International Journal of Advanced Research and Review

www.ijarr.in

RP-HPLC METHOD DEVELOPMENT AND VALIDATION USING EXPERIMENTAL DESIGN APPROACH FOR SIMULTANEOUS ESTIMATION OF THIOCOLCHICOSIDE AND DEXKETOPROFEN WITH FORCED DEGRADATION STUDIES

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ABSTRACT

Introduction: In the present work, sensitive RP-HPLC method been developed for the quantitative estimation of Thiocolchicoside (THC) and Dexketoprofen (DKP) combined dosage form.

Material & methods: Determination of THC and DKP was carried on a reverse phase C_{18} (250×4.6mm, 5µ) column using a mobile phase consisting of Methanol: Sodium Phosphate Buffer (70:30 v/v) pH 4.5, Flow rate of 1.0 ml/min and the detection was carried out at 280 nm.

Result: The linearity was found to be in the range of 5-40 µg/ml and 30-240 µg/ml with (r^2=0.9991, and r^2=0.9992) for THC and DKP respectively. The sharp peaks obtained were having clear baseline separation with a retention time of 3.20 min and 8.40 min for THC and DKP respectively.

Discussion: The forced degradation studies performed in acidic, basic, oxidative, photolytic and thermal conditions at different time intervals. The method was validated as per the International Conference on Harmonization (ICH) guidelines as well as for the ROBUSTNESS studies the Quality by Design approach used based on 3-Level Factorial Design of Experiment. On the basis of Designs the three chromatographic parameters (Flow Rate, pH and Mobile phase composition) were changed and study were carried out with effects on Retention time and Peak Area.

Conclusion: The proposed validated method was successfully used for the quantitative analysis of commercially available dosage form.

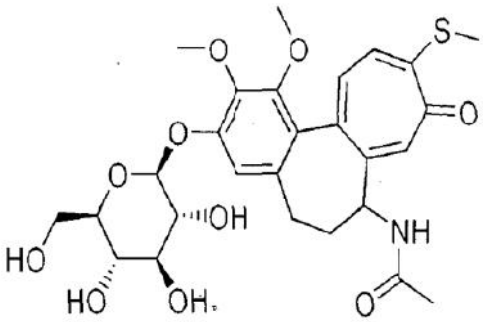
Key words: Thiocolchicoside, Dexketoprofen, QbD based RP-HPLC, Factorial design

INTRODUCTION

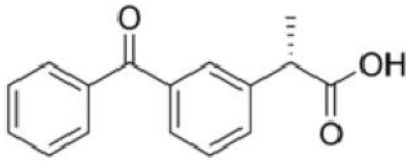
Thiocolchicoside, Chemically *N* - [(7*S*) -3- (beta-D-glucopyranosyloxy) -1, 2- dimethoxy- 10- (methylsulfanyl) -9 -oxo -5,6,7,9 -tetrahydrobenzo [a] heptalen-7-yl] acetamide. Muscle relaxant with anti-inflammatory and analgesic activity. Thiocolchicoside is a 3-demethyl-thiocolchicine glucoside, derivative of colchicines. It acts as a competitive $GABA_A$ receptor antagonist and also

inhibits glycine receptors with similar potency and nicotinic acetylcholine receptors to a much lesser extent. It has powerful convulsant activity. Thiocolchicoside's activity is ascribed to its ability to interact with the strychnine sensitive glycine receptor and therefore being endowed with glycino-mimetic activity and produce myorelaxant effect.

Dexketoprofen, Chemically (2S)-2-[3-(benzoyl)phenyl] propanoic acid. Dexketoprofen belongs to a class of medicines called non-steroidal anti-inflammatory drugs (NSAIDs). It works by blocking the action of a substance in the body called cyclo-oxygenase. Cyclo-oxygenase is involved in the production of chemicals in the body called prostaglandins. Prostaglandins are produced in response to injury or certain diseases and would otherwise go on to cause swelling, inflammation and pain. By blocking cyclo-oxygenase, Dexketoprofen prevents the production of prostaglandins and therefore reduces inflammation and pain. Along with Peripheral analgesic action it possesses central analgesic action.



Thiocolchicoside (THC)



Dexketoprofen (DKP)

Thiocolchicoside 4mg + Dexketoprofen Trometamol 25mg are manufactured by "Emcure pharma" under the brand name **Infen MR**.

Thiocolchicoside is official in IP 2014 and includes HPLC method for estimation of THC. Dexketoprofen Trometamol is not official in any pharmacopoeia. The combination of these two drugs is not official in any pharmacopoeia (Indian pharmacopoeia, 2014). Literature review shows that numbers of analytical methods are available for estimation of both the drugs either alone or in combination with other drugs (Bhusari V. K et al, 2012; Chaudhari B. G. and Trivedi J. B, 2012; Harde M. T, 2012) Based on our current and ongoing referencing work, till date, we have came across to first order derivative spectroscopic method and RP-HPLC method for simultaneous estimation of both the drugs in their combined dosage form. Therefore, the objective is to develop QbD based RP-HPLC method with forced degradation studies for simultaneous estimation of THC and DKP in their formulation and to validate the developed method according to ICH guidelines and for the ROBUSTNESS studies the Quality by Design approach used based on 3-Level Factorial Design of Experiment. On the basis of Designs the three chromatographic parameters (Flow Rate, pH and Mobile phase composition) were changed and study were carried out with effects on Retention time and Peak Area.

MATERIALS AND METHODS

1. Instruments

Chromatographic analysis was carried out on a **LC-2010 CHT** series, Auto injection system, temperature controller (system controller and a UV detector, **LC solution** software was used to acquire and process the data.

2. Reagents and Chemicals

Standard APIs were kindly gifted by Emcure Pharma, Pune and Tablets **Infen MR** were procured from local market. HPLC grade Methanol, Water and other chemicals used in Mobile phase preparation (Rankem, RFCL chemicals Pvt Ltd.)

3. Methodology (RP-HPLC Method)

3.1 Preparation of Dilutions

THC (10 mg) and DKP (10 mg) were accurately weighed and transferred to a 100 ml volumetric flask, dissolved in sufficient quantity of methanol and then diluted to the mark with mobile phase. The stock solution contains 100 µg/ml for both drug. 0.4 ml aliquot from stock solution of THC was transferred to a separate 10 ml volumetric flask and volume was adjusted to the mark with mobile phase to give the final concentration 4 µg/ml for THC. 2.5 ml aliquot from stock solution of DKP was transferred to a separate 10 ml volumetric flask and volume was adjusted to the mark with mobile phase to give the final concentration 25 µg/ml for DKP. The Solutions were filtered through 0.45 µm Nylon 66 (N66) 47 mm membrane filter paper and first few drops of filtrate were discarded. An aliquot of sample was transferred to a separate 10 ml volumetric flask and volume was adjusted to the mark with mobile phase to give the final concentration of 4 µg/ml for THC and 25 µg/ml for DKP.

