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RP-HPLC Method Development and Validation using Factorial Design for Simultaneous Estimation of Thiocolchicoside and Etodolac with Forced Degradation Studies

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ABSTRACT:

In the present work, sensitive, accurate, precise and robust RP-HPLC method been developed for the quantitative estimation of Thiocolchicoside (THC) and Etodolac (ETD) in combined tablet dosage form. Determination of THC and ETD was carried on a reverse phase C18 (250×4.6mm, 5μ) column using a mobile phase consisting of Acetonitrile: Phosphate Buffer (60:40 v/v) pH 5, Flow rate of 1.0 ml/min and the detection was carried out at 259 nm. The linearity was found to be in the range of 10-50 μg/ml and 50-250 μg/ml with (r²=0.9999, and r²=0.9993) for THC and ETD respectively. The sharp peaks obtained were having clear baseline separation with a retention time of 2.45 min for THC and 7.10 min for ETD with resolution 16.55. 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. The proposed validated method was successfully used for the quantitative analysis of commercially available dosage form.

KEY WORDS: Thiocolchicoside, Etodolac, QbD based RP-HPLC, Factorial design, Forced degradation studies

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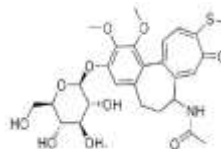
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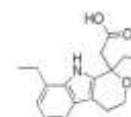
INTRODUCTION:

Thiocolchicoside, Chemically N - [(7S) -3- (beta-D-glucopyranosyloxy) -1, 2-dimethoxy- 10-(methylsulfonyl) -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 GABAA 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.

Etodolac, Chemically (R, S)-2-[1, 8-Diethyl -4,9-dihydro -3H-pyrano (3,4- b) indol-1-yl] acetic acid. It belongs to the class of Pyranocarboxylic acid of the class of drugs called nonsteroidal anti-inflammatory drugs (NSAIDs). This drug is also used for the management of mild to moderate pain, fever and inflammation.



Thiocolchicoside (THC)



Etodolac (ETD)

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Thiocolchicoside 8mg + Etodolac 300mg Tablets are manufactured by "Emcure pharma" under the brand name Proxym-MR Forte (Etodolac 300 gm + Thiocochicoside 8 mg).

Thiocolchicoside is official in IP 2014[1] and includes HPLC method for estimation of THC. Etodolac is not official in any pharmacopoeia.[1-4] The combination of these two drugs is not official in any pharmacopoeia.[1-4] Literature review shows that numbers of analytical methods are available for estimation of both the drugs either alone or in combination with other drugs.[5-14] Based on our current and ongoing referencing work, till date, we have come across to first order derivative spectroscopic method and RP-HPLC method for simultaneous estimation of Thiocolchicoside and Etodolac 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 ETD 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

Optimization of Chromatographic conditions

Stationary phase : RP C18 (250×4.6mm) 5μ

Mobile phase : Acetonitrile : Phosphate buffer (60:40 v/v)

pH : pH adjusted to 5.0 by orthophosphoric acid

Flow rate : 1.0 ml/min

Wavelength : 259 nm

Preparation of standard solution

Standard stock and Working standard solution of THC

Accurately weighed THC (100 mg) was transferred to 100 ml volumetric flask and dissolved in methanol and diluted up to the mark with methanol to give a stock solution having strength 1 mg/ml (1000 μg/ml). 100 μg/ml of THC working standard solution was prepared by diluting 1 ml of stock solution with methanol to 10 ml with methanol.

Standard stock and Working standard solution of ETD

Accurately weighed ETD (100 mg) was transferred into 100 ml volumetric flask and dissolved in methanol and diluted up to the mark with methanol to give a stock solution having strength 1 mg/ml (1000 μg/ml). 100 μg/ml of ETD working standard solution was prepared by diluting 1 ml of stock solution to 10 ml with methanol.

Preparation of Calibration Curve for THC and ETD

Calibration curve for THC

The calibration curve was constructed with five concentrations ranging from 10-50 μg/ml for THC. The solutions were prepared by transferring 1, 2, 3, 4 and 5 ml aliquots of the working standard solution of THC (100 μg/ml) into series of 10 ml volumetric flasks and the volume was adjusted to mark with methanol to get the final concentration of 10, 20, 30, 40 and 50 μg/ml. All the solutions were injected. The data of peak area versus concentration were treated by linear least square regression analysis.

