IJBPAS, August, 2022, 11(8): 3934-3943

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

QUALITY-BY-DESIGN (QBD) APPROACH TO CHROMATOGRAPHIC CONDITIONS APPLIED FOR DETERMINATION OF ROBUSTNESS IN SILYMARIN EXTRACT

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Received 16th Sept. 2021; Revised 20th Oct. 2021; Accepted 19th Dec. 2021; Available online 1st Aug. 2022

https://doi.org/10.31032/IJBPAS/2022/11.8.6336

ABSTRACT

In the present study Quality by design approach was implemented for HPTLC method development and validation for robustness. Silymarin found in highest concentration in fruit portion. Silymarin is a flavolignan from *Silybum marinum* plant used for hepatoprotection. Silymarin extract and Silybin were used in the study. Chloroform, ethyl acetate, acetone, formic acid was used for mobile phase optimization.HPTLC method developed and validated. All data analysis of experimental design were performed by using the Design-Expert trial version 7.0.0 (Stat-Ease Inc., Minneapolis, MN,USA) and Box-Behnken method was used for the optimization of chromatographic conditions. Mobile phase ratio, solvent front and chamber saturation time were determinate as critical method parameters.Method was developed and validated using solvent on silica as chloroform: ethyl acetate: acetone: formic acid (40:30:20:10, v/v) (R_f of silybin **0.15 ± 0.03**) At 296 nm in the absorbance mode. The technique demonstrated a strong linear relationship ($r^2 = 0.9983$) in the concentration 250, 500, 750, 1000, 1250 and 1500 ng/ band of Silybin, respectively.It was found that it was linear, accurate, precise and robust.

Keywords: Silybin, silymarin extract, Box- Behnken, flavolignan, hepatoprotection

INTRODUCTION

The terms milk thistle, silymarin, and silybin are generally used interchangeably **[1]**. Silymarin is a C25 containing flavonoid mixture having flavonoligans (silybin A and silybin B, isosilybin A, isosilybin B, silychristin and silydianin) with approximately small amount of 20%-35% of fatty acids and polyphenolic compounds **[2]**. Silybin was predominant and primary ingredient in silybum marinum antioxidant and chemoprotective effect on liver. Therefore it is used as a complementary and alter-native hepato-protective medicine **[3]**.

Product quality should not be tested; it should be built-in or design-based."According to the ICH Q8 (R2) standard, "a systematic approach to development that begins with specified objectives and emphasizes product and process understanding and process control, based on good science and quality risk management **[4]**. "An integral part of the QbD is the analytical methods used for the study of active pharmaceutical ingredients (API) and drug products **[5]**. The consequence of the use of QbD principles is a well-known product and process that achieves its expected output consistently **[6]**. QbD offers insights upstream in the production process instead of focusing on finished product testing alone. As a consequence, it is possible to accurately evaluate a quality problem and determine its root cause quickly **[7]**. A few new drug applications (NDA) have been approved by the Food and Drug Administration (FDA) with regulatory flexibility centered on the analytical approach to quality by design (QbD**) [8]**. Box- Behnken method is used for optimization of chromatographic conditions **[9]**. Literature survey revealed that UV-spectroscopy method has been reported. To our notice, quality by design approach was not implemented for HPTLC therefore made attempt to apply QbD approach to develop simple, robust, sensitive, effective HPTLC method for estimation of silybin in silymarin extract. Structure of silymarin shown in **Figure 3**.

