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Original Article

Application of Box-Behnken Design for Validation of High-Per-Formance Thin-Layer Chromatography/Densitometry Method for Robustness Determination of Apremilast in Bulk and *in-house* Tablets

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ABSTRACT

Background: Apremilast is small molecule inhibitor of phosphodiesterase-4 (PDE-4) and an immunomodulating agent which is used for management of refractory psoriatic arthritis. **Material and Methods:** High-Performance Thin-Layer Chromatography (HPTLC) method for the analysis of apremilast was developed and validated as per ICH guidelines. Apremilast was chromatographed on silica gel 60 F_{254} TLC plates using toluene: methanol (8:2 *v/v*) as a mobile phase. A Compact spot for apremilast was observed with Rf 0.64 ± 0.05, when the densitometric scanning was implemented at 230 nm. The linear regression analysis data for the calibration plots showed $r^2 > 0.99$ with a concentration range from 250 – 1500 ng/band. 'Design of Experiments' (DoE) employing 'Box-Behnken Design' (BBD) and 'Response Surface Methodology' (RSM) were studied as an advancement to traditional 'One Variable at Time' (OVAT) approach to assess the effects of variations in selected factors particularly (development distance, saturation time, activation time of plate and mobile phase ratio) as graphical interpretation for robustness. The statistical insight was achieved with Multiple Linear Regression (MLR) and ANOVA. **Results:** The method was validated for precision, accuracy, detection limit and quantitation limit, and robustness. **Conclusion:** The method was successfully employed for the determination of apremilast from its *in-house* tablet formulation.
Key words: Apremilast (APL), High-Performance Thin-Layer Chromatography (HPTLC), Design of Experiments (DoE), Validation.

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INTRODUCTION

Psoriasis is classified in a group of chronic inflammatory disease troubling approximately 2 – 3% of the wide-reaching people. In psoriasis, the augmented the levels of pro-inflammatory mediators, such as, tumor necrosis factor, interleukin IL-17 and IL-23, and decreases the level of anti-inflammatory mediators, viz. IL-10. Phosphodiesterase 4 (PDE4) a principle enzyme dominant in immune cells and modulates the production of this cytokinines.[1]

Apremilast (APL), [N-[2-[(1S)-1-(3-ethoxy-4-methoxyphenyl)-2-methylsulfonylethyl]-1,3dioxoisoindol - 4- yl] acetamide] (Figure 1) is an orally existing, small molecule inhibitor of phosphodiesterase-4 (PDE-4) and an immunomodulating agent which is used for management of refractory psoriatic arthritis. Apremilast has been linked to a low rate of serum enzyme elevations during therapy, but has not been given in cases of clinically apparent acute liver injury.[2] Literature revealed few methods for analysis of apremilast in bulk, pharmaceutical formulations and biological fluids which includes UPLC–MS/MS,[1] High-Performance Liquid -Chromatography method for quantification of impurities of Apremilast[3] and stability-indicating UV- Spectrophotometric method.[4] Robustness can be interpreted as the capability to reproduce the (analytical) method in diverse laboratories or under different conditions without the occurrence of unexpected differences in the obtained result(s), and a robustness test as an experimental set-up to evaluate the robustness of a method.[5] The implication of use of 'Quality by Design' (QbD) or 'Design of Experiments' (DoE) approach suggested achieving these goals.[6] The robustness test ensures the possible causes of changeability in one or a number of responses of the method.[7,8] To assess likely sources of changeability, a number of factors are selected from the working procedure and examined in an interval that to some extent exceeds the differences which can be anticipated when a method is conveyed from one instrument to another or from one laboratory to another, these factor are observed in an experimental design and the effect of the factors on the response(s) of the method is assess in this way the factors that possibly will harm the method performance are discovered. The analysts subsequently know that such factors should be more stringently forbidden throughout the implementation of the method.[9]