3.2 Chromatographic conditions

Stationary phase: C_{18} column (250 X 4.6 mm, 5 µm).
Mobile phase: Methanol: Sodium Phosphate Buffer (70:30 v/v) (pH - 4.5)
Flow rate: 1.0 ml/min.
Wavelength: 280 nm.

4. Method validation

i. Linearity and Range (n = 3): The linearity response was determined by analyzing 5 independent levels of calibration curve in the range of 5-40 µg/ml (5, 10, 20, 30 and 40 µg/ml) for THC and 30-240 µg/ml (30, 60, 120, 180 and 240 µg/ml) for DKP. The plot of peak area against concentration was plotted. Correlation coefficient and regression line equations for THC and DKP were calculated.

ii. Accuracy (n = 3)

Accuracy was determined by calculating the % Recovery of THC and DKP from the marketed formulation by the standard addition method in which, known amounts of standards powders of THC and DKP at 80%, 100% and 120% levels were added to the pre-analyzed samples. The recovered amounts of THC and DKP were calculated at each level and % Recovery was reported.

iii. Precision

A) Repeatability (n = 6)

For the repeatability study, 20 µg/ml of THC and 120 µg/ml of DKP were injected into the system. The peak areas of THC and DKP were observed. The procedure was repeated six times and % CV was calculated.

B) Intraday Precision (n = 3)

Three concentrations levels injected into the HPLC system and analyzed three times on the same day and % CV was calculated of 10, 20 and 30 µg/ml for THC and 60, 120 and 180 µg/ml for DKP.

C) Interday Precision (n = 3)

Three concentrations levels injected into the HPLC system and analyzed on three different days and % CV was calculated of 10, 20 and 30 µg/ml for THC and 60, 120 and 180 µg/ml for DKP.

iv. Specificity

In the case of assay, demonstration of specificity is required to show that the procedure is unaffected by the presence of impurities or excipients. Specificity of an analytical method indicates that the analytical method is its able to measure accurately and specifically the analyte of interest without any interference from blank. So here, the specificity was determined by the comparison of the chromatograms of

a) Standard sample solutions of THC and DKP
b) Blank (mobile phase) and
c) Sample solution of THC and DKP

v. LOD and LOQ

The LOD and LOQ were estimated from the set of 5 calibration curves.
They were calculated as,
LOD = 3.3 × (SD/Slope)
LOQ = 10 × (SD/Slope)
Where,
SD = Standard deviation of the Y- intercepts of the 5 calibration curves.
Slope = Mean slope of the 5 calibration curves.

vi. Robustness

2^3 Factorial Design of Experiments consisting of 6 experiments varying different analytical parameters, Flow rate, pH and mobile phase composition used for study. The responses were assessed using statistic software, Plots for different chromatographic parameters as a function of operational variables levels were presented. Finally from the analysis of data using statistical software for optimization of analytical method, hence proved that the method is robust and not affected by change in flow rate, pH and mobile phase composition.

5. Forced Degradation studies

Solution used are: 20 µg/ml of TCC and 120 µg/ml of DKP

(i) Acid induced degradation

Condition: 1M HCl at 60°C

(ii) Base induced degradation

Condition: 1M NaOH at 60°C

(iii) Oxidative-stress induced degradation

Condition: 3% H_2O_2 at 60°C

(iv) Photolytic Degradation:

a. Condition (1): In UV-Light for 30 min.
b. Condition (2): In Sun-Light for 30 min.

(v) Thermal Degradation

Condition: In Hot Air Oven at 60°C for 30 min.

Estimation of Thiocolchicoside and Dexketoprofen in the marketed formulation by the proposed method (n = 5)

Twenty tablets were weighed and finely powdered. The powder equivalent to 4 mg of THC and 25 mg of DKP was weighed accurately and mixed, diluted with methanol (50 ml) in 100 ml volumetric flask, kept in ultrasonic water bath for 10 min to get optimum dissolution of the active ingredients and diluted up to mark with mobile phase (40 µg/ml of THC and 250 µg/ml of DKP). The final solution was filtered using 0.45 µm Nylon 66 (N66) 47 mm membrane filter paper and first few drops of filtrate were discarded. 5 ml of aliquot of this solution was diluted to 10 ml with mobile phase 20 µg/ml of THC and 125 µg/ml of DKP). The peak areas of THC and DKP were obtained from the chromatogram and utilized for estimation the concentration of each drug was calculated using equation of regression line.

RESULTS AND DISCUSSION

The mobile phase used consisted of Methanol: Sodium Phosphate Buffer (70:30 v/v) (pH-4.5). The peaks were well resolved with a resolution factor of 15.20. The estimation was carried out at 280 nm using a UV detector keeping the flow rate of 1.0 ml/min. Results of the validation of the above method indicate that the method was linear in the range of 5-40 µg/ml for THC and 30-240 µg/ml for DKP. The % recoveries for THC and DKP obtained in the accuracy study were 99.58 – 99.90% and 98.87 - 99.93% respectively. The results of the precision study indicate that the proposed method showed good repeatability for THC and DKP with a % CV of 0.29 and 0.24 respectively. The % CV from the intraday precision data were found to be 0.27 - 0.43 for THC and 0.21 - 0.33 for DKP. Similarly % CV from the interday precision data were found to be 0.38 – 0.57 for THC and 0.25 – 0.43 for DKP. The LOD for THC and DKP was found to be 0.0442 µg/ml and 4.0156 µg/ml respectively. Similarly LOQ for THC and DKP was found to be 0.1341 µg/ml and 12.1686 µg/ml respectively. The % assay results of 99.21% for THC and 99.85% for DKP indicate that the developed method was successfully utilized for the estimation of THC and DKP in their combined dosage form.