MATERIALS AND METHODS

Sami Laboratories (Bangalore, India) gave Silybin as a gift sample, and Ranbaxy provided Silymarin (Gurgaon, India). The other analytical (AR) grade chemicals and reagents used were obtained from Merck (India) and S. D. Fine Chemicals (India, New Delhi). Using a Camag Linomat V applicator (Switzerland) and a Camag microliter syringe, the samples were spotted on a pre-coated silica gel aluminium plate 60F-254 (20 x 10 cm, 0.2 mm thickness, E. Merck, Germany) as a 3 mm wide band. Throughout the study, the application rate of 150 nL / s was constant and a space of 6.2 mm was kept between the two bands.The slit dimension was kept at 4 × 0.1 mm, and the scanning speed was 20 mm/s. chloroform: ethyl acetate: acetone: formic acid (40:30:20:10, v/v)was used as the solvent system. In a twin-trough glass chamber (20 x 10 cm) saturated with the mobile phase for 20 min at room temperature, linear ascending development was carried out. The solvent front was run up to 80 mm. Subsequent to the development; TLC plates were dried with the help of an air-dryer after development. Using the TLC scanner III (CAMAG, Switzerland) in absorbance mode at 296 nm, densitometric scanning was carried out.Deuterium and tungsten lamps used as radiation source.

Stock standard solution and study of linearity curve

For the stock standard solution, weigh 10 mg of Silybin. The weighed powder was transferred to a 10 mL volumetric flask and dissolved and diluted to a methanol mark to obtain 1 mg / mL concentration. With the aid of the previously calibrated pipette, a sufficient volume of 0.5 to 3 mL from the stock standard solution was transferred into a series of 10 mL volumetric flasks. A fixed volume of 5 µL was applied to the HPTLC plates to obtain 250, 500, 750, 1000, 1250 and 1500 ng / band concentrations.

RESULT AND DISCUSSION

Mobile phase optimized successfully. Initially, chloroform: ethyl acetate: acetone: formic acid in the ratio of (40:30:20:10, v/v) was found to give spots for silybin in different concentration levels at an R_f value of 0.15 ± 0.03. Result shown in **Table 1**. HPTLC densitogram of Silybin shown in **Figure 2**.

Linearity

The linear regression analysis data for the calibration plots in the 250- 1500 ng/ band concentration range showed a good linear relationship (r^2= 0.9983) with respect to peak area. Result shown in **Table 2** and **Figure 3, 4, 5, 6, 7**.

Precision

The intra- and inter-day precisions of silybin were studied using concentration 500, 700 and 1000 ng/band. The % RSD was found in the range of 0.35-0.61% and 0.31 -0.48% .Result shown in **Table 3**.

Accuracy

The method's accuracy was estimated by spiking the drug standard at concentration levels of 80 percent, 100 percent and 120 percent in the pre-determined laboratory mixture solution.The mean recovery was 99.724%. The %RSD values after spiking with 80 %, 100 % and 120 % were found in the range of 0.53-0.85. Result shown in **Table 4**.

Limit of Quantitation(LOQ) and Limit of Detection

LOD and LOQ have been discovered to be 11.33 ng/spot and 67.15 ng/spot respectively, which indicate sensitivity of the method. Result shown in **Table 5**.

Robustness

The low values of % RSD was obtained after introducing small deliberate changes in ethyl acetate concentration, solvent front and chamber saturation time at a time at concentration of 500,1250 and 1500ng/band. The coded equation by software is Retention factor (R_f) = +0.15 +0.32A -0.15B -0.050C -0.14AB +0.095AC +0.080BC +0.25A^2 -0.025B^2 +0.11C^{2}The coded equation by software is Peak area= +9362.00 +1288.38A-1035.25B-143.38C -556.50AB+412.75AC+ 720.50BC+ 132.37A^2+ 354.62B^2 +826.88C**.** Retention factor (R_f) = +0.15 +0.32A -0.15B -0.050C -0.14AB +0.095AC +0.080BC +0.25A^2 -0.025B^2 +0.11C^2. Peak area= +9362.00 +1288.38A-1035.25B-143.38C -556.50AB+412.75AC+ 720.50BC+ 132.37A^2+ 354.62B^2 +826.88C^2 .Result shown in **Table 6, 7**.

Table: 1 Experimental factors and levels used in BBD.

