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Evaluation and method development of promising hepatoprotective and cytoprotective antioxidant (Silymarin) by chromatography

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Abstract
In recent days, application of herbal drugs is increasing for curing many diseases all over the world. Silymarin, a herbal compound used worldwide from ancient times have shown protective effects in the fields of nepherotoxicity, hepatotoxicity, viral hepatitis, neurotoxicity, lung diseases, depression and cancer. Chromatographic methods play significant role in the drug discovery, development, formulations and quality control in pharmaceuticals industry Validation of an analytical procedure is used to demonstrate that it is suitable for its intended purpose. Hence in the proposed study, we established a new reliable gradient HPLC method for the determination of Silymarin from herbal medicine. The method was validated with respect to specificity, accuracy, precision, linearity and range, limit of detection, limit of quantification, and robustness. This also includes the details ofsample preparation and method of analysis.

Keywords: HPLC, method validation, silymarin, linearity, precision, hepatoprotective

1. Introduction
Silybum marianum L. (Milk Thistle) a member of Carduus Marianum family is an anti-hepatotoxicpolyphenolic substance, isolated from the milk thistle(ref WJOH). It belongs to the family Asteraceae [1, 2]. It(Figure 1), is 3, 5, 7-trihydroxy-2-[3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxy-methyl)-1,4-benzodioxan-6-yl]-4-chromanone [2].

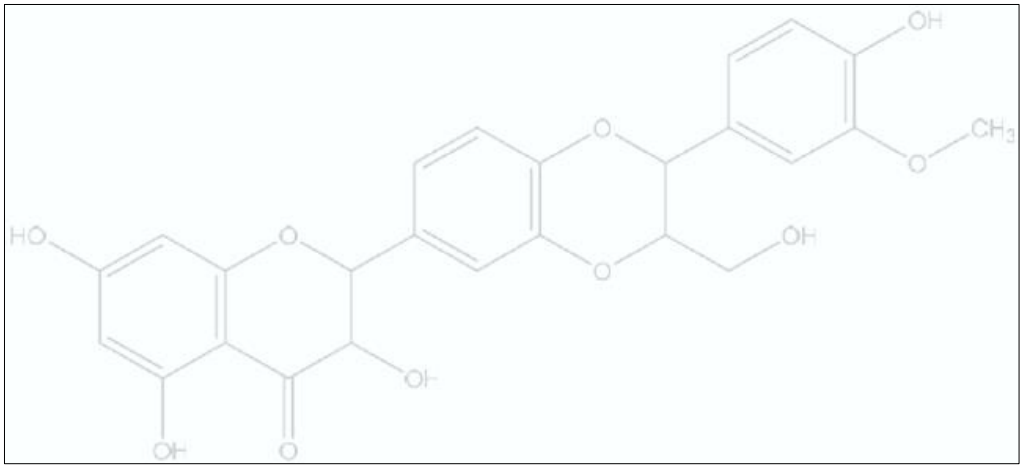


Fig 1: Structure of Silymarin

From centuries Silymarin has been used to treat various liver disorders as well as to prevent hepatotoxicity associated with poisoning [3]. It is an antioxidant, protecting liver from free radical damage produced by alcohol metabolism.Recently Silymarin is gaining attention due to its alternative beneficial effects like anticancer and chemopreventive properties, as well as hypocholesterolemic, cardioprotective, neuroactive and neuroprotective activities [2]. Silymarin increases Superoxide dismutase (SOD) activity of lymphocytes and erythrocytes, as well as the expression of SOD in lymphocytes in patients of liver diseases [4]. Silymarin has also been shown to increase patient serum levels of glutathione and glutathione peroxidase [4]. It is a

widely used compound among large number of herbal products having therapeutics benefits as well as antioxidant potential [5].
Literature survey showed that different analytical techniques have been attempted for the analysis of Silymarin. The present method is very simple, accurate, precise, rapid and cost effective and also able to overcome the different interferences of matrixes.

2. Material and methods

2.1. Instrumentation

Agilent HPLC system of HPLC -1200 series with Ezee chrome elite software was used for all chromatographic runs. The detector used is UV with C18 column of dimensions 100 mm X 4.6 mm and particle size of 3 µm.