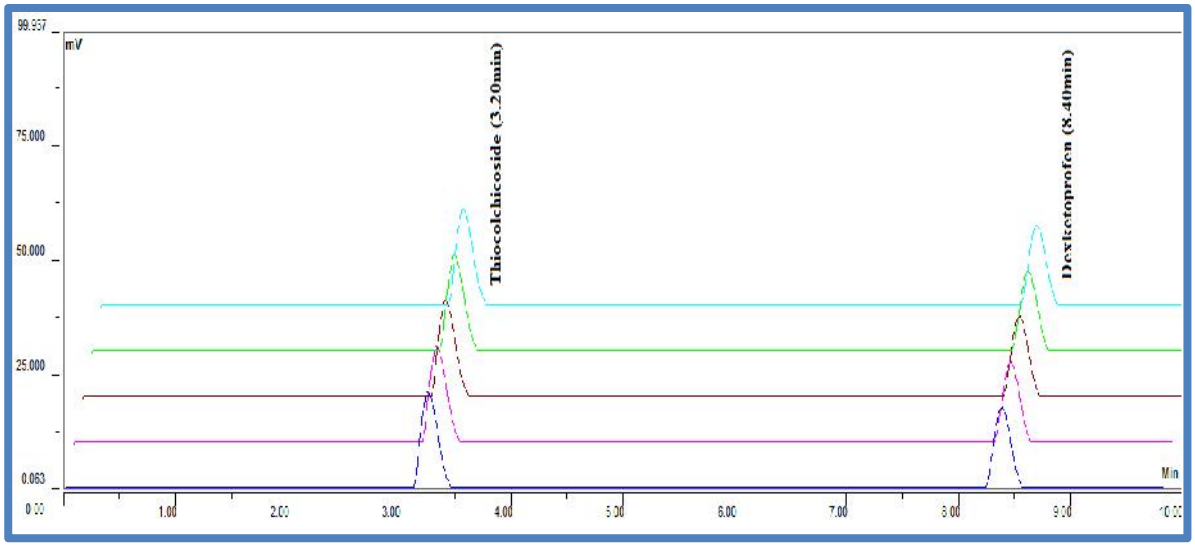


Fig-1: Linearity (5-40 µg/ml for THC and 30-240 µg/ml for DKP)

Table 1: Linearity Data for Thiocolchicoside and Dexketoprofen

Concentration of Thiocolchicoside (µg/ml)	Peak Area	Concentration of Dexketoprofen (µg/ml)	Peak Area
5	52868	30	51880
10	135470	60	163574
20	342825	120	371923
30	555625	180	565537
40	768248	240	754805

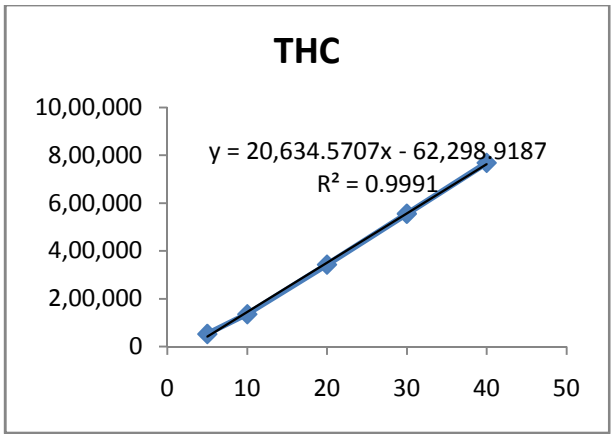


Fig-2: Linearity (5-40 µg/ml for THC)

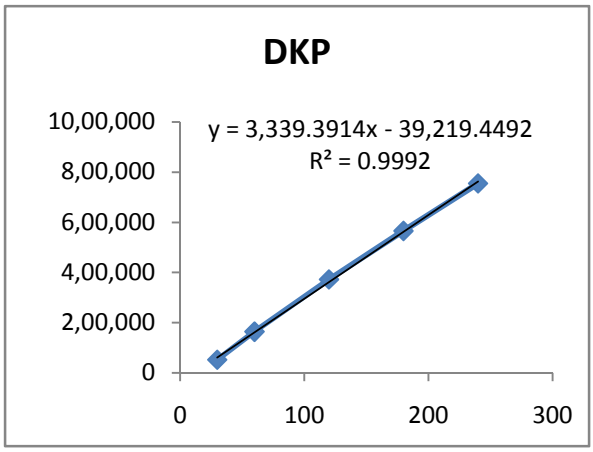
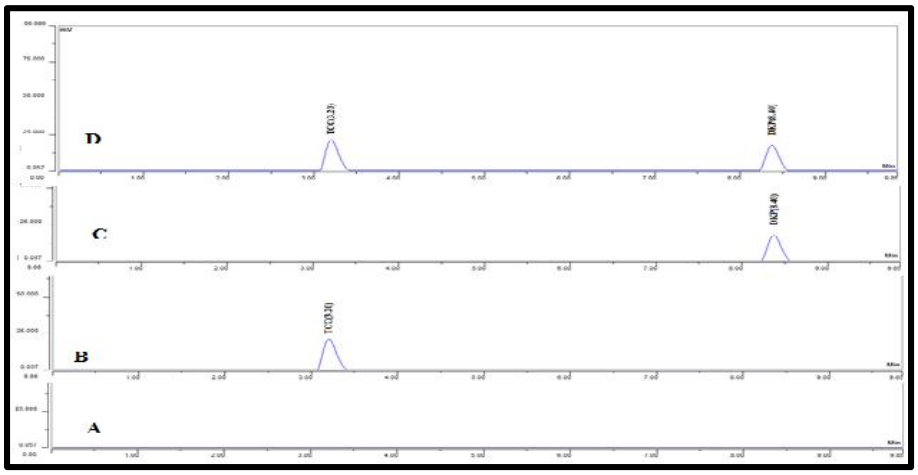


Fig-3: Linearity (30-240 µg/ml for DKP)

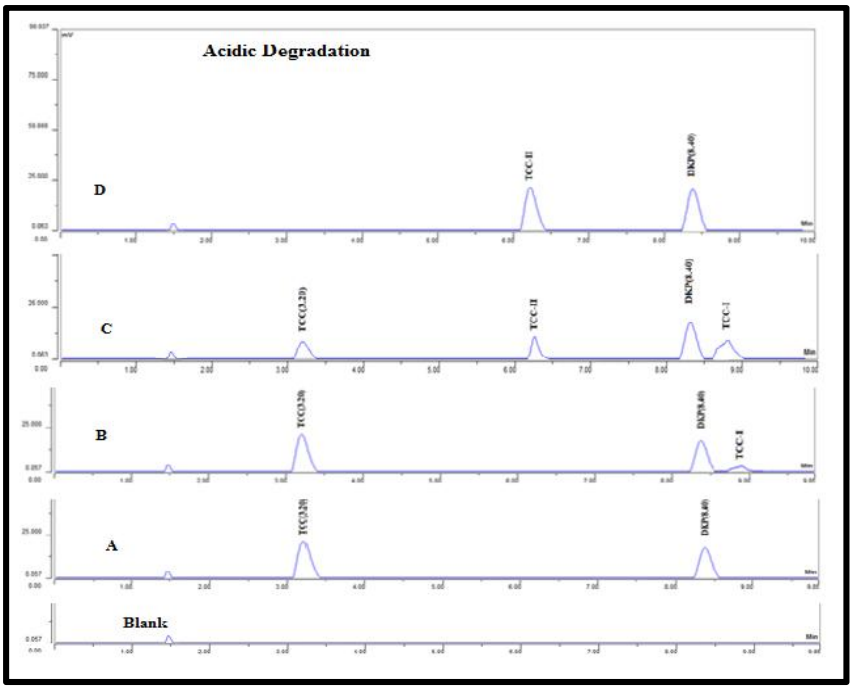
Table 2: System Suitability Parameters For THC and DKP