Factors	Levels		
	IB fact (−1)	Center point	IB fact (+1)
Ethyl acetate Concentration (% V/V)	20	30	40
Solvent front [mm]	70	80	90
Chamber saturation time [min]	15	20	25

Table 2: Linearity data of silybin by HPTLC

Sr. no.	concentration (ng/ band) R_f of 0.15 ± 0.03	Peak area (AU)
1.	250	3289
2.	500	5298
3.	750	7532
4.	1000	9362
5.	1250	12001
6.	1500	13979

Table 3: Interday and intraday precision study results

Drug Conc. (ng/ band)	% Amount found (ng/ band)			Mean	SD	%RSD
		Intra-day				
500	101.03	100.82	99.87	100.5733	0.618088	0.614565
1000	99.36	99.46	100.01	99.61	0.35	0.35137
1250	100.32	101.02	100.22	100.52	0.43589	0.433635
		Inter-day				
500	99.34	99.27	100.04	99.55	0.425793	0.427718
1000	100.54	100.23	99.91	100.2267	0.315013	0.314301
1250	99.12	99.87	100.01	99.66667	0.478574	0.480175

Table 4: Accuracy results of Silybin by HPTLC

Initial Amount (ng/ band)	Amount of drug added (%)	% Amount recovered			% Recovery	SD	%RSD
1000	80	99.23	100.02	99.01	99.42	0.531131	0.534229
1000	100	100.82	100.21	99.15	100.06	0.845044	0.844538
1000	120	99.34	99.12	100.62	99.693333	0.810021	0.812512

Table 5: Data generated using Box Behnken design

Run	Factor 1: Ethyl acetate concentration (%v/v)	Factor 2: Solvent front (mm)	Factor 3: Chamber saturation time (min)	Response 1: Retention factor	Response 2: Peak area
1	30	80	20	0.15	9362
2	40	80	15	0.75	10872
3	40	80	25	0.94	11723
4	40	90	20	0.36	10023
5	30	80	20	0.15	9362
6	20	80	25	0.07	8945
7	20	90	20	0.05	7935
8	20	80	15	0.26	9745
9	40	70	20	0.97	12876
10	30	80	20	0.15	9362
11	30	70	15	0.53	12764
12	30	90	25	0.09	9764
13	30	80	20	0.15	9362
14	20	70	20	0.12	8562
15	30	70	25	0.17	10724
16	30	80	20	0.15	9362
17	30	90	15	0.13	8922

Response: Retention factor (Rf)

ANOVA for Response Surface Quadratic Model

Table 6: Response: Retention factor (Rf) ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1.44	9	0.16	37.30	< 0.0001	Significant
A-Ethyl acetate Concentration	0.79	1	0.79	185.22	< 0.0001	
B-Solvent front	0.17	1	0.17	39.25	0.0004	
C-Chamber saturation time	0.020	1	0.020	4.67	0.0676	
AB	0.073	1	0.073	17.01	0.0044	
AC	0.036	1	0.036	8.42	0.0229	
BC	0.026	1	0.026	5.97	0.0445	
A^2	0.26	1	0.26	61.40	0.0001	
B^2	2.632E-003	1	2.632E-003	0.61	0.4590	
C^2	0.046	1	0.046	10.83	0.0133	
Residual	0.030	7	4.286E-003			
Lack of Fit	0.030	3	0.010			
Pure Error	0.000	4	0.000			
Cor Total	1.47	16				

Response: Peak area
ANOVA for Response Surface Quadratic Model

Table 7: Response: Peak area ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2.973E+007	9	3.303E+006	19.38	0.0004	significant
A-Ethyl acetate Concentration	1.328E+007	1	1.328E+007	77.93	< 0.0001	
B-Solvent front	8.574E+006	1	8.574E+006	50.32	0.0002	
C-Chamber saturation time	1.645E+005	1	1.645E+005	0.97	0.3586	
AB	1.239E+006	1	1.239E+006	7.27	0.0308	
AC	6.815E+005	1	6.815E+005	4.00	0.0857	
BC	2.076E+006	1	2.076E+006	12.19	0.0101	
A^2	73781.64	1	73781.64	0.43	0.5316	
B^2	5.295E+005	1	5.295E+005	3.11	0.1213	
C^2	2.879E+006	1	2.879E+006	16.89	0.0045	
Residual	1.193E+006	7	1.704E+005			
Lack of Fit	1.193E+006	3	3.976E+005			
Pure Error	0.000	4	0.000			
Cor Total	3.092E+007	16				

