

### **Analytical Method Development and Validation for Estimation of Silymarin in tablet dosage form by HPLC**

#### **Abstract**

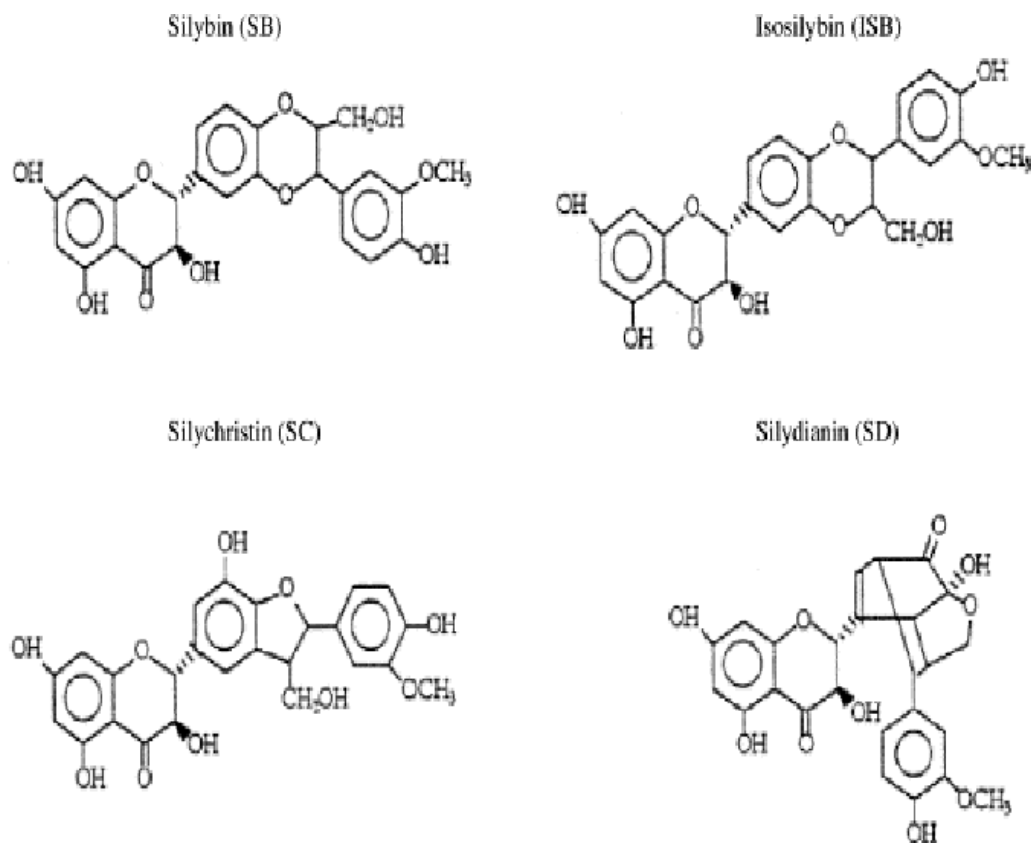
Silymarin is extracted from the *Silybum marianum* (milk thistle) plant having C<sub>25</sub> containing flavonoid mixture. It is mainly used for its effect in liver disease. The HPLC method for determination of assay of silymarin tablet had been validated for precision, accuracy (recovery) & Linearity. In the present study, an attempt was made to provide a newer, simple, sensitive, precise, accurate stability and low cost HPLC method for the effective quantitative determination of silymarin as an active pharmaceutical ingredient as well as in pharmaceutical preparations without the interferences of other constituent in the formulations. HPLC method is developed and validated for various parameters as per ICH guidelines. The validated method was effectively useful to the commercially accessible pharmaceutical dosage form, yielding extremely good and reproducible result.