Calibration curve for ETD

The calibration curve was constructed with five concentrations ranging from 50-250μg/ml for ETD. The solutions were prepared by transferring 0.5, 1, 1.5, 2.0 and 2.5 ml aliquots of the standard stock solution of ETD (1000 μg/ml) into series of 10 ml volumetric flasks and the volume was adjusted to mark with methanol to get the final concentration of 50, 100, 150, 200, 250 μg/ml. All the solutions were injected. The data of peak area versus concentration were treated by linear least square regression analysis.

Preparation of binary mixtures of THC and ETD

Accurately weighed 30 mg THC and 150 mg of ETD were transferred to 100 ml volumetric flask. It was dissolved with sufficient methanol and diluted up to mark with methanol to give concentration of 300 µg/ml of THC and 1500 µg/ml of ETD. Above solution was diluted further to get the concentration range of 10, 20, 30, 40 and 50 µg/ml for THC and 50, 100, 150, 200 and 250 µg/ml for ETD.

Forced Degradation Study:

In order to establish whether the analytical method for the assay was stability-indicating, pure active pharmaceutical ingredient (API) and tablet content of THC & ETD was subjected to various stress conditions to conduct forced degradation studies. Stress studies were carried out under the conditions of acid/base hydrolysis, oxidation, Thermal as mentioned in ICH Q1A (R2). UV light degradation of drug substances and drug product was performed in the solid state as mentioned in ICH Q1B.

1. Acid Degradation:

To the different 10 ml volumetric flask, 1 ml stock solutions of THC and ETD were taken and 2 ml of 1 M HCl for THC and ETD was added respectively. Both flasks were heated at 60°C for 0 min, 30 min, 1 hr and 2 hr and allowed to cool to room temperature. Solutions were neutralized with 1 M NaOH and diluted up to the mark with mobile phase. Appropriate aliquots were taken from the above solutions and diluted with mobile phase to obtain final concentration of 20 µg/ml THC and 100 µg/ml ETD separately and in the mixture.

2. Alkali hydrolysis:

To the different 10 ml volumetric flask, 1 ml stock solutions of THC and ETD were taken and 2 ml of 1 M NaOH for THC and ETD was added respectively. Both flasks were heated at 60°C for 0 min, 30 min, 1 hr and 2 hr and allowed to cool to room temperature. Solutions were neutralized with 1 M HCl and diluted up to the mark with mobile phase. Appropriate aliquots were taken from the above solutions and diluted with mobile phase to obtain final concentration of 20 µg/ml THC and 100 µg/ml ETD separately and in the mixture.

3. Oxidative stress degradation:

To perform oxidative stress degradation, appropriate aliquots of stock solutions of THC and ETD were taken in two different 10 ml volumetric flasks and 2 ml of 3 % hydrogen peroxide was added respectively. Both flasks were heated in a water bath at 60 oC for 0 min, 30 min and 1 hr and allowed to cool

to room temperature and diluted up to the mark with mobile phase. Appropriate aliquots were taken from above solutions and diluted with mobile phase to obtain final concentration of 20 µg/ml THC and 100 µg/ml ETD separately and in the mixture.

4. Photolytic Degradation:

4.1. Condition (1): In UV-Light for 30 min.

Analytically 10mg of pure samples of THC and ETD were exposed in UV-light at 80o C for 30 min separately in 10 ml volumetric flasks. The solids were allowed to cool and dissolved in few ml of methanol. Volumes were made up to the mark with the methanol. Solutions were further diluted by mobile phase taking appropriate aliquots in 10 ml volumetric flask to obtain final concentration of 20 µg/ml THC and 100 µg/ml ETD separately and in the mixture.

4.2 Condition (2): In Sun-Light for 30 min.

Analytically 10mg of pure samples of THC and ETD were exposed in Sun-light for 30 min separately in 10 ml volumetric flasks. The solids were allowed to cool and dissolved in few ml of methanol. Volumes were made up to the mark with the methanol. Solutions were further diluted by mobile phase taking appropriate aliquots in 10 ml volumetric flask to obtain final concentration of 20 µg/ml THC and 120 µg/ml ETD separately and in the mixture.

5. Thermal Degradation:

Analytically 10mg of pure samples of THC and ETD were exposed in in Hot Air Oven at 60°C for 30 min separately in 10 ml volumetric flasks. The solids were allowed to cool and dissolved in few ml of methanol. Volumes were made up to the mark with the methanol. Solutions were further diluted by mobile phase taking appropriate aliquots in 10 ml volumetric flask to obtain final concentration of 20 µg/ml THC and 120 µg/ml ETD separately and in the mixture.

