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Simultaneous Estimation of Stigmasterol and Withaferin A in Union Total Herbal Formulation Using Validated HPTLC Method

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ABSTRACT

Union Total is herbal formulation made in the form of capsule which contains two standardized plant extracts *Cissus quadrangularis* (CQ) and *Withania somnifera* (WS). The present work describes development and validation of High Performance Thin Layer Chromatographic method for simultaneous analysis of Stigmasterol (STG) in *Cissus quadrangularis* (CQ) and Withaferin A (WFA) in *Withania somnifera* (WS). Stigmasterol and Withaferin A were identified on silica G60 F_{254} HPTLC plates by post derivatization technique and robustness study was performed by applying a central composite design (CCD) with k factor having 2^k factorial runs, 2k axial experiments and five center points. In HPTLC good separation was obtained with chloroform: methanol: toluene: formic acid (6.5: 0.5: 3: 0.25 v/v/v/v) as mobile phase and anisaldehyde sulphuric acid as a derivatizing reagent at detection wavelength 530 nm. Linearity was obtained in the concentration range of 100-200 ng/band for WFA and 200-700 ng/band for STG and the % recoveries were found in the range of 100.06 % to 100.46 % for WFA and 99.97 % to 100.94 % for STG respectively. HPTLC method was found to be sensitive, precise, accurate and reproducible, which would be of use in quality control of these tablets.

INTRODUCTION

Osteoporosis, a silent epidemic, is characterized by decreased bone mineral density (BMD), increased risk of fractures and is associated with micro architectural deterioration of bone tissue that results in low bone mass. It has become major health hazard affecting more than 2000 million people, of both sexes and all races worldwide in recent years (Kumar at el., 2010; Jia *et al.*, 2012). According to World Health Organization (WHO), osteoporosis is defined as a bone mineral density that lays 2.5 standard deviations or more below the average value for young healthy women. In osteoporosis, bone loss occurs especially at the trabecular area when the balance of bone remodeling is tipped towards bone resorption. The bone loss is associated with bone biochemical marker changes such as reduction in osteocalcin level, the marker for bone formation and elevation in crosslink C-telopeptide, the marker for bone resorption (Shuid *et al.*, 2012). Last few decades have seen a tremendous increase in the use of traditional medicines globally. According to an estimate about 80% of population the world over depends on traditional medicines (Bhondave *et al.*, 2014). Eco-friendly, bio-friendly, cost-effective and relatively safe herbal medicines have moved from the fringe to the mainstream with increased research in the field of traditional medicine (Sen *et al.*, 2011). Natural products for the management of osteoporosis are largely phytoestrogens which include isoflavones, lignins, flavonoids, and coumestans that share structural and functional similarities with naturally occurring or synthetic estrogens. Phytoestrogens exhibit estrogen-like effects at various reproductive and non-reproductive tissues (Compston, 2014). *Cissus quadrangularis* is the most common species belonging to the family Vitaceae, commonly known as Hadjod or bonesetter in Hindi due to its bone fracture healing property. The plant is prescribed in the ancient Ayurvedic literature as general tonic and analgesic, with specific bone fracture healing properties due to presence of several new compounds like lipids, stilbenoids, steroids, irridoids and flavanoids (Gupta, 2005; Shah, 2011). Because being an effective remedy in treatment of bone fractures, a range of formulations containing CQ extract either alone or in combinations with other hearths have been marketed (Udupa, 1964; Chopra, 1975). *Withania somnifera* belonging to family Solanaceae; is also known as Ashwagandha.

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The main constituents of Ashwagandha are alkaloids and steroidal lactones from which withaferin A help to increase expression of osteoblastogenesis and decrease expression of osteoclastogenesis (Gupta, 2011; Anonymous, 1998). Union total capsule is a herbal formulation contains two standardized plant extracts i.e. *Cissus quadrangularis* 750 mg and *Withania somnifera* 100 mg. It is mainly indicated for fracture healing and osteoporosis. The key challenge in integrating Ayurvedic medicines with the current clinical practice is lack of scientific data and better understanding of efficacy and safety of the herbal formulations. The need of the hour is to evolve a systematic approach and to develop well-designed methodologies for standardization of raw material as well as herbal formulations (Marcus and Grollman *et al.,* 2002; Vaidya *et al.,* 2003). HPTLC has recently emerged as a preferred analytical tool for fingerprints and quantification of marker compounds in herbal drugs because of its simplicity, sensitivity, accuracy, suitability for high throughput screening analysis of herbal medicine. Previously, different methods have been reported for HPTLC estimation of stigmasterol and withaferin A individually or in combination with other markers (shah *et al.,* 2010; Hussains *et al.,* 2012; Badole *et al.*, 2011; Gauttam, 2013). To the best of our knowledge this is the first report on a method for concurrent analysis and estimation of these marker compounds. Therefore, the current study was aimed to determine STG and WFA by HPTLC method using Design of Experiment (DoE) approach for method validation, which will be useful for the proper identification of Union total herbal formulation.