**Key words:** Silymarin, HPLC, Precision, Linearity & Accuracy.

#### **Introduction**

Herbal drugs play an vital role in health care programs primarily in developing countries. Ancient Indian text incorporates an extremely broad definition of medicinal plants to be potential sources of medicinal substances<sup>1</sup>. Silymarin extracted from the *Silybum marianum* (Milk Thistle) plant having a C<sub>25</sub> containing flavonoid mixture. Silymarin has been the standard drug to treat liver disorders of diverse etiologies and their extracts have been used as conventional herbal remedies (“liver tonics”) for almost 2000 years<sup>2</sup>. Silymarin is the extract consists of seven flavonoglignans (silibinin, isosilibinin, silychristin, silydianin and isosilychristin) and a flavonoid (taxifolin). Amongst these substances, silybin is chiefly prevalent and has the mainly important

biological consequence. The 70% of the total silymarin in the form of two diastereoisomeric compounds: silybin A and silybin B<sup>3-5</sup>.



**Image 1: Molecular Structure**

Silymarin is mainly used for its effect in liver disease. It has also been mentioned in homeopathy for treatments of jaundice, bronchitis<sup>6</sup>. HPLC is extensively considered to be a technique chiefly for biotechnological, biomedical and biochemical research as well as for the pharmaceutical industry, these fields currently comprise only about 50 % of HPLC users. At present HPLC is used by a variety of fields including cosmetics, energy, food, and environmental industries. New methods including Reverse Phase Liquid Chromatography allowed for improved separation between very analogous compounds. Innovative techniques improved separation, identification, purification and quantification far above the previous techniques. Computers and automation added to the ease of HPLC. Improvements in type of columns and thus reproducibility were

made as terms such as micro-column, affinity columns and fast HPLC began to come out<sup>7</sup>.The objective of our research is to develop a method and validated that is more precise ,accurate, specific ,and sensitive ,had shortest retention time and give better resolution. Result the validation of that developed method is done to ensure that is suitable for its intended use. The aim of our research is to develop a dissolution and an assay analytical method for the estimation of Silymarin tablets by HPLC that is more precise, accurate, specific and give better resolution results.

## Experimental Section

### Instrument & Chemicals

For the estimation of Silymarin (URI DOX tablet) various chemical and instrument required are tabulated in table 1 & 2.

**Table 1.Instrument Required**

S.NO.	INTSTRUMENT	MAKE	MODEL
1	HPLC equipped with pump,injector and UV/ PDA detector	SHIMANZU	LC-2010 AHT
2	UPLC equipped with pump,injector and PDA detector	SHIMANZU	ACQUITY UPLC
3	Balance	METTLER	AB204-5
4	PH meter	Eutech	PH meter
4	PH meter	Eutech	-

5	Sonicator	Power Sonic 410	-
6	Analytical Balance	Sartorius	-

**Table 2. Reagent and Chemicals Required**

S.No.	Reagent/chemicals	Grade
1	Methanol	HPLC
2	Acetonitrile	HPLC
3	Water	Milli-Q
4	Orthophosphoric acid	AR
5	Formic acid	HPLC

### **Preparation of Mobile Phase**

#### **Buffer Preparation**

1.0% v/v formic acid in water (Take 10 ml of formic acid min with 500 ml water and make up volume 1000 ml with water).

### Mobile Phase

Min 65 ml buffer solvent and 35 ml methanol and sonicate

### Diluents

Methanol and 30 ml H<sub>2</sub> O .

### Reference Preparation

Weight accurately about 50 mg of Silymarin (Silymarin extract at 70%) working standard transfer in 50 ml volumetric flask and add about 40 ml methanol sonicate for 20 minute make the volume 50 ml with diluents with 0.45 ml micro nylon membrane filter (concentration:100mg/ml).

### Test Solution

Crush 20 tablets, weight accurately equivalent to 250 ml of Silymarin and transfer 250 ml of volumetric and make the volume 250 ml with methanol. Dilute 5 ml of the solution for 50 ml with diluents and filter with 0.45 ml micro nylon filter (Concentration: 100 mg/ml).

### Chromatographic parameters

Use suitable high performance liquid Chromatography equipped with UV Detector.

