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RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF TETRAHYDROCURCUMIN USING MULTILEVEL FULL-FACTORIAL DESIGN IN BULK, NANOEMULSION AND LIPOSOMES

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ABSTRACT

In this paper, an application of Design of Experiment (DoE) concept for the development of a simple yet precise reverse phase HPLC method for analysis of tetrahydrocurcumin in bulk drug and dermal formulations including nanoemulsion and liposomes is described. The method optimization was accomplished by applying multi-level full-factorial design model using Design Expert® (Version 10) software. 3 fators (F1, F2 and F3) and 2 responses (R1 and R2) were used viz mobile phase ratio of acetonitrile and methanol, flow rate, % v/v acetic acid, Retenton time and number of theoretical plates respectively to obtain the right combination that gave single sharp peak. The optimized HPLC conditions (acetonitrile and methanol in the ratio of 53:47, 0.26 % v/v with glacial acetic acid, at a flow rate of 0.6 ml/min and temperature maintained at 25˚C ± 0.5˚C gave single sharp peak at 4.5 minutes. The developed method was validated using ICH guidelines (for linearity, precision, robustness, Limit of quantification and limit of detection) and then analysed using ANOVA and 3-dimensional plots. Values of adequate precision 21.614 indicated that the model could be used to navigate the design space. "Prob>5" value less than 0.0500 indicated that the model terms were significant. It was concluded that the proposed method was simple, sensitive, and highly robust for routine analysis of bulk drug and other dermal formulations of tetrahydrocurcumin.

KEYWORDS: Tetrahydrocurcumin, RP-HPLC, Method development, Validation, Full factorial design.

1. INTRODUCTION

Tetrahydrocurcumin is 1,7-bis (4-hydroxy-3-methoxyphenyl) heptane-3,5-dione (Fig. 1), a colorless hydrogenated product derived from the yellow curcuminoids, (the biologically active principles from the rhizomes of *Curcuma longa*). Tetrahydrocurcumin (THC) is an anti-inflammatory, anti-fungal, anti-oxidant as well as a skin lightening agent. Tetrahydrocurcumin inhibits the cyclooxygenase-2 (COX-2) in lipopolysaccharide to exhibit anti-inflammatory action. Structurally, both curcumin and THC have diketone structures and phenolic groups, but THC lacks the double bonds.[1-5] THC inhibits the growth of dermatophytes *Trichophytonrubrum, Microsporum gypseum* and *Epidermophyton flocossum.* The free radical scavenging ability of THC, evaluated by using the DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging method, gave IC_{50} value of 1.3 µg/ml.[6,7] Curcuminoids act by inducing antioxidant enzymes (glutathione peroxide, glutathione *S*-transferase and NADPH:quinone reductase). In a study, it was found that THC was found to be more active than curcumin and scavenged Fe-NTA-induced free radicals more effectively than curcumin *in vitro*.[8] At 50µg/ml, it exhibits maximum radical scavenging activity (74.35% inhibition) among different concentrations. The diketone moiety in the heptane side chain of THC, undergoes keto-enol tautomerism and donate electrons to quench the DPPH$^{\cdot}$ radical. This could possibly be the reason for its antioxidant activity.[9] THC efficiently inhibits tyrosinase, the rate limiting enzyme in melanogenesis. In a patent issued by Sabinsa Corporation®, the IC_{50} (µg/ml) value for tyrosinase and melanin inhibition was 1.8 and 3.2 respectively, which is significantly lesser than kojic acid and arbutin (well known skin lightening agents), thus indicating higher activity.[4,6] THC is available in USA in the form of gel, serum and mask for cosmetic purpose. Thus aim of the present study was to establish an easy yet robust HPLC method for quantification of tetrahydrocurcumin from bulk drug, liposomes and nanoemulsion. Though a method has been reported before, it stands for plasma and urine estimation, whereas we have applied the concept of Design of experiment and that too for dermal pharmaceutical formulations as tetrahydrocurcumin is a very effective anti-oxidant and skin-lightening agent.[11] With this background the work was undertaken to

develop an analytical method for quantitative determination of the same from dermal formulations, using design of experiment approach, which has not been reported before.