All the reaction solutions were injected in the liquid chromatographic system and chromatograms were recorded.

Validation of Forced degradation RP-HPLC method

1. System suitability studies

The system suitability was evaluated by five replicate analyses of THC and ETD mixture at concentration of 30µg/ml of THC and 150µg/ml of ETD. The column efficiency (Numbers of Theoretical Plates), Resolution and Peak Asymmetry (Tailing Factor) were calculated for the standard solutions.

2. Linearity

Appropriate aliquots of THC and ETD working standard solutions were taken in different 10 ml volumetric flasks and diluted up to the mark with mobile phase to obtain final concentrations of 10, 20, 30, 40 and 50 µg/ml for THC and 50, 100, 150, 200 and 250 µg/ml for ETD. The solutions were injected using a 20 µL fixed loop system and chromatograms were recorded. Calibration curves were constructed by plotting average peak area versus concentrations and regression equations were computed for both the drugs.

3. Precision

3.1 Repeatability

Repeatability was carried out by estimating response of 30 µg/ml of THC and 150 µg/ml of ETD, six times and results are reported in terms of relative standard deviation.

3.2 Intra-day precision

Mixed solutions containing THC (20, 30, 40 µg/ml) and ETD (100, 150, 200 µg/ml) were analyzed three times on the same day and % CV was calculated.

3.3 Inter-day precision

Mixed solutions containing THC (20, 30, 40 µg/ml) and ETD (100, 150, 200 µg/ml) were analyzed three times on three different days and % CV was calculated.

4. Accuracy

It was carried out to determine the suitability and reliability of the proposed method. Accuracy was determined by calculating the % Recovery. Twenty tablets were weighed and powdered. Powder equivalent to 30 mg of THC and 150 mg of ETD was weighed and transferred into a 100 ml of volumetric flask, standard was added on the basis of 80%, 100% and 120 % and diluted up to mark with mobile phase. The amount of THC and ETD was calculated at each of three level and % recoveries were computed at three different levels (80%, 100% and 120%). For THC the accuracy study performed on 30 µg/ml concentration and 150 µg/ml for ETD.

5. Limit of Detection

From the linearity curve equation, the standard deviation (SD) of the intercepts (response) was calculated. Then LOD was measured by using mathematical expressions given in section. The limit

The limit of detection (LOD) of the drug was calculated by using the following equations designated by ICH guideline:

$$\text{LOD} = 3.3 \times \sigma / S,$$

Where, σ = the standard deviation of the Intercept

S = Mean slope of the calibration curve

6. Limit of Quantification

From the linearity curve equation, the standard deviation (SD) of the intercepts (response) was calculated. Then LOQ was measured by using mathematical expressions given in section. The limit of quantification (LOQ) of the drug was calculated by using the following equations designated by ICH guideline:

$$\text{LOQ} = 10 \times \sigma / S$$

Where, σ = the standard deviation of the Intercept

S = Mean slope of the calibration curve

7. Robustness

Robustness and Ruggedness of the method was determined by subjecting the method to slight change in the method condition, individually, the:

- Pump flow rate,
- pH Change.
- Mobile Phase Composition change

Three replicates were made for the same concentration (30µg/ml of THC and 150µg/ml of ETD). % CV was calculated.

8. Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities and degradation products. Commonly used excipients (starch, microcrystalline cellulose and magnesium stearate) were spiked into a pre weighed quantity of drugs. The chromatogram was taken by appropriate dilutions and the quantities of drugs were determined.

Analysis of Tablet Formulation

Twenty tablets were weighed. Average weight was calculated and tablet powder equivalent to about 8 mg THC and 300 mg ETD was transferred in 100 ml volumetric flask. Methanol (50 ml) was added to the above flask and the flask was sonicated for 15 minutes. The solution was filtered using whatman filter

paper No.41 and volume was made up to the mark with the mobile phase. Appropriate volume of the aliquot was transferred to a 10 ml volumetric flask and the volume was made up to the mark with the mobile phase to obtain a solution containing 30 µg/ml of THC and 150 µg/ml of ETD. The solution was sonicated for 10 min. It was injected as per the above chromatographic conditions and peak areas were recorded. The quantifications were carried out by keeping these values to the straight line equation of calibration curve.

