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Development and Validation of Stability Indicating RP-HPLC Method for Simultaneous Determination of Anti-Haemorrhagic Ethamsylate and Anti-Fibrinolytic Tranexamic Acid in Combined Formulation using Green Assessment

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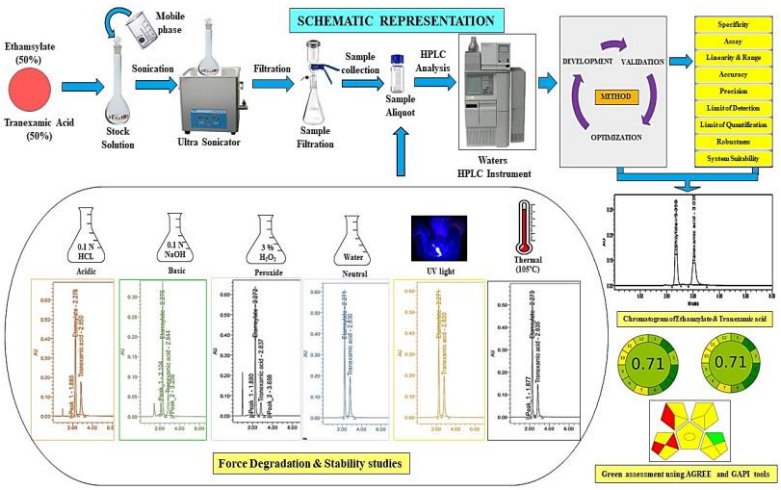
KEYWORDS
Ethamsylate, Green Profile, Method Validation, RP-HPLC, Tranexamic Acid.

Abstract

The present study demonstrates the method development and validation for simultaneous estimation of Ethamsylate & Tranexamic acid in combined pharmaceutical dosage form by using RP-HPLC along with the stability studies. According to ICH guidelines for stability testing of new drug substances and products, both the drugs were subjected to various stress conditions. The chromatographic separation was carried out on ALTIMA C18 column with the dimensions of 150 mm x 4.6 mm x 5µm using Waters 2695 HPLC instrument equipped with 2998 series of PDA detector. The isocratic mobile phase used was made up of Phosphate Buffer with pH adjusted at 3.1 & Acetonitrile (ACN) in the ratio 80:20 v/v with the flow rate of 1.0 ml /min and both the drugs have been detected using UV detector at 249 nm. At room temperature Ethamsylate and Tranexamic acid were found to have retention times of 2.353 & 3.033 minutes respectively with a total run time of 6 minutes. The developed method was found to be linear at the concentration range of 25 µg/ml – 150 µg/ml with coefficient of determination observed at 0.09993 for Ethamsylate & 0.9998 for Tranexamic acid respectively. The developed method was validated by utilizing various validation parameters and the force degradation & stability studies were applied to analyse the stability and to identify settlement of the degradation products. The developed method was also analysed using two tools like AGREE and GAPI for assessing the green profile of the developed method. The established method can be applied for the simultaneous determination of ETS & TXA in combined dosage form.

Graphical Abstract



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1. Introduction

The blood loss during the caesarean delivery is more than that of the vaginal delivery. And also for reducing maternal and foetal mortality, caesarean delivery plays an important role in saving the lives. The main cause for maternal mortality is the postpartum haemorrhage. To minimise the intraoperative and postoperative blood loss during caesarean delivery medicines such as ergometrine, oxytocin, prostaglandin and misoprostol are used. In addition to the above medicines pro-haemostatic drugs such as Ethamsylate an Tranexamic acid are also used [1-3].

Ethamsylate (ETS) is a synthetic haemostatic drugs used in case of increasing capillary endothelial resistance and also promotes platelet adhesion. The molecular formula of ETS is $C_{10}H_{17}NO_5S$ and its structure is shown in the Fig. 1. [4]. **Tranexamic acid (TXA)** is a synthetic derivative of lysine (an amino acid) which exerts anti-fibrinolytic action. The molecular formula of TXA is $C_8H_{15}NO_2$ and its structure is shown in the Fig. 2. [5]. The Etosys tablet (marketed formulation) is mainly composed of 50% ETS & 50% TXA. The ETS & TXA drugs are used in the treatment of heavy blood loss during dental extractions, operations, abortions and deliveries [6].

The extensive literature review on ETS & TXA evidenced that there are few related works in the simultaneous determination and analysis in bulk, marketed formulations, and individual determinations which have been reported by using RP-HPLC techniques [7], [8-11]. UV Spectrophotometric individual estimation of Ethamsylate was also carried out [12]. UPLC work was also conducted on the simultaneous estimation of Ethamsylate and Tranexamic acid. [13]. The above reported methods don't meet the requirements as per ICH guidelines as they revealed uncertain resolution and non-specificity using expensive chemicals and costly methods. Hence, the main objective of the present study is to develop a rapid, sensitive, precise, economical, simple and green method using environmental friendly chemicals with quick elution times, specificity and good resolution between peaks for the simultaneous estimation of ETS & TXA using RP-HPLC as per the ICH guidelines.

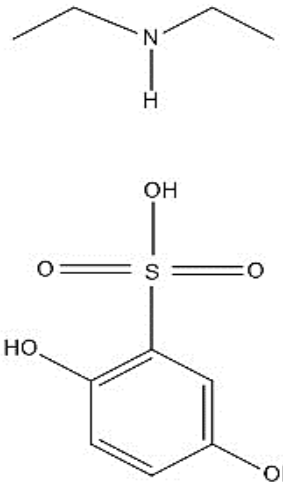

Figure 1. Chemical Structure of ETS

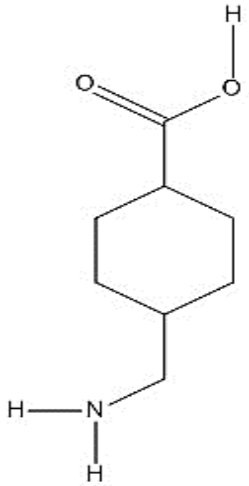

Figure 2. Chemical Structure of TXA

2. Experimental

2.1 Pharmaceutical drugs, materials and reagents used

ETS, TXA were obtained as gift samples from Aurobindo Pharma Ltd, Hyderabad, Telangana. Etosys (ETS 250 mg & TXA 250 mg) tablets were obtained from local pharmacy. HPLC grade Acetonitrile and Analytical grade Triethylamine, HCl, NaOH, Hydrogen peroxide were purchased from SD Fine Chemicals, Mumbai, Maharashtra. Ultrapure grade Sodium dihydrogen phosphate, Orthophosphoric acid & Milli-Q water was bought from Merck Ltd, Mumbai, Maharashtra.

2.2 RP-HPLC Instrumentation & Apparatus

HPLC instrument used for the method development, method validation for the simultaneous determination of ETS & TXA along with the force degradation and stability studies was Waters 2695 HPLC (Waters Alliance, Milford, Massachusetts, USA) equipped with an inline degasser, auto sampler, 2998 series of Photo

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Diode Array detector accompanied with a spectral band-pass filter of 1.2 nm. The chromatographic separation was achieved on an ALTIMA C18 column with the dimensions of 150 mm column length x 4.6 mm internal diameter x 5µm particle size at 25°C temperature in an air conditioned lab for all the chromatographic runs. The processing, chromatographic integration, data acquisition and data recording was achieved by Empower 2^{TM} software. Electronic weighing machine (Avis Ener Tech, Chennai, Tamil Nadu), Ultrasonic water bath (Elico, Hyderabad, Telangana) & pH meter (Elico, Hyderabad, Telangana) were used in the study.

2.3 Preparation of Buffer solution

The buffer solution preparation was carried out by accurately weighing 1.41gms of Sodium dihydrogen phosphate in 1000 ml graduated flask and dissolving it with HPLC grade water. By adding 1ml Triethylamine to the same solution, it is further made up to the mark with HPLC water. The adjustment of the pH is done by using Elico pH meter at 3.1 by adding Orthophosphoric acid.

After the preparation of the solution, it is degassed by sonicating in ultrasonic water bath for 10 minutes and then the filtration is carried out through 0.45 µm nylon membrane.