SR.NO	SYSTEM SUITABILITY PARAMETER	OBSERVED VALUE		IP 2014 SPECIFICATION
		THC	DEX	
1	NUMBER OF THEORITICAL PLATES (N)	2545	8940	> 2000
2	RESOLUTION	15.20		> 2
3	TAILING FACTOR	1.02	1.35	< 2



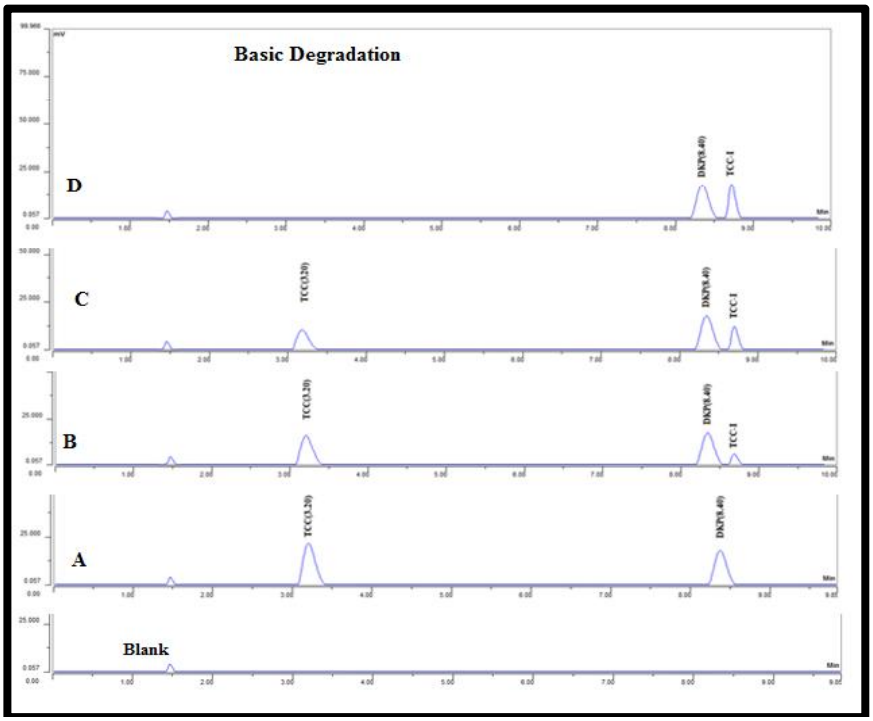
Where, A= blank, B= THC single, C= DKP single, D= in combination

Fig-4: Chromatogram for Untreated sample solution



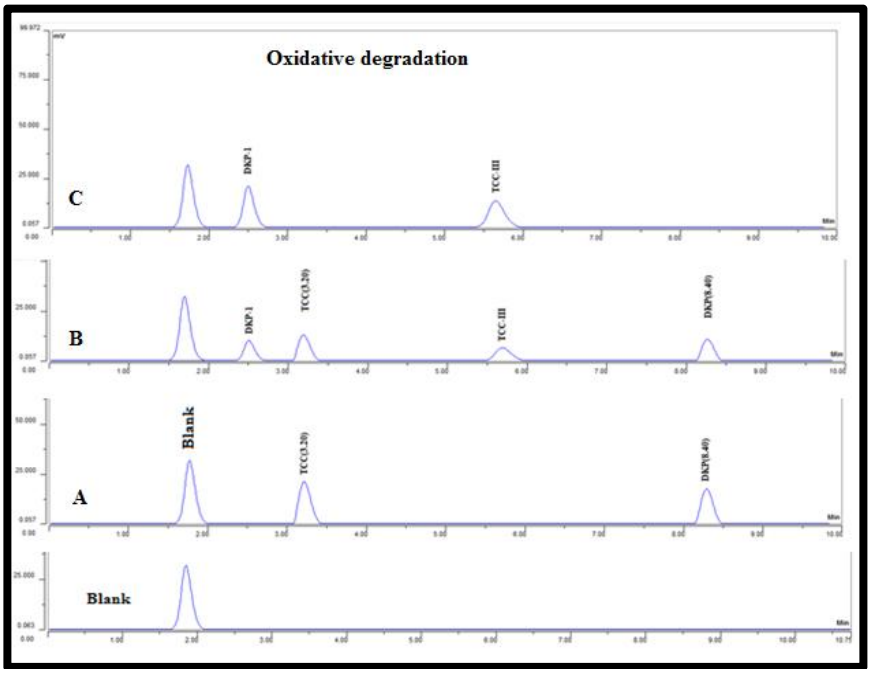
Where A= at 0 min, B= at 30 min, C= at 1 hr, D= 2 hr

Fig-5: Acid degradation



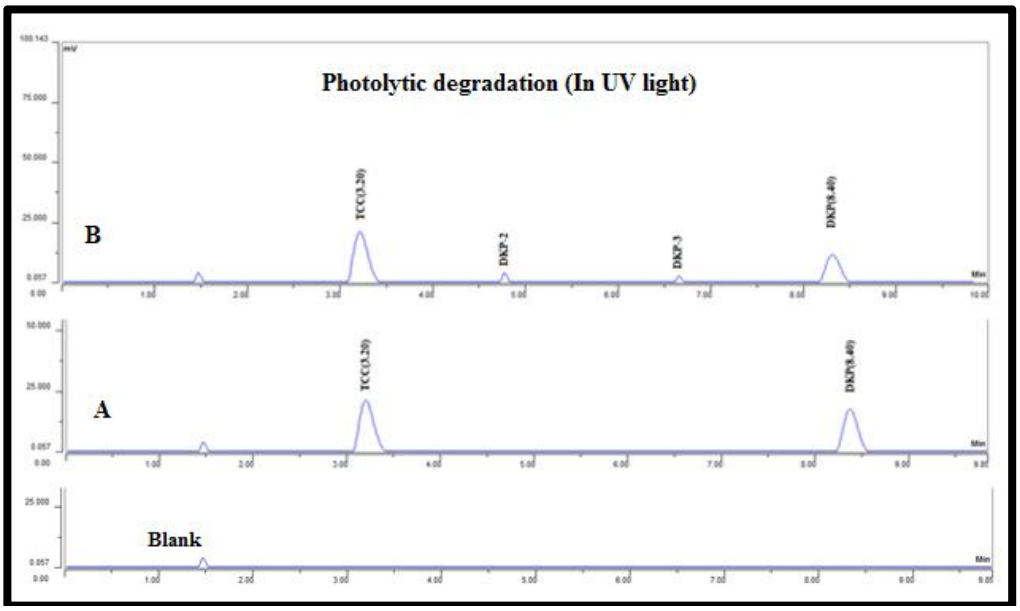
Where A= at 0 min, B= at 30 min, C= at 1 hr, D= 2 hr