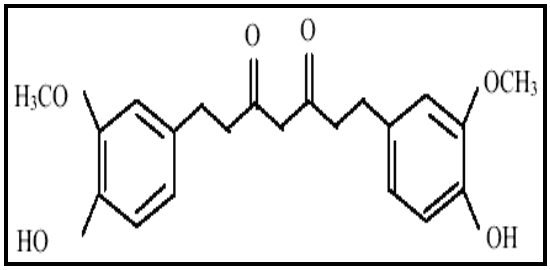

Fig. 1: Chemical structure of tetrahydrocurcumin.

2. MATERIALS AND METHODS

2.1. Reagents

Tetrahydrocurcumin (THC) was received as a gift sample from Sami Labs, Bengaluru, Karnataka, India. Methanol, acetonitrile and acetic acid of HPLC grade were purchased from S.D. Fine Chemicals Ltd., Mumbai, India.

2.2. Chromatographic conditions

The analysis of tetrahydrocurcumin (THC) was carried out on Agilent 1260 Infinity HPLC instrument (Agilent Technologies, India) using *EZchrome Elite* software. C_{18} Qualisil BDS reverse phase analytical column (5 µm, 4.6 mm x 250 mm) was used. The column temperature was maintained at 25˚C ± 0.5˚C. Injection volume was 20 µl with the flow rate of 0.6 ml/min. The detection by UV was at λ_{max} of 280 nm. The mobile phase consisted of acetonitrile and methanol in the ratio of 53:47, 0.26 % v/v with glacial acetic acid, at a flow rate of 0.6 ml/min and temperature was maintained at 25˚C ± 0.5˚C. The calculations of quantitative analysis were performed with external standardization by measurement of peak areas.

2.3. Preparation of sample

2.3.1. Determination of detection wavelength for tetrahydrocurcumin API

Appropriate dilutions of the standard drug solutions were prepared for 10 µg/ml of tetrahydrocurcumin API. Stock solution of tetrahydrocurcumin (10µg/ml) in methanol was used. Solution was scanned using double beam UV-VIS spectrophotometer between the range of 200 to 400 nm, λ_{max} being observed at 280 nm.

2.3.2. Estimation of bulk drug

Stock solution of pure tetrahydrocucumin drug was prepared in HPLC grade methanol in a concentration of 0.01% w/vol. This stock solution was sonicated prior to use. The stock solution was further diluted with the mobile phase to form a concentration of 0.2µg/ml. This solution (20µl) was injected into HPLC to obtain area under the curve (AUC).

2.3.3. Nanoemulsion

The nanoemulsion of tetrahydrocurcumin was developed. For its sample preparation, weighed amount of the nanoemulsion was dissolved in HPLC grade methanol in a concentration of 25% v/v. This mixture was vigorously shaken using a vortex for 5 min and further subjected to centrifugation for 20 min at 1000 rpm. The supernatant was separated and further diluted using the mobile phase to get 44 µg/ml of the concentration. This solution (20µl) was then injected into the column to obtain area under the curve (AUC).

2.3.4. Liposome

Liposomes, equivalent to 60 µg of the drug were dissolved into 10 ml of methanol and kept for stirring for 20 min. 1 ml of the above solution was diluted up to 3 ml by using the mobile phase. It was further centrifuged at 4000 rpm for 40 min. The supernatant (20 µl) was injected into the column.

2.4. Method development by Design of Experiment (DoE) approach

Step 1: Intention of method

The HPLC method development was performed using the approach of Design of experiment (DoE), as it is a knowledge rich, scientific, risk based and holistic and approach that begins with predefined objective which is to separate and quantify the API and emphasizes on the product and process understanding.

Step 2: Performing experimental design

Quality by design is needed to assist with obtaining in-depth method understanding and performing optimization. Here, an efficient and comprehensive design of experiment based on systematic assortment of flow rate, concentration of acetic acid in mobile phase and ratio of different solvent as mobile phase is presented. It forms a chromatographic database, which assists method understanding, optimization, and selection. The scouting of the above three parameters are shown below. The experimental design comprised of different percentages of (0.13% and 0.26%) of acetic acid, flow rates (0.6 ml/min, 0.8 ml/min and 1.0 ml/min) of mobile phases with different ratios of methanol and acetonitrile (as shown in Table 1). After feeding these factors and variables in multi-level, full-factorial option in *Design Expert*® Version 10, it led to a total of 42 method conditions (2 acetic acid percentages x 3 flow rates x 7 mobile phases) or runs. For each combination, a 10 minutes run time was prefixed. The parameters are tabulated in Table 1.