RESULTS

The chromatographic conditions optimized for separation of THC and ETD with accuracy and precision. The mobile phase consisted of Acetonitrile: Phosphate Buffer (60:40 v/v) pH 5, Flow rate of 1.0 ml/min and the detection was carried out at 259 nm. The linearity was found to be in the range of 10-50 µg/ml and 50-250 µg/ml with ($r^2=0.9999$, and $r^2=0.9993$) for THC and ETD respectively. The sharp peaks obtained were having clear baseline separation with a retention time of 2.45 min for THC and 7.10 min for ETD with resolution 16.55 (Which was more than 2). Numbers of Theoretical Plates (N) were found to be 5852 for THC and 2110 for ETD (Which was more than 2000), Tailing Factor (TF) was found to be 1.25 for THC and 1.40 for ETD (Which was less than 2). The % recoveries for THC and ETD obtained in the accuracy study were 99.56 - 99.92% and 99.75 - 99.87% respectively. The results of the precision study indicate that the proposed method showed good repeatability for THC and ETD with % CV of 0.21 and 0.40 respectively. The % CV from the intraday precision data were found to be 0.12 - 0.35 for THC and 0.14 - 0.31 for ETD. Similarly % CV from the interday precision data were found to be 0.24 - 0.42 for THC and 0.21 - 0.37 for ETD. The LOD for THC and ETD was found to be 0.1534 µg/ml and 0.1215 µg/ml respectively. Similarly LOQ for THC and ETD was found to be 0.4649 µg/ml and 0.3683 µg/ml respectively. The % assay results of 99.34% for THC and 99.89% for ETD indicate that the developed method was successfully utilized for the estimation of THC and ETD in their combined dosage form. Forced degradation study was performed in different degradation conditions and samples were analyzed at different time of interval. Thiocolchicoside was degraded completely in acidic, basic and oxidative condition and stable in photolytic and thermal condition while

Etodolac was not stable in all conditions. It was partially degraded in all conditions.

DISCUSSION

The developed RP-HPLC method was found to be simple,

rapid, accurate, sensitive and robust for simultaneous estimation of Thiocolchicoside and Etodolac. The % assay results of 99.34% for THC and 99.89% for ETD indicate that the developed method was successfully utilized for the estimation of THC and ETD in their combined dosage form in routine analysis. Forced degradation studies showed that Thiocolchicoside is not affected in Photolytic and thermal degradation conditions while easily degraded in acidic, basic and oxidative conditions. Etodolac was affected in all degradation conditions. Method was proved to be Robust by applying three level Factorial design of experiment using quality by design approach that the retention time and area were affected by critical parameters like change in flow rate, pH and mobile phase composition.

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TABELS:

Table 1: Summary of Validation Parameters

Parameters	Thiocolchicoside	Etodolac
Linearity	10-50 µg/ml	50-250 µg/ml
Accuracy (%)	99.56 – 99.92 %	98.75 – 99.87
Recovery (n=3)		%
Precision (% CV)		
Repeatability (n=6)	0.21	0.40
Intraday (n=3)	0.12 - 0.35	0.14 - 0.31
Interday (n=3)	0.24 – 0.42	0.21 – 0.37
LOD (µg/ml)	0.1534	0.1215
LOQ (µg/ml)	0.4649	0.3683

Table 2: Summary of Degradation studies

Degradation Conditions	Thiocolchicoside	Etodolac
Acidic (1M HCl at 60°C, 2hrs)	100%	22.90%
Basic (1M NaOH at 60°C, 2 hrs)	100%	6.25%
Oxidative (3% H₂O₂ at 60°C, 1 hr)	100%	22.70%
Photolytic		
a. UV light (30 min)	0%	1.76%
b. Sunlight (30 min)	0%	1.90%
Thermal (Hot Air Oven, 60°C, 30 min)	0%	6.85%

FIGURES



Figure 1: Chromatographic separation of THC and ETD

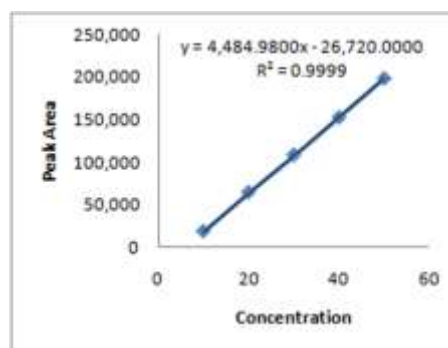


Figure 2: Calibration curve for THC (10-50 µg/ml)