2.4 Preparation of Mobile Phase

The mobile phase mixture was prepared by combining 800 ml of Phosphate buffer and 200 ml of Acetonitrile. After combing both the solutions, mobile phase was degassed in the ultra-sonicator for twenty minutes using Elico ultrasonic water bath. Further the solution was filtered using 0.45 µm filter under vacuum filtration.

2.5 Preparation of Diluent

The diluent preparation is performed by assimilating mobile phase blend of Phosphate buffer with pH adjusted to 3.1 & ACN in the ratio 80:20 v/v.

2.6 Preparation of Standard stock solution of ETS & TXA

Final concentration – ETS-1mg/ml & TXA-1 mg/ml

Accurately weighed 100 mg of ETS & 100 mg TXA standard and transferred into 100 ml graduated flask. 70 ml of the diluent was added to the same graduated flask and sonicated for 10 minutes, the final solution was made up to 100 ml using the diluent and labelled as standard stock solution.

2.7 Preparation of Working standard solution of ETS & TXA

Final concentration – ETS-100 µg/ml & TXA-100 µg/ml

To obtain the concentration of 100 µg/ml of ETS & 100 µg/ml TXA, 10 ml of the above prepared Stock Solution was pipetted into 100 ml graduated flask and made up to the final volume and labelled as working standard solution.

2.8 Preparation of sample (tablet) stock solution of ETS & TXA

20 tablets of Etosys (ETS 250 mg & TXA 250 mg) containing ETS and TXA were weighed and crushed into powder.

Final Concentration – ETS-1 mg/ml & TXA-1 mg/ml

Average weight of 20 tablets was calculated and then the powder weight equivalent to ETS & TXA drugs (100 mg ETS & 100 mg TXA) was transferred to 100 ml graduated flask. 70 ml of the diluent was then added to the same flask and sonicated for 10 minutes. The final concentration was made up to 100 ml using the diluent and labelled as sample stock solution.

2.9 Preparation of sample (tablet) working solution of ETS & TXA

Final Concentration – ETS-100 µg/ml & TXA-100 µg/ml

To obtain the final concentration of 100 µg/ml of ETS & TXA, 10 ml of the above prepared Stock Solution was pipetted into 100 ml graduated flask and made up to the final volume and labelled as working sample solution.

2.10 Development and optimization of method with trials

Both ETS & TXA have significant differences in their physical and chemical properties. As a result of which simultaneous estimation of ETS & TXA on the same chromatogram was initially conducted using different mobile phases and columns. By regulating the mobile phase and column trials the chromatographic parameters were optimised for reducing the noise, removing all the interfering and unwanted peaks and to further increase the selectivity and sensitivity of the developed method.

2.11 Method Validation

For simultaneous determination of ETS & TXA the developed method has been validated using various validation parameters viz., Specificity, linearity,

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accuracy, precision, Limit of detection, limit of quantification and robustness as per ICH guidelines Q2 (R1) [14].

2.11.1 **Selectivity/ Specificity**

Selectivity or Specificity is the ability to assess the presence of the analyte of interest which may be present in the sample. Triplicates of blank injections with diluent and injections of ETS & TXA standard and sample solutions (conc. 100µg/ml of ETS & 100µg/ml of TXA) were injected into the HPLC system to assess the selectivity/ specificity of the developed method for the simultaneous determination of ETS & TXA.

2.11.2 **Assay**

The working standard and sample solutions consisting of 100 µg/ml of ETS & 100 µg/ml TXA was considered for assay studies. 20 µl of the solution was injected in triplicates into the HPLC instrument to evaluated the % assay. To calculate the % assay the formula used is mentioned below:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_S}{D_S} x \frac{D_T}{W_T} x \frac{P}{100} x \frac{\text{Avg. Wt}}{\text{Label Claim}} x 100$$

Where:

A_T = Average area counts of Sample preparation

A_S = Average area counts of Standard preparation

W_S = Weight of Working Standard (mg)

D_S = Dilution of standard solution

D_T = Dilution of sample solution

W_T = Weight of sample (mg)

P = Percentage purity of Working Standard

2.11.3 **Linearity & Range**

The desired concentration ranges from 25 µg/ml – 150 µg/ml were prepared in triplicates for both ETS & TXA (tablet) and plotted against the calibration curve. The linearity & range were assessed by establishing slope, correlation coefficient and intercept of the calibration curve.

2.11.4 **Accuracy along with recovery**

❖ **Accuracy Solution Preparation**

2.11.4.1 **Preparation of Placebo**

49 mg of placebo powder was weigh accurately and transferred into a 100 ml graduated flask and made up to the volume with the diluent to get the final concentration 490 µg/ml

2.11.4.2 **Preparation of 50% Standard with placebo**

To obtain the final concentration of 500 µg/ml, 50 mg of ETS and TXA (tablet) were weighed precisely and transferred to 100 ml graduated flask. 1 ml of the above prepared stock solution along with 1 ml of placebo solution were taken in 10 ml graduated flask and made up to the volume with the diluent to get the final concentration of 50 µg/ml of the sample and 49 µg/ml of the placebo sample.

2.11.4.3 **Preparation of 100% Standard with placebo**

To obtain the final concentration of 1000 µg/ml, 100 mg of ETS and TXA (tablet) were weighed precisely and transferred to 100 ml graduated flask. 1 ml of the above prepared stock solution along with 1 ml of placebo solution were taken in 10 ml graduated flask and made up to the volume with the diluent to get the final concentration of 100 µg/ml of the sample and 49 µg/ml of the placebo sample.

2.11.4.4 **Preparation of 150 % Standard with placebo**

To obtain the final concentration of 1500 µg/ml, 150 mg of ETS and TXA (tablet) were weighed precisely and transferred to 100 ml graduated flask. 1 ml of the above prepared stock solution along with 1 ml of placebo solution were taken in 10 ml graduated flask and made up to the volume with the diluent to get the final concentration of 150 µg/ml of the sample and 49 µg/ml of the placebo sample.

Accuracy of analytical procedure is the closeness of the agreement of the true value and the value found.

Triplicate injections of ETS & TXA solution at three different concentrations of 50%, 100% & 150% along with placebo solution were introduced into the HPLC instrument in order to evaluate the accuracy of the developed method & % recovery of the sample.

2.11.5 **Precision – System Precision, Method Precision and Ruggedness (Intermediate Precision)**

2.11.5.1 **System Precision**

Six replicates of ETS & TXA tablet mixture at 100 % concentration were injected into the HPLC system on the same day to assess the system precision.

2.11.5.2 **Method Precision**

Six replicates of ETS & TXA tablet mixture at 100 % concentration were injected into the HPLC system on the same day to assess the method precision.

2.11.5.3 **Intermediate Precision – (Ruggedness)**

Six replicates of ETS & TXA tablet mixture at 100 % concentration were injected into two different HPLC system on two different days with the help of two

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different analysts at two different laboratories to assess the intermediate precision (ruggedness).

2.11.6 **LOD & LOQ**

On the basis of the standard deviation of the response and the slope of the calibration curve, the LOD & LOQ for the developed method of determining ETS & TXA were calculate using the below mentioned formulas:

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

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

Where, σ: The standard deviation of the response;
S: Slope of the calibration curve.

2.11.7 **Robustness**

Variation in the chromatographic conditions such as change in the flow rate and composition of the mobile phase have been made. The evaluation of the resolution, retention time & % RSD values of ETS & TXA (tablet) are calculated to assess the robustness of the developed method. Six replicates of the ETS & TXA mixture at 100% concentration is injected into the HPLC system to evaluate the robustness of the developed method.

2.11.8 **System Suitability**

To measure the system suitability parameters viz., Theoretical plate count, retention time, tailing factor and resolution between ETS & TXA, a blank injection with diluent along with six replicates of ETS & TXA mixture at 100 % concentration were injected into the HPLC system.

2.12 **Force degradation and stability studies of ETS & TXA**

Force degradation studies were carried out under various stress conditions such as acidic, alkaline, neutral, oxidative, thermal and photolytic for the prepared sample solutions under optimized conditions with the blank as control for studying the dissociation of ETS & TXA. By following the ICH guidelines defining the stability studies all the force degradation studies were carried out [15].