Table 1: Scouting of HPLC parameters

PARAMETERS	DESCRIPTION OF PARAMETERS		
Concentration of acetic acid (% v/v)	0.13	0.26	
Ratio of Actonitrile:Methanol in mobile phase	70:30	40:60	50:50
	60:40	47:53	
	45:55	53:47	
Flow rate (ml/min)	0.6	0.8	1.0

Step 3: Validation of the method

Linearity

A stock solution of tetrahydrocurcumin 100 µg/ml concentration was prepared. This stock solution was sonicated prior to use. Appropriate amounts of these stock solutions were diluted yielding concentrations ranging from 0.5 to 3µg/ml using the mobile phase. Every concentration was injected into the column and peaks were obtained with area under the curve (AUC). The standard curve was plotted by using this AUC verses the concentration of tetrahydrocurcumin. Triplicate injections were made for each concentration. The linearity of the calibration curves [peak area (*y*) v/s concentration (*x*)] was determined for intra- and inter-day precision on 3 different days.

Selectivity and specificity

The selectivity of the developed analytical method was determined from the chromatogram obtained with mobile phase consisting of acetonitrile and methanol in the ratio of 53:47, contains 0.26% glacial acetic acid, at a flow rate of 0.6 ml/min and temperature of 25˚C ± 0.5˚C, which showed that tetrahydrocurcumin was completely detected and the method was selective. The specificity of developed analytical method was determined from the observation of no other peaks at the retention time of the drug.

Precision

The precision of the developed analytical method was determined by analyzing standard drug solution in a set of three replicate. The area under curve was recorded for each concentration and standard deviation (SD) and percent relative standard deviation (% RSD) were calculated. Precision is considered as three levels: repeatability (intra-day), intermediate precision (inter-day) and reproducibility.

a) Repeatability studies: Repeatability is the precision under the same operating conditions within a short period of time. The study was carried out by measuring the AUC for 4 solutions of different concentrations, three times in a day.

b) Intermediate Precision: Intraday study was carried out by measuring the AUC for 4 solutions of different concentrations, three times in a day.

Standard deviation (S.D.) was calculated by using the formula:

$$S.D. = \left[\frac{\Sigma(X - x)}{n - 1}\right]^{1/2}$$

where, X is individual measurement in a set

x is arithematic mean of the set of values

n is the total number of replicated measurements taken in a set

Per cent relative standard deviation was calculated using the following formula:

$$\% RSD = \frac{S.D.}{Mean} \times 100$$

Limit of quantification and limit of detection

The limit of quantification (LOQ) (taken as the lowest concentration of an analyte in a sample, which can be determined with acceptable precision and accuracy) and the limit of detection (LOD) (taken as the lowest absolute concentration of an analyte in a sample, which can be detected but not necessarily quantified) were calculated based on the ICH guidelines.

$$\textbf{LOQ} = \frac{3.3\sigma}{S} \quad \textbf{LOD} = \frac{10\sigma}{S}$$

where, σ = Standard deviation of the response

S = Slope of calibration of curve

Robustness

Robustness is the capacity of the method to remain unaffected by deliberate variation in the method parameters. The robustness of the developed analytical method was determined by analyzing four different concentrations of drug solution by two different analysts. From the data obtained, the standard deviation and the % RSD were calculated.

3. RESULTS AND DISCUSSION

Method development

The HPLC method of analysis was developed for the analysis of tetrahydrocurcumin and validated as per the ICH guidelines Q2 (R1).[10] All the studies were carried out at same chromatographic conditions. The calibration curve of the drug was developed in methanol. The plot was found to be linear and found to obey Beer-Lambart law. The detection of the peak was at λ_{max} = 280 nm.

The optimum mobile phase was composed of acetonitrile and methanol in the ratio of 53:47 with 0.26 % v/v of glacial acetic acid and flow rate of 0.6 ml/min. With this mobile phase, the best results were obtained in terms of shape of peak, selectivity and retention time for bulk drug, nanoemulsion, gel and liposomal formulations. The retention time for tetrahydrocurcumin was 5.4 min. No interference from the solvent (used for dilution) and dosage form excepients was observed at the detection wavelength (280 nm). The observation and remarks of

method development using *Design of Experiment* approach are shown in Table 2. Table for executing HPLC runs as per *Design of Experiment* with retention time (Rt) and Number of theoretical plates of each are shown in Table 3. The responses were statistically evaluated using the ANOVA procedure as shown in Table 4.

