



NEW VALIDATED RP-HPLC METHOD FOR THE ESTIMATION OF SILYMARIN IN PHARMACEUTICAL FORMULATION

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ABSTRACT

A simple, selective, linear, precise and accurate RP-HPLC method was developed and validated for rapid assay of Silymarin in Bulk and Pharmaceutical tablet Formulation. Isocratic elution at a flow rate of 1ml/min was employed on symmetry Shimadzu LC-20 AT_{VP} Kromasil C-18 column at ambient temperature. The mobile phase consisted of Methanol: Water: Acetonitrile (80:15:5 v/v). The UV detection wavelength was 285nm and 20μl sample was injected. The run time for Silymarin is 15min. The flow rate was found to be 1.5ml/min. The percentage recovery of the method was found to be 100.47%. The LOD and LOQ for Silymarin was found to be 10μg/ml and 40μg/ml respectively. The method was validated as per the ICH guidelines. The method was successfully applied for routine quality control analysis of pharmaceutical formulation. The HPLC method can be successfully applied for the routine quality control analysis of Silymarin formulations, which could be the better choices compared to the reported methods of literature

KEYWORDS: Silymarin, Rp- HPLC, UV detection, Recovery, Precise.

INTRODUCTION

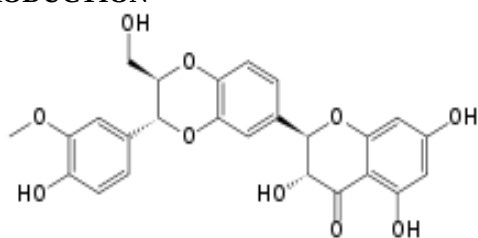


Fig: 1 Structure of Silymarin

IUPAC Name for Silymarin^[1] is (2R,3R)-2-[(2R,3R)-2-3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzo-dioxin-6-yl]-2-3-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one.

Silymarin/silibinin provide an alternative treatment option for anti-cancer treatment.^[2] Silibinin has also demonstrated anti-cancer effects against human prostate adenocarcinoma cells, estrogen-dependent and -independent human breast carcinoma cells, human ectocervical carcinoma cells, human colon cancer cells, and both small and nonsmall human lung carcinoma cells.^[3] There is also clinical evidence for the use of silibinin as a supportive element in alcoholic and grade Child 'A' liver cirrhosis.^[4] Silipide (trade name Siliphos), a complex of silymarin and phosphatidylcholine (lecithin), is about ten times more bioavailable than silymarin.^[5] Tian-ming Ding, et al^[6], Determined by RP-

HPLC consisted of column: Shim-pack VP-ODS (150×4.6mm i.d. 5μm) and Pre-column (10×4.6 mm i.d. 5μm); mobile phase: methanol and solvent mixture (water: DIOXANE=9:1) by gradient; flow rate: 1.5ml/min; column temp.: 40°C; detector wavelength: 288nm; The recovery of 99.66% for silycristin, 99.48% for silydianin, 100.0% for silybin and 98.72% for isosilybin. F. M. Hammouda et al^[7], An HPLC assay for the determination of silymarin in the fruits of *Silybum marianum* is given. T. radjabian, et al^[8], aim of this research was to study the variations in composition and content of flavonolignans of silymarin samples from seeds of some native milk thistle ecotypes of Iran, along with a foreign cultivar. Silymarin was extracted by a two-step (defatting and extraction) process using n-hexane, ethyl acetate and methanol in a Soxhlet extraction from the seeds. A.V.D. Nagendra kumar et.al,^[9] proposed the expected separation and peak shapes were obtained on chromosil C18 (250mm x 4.6mm, 5μm) column. A mixture of methanol: 0.1% orthophosphoric acid: acetonitrile in the ratio of 80:05:15v/v/v was proved to be the most suitable of all the combinations since the chromatographic peak obtained was better defined and resolved and was almost free from tailing. The flow rate was 1.5ml/min and effluents were monitored at 256nm. The Rt for telmisartan was 2.7min. Recovery of telmisartan from tablet formulation was found to be 99.41%. A.V.D.