Experimental design procedures are very useful in pharmaceutical development including formulation development and analytical method optimization and validation, and are more effective than the traditional one variable-at-a-time approach (Goupy, 2005). Experimental design methodology has proved to be a useful tool for method validation, as it allows the investigation of simultaneously changing factors. During method validation, ruggedness (different normal conditions) or robustness (small changes introduced deliberately) studies are typically performed with the expected outcome that there is no significant change of the response, allowing the claim of a rugged/robust method.

Moreover, many factors can be screened simultaneously without concerns about interacting and non-interacting effects, as they are usually considered negligible. On evaluation of obtained results, when a factor is not robust, the proposed method can be changed, revalidated or the factor can be controlled. Various experimental designs for robustness study includes Plackett Burman design, factorial, fractional factorial and response surface designs. (Shrinubanu *et al.,* 2007; Hibbert, 2012; Fabre, 1996). This research article focuses on the determination of robustness of HPTLC analytical method by central composite design. Among the various experimental designs, central composite design (CCD) as a response surface design was preferred for prediction of nonlinear response due to its flexibility, in terms of experimental runs and information related to factor's main and interaction effects. CCD combines two level factorial design with a star design and centre points covers the factor space near the centre with more points than at the periphery and allows more number of levels without performing experiments at every combination of factor levels (Beer *et al.,* 1996; Valdimir *et al.,* 2002).

MATERIAL AND METHODS

Materials and reagents

Standards namely Stigmasterol (STG) and Withaferin A (WFA) were provided as gift sample from Sigma Life Science (Mumbai) and Pharmanza Herbal Pvt. Ltd (Dharmaj) respectively. Herbal extracts i.e. *Cissus quadrangularis* (CQ) and *Withania somnifera* (WS) present in Union total Herbal formulation and Union total capsules were procured from Pharmanza Herbal Pvt. Ltd.; all chemicals and reagents used in the study were of analytical grade and purchased from Merck specialties Pvt. Ltd. Mumbai, India.

Instrumentation and software

A Camag HPTLC system (Switzerland) comprising of Camag Linomat V semi automatic sample applicator, Camag TLC Scanner IV, Camag (Muttenz, Switzerland) flat bottom and twin-trough developing chamber (10 x10 cm), UV cabinet with dual wavelength UV lamp, Hamilton syringe (100 µl), Ultrasonic bath (Frontline FS-4, Mumbai, India) and Camag winCATS software were used in the study. Experimental design based robustness testing was carried out by using Design expert software (v 9.0.0.7) Stat-Ease Inc., Minneapolis and remaining calculations were performed by use of Microsoft Excel 2007 software.

Preparation of standard solutions

A stock solution of STG and WFA were prepared separately by weighing accurately 10 mg of each marker followed by dilution in methanol in 10 ml volumetric flask and volume was made up to the mark with methanol, to obtain a concentration of 1000 µg/ml. The standard stock solutions were suitably diluted with methanol to obtain the working standard solutions of STG and WFA.

Preparation of sample solution

Ten Union total capsules (Herbal formulation) were taken, shell of capsules was removed to get powder and grind into a clean and dry mortar-pestle. An equivalent to 750 mg CQ extract powder and 100 mg of WS extract powder was transferred to a 10 ml volumetric flask followed by addition of 10 ml of methanol and sonicated for 30 minutes. This solution will give 500 µg/ml of STG and 120 µg/ml of WFA which was directly used for analysis.

Method Development

Different solvents in different ratios like methanol, chloroform, ethyl acetate, toluene, glacial acetic acid, formic acid, ammonia, triethylamine and hexane were tried for optimization of

mobile phase. Suitable volumes of standard and sample solutions were applied to the Pre-coated silica gel 60 F_{254} \ HPTLC plates (E. Merck) as bands of 6 mm using Linomat V. Application positions were at least 15 mm from the sides and 8 mm from the bottom of the plates. Mobile phase components were mixed prior to use and the development chamber was left to saturate with mobile phase vapour for 25 min before each run. Development of the plate was carried out by the ascending technique to a migration distance of 8 cm. Then the plates were dried in an oven. After drying plate was dipped in anisaldehyde sulphuric reagent and then plates were dried in oven at 120°C ± 2°C. Densitometric scanning was done in absorbance reflectance mode at 530 nm using a deuterium lamp. The slit dimensions were set at 6 mm × 0.30 mm, the scanning speed at 20 mm/s, and the data resolution of 100 µm/step. The results were evaluated with the aim of achieving an optimum separation between spots (Rs ≥1.5), and a migration of spots with R_f values between 0.2 and 0.8, in order to ensure separation reproducibility.