**Table 3.Chromatographic parameters**

Column	Acquity UPLC BEHC18, 2.1x100mm, 1.7 m (make : water)
Column Temperature	40°C
Flow rate	1.0 ml/min
Wave length	288 nm
Injection volume	2 ul

Run Time	10 minute
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### Calculation of Silymarin (mg/tablets)

$$A = \frac{AT}{AS} \times \frac{WS}{50} \times \frac{5}{50} \times \frac{250}{WT} \times \frac{50}{5} \times \frac{P}{100} \times \text{Avg. Wt.}$$

$$\text{Silymarin (\%)} = \frac{A}{140} \times 100$$

Where:

AT- Average area of Silymarin A and Silymarin B in the test solution.

Ws= Average area of Silymarin A and

Silymarin B in the standard solution Ws=

Weight of the reference standard

Wt= Weight of the test sample

Methods validation is the procedure of demonstrating that analytical procedures are suitable for their intended use<sup>8-10</sup>. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to sustain analytical procedures. The following are typical analytical performance characteristics which may be tested during methods validation<sup>11-14</sup>.

### Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a process with high accuracy, a sample (whose —true value is known) is analyzed and the measured value is identical to the true value. Typically, accuracy is represented and determined by recovery studies, but there are three ways to determine accuracy: comparison to a reference standard recovery of the analyte spiked into blank matrix, or Standard addition of the analyte.<sup>15-18</sup>

### Precision

The precision of an analytical procedure articulates the proximity of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the identical homogeneous sample under the prescribed conditions. Precision may be considered at three levels; Repeatability, Intermediate precision and Reproducibility. Precision should be obtained preferably using authentic samples. As parameters, the standard deviation, the relative standard deviation (coefficient of variation) and the assurance interval should be calculated for each level of precision.

### **Linearity**

The linearity of an analytical procedure is its aptitude (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. It may be demonstrated directly on the analyte, or on spiked samples using at least five concentrations over the whole working range. Moreover a visual evaluation of the analyte signal as a function of the concentration, suitable statistical calculations are recommended, such as a linear regression. The parameters slope and intercept, residual sum of squares and the coefficient of correlation should be reported.

### **Limit of Detection**

The detection limit (DL) or limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. In analytical procedures such as HPLC that reveal baseline noise, the LOD can be based on a signal-to-noise (S/N) ratio (3:1), which is usually expressed as the concentration (e.g., percentage, parts per billion) of analyte in the sample.

### **Limit of Quantitation**

The quantitation limit (QL) or limit of quantitation (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively

determined with suitable precision and accuracy. It is usually expressed as the concentration (e.g., percentage, parts per million) of analyte in the sample. For analytical procedures such as HPLC that demonstrate baseline noise, the LOQ is generally estimated from a determination of S / N ratio (10 : 1) and is usually confirmed by an acceptable percent relative standard deviations (% RSD)

### **Range**

The variety of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

### **Robustness**

Robustness is defined as the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g., pH, mobile-phase composition, warmth, and instrument settings) and provides an indication of its reliability during normal usage.

### **Specificity**

Selectivity is defined as the ability of the method to separate the analyte from other components that may be present in the sample, including impurities. Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products and excipients. To determine specificity during the validation blanks, sample matrix (placebo), and known related impurities are analyzed to determine whether interferences occur.

### **System Suitability Determination**

System suitability is the assessment of the components of an analytical system to show



that the performance of a system meets the standards required by a method. For chromatographic assays, these may include tailing factors, resolution, and precision of standard peak areas, and comparison to a confirmation standard, capacity factors, retention times, and theoretical plates.

**Table 4. Proposed use acceptance criteria for the different parameters specified by ICH**

Parameters	Proposed use acceptance criteria
Linearity	$r > 0.99$ , similar response ratio
Precision- system	RSD < 2%
Precision- method	RSD < 2%
Precision-repeatability/reproducibility	%R & R < 20%
Accuracy	FDA 98-102%, EPA 50-150%
Specificity	No interference
Detection limit	>2 times baseline
Quantitation limit	Signal to-noise ratio=10:1
Range	Conc. Where data can be reliably determined

## Result and Discussion

### Precision Method

The precision of an analytical procedure is the degree of conformity among individual test results when the procedure is applied repeatedly to multiple sampling of homogenous sample. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (Coefficient of variation) of a series of measurements.