Fig-6: Base degradation



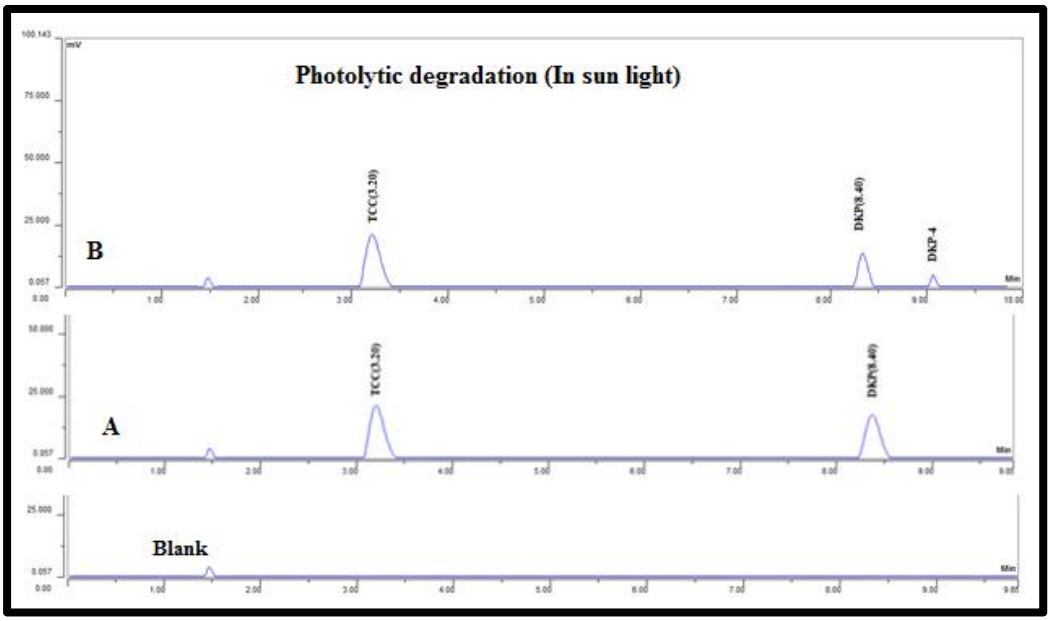
Where A= at 0 min, B= at 30 min, C= at 1 hr

Fig-7: Oxidative Degradation



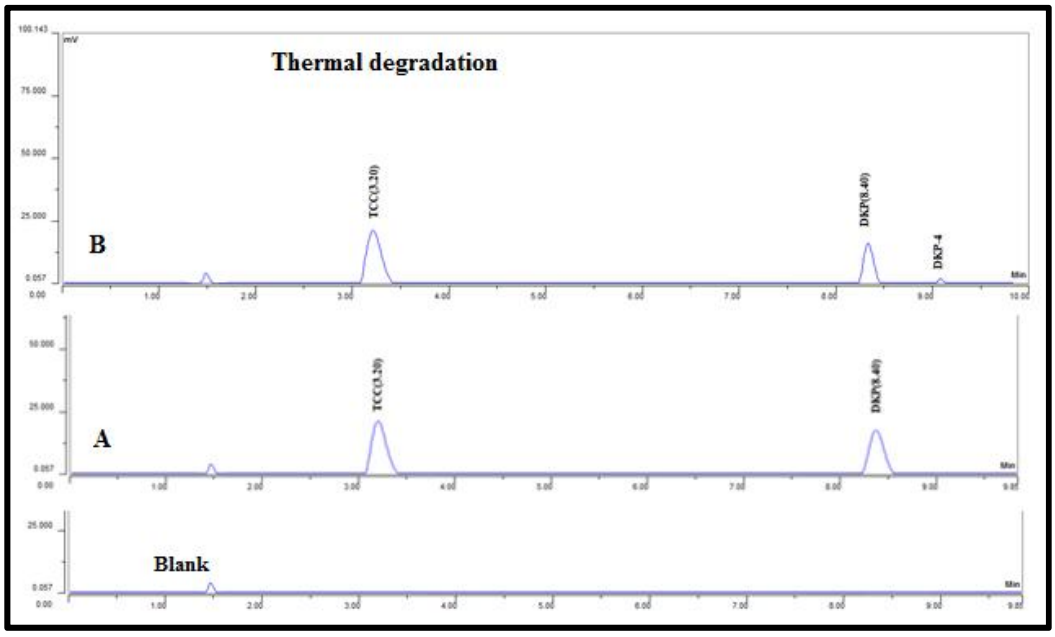
Where A= at 0 min, B= at 30 min

Fig-8: Photolytic Degradation (i) UV-Light



Where A= at 0 min, B= at 30 min

Fig-9: Photolytic Degradation (ii) Sun-Light



Where A= at 0 min, B= at 30 min

Fig-10: Thermal Degradation:

Table 3: Summary of Degradation studies

Degradation Conditions	**Thiocolchicoside**	**Dexketoprofen**
Acidic (1M HCl at 60°C, 2hrs)	100%	0%
Basic (1M NaOH at 60°C, 2 hrs)	100%	0%
Oxidative (3% H_2O_2 at 60°C, 1 hr)	100%	100%
Photolytic		
a. UV light (30 min)	0%	23.25%
b. Sunlight (30 min)	0%	20.50%
Thermal (Hot Air Oven, 60°C, 30 min)	0%	6.02%

Statistical analysis of 2^3 Factorial Design
Fitting of data to the model for Thiocholchicoside

