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DEVELOPMENT AND VALIDATION OF A STABILITY INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF TRANEXAMIC ACID AND ETHAMSYLATE IN BULK AND TABLET DOSAGE FORM

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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 ALLIANCE C18 column with the dimensions of 250 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 250 nm. At room temperature Ethamsylate and Tranexamic acid were found to have retention times of 2.522 & 4.739 minutes respectively with a total run time of 7 minutes. 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 established method can be applied for the simultaneous determination of ETS & TXA in combined dosage form.

Keywords: *ETS (Ethamsylate), TXA (Tranexamic acid)* ***ACN****(Acetonitrile).*

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INTRODUCTION:

Chromatography: [1-3]

For the separation of mixtures, chromatography is a family of analytical chemistry procedures. There is a "stationary phase" in which the mixture containing the analyte is passed via a channel in which the specimen is in the "mobile phase." Components of the specimen are slowed down by the stationary phase. Components are split in time like marathon runners as they go through the system at various paces. Each part of the system should, in theory, have a certain time it takes to go through it. "Retention time" is the term for this.

One phase is immobile, while another passes across it in a certain direction. This is a physical isolation technique that uses variances in the distribution of a mixture between the two phases to extract the constituents. In order to maintain and separate the chemicals, the stationary phase must have some kind of interaction with them.

A chromatograph separates a chemical mixture into its component parts as solutes flow around or over a stationary liquid or solid phase. Because of the varying affinities that various chemicals have for a gaseous or liquid movable medium and a stationary adsorbing media in use today, adsorbing mediums like as paper, gelatin or magnesium silicate gel are utilized in a variety of ways to separate complicated mixtures. Individual molecules in a solution may be identified and quantified using analytical chromatography.

This approach may be used to purify molecular species.

Types of Chromatography:

- ❖ Depending on the chromatographic modes
  - Normal phase
  - Reverse phase
- ❖ Depending on principles of isolation chromatography is of
  - Adsorption
  - Ion exchange
  - Ion pair
  - Size exclusion
  - Affinity
  - Chiral phase
- ❖ Based on the elution process
  - Iso critic isolation
  - Gradient isolation
- ❖ Depending on the scale of operation
  - Analytical HPLC
  - Preparative HPLC

Depending on modes of chromatography:

Normal Phase HPLC:

The stationary step and mobile step in normal-phase liquid-liquid extraction are both non-polar. Heptane's pure hydrocarbon mobile phase is a good place to start when looking for the best mobile phase. Adding methanol or dioxin to the mobile phase may help enhance the polarity if the sample is heavily retained. Alcohol/heptane has been used as the mobile phase in oil-soluble vitamin, essential oil, nitro phenol, and other homologous series separations in the normal phase mode. Chiracel OJ and Chiracel OD are chiral separation columns used in normal phase chromatography.

Reverse Phase HPLC:

A polar mobile step, frequently a partly or completely water-based mobile phase, is used in reverse phase chromatography, which often employs hydrophobic bonded packing.

Polar compounds elute first in the movable phase. Retention improves with increasing solute hydrophobicity. The greater the eluent strength, the lower the movable phase's polarity should be. Compound classes in the table are eluted in reverse order (thus the name reverse-phase chromatography).

A simple definition of pharmaceutical analysis is the study of drugs. A pharmaceutical, according to Webster's, is a prescription medication. Active pharmaceutical ingredient (API) is the better phrase to use when describing a drug component that has been combined with inert ingredients (excipients) in order to create an acceptable medication for administration to patients. To make sure a new medication product fulfils the set criteria, R&D plays a critical role in its development and follow-up, ensuring that all batches of the drug product are created in accordance with the particular requirements. Medicinal assessors in the QC or QA department are accountable for the use of permitted substances and production techniques. R&D departments typically develop processes and then transfer them to QC or other departments as needed. Sometimes, they may be moved to separate divisions. It is crucial for pharmacological analyzers to make sure that drugs are correctly identified, safe, efficacious, and of high quality.

Studies on safety and efficacy demanded that medication substance and medication product satisfy 2 crucial conditions.

1. A clear sense of self and purity.
2. Biological availability/dissolution has been established.

HPLC NP vs RP Comparison:

Table.1: Comparison of Normal Phase and Reverse Phase HPLC

Properties	NP	RP
Parallelism in Phase Polarity	High	Low
Movable step Polarity	Low to medium	Low to high
Specimen elution order	Least polar First	Most polar First
The rate of retention will get increased by	Increasing the stationary phase's surface area Increasing the stationary phase's n-alkyl chain length. The polarity of the mobile phase is decreasing. The polarity of the sample Molecules are being increased.	Increasing the stationary phase's surface. Increased mobility in the phase diagram Sample molecule polarity is waning.