To conduct the force degradation studies initial concentration of 1 mg/ml tablet stock solution of ETS & TXA was utilized.

2.12.1 Acidic degradation

Acidic degradation was carried out by adding 10 ml of the above prepared stock solution & 10 ml of 0.1N HCl to a 100 ml graduated flask and refluxed for a time period of 30 minutes at 80 °C temperature. After cooling to the same graduated flask, diluent was added up to the mark to obtain the final concentration of the stress sample. The sample thus prepared is filtered and 10 µl of the same is injected onto the HPLC system as triplicates for establishing the stability of the sample.

2.12.2 Alkaline degradation

Alkaline degradation was carried out by adding 10 ml of the above prepared stock solution & 10 ml of 0.1N NaOH to a 100 ml graduated flask and refluxed for a time period of 30 minutes at 80 °C temperature. After cooling to the same graduated flask, diluent was added up to the mark to obtain the final concentration of the stress sample. The sample thus prepared is filtered and 10 µl of the same is injected onto the HPLC system as triplicates for establishing the stability of the sample.

2.12.3 Neutral degradation

Neutral degradation was carried out by adding 10 ml of the above prepared stock solution & 10 ml of distilled water to a 100 ml graduated flask and refluxed for a time period of 30 minutes at 80 °C temperature. After cooling to the same graduated flask diluent was added up to the mark to obtain the final concentration of the stress sample. The sample thus prepared is filtered and 10 µl of the same is injected onto the HPLC system as triplicates for establishing the stability of the sample.

2.12.4 Oxidative degradation

Oxidative degradation was carried out by adding 10 ml of the above prepared stock solution & 10 ml of 3% H_2O_2 to a 100 ml graduated flask and refluxed for a time period of 30 minutes at 80 °C temperature. After cooling to the same graduated flask diluent was added up to the mark to obtain the final concentration of the stress sample. The sample thus prepared is filtered and 10 µl of the same is injected onto the HPLC system as triplicates for establishing the stability of the sample.

2.12.5 Thermal degradation

Thermal degradation was carried out by adding 10 ml of the above prepared stock solution to a 100 ml graduated flask and place it in the oven at 105 °C temperature and refluxed for a time period of 30 minutes. After cooling to the same graduated flask diluent was added up to the mark to obtain the final concentration of the stress sample. The sample thus prepared is filtered and 10 µl of the same is injected onto the HPLC system as triplicates for establishing the stability of the sample.

2.12.6 Photolytic degradation

Photolytic degradation was carried out by adding 10 ml of the above prepared stock solution to a 100 ml

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graduated flask and place it in UV chamber for a time period of 4 hours for photo stability testing. After 4 hours to the same graduated flask diluent was added up to the mark to obtain the final concentration of the stress sample. The sample thus prepared is filtered and 10 µl of the same is injected onto the HPLC system as triplicates for establishing the stability of the sample.

3. Results and discussion

3.1 Method development and optimization

The present study focuses on the development of simple, rapid, cost effective, accurate, precise and stability indicating RP-HPLC method for the simultaneous estimation of ETS & TXA with acceptable resolution and quick retention time. To optimize the chromatographic conditions viz., selection of wavelength, stationary phase with different column dimensions and mobile phase ratios, different initial trials were carried out. As the wavelength selection is carried out, the combined UV Spectrum of ETS & TXA is shown in the Fig 3. The outcome of the optimized chromatographic conditions and their respective chromatograms are shown in the Figs. 4-7.

3.1.1 Optimization of the method

3.1.1.1 Determination of λ max of ETS & TXA

- UV determination was carried out by Shimadzu 1800 instrument.
- Accurately weighed 100 mg of ETS into a 100 ml graduated flask and add 70 ml of diluent for dissolving the drug and make up the volume up to the mark with the diluent. 1ml was pipetted out from the prepared solution and transferred to 10 ml graduated flask and made up the volume up to the mark with the diluent to get a concentration of 100 µg /ml of ETS.
- Accurately weighed 100 mg of TXA into a 100 ml graduated flask and add 70 ml of diluent for dissolving the drug and make up the volume up to the mark with the diluent. 1ml was pipetted out from the prepared solution and transferred to 10 ml graduated flask and made up the volume up to the mark with the diluent to get a concentration of 100 µg /ml of TXA.
- Both the drugs are scanned in the UV-VIS Spectrophotometer and the Isosbestic point was determined by overlaying the spectrum of ETS & TXA at 249 nm.

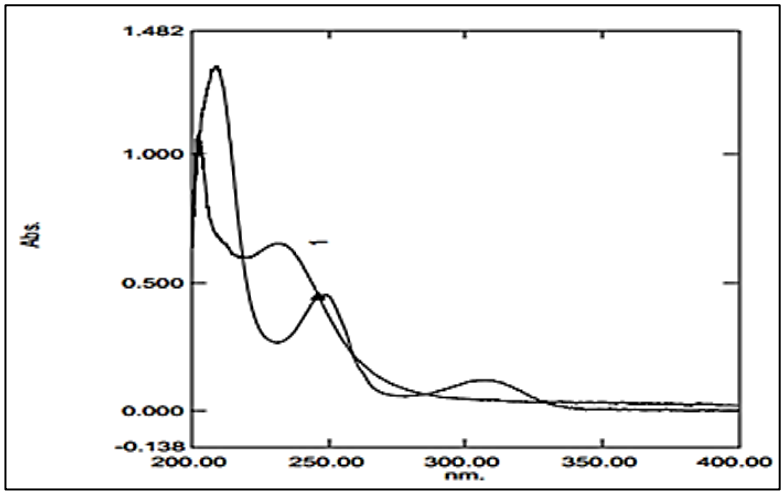

Figure 3. Combined UV Spectrum of ETS & TXA

3.1.2 Optimized chromatographic conditions and their respective chromatograms

Optimized Conditions of ETS & TXA

Mobile Phase	: KH_2PO_4 (pH:3.1): Acetonitrile (80:20)
Flow Rate	: 1.0 ml/min
Column	: ALTIMA, C18 (150 X 4.6 X 5µm)
Column Temperature	: Ambient
Volume	: 20µl

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Sample Temperature	: Ambient
Retention Time	: 2.353 & 3.033
Run Time	: 6 min
Detector	: PDA