**Table 5. Method Precision**

<b>Sample No.</b>	<b>% Assay of Silymarin</b>
1	99.44
2	98.99
3	99.37
4	99.85
5	100.97
6	99.52
Mean	99.69
SD	0.685
RSD (%)	0.687

**Acceptance Criteria**

The % relative standard deviation (RSD) for % assay of silymarin per the six samples should not be more than 2.

**Linearity**

For linearity, standard, solution was prepared in the range of 70 % to 130% of the test concentration. A graph was plotted with injection volume on X axis and response (area) of the analytical on Y axis and the co-relation co-efficient was determined. The result obtained is summarized in the table below.

**Table 6. Linearity for Silymarin**

<b>S.No</b>	<b>Injection value (ul)</b>	<b>Response (area)</b>
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1	1.4	187124
2	1.6	216280
3	1.8	245030
4	2.0	271745
5	2.2	299774
6	2.4	356373
7	2.6	356373
<b>Slope</b>		<b>140834</b>
<b>Intercept</b>		<b>-9473.4</b>
<b>Co-relation co-efficient</b>		<b>0.99993</b>

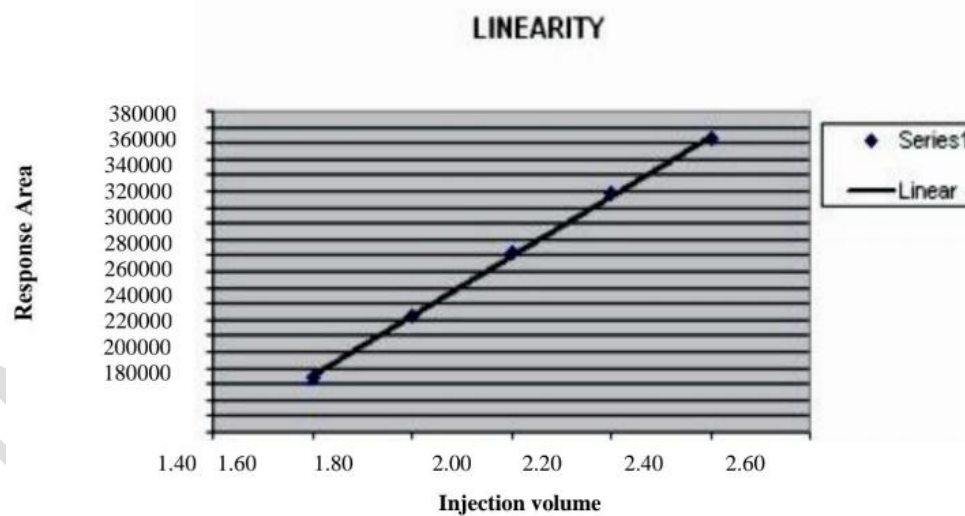


Image 2: The method linear from the injection volume 1.4 µl to 2.6 µl for the estimating of Silymarin. The co-relation co-efficient (CC) value should be less than 0.999.

#### **Accuracy (Recovery)**

The accuracy of an analytical producer express the closeness of agreement between the

volumes which is accepted either as a conventional true value or an accepted reference value and the value found. For known amount of Silymarin raw material was spiked in the placebo in triplicate at 80% 100% & 120% of test concentration. The amount of silymarin is quantified as per the test method. The percentage recovery is calculated from the amount found and actual added and the resulted are summarized in the table below.

