Quantitative estimation of silybin in *Silybum marianum* mother tincture using high performance liquid chromatography

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Silybin is a characterising compound present in the plant *Silybum marianum,* used as an hepatoprotective. A simple, accurate and sensitive HPLC method was developed for estimation of silybin. The chromatographic separation was carried out on RP C18 (Waters) column (250 × 4.6 mm, 10 µm) using mobile phase, phosphate buffer: water (65:35 % v/v) pumped at a flow rate of 1 ml/min. The eluent was monitored at 288 nm. The method is specific and linear over the range of 10-200 ng/ml. The method was statistically validated for precision, accuracy, robustness and recovery. The HPLC method can be applied for identification and on of silybin in herbal extracts of *Silybum marianum.*

Key words: HPLC, mother tincture, quantitative estimation, silybin, *Silybum marianum*

INTRODUCTION

In the area of hepatoprotective drugs, *Silybum marianum* of family Asteraceae (Compositae) is one of the most widely used drugs.[1] The drug has been recognised in the Homeopathic pharmacopoeia of India, volume I under *Carduus marianus*.[2] In folk medicine, flowering heads of *S. marianum* are consumed by diabetics. Young leaves of the plant are used as salads. Roots are eaten boiled as an potherb[3] The key constituent of *S. marianum* is silymarin, a mixture of flavanolignans, which is produced in the plant by a radical coupling of a flavanoid, taxifolin and coniferyl alcohol.[4]

The major constituent of silymarin is silybin [Figure 1] which shows inhibitory activity on several peroxidative enzymes.[4,5] Silybin inhibits liver damage induced by rare earth metal (praseodium).[6] It also stimulates the formation of new hepatocytes[7] and inhibits the cholesterol biosynthesis.[8]

The methods to analyse the flavonolignan content of *S. marianum* consist of a spectrometric method[9] and a colourimetric method, but these methods lack sensitivity and specificity. The chromatographic methods of analysis include TLC,[10,11] HPLC methods and LC-MS methods. The existing HPLC methods[12-16] for determination of silybin, either did not have sufficiently low level of detection or they required column-switching technique[13] or have a long run time[14,15] or did not result in sufficient resolution.[16] Thus, an attempt has been made to develop and validate HPLC method for the analysis of silybin, which would be highly sensitive, having good resolution, shorter retention time and reproducible.

MATERIALS AND METHODS

Plant Material

Seeds of *Silybum marianum* were imported from Europe via Unicorn remedies and authenticated at Agharkar Research Institute, Pune, India.

Chemicals and Reagents

The silybin procured from Sigma Aldrich, Switzerland was subjected to Ultra-violet (UV), Infrared Proton Nuclear Magnetic Resonance (NMR) spectral analysis to confirm their identity and purity. HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate and o-phosphoric acid of AR (Analytical Reagent) grade were procured from Qualigens Fine Chemicals (Mumbai, India). Deionised and ultra pure water used in all experiment was obtained from Milli-Q system (Millipore, USA).

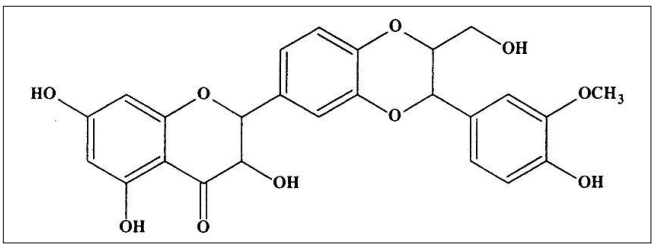

Figure 1: Structure of silybin

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Equipments

pH of the mobile phase was checked on a pH/ion analyser (Lab India PHAN, India). The HPLC system employed in the method development and validation was Jasco PU 1580 intelligent HPLC pump with a Rheodyne 7725 loop injector, Jasco UV-Vis 1520 detector and Borwin Chromatographic Software (version 1.21) as data integrator.

Optimised Chromatographic Conditions

The chromatographic separation was achieved on RP Spherisorb C-18 (Waters) column (250 × 4.6mm, 10 μm), using a mobile phase consisting a mixture of 0.1M sodium dihydrogen phosphate buffer (pH3.3): water (65:35 % v/v). All reagents were filtered through 0.45 μm filter paper and sonicated before use. The injection volume was 100 μl. The UV-Vis detector was set at of wavelength 288 nm. The experiment was performed at room temperature and the flow was fixed at 1 ml/min.

Preparation of Standard Solution

A stock solution of silybin (1 mg/ml) was prepared in methanol from the standard procured. Standard solutions were prepared by dilution of the stock solution with mobile phase to give solution in concentration range of 10–200 ng/ml.

Sample Preparation

Preparation of in-house mother tincture

Strong alcohol (53.7 ml) and purified water (50 ml) was added to powdered seeds of *S. marianum*. After four weeks, the supernatant fluid was decanted. The total juice was then finally filtered through G-3 sintered glass filter to obtain the mother tincture of the plant drug.

