RESEARCH ARTICLE

AQbD Assisted Method Optimization for Simultaneous Quantification of Potential Phytosterols in Novel Polyherbal Formulation by LC-PDA Method

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ABSTRACT

Background: Herbal medications are preferred by individuals with cardiovascular disorders (CVDs) worldwide for their distinct benefits in preventing and treating illnesses and lesser side effects. A novel polyherbal formulation compiled of ten plants (*Allium sativum, Rubia cordifolia, Garcinia cambogia, Terminalia arjuna, Acacia catechu, Helianthus annus, Vitis vinifera, Commiphora mukul, Linum usitatissimum*, and *Piper nigrum*) was developed for treating cardiovascular diseases. As a part of standardization, marker analysis using high-performance liquid chromatography (HPLC) will be highly encouraged.

Materials and Methods: In the present study, we selected lupeol, stigmasterol, and β-sitosterol and went for simultaneous quantification using design of experiments (DoE) software (AQbD-Analytical quality by design) to optimize the experimental parameters. The phenomenex RP C18 column, with 250 mm length, 4.6 mm internal diameter, 5 µm particle size, was preferred for the study. Mobile phase with methanol and 0.01% formic acid solution in water surged with a flow rate of 1.5 mL/min. using a maximum detection wavelength of 208 nm.

Results and Discussion: Under the specified circumstances, we determined the retention times of lupeol, stigmasterol, and β-sitosterol to be 14.056, 15.544, and 17.431 minutes, respectively, with the desirability of 0.975. The concentrations of lupeol, stigmasterol, and β-sitosterol in the extract were determined to be 30.72, 370.6, and 212.64 µg/10 mg, respectively. The linear range of 31.25 to 500 µg/mL was found to have an R^2 value of 0.9972, 0.9976 and 0.9968, respectively. 0.2534, 0.754, 0.421 µg/mL and 0.7681, 2.29, 1.28 µg/mL were the limit of detection (LoD) and limit of quantitation (LoQ) values, respectively. The RSD for precision was determined to be 0.92, 0.98, and 0.96%, respectively. Recovery experiments were conducted, and the accuracy of the suggested approach was between 99 and 102%.

Conclusion: Using the modernized software tool- AQbD (analytical quality by design), the study was first scientifically validated for the quantification of three phytomarkers in the novel polyherbal formulation.

Keywords: HPLC, Lupeol, Stigmasterol, β-sitosterol, Analytical quality by design, Design Expert.

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INTRODUCTION

Herbal medications are preferred by individuals with cardiovascular disorders (CVDs) worldwide for their distinct benefits in preventing and treating illnesses, rehabilitation, and health care, as self-care knowledge and worry about the unavoidable side effects of traditional therapy have increased.[1] Usage of herbal medicines is consistently rising, and many patients with cardiovascular diseases often utilize herbal medicines in combination with prescribed cardiovascular drugs. There is mounting evidence that several herbal medications and the active components in them even in low quantities, support the conventional treatment for CVDs. In European nations, the use of dietary supplements and functional foods to maintain normal plasma cholesterol values is rising gradually because of their effectiveness in decreasing cholesterol.[2-4] Phytosterols (also known as plant sterols) have lately acquired popularity among these products.[5] Triterpene-family fat-soluble substances known as phytosterols are found in most plant cells, where they

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support the integrity and structure of membranes. They are distinguished by having a side chain at the D ring's position 17 and a tetracyclic structure.[6] They have a lot of structural similarities with cholesterol, which is by far the most prevalent sterol in animal cells and has a comparable structural function. Compared to cholesterol, phytosterols have a side chain bound at their C-17 position; for instance, sitosterol has an ethyl group connected to its side chain at position C-24, but campesterol has a methyl group, which is empty compared to cholesterol.[7] In plant cells, there are hundreds of distinct phytosterol molecules; the most prevalent ones are beta-sitosterol, campesterol, stigmasterol, brassicasterol, and avenasterol.[8]

Analytical instruments are crucial for qualitative, semi-quantitative, and quantitative phytochemical analyses of herbal medications and formulations. Chromatographic techniques such as high-performance liquid chromatography (HPLC) is used in order to successfully determine the quality of the herbs. These techniques include assessing fingerprints and estimating biomarkers.[9] For the purpose of quantification in the methanolic extract of a novel polyherbal formulation of lupeol, stigmasterol and β-sitosterol, an HPLC technique has been devised as a quality control tool (Figure 1).