2.2. Chemicals and reagents

Marketed formulation of Silymarin was used for the throughout validation process. The solvents and chemicals used are methanol, o- phosphoric acid and water of HPLC grade.

2.3. Preparation of standard stock solution (1000 µg/ml)

Stock solution was prepared by dissolving accurate amount of Silymarin reference standard in methanol to obtain a solution having concentration 1000 µg/ml. This solution was stable in the refrigerator at 5^0C for a week.

2.4. Preparation of Working Standard Solutions

Working Silymarin standard solutions were prepared by accurately transferring different volumes (0.75-15 ml) of stock solution into 100ml volumetric flasks, completing to volume upto the mark with methanol in order to obtain different working standard solutions of Silymarin in the range of (7.5 to 150µg/ml).These solutions were directly injected into the HPLC chromatograph.

2.5. Test Preparation

Tablets received as the labelled claim of 300 mg Silymarin per tablet were used. Test solution was prepared by weighing 20 tablets to determine their average weight, then an amount of powdered tablets equivalent to 100 mg of Silymarin was transferred into a 100-mL volumetric flask, 40 mL of methanol was added, then the solution was sonicated for 15 min and the volume was completed with methanol to obtain a stock test solution having a concentration of 1000µg /ml. The stock test solution was filtered, 5 ml of this solution was transferred into a 50-mL volumetric flask and completed to volume with methanol and mixed well to obtain the working test solution. This solution was injected directly into the HPLC chromatograph.

2.6. Validation parameters [6, 7]

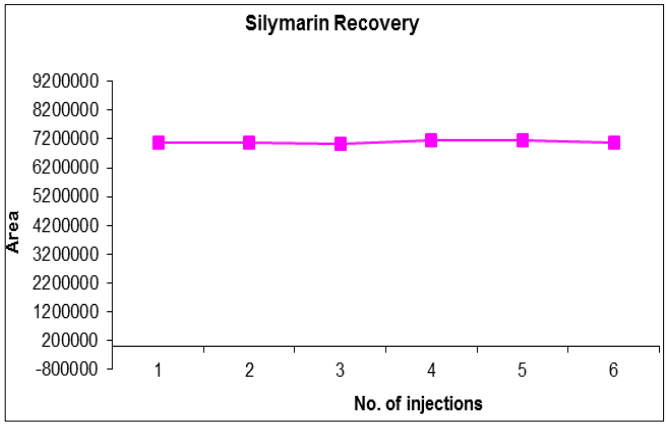
The following are typical analytical performance parameters which were validated during method verification:

2.6.1. Specificity

Selectivity of the analytical method is defined as the degree to which a method can quantify the analyte in the presence of interferences, impurities, degradants and different matrixes. To assess the specificity of our method, we have used blank solvent as injection and there was no interference observed at the RT (retention time) of Silymarin. Hence the method is specific to our analyte of interest.

2.6.2. Accuracy/ Recovery

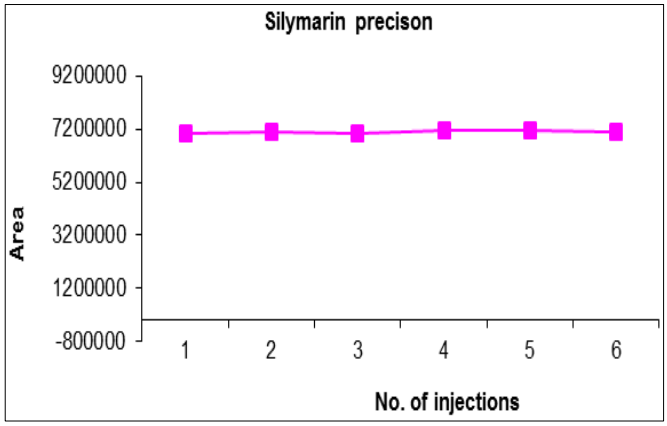
It is the nearness of a measured value to the true or accepted value. It is determined by applying the method in which known amount of analyte have been added to samples. This should be analysed against standard and blank solutions to ensure that no interference exists. Accuracy is done by spiking the known amount of reference standard in the sample at the start of preparation and same method was followed as per the test preparation (2.5) and the final solution was injected into HPLC. Six injections of the final solution were given and RSD was calculated (presented in Graph 1). The accuracy is calculated from the test results as percentage of the analyte recovered by the assay. The Recovery is 88%. Hence the method is accurate.