Nagendra kumar et.al.,^[10] proposed a New Validated HPLC Method for the Estimation of Oxazepam In Pharmaceutical Formulation. Isocratic elution at a flow rate of 1.0ml/min was employed. The chromatographic analysis was performed on a ODS 5 μ m (4.6mm x 15cm) or equivalent column at ambient temperature. The mobile phase consisted of Methanol: Water in the ratio of 65:35v/v. The UV detection wavelength was 230nm and 100 μ l sample was injected. Flow rate was found to be 1.0. The retention time for Oxazepam was identified. The Average percentage recovery of the method was in the range of 0.5.

EXPERIMENTAL

2.1 Instrumentation

Peak HPLC containing LC 20AT pump and variable wavelength programmable **PDA detector** and Rheodyne injector was employed for investigation. The chromatographic analysis was performed on a Kromasil C₁₈ column 250 x 4.6mm ID with 5 μ particle size and the column were maintained at ambient temperature. Degassing of the mobile phase was done using a Loba ultrasonic bath sonicator. A Denwar analytical balance was used for weighing the materials.

2.2 Chemicals and solvents

The reference sample of levalon was obtained from Cipla, Mumbai. The Formulation was procured from the local market. Water, Methanol and Acetonitrile used were of HPLC grade and purchased from the chemicals were procured from E-Merck, India, Limited.

2.3 The mobile phase

The mobile phase was prepared by mixing Methanol: Water: Acetonitrile (80:15:5v/v). Prepared mobile phase was filtered through 0.45 μ membrane filter and sonicated. Sample solution was prepared by dissolving the drug in mobile phase and sonicated for 30 minutes.

2.4 Preparation of solutions

2.41 Preparation of mobile phase solution

The mobile phase was prepared by mixing Methanol and Water and Acetonitrile (80:15:5 v/v) by ultra bath sonicator for 30min.

2.42 Preparation of standard solution

Stock solution of Silymarin was prepared by dissolving accurately weighed 5mg of drugs in 10ml Methanol. The prepared stock solutions were stored away from light. From the stock, standard solutions was freshly prepared during the day of analysis.

2.43 Preparation of working standard solution (A.P.I)

From the stock solution 20 μ g/ml solution was prepared.

2.44 Preparation of working standards for linearity

Solutions in the concentration range of 0.5-2.5 μ g/ml were prepared from the standard working solution.

2.45 Preparation of formulation sample solution

10mg of formulation powder was taken from LEVALON (70 mg formulation) and dissolved in 10ml of mobile phase and injected into HPLC and chromatogram was recorded. The amount of drug present in the 1mg formulation was calculated from linearity graph.

3. Method Development

For developing the method, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all other conditions constant. Method development consists of selecting the appropriate wave length and choosing stationary and mobile phases. The following studies were conducted for this purpose:

3.1 Detection of wavelength

The spectrum of 10ppm solution of Silymarin was recorded separately on UV spectrophotometer. The peak of maximum absorbance wavelength 285nm was observed.

3.2 Choice of stationary phase and mobile phase

Finally the expected separation and peak shapes were obtained on Kromasil C₁₈ column 250 x 4.6mm ID with 5 μ particle size.

3.3 Flow rate

Flow rates of the mobile phase were changed from 1.0-2.0ml/min for optimum separation. It was found from experiments that 1.5ml/min flow rate was ideal for elution of analyte.

4. Validation Procedure and Requirements

The analytical performance of the method of analysis was checked for specificity, System suitability, detection limit, and method precision.