Recently, analytical techniques for bulk pharmaceutical drugs were developed using the systematic process called Analytical Quality by Design (AQbD).[10] As an early risk assessment tool, AQbD is crucial in the development of robust methods since it aids in identifying the crucial analytical parameters and helps method developers concentrate on these elements.[11, 12] Due to the intricacy of herbs, quality control and product standardization of the active components responsible for the pharmacological action of the product should be continuously maintained as these factors directly affect how well a therapy works. To ensure product quality and observe the whole life cycle of the medicine, standardizing herbal drugs is done using the modern QbD approach. Too far, articles examining simultaneous estimation of lupeol, stigmasterol and β-sitosterol using intergraded experimental design of experiments (DoE) methods are very limited. An innovative, enhanced and contemporary technique was used to design and validate a reverse-phase high-performance liquid chromatography (RP-HPLC) system to standardize lupeol, stigmasterol, and β-sitosterol from the formulation subjected for the current article.

MATERIALS AND METHODS

Preparation of Formulation

As per the Ayurvedic Formulary of India, the polyherbal formulation (PHF) was prepared. Each plant parts of *Allium sativum, Rubia cordifolia, Garcinia cambogia, Terminalia arjuna, Acacia catechu, Helianthus annus, Vitis vinifera, Commiphora mukul, Linum usitatissimum*, and *Piper nigrum* was dried in the shade and ground. Further, the ground powders were mixed in equal proportions to form as a formulation.[13]

Preparation of Plant Extract from Formulation

The extraction method was preferred, as was the ultrasonicated technique, and the solvent used was methanol. After occasional shaking, the extract was filtered using a muslin cloth and concentrated on a rotary evaporator.[14]

HPLC Instrumentation

The HPLC was carried out using a Shimadzu Prominence model equipped with a manual injector (Rheodyne - model 7125, USA), a binary solvent supply module (LC20AD), a photodiode array (PDA) detector, and a CT0-20A Column oven. The 7.1 Version of the Lab Solutions software was used during the process of data gathering.

Software

The experimental design, analysis of data, and establishment of desirability for enhancing the RP-HPLC technique were analyzed using Design Expert® software of version 22.0.6.0 (Stat-Ease Inc., Minneapolis, MN, USA).

Experimental Design

When confronted with challenges involving multiple independent variables, such as buffer strength, pH, flow rate, organic phase, and wavelength, we have chosen mobile phase ratio, flow rate, and pH. The responses we are interested in are the capacity factor, the retention time of lupeol, and resolution. The response surface methodology (RSM) is used to optimize the amounts of several variables to get optimum system performance. Quadratic models can effectively describe the chromatographic conditions' response values inside the experimental design. In order to get the coefficients of the quadratic regression model, it is necessary to examine each variable that has a minimum of three independent levels.[15,16] A central composite design (CCD) was applied in this optimization investigation as it is the most often used fractional factorial design in the response surface model. The focal points in this design have been enhanced by the addition of a cluster of axial points referred to as star points. The three chromatographic parameters, A- Methanol percentage, B- Flow rate, and C- pH and three response levels that were taken into account while adjusting the experimental conditions are shown in Table 1 and 2.

Standards and Reagents

Natural Remedies Pvt. Ltd. in Bangalore, India, provided the standards for lupeol, a triterpenoid and the sterols stigmasterol and β-sitosterol for these measurements. We bought formic acid (analytical grade) and methanol (HPLC quality) from Ranchem Private Limited. Entire analysis was made using Millipore Mill-Q-water. The Merck Specialities Private Limited Company in Mumbai supplied the analytical-grade chemicals.