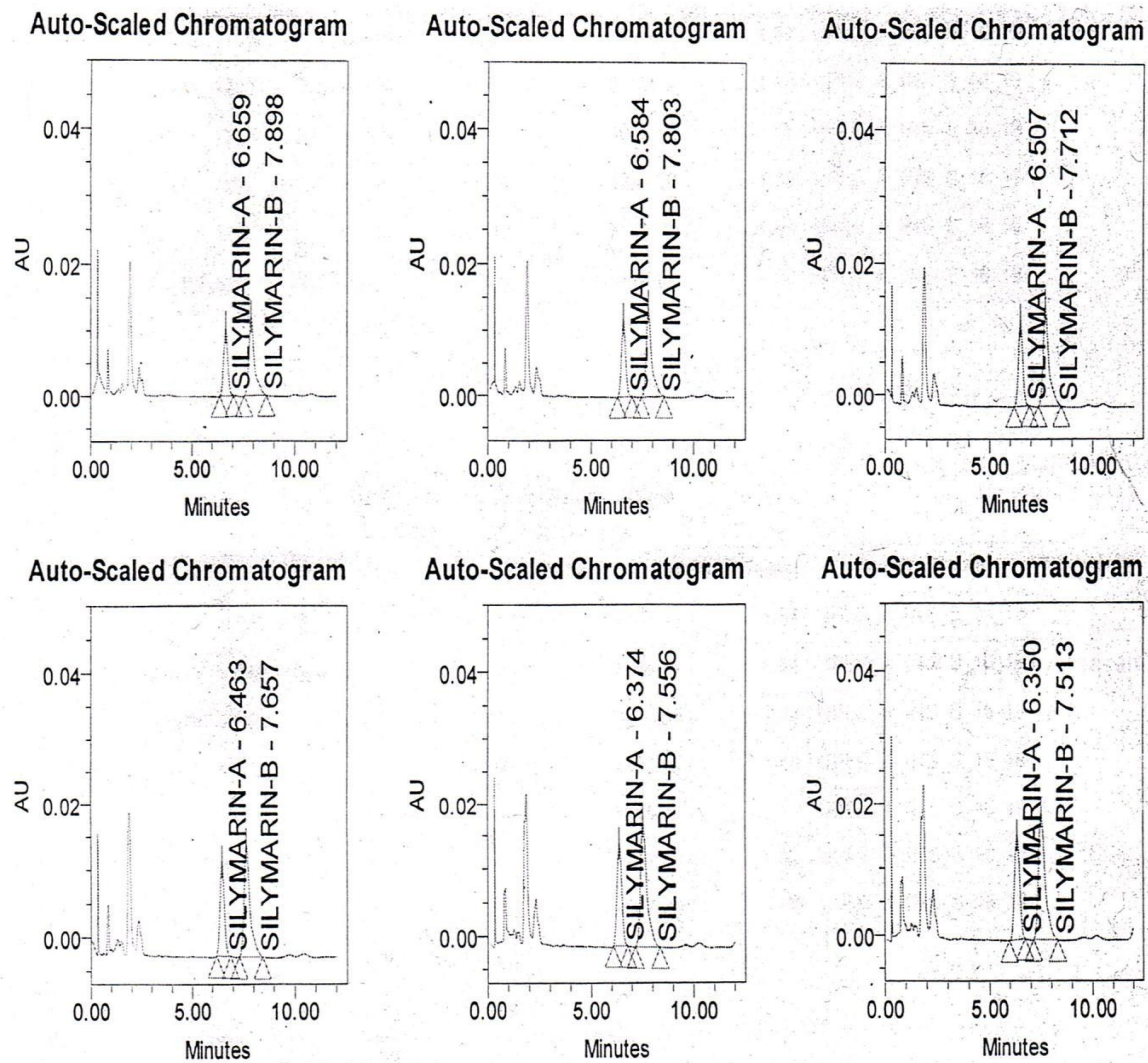
**Table 7. Accuracy (Recovery)**

S. No.	Known amount added in Placebo (in%)		Recovery		% of recovery
			Individual value	Average values	
1	1	80%	80.00	80.64	101.19
	2	80%	80.00	80.63	
	3	80%	80.00	81.59	
2	1	100 %	100.0 0	98.43	98.6 1
	2	100 %	100.0 0	98.33	
	3	100 %	100.0 0	99.08	
3	1	130 %	120.0 0	119.71	99.0 1
	2	130 %	120.0 0	118.47	
	3	130 %	120.0 0	118.26	

The analytical method convene the pre-established acceptance criteria for recovery study as per protocol, hence the method is accurate.

