUC_{0-t}) were calculated individually for each treatment group, and the values were expressed as mean ± SEM (n = 6). Plasma samples obtained from rats were assayed for the determination of silymarin (silybin) through the validated HPLC method described above. The mean plasma silybin (A+B) concentration (µg/mL) was plotted as a function of time (h), as shown in Figure 3. The pharmacokinetic parameters of various silymarin formulations were compared in rats and are presented in Table II. The net silybin plasma levels in the silymarin + bioenhancer groups were high, suggesting that lysergol, piperine, and fulvic acid exerted bioenhancing effects on the absorption of silymarin.

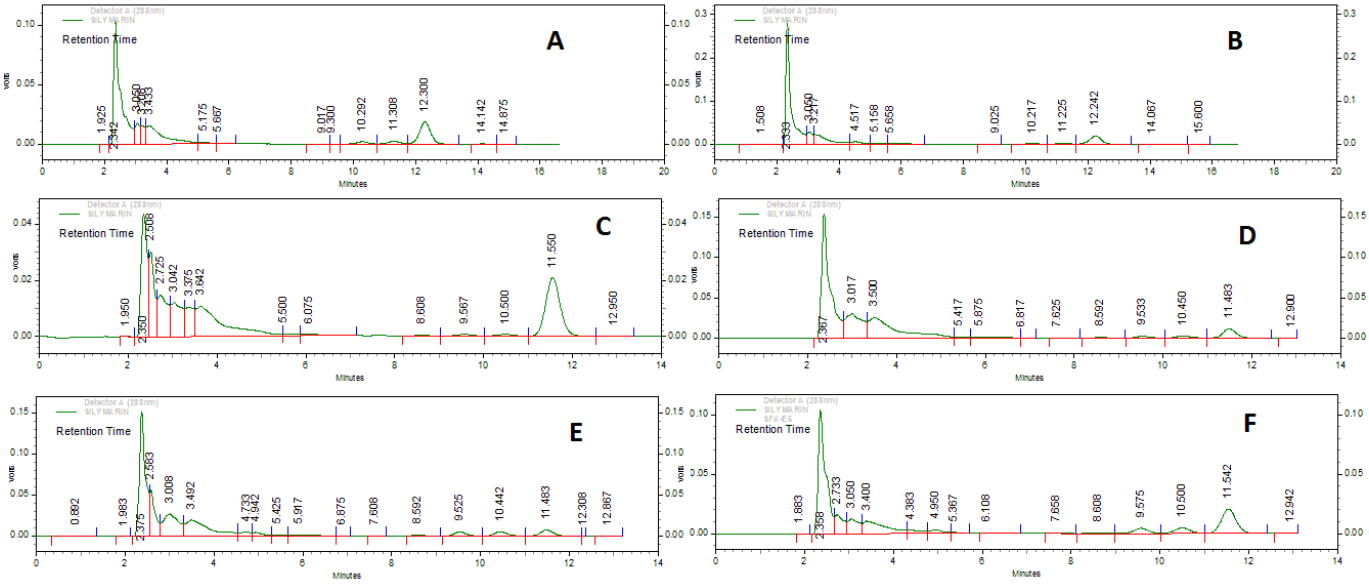

FIGURE 2 - Chromatograms of silymarin obtained for A) spiked plasma, B) plain silymarin (control), C) silymarin + lysergol, D) silymarin + piperine, E) silymarin + fulvic acid, and F) silymarin + fulvic acid + piperine formulations.