Method validation

The method was validated in accordance with ICH guidelines Q2 (R1) for evaluation of various parameters; linearity, precision, accuracy, LOD and LOQ and robustness.

Linearity

Linear relationship between peak area and concentration of two markers were evaluated by making five replicate measurements in the concentrations range of 100-600 ng/band for STG and 200-700 ng/band for WFA. Each calibration plots were constructed by plotting the peak area of band versus the concentration of the markers and treated using the method of ordinary least squares regression analysis.

Precision

Precision of the developed method was studied by performing repeatability, intermediate precision. The repeatability of sample application and measurement of peak area was determined by performing 3 replicate measurements of the same band. The various solutions were applied on HPTLC plates to form bands with 100, 300, and 500 ng/band of WFA and 200, 400, and 600 ng/band of STG.

Accuracy

The accuracy was assessed by the methodological recovery studies to check the recovery of the drug at different levels in the formulations by optimized method. It was carried out by adding known amount of standard to samples at 80, 100 and 120 % level and analyzed by the proposed method, in triplicate.

Limit of detection (LOD) and limit of quantitation (LOQ)

As per ICH guideline, limit of detection and quantitation of the developed method were calculated from the standard deviation of the response (σ) and slope of the calibration curve (S) of each drug using the formula; Limit of detection=3.3*σ/S and Limit of quantitation=10*σ/S

Specificity

Specificity of method was ascertained by comparing spectra of formulation, extract and standard. The peak purity was assessed by comparing spectra at three levels, i.e., peak start (S), peak apex (M) and peak end (E) of spot.

Robustness study using experimental design

The robustness of an analytical procedure refers to its capability to remain unaffected by small and deliberate variations in method parameters. A CCD with k factors requires 2k factorial runs, 2k axial experiments, which is symmetrically spaced at along each variable axis, and at least one center point. The factors and ranges selected for consideration were based on previous univariate studies of method development and chromatographic intuition.

In the present study three factors viz. developing distance (A), band size (B) and Chloroform in total mobile phase (C) were selected. The nominal value for all these three factors, A, B and C, were 8 cm, 6 mm and 6.5 ml, respectively. The ranges examined were small deviations from the method settings and the considered response was the R value of different drugs. Here five different levels (-α, 1, 0, +1, + α) of study were carried out by using rotatable CCD. The coded value of α is 1.41. A three-factor small type CCD requires 15 experiments, including five center points.
The experimental plan of selected factors is reported in Table 2. All experiments were performed in randomized order to minimize the bias effects of uncontrolled factors.

Analysis of union total formulation

Ten Union total capsules (Herbal formulation) were taken, shells of capsules were removed to get powder and grind into a clean and dry mortar-pestle. An equivalent to 750 mg CQ extract powder and 100 mg of WS extract powder was transferred to a 10 ml volumetric flask followed by addition of 10 ml of methanol and sonicated for 30 minutes. This solution will give 500 µg/ml of STG and 120 µg/ml of WFA which was then filtered through Whattman filter paper No. 42 wetted with methanol. The resulting solution was directly used for analysis.

RESULT AND DISCUSSION

Method development

UV scanning at 400-800 nm shows that 530 nm is the suitable wavelength for detection of both markers (Fig. 1).
Various combinations of mobile phase were tried for optimizing the peaks of mentioned markers by trial and error. The final optimized mobile phase used was Chloroform: Methanol: Toluene: Formic acid (6.5: 0.5: 3.0: 0.25; v/v/v) which gave highest resolution and R_f values of 0.22 and 0.57 for WFA and STG respectively (Fig. 2)