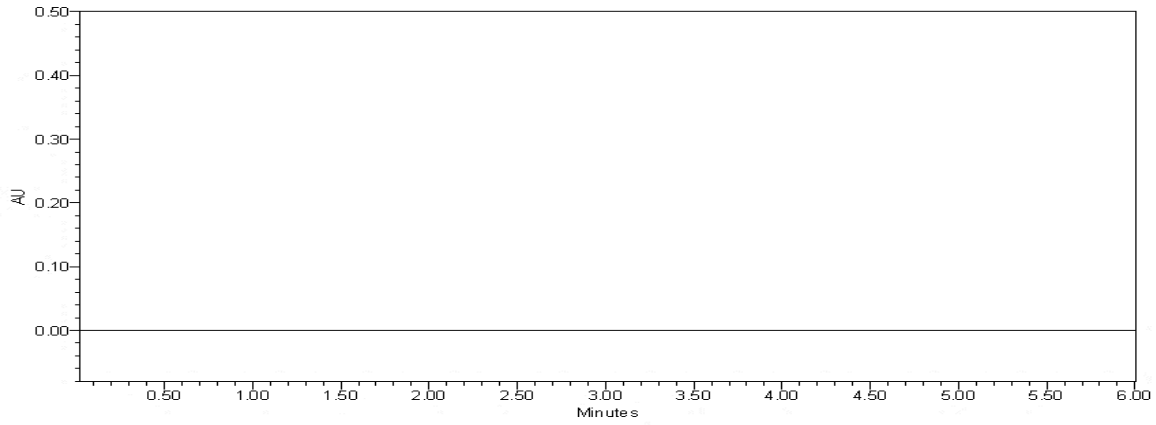

Figure 4. The Chromatogram of Blank

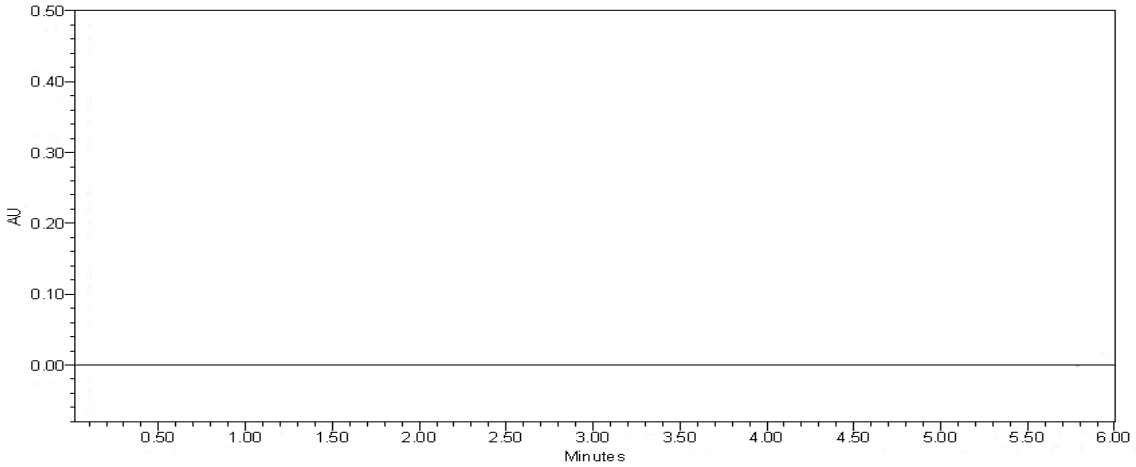

Figure 5. The Chromatogram of Placebo

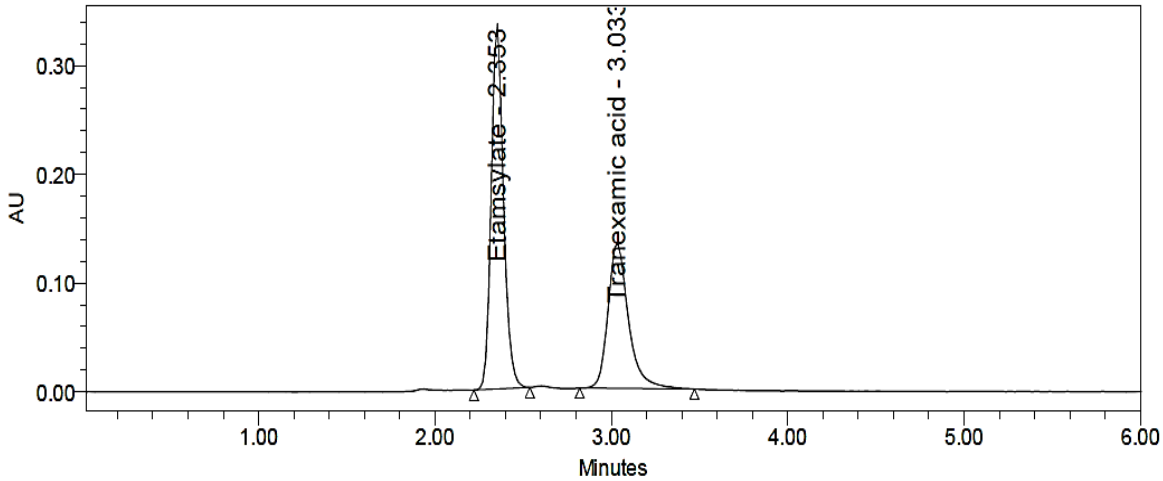

Figure 6. Typical Chromatogram of Sample ETS & TXA

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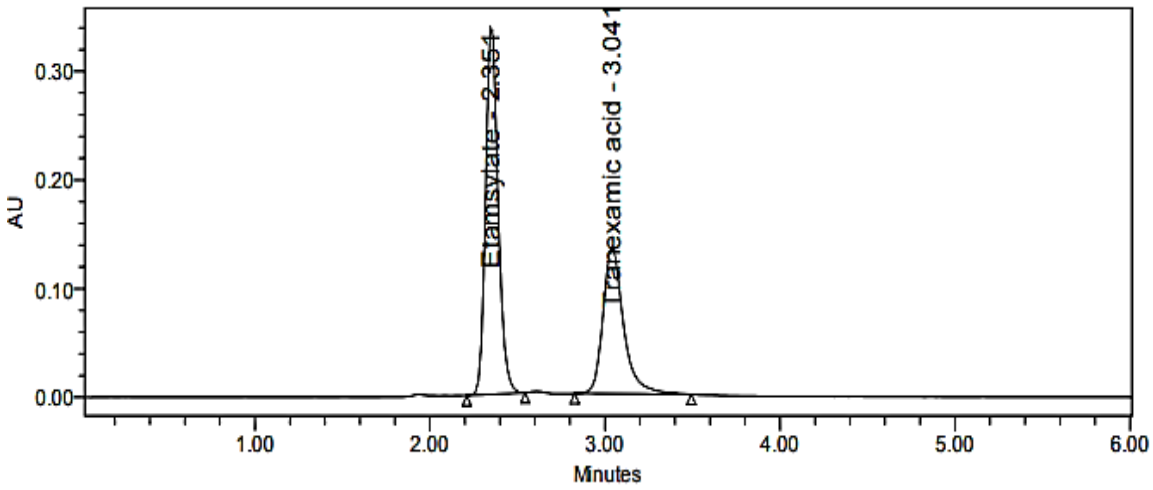

Figure 7. Typical Chromatogram of Standard ETS & TXA

3.2 Method validation and its corresponding parameters

The above developed and optimized method is validated using various validation parameters viz., specificity, assay, linearity & range, accuracy, precision, LOD, LOQ, robustness & system suitability as per ICH guidelines Q2 (R1).

3.2.1 Selectivity / Specificity

To evaluated the selectivity/ specificity of the developed analytical method, the optimized conditions were applied to detect the standard & sample solutions (in triplicates) of ETS & TXA with reference to the blank & placebo samples. The method which is developed is found to be selective for ETS & TXA. The values obtained for selectivity/ specificity are shown in the Table 1.

Table 1. Selectivity / Specificity

S. No.	Injection (n=3)	RT of analyte	Remarks
1.	Blank	-	No compound found in blank
2.	Placebo	-	No compound found in placebo
3.	Sample ETS & TXA	2.353, 3.033	RT of Sample ETS & TXA values are not interfering with each other and with the blank sample Resolution was good for both ETS & TXA peaks
4.	Standard ETS & TXA	2.351, 3.041	RT of Standard ETS & TXA values are not interfering with each other and with the blank sample Resolution was good for both ETS & TXA peaks

3.2.2 Assay

Triplicate injections of 100 µg/ml of ETS & 100 µg/ml TXA were injected into the system and % Assay, SD & % RSD values were calculated for both ETS & TXA. The assay results are shown in the Table 2.

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Table 2. Assay report of tablet dosage of ETS & TXA

Drug Name	% Assay (n=3)	SD (n=3)	% RSD (n=3)
Ethamsylate	100.02	0.42	0.4
Tranexamic acid	99.73	0.8	0.8

3.2.3 Linearity & Range

The linearity was evaluated by injecting samples in triplicates from low concentration to high concentration into the HPLC system and determining the calibration curve for ETS & TXA. The drug concentration ranges for ETS ranged from 25 µg/ml – 150 µg/ml and for TXA it ranged from 25 µg/ml – 150 µg/ml. The peak area of each concentration was measured to calculate correlation coefficient which was found to be 0.9993 & 0.9998 for ETS & TXA respectively. The method which is developed is found to be linear and within the range for ETS & TXA. The values are shown in the Table 3 and Figs. 8 & 9.