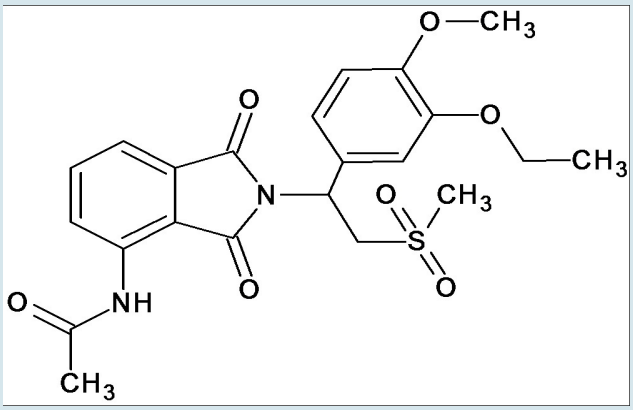

Figure 1: Chemical structure of Apremilast.

Various design methodologies to assess robustness of method such as; full factorial design, fractional factorial designs, Asymmetrical Facto-

rial Designs (AFD),[10] Central Composite Design (CCD) either as Circumscribed or Face-centred, Doehlert Designs, Box–Behnken Design (BBD), Plackett–Burman Design (PBD), Star Designs;[11] supported with graphical methods of interpretation such as Normal probability plot, half-normal probability plot, bar plot with or without limit value, counter plot, standardized pareto chart and response surface method (RSM).[12]

The Box-Behnken is an excellent design for response surface methodology because it permits: (I) estimation of the parameters of the quadratic model; (II) structure of sequential designs; (III) recognition of lack of fit of the model; (IV) use of blocks. None of these methods have been found to be cost-effective.[13] However, to our endeavor no HPTLC method has been studied so for the estimation of apremilast in bulk material and in-house tablets formulation. The present research illustrates a simple, sensitive, effective and economical Normal Phase (NP) - HPTLC method for estimation of apremilast in bulk material and in-house tablets formulation giving emphasis on application of DoE approach to evaluation of robustness of method.

MATERIALS AND METHODS

Experimental

Chemicals and Reagents

Pharmaceutical grade Apremilast working standards were obtained as generous gifts from Intas Pharmaceuticals Ltd, Ahmedabad, India. Methanol (HPLC Grade) and aluminium backed TLC plates pre-coated with silica gel 60 F_{254} (0.2 mm thick) were purchased from E. Merck Ltd., Mumbai (India).

HPTLC instrumentation and Chromatographic conditions

Instrumentation

HPTLC system : Camag TLC system (Muttenz, Switzerland)

Sample applicator : Linomat 5

Scanner : TLC scanner 3

Data processor : winCATS (version 1.3.0)

Development chamber : Camag twin trough chamber (20 x 10 cm)

Syringe for application : Hamilton syringe (100 µL)

Ultrasonicator : ENERTECH Electronics Pvt. Ltd., India

Chromatographic conditions

Stationary phase : Aluminium backed precoated silica gel 60-F_{254} (20 x 10 cm)

Mobile phase : Toluene : Methanol (8:2 *v/v*)

Development distance : 8 cm

Saturation time : 25 mins

Scanning wavelength : 230 nm

Densitometry scanning mode : Absorbance–Reflectance.

Preparation of Stock Standard Solution and study of linearity curve

Stock standard solution was prepared by weighing 10 mg of apremilast. Weighed powder was transferred into volumetric flask of 10 mL and dissolved and diluted to mark with methanol to obtain concentration 1 mg/mL.

An appropriate volume of 0.5 – 3 mL from stock standard solution was transferred with the help of previously calibrated pipette into series of 10 mL volumetric flasks. A fixed volume of 5 µL was applied on the HPTLC plates to obtain concentrations 250, 500, 750, 1000, 1250 and 1500 ng/band of APL, respectively.

Preparation of Sample

As the pharmaceutical formulation of apremilast is not available in the local Indian market; therefore, in-house tablets were prepared with 30 mg of apremilast and common excipients. The sample solution was prepared from in-house formulated apremilast tablets.