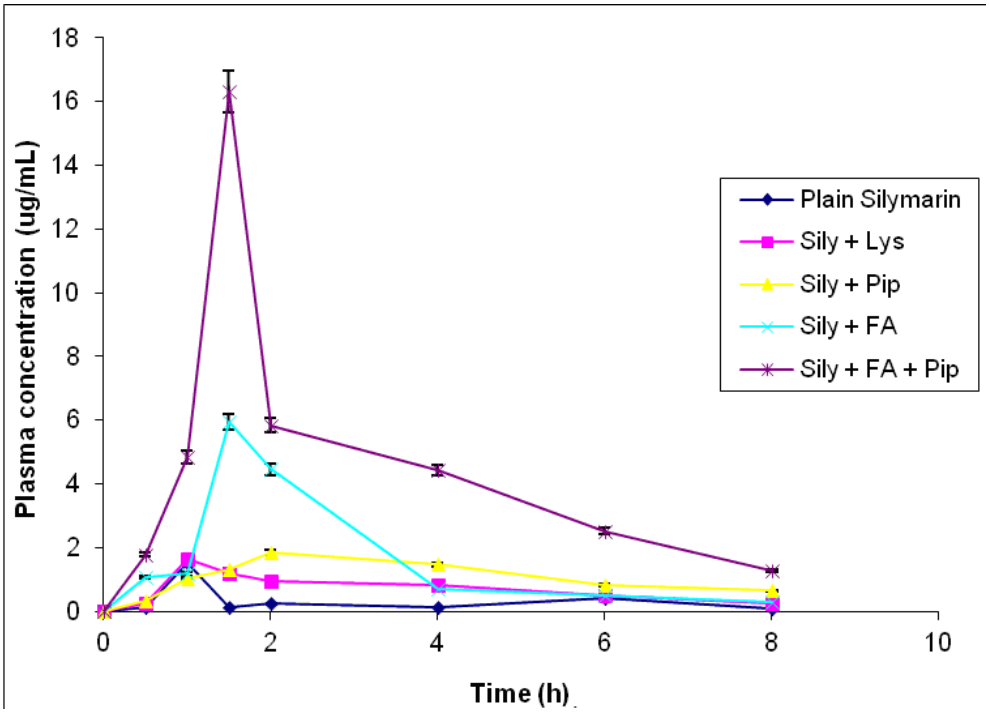

FIGURE 3 - Mean (± S.E.M) plasma concentration versus time curve of a single oral dose (140 mg/kg) of control and silymarin with bioenhancer formulations in rats (the standard error bars were omitted from the graph for the better elucidation of the pharmacokinetic profiles).

TABLE II - Pharmacokinetic parameters of all silymarin formulations obtained in rat plasma (Mean ± SEM, n = 6)

PK Parameters	Plain silymarin Tablet (Control)	Silymarin-Lysergol Tablet	Silymarin-Piperine Tablet	Silymarin-Fulvic Acid Tablet	Silymarin –Fulvic Acid-Piperine Tablet
C_{max} (µg/mL)	1.51 ± 0.21	1.64 ± 0.15	1.84 ± 0.32	5.93 ± 1.23	16.32 ± 1.85
T_{max} (hr)	1.0 ± 0.12	1.0 ± 0.15	2.0 ± 0.18	1.5 ± 0.18	1.5 ± 0.14
$AUC_{(0\text{-}8h)}$ (µg-hr/mL)	2.31 ± 1.35	5.69 ± 1.45	8.45 ± 1.65	12.37 ± 1.87	33.44 ± 1.98
MRT (hr)	2.91 ± 1.43	3.01 ± 1.12	3.79 ± 1.32	3.24 ± 1.43	3.95 ± 1.41
$T_{1/2}$ (hr)	1.84 ± 0.55	3.15 ± 1.63	3.36 ± 1.54	1.00 ± 0.17	3.75 ± 1.56
$AUC_{(0\text{-}\infty)}$ (µg-hr/mL)	2.55 ± 1.21	8.47 ± 1.41	11.56 ± 1.63	13.47 ± 1.78	37.27 ± 1.89
Folds increase in bioavailability	**--**	**2.4**	**3.65**	**5.35**	**14.53**

Pharmacokinetics of control formulation

When silymarin tablet (control) was administered at a single oral dose of 140 mg/kg to the rats, C_{max} of 1.517 ± 0.21 µg/mL, $AUC_{(0\text{-}8h)}$ of 2.3183 ± 1.35 µg-h/mL, and and $AUC_{(0\text{-}\infty)}$ of 2.5523 ± 1.21 µg-h/mL were obtained. Given that silymarin has poor solubility in aqueous media (40–50 µg/mL), these low *AUC* values might be due to the dissolution limited absorption. T_{max} was obtained within 1.0 h after oral administration (Figure 3). A short half-life of 1.84 ± 0.55 h suggested the rapid elimination of silymarin.

