DEVELOPMENT AND VALIDATION OF A BIOANALYTICAL METHOD FOR DETERMINATION OF SILYMARIN IN PLASMA

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ABSTRACT

Silymarin is the active component of milk thistle plant which is a standardized extract consisting of silymarin flavonolignans (70-80 %), and a chemically undefined mixture composed of polymeric and oxidized polyphenolic compounds (20-30 %). Various analytical methods have been reported for its determination in plasma. Reported methods involved sample pretreatment using liquid-liquid extraction which makes them more time consuming because of frequent evaporation and reconstitution steps. Present study was aimed to develop a simple High-Performance Liquid Chromatography method with minimum time for sample pretreatment. This method was developed and validated using ultra violet detection for the determination of silymarin in plasma. The method was based on protein precipitation using methanol. Analysis was performed on ACE C18 column (150 × 4.6mm, 5µ) with gradient elution using solutions containing orthophosphoric acid: methanol: water in two different ratios 0.5:35:65 V/V/V and 0.5:50:50 V/V/V, pumped at a rate of 0.8 mL/min. The UV detection was performed at 288 nm. The retention time for the analysed comonents; silycristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B were found to be 7.97, 9.50, 17.04, 18.40, 22.45 and 23.35 min, respectively. The standard curve was linear over the range of 2 to 40 µg/mL with the regression coefficient >0.99. The intraday and interday precision was less than 3.06 and 6.56%, respectively. The results of analysis have been validated statistically and by recovery studies. The developed method was successfully applied for preclinical pharmacokinetic studies of silymarin in plasma samples of rabbit that were obtained at different time intervals after oral administration of silymarin.

Key Words: Silymarin, bioanalytical method, determination, plasma, pharmacokinetic

1 INTRODUCTION

Silybum marianum is one of the ancient and thoroughly researched plants. It is popularly called as milk thistle as common name. Active component of this plant is silymarin which is a standardized extract composed of approximately 70-80 percent silymarin flavonolignans including silybin A & B, isosilybin A & B, silydianin and silychristin as shown in Figure 1, and flavonoids (taxifolin and quercetin). Remaining 20-30 percentage part is a chemically undefined mixture comprising of polymeric and oxidized polyphenolic compounds. The isomers are collectively known as silymarin [1, 2]. Silymarin is a flavonolignans derived from the plant *Silybum marianum.* It is official in Indian Pharmacopoeia [3], United States Pharmacopoeia [4] and British Pharmacopoeia [5]. As per Indian Pharmacopoeia, it is indicated for hepatoprotective and chemoprotective activities [3]. It exhibits different pharmacological activities, like antioxidant, cardioprotective [6], antiinflammatory, anticancer, and hepatoprotective [7-11]. It has been used for the treatment of liver cirrhosis [12] and viral hepatitis [13]. Effect of Silymarin is due to several activities likes regulation of cell membrane permeability, leukotriene inhibition and reactive oxygen species scavenging properties [14]. Silymarin has an elimination half life of approximately 6-7 h and it is more than 70% bound to plasma proteins [15]. The major constituent of silymarin is silybin (also known as silibinin), a mixture of two diastereomers, silybin A and silybin B, in approximately equimolar ratio [16]. Silymarin is herbal medicine hence collectively all compounds are active and used for the treatment of liver cirrhosis and viral hepatitis [17] hence all the components were analyzed in the present method.