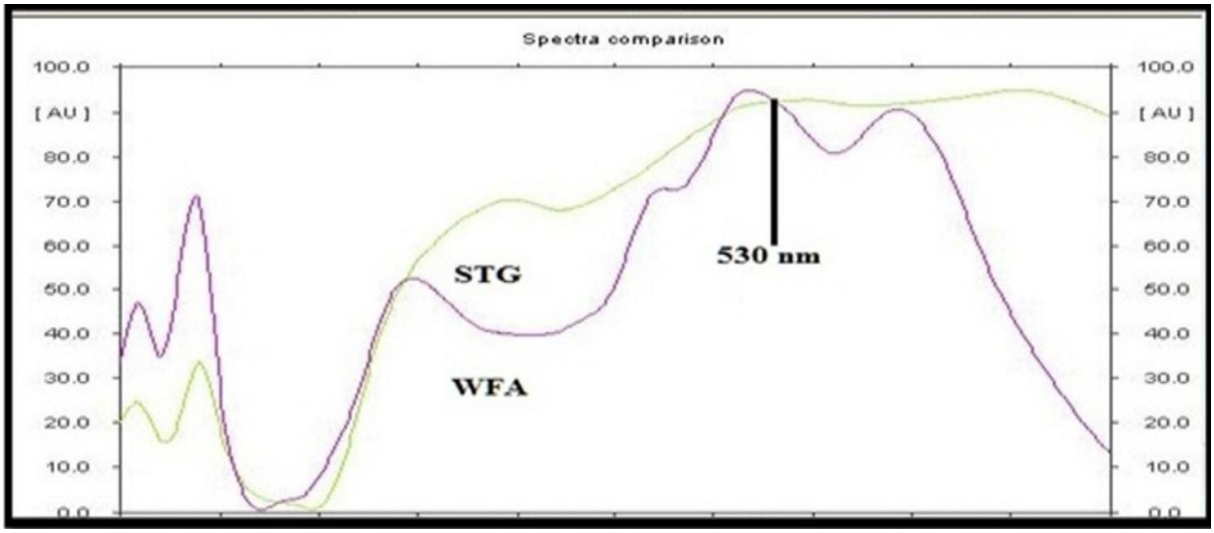


Fig. 1: overlain spectra of stigmasterol and withaferin A at 530nm.

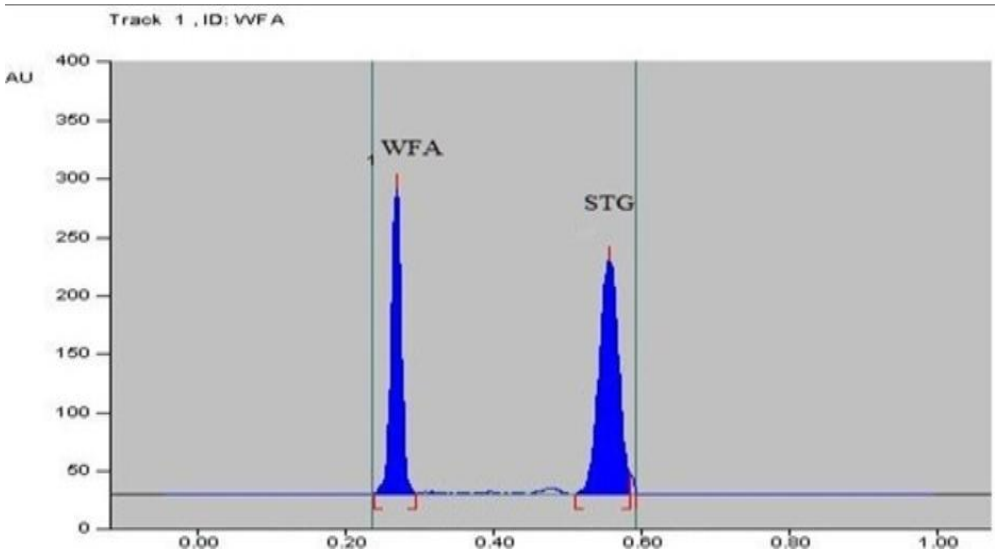


Fig. 2: HPTLC chromatogram of withaferin A and stigmasterol using chloroform: methanol: toluene: formin acid (6.: 0.5: 3: 0.25: v/v/v) optimized mobile phase.