Accurately weighed power drug equivalent to 30 mg apremilast was quantitatively transferred into 100 mL volumetric flask dissolved and diluted volume with methanol. The resulting solution was filtered through a 0.45 µm filter (Millifilter, Milford, MA, USA). An appropriate volume of 4 µL from filtrate was applied on HPTLC plates were subjected to proposed method for further analysis.

Statistical tools

Experimental design for robustness study was performed using Design Expert® (Version 8.0.4.1), Stat-Ease Inc., Minneapolis, MN, USA statistical software. The rest of the calculations for the analysis were performed by use of Microsoft Excel 2007 software (Microsoft, USA).

Validation of HPTLC Method

The anticipated method was validated as to ensure it for precision, accuracy, sensitivity and robustness as per recommendations of International Conference on Harmonization (ICH) guidelines.[14]

Precision and Accuracy

Method precision was performed as repeatability, intra-day and inter-day deviation. Repeatability was accessed at concentration of 1000 ng/band of APL using six replicates. Intra-day deviation was studied using concentration 500, 700 and 1000 ng/band of APL; analyzed it for three times in the same day while it was analyzed for the three different days over a period of week for inter-day studies.

Accuracy of the method was estimated by spiking the drug standard in pre-determined laboratory mixture solution at concentration levels of 80 %, 100 % and 120 % and determined as percent recovery studies.

Sensitivity

The sensitivity of proposed methods was estimated in terms of Detection Limit (DL) and Quantification Limit (QL) determinations for both specified methods were based on the standard deviations of the responses and slopes of constructed calibration curves (n = 3) as described by the International Conference for Harmonization guidelines Q2(R1). For the determination of DL and QL during HPTLC method validation; APL solutions of, 250, 300, 350, 400,450 and 500 ng /band were applied on HPTLC plates. The DL and QL were calculated using equations DL = 3.3·N/B and QL = 10·N/B; where, 'N' is standard deviation of peak areas of the drug (n = 3) taken as a measure of noise and 'B' is the slope of corresponding calibration curve.

Experimental design methodology for robustness

A Box-Behnken statistical screening design was used to optimize the compositional parameters and to evaluate quadratic effects of the mobile phase composition, saturation time, development distance and activation time of plate on the retention factor (R*f*) and peak area. The linear polynomial equations generated from ANOVA are in the form, depicted below.

$$Y = b_o + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{12}x_1x_2 - b_{13}x_1x_3 - b_{14}x_1x_4 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{34}x_3x_4 - b_{11}x^2_2 + b_{22}x^2_2 - b_{33}x^2_3 + b_{44}x^2_4 \quad (1)$$

Where, 'y' is the measured response (dependent variable) associated with the each factor level combination; 'b_0' represents the polynomial equation intercept representing average arithmetic mean of all quantitative outcomes of twenty-nine runs and 'b_1 – b_{44}' is regression coefficients computed from the observed experimental values of 'y' and 'y_1' 'x_1', 'x_2' ,'x_3' and 'x_4' represent the coded levels of independent variables; Where, x_1: development distance, x_2: saturation time, x_3: activation time of plate and x_4: proportion of methanol in mobile phase; ranges selected for independent variables during determination of method robustness were 7 to 9 cm, 20 – 30 minutes, 8-10 minutes and 1.80 – 2.20 mL, respectively for x_1, x_2, x_3 and x_4. The x_1x_2, x_1x_3 and x_1x_4 represent the interaction terms. Polynomial terms x^2_1, x^2_2, x^2_3 and x^2_4 are including investigating the type of model. The considered responses were retention factor (y_1) and peak area (y_2).

RESULTS AND DISCUSSION

Development of optimum mobile phase

To obtain high resolution and reproducible peaks, various mobile phase compositions were experimented. The essential parameters were found optimum with use of toluene - methanol (8:2 *v/v*) as mobile phase. The wavelength of 230 nm was selected to be optimal for the highest sensitivity. A sharp and well resolved peak was obtained for APL at R*f* of 0.64 ± 0.05 when the chamber was saturated with mobile phase for 25 min at room temperature.