4.1 Linearity and Calibration

Linearity was assessed by performing single measurement at several analyte concentration varying quantities of stock standard solution diluted with the mobile phase to give a concentration of 0.5,1.0,1.5,2.0,2.5 μ g/ml Injection was made at intervals of 10min. The linearity was tested for the concentration ranging from 0.5 μ g/ml to 2.5 μ g/ml. The peak area ratio of the drug was plotted against concentration. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

4.2 Precision

Reproducibility was performed by injecting three replicates concentrations of standard and sample solutions which were prepared and analyzed by same analyst on same day. Inter-day variations in the peak area of drug solutions and the amount of drug were calculated in terms of Percentage Relative Standard Deviation. The sample concentration is 20 μ g/ml.

4.3 Accuracy

Recovery assessment was obtained by using standard addition technique which was by adding known quantities of pure standards at three different levels in 80%, 100% and 120% to the pre analysed sample formulation.

4.4 ASSAY

The estimation of drug in pharmaceutical dosage forms. LEVALON 70mg strength was evaluated for the amount of Silymarin present in the formulation. Each sample was analyzed in triplicate after extracting the drug. The amount of drug present in formulation was calculated by comparing the mean peak area from standard.

4.5 Intermediate Precision or Ruggedness

Inter-day variations were performed by using six replicate injections of standard and sample solutions of concentrations which were prepared and analyzed by different analyst on three different days over a period of one week. Ruggedness also expressed in terms of percentage relative standard deviation.

4.6 Robustness

Robustness was carried out by varying two parameters from the optimized chromatographic conditions.

4.7 Specificity

The method was determined as specific by comparing test results obtained from analyses of sample solution containing excise ingredients with that of test results those obtained from standard drug.

4.8 System Suitability Parameter

System suitability tests were carried out on freshly prepared standard stock solutions of Silymarin and it was calculated by determining the standard deviation of Silymarin standards by injecting standards in five replicates at 6 minutes interval and the values were recorded.

5. Silymarin Analysis In Serum

From a local hospital blood was collected and serum was separated. 10ml of this serum was taken in a test tube and added 100 μ l of diltizem hydrochloride (1 μ g/ml) and 0.1ml of 1M NaOH and 5ml of dichloromethane and mixed about 20min in vortex mixer and centrifuged at 3000rpm for 10min. From this centrifuged solution 4ml of organic layer was separated and evaporated to dryness to get residue. To this residue 100 μ l of 1M acetic acid and 3ml of n-Hexane and mixed for 5min by vortex

mixer and evaporated the organic layer and finally the remaining sample was injected into HPLC and chromatogram was recorded. The amount of drug present in the blood sample was calculated from linearity graph.

5.1 Serum Data of Silymarin

Drug estimation in human serum by developed protocol. From linearity graph we can estimate amount of drug present in the sample. Amount of Silymarin present in serum is 9.24 μ g/10ml.