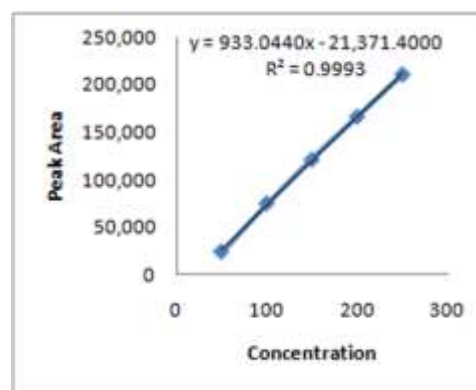
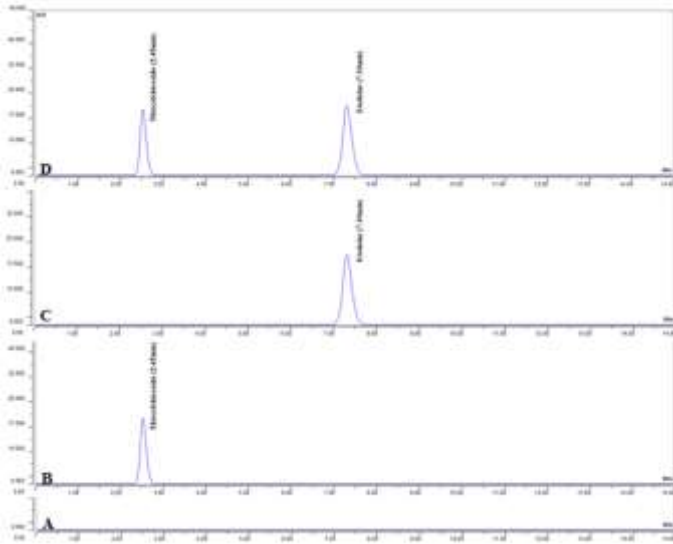
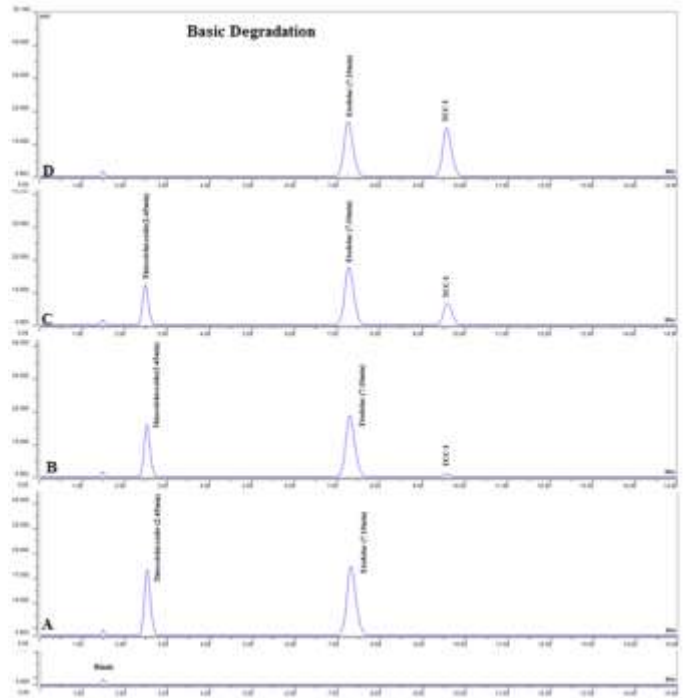


Figure 3: Calibration curve for ETD (50-250 µg/ml)



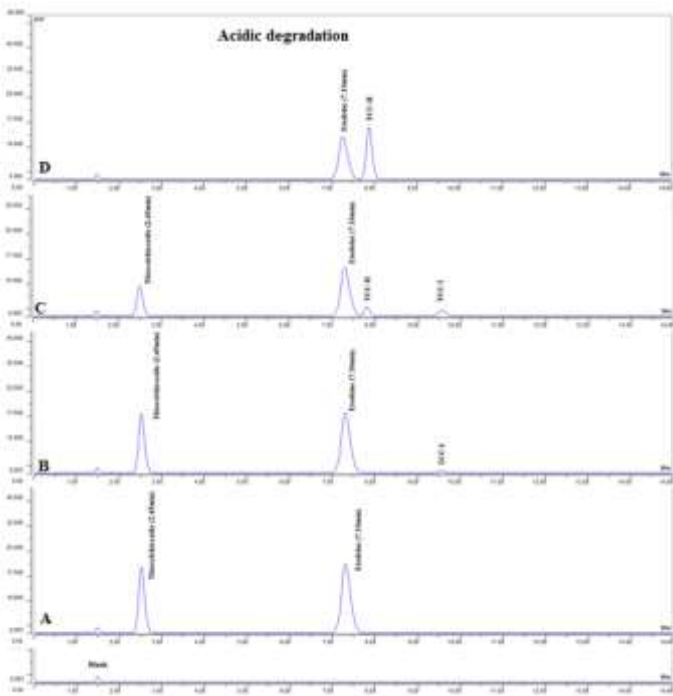
Where, A= blank, B= THC single, C= ETD single, D= in combination