Lysergol as the bioenhancer of silymarin

The pharmacokinetic parameters of silymarin improved when lysergol was used as a bioenhancer, and C_{max} (1.64 ± 0.15 µg/mL) and $AUC_{0\text{-}8h}$ (5.69 ± 1.45 µg-h/mL) increased and were approximately 1.0- and 2.4-fold those of the control. The mean residence time (MRT) increased to 3.01 ± 1.12 h, resulting in the increased of elimination half-life to 3.15 ± 1.63 h. However, T_{max} showed no change in both formulations. Relative bioavailability was enhanced by 2.4-fold after the administration of silymarin with lysergol at a bioenhancing dose of 10% *w/w* of silymarin in the formulation. As shown in Figure 3, lysergol improved the oral bioavailability of silymarin and it altered the elimination pattern of silymarin.

Piperine as the bioenhancer of silymarin

A comparative plasma profile of control versus silymarin with piperine formulation revealed that piperine enhanced the extent and the absorption rate profile of silymarin by increasing C_{max} (1.84 ± 0.32 µg/mL) and $AUC_{(0\text{-}8h)}$ (8.45 ± 1.65 µg-hr/mL). The values increased approximately 1.21- and 3.65-fold compared with those of the control. MRT increased to 3.79 ± 1.32 h, and the elimination half-life increased to 3.36 ± 1.54 h. T_{max} increased to 2 h compared with that of the control (1 h). Therefore, the relative bioavailability of silymarin was enhanced up to 3.65-fold by the administration of piperine at a bioenhancing dose of 10% *w/w* of silymarin in the formulation. $T_{1/2}$ in silymarin with the piperine group was longer than that in the control, suggesting that the overall rate of elimination of silymarin slowed down during the terminal phase and bioavailability was enhanced (Figure 3).

Fulvic acid as a bioenhancer of silymarin

Silymarin was complexed with fulvic acid through a phase solubility method described previously (Javed, Kohli, Ahsan, 2016). When silymarin complexed with fulvic acid (1:1 w/w) in a tablet dosage form was administered, the pharmacokinetic parameters of silymarin further improved, which C_{max} of 5.93 ± 1.23 µg/mL and $AUC_{0\text{-}8h}$ of 12.37 ± 1.87 µg-h/mL. Bioavailability was approximately 3.90- and 5.35-fold that in the control. The MRT value obtained was 3.24 ± 1.43 h, the elimination half-life was 1.00 ± 0.17 h, and T_{max} increased to 1.5 h. Interestingly, $T_{1/2}$ decreased from 1.84 ± 0.55 h in the control to 1.00 ± 0.17 h in silymarin with fulvic acid group possibly because of the rapid elimination of solubilized silymarin with decreased molecular size. The enhanced solubilization of silymarin by fulvic acid led to the elevated levels of the drug in the plasma (Figure 3).

Fulvic acid and piperine in combination as bioenhancers of silymarin

C_{max} (16.32 ± 1.85 µg/mL) and $AUC_{0\text{-}8h}$ (33.44 ± 1.98 µg-h/mL) improved compared with those in the control. The increase was approximately 10.0- and 14.47-fold those of the control, respectively. MRT also increased to 3.95 ± 1.41 h, and elimination half-life increased to 3.75 ± 1.56 h. T_{max} also increased to 1.5 h. The pharmacokinetic profiles of the control and optimized silymarin–fulvic acid (1:1 *w/w*)–piperine (10% w/w) tablets are compared in Figure 3.