Solution Preparations

Preparation of standard stock solution

About 250 µg standards of lupeol with a purity 99.96%, stigmasterol with a purity 99.98%, and β -sitosterol with a purity of 99.99%, were precisely weighed and transferred to a 1-mL Eppendorf tube. Standards were dissolved in methanol and then sonicated for ten minutes. The final volume was made up with methanol to get stock solutions containing 250 µg/mL of lupeol, stigmasterol, and β -sitosterol.

Preparation of sample solution

Precisely 50 mg of the methanolic extract from the formulation was measured and placed into a 1-mL Eppendroff tube. A volume of 1-mL of methanol was added as a diluent and agitated using sonication for about 5 minutes.

HPLC Chromatographic Conditions

An RP-Phenomenex C18 reverse phase column was used as the stationary phase in the HPLC analysis, and a 0.01% formic acid solution diluted in Milli-Q® water (solvent A) and methanol (solvent B) at a flow rate of 1.5 mL/min. was used as the mobile phase. The temperature of the column was maintained constant during the study. The detection wavelength selected was 208 nm. A blank analysis was also performed to identify the interference of methanol during analysis.

Validation

According to International Council of Harmonization (ICH) requirements, the optimized technique was verified for linearity, limit of detection (LoD), limit of quantitation (LoQ), accuracy, precision, and robustness.

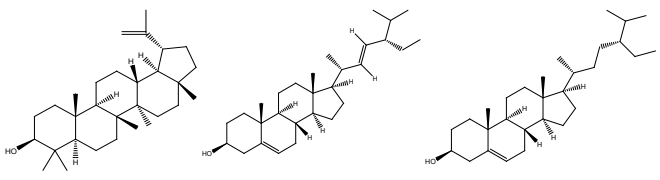
RESULT AND DISCUSSION

Optimization of the Chromatographic Method

Under chromatographic conditions, RSM is able to explain the quadratic model response in the experimental set-up for all values. The quadratic regression model's coefficients were obtained for the optimization study using a CCD, which requires examining each design variable at a minimum of three distinct levels (Table 1). In order to maximize chromatographic responses such as the capacity factor, retention time of lupeol, and resolution, the ideal methanol percentage, flow rate, and pH, were found using the CCD design. The experimental set-up was optimized by selecting and applying chromatographic parameters and levels. About 20 runs were designed, was indicated in Table 2. Figure 2 and Figure 3 displays the favorable, optimal responses for all three of the marker compounds and chromatograms of Blank, Standard and sample solutions.

Effect of variables on capacity factor

Table 2 shows how the independent factors affected the capacity factor (Response 1). In the instance of the capacity factor equation (1), the positive coefficients for A, B and C were found. As a result, it is evident that when there is an increase in methanol percentage, flowrate and pH, the capacity factor values also increase. We can also observe from the equation that the factors AB, AC and BC had positive regression coefficients,



(a) (b) (c)

Figure 1: Structures of (a) Triterpenoid - Lupeol, (b) Plant sterol- Stigmasterol and (c) Plant sterol- β- Sitosterol

Table 1: Selected experimental factors and levels in the central composite design

Selected factor	*Factor code*	*Level*		
		Low (-)	*Medium (0)*	*High (+)*
Methanol (% v/v)	A	8	10	12
Flowrate (mL/min.)	B	1.6	1.8	2.0
pH	C	3.0	3.5	4.0

Table 2: Optimization method parameters for central composite design

Run	*Factor A*	*Factor B*	*Factor C*	*Capacity factor- Response 1*	*Retention time of lupeol- Response 2*	*Resolution of lupeol and stigmasterol- response 3*
1	10	2.13	3.5	1.63	11.986	2.6
2	10	1.46	3.5	1.15	15.632	3.03
3	8	1.6	3	1.53	14.863	3.12
4	13.3	1.8	3.5	0.92	15.346	3.12
5	10	1.8	3.5	1.15	14.009	4.42
6	8	2	3	0.63	13.987	3.01
7	10	1.8	4.3	0.82	14.532	3.39
8	8	2	4	1.09	13.756	3.09
9	12	2	3	1.06	13.546	3.45
10	10	1.8	3.5	0.73	15.023	4.09
11	10	1.8	3.5	1.01	14.056	4.21
12	10	1.8	3.5	1.15	13.903	4.42
13	6.6	1.8	3.5	0.76	12.563	3.42
14	12	2	4	1.63	12.936	3.36
15	10	1.8	2.6	0.68	14.812	3.13
16	12	1.6	4	0.72	14.462	3.06
17	10	1.8	3.5	1.05	14.056	4.33
18	10	1.8	3.5	1.3	14.056	3.54
19	8	1.6	4	0.89	15.023	3.39
20	12	1.6	3	1	16.09	3.03