Figure 4: Chromatograms for untreated Stock Solution



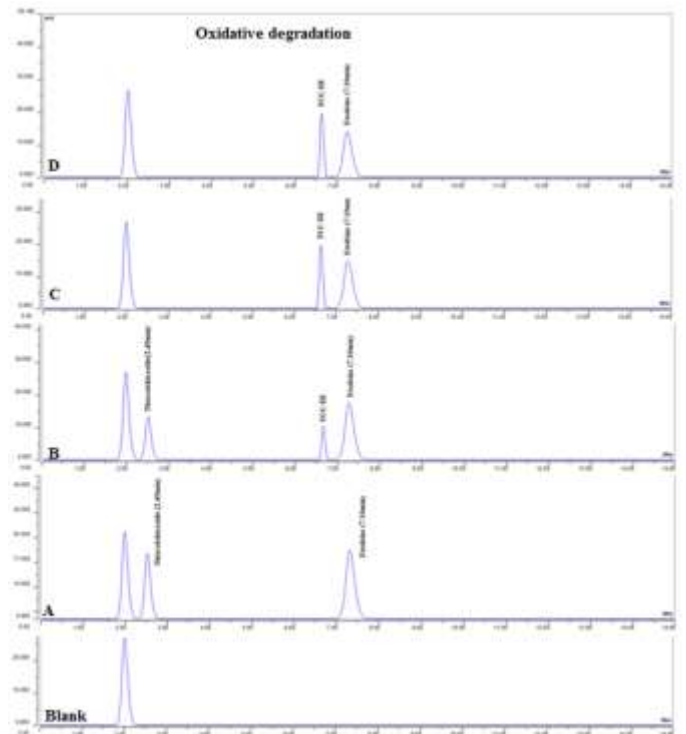
Where A= at 0 min, B= at 30 min, C= at 1 hr, D= 2 hr

Figure 6: Chromatograms for Alkali treated Stock Solution



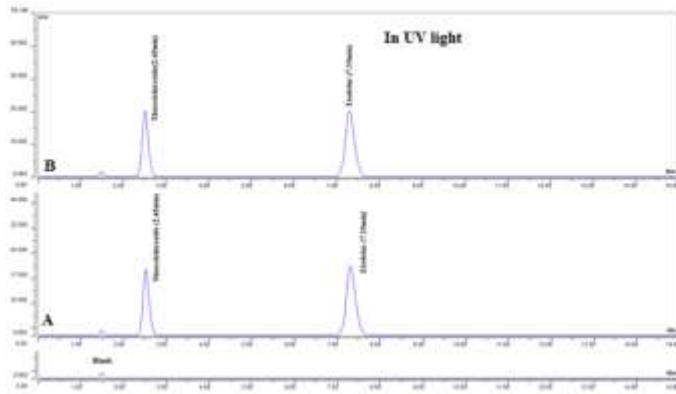
Where A= at 0 min, B= at 30 min, C= at 1 hr, D= 2 hr

Figure 5: Chromatograms for Acid treated Stock Solution



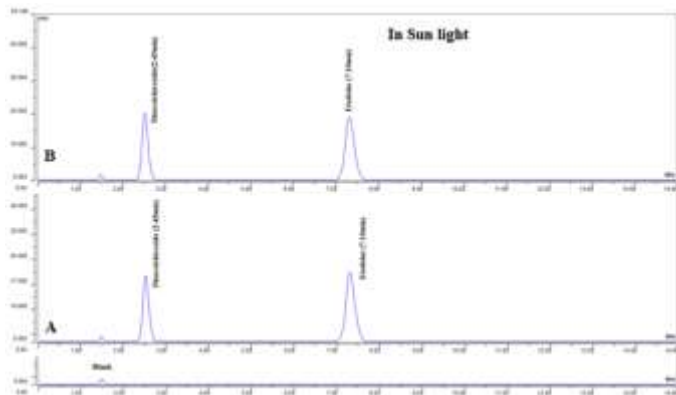
Where A= at 0 min, B= at 30 min, C= at 1 hr