Table 2: Parameters and their values in *Design of Experiment*

PARAMETER	OBSERVATION	REMARKS
Concentration of acetic acid (%v/v)		
0.13	Peak was found to be asymmetrical	Not satisfactory
0.26	Sharp and symmetrical peak was obtained	Satisfactory
Mobile phase ratios (Actonitrile:Methanol)		
70:30, 60:40, 47:53, 45:55, 50:50	Two peaks were observed	Not satisfactory
53:47	Sharp peak was obtained	Satisfactory
Flow rate (ml/min)		
0.6	Sharp and single peak was obtained at Rt 5.4 min	Satisfactory
0.8	Rt was very less	Not satisfactory
1.0	Rt was very less and unacceptable	Not satisfactory

Table 3: Chromatographic conditions/run as per Design of experiment

Runs	Factor 1 (F1): Methanol:Acetonitrile (%)	Factor 2 (F2): Flow rate (ml/min)	Factor 3 (F3): Acetic acid (% v/v)	Response 1 (R1):Rt (min)	Response 2 (R2): Number of theoretical plates (NTP)
1	70:30	0.6	0.26	5.201	18985
2	70:30	1.0	0.26	2.79	24456
3	70:30	1.0	0.13	2.73	24905
4	70:30	0.6	0.13	5.1664	26744
5	70:30	0.8	0.26	3.4175	19898
6	70:30	0.8	0.13	3.4033	27197
7	60:40	0.6	0.13	5.42	14156
8	60:40	1.0	0.26	4.57	28452
9	60:40	0.8	0.26	5.29	26516
10	60:40	0.6	0.26	6.353	14196
11	60:40	0.8	0.13	5.34	15489
12	60:40	1.0	0.13	4.58	19987
13	45:55	1.0	0.13	2.70677	22502
14	45:55	0.8	0.26	3.37	18625
15	45:55	1.0	0.26	2.71245	23458
16	45:55	0.8	0.13	3.36333	17051
17	45:55	0.6	0.13	6.359	26547
18	45:55	0.6	0.26	4.48	18675
19	40:60	1.0	0.13	2.72	15247
20	40:60	0.6	0.13	4.51333	17514
21	40:60	0.6	0.26	4.47667	17552
22	40:60	0.8	0.13	3.39	16213
23	40:60	1.0	0.26	2.73667	18174
24	40:60	0.8	0.26	3.40667	21863
25	47:53	0.6	0.13	4.3033	24307
26	47:53	0.8	0.13	3.38333	22459
27	47:53	0.6	0.26	4.507	14547
28	47:53	1.0	0.13	2.71333	26031
29	47:53	0.8	0.26	3.38	15429
30	47:53	1.0	0.26	2.726	12793
31	50:50	1.0	0.13	2.72	18962
32	50:50	0.8	0.26	3.38	17059
33	50:50	0.6	0.13	4.502	18498
34	50:50	0.8	0.13	3.383	16484
35	50:50	1.0	0.26	2.7133	23175
36	50:50	0.6	0.26	4.49667	18802
37	53:47	0.8	0.13	3.37	14243
38	53:47	0.6	0.13	4.5	19898
39	53:47	0.6	0.26	4.49	21793
40	53:47	1.0	0.13	2.71	20189
41	53:47	0.8	0.26	3.44333	21694
42	53:47	1.0	0.26	2.70333	20405