Table 3. Linearity & Range of ETS & TXA

S. No.	Linearity Levels	ETS		TXA	
		Concentration (µg/ml) (n=3)	Peak area (Mean)	Concentration (µg/ml) (n=3)	Peak area (Mean)
1.	I	25	402470	25	261869
2.	II	50	861009	50	551532
3.	III	75	1253144	75	793527
4.	IV	100	1636000	100	1074644
5.	V	125	2112250	125	1340745
6.	VI	150	2473244	150	1603286
Correlation coefficient		**0.9993**		**0.9998**	

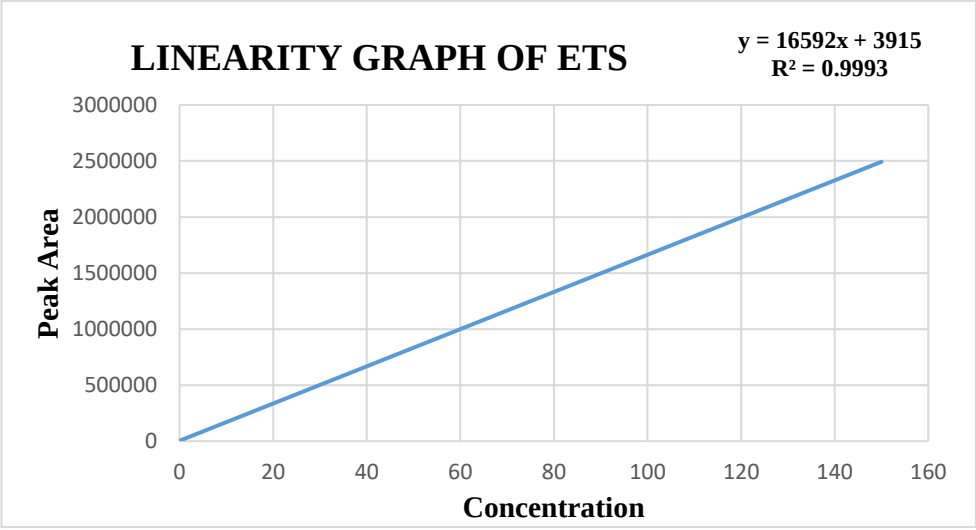

Figure 8. Linearity & Range graph of ETS

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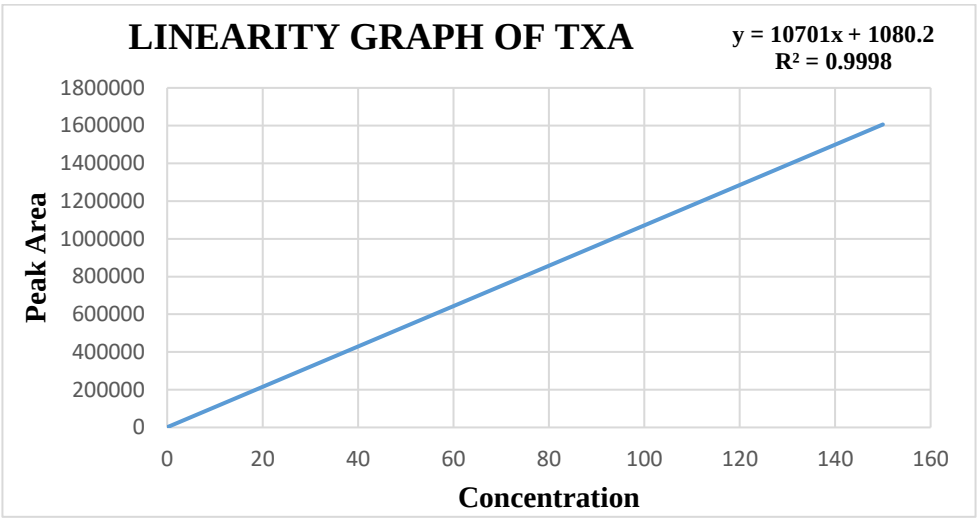

Figure 9. Linearity & Range graph of TXA

3.2.4 **Accuracy along with recovery**

Accuracy and recovery studies were evaluated at three different concentrations such as 50%, 100% & 150% in triplicate injections. The method which is developed is found to be accurate for ETS & TXA estimation. The accuracy along with recovery results are mentioned in the Table 4.

Table 4. Accuracy and Recovery report

Drug name	Amount considered (µg/ml)	Recovery level	Amount added (µg/ml) (n=3)	Placebo added (µg/ml) (n=3)	Amount recovered (µg/ml)	% Recovery (n=3)	Acceptance Criteria
ETS	100	50%	50	49	50.375	100.75	98-102%
					50.06	100.12	
					49.98	99.69	
					Mean	**100.18**	
					SD	**0.53**	
					% RSD	**0.53**	
	100	100%	100	49	98.48	98.48	98-102%
					101.27	101.27	
					99.98	99.98	
					Mean	**99.91**	
					SD	**1.40**	
					% RSD	**1.40**	
	100	150%	150	49	149.05	99.37	98-102%
					149.01	99.34	
					150.99	100.66	
					Mean	**99.79**	
					SD	**0.75**	
					% RSD	**0.75**	
TXA	100	50%	50	49	50.295	100.51	98-102%
					49.94	99.88	
					49.595	99.19	
					Mean	**99.86**	

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				SD	**0.66**	
				% RSD	**0.66**	
100	100%	100	49	99.78	99.78	98-102%
				100.77	100.77	
				100.95	100.95	
				Mean	**100.5**	
				SD	**0.63**	
				% RSD	**0.62**	
100	150%	150	49	149.7	99.80	98-102%
				151.095	100.73	
				151.035	100.69	
				Mean	**100.40**	
				SD	**0.52**	
				% RSD	**0.51**	

3.2.5 **Precision – System Precision, Method Precision and Ruggedness (Intermediate Precision)**

To evaluate the precision, the peak areas of the injected six replicates of ETS & TXA mixture were evaluated. The results for system precision, method precision & ruggedness (intermediate precision) data are calculated in the form of % RSD. The method which is developed is found to be precise for ETS & TXA estimation. The values obtained for the system precision, method precision & ruggedness (intermediate precision) are shown in the Tables 5 – 8.

Table 5. System precision results for both ETS & TXA

Injection No. (100 µg/ml)	**Values obtained for ETS Peak area (n=6)**	**Values obtained for TXA Peak area (n=6)**
1.	1679824	1115280
2.	1679824	1113611
3.	1690212	1109345
4.	1679701	1099931
5.	1685616	1099931
6.	1684543	1100121
Mean	**1683287**	**1106370**
SD	**4284.89**	**7247.65**
% RSD	**0.25**	**0.65**

Table 6. Method precision results for both ETS & TXA

Injection No. (100 µg/ml)	**Values obtained for ETS Peak area (n=6)**	**% Assay**	**Values obtained for TXA Peak area (n=6)**	**% Assay**
1.	1680342	99.43	1092248	98.33
2.	1695290	100.30	1104626	98.44
3.	1694731	100.28	1111226	100.04
4.	1681792	99.51	1117087	100.56

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5.	1693564	100.21	1114837	100.36
6.	1696247	100.37	1106892	99.65
Mean	**1690328**	**100.02**	**1107819**	**99.73**
SD	**7240.1**	**0.4284**	**8947.2**	**0.80**
% RSD	**0.42**	**0.42**	**0.80**	**0.80**

Table 7. Ruggedness (intermediate precision) results of ETS

Injection No	**Concentration (µg/ml) (n=6)**	**Laboratory-1, Analyst-1 & HPLC-1 (% Assay)**		**Laboratory-2, Analyst-2 & HPLC-2 (% Assay)**	
		Day-1	**Day-2**	**Day-1**	**Day-2**
1.	100	100.983	99.521	100.976	100.150
2.	100	100.399	100.566	100.751	100.110
3.	100	100.616	99.34	100.463	100.600
4.	100	100.404	100.01	99.03	99.65
5.	100	100.759	100.67	100.060	100.140
6.	100	100.981	100.751	100.389	100.467
Mean		**100.690**	**100.143**	**100.27**	**100.18**
SD		**0.26**	**0.61**	**0.68**	**0.33**
% RSD		**0.25**	**0.60**	**0.67**	**0.33**

Table 8. Ruggedness (intermediate precision) results of TXA

Injection No	**Concentration (µg/ml) (n=6)**	**Laboratory-1, Analyst-1 & HPLC-1 (% Assay)**		**Laboratory-2, Analyst-2 & HPLC-2 (% Assay)**	
		Day-1	**Day-2**	**Day-1**	**Day-2**
1.	100	100.933	99.541	99.80	100.05
2.	100	100.349	100.562	100.23	100.08
3.	100	100.442	99.14	100.422	100.10
4.	100	100.410	100.23	99.01	99.25
5.	100	100.751	100.01	100.071	100.03
6.	100	100.859	100.555	100.546	100.320
Mean		**100.624**	**100.006**	**100.013**	**99.97**
SD		**0.25**	**0.57**	**0.55**	**0.36**
% RSD		**0.25**	**0.57**	**0.55**	**0.36**

3.2.6 LOD & LOQ

The LOD & LOQ were calculated for ETS & TXA. The limit of detection was found to be 0.78 µg/ml for ETS and 0.33 µg/ml for TXA respectively. The limit of quantification was found to be 2.36 µg/ml for ETS and 1.01 µg/ml for TXA respectively. The LOD & LOQ values are shown in the Table 9 and the Chromatograms are shown in the Figs. 10 & 11 respectively.