DISCUSSION

Silymarin and various formulations of silymarin in combination with bioenhancers were tested on animal models, and their pharmacokinetic parameters were evaluated. Plain silymarin tablet was considered the control and compared with all the other formulations. The results of the control formulation were concordant with the literature. Relatively low C_{max} and *AUC* and short half-lives (1–3 h) for free/unconjugated silymarin (silybin) have been reported (Wen *et al*., 2008). It was considered to be the characteristic of enterohepatic circulation in the sequence: intestinal absorption, conjugation in the liver, excretion in the bile, hydrolysis by the intestinal flora and finally uptake in the intestine.

After the oral administration of legalon (plain silymarin) capsule to human volunteers at a dose of 560 mg, C_{max} ranged from 0.18 µg/mL to 0.62 µg/mL (Lorenz *et al*., 1984). In another study, after a single oral dose of silymarin (200 mg/kg as silybin) in rats, *AUC* and

C_{max} were 77.1 µg.h/mL and 6.7 µg/mL, respectively (Morazzoni *et al*., 1993). Furthermore, when legalon capsule was administered orally at a dose of 140 mg/kg, C_{max} (3.47 ± 0.20 µg/mL) and *AUC* (22.75 ± 3.19 µg.h/mL) remarkably decreased (Woo *et al*., 2007.

Lysergol, a bioenhancer of silymarin, was used in preparing a formulation. The low bioavailability of silymarin is due to its extensive metabolism in the liver (Jancova *et al*., 2007), efflux by P-glycoprotein (Wu *et al*., 2008), or its low permeability across intestinal epithelial cells. Hence, any compound that can inhibit the metabolism of silymarin or alter membrane transport via the P-glycoprotein or alter membrane permeability across intestinal epithelial cells might be helpful in enhancing the oral bioavailability of silymarin. Lysergol has hypotensive, psychotropic, and analgesic effects and uterus- and intestine-stimulating activities (Ferrari, 975). In this study, we attempted to improve the oral bioavailability of silymarin by using lysergol as a bioenhancer. This can be explained by the fact that lysergol might enhance the permeation of silymarin across the monolayers of intestinal epithelial cells, which are the rate-limiting barriers for drug absorption/diffusion. Although lysergol is a bioenhancer according to *in vitro* studies, no mechanistic studies have been conducted yet to elucidate its bioenhancing property. Further studies are required to confirm the mechanistic approach underlying the bioenhancing property of lysergol.

Piperine is a well-known drug metabolism inhibitor and was therefore selected as another bioenhancer for silymarin. When co-administered with coenzyme Q10 once daily for 21 days, it showed significant increase in the plasma levels of coenzyme Q10 compared with the control group, showing 30% increase in the *AUC* values (Atal, Dubey, Singh, 1985; Badmaev, Majeed, Prakash, 2000). In another study, a piperine analogue (1-piperoyl piperidine) was used to enhance the oral bioavailability of etoposide (an anticancer agent; Najar *et al*., 2011). Bioenhancement was possibly due to its ability to modify P-gp/CYP3A4-mediated drug disposition mechanism. Similar results were observed in another study, where the piperine analogue (PA-1) bioenhanced the oral bioavailability of etoposide in mice 2.32-fold (Sachin *et al*., 2010). Thus, the progressive inhibition of drug metabolism leads to the elevated levels of the drug in the plasma and piperine, a natural product, might be useful in enhancing the bioavailabilty of silymarin through various mechanisms, including hepatic and intestinal glucoronidation inhibitor and enhanced mucosal uptake by P-gp inhibition, as both of them are responsible for the rapid metabolism of silymarin *in vivo*.