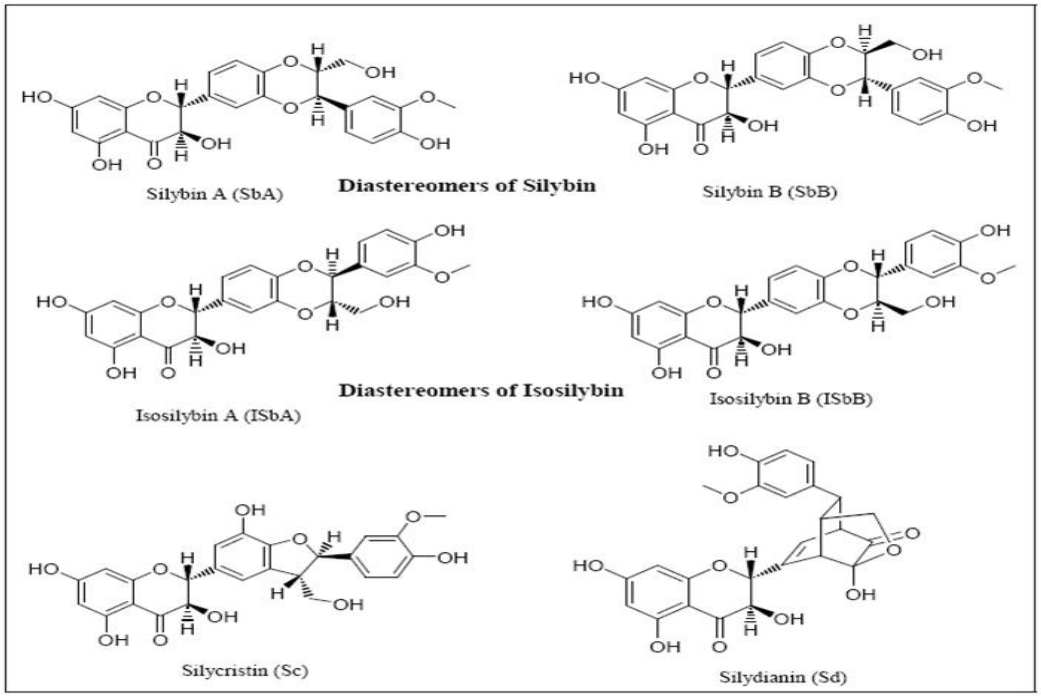

Figure 1: Chemical structure of Silymarin components

Most pharmacopoeial methods for assay of silymarin described HPLC with spectrophotometric detection at 288 nm [18-20]. Some non-pharmacopoeial methods are also reported for analysis of silymarin components in non-biological samples [21-24]. Few methods are reported for analysis of silymarin in biological matrices [25-27]. However, in the reported bioanalytical methods, the sample pretreatment consisted of liquid-liquid extraction. The liquid-liquid extraction protocols, evaporation and reconstitution steps used in such methods makes them more time consuming. Therefore, the present study was aimed to develop a simple HPLC method for determination of silymarin in plasma with minimum sample pretreatment.

2 MATERIALS AND METHODS

2.1 Chemicals

Silymarin was a generous gift from Centaur Pharmaceutical Private Limited (Goa, India). Methanol of HPLC grade and phosphoric acid (85%) of analytical grade were obtained from Finar Chemicals Ltd (Ahmedabad, India). Double distilled water prepared in the laboratory was used during the entire HPLC procedure.

2.2 Apparatus

2.2.1 HPLC system

The chromatographic separation was carried out using integrated Shimadzu HPLC instrument (Model: LC 2010 CHT) equipped with an auto injector and UV Visible detector. A reversed column and gradient elution of a mobile phase were used for chromatographic separation. Spectrophotometric detection was done at 288 nm. The output signal was monitored and processed using LC solution software.

2.2.2 Column

ACE C18, 5 µm, 4.6 x 150 mm analytical column with ODS 5 µm, 4.6 x 100 mm guard column was used for the method development.

2.2.3 Software

LC solution software was used for acquisition, reporting and analysis of data generated in the experimentation.

2.3 HPLC Conditions

Samples were eluted using a mobile phase consisting of solution A containing orthophosphoric acid: methanol: water (0.5:35:65 V/V/V) and solution B containing orthophosphoric acid: methanol: water (0.5:50:50 V/V/V). The mobile phase was filtered through a membrane filter (0.45 µm pore size, 47 mm diameter, Sartorius India Pvt. Ltd., Mumbai, India) and degassed in a sonicator (Enertech Fast Clean, Mumbai, India). Pumping of the mobile phase at a flow rate of 0.8 mL/min was carried out in the gradient mode for the run time of about 51 min. The temperature of column oven was set at 45 °C and UV detector was set at 288 nm. Samples of 10 µL were injected into column using an auto injector.

2.4 Preparation of Standard Solutions

Silymarin primary stock solution was prepared in methanol. For the preparation of solution, an accurately weighed 10 mg of silymarin was transferred to a 10 mL volumetric flask. Then 7 mL of methanol was added to the flask and stirred well to dissolve the drug. The volume was made up to the mark with methanol to yield a concentration of 1 mg/mL. Working standard solutions were prepared by dilution with methanol. Calibration standards were prepared by spiking stock solutions into drug-free plasma to yield concentrations of 2, 4, 8, 20, 32 and 40 µg/mL. External standard method was used to analyze the drug samples.

2.5 Preparation of Samples

The storage of collected plasma samples was done at -20 °C. The stored plasma samples were taken out and allowed to attain a room temperature before further processing. Methanol (2.5 mL) was added to 500 µL plasma and then mixture was vortexed for 30 s. The tube was centrifuged for 5 min at 2000 rpm (Remi, Cooling Centrifuge, Model C- 24BL, Remi Elektrotechnik Limited, Vasai, India) and supernatant was filtered through polyvinylidene difluoride (PVDF) syringe filter (Millipore Millex-HV Hydrophilic, 0.45 µm pore size, 33 mm diameter, Millipore India Pvt. Ltd., Mumbai, India) and transferred into a clean tube. The resulting solution (10 µL) was injected into the chromatographic system for analysis.