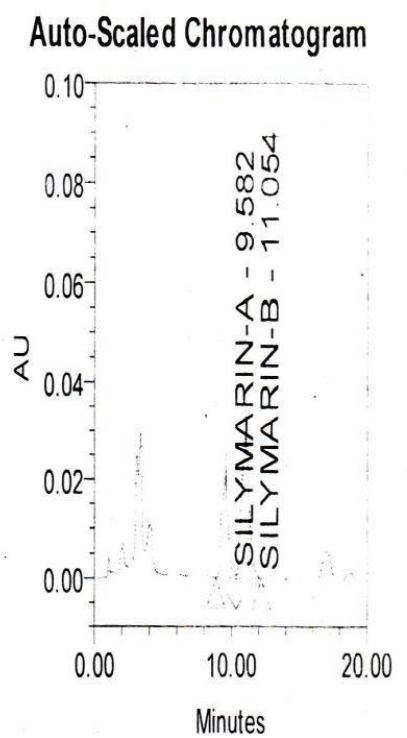
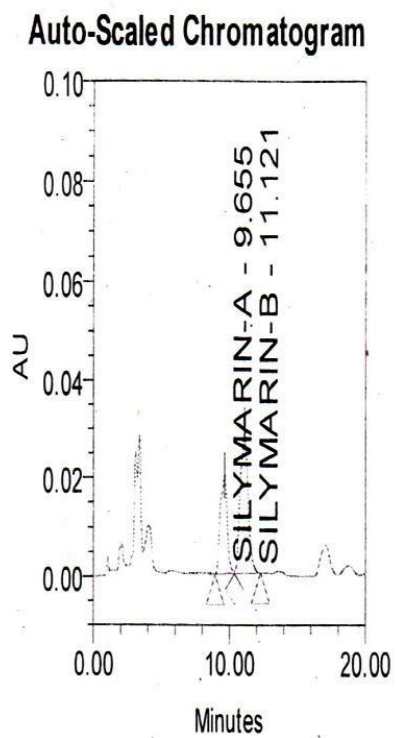
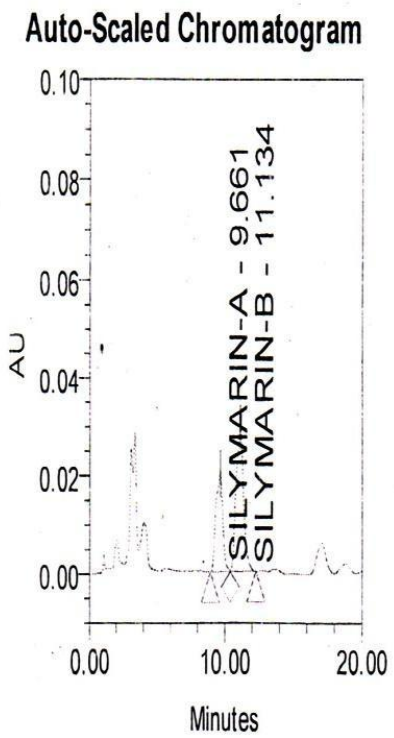
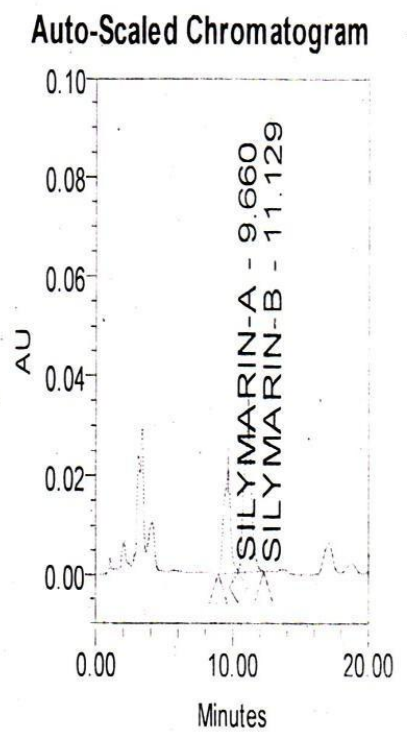