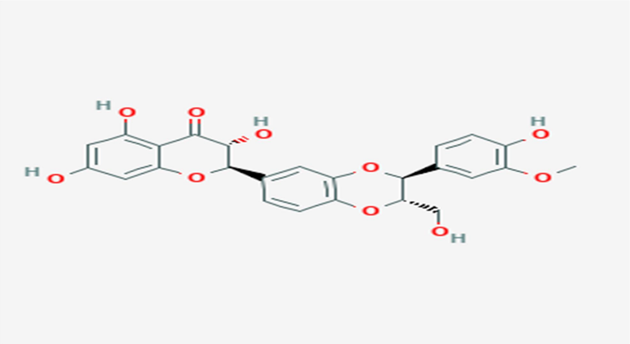
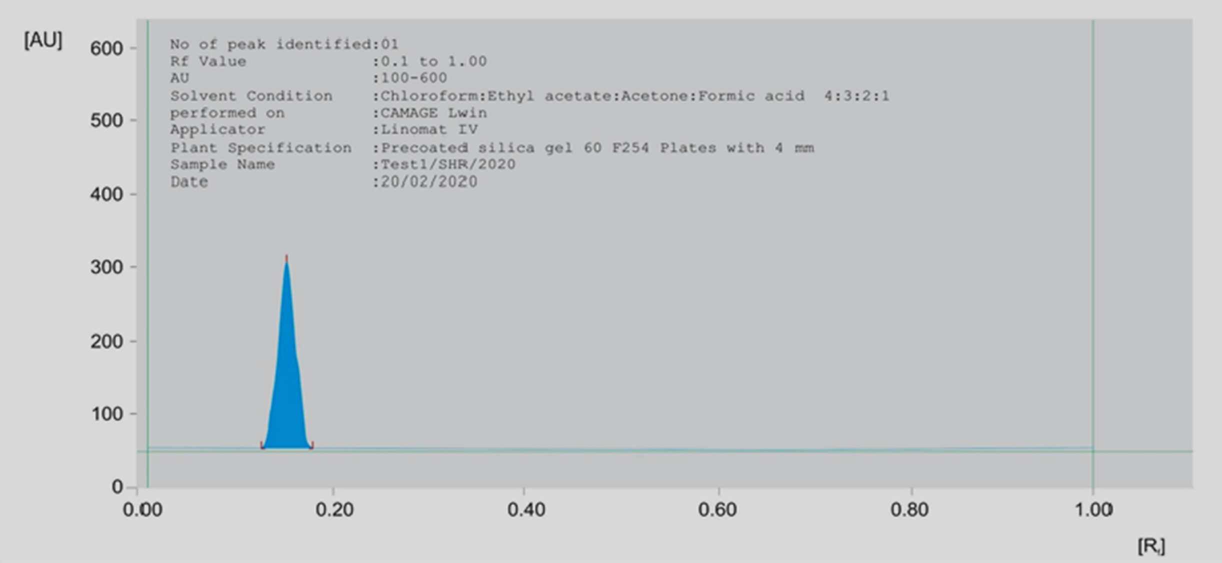


Figure 1: Structure of silymarin



```
No of peak identified:01
Rf Value              :0.1 to 1.00
AU                    :100-600
Solvent Condition     :Chloroform:Ethyl acetate:Acetone:Formic acid  4:3:2:1
performed on          :CAMAGE Lwin
Applicator            :Linomat IV
Plant Specification   :Precoated silica gel 60 F254 Plates with 4 mm
Sample Name           :Test1/SHR/2020
Date                  :20/02/2020
```