2.6 Selectivity

The developed method was investigated for its selectivity of the analytes over the potential interference of endogenous substances by analyzing blank plasma and comparing with LOQ solution samples from at least six sources.

2.7 Calibration Curves

The calibration curve was constructed in the range of 2-40 µg/mL to encompass the expected concentrations in measured samples. The calibration range was selected to cover the expected Cmax (2-6 µg/mL) of silymarin in tested formulation. The calibration curve was obtained by plotting silymarin peak area versus the silymarin concentration. Linear regression of the data was performed to determine correlation.

2.8 Accuracy, Precision and Recovery

The accuracy, precision (within-day and between-day) and recovery values of the method were determined using the quantification of five QC samples with three different concentrations within the calibration range. The food and drug administration (FDA) criterion for acceptability of accuracy and precision is ± 15% deviation from the nominal value, except for the LOQ, which should not exceed 20% [28].

2.9 Stability Study

Short-term, long-term, freeze-thaw and processed sample stability study of silymarin in plasma were carried out. For the purpose of stability study, spiked QC samples of 2 µg/mL (LQC) and 40 µg/mL (HQC) were used. Silymarin QC samples were kept room temperature for 24 h before protein precipitation to perform short-term stability study. For long-term stability study, the plasma QC samples were stored at -20 °C for 16 days before analysis. Freeze-thaw stability study was conducted by cycling of the plasma QC samples for three cycles between -20 °C and room temperature. The plasma sample were analyzed after each cycle. Analysis of freshly prepared plasma QC samples and samples kept in the auto injector for 6 h before injection was carried out for the assessment of processed sample stability.

2.10 Application of Developed Method

Developed method was applied for determination of silymarin in plasma following oral administration of 300 mg/kg dose of silymarin in New Zealand white rabbits (three). The study protocol was approved by Institutional Animal Ethical Committee (IAEC) of Shri Sarvajanik Pharmacy College, Mehsana, Gujarat vide Proposal No.: SSPC/IAEC/17/08/2013 as per the guidance of Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA). Blood samples were taken from a rabbit prior to dosing and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12 and 24 h after drug administration. The blood samples were centrifuged at 5000 rpm for 10 min, and plasma was collected in polyethylene tubes and stored frozen at -20 °C until analysis.

3 RESULTS AND DISCUSSION

3.1 Selection of Extraction Solvent

Extraction of silymarin isomers from plasma was tried using extracting solvents like methanol, ethanol, ethyl acetate, diethyl ether, acetone, and dichloromethane. The solubility in methanol, ethanol, ethyl acetate, diethyl ether, acetone and dichloromethane were found to be 237.7, 225.3, 198.1, 185.4, 168.5 and 102.9, respectively expressed in mg/mL. From the tested solvents methanol was used due to high solubility of silymarin isomers in it.

3.2 Chromatographic Selectivity

Typical chromatograms of drug-free plasma and spiked plasma of silymarin (2µg/mL) are shown in Figure 2. The method selectivity was demonstrated on six blank plasma samples. Chromatograms were found to be free of interfering peaks. The retention time of silycristin (Sc), silydianin (Sd), silybin A (SbA), silybin B (SbB), isosilybin A (ISbA) and isosilybin B (ISbB) were 7.97, 9.50, 17.04, 18.40, 22.45 and 23.35 min, respectively under the optimized chromatographic conditions.