Linearity and calibration curve

The calibration curve constructed was assessed by its correlation coefficient. A fixed volume in the range of 0.5 - 3 mL was transferred from stock solution into series of 10 mL volumetric flasks and volumes were adjusted up to mark with methanol. From each volumetric flask, 5 µL of solution was applied on HPTLC plate to get concentration in the range of 250 – 1500 ng/band. After evaporation of solvents at room temperature for 25 min, chromatography was performed as described above. The linearity of APL was shown in (Figure 2). Calibration curve was developed by plotting Peak-area against drug quantity per band. Calibration equations were determined by use of linear regression analysis and correlation coefficients (r^2) were calculated. No significant difference was observed in the slope of standard curve. The HPTLC chromatogram of standard for APL was shown in (Figure 3).

METHOD VALIDATION

Precision and Accuracy

Repeatability, Intra-day and inter-day precisions were perceived using six repetitive measurements in target concentration level. The precision of developed method was evaluated in terms of % RSD.

For Repeatability, intra-day and inter-day precision % RSD values were found to be in range of 1.35, 0.19 – 0.29 and 0.28 - 0.85, respectively. Results for the precision studies are represented in (Table 1).

Accuracy study was executed by standard addition method using three different levels. Recovery experiment was evaluated by over spotting the drug standard at 80 %, 100 % and 120 % to the pre- analyzed sample and the results were re-analyzed by proposed HPTLC method; shown in (Table 2).

Detection limit (DL) and Quantification limit (QL)

The determination of DL and QL was based on the standard deviations of the responses and slopes of constructed calibration curves (n = 3) as described by ICH guidelines Q2 (R1). The DL and QL values found were 0.35 ng and 1.068 ng, respectively.

Robustness and design analysis for robustness

A Box-Behnken Design (BBD) comprising a total twenty-nine experiment runs obtained from the design matrix were subjected to experiment in order to generate the response variables (y_1 and y_2) shows in (Table 3). All experimental runs were performed in randomized order to minimize the effects of uncontrolled factors that may introduce biased responses. Rather than analysis of single coefficient whole model equation was used and for response surface analysis; crucial focus was given to factors whose responses are with or without significance and are considered too.

$$Y_1 = 0.65 + 0.083x_1 + 0.022x_2 + 5.83E\text{-}003x_3 + 5.83E\text{-}0.003x_4 + 0.013x_1x_2 - 5.00E\text{-}0030x_3 - 7.500E\text{-}003x_1x_4 + 7.500E\text{-}003x_2x_3 + 0.00x_2x_4 + 0.00x_3x_4 - 5.583E\text{-}003x^2_1 + 4.417E\text{-}003x^2_2 - 4.333E\text{-}003x^2_3 + 0.011x^2_4 \quad (2)$$

$$Y_2 = 8706.44 + 498.05\,x_1 - 38.15\,x_2 - 43.99x_3 + 97.04\,x_4 + 7.86\,x_1x_2 + 223.95\,x_1x_3 - 294.55\,x_1x_4 + 64.10\,x_2x_3 - 8980\,x_2x_4 - 34.43\,x_3x_4 + 163.02\,x^2_1 - 16.63\,x^2_2 - 173.55\,x^2_3 - 20.75\,x^2_4 \quad (3)$$

Table 1: Precision studies for Apremilast.

Drug Conc. (ng/band)	Intra-day		Inter-day	
	% Amount found (ng/band)	% RSD	% Amount found (ng/band)	% RSD
500	100.12	0.19	101.23	0.28
1000	98.94	0.38	99.45	0.85
1250	99.00	0.29	100.23	0.32

n- number of determinations

Table 2: Recovery studies.