Figure 7: Chromatograms for Oxidative Degradation



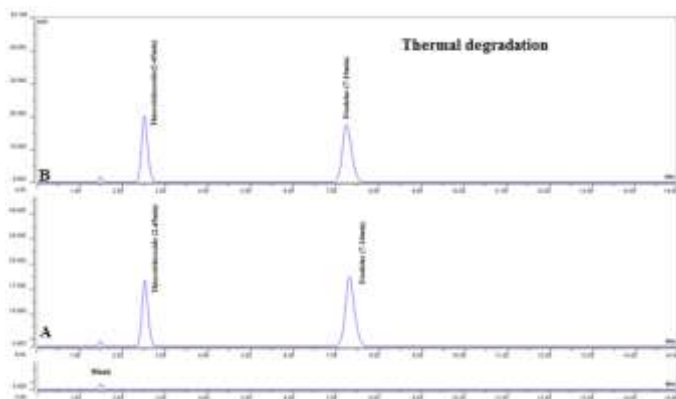
Where A= at 0 min, B= at 30 min

Figure 8: Chromatograms for UV-light Degradation



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

Figure 9: Chromatograms for Sun-light Degradation



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

Figure 10: Chromatograms for Thermal Degradation

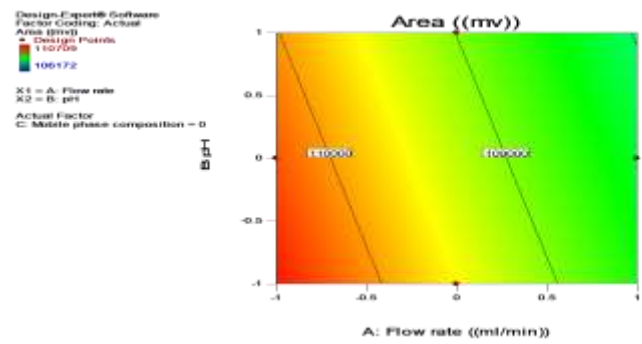


Figure 11: Two dimensional Contour plot for THC showing the effect on Y1 (AREA)

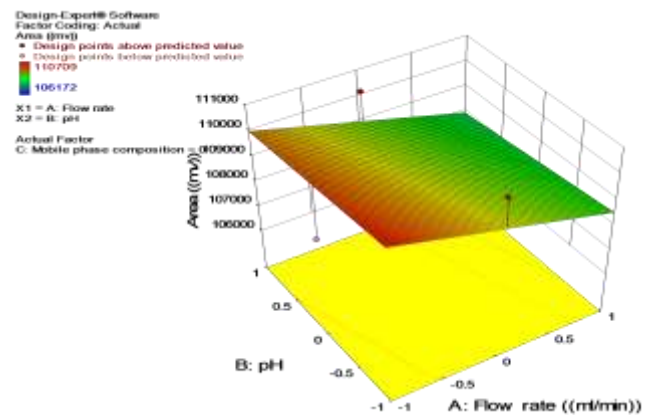


Figure 12: 3D surface plot for THC showing the effect on AREA (Y1)

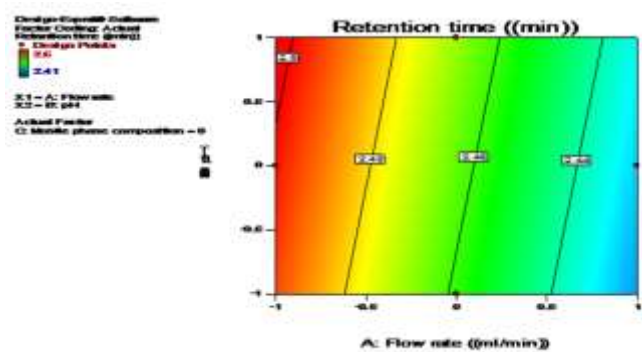


Figure 13: Two dimensional Contour plot for THC showing the effect on Retention Time (Y2)