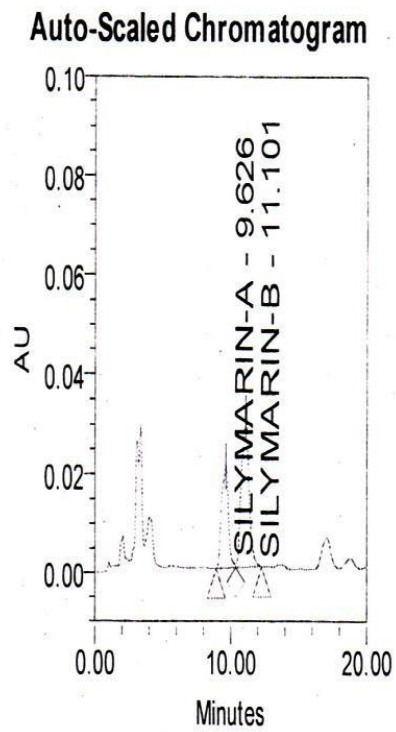
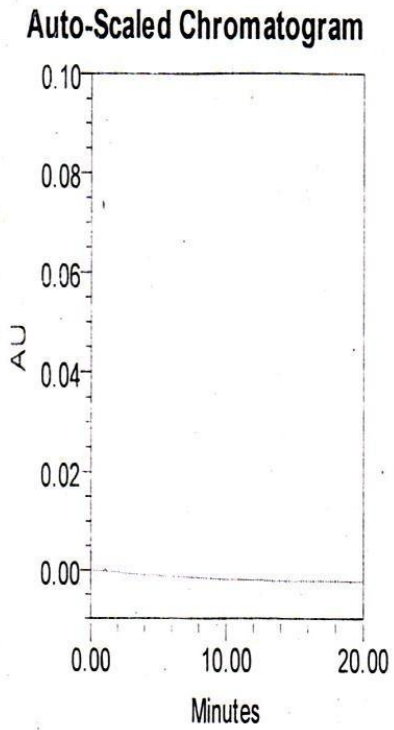
**Table 8. Summary of Validation Parameters**