clearly saying that there is an increase in 'K' value when there is an increase in AB, AC and BC. Considering all factors, we had optimized flow rate to reduce the capacity factor.

Capacity factor = 1.06242 + 0.0394737A + 0.0788806B + 0.0252951C + 0.20875AB + 0.05875AC + 0.24375BC -0.0627174A^2 + 0.131737B^2 -0.0945372 C^2…… (1)

Effect of variables on retention time of lupeol

From equation 2, the possibility of changing the factors to optimize response 2 has been identified. It was noted that A and B had negative regression coefficients, whereas C had a positive regression coefficient. The findings unequivocally show that when methanol percentage and flow rate decrease, the retention time increases. It clearly demonstrates an improvement in retention time with a concurrent decrease in AB, in contrast to the positive regression coefficient for AC and BC(Figure 4).

Retention time of lupeol = 15.5501 -0.247494 A -1.05594 B + 0.047628 C -0.22375 AB + 0.01625 AC + 0.05625 BC -0.147193 A^2 -0.196691 B^2 -0.0853214 C^2…… (2).

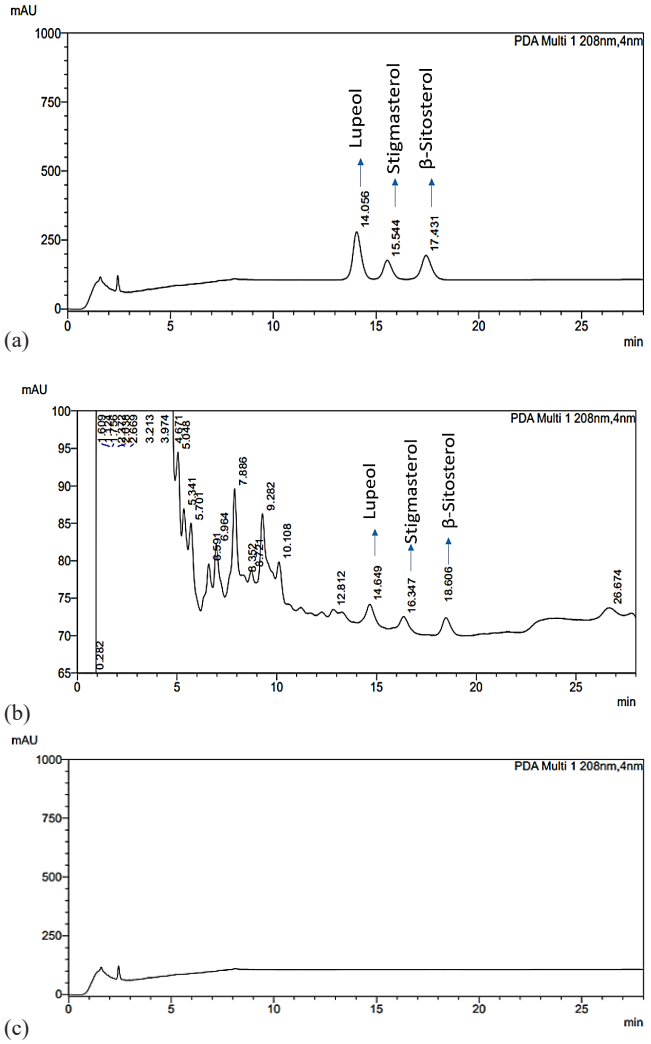


Figure 2: a) Chromatogram of standard lupeol, stigmasterol, and β-Sitosterol solutions. b) Chromatogram of sample solution of polyherbal formulation c) Blank chromatogram