Initial Amount	Amount of drug added (%)	Amount recovered ± SD (ng/band) n=3	% Recovery	% RSD
500	80	907.07 ± 1.82	101.76	0.44
500	100	1000.60 ± 0.33	100.12	0.66
500	120	1111.01 ± 8.34	101.83	1.36

n- number of determinations

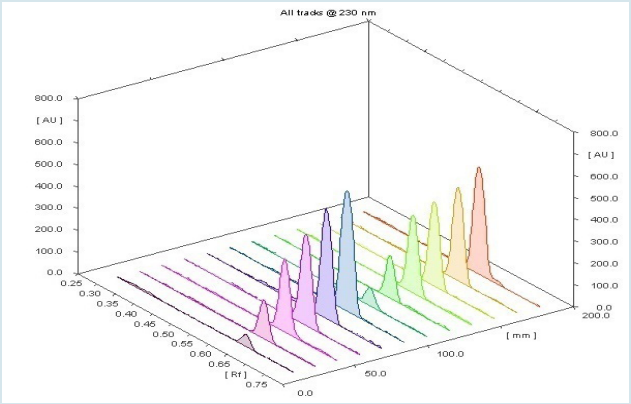

Figure 2: Linearity curve of Apremilast.

Table 4: The estimates of BBD regression analysis and statistical parameters of ANOVA for robustness determination of APL.

Statistical Parameters	Y_1 Retention factor	Y_2 Peak Area
Coefficient of regression (r^2)	0.8301	0.7578
Adjusted coefficient of regression (r^2 adj.)	0.6603	0.5155
Standard deviation (SD)	0.037	309.26
% Coefficient of variation (% CV)	5.62	3.56
Degree of freedom (DF)	14	14
Sum of squares (SS)	0.092	4.189
Mean of square (MS)	6.600E-003	2.992E + 005
Fischer's ratio (F-ratio)	4.89	3.13
P-value	0.0027	0.0205

Table 5: Summary of Regression, validation and laboratory mixture assay parameters for NP-HPTLC

Validation Parameters	Results
Regression coefficient	0.998
Slope	8.996
Intercept	460.3
Linearity range (ng/band)	250 – 1500
Intra-day precision (n= 3, RSD, %)	0.19 - 0.29
Inter-day precision (n= 3, RSD, %)	0.28 – 0.85
Repeatability (n= 3, RSD, %)	1.35
Accuracy	100.23 %
DL	0.35 ng
QL	1.068 ng
Ruggedness	
Analysts I (n= 3, RSD, %)	98.80, 1.49
Analysts II (n= 3, RSD, %)	100.52, 1.72
Robustness	Robust
Specificity	Specific
in-house tablet assay	99.13 ± 0.75

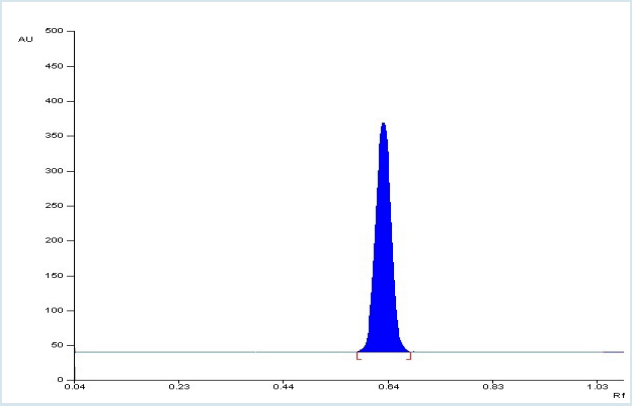

Figure 3: Standard of APL.