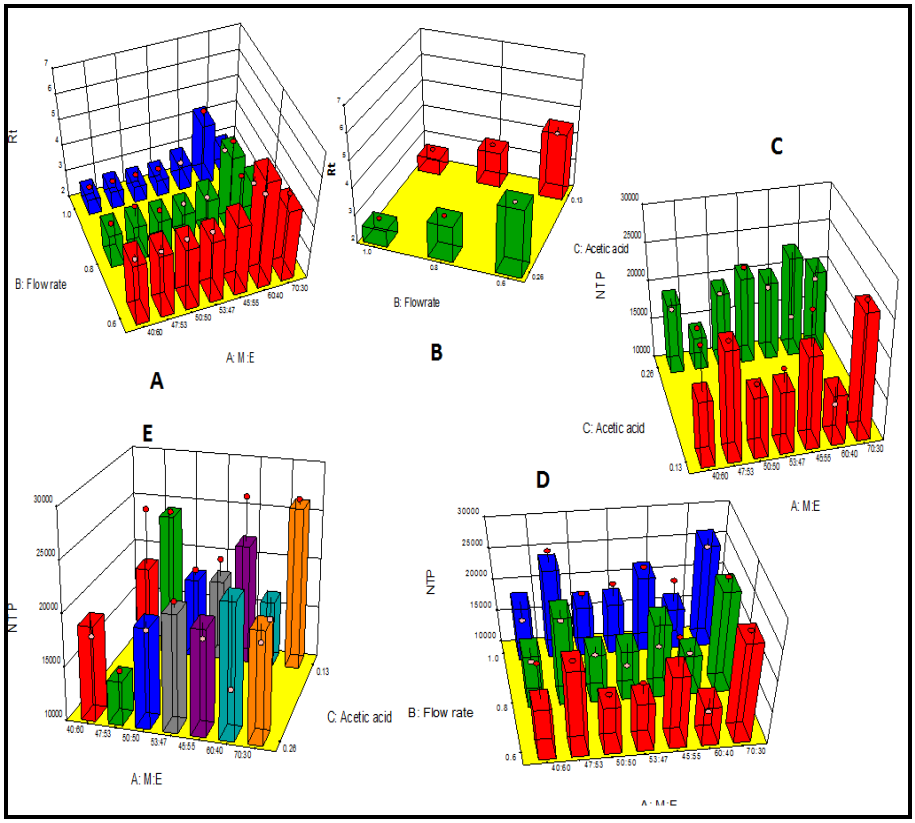

Figure 2: 3-D graphical representation of responses with respect to various combinations of parameters. *Effect on retention time (Rt)* A: flow rate (ml/min) v/s Methanol:Acetonitrile ratio; B: flow rate 9 ml/min) v/s acetic acid (%v/v). *Effect on number of theoretical plates.* C: Validation of the method: acetic acid (%v/v) v/s Methanol:Acetonitrile ratio; D: Methanol:Acetonitrile ratio v/s acetic acid (%v/v); E:Flow rate (ml/min) v/s Methanol:Acetonitrile ratio.

Table 4: Summary analysis of ANOVA results of tetrahydrocurcumin.

ANOVA parameters	R1:Rt	R2:NTP
DF	8	13
F value	38.61269	2.445394
Prob>F	< 0.0001	0.0231
Predicted R^2 value	0.843802	0.05369
Adjusted R^2 value	0.880082	0.314268
Adequate precision	21.61363	5.983571

Analysis of ANOVA parameters for Retention time and Number of theoretical plates

Retention Time: The Model F- value of 38.61 implied that the model was significant and there was only 0.01% chance that such a large value of 38.61 could have occurred due to noise. Values of "Prob > F" less than 0.0500 indicated that the model terms were significant. In this case A and B were significant model terms. The "Predicted value of R^2" of 0.8438 was in reasonable agreement with the "Adjusted R^2" of 0.8801. "Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 21.614 indicated an adequate signal. All these results showed that this model can be used to navigate the design space.

Number of theoretical plates: The Model F-value of 2.45 implies that the model is significant. There is only a 2.31% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicated that the model terms are significant. A negative "Predicted R^2" implies that the overall mean is a better predictor of "Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 5.984 indicated an adequate signal. Hence this model can be used to navigate the design space.

Validation of the method

Linearity

The values of the area under the curve versus the concentration are shown in Table 4 and plotted in Figure 2.

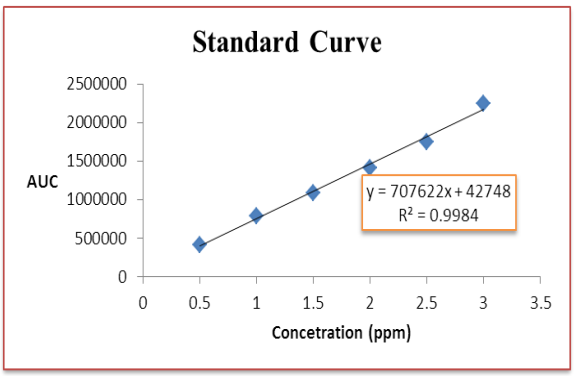

Figure 2: Standard curve of tetrahydrocurcumin.

Table 4: Values obtained for linearity range for tetrahydrocurcumin

Sr. No.	Concentration (µg/ml)	Area under curve
1	0.5	410050
2	1	789374
3	1.5	1083623
4	2	1406922
5	2.5	1752152
6	3	2244401

The linearity curve constructed for THC by plotting concentration versus peak area was found to be linear in the range of 0.5-3 µg/ml as the regression coefficient was found to be > 0.99. The slope was found to be 707622 with y intercept of 42748 as shown in Figure 3 and Table 4.