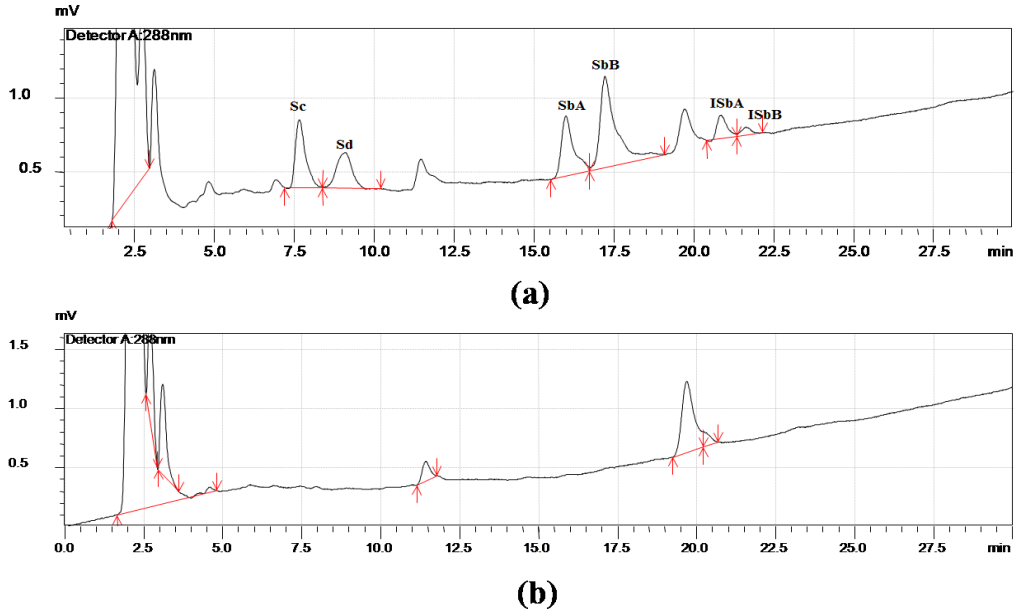

Figure 2: Chromatograms of (a) spiked plasma of Silymarin (2 µg/mL) and (b) a drug-free plasma

3.3 Linearity

The standard curve of silymarin in plasma was linear over the range of 2 to 40 µg/mL as shown in Table 1. The linearity of the calibration data was evaluated using regression coefficient of 0.999 as shown in Table 2.

3.4 Accuracy, Precision and Recovery

Accuracy, intraday and interday precision and recovery were assessed by analyzing the LOQ and QC samples prepared by spiking the plasma with known amount of silymarin. Results of accuracy data and intraday precision are shown in Tables 3 and 4, respectively. The accuracy of the method was higher than 86% for all the concentrations. The mean intraday precision was found to be 3.06%.

Table 1: Calibration Curve of Silymarin in Plasma

Sr. No.	Concentration in spiked plasma (µg/mL)	Concentration of Silymarin Std (µg/mL)	Peak area ± S.D. (n=3)		% Recovery from spiked plasma	% CV
			Silymarin in Spiked plasma	Silymarin Std in Methanol		
1	2	2	29627 ± 1608	31813 ± 1137	93.13	5.42
2	4	4	55873 ± 1534	65029 ± 2154	85.92	2.75
3	8	8	117272 ± 11330	137563 ± 9773	85.25	9.66
4	20	20	302410 ± 10138	320991 ± 13379	94.21	3.35
5	32	32	494199 ± 9859	527288 ± 18154	93.72	1.99
6	40	40	615364 ± 42182	698450 ± 47498	88.10	6.85

Table 2: Linearity Results of Silymarin in Plasma and Methanol

Pearameters	Results	
	Silymarin in Plasma	Silymarin in Methanol
Linearity Range	2-40 µg/mL	2-40 µg/mL
Regression Line Equation	y = 15519x + 5041	y = 17152x + 6165
Correlation Coefficient (R^2)	0.999	0.997

3.5 Stability Study

The stability study was performed as per bioanalytical validation guidelines. From the results of short-term stability study performed at room temperature, it was observed that low and high QC samples were stable for 24 h with assay of 90.63 ± 0.67%. From the results of long-term stability, it was found that silymarin samples stored at -20 °C for 16 days were stable with assay of 95.96 ± 0.95%. The decrease of silymarin concentration in plasma samples detected after exposing samples to three freeze-thaw cycles was insignificant, with mean assay of 95.15 ± 0.53%. The results of stability of processed samples in the autosampler showed that QC samples were stable for at least 6 h at room temperature, without significant loss of silymarin.

Based on the preliminary stability results, it was concluded that the processing and analysis of the plasma samples shall be completed within 16 days after animal experiments. The collected plasma samples were frozen and thawed only once and were processed immediately after thawing. After processing, the samples were kept in the auto sampler for a time not longer than 6 h.

Table 3: Accuracy Data of Silymarin in Plasma Samples

Sr. No.	Concentration in spiked plasma (µg/mL)	Peak area of Silymarin in Spiked plasma	Concentration Recovered from plasma (µg/mL)	Mean Concentration (µg/mL)	% Deviation
1	2	31627	1.71	1.69	15.27
		30422	1.64		
		31245	1.69		
		30945	1.67		
		32456	1.77		
		119272	7.36		
2	8	124585	7.70	7.52	6.03
		122354	7.56		
		118737	7.33		
		123562	7.64		
		302410	19.16		
3	20	295451	18.71	19.07	4.64
		311424	19.74		
		304428	19.29		
		291452	18.46		
		474199	30.23		
4	32	492145	31.39	30.66	4.19
		482542	30.77		
		472962	30.15		
		482456	30.76		