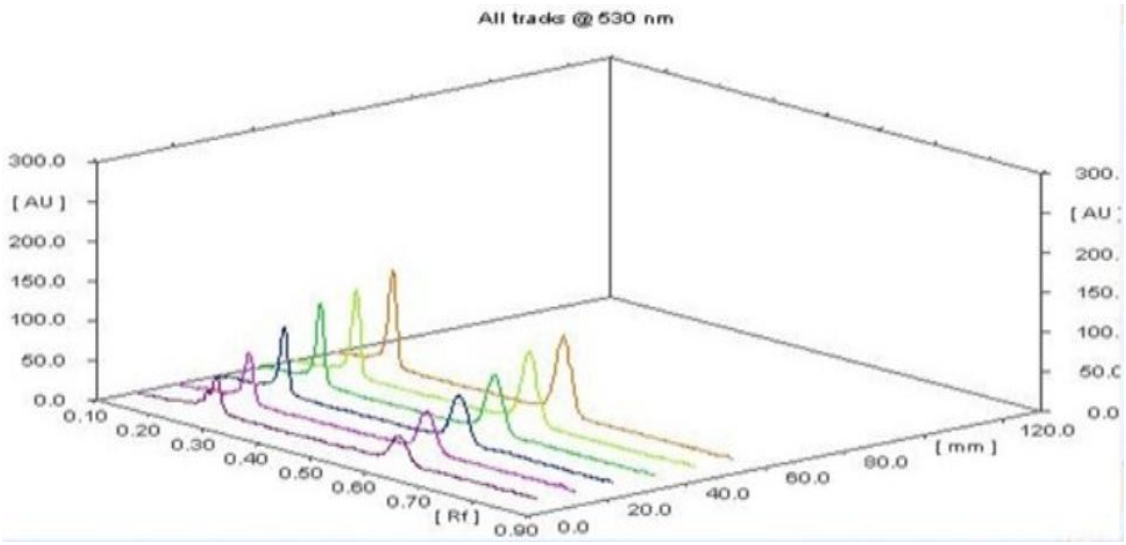


Fig. 3: 3D chromatogram showing Linearity of withaferin a and stigmasterol

Method Validation

Linearity

The STG and WFA showed good correlation coefficient (r^2 = 0.99681 for STG and $r^{2=}$0.9960 for WFA) in the mentioned concentration range 200-700 ng/band for STG and 100-600 ng/band WFA (Fig. 3 and Table 1)

Precision

Precision of developed method was evaluated by repeatability and intermediate precision, and was expressed as % relative standard deviation (%RSD) of the peak area. Repeatability and intermediate precision when carried out by performing three replicates of three different concentrations (100, 300 and 500 ng/band for STG and 200, 400 and 600 ng/band for WFA) showed %RSD less than 2% (Table 1) and meet the criteria as per ICH and USP guideline indicating acceptable precision of method.

Accuracy

The proposed method when used for evaluation of recovery at three concentrations levels, 80%, 100% and 120% after spiking with standard, showed percentage recovery between100.06 % to 100.46 % for WFA and 99.97 % to 100.94 % for STG respectively, with acceptable % RSD, less than 2 (Tab 2).

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ for WFA were 23.818 ng/band and 72.175 ng/band and for STG were 10.049 ng/band and 30.454 ng/band respectively.

Specificity

The peak purity of STG and WFA assessed by comparing their respective spectra at the peak start (S), apex (M), and peak end (E) positions of the band and results obtained were depicted in Figure 4.

Robustness by using experimental design

Experimental results were computed by design expert trial version 9.0.0.7. The coefficients of the second-order polynomial model were estimated by the least squares regression. The equation models for Y for different drugs are shown in Tab. 3. Here positive sign indicates synergistic effect, while a negative sign indicates antagonistic effect. The model was validated by the analysis of variance (ANOVA). In the present study, the value of adequate precision, (depicts the value of signal to noise ratio) greater than 4 % is desirable; Ratio of 18.074 indicates an adequate signal and model can be used to navigate the design space. The value coefficient of variation (CV) (measures the reproducibility of the model) less than 10% is desirable and the P value of the models ($p < 0.05$ is required) these all were in desirable limits (Table 3). It reveals that the model represents the phenomenon quite well and the variation of the response was correctly related to the variation of the factors, showing a good agreement between experimental and predicted values. The interpretation of the results has to start from the analysis of the whole model equation rather than from the analysis of the single coefficients; it is important, for the RSM, to consider also the factors whose coefficients are statistically non-significant. For this reason the analysis of the response surface plot is necessary. Here, predicted models are presented in the form of perturbation plots for better understanding of results (Figure 5). These graphs give the idea about how the response changes as each factor moves from its defined reference value, with all other factors held constant. A steep slope or curvature in a factor indicates that the response is sensitive to that factor. Here, the plots show that factor C is mostly influencing the R_f value of all drugs compare to factor A and B. As shown in Figure 6 (A–D), the analysis produces three-dimensional graphs of RSM by plotting the response model against two of the factors, while the third is held constant at a specified level. Figure 6(A) and 6(C) shows a graphical representation of the variation of response as the function of A and C, while B is maintained constant. An increase in the chloroform content results in an increase in R_f value of WFA (Y) and STG (Y), while developing distance and band size had no significant effect on the response shown in fig 6 (B). Here, we selected the plots which represent the pronouncing effect on the response. Similarly, analogous interpretation may be derived by examining different plots from Figure 6 (A-D). In conclusion, the analysis of response surface confirms that Y is not robust for factor C, thus a precautionary statement should be included in the analytical procedure for the same.