Table 4: Experimental values of dependent variables for optimization

Exp. Trial	Variables			Area	Retention Time
	Flow Rate (ml/min)	pH Change	Mobile Phase Composition (v/v)		
1	-1	0	0	346574	3.16
2	+1	0	0	340673	3.24
3	0	-1	0	349758	3.23
4	0	+1	0	348963	3.25
5	0	0	-1	345110	3.18
6	0	0	+1	346010	3.26

Table 5**:** Summary of results of multiple regression analysis for Y_1 and Y_2

Dependent Variable	Area(Y1)		Retention Time (Y2)	
	P value	**Coefficient**	**P value**	**Coefficient**
Intercept	0.0301	+3.462	0.0277	+3.22
X_1	0.0410	-2950.50	0.0183	+0.040
X_2	0.0255	-397.50	0.0200	+0.010
X_3	0.0344	+450.00	0.0185	+0.040

Table 6: Summary of results of regression analysis for responses Y1-Y2 for fitting to quadratic model

Quadratic model	R^2	Adjusted R^2	Predicted R^2	Adequate precision	%CV
Y1	0.9473	0.8317	0.48	83.24	0.95
Y2	0.9049	0.8025	0.60	81.78	0.50

Table 7**:** Summary of Quadratic polynomial equation for responses Y1 and Y2 for fitting to quadratic model.

Quadratic model	Quadratic polynomial equation
Y_1	$Y_1 = 3.462 + 2950.50X_1 + 397.50X_2 + 450.00X_3$
Y_2	$Y_2 = 3.22 + 0.040X_1 + 0.010X_2 + 0.040\ X_3$

Contour Plots and Response Surface Analysis

A) 2D Contour plot and 3D surface plot for response Y_1 (AREA)

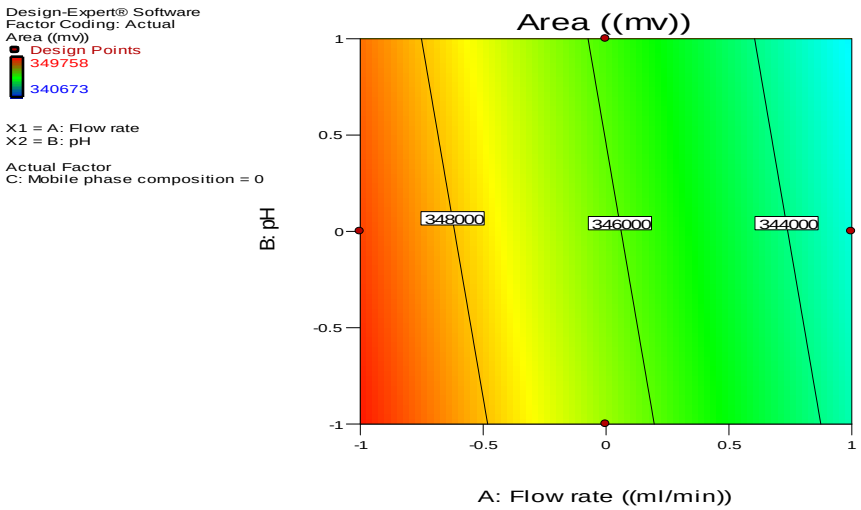


Fig-11**:** Two dimensional Contour plot showing the effect on Y1 (AREA)

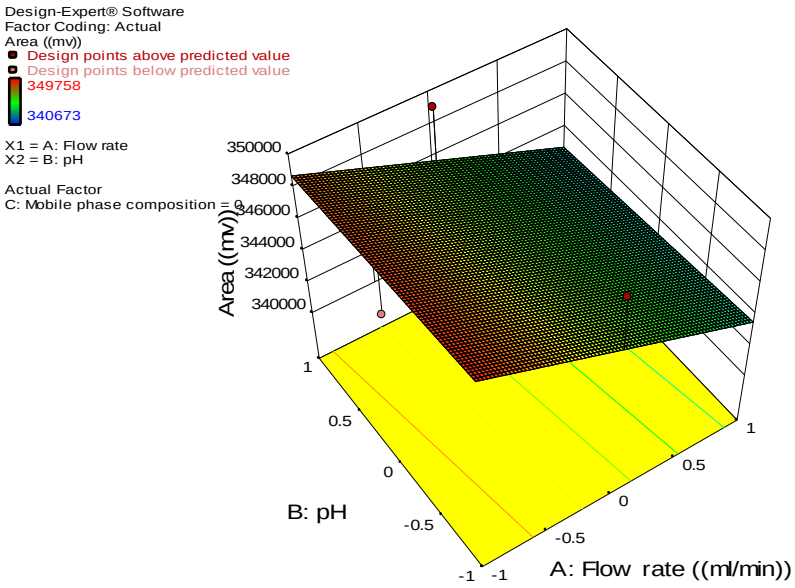


Fig 12**:** 3D surface plot showing the effect on AREA (Y_1)

B) 2D Contour plot and 3D surface plot for response Y_2(Retention Time)

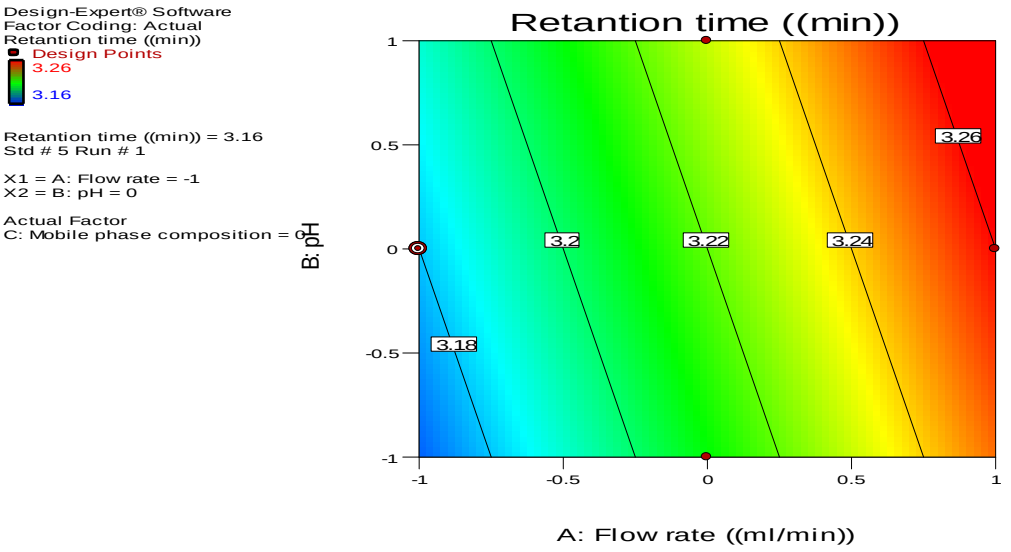


Fig 13**:** Two dimensional Contour plot showing the effect on Retention Time (Y_2)