6. RESULT AND DISCUSSION

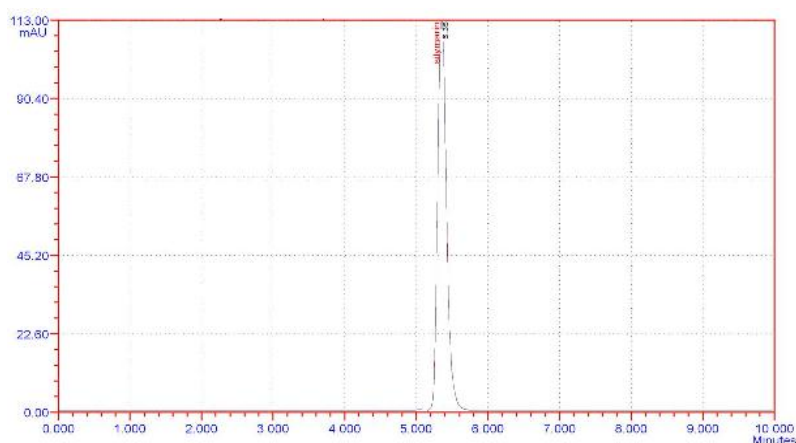
The Reverse Phase High Performance Liquid Chromatography method was developed a stability indicating assay method. Pure drugs chromatogram was run in different mobile phases containing methanol, acetonitrile, THF, and different buffers like potassium dihydrogen phosphate, sodium dihydrogen phosphate, Ortho phosphoric acid in different volumes ratios. Different columns like C₈, C₁₈, phenyl, cyano with different dimensions were used. Then retention time and tailing factor were calculated. Finally Methanol and Water and Acetonitrile in the volume of ratio 80:15:5 v/v (P^H: 5.0) and Kromosil C₁₈ analytical column was selected which gave a sharp and symmetrical peak with 1.76 tailing. Calibration graph was found to be linear at range 0.5 μ g/ml to 2.5 μ g/ml. five different concentrations of Silymarin in range given above were prepared and 20 μ l of each concentration injected in HPLC as shown in the Figure no: 2. The slope (m) and intercept (c) obtained were found to be 94780.08 and -0.087404319. The correlation coefficient (r²) obtained was found to be 0.9992 as shown in the Table no: 2. It was observed that the concentration range showed a good relationship. The limit of detection for Silymarin was found to be 10 μ g/ml and the limit of quantification was found to be 40 μ g/ml. It proves the sensitivity of the method. The Percentage assay of Silymarin in formulation was found to be 100.47% as shown in the Table no: 1 and figure no: 4. The relative standard deviation value obtained was below 1 which indicates the precision of the method. The validation of the proposed method was further verified by recovery studies. The data was presented by in the Table no: 2 and figure no: 3. The percentage recovery was found to be 99.76% which shows a good index of accuracy of the developed method. The amount of drug present in the human serum sample was calculated from the linearity graph was found to be 9.24mg/10ml as shown in Figure no: 5.

Table1: Optical Characterisation of Silymarin

PARAMETERS	SILYMARIN
Linearity range($\mu\text{g/ml}$)	0.5 – 2.5
Correlation coefficient (r)	0.9992
Slope(m)	94780.08
Intercept (c)	-0.087404319
Limit of detection (LOD; $\mu\text{g/ml}$)	10
Limit of Quantification (LOQ; $\mu\text{g/ml}$)	40
Tailing factor	1.76
Retention time (min)	5.354
Theoretical plates	6540
(%) R.S.D	0.804
(%) Accuracy	99.76
(%) Assay	100.47
Serum ($\mu\text{g/10ml}$)	9.24

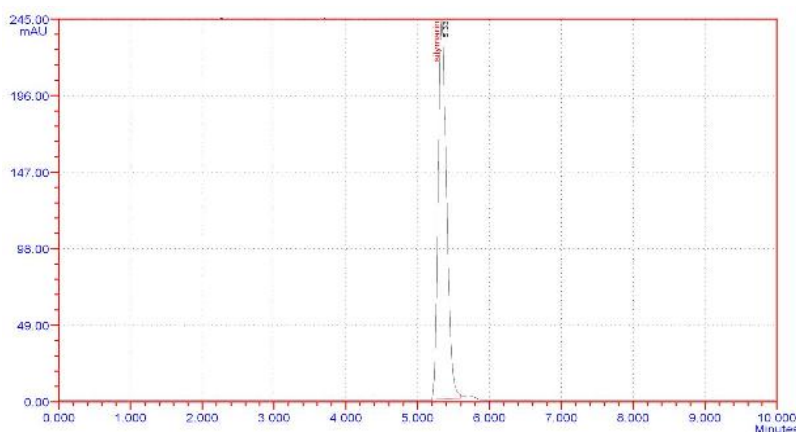
Table 2: Recovery Data of Silymarin

Pharmaceutical formulation (brand name)	Labeled amount (mg)	Percentage Assay	Percentage recovery
LEVALON	70 mg	100.47	99.76