Table. 1: Analytical parameters of proposed HPTLC method for simultaneous estimation of WFA and STG.

PARAMETERS	WFA	STG
LINEARITY [a]		
Calibration range (ng/band)	100-600	200-700
Correlation coefficient	0.9960	0.9931
Slope ± SD [b]	1.7478 ± 0.0123	3.1524 ± 0.0046
CI of slope [c]	1.595 – 1.900	2.788 – 3.518
Intercept ± SD [b]	769.430 ± 12.615	257.680 ± 9.600
CI of intercept [c]	709.896 – 828.962	81.951 – 433.409
Bartlett's test [d] ($\chi 2$)	0.002181	0.000089
SENSITIVITY (ng/band)		
Limit of detection	23.819	10.0499
Limit of quantification	72.176	30.455
PRECISION (%RSD, n=3)		
Repeatability	0.235 – 1.260	0.632 – 1.228
Intermediate precision	0.744 – 1.507	0.749 – 0.952
ACCURACY (Mean ± SD)	100.06 - 100.46	99.97 - 100.94

[a] n=5 replicates, [b] standard deviation, [c] Confidence interval at 95% confidence level and five degree of freedom (t=2.57) , , [d] Calculated value $\chi 2$ less than critical value $\chi 2$ (0.05, 5) = 11.070, SD = standard deviation, RSD = relative standard deviation

Table. 2: CCD model for robustness study with obtained responses.

Run	Space Type	FACTORS			RESPONSE	
		Developing distance (cm)	Band size (mm)	Chloroform content in total mobile phase (ml)	R_f value of WFA	R_f value of STG
1	Factorial	-1	1	1	0.24	0.59
2	Factorial	-1	-1	-1	0.2	0.55
3	Center	0	0	0	0.22	0.57
4	Axial	-1.41	0	0	0.22	0.56
5	Axial	0	-1.41	0	0.22	0.57
6	Factorial	1	-1	1	0.24	0.59
7	Axial	1.41	0	0	0.23	0.59
8	Center	0	0	0	0.22	0.57
9	Axial	0	0	1.41	0.25	0.62
10	Center	0	0	0	0.23	0.58
11	Axial	0	0	-1.41	0.18	0.52
12	Center	0	0	0	0.21	0.56
13	Factorial	1	1	-1	0.2	0.55
14	Center	0	0	0	0.22	0.57
15	Axial	0	1.41	0	0.23	0.58

n=3 replicates, WFA: withaferin A, STG: stigmasterol, CCD: central composite design

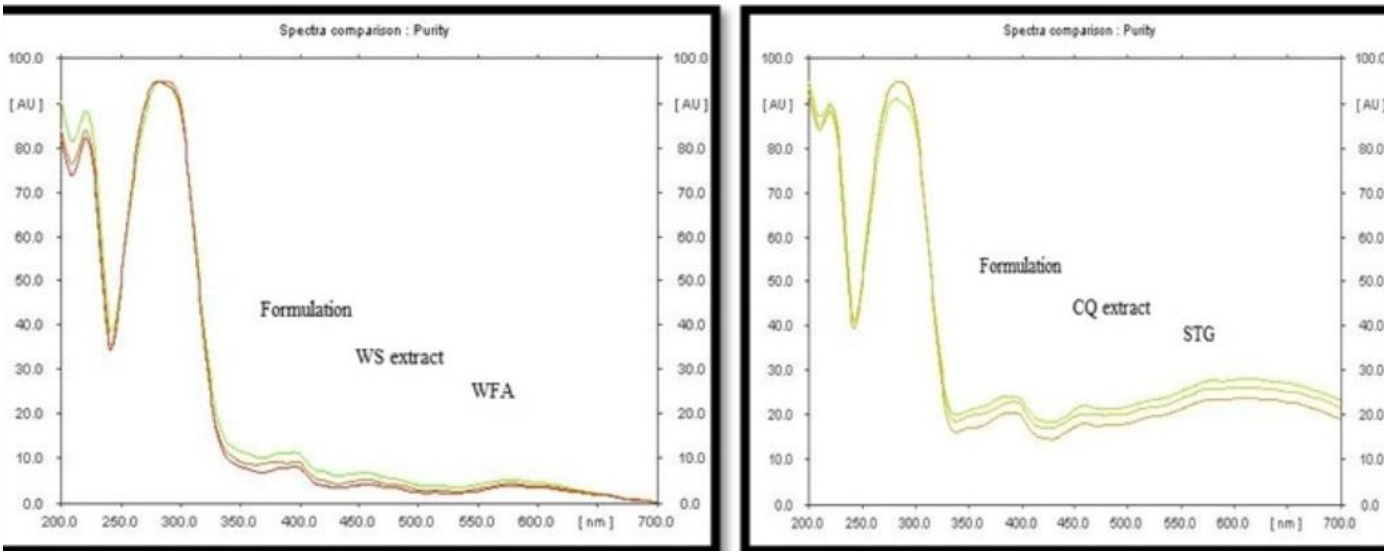
Table. 3: Predicted response models and statistical parameters obtained from ANOVA for CCD.