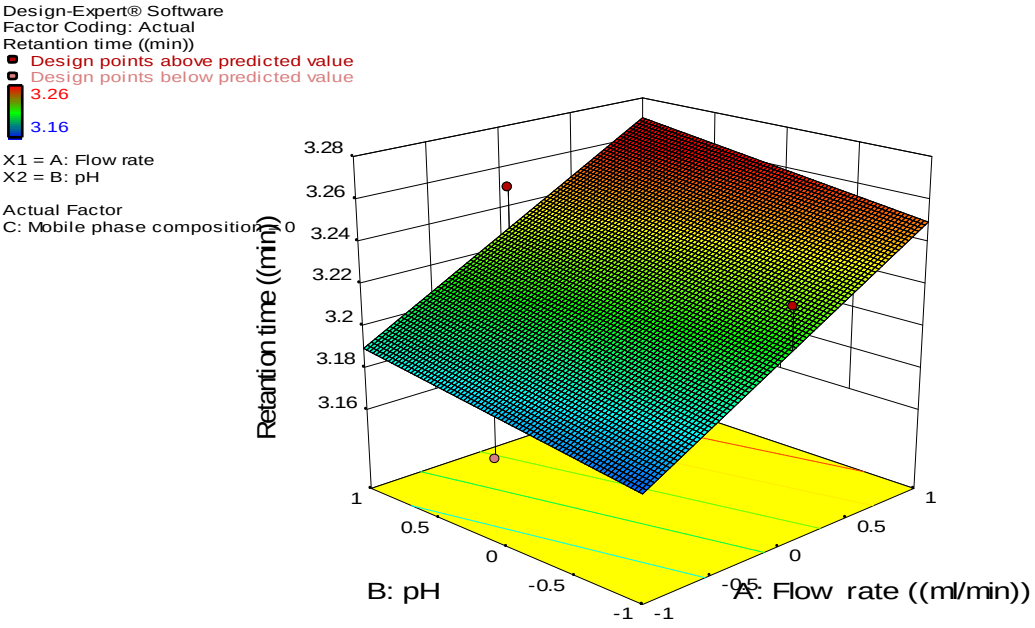


Fig 14**:** 3D surface plot showing the effect on response Retention Time (Y_2)

Fitting of data to the model for Dexketoprophen

Table 8**:** Experimental values of dependent variables for optimization

Exp. Trial	Variables			Area	Retention Time
	Flow Rate (ml/min)	pH Change	Mobile Phase Composition (v/v)		
1	-1	0	0	387973	8.51
2	+1	0	0	380124	8.31
3	0	-1	0	389512	8.56
4	0	+1	0	388397	8.52
5	0	0	-1	387945	8.49
6	0	0	+1	388978	8.34

Table 9**:** Summary of results of multiple regression analysis for Y_1 and Y_2

Dependent variable	Area(Y1)		Retention Time (Y2)	
	P value	**Coefficient**	**P value**	**Coefficient**
Intercept	0.0375	+3.872	0.0430	+8.45
X_1	0.0283	-3924.50	0.0307	-0.100
X_2	0.0440	-557.50	0.0213	-0.020
X_3	0.0130	-516.50	0.0415	-0.075

Table 10: Summary of results of regression analysis for responses Y1-Y2 for fitting to quadratic model

Quadratic model	R^2	Adjusted R^2	Predicted R^2	Adequate precision	%CV
Y1	0.9226	0.8934	0.30	41.20	0.60
Y2	0.9563	0.8680	0.50	49.42	0.79

Table 11**:** Summary of Quadratic polynomial equation for responses Y1 and Y2 for fitting to quadratic model.

Quadratic model	Quadratic polynomial equation
Y_1	$Y_1 = 3.872 - 3924.50X_1 - 557.50X_2 + 516.50X_3$
Y_2	$Y_2 = 8.45 - 0.100\ X_1 - 0.020X_2 - 0.075X_3$

Contour Plots and Response Surface Analysis

B) 2D Contour plot and 3D surface plot for response Y_1 (AREA)

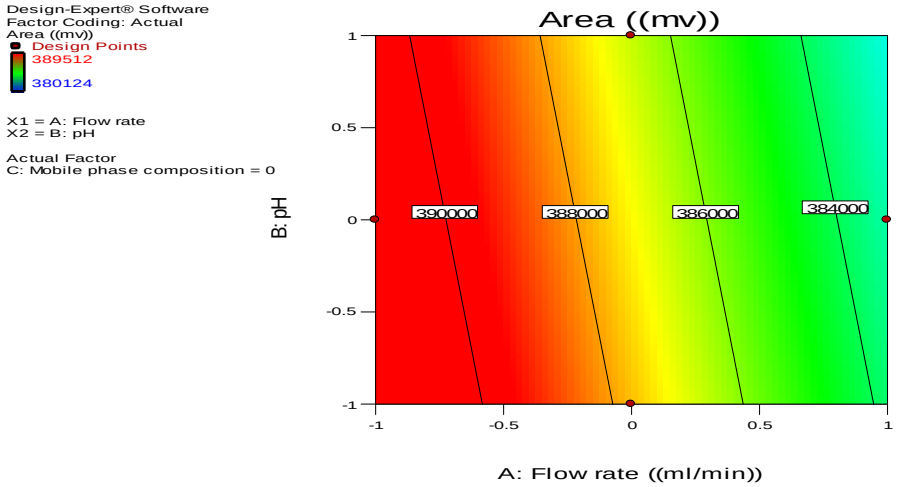


Fig 15**:** Two dimensional Contour plot showing the effect on Y1 (AREA)

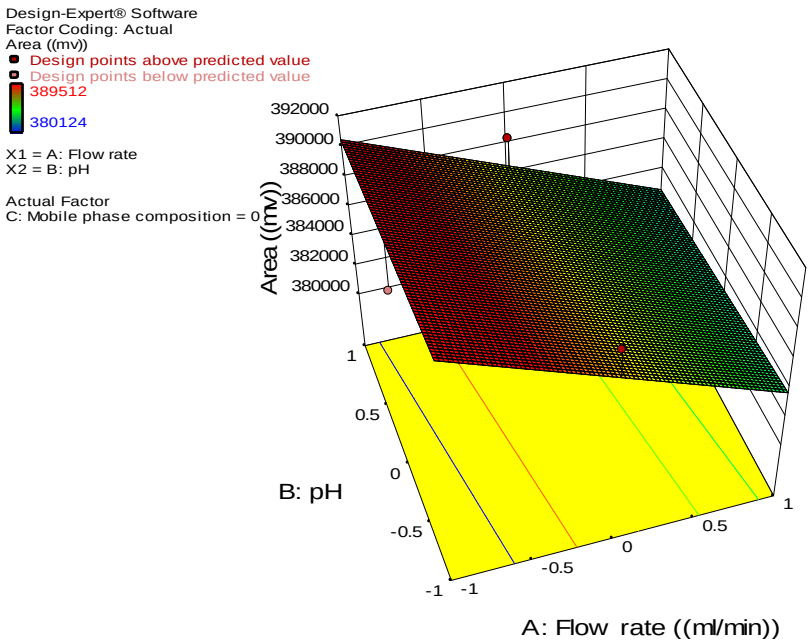


Fig 16**:** 3D surface plot showing the effect on AREA (Y_1)