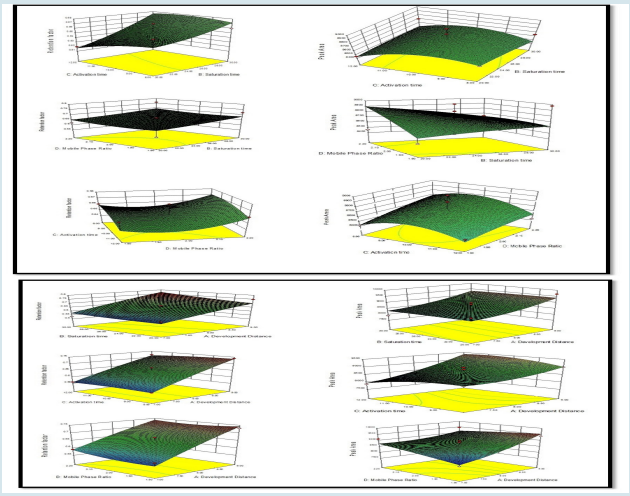

Figure 4: 3D response surface plots for impact of mobile ratio, development distance, activation time and saturation time on retention factor and peak area.

Table 3: Box-Behnken Design BBD consisting of twenty-nine experiment runs.

Runs	Development Distance	Saturation Time	Activation Time	Mobile Phase Ratio	Retention Factor	Peak area
1	9	25	12	2	0.72	9297
2	8	30	12	2	0.65	8698.2
3	9	25	10	1.8	0.73	9356
4	7	25	10	2.2	0.59	9124.2
5	8	25	8	2.2	0.66	8534.2
6	7	20	10	2	0.58	8626.45
7	8	25	10	2	0.64	8426.2
8	8	20	8	2	0.66	8656.2
9	8	25	12	2.2	0.66	8425.5
10	7	25	12	2	0.56	7859
11	7	25	10	1.8	0.58	7845
12	9	25	10	2.2	0.71	9457
13	8	25	10	2	0.65	8642
14	8	25	12	1.8	0.66	8452
15	8	25	10	2	0.65	8845
16	8	25	8	1.8	0.66	8423
17	7	30	10	2	0.53	7896
18	8	30	8	2	0.66	8645
19	8	25	10	2	0.67	8645
20	8	30	10	1.8	0.72	8975
21	8	25	10	2	0.65	8974
22	9	30	10	2	0.75	8988
23	7	25	8	2	0.56	8456
24	8	20	10	2.2	0.62	8456
25	8	20	10	1.8	0.58	8426.6
26	8	20	12	2	0.62	8453
27	9	20	10	2	0.75	9687
28	9	25	8	2	0.74	8998.2
29	8	30	10	2.2	0.76	8645.2

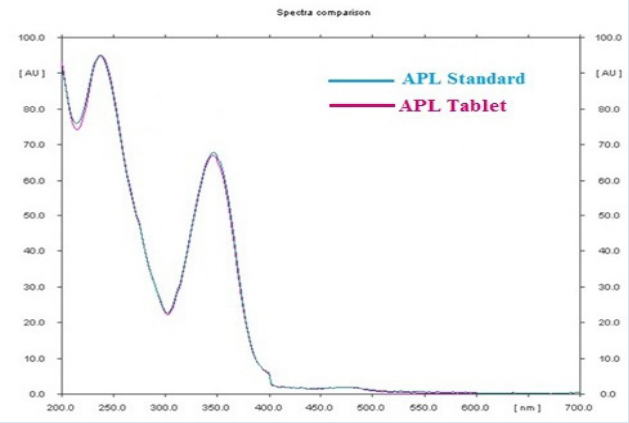

Figure 5: Peak - purity spectrum of Apremilast.

Table 4: The estimates of BBD regression analysis and statistical parameters of ANOVA for robustness determination of APL.

Statistical Parameters	Y_1 Retention factor	Y_2 Peak Area
Coefficient of regression (r^2)	0.8301	0.7578
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Standard deviation (± SD)	0.037	309.26
Coefficient of variations (C.V.%)	5.62	3.56
Degree of freedom (DF)	14	14
Sum of squares (SS)	0.092	4.189
Mean of squares (MS)	6.600E-003	2.992E + 005
Fischer's-ratio	4.89	3.13
P-value	0.0027	0.0205