Response (R_f value)	**WFA**	**STG**
Type of model	**Linear**	**2 factor interaction (2FI)**
Polynomial equation model for Y	0.22 +1.768E-003 A +1.768E-003 B +0.022 C	0.57 + 0.011 A + 3.536E-003 B + 0.035 C + 0.015 AB + 3.536E-003 AC+ 0.011 BC
Model p value	0.0001	0.0001
% CV	2.86	1.02
Adequate precision	19.411	31.429
R-Squared	0.902	00.963
Adj R-Squared	0.876	0.935
Pred R-Squared	0.824	0.897
PRESS	7.906E-004	7.567E-004

Table. 4: Analysis of Union Total herbal formulation.

Standards	Label claim (mg)	% w/w Amount found (Mean)[a]	% RSD
WFA	1.35	100.239	0.722
STG	5.625	99.667	0.116

%RSD= relative standard deviation, [a] n=3 replicates



A **B**

Fig. 4: overlain peak purity spectra of (A) formulation, WS extractand WFA (B) formulation, CQ extract and STG.

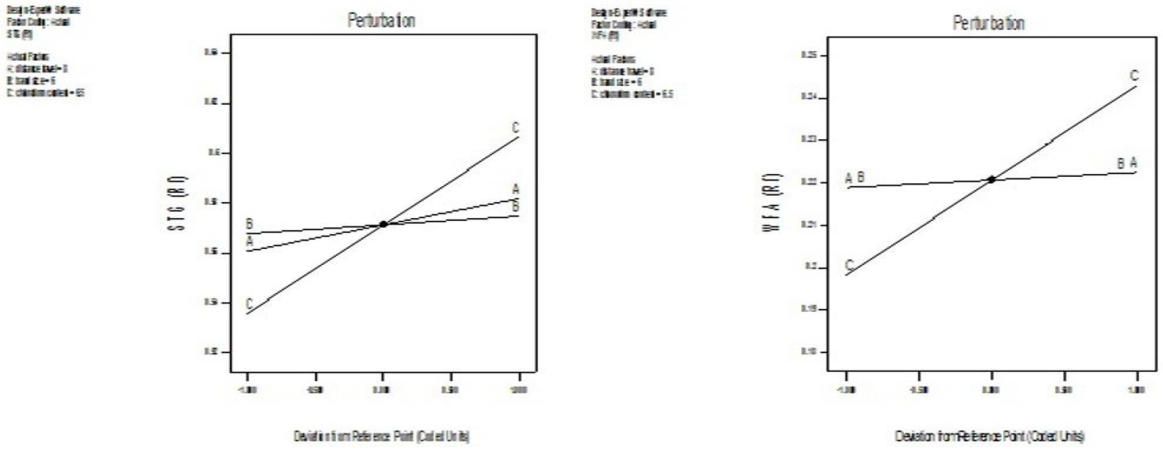
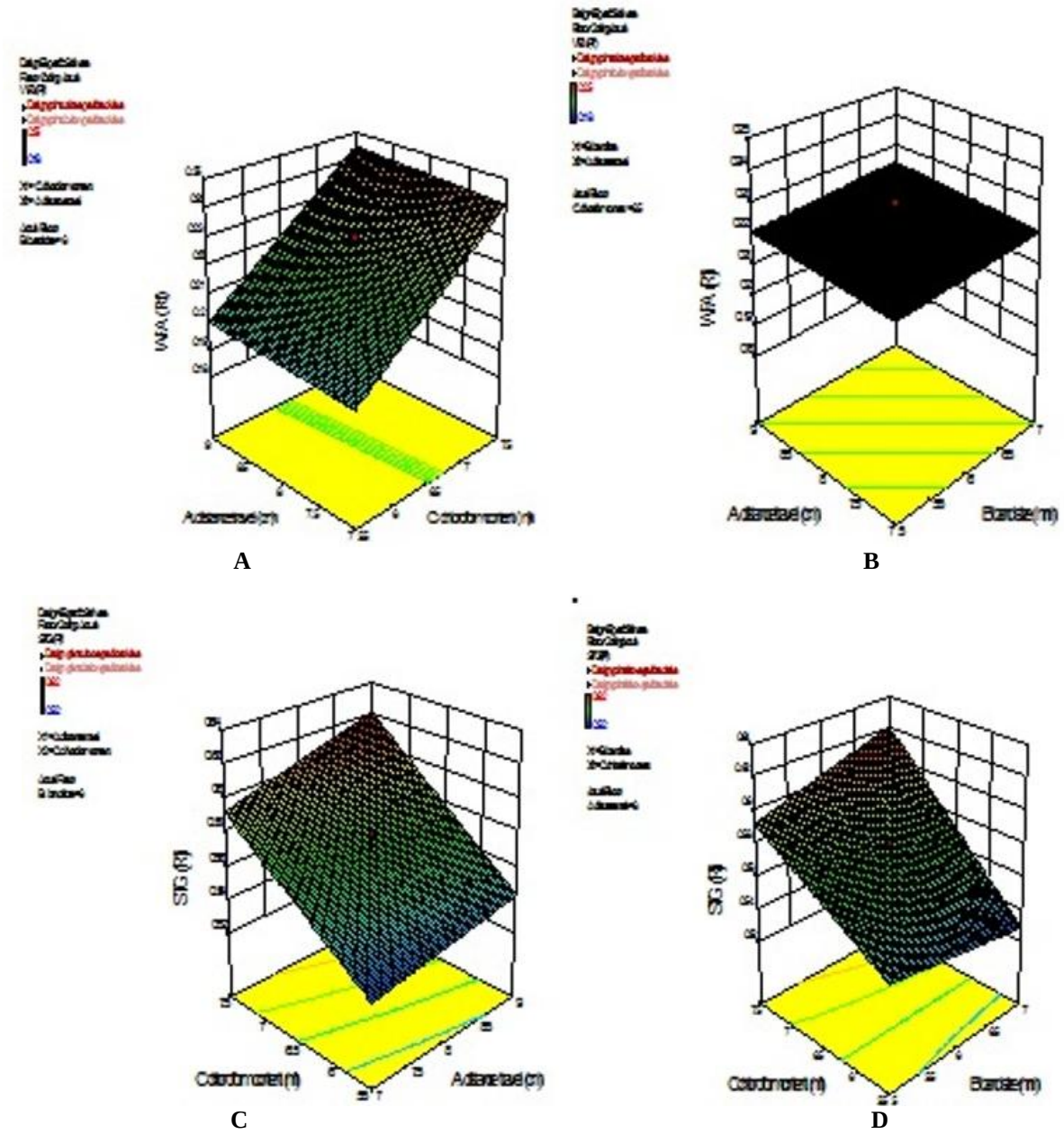


Fig. 5: Perturbation graph showing the effect of each factor A,B, and C on (A)R_f value of WFA, (B)R_f value of STG.



A **B**

C **D**

Fig. 6: three- dimensional plot of the RSM for Y (R fvlue)

Analysis of Union Total formulation

Herbal formulations; *Withania somnifera* 100 mg (WFA 1.35 mg) and *Cissus quadrangularis* 750 mg (STG 5.625 mg) when analyzed in triplicate using the developed method, showed only two peaks of WFA and STG at Rf value 0.22 and 0.57 respectively in the chromatogram of formulation indicating no interference of the excipients.

SUMMARY AND CONCLUSION

The results indicate that the proposed HPTLC method is novel, rapid, simple, specific, economical and reliable for simultaneous analysis of WFA and STG. The method was validated in accordance with ICH guidelines.

The measured signal was shown to be precise, accurate, and linear over the concentration range (100-600 ng/band and 200-700 ng/band) with a correlation coefficient better than 0.9960. The mean recovery found for mentioned drugs are within the range of 99.66 to 100.239 using HPTLC method. The %RSD was also less than 2% showing high degree of precision of the proposed method. The application of CCD on robustness was to study simultaneous variation of effects on responses. CCD was applied to design the experimental program by evaluating the effects of developing distance, chloroform content in total mobile phase, and band size. The results reveal that the chloroform content of total mobile phase has a significant effect on R_f value of each drug.

This study reveals that highly precise, accurate, sensitive, and specific HPTLC method was developed for simultaneous estimation of WFA and STG. Furthermore, the same methodology can be applied for quality control testing and routine analysis of union total formulation.

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