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Table 9. Limit of Detection & Limit of Quantification for ETS & TXA

Drug name	SD	Slope	LOD (µg/ml)	LOQ (µg/ml)
ETS	3915	16592	0.78	2.36
TXA	1080	10701	0.33	1.01

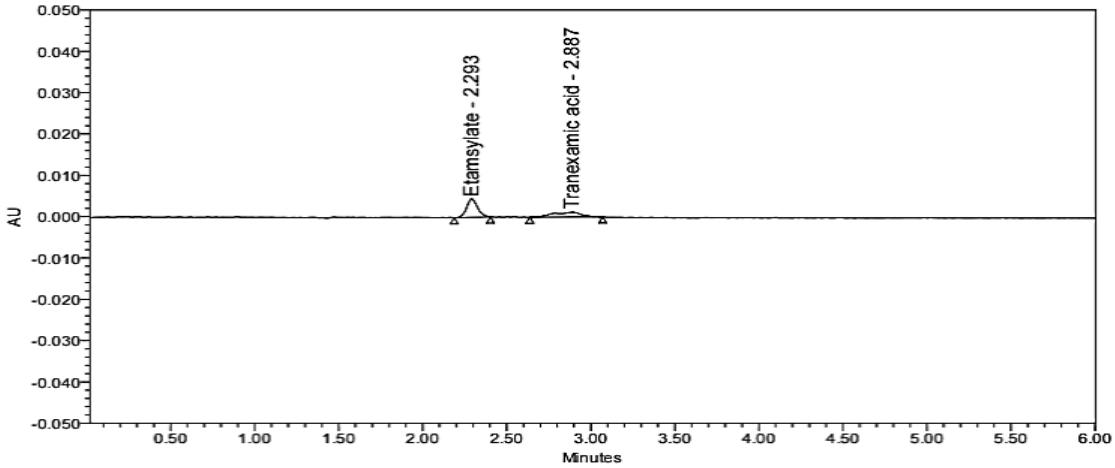

Figure 10. LOD Chromatogram of ETS & TXA

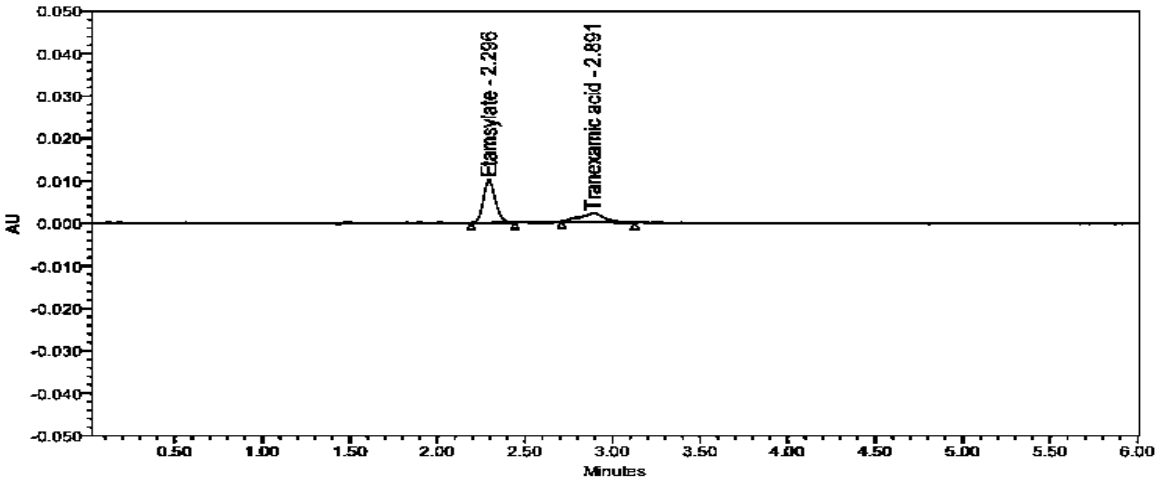

Figure 11. LOQ Chromatogram of ETS & TXA

3.2.7 **Robustness**

There were no significant changes which were observed in the developed stability indicating method when the chromatographic conditions viz., Change in the flow rate & Composition of mobile phase were calculated for six injections of ETS & TXA samples. Therefore, the method which was developed was found to be robust for ETS & TXA determination. The robustness values and the % RSD results are mentioned in the Table 10.

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Table 10. Robustness values of ETS & TXA

Parameters	Optimized conditions	Chromatographic conditions	ETS			TXA			Remarks
			Peak Area (n=6)	SD	% RSD	Peak Area (n=6)	SD	% RSD	
Flow Rate	1.0 ml/min	0.9	1910031	4686.6	**0.24**	1150773	6357	**0.55**	Robust
		1.0	1683287	4284.9	**0.25**	1106370	7247.6	**0.65**	--
		1.1	1717216	3301.8	**0.19**	1061202	5577.2	**0.52**	Robust
Mobile phase composition	80:20	72:28	1733623	1698.6	**0.10**	1015988	7247.6	**0.71**	Robust
		80:20	1683287	4284.9	**0.25**	1106370	7247.6	**0.65**	--
		88:12	1679212	5861.0	**0.34**	1003563	9784.4	**0.97**	Robust

3.2.8 System Suitability

To evaluate the system suitability six replicates of 100 % concentration of the sample were injected onto the HPLC ALTIMA C18 column with the dimensions of 150 mm column length x 4.6 mm internal diameter x 5µm particle size; carried forward with the help of the mobile phase Phosphate Buffer with pH adjusted at 3.1 & Acetonitrile in the ratio 80:20 v/v with the flow rate of 1.0 ml /min. The values obtained for USP plate count, tailing factor, retention times & resolution are shown in the Table 11.

Table 11. System suitability results of ETS & TXA

S. No	Parameters	Values obtained for ETS (n=6)	Values obtained for TXA (n=6)	Acceptance criteria
1.	Retention time	2.349	3.038	Resolution was good
2.	USP Plate count (N)	5070	3473	> 2000
3.	Tailing factor (T)	1.18	1.36	≤ 2.0
4.	USP Resolution	3.8		> 1.5

3.3 Force degradation and stability studies of ETS & TXA

Both ETS & TXA were subjected to force degradation conditions viz., acidic, alkaline, neutral, oxidation, photolytic. The stress sample of all the degradations were diluted with the prepared diluent to obtain a final concentration of 100 µg/ml. The % degradation & % recovery both are calculated and the values are shown in the Table 12 & Fig 12 respectively.