Table 4: Intraday Precision Data of Silymarin in Plasma Samples

Sr. No.	Concentration in spiked plasma (µg/mL)	Average Peak Area (n=5)					S.D.	% CV
1	2	29627	30422	28245	28746	30456	991	3.36
2	8	117272	124585	122354	114737	123562	4275.88	3.55
3	20	302410	295451	315424	304428	291452	9223.30	3.06
4	32	494199	502145	482542	492962	512456	11163	2.25

Results of interday precision and recovery are shown in Tables 5 and 6, respectively. The mean interday precision was found to be 6.56%. To assess the extraction recovery of silymarin from plasma, protein precipitation method was chosen due to simplicity. The mean extraction recovery was 91.24 ± 1.27%.

Table 5: Interday Precision Data of Silymarin in Plasma Samples

Sr. No.	Concentration in spiked plasma (µg/mL)	Average Peak Area (n=5)			S.D.	% CV
		Day 1	Day 2	Day 3		
1	2	29499	27134	30945	1923	6.59
2	8	120502	102452	112145	9033	8.08
3	20	301833	274154	295412	14487	4.98
4	32	496861	435452	462515	30776	6.62

Table 6: Recovery Data of Silymarin in Plasma and Methanol

Sr. No.	Concentration in spiked plasma (µg/mL)	Concentration of Silymarin Std (µg/mL)	Average Peak Area ± S.D. (n=5)		% Recovery
			Silymarin in Spiked plasma	Silymarin Std in Methanol	
1	2	2	28367 ± 990	31564 ± 1265	89.55
2	8	8	127272 ± 11330	137563 ± 9773	92.51
3	20	20	292410 ± 10138	320991 ± 13379	91.09
4	32	32	484199 ± 9859	527288 ± 18154	91.82

3.6 Applicability of Developed Method

Suitability of the developed method was investigated in preclinical pharmacokinetic study of silymarin. Figure 3 shows mean plasma concentration time profile of silymarin after a 300 mg/kg oral dose administration to New Zealand white rabbit (three). Table 7 shows the pharmacokinetics parameters determined by noncompartmental analysis of the plasma drug concentration time profile.

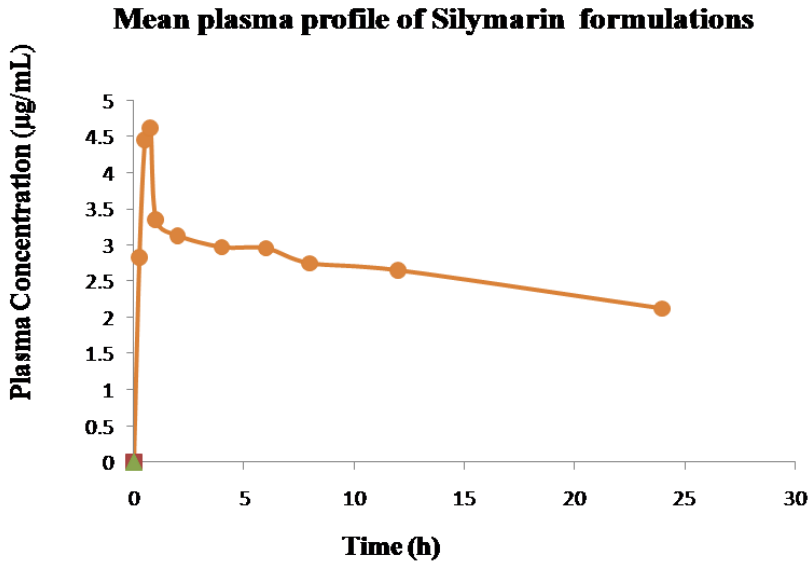

Figure 3: Mean plasma concentration Vs time profile of silymarin following a single dose of 300 mg/kg to rabbit

Table 7: Pharmacokinetic Parameters of Silymarin following a single oral dose of 300 mg/kg to New Zealand White Rabbit

Pharmacokinetic parameters	Average ± SD (n=3)
T_{max}, h	0.75 ± 0.011
C_{max}, µg/mL	4.627 ± 0.058
AUC, µg.h/mL	63.953 ± 1.720

4 CONCLUSION

A simple, sensitive, and reproducible HPLC method that is applicable for preclinical pharmacokinetic study of silymarin in plasma was developed and validated. The stability studies demonstrated that

silymarin was stable during assay procedures and for 16 days in frozen storage conditions. The developed method is selective without any interference of endogenous substances. Developed method require less time for sample pretreatment and cost effective compared to LC-MS method.

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