<b>Validation parameters</b>	<b>Observation</b>	<b>Acceptance criteria</b>
Precision		
Method precision	0687%	RSD: NMT 20%
Linearity	0.99993	correlation coefficient : NLT 0.999
Accuracy (Recovery)	98.61% to 101.19 %	98% to 102%

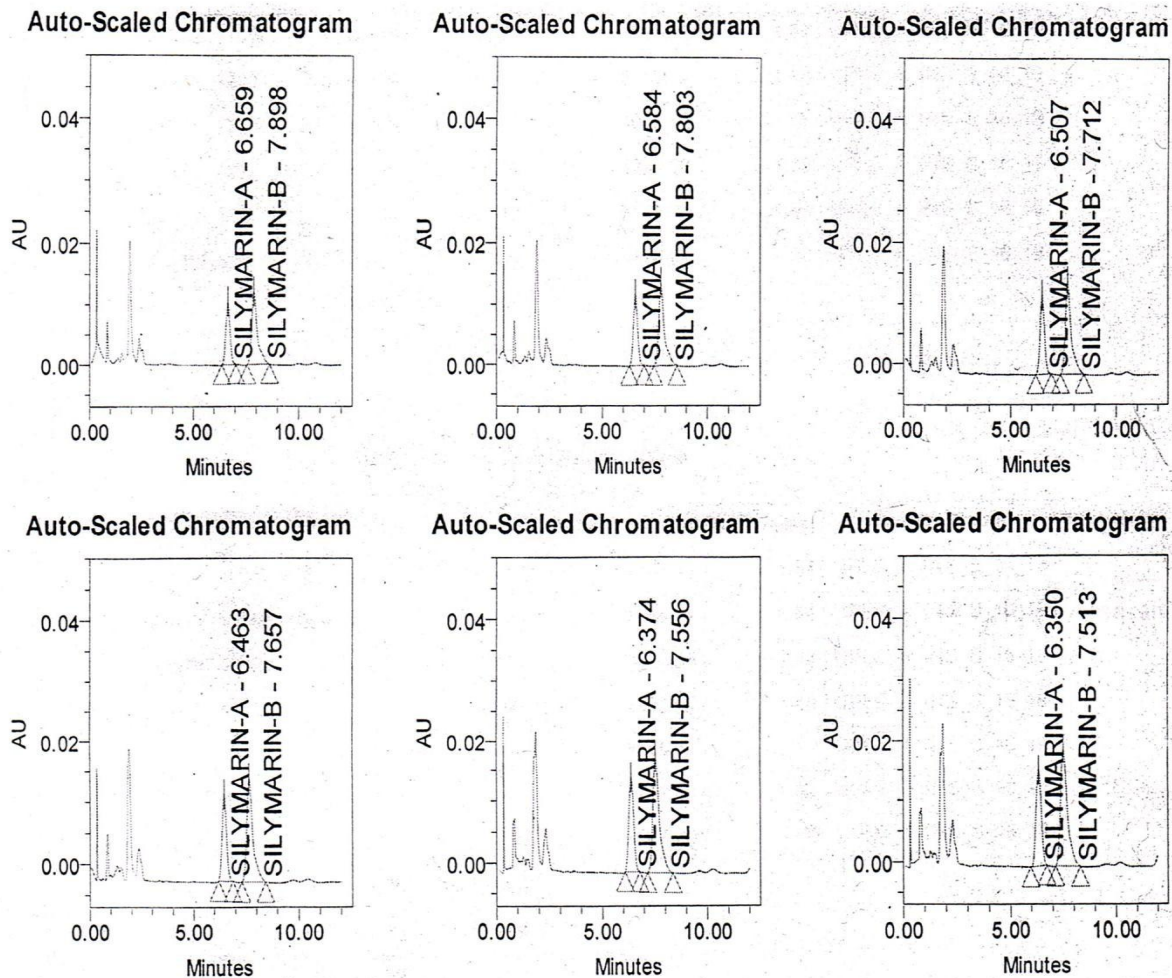


**Fig 1. Linearity : Blank, injection Volume: 2.00 ul, Run Time: 12:00 min.**

UNDER



**Fig 2. Linearity : Standard, injection Volume: 1.8 ul, Run Time: 12:00 min.**



**Fig 3. Linearity : Sample, injection Volume: 1.6 ul, Run Time: 12:00 min.**

### Summary and Conclusion

Silymarin is a vanishing bile duct syndromes (VBDS) are characterized by progressive loss of small intra-hepatic ducts caused by a variety of different diseases leading to chronic cholestasis, cirrhosis, and premature death from liver failure. From the reported literature, there was no validation method developed by HPLC for the determination of silymarin. So in the present study we develop and validated a chromatographic method by HPLC for the estimation of silymarin tablet dosage form. For the optimization of Chromatography a number of preliminary trials were conducted and the effects of mobile phase, flow rate, solvent ratio were studied to check the retention time, shape, resolution, and other chromatographic parameters. From these trials the mobile phase selected was mixture of phosphate buffer methanol and acetonitrile in the



ratio of 50:35:15. Orthophosphoric acid this was found to be ideal mobile phase. The flow rate was optimized at 1.5 mL/ min. and the column temperature is 40<sup>0</sup> C detection was carried out at 288 nm for Silymarin. This system produced symmetric peak shape, good resolution and shorter time for silymarin. The telling factor is not more than 2.0 and the RSD of Replicate injection is not more than 2.0%. In the present study, an attempt was made to provide a newer, simple, sensitive, precise, accurate stability and low cost HPLC method for the effective quantitative determination of silymarin as an active pharmaceutical ingredient as well as in pharmaceutical preparations without the interferences of other constituent in the formulations. HPLC method is developed and validated for various parameters as per ICH guidelines.

#### COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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