Evaluation of experimental fault and quantity of validity of polynomial models (lack of fit) were obtained through replication of experimental points (optimized level of variables). ANOVA was applied to obtain regression lack of fit models and the models were provided with adequate representation of data. Considering the degrees of freedom, it was indicated that the data is well fitted to regression models as depicted in (Table 4). The values of coefficients from the polynomial models (Eqs. (2) And (3)) and their signs indicates that, x_1 (development distance) has negative effect on the responses y_1 (retention factor) and y_2 (peak area) while x_2 (saturation time) x_3 (activation time) and x_4 (mobile phase ratio) has positive effect on the retention factor and peak area. Response surfaces from DoE showed that quadratic model suggested for the entire four variable x_1, x_2, x_3 and x_4 and depicted least influence of the saturation time, activation time and mobile phase ratio. Whereas development distances show the highest influence on peak area and retention time, respectively. When the saturation time, activation time and mobile phase ratio was kept constant and increases in the development distance to increases in the peak area (y_2) and retention time (y_1).

The 3D response surface plots (Figure 4) - (a - a_1, b – b_1, c – c_1, d – d_1, e - e_1 and f - f_1) shows the impact of mobile ratio, development distance, activation time and saturation time on retention factor and peak area.

Ruggedness

Ruggedness of HPTLC method was performed at a concentration of 1000 ng/band. Methods were found to be rugged when analysis was performed by two different analysts under the same experimental and environmental conditions.

Assay of *in-house* tablets of APL

A distinct peak at R*f* of 0.64 ± 0.05 was observed in the chromatogram for APL throughout HPTLC analysis. There was no obstruction observed from the excipients used in the in-house tablets of APL. The drug content ± SD found for HPTLC analysis was found to 99.13 ± 0.75.

Specificity

A typical absorption spectrum of APL was shown in (Figure 5) the peak - purity of APL was hardened by correlating the spectra of APL added to laboratory at the peak-start (S), peak - apex (A) and at the peak - end (E) positions. Correlation between these spectra indicated purity of APL peak {correlation r (S, M) = 0.9995, r (M, E) = 0.9806}.

The summary of regression, validation and laboratory mixture assay parameters is represented in (Table 5), for developed NP-HPTLC analysis.

CONCLUSION

The method was successfully developed and robustness determination through DOE. Application of Box-Behnken design was used for evaluation of robustness of the method exhibited slight changes in different factors such as development distance, saturation time, activation time and mobile phase ratio had a exactly effect on a peak area and retention factor. Accordingly, specific consideration required for stringent control of this factors during the analysis of APL in chromatography. Validated method was simple, precise and rugged. Further, the method is found to be accurate and sensitive. The developed method can be used for regular analysis of APL in bulk and in pharmaceutical formulation.

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CONFLICT OF INTEREST

Authors has no conflict of interest

ABBREVIATIONS USED

APL: Apremilast; **DoE:** Design of Experiment; **HPTLC:** High - Performance Thin - Layer Chromatography; **BBD:** Box - Behnken Design.

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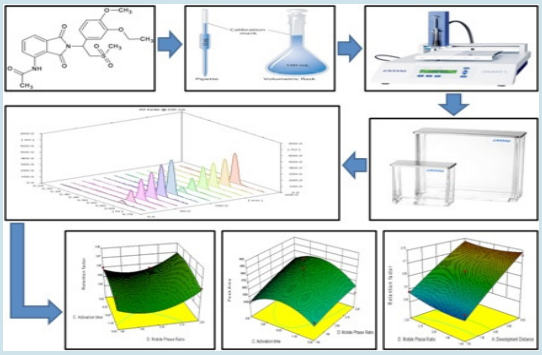
PICTORIAL ABSTRACT



SUMMARY

- Apremilast is immunomodulating agent which is used for management of refractory psoriatic arthritis.
- Simple, robust, specific and rapid HPTLC method developed for has been established for the determination of Apremilast in bulk and *in-house* tablets.
- Design of experiment (DoE) successfully applied for determination of robustness studies.
- The methods were validated as per International Conference on Harmonization (ICH) guidelines.

ABOUT AUTHORS

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