Precision: Precision studies were performed in methanol. The standard deviation and % RSD were found to be less than 2%. Inter-day and intra-day studies were carried out to study the variation in the developed method for 3 days. The results demonstrated that the developed method showed very low variation in the inter-day and intra-day studies. Thus it can be concluded that the drug was stable in methanol. The results are shown in Tables 6 and 7.

The values of Inter-day and Intra-day repeatability are shown in Table 6 and 7.

Table 6: Inter-day repeatability studies

Conc (µg/ml)	AUC	Conc (µg/ml)	AUC	Conc (µg/ml)	AUC	Conc (µg/ml)	Mean Conc	S. D. (±)	%RSD
	Day 1		Day 2		Day 3				
1	783947	1.0551	789374	1.0333	773947	1.0474	1.04529	0.0110	1.05798
1.5	1188326	1.4709	1083623	1.4310	1138321	1.4185	1.44018	0.0273	1.90031
2	1460792	1.9017	1406922	1.8979	1370992	1.9339	1.91114	0.0198	1.03916
2.5	1799527	2.4157	1752152	2.5022	1825125	2.4426	2.4535	0.0443	1.80553

Table 7: Intra-day repeatability studies

Conc (µg/ml)	AUC	Conc (µg/ml)	AUC	Conc (µg/ml)	AUC	Conc (µg/ml)	Mean Conc	S. D. (±)	%RSD
	Morning		Afternoon		Evening				
1	789374	1.0695	773947	1.0397	783947	1.0496	1.0529	0.01516	1.44009
1.5	1083623	1.6290	1138321	1.6419	1188326	1.6000	1.6236	0.02146	1.32227
2	1406922	1.8695	1370992	1.9437	1460792	1.9159	1.9097	0.03752	1.96484
2.5	1752152	2.4810	1825125	2.5598	1799527	2.5040	2.5138	0.04097	1.62982

Limit of quantification and limit of detection: The values for LOD and LOQ are show below in Table 8.

Table 8: Limit of detection (LOD) and limit of quantification (LOQ) of tetrahydrocurcumin.

S. No.	Parameter	Concentration (µg/ml)
1	LOD	0.280
2	LOQ	0.850

Robustness

The robustness study was carried out by analyzing four different concentrations of drug solution by two different analysts. From the data obtained, the standard deviation and the % RSD were calculated. The S.D. and % RSD were found to be less than 2% as shown in Table 9. Hence, the developed and validated analytical method was robust as it meets specific acceptance criteria of ICH Q2 (R1) guidelines.

Thus, the developed method was found to be sensitive, linear, precise reproducible and robust and hence, this method can be used for the intended purpose.

Table 9: Robustness data for analytical method of tetraydrocurcumin

Conc (µg/ml)	AUC	Conc (µg/ml)	AUC	Conc (µg/ml)	Average Conc	S. D. (±)	%RSD
	Analyst 1		Analyst 2				
1	799584	1.06954	778526	1.03971	1.0581	0.02516	1.54009
1.5	1195487	1.62906	1204632	1.64193	1.6673	0.01146	1.12227
2	1365658	1.86508	1418216	1.9432	1.9754	0.02752	1.544844
2.5	1798418	2.48453	1854123	2.55994	2.3877	0.03097	1.149826

4. CONCLUSION

The research work was carried out to develop a RP-HPLC method development using *Design of experiment* approach using Design Expert® Version 10 that has the advantage of being not just accurate, precise and robust but also systematically scouted for various factors and

variables. The intention of the method was explained followed by the scouting of the key parameters including mobile phase, acetic acid concentration and flow rate. *Design of Experiment* was applied to study the interrelationships between the parameters and preliminary optimized conditions were obtained using flow rate, concentration of acetic acid and adjusting the flow rate. ANOVA parameters for retention time and number of theoretical place showed that the values were significant and the model can be used to navigate the design space. All the validated parameters were found within acceptance criteria as per ICH guidelines Q2 (R1). The validated method was found to be specific, linear, precise, accurate, robust and rugged for determination. Hence, the method that can be used to estimate THC levels in bulk drug and various kinds of topical formulations ranging from conventional gels to advanced lipid based vesicular systems, liposomes and nanoemulsion was developed successfully.

5. ACKNOWLEDGEMENTS

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