Table 12. Force degradation and Stability studies of ETS & TXA

Type of degradation	Time (Hrs. /Min)		Peak area Mean ± S.D (n=3)	ETS		Peak area Mean ± S.D (n=3)	TXA	
		Temp (°C)		% Recovered	% Degraded		% Recovered	% Degraded

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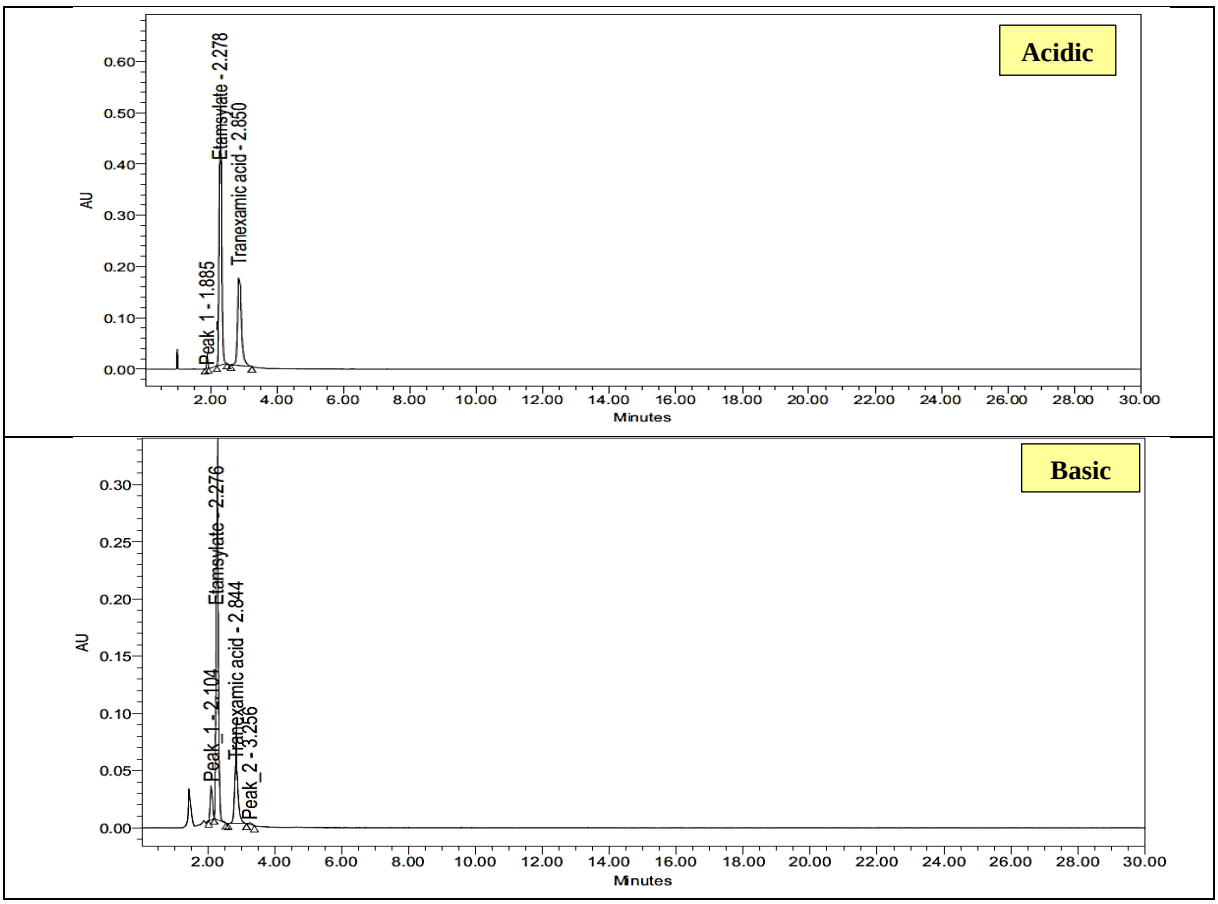
Acid (0.1 N HCl)	30 min	80ºC	1580164	93.50	6.5	1044058	93.99	6.01
Base (0.1 N NaOH)	30 min	80ºC	1542927	91.29	8.71	1054941	94.97	5.03
Neutral	30 min	80ºC	169692	99.39	0.61	1099852	99.01	0.99
Peroxide	30 min	80ºC	1597978	94.55	5.45	1054261	94.91	5.09
Thermal	30 min	105ºC	1621854	95.97	4.03	1063636	95.75	4.25
Photolytic	4 hrs	--	1653273	97.82	2.18	1086633	97.82	2.18



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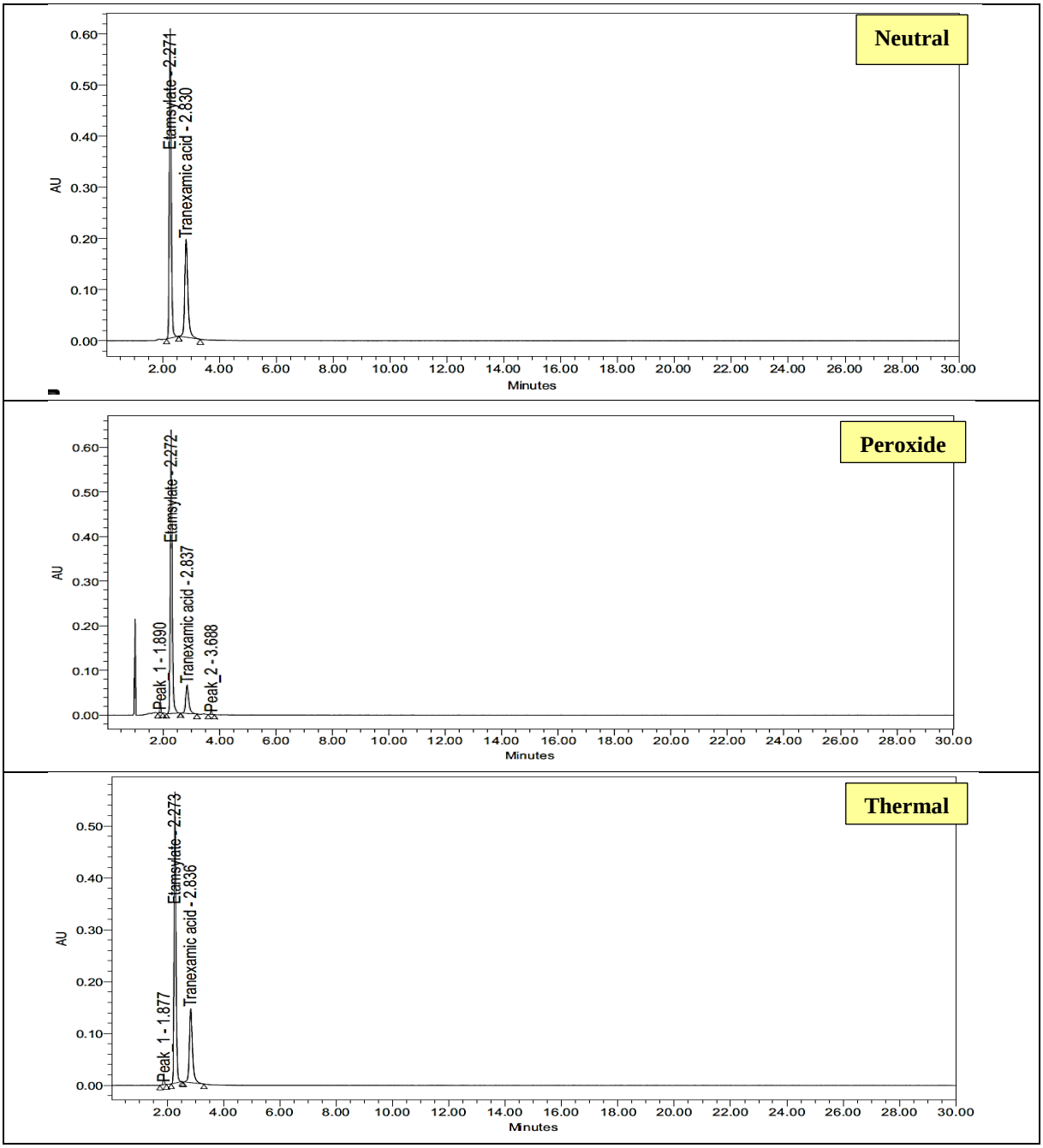