Figure 2: HPTLC densitogram of Silybin

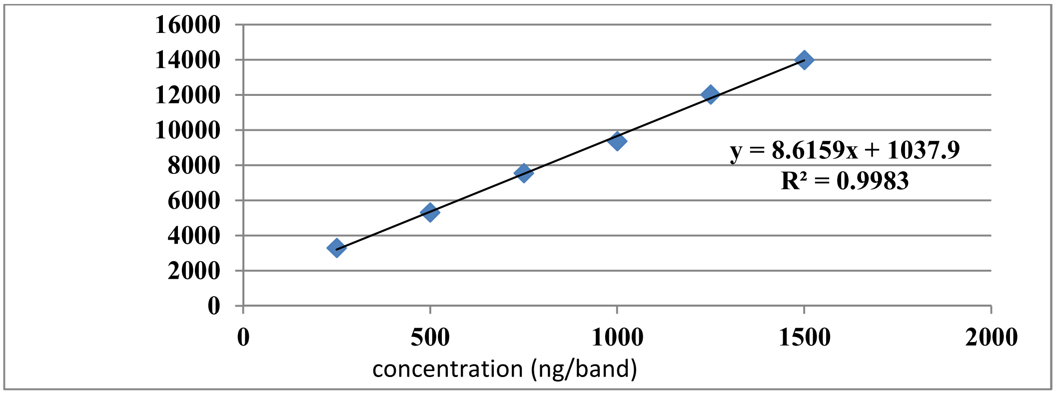


Figure 3: Calibrations curve of silybin

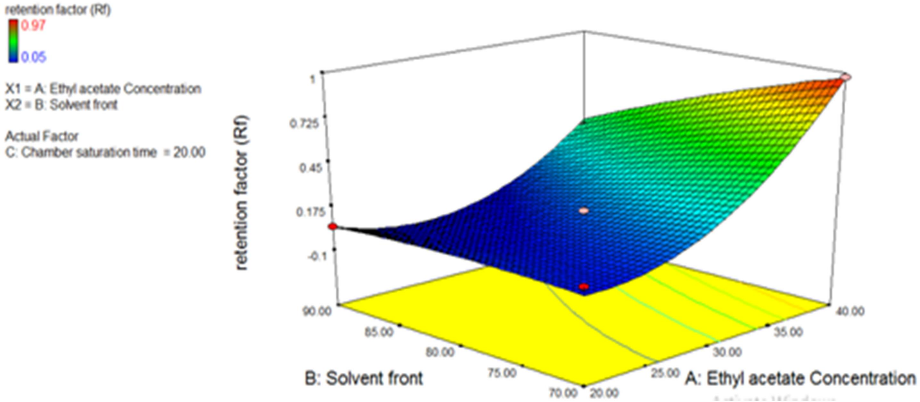


Figure 4: 3D plot of retention factor

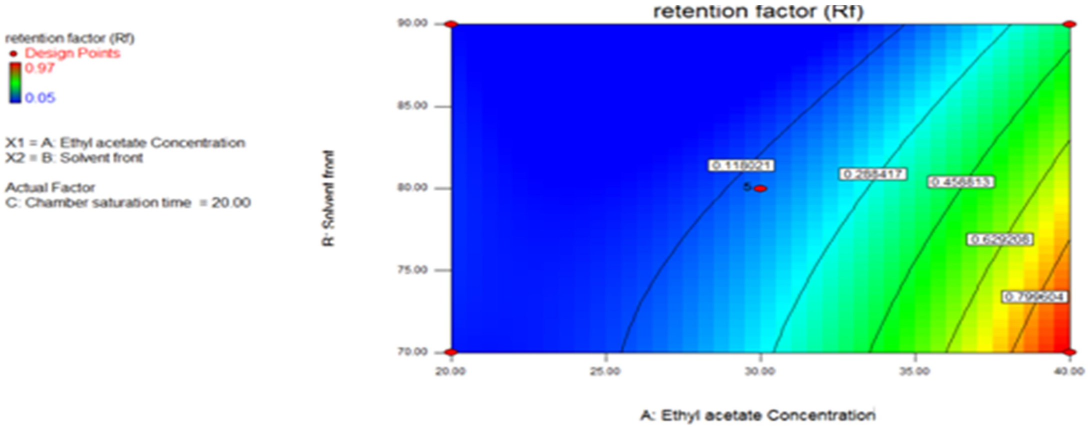


Figure 5: Contour plot of retention factor

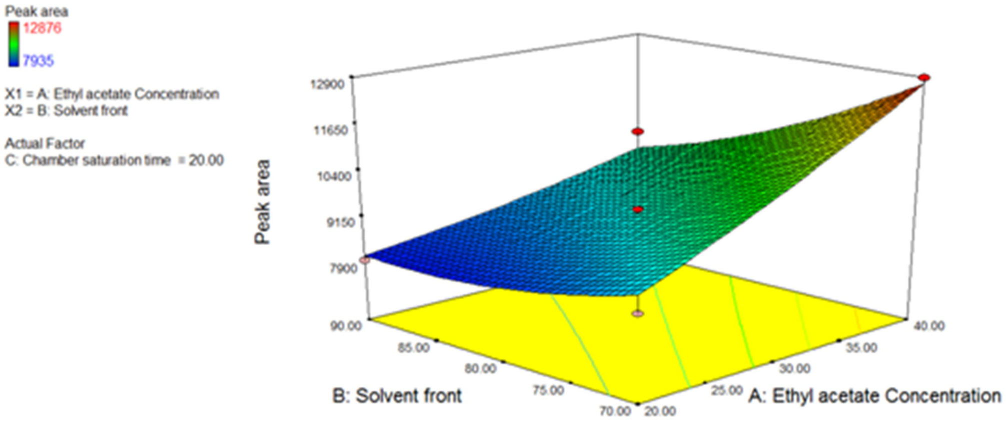


Figure 6: 3D plot of peak area

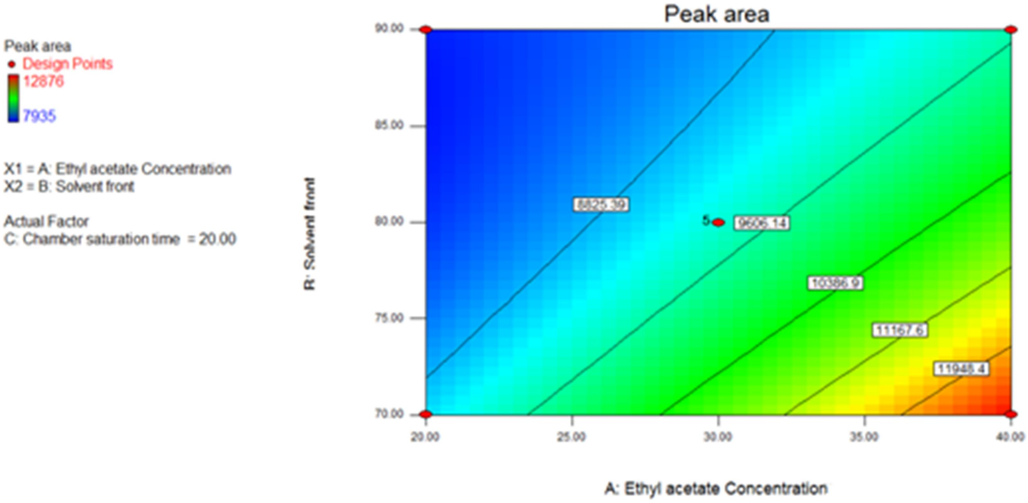


Figure 7: Contour plot of peak area

CONCLUSION

The proposed HPTLC methods gives well symmetric peaks for silybin. The concentration of Silybin in Silymarin extract is determined by HPTLC method.Statistical analysis has shown that for the determination of silybin, the HPTLC method developed in this study is specific, accurate, robust, reproducible and precise.

ACKNOWLEDGEMENT

The Author thanks the authorities of Modern college of pharmacy, Nigdi for permitting them to carry out this work at Pharmaceutical quality assurance Techniques laboratory, Department of Pharmaceutical Quality Assurance Techniques.

CONFLICT OF INTEREST

No conflict of interest exists.

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