Graph 1: Accuracy/Recovery of Silymarin

2.6.3 Precision

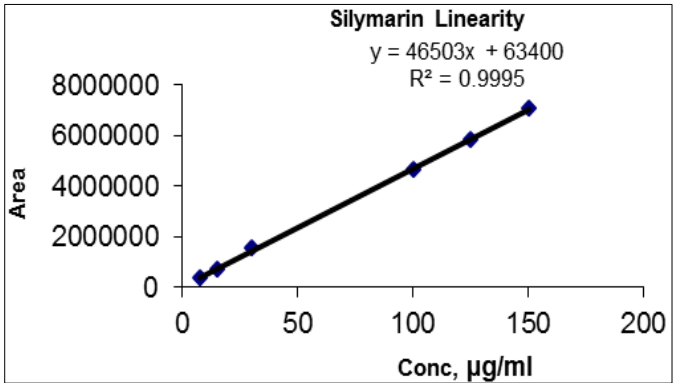
Precision is a measure of the reproducibility and repeatability of the whole analytical method. Silymarin standard solution (150µg/ml) was prepared and injected in six replicates. The value of % relative standard deviation (RSD) obtained for area response is 0.7, hence the system and method is precise. The RSD calculation for area response is graphically presented in Graph 2.



Graph 2: Precision of Silymarin at 150µg/ml

2.6.4. Linearity and Range

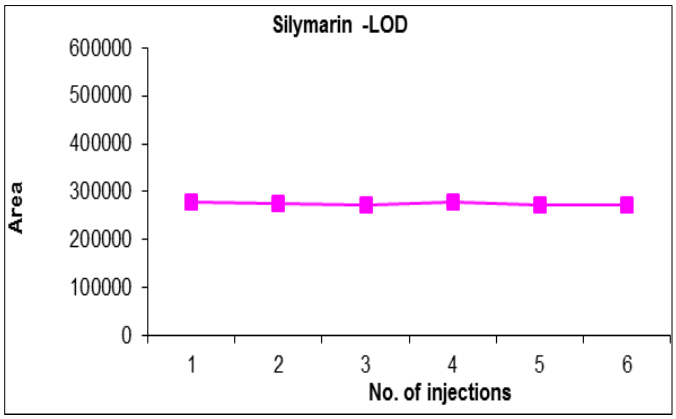
Six preparations of Silymarin standard solution ranging from 7.5 to 150µg/ml concentration were prepared and injected in to HPLC. The Correlation Co-efficient is more than 0.99, hence the method is linear in the range of 7.5 to 150µg/ml concentration. It is illustrated by the graphical presentation between the sample concentration and area in Graph 3.



Graph 3: Calibration curve of Silymarin

2.6.5. Limit of Detection (LOD)

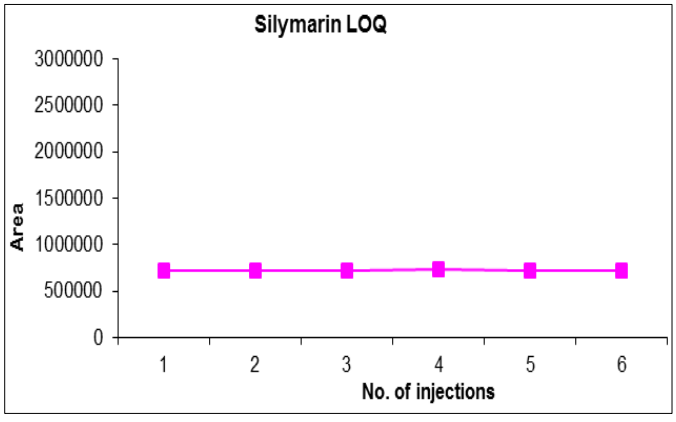
The detection limit or limit of detection of an individual procedure is the lowest amount of analyte in a sample that can be analyzed but not necessarily quantified. It is calculated by the Signal to Noise (3:1) ratio method, which is usually expressed as the concentration of analyte in the sample. To establish the LOD value six runs of blank were given and then the LOD was found to be 5µg/ml. The RSD value of the six injections of LOD (5µg/ml) solution was found to be 1.1%. It is presented in Graph 4.