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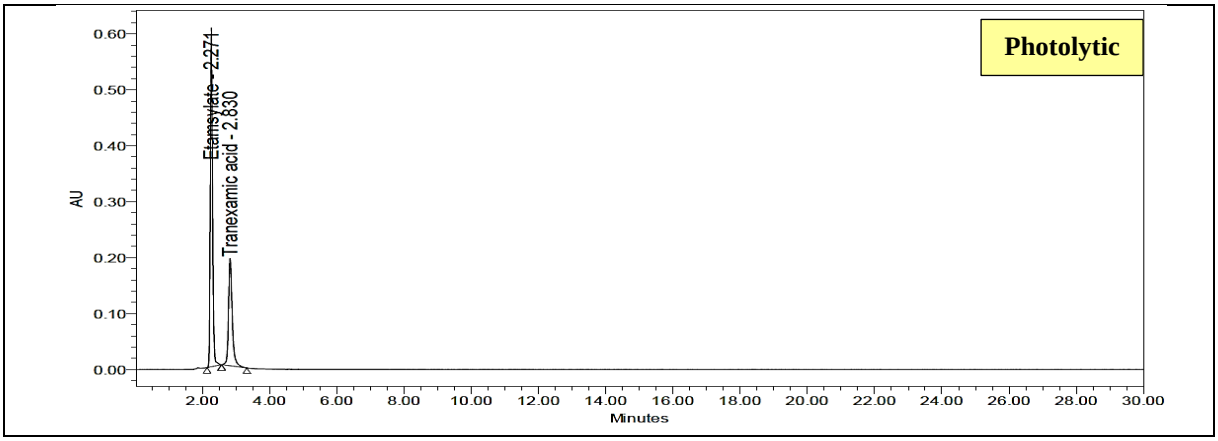

Figure 12. Chromatograms of Force degradation and stability studies of ETS & TXA

3.4 Green Evaluation of the developed method using AGREE & GAPI tools

The primary areas of interests among the analytical chemists are environmental safety and sustainability. As regular application of analytical procedures during research and quality control activities results in significant amount of long-lasting environmental concerns. For this reason, evaluation of the ecological impact of newly developed analytical procedure has become an important aspect. For green evaluation several criteria have been documented over the past ten years. [16-17] The most frequently and efficiently used metrics are AGREE & GAPI. AGREE metrics consists of a clockwise shaped pictogram and its perimeter is divided into 12 areas representing 12 principle of Green Analytical Chemistry (GAC). The middle part of the pictogram shows a score number from 0-1. The value closer to 1 indicates greenness of the developed method. In the current study, the developed method has a total score of 0.71 for ETS & 0.71 for TXA respectively. Taking in consideration all the 12 principles the total score for both the drugs is represented as environmental friendly and the AGREE metrics in the shape of the pictograms for the drugs ETS & TXA are shown in the Fig. 13.

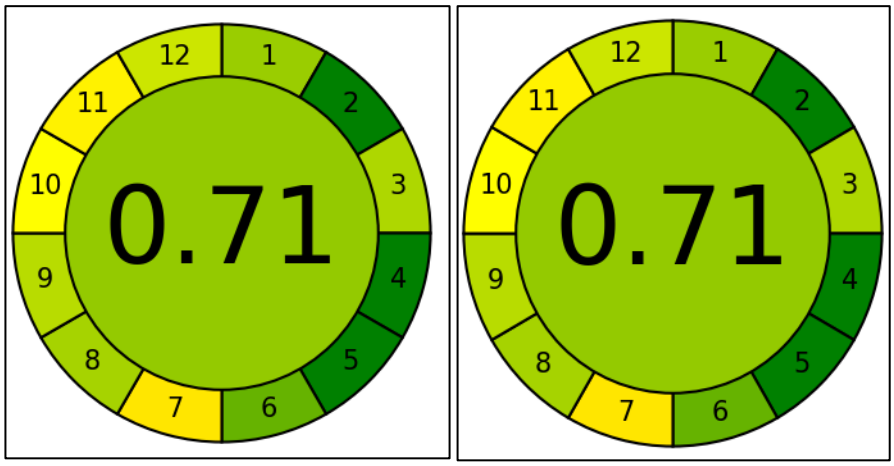

Figure 13. AGREE Metrics for ETS & TXA of the developed method

One of the early evaluation techniques among all the metrics is the GAPI. Five pentagons make up the GAP pictogram. The five pentagons represent five key steps along the analytical methodology, starting with sample approach, sample preparation, reagents and compounds used, Instrumentation and General method type which are further divided into 15 various areas which represent a stage of the analytical process [18-

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20]. The GAP pictogram is represented in the form of colours like red, yellow & green evaluating the nature of the analytical procedure used for the sample approach to interpretation of the results. The GAPI metrics for both the drugs is shown in the Fig 14. Three of the GAPI pictogram which are in red zone indicate offline collection of the sample, transportation of the sample & use of Acetonitrile solvent.

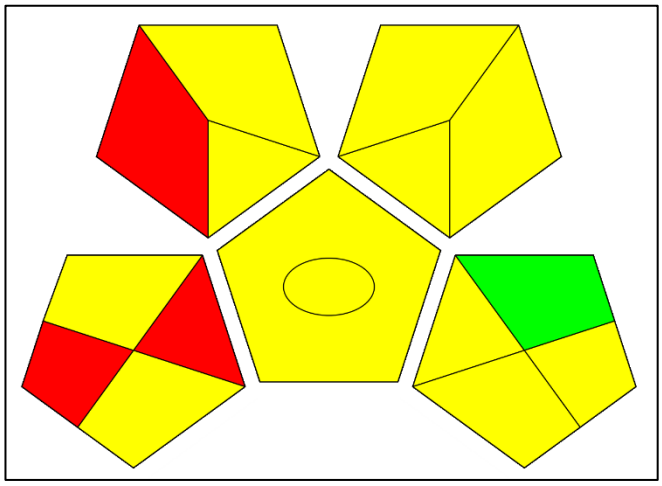

Figure 14. GAPI Evaluation for the developed method

3.5 **Summary of the optimised validation parameters**

The validation results along with the acceptance criteria for ETS & TXA for all the conducted validation parameters and their results are mentioned in the Table 13.

Table 13. Optimised Validation parameters table for ETS & TXA

Parameters			**ETS**	**TXA**	**Acceptance Criteria**
Specificity	**Retention time**		2.353	3.033	Specific
Assay	**% Assay**		100.02	99.73	98-102%
	% RSD		0.4	0.8	< 2%
Linearity & Range	**Concentration (µg/ml)**		25-150	25-150	Linear
	Correlation coefficient		0.9993	0.9998	$r^2 \geq 0.998$
Accuracy	**50 % (% Recovery)**		100.18%	99.86%	98-102%
	100 % (% Recovery)		99.91%	100.5%	98-102%
	150 % (% Recovery)		99.79%	100.40%	98-102%
Precision	**System Precision (% RSD)**		0.25	0.66	< 2%
	Method Precision (% RSD)		0.42	0.80	< 2%
	Ruggedness (Intermediate precision) (% RSD)		0.25-0.67	0.25-0.57	< 2%
LOD (µg/ml)			0.78	0.33	LOD<LOQ
LOQ (µg/ml)			2.36	1.01	LOQ>LOD
Robustness	**Flow rate (ml/min) (% RSD)**	**0.9**	0.24	0.55	Robust
		1.1	0.19	0.52	Robust

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	Mobile phase composition (% RSD)	**72:28**	0.10	0.71	Robust
		88:12	0.34	0.97	Robust
System suitability	**Retention time**		2.349	3.038	Resolution was good
	USP Plate count (N)		5070	3473	> 2000
	Tailing factor (T)		1.18	1.36	≤ 2.0
	USP Resolution		3.8		> 1.5

4. CONCLUSION

Conventional HPLC methods are mostly used in pharmaceutical analysis which have increased negative impact on the environment. To reduce the impact on the environment, conventional use must be minimised and green methods must be focused. The present study focuses on the development and validation of RP-HPLC method for simultaneous determination of Ethamsylate and Tranexamic acid along with stability studies in combined dosage form using green assessment tools like GAPI & AGREE. The developed, optimised and validated method is economic, environment safe, simple, quick, precise, accurate, robust etc. This is the first method which has been reported with green analysis of the developed and validated method for estimation of ETS & TXA in combined form with good resolution, fast elution time and good peak resolution. The present developed method can be easily applied for quality control tests and routine analysis of ETS & TXA in combined pharmaceutical formulations.

Conflicts of Interest

The authors declare no conflicts of interest.

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