Validation of method

A stock solution of the drug was prepared at strength of 1 mg/ml. It was diluted to prepare solutions containing 10–200 ng/ml of silybin. The solutions were injected in triplicate into the HPLC column, keeping the injection volume constant (100 μl).

Five injections, of three different concentrations (10, 50 and 200 ng/ml), were given on the same day and the values of relative standard deviation (R.S.D) were calculated to determine intra-day precision. These studies were also repeated on different days to determine inter-day precision.

Accuracy was evaluated for the known concentration of the drug. The recovery of the added drug was determined. The specificity of the method was ascertained by analysing standard silybin and then comparing the sample retention time (RT) of silybin in herbal extract with the RT of the standard.

The LOD and LOQ were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations.

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution was recorded. The flow rate of the mobile phase was 1 ml/min.

The solution stability was carried out by leaving both the test solution of sample and standard in tightly capped volumetric flasks at -20^0 for seven days. The sample solution was assayed after seven days against fresh samples.

Application of the Method

The developed solvent system was validated and was successfully applied for quantitating the silybin content of the mother tinctures (one in house and two marketed). 1 ml of in-house mother tincture was diluted to 100 times in methanol, 1 ml of this dilute solution was further diluted to 20 ml with methanol and 100 μl was injected on to the column. And 1 ml of marketed mother tincture 1 and 2 (separately) were diluted 100 times in methanol, 1 ml of this dilute solution was further diluted to 40 ml with methanol and 100 μl was injected on to the column.

RESULTS AND DISCUSSION

A simple and sensitive HPLC method was developed which required a short run time of 15 min. The mobile phase composition was optimised to give better resolution and shorter retention time. The pH of the mobile phase was kept acidic, as silybin is acidic in nature due to the presence of phenolic groups in the structure. For the separation of the drug pH of the mobile phase was optimised to 3.3. Since the method was reversed phase HPLC, C8 and C18 column were tried. But C18 column was preferred due to the moderately polar nature of the drug. Sufficient separation was achieved using C18 column.

For the detection purpose spectroscopic characteristics of the compound were considered. Silybin has UV chromophore properties due to the presence of benzopyran ring. The UV absorbance was found to be 288 nm; hence the detector wavelength was set at 288 nm. The mobile phase comprising of phosphate buffer: water (65:35 % v/v) was found to be optimum which gave the best resolution and sensitivity. Figure 2 shows a representative chromatogram of standard silybin using the proposed method. As shown in the figure, silybin was eluted forming symmetrical peak and well separated from the solvent front. Observed retention time (8.1 min) allowed a rapid determination of silybin.

The method was validated with respect to parameters like linearity, precision, accuracy, specificity and robustness.

The method developed was validated for limit of detection

(LOD) and limit of quantitation (LOQ) in order to determine and ensure sensitivity of the developed method. The limit of detection (LOD) and limit of quantitation (LOQ) were found to be 5 and 10 ng/ml respectively. The method was found to be linear over the range of 10–200 ng/ml with a regression value of 0.99.

Table 1 provides data obtained from the precision experiments. The R.S.D. values for intra and inter-day precision were <2%, respectively, thereby indicating that the method was sufficiently precise. The method was found to be specific to the drug. The drug peak was free from any coeluting peak. The result indicated that the method was capable of analytical application with high precision.

The % R.S.D. of the assay of silybin during solution stability experiments were within 2%. No significant changes were observed during solution stability. The solution stability data confirms that the sample solutions were stable at least for seven days, thus, conforming the stability of sample solution, prior to development and after development.

Thus the validated method was applied to determine the percentage content of silybin in *S. marianum*. The percentage content of silybin per 100 ml of mother tinctures (one in house and two marketed) was determined successfully. The results are summarised in Table 2. The representative chromatograms of in house mother tincture, marketed mother tincture 1 and marketed mother tincture 2 are shown in Figures 3, 4 and 5 respectively.

CONCLUSION

The developed and validated method reported was found to be specific, accurate, simple, precise and reproducible. Apart from the greater precision and sensitivity attained using the HPLC method, the specificity offered is undoubtedly another advantage compared to the colourimetric, spectrometric and other methods/techniques of analysis. The outcome justifies the use of this HPLC method and the validation data convincingly demonstrates the adequate performance of the proposed method of analysis. Thus, the method presented is of considerable importance and has great industrial applicability for