Graph 4: Precision at LOD (5µg/ml)

2.6.6. Limit of Quantification (LOQ)

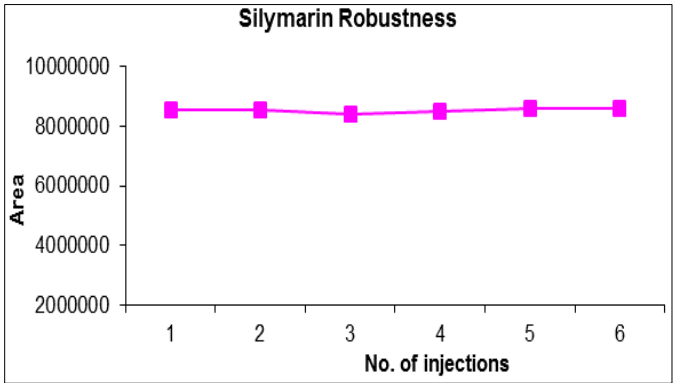
The limit of quantification of an individual procedure is the lowest amount of analyte in a sample that can be quantified. It is calculated by the Signal to Noise (10:1) ratio method, which is usually expressed as the concentration of analyte in the sample. LOQ was found to be 15µg/ml. The RSD value of the six injections of LOQ (15µg/ml) solution was found to be 1.0%. It is presented in Graph 5.



Graph 5: Precision of Silymarin at LOQ level (15µg/ml)

2.6.7. Robustness

It is defined as the measure of ability of an analytical method to remain unaffected by small but deliberate variation in method parameters e.g. change in pH, mobile phase composition, temperature and instrumental settings. Active ingredient are analyzed by two different analysts and chromatographed. The overall results RSD% was calculated and it was found to be 0.98 which is presented in Graph 6.



Graph 6: Robustness of Silymarin

2.7. Chromatographic conditions

The below mentioned chromatographic conditions were maintained throughout the validation process:
Reverse phase mode of chromatography
UV DAD-288nm

Column: Princeton (C18) column.

Dimension: (100X 4.6 mm) and Particle size: 3µm.

Mobile phase: Gradient elution of mobile phase

Mobile phase A: 80% water: 20%Methanol: 0.5%Orthophosphoric acid

Mobile phase B: 20% water: 80%Methanol: 0.5%Orthophosphoric acid

Flow rate: 1 ml/min.

Injection Volume: 20µl

Temperature: Column oven was maintained at 40 ^{0}C.

3. Results and Discussion

An attempt was made to develop a simple and specific HPLC method for the determination of Silymarin in bulk and tablet dosage form. Using C18 column the Silymarin was eluted in the sum of six peaks (Silychristin, Silydianin, Silybin-A, Silybin-B, Iso Silybin-A, Iso Silybin-B) which are then integrated. The elution profile is represented in the following chromatograms (Figure 2, Figure 3 and Figure 4)