B) 2D Contour plot and 3D surface plot for response Y_2(Retention Time)

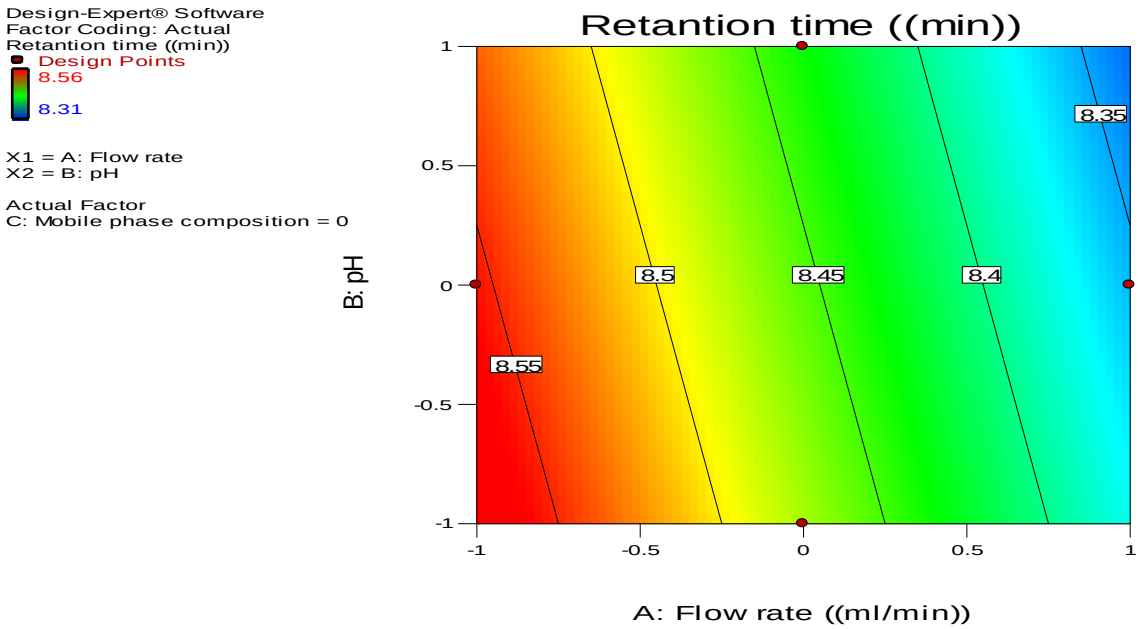


Fig 17: Two dimensional Contour plot showing the effect on Retention Time (Y_2)

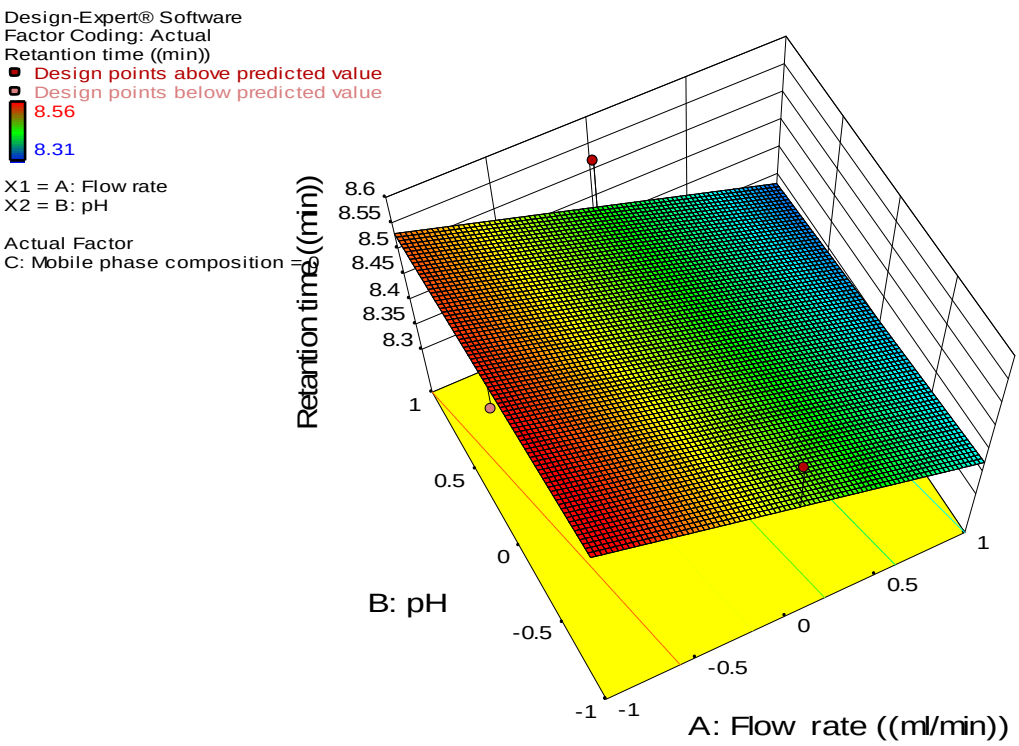


Fig 18**:** 3D surface plot showing the effect on response Retention Time (Y_2)

CONCLUSION

The developed RP-HPLC method was found to be simple, rapid, accurate, sensitive and specific methods for the estimation of THC and DKP. The % assay results of 99.21% for THC 99.85% for

DKP indicate that the developed method was successfully utilized for the estimation of THC and DKP in their combined dosage form in routine analysis.

ACKNOWLEDGEMENT

The authors are thankful to Emcure pharma, pune, India. For providing standard sample of drugs. Authors are thankful to shri Jagdishprasad Jhabarmal Tibrewala University and also to the ROFEL shri G.M. Bilakhia College of Pharmacy for providing facilities to carry out studies and research work. No uses of animal and human subjects were included to carry out research work.

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