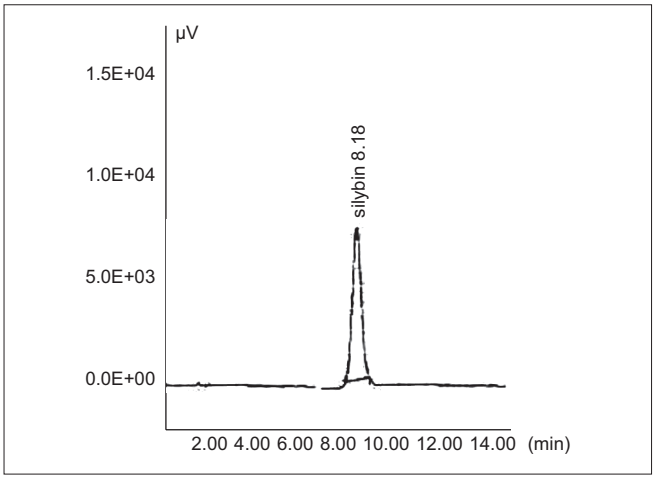

Figure 2: Chromatogram of standard silybin

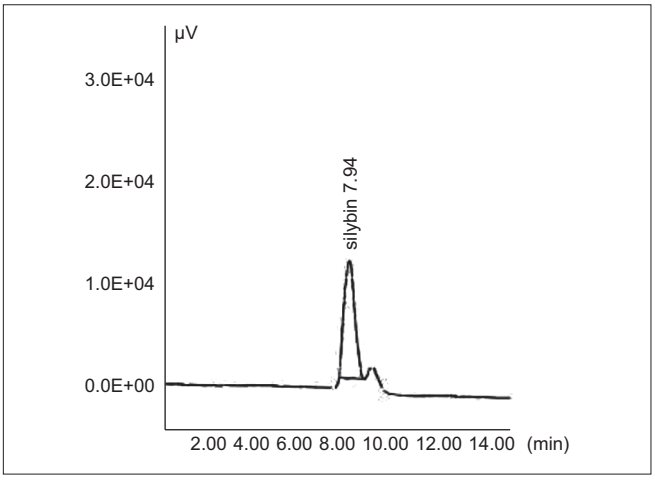

Figure 3: Chromatogram of in house mother tincture

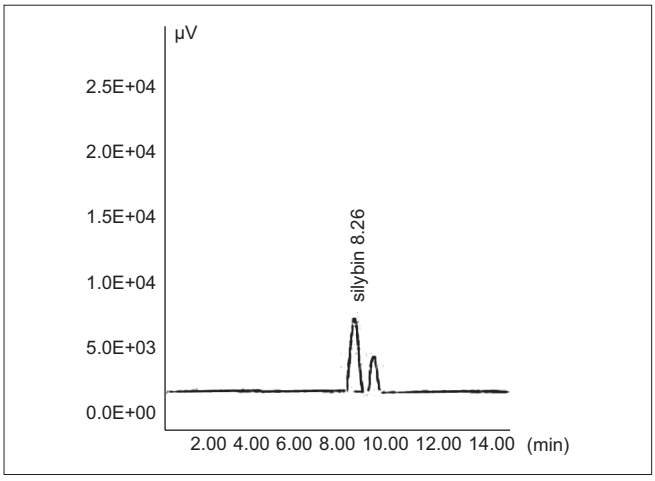

Figure 4: Chromatogram of marketed mother tincture 1

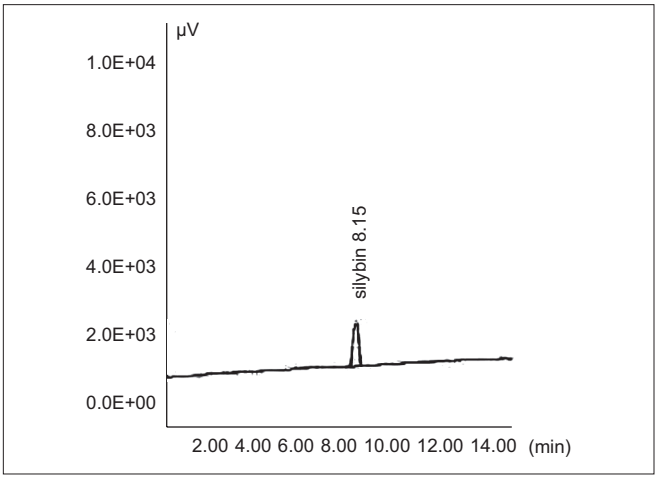

Figure 5: Chromatogram of marketed mother tincture 2

Table 1: Summary of validation parameters for the proposed method

Parameters	Values
Detection limit	5 ng/ml
Quantitation limit	10 ng/ml
Calibration range	10–200 ng/ml
Accuracy (%)	98.92–99.23
Precision (RSD, %)	
Intraday (n=5)	0.74–1.83
Interday (n=5)	0.68–1.81
Correlation coefficient (r)	0.99

Table 2: Silybin content

Mother tincture	Mg of silybin per 100 ml of mother tincture
In-house mother tincture	37.02
Marketed mother tincture 1	25.60
Marketed mother tincture 2	4.03

routine analysis of extract of *Silybum marianum* for silybin content and quality control.

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