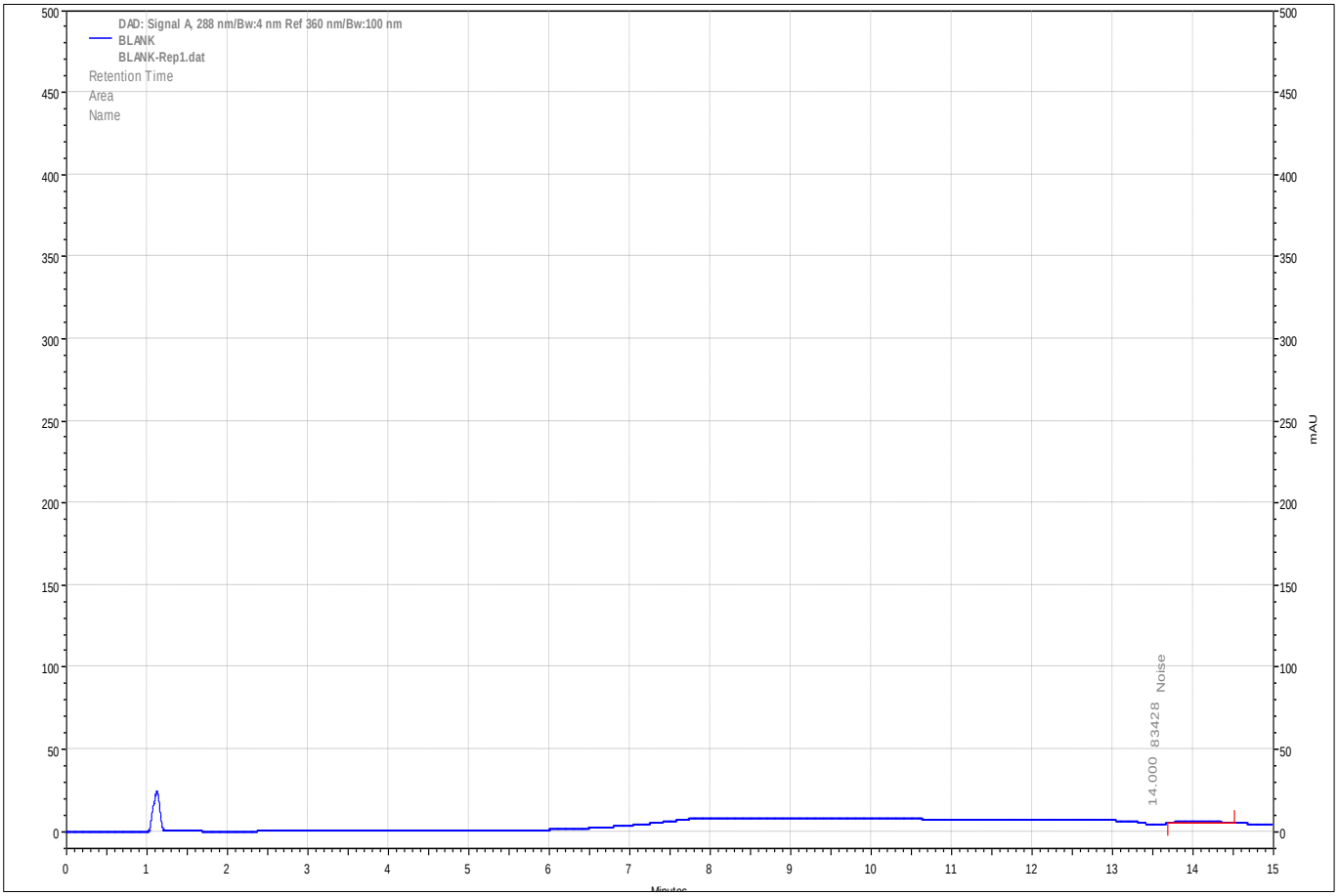


Fig 2: HPLC chromatogram of blank solution

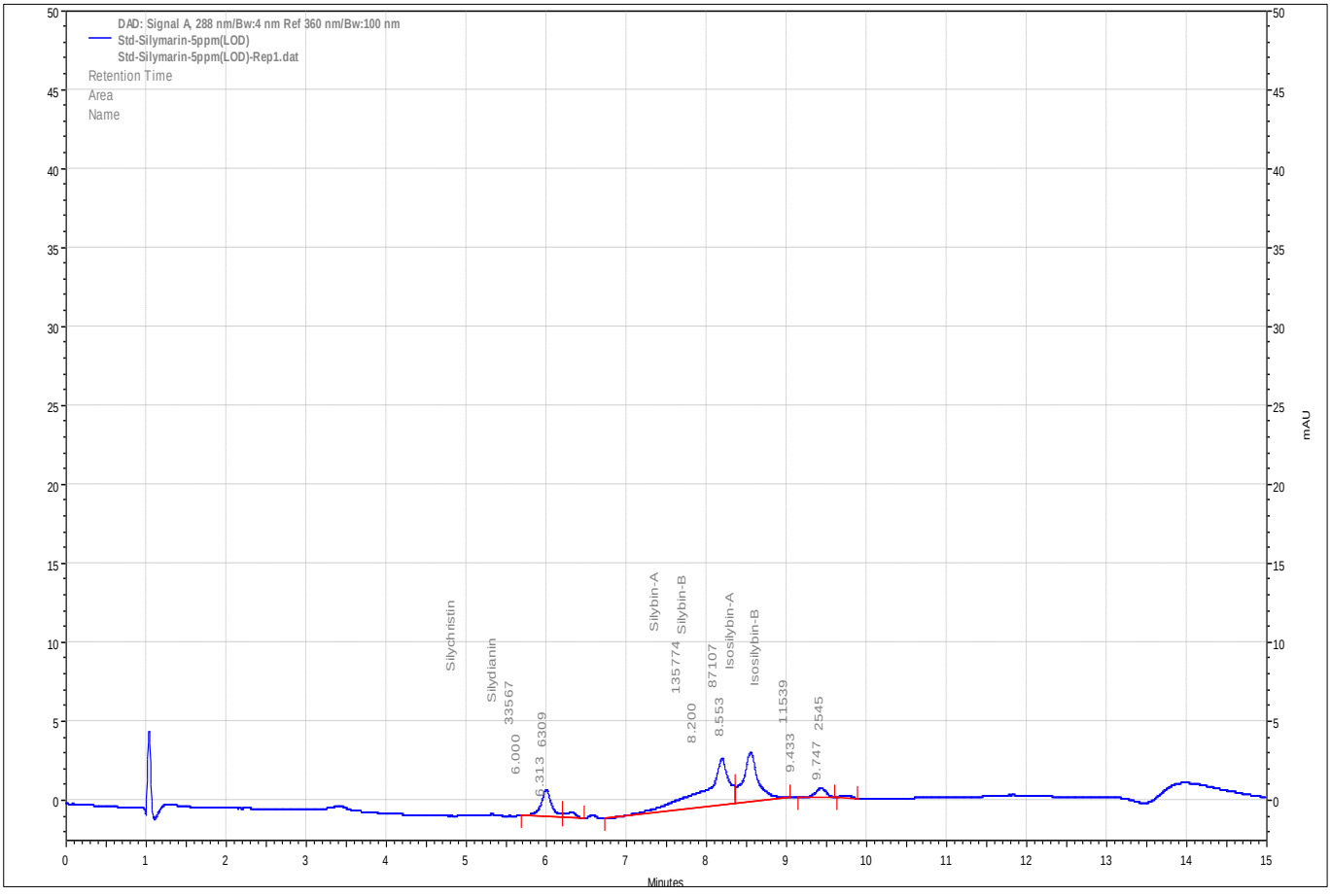


Fig 3: HPLC chromatogram of 5µg/ml standard solution of Silymarin

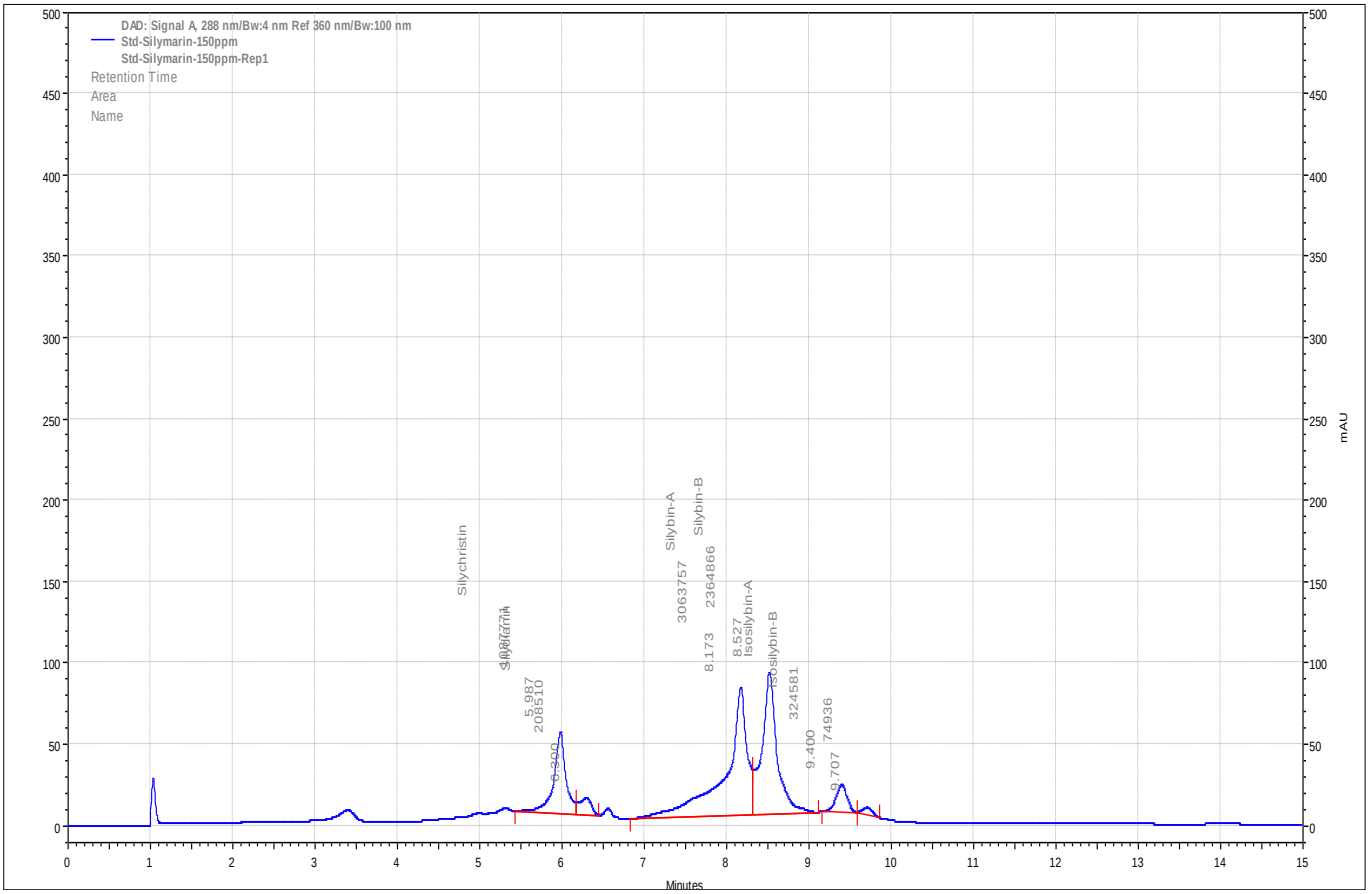


Fig 4: HPLC chromatogram of 150µg/ml standard solution of Silymarin

The Linearity range for Silymarin was 7.5 to 150 µg/ml at wavelength 288nm. The coefficient of correlation for Silymarin is 0.999. This shows that Silymarin has good regression values at its wavelength and the results of recovery studies revealed that small changes in drug concentration in the solutions could be accurately determined by this validated method. The relative standard deviation (RSD) for all the validation parameters is found to be less than 2%. The validity and reliability of the proposed method is assessed by the recovery studies.

Summary of the validation parameters results is presented in the below mention table1.

Table 1: Summary of results of Silymarin validation parameters

Sl. No.	Parameters	Result
1	Specificity	No interference was observed at the RT of Silymarin in the blank with respect to standard peak
2.	Accuracy/Recovery	88%
3.	Precision	0.7% (RSD)
4.	Linearity and range	0.999 (correlation coefficient)
5.	LOD	1% (RSD) @ 5 ppm
6.	LOQ	1%(RSD) @ 15 ppm
7.	Robustness	0.9%(RSD)

4. Conclusions

The validated method was accurate, precise and consistent for the determination of Silymarin in tablet dosage form. The advantages of the proposed method over the previously reported ones is the use of a DAD detector which is user friendly compared to the more sophisticated mass or fluorescence detectors[8]. We have used diode array detector as a tool for peak identification and purity confirmation. The method was validated with correlation coefficient of 0.999, accuracy of 88% along with RSD value of 0.7, 1.1, 1.0 and 0.9% for precision, LOD, LOQ and robustness respectively. Hence the method is used for the estimation of Silymarin by HPLC.

5. Acknowledgements

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