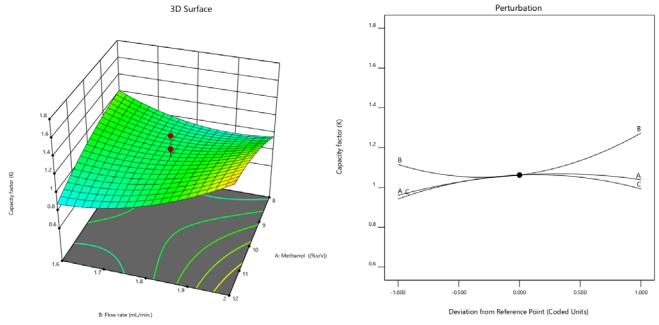


Figure 3: (a) 3D Response surface plot and (b) Perturbation graph of capacity factor

Effect of variables on of resolution factor

The impact of various parameters on response 3 has been evaluated using equation 3. The negative regression coefficient was seen for A, B, and C factors. The findings unequivocally

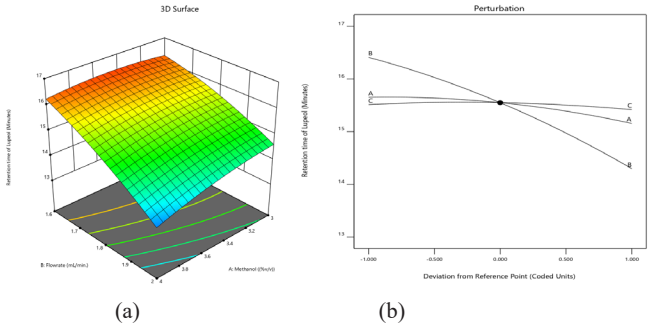


Figure 4: (a) 3D Response surface plot and (b) Perturbation graph of retention time of lupeol

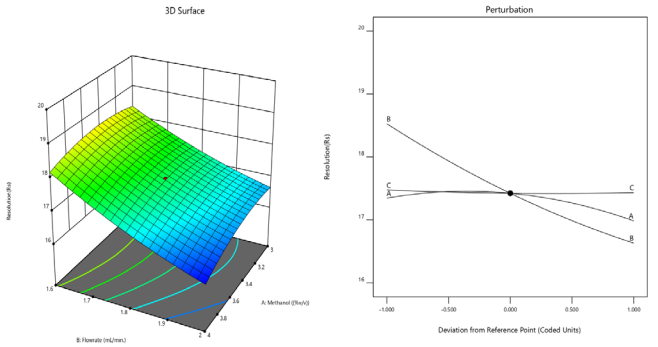


Figure 5: (a) 3D Response surface plot and (b) Perturbation graph of resolution

show that the resolution decreases when methanol percentage, flow rate, and pH increase. The positive regression coefficient for AC and BC was observed, which clearly explains that resolution increased along with the increase in AC and BC. On the other hand, the negative coefficient for AB indicates a reduction in resolution with a concurrent rise in AB (Figure 5).

Resolution = 17.4239 -0.180994A -0.946772B -0.0214678C -0.09875AB + 0.08125AC + 0.04625BC -0.257333A^2 + 0.156324 B^2 + 0.0308128 C^2……..... (3)

Desirability function

The optimization of capacity factor (Response 1), Retention time of lupeol (Response 2), and resolution (Response 3) utilizing various criteria was accomplished using Derringer's desirability function. The Derringer's desirability function D refers to the geometric mean of the several desire functions, whether they are normalized or not.[17] A desirability function provides a numerical value between 0 and 1 to each possible response, where 0 represents the least desirable value and 1 represents the most desirable or ideal value. The distinct desirabilities are then merged using the geometric mean, resulting in the overall desirability D. The obtained value for desirability was 0.975, which shows that the selected strategy is the best approach (Figure 6).