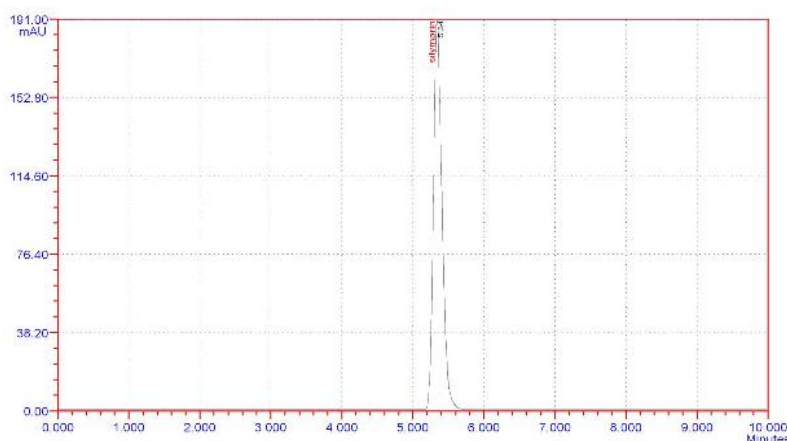
S.No	Name	Rt.	Height	Area	Conc.	Tailing factor	Theoretical plates
1	Silymarin	5.352	8361	53743.0	100.000	0.99	13818
	SUM		8361	53743.0	100.0000		

Fig: 2 Chromatogram of Silymarin and their values (Standard)



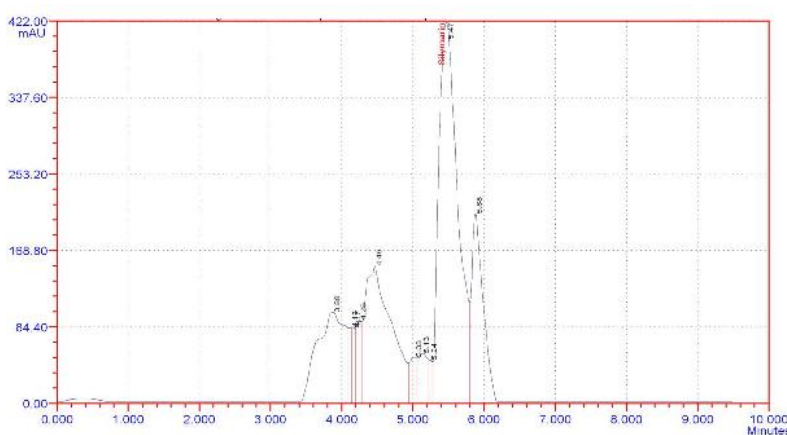
S.No	Name	Rt.	Height	Area	Conc.	Tailing factor	Theoretical plates
1	Silymarin	5.332	24276	200565.5	100.000	1.53	8301
	SUM		24276	200565.5	100.0000		

Fig: 3 Chromatogram of Silymarin and their values (Accuracy)



S.No	Name	Rt.	Height	Area	Conc.	Tailing factor	Theoretical plates
1	Silymarin	5.340	18564	147179.7	100.000	1.18	9041
	SUM		18564	147179.37	100.0000		

Fig: 4 Chromatogram of Silymarin and their values (Formulation Assay)



S.No	Name	Rt.	Height	Area	Conc.	Tailing factor	Theoretical plates
1	Silymarin	3.882	12581	382638.5	16.120	0.68	325
		4.130	10973	28568.2	1.204	0.68	50153
		4.167	11050	32925.2	1.387	1.03	38974
		4.247	11877	59816.0	2.520	0.83	14171
		4.465	17898	501547.7	21.129	1.80	506
		5.027	8029	54812.6	2.309	0.68	10805
		5.135	8484	80978.6	3.411	1.10	5768
		5.243	7806	23915.9	1.008	1.60	58372
		5.472	45242	876028.7	36.905	1.34	1591
	Sum		158110	2373745.0	100.0000		

Fig: 5 Chromatogram of Silymarin and their values (Serum)

6. CONCLUSION

The RP-high performance liquid chromatographic method developed and validated for the analysis of Silymarin and for the serum studies from their formulations was found to be accurate and precise. This method is more useful than the reported methods of analysis in the literature. Thus, the proposed HPLC method can be successfully applied for the routine quality control analysis of Silymarin formulations.

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