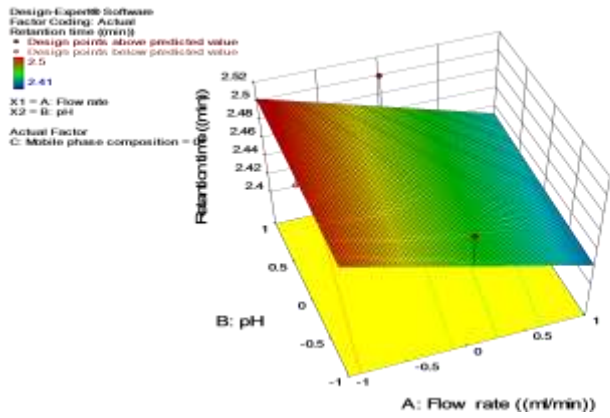


Figure 14: 3D surface plot for THC showing the effect on Retention Time (Y_2)

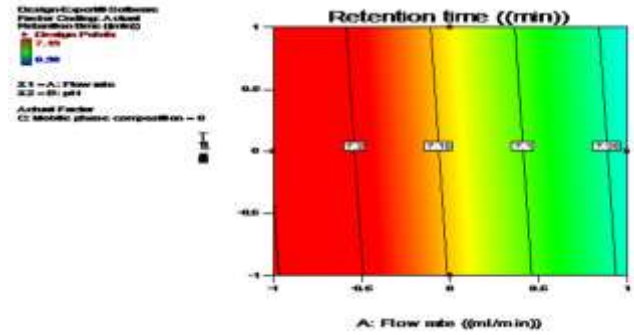


Figure 17: Two dimensional Contour plot for ETD showing the effect on Retention Time (Y_2)

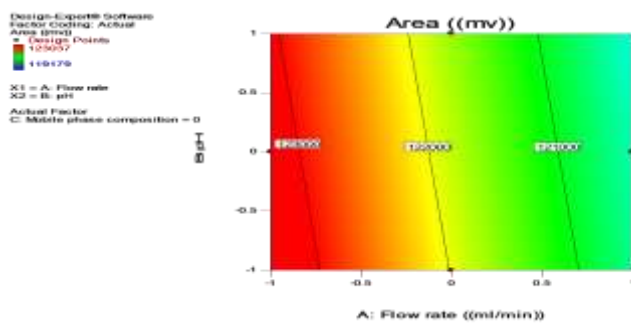


Figure 15: Two dimensional Contour plot for ETD showing the effect on Y_1 (AREA)

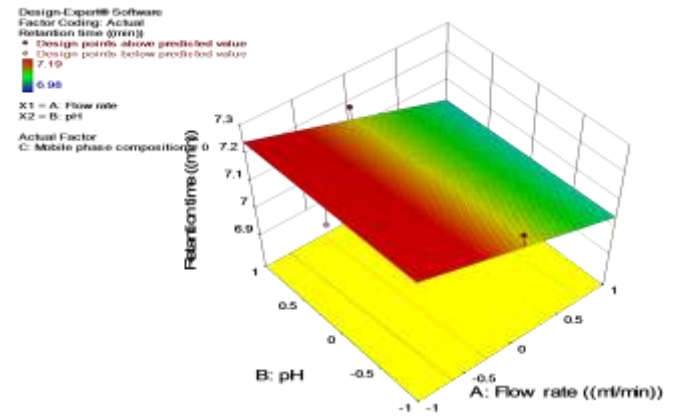


Figure 18: 3D surface plot for ETD showing the effect on Retention Time (Y_2)

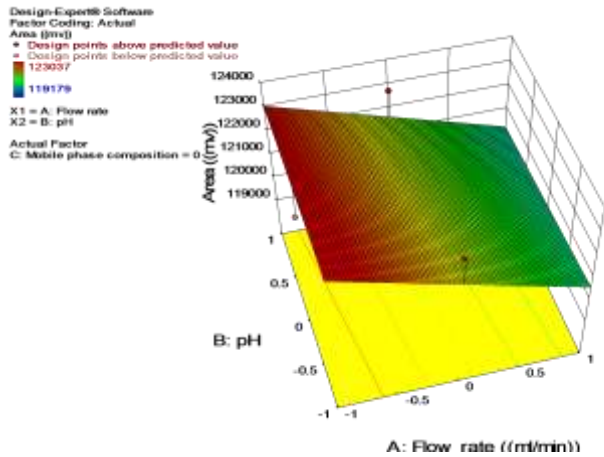


Figure 16: 3D surface plot for ETD showing the effect on AREA (Y_1)