The third bioenhancer selected for this study was fulvic acid owing to its many advantages. Fulvic acid is a naturally occurring organic product derived from humus, a soil organic material produced by the decomposition of organic matter (Dyke *et al*., 2014). Humic substances are naturally occurring high-molecular-weight compounds and the major organic constituents of native shilajit, a blackish brown exudation obtained from the steep rocks of different formations found in the Himalayas at altitudes between 1000 and 5000 m (Ghosal, 2003). In general, fulvic acid is a stable water-soluble carrier moiety characterized by having a sponge-like structure punctured by voids of about 200–1000 Å in diameter and 700–2500 daltons in average molecular weight. It is a type of solubilizers and acts as a carrier for poorly bioavailable drugs, such as glibenclamide, insulin, pentazocine, folic acid, multivitamins, and coenzyme Q10, and increases their solubility through its great complexing ability (Javed, Kohli, Ahsan, 2016). Fulvic acid was previously extracted from the raw shilajit extract of a marketed shilajit capsule formulation through a microwave-assisted extraction technique (Javed, Kohli, Ali, 2013). To obtain the concentration of fulvic acid, phase solubility studies were carried out previously, and the results revealed that a stoichiometric ratio of 1:1 is the most suitable for a silymarin formulation (Javed, Kohli, Ahsan, 2016).

The final combination tested was silymarin along with fulvic acid (1:1) and piperine (10%). The purpose of selecting this combination is based on our previous studies. Previously, we carried out *ex vivo* intestinal permeability test on a non-averted rat gut sac model to check the enhancement of permeability of silymarin and used different combinations of bioenhancers (Javed, Kohli, Ahsan, 2018). The best results were obtained when silymarin was used in combination wotj fulvic acid in 1:1 ratio along with piperine with 10% *w/w* concentration. These results were further supported by

the findings obtained in the pharmacodynamic studies on carbon tetrachloride (CCl_4)-induced hepatotoxicity animal model (Javed, Ahsan, Kohli, 2018). This combination showed extremely good potential in reducing the elevated liver enzymes and reducing liver necrosis caused by CCl_4.

To further confirm the potential of this combination, we correlated the pharmacodynamic results with pharmacokinetic study. Interestingly, this formulation augmented the pharmacokinetic parameters remarkably, and the results obtained were encouraging. The reason for this improvement is the solubilization effect of fulvic acid. The release of entrapped silymarin from the complex increased C_{max}. A higher jump in C_{max} can also be because of the pH independent solubility profile of fulvic acids. The increase in $T_{1/2}$ of silymarin to 3.75 ± 1.56 h in the formulation with fulvic acid and piperine might be due to the inhibition of the metabolism of silymarin by piperine either by inhibiting the hepatic and intestinal glucoronidation or by inhibiting the P-glycoprotein efflux pump. Hence, the dual mechanism of action exerted by fulvic acid and piperine on silymarin resulted in the significant enhancement in bioavailability. Fulvic acid improved the solubility and dissolution of silymarin in the gastrointestinal tract, whereas piperine inhibited silymarin metabolism.

CONCLUSION

Advances in the understanding of the cause of poor bioavailability have led to significant improvements in the design of technologies for addressing deficiencies. In oral drug delivery, many scientific challenges and breakthrough technologies are required to generate novel dosage forms that can increase the efficiency of drug delivery. Many strategies for improving oral bioavailability are available. The current work proposes the application of formulation design approaches that can enhance the oral bioavailability of silymarin. Formulation strategies, such as improvement in solubility and dissolution rate through solid dispersion technique, permeability enhancement with the use of permeability enhancers, and incorporation of metabolism inhibitors to decrease metabolism, have been used in modifying the oral bioavailability of silymarin, are low cost, and offer rapid solutions. As revealed by this pharmacokinetic study, lysergol, piperine, and fulvic acid were ranked according to performance as follows: fulvic acid and piperine in combination (14.53-fold) > fulvic acid alone (5.35-fold) > piperine alone (3.65-fold) > lysergol (2.40-fold) compared with the control.

ACKNOWLEDGEMENTS

S. Javed wishes to thank the Indian Council of Medical Research, New Delhi, India for providing financial assistance in the form of Senior Research Fellowship (Award no. 45/33/009-PHA/BMS) during her doctorate.

CONFLICT OF INTEREST

Authors declare no conflict of interest involved

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Received for publication on 14th March 2020
Accepted for publication on 16th June 2020