Method validation

The proposed RP HPLC technique was verified in accordance with ICH Q2b validation criteria for characteristics including linearity, LoD, LoQ, accuracy, precision, and robustness.[18]

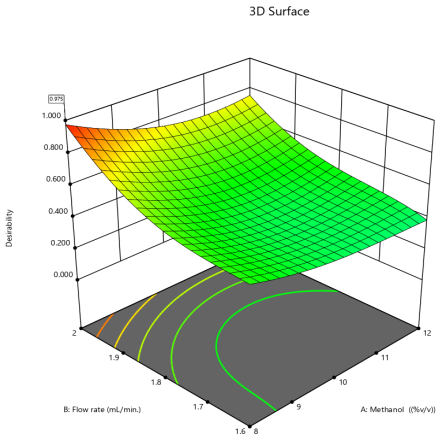


Figure 6: 3D Response surface graph for desirability function

Table 4: Results of validation for the developed method

Criteria	Lupeol	Stigmasterol	*β-Sitosterol*
Linearity range (µg/mL)	31.25–500	31.25–500	31.25–500
Regression equation	y = 100.96x + 650.67	y = 45.057x + 109.29	y = 63.469x + 179.93
Regression coefficient	0.9972	0.9976	0.9968
LoD (µg/mL)	0.2534	0.754	0.421
LoQ (µg/mL)	0.7681	2.29	1.28
Recovery (%) 80	99.81	100.32	99.64
100	101.23	100.65	99.62
120	100.51	99.96	101.44
Precision (%RSD)	0.92	0.98	0.96
Content of compound in 10 mg of extract (µg)	30.72	370.6	212.64

Table 5: Robustness data of lupeol, stigmasterol, β- sitosterol

Parameters	*Conditions*	*%RSD of lupeol*	*%RSD of stigmasterol*	*%RSD of β-sitosterol*
Methanol percentage	Low (8.0)	0.46	0.38	0.57
	High (12.0)	0.28	0.91	0.53
Flow rate (mL/min.)	Low (1.6)	0.35	0.85	0.26
	High (2.0)	0.37	0.66	0.94
pH	Low (3)	0.18	0.86	0.72
	High (4)	0.26	0.54	0.87

Linearity

The stock solution was chosen for the calibration curve and was examined in triplicate with concentration ranges of 31.25 to 500 µg/mL for lupeol, stigmasterol, and β-sitosterol, respectively. The linear response throughout the range was found to be elicited by the regression coefficient (R^2), which was 0.9972, 0.9976 and 0.9968 for lupeol, stigmasterol, and β-sitosterol, respectively and shown in Table 3.

LoD and LoQ

The LoD and LoQ values were determined using the regression equation of the calibration curve, which was established within the concentration range of 31.25 to 500 µg/mL. It was found to be 0.2534 and 0.7681 µg/mL for lupeol, 0.754 and 2.29 µg/mL for stigmasterol, and 0.421 and 1.28 µg/mL for β-sitosterol.

Accuracy

The recovery investigations were carried out using the standard addition technique, which involves spiking the standards at a concentration of 100 ± 20% to the nominal concentration of test solution of 30.72, 370.6 and 212.64 µg/10 mg to confirm the procedure's consistency and accuracy. Table 4 includes the findings of the study in which triplicate analysis at each level was done.

Precision

The precision of the technique was determined by calculating the %RSD using six replicates at a concentration of 100% for the standards. Table 4 presents the data. The repeatability of the proposed technique are specified by %RSD not more than 2. The results tabulated show that it was within limits.

Robustness

Robustness has been analyzed by slightly changing the selected factor conditions like pH (± 2), flow rate with high and low values as ± 0.2 mL/min and wavelength with above 2 nm and below 2 nm. The results are tabulated in Table 5.

CONCLUSION

Lupeol, stigmasterol, and β-sitosterol determination were made possible by a new AQbD-assisted RP-HPLC technique that was developed and validated. The capacity factor and resolution were optimized while retention time was kept to a minimum throughout the development of the method. The predicted values and actual values were well-aligned according to the model equation. The approach for concurrently evaluating the three markers was found to be efficient and effective. The findings of each validation parameter were good. The research showed that AQbD-assisted chromatography successfully improved the separation process. The proposed method can be considered suitable for the determination of lupeol, stigmasterol, and β-Sitosterol measurement in novel polyherbal formulations owing to its high selectivity, repeatability, and reproducibility.

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