Basic principle of HPLC:

- HPLC is a isolation process that makes use of variations in the distribution of chemicals in two phases, referred to as the stationary step and the movable phase.
- Stationary phases are thin layers formed on the surface of small particles, whereas movable phases are liquids that move across those layers. Depending on solubility and/or molecular size, each component in a specimen has a various equilibrium distribution.
- As a result, during the stationary time, the components move at different rates and are consequently separated from one another.
- Metal or resin tubing is used to hold the sphere-shaped solid particles.
- Consistent mobile phase delivery is ensured by an on-demand liquid pump. To supply a sample, a specimen injector is located near the column inlet.
- When a specimen is introduced into the column, its components move between the stationary and the movable phases, resulting in the separation.
- It is only during the mobile phase that compounds travel along the column. As a result, mobile phase-distributed compounds move across the column more quickly whereas stationary phase-distributed compounds move more slowly.
- Thus, each component is isolated and elutes successively from the column.

Selectivity of HPLC-Method Development:

HPLC-method may be used to evaluate the majority of pharmaceuticals because of its speed, specificity, accuracy, and precision, as well as the fact that it can be automated, which removes the need for time-consuming extraction and isolation processes. Some of the benefits include:

- Effortless.
- Sensitivity has increased
- Enhanced clarity
- Reliable columns

✓ Suitable for low-volatility compounds
✓ Recovering, managing, and maintaining samples are made simple.

Method Development Guide:

Defining separation objectives based on sample information.

↓

Special procedures and sample handling are required. Decide on the detector and the detector settings.

↓

Select a method of payment: preliminary run: determine the ideal separation conditions.

↓

Optimize the circumstances for separation.

↓

Verify the procedure.

Table.2: HPLC Parameters limits

S.no	Parameters	Recommendations
1	Theoretical plates(N)	>2000
2	Tailing factor(T)	2
3	Resolution(Rs)	>2 between peak of interest and the closest Eluting potential interference
4	Repeatability	RSD 2%
5	Capacity factor (k^1)	>2.0

Table.3: Performance characteristics to be considered during the validation of a quantitative method in analysis.

Method parameters	Short description
Precision	Random error of the method
Repeatability	Precision measured under the best condition possible (short period, one analyst.)
Intermediate Precision	Precision measure of the within-laboratory variation due to different days, analysts, equipment's, etc.
Robustness	Capacity of a method to remain unaffected by small variations in the method parameters as could Possibly occur during the normal use of the method (pH,mobile Phase composition,)
Reproducibility	Precision measure determined by inter-laboratory studies
Specificity	Ability to determine the analyte in presence of other compounds
Limit of detection	Lowest sample concentration that can be detected
Limit of quantitation	Lowest sample concentration that can be quantified with suitable bias and precision
Linearity	Ability of the method to obtain test results which are proportional to the concentration in the sample

Limit of Detection:

These limits are normally applied to related substances in the drug substance or drug product. Specifications on these limits are submitted with the regulatory impurities method relating to release and stability of both drug substance and drug product[8].

Limit of detection is the lowest concentration of analyte in a sample that can be detected, but not necessarily Quantified, under the stated experimental conditions. With UV detectors, it is difficult to assure the detection precision of low-level compounds due to potential gradual loss of sensitivity of detector lamps with age, or noise level variation by detector manufacturer.

A crude method to evaluate the feasibility of the extraneous peak detection is to use the percentage claimed for detection limit from the area counts of the analyte. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

- Based on Visual Evaluation
- Based on Signal-to-Noise
- Based on the Standard Deviation(SD)of the Response and the Slope The Limit of detection limit **(LOD)** may be expressed as:

Where, σ=the standard deviation of the response

$$LOD = 3.3\sigma/S$$

S = the slope of the Calibration curve

The slope S may be estimated from the Calibration curve of the analyte.

Limit of Quantification:

Limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental [9].

- Based onVisual Evaluation
- Based on Signal-to-Noise Approach
- Based on the SD of the response and the slope

The Limit of Quantitation **(LOQ)** may be expressed as:

$$LOQ = 10\sigma/S$$

Where, σ=the standard deviation of the response

S = the slope of the Calibration curve

Robustness:

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters." A good practice is to vary, important parameters in the method, systematically and measure their effect on separation.

Ruggedness:

Degree of reproducibility of test results obtained by the analysis of the same samples under a variety of condition such as different laboratories, different analysts, different instruments etc, normally expressed as the lack of influence on test results of operational and environmental variable of the analytical method. Ruggedness is a measurement of reproducibility of test results under the variation in condition normally expected from laboratory to laboratory and from analyst to analyst.

Stability:

To generate reproducible and reliable results, the samples, standards, and reagents used for the UV method must best able for a reasonable time (e.g.,one day,one week,and one month, depending on need). For example, the analysis of even a single sample may require 10 or more Chromatographic runs to determine system suitability, including standard concentrations to create a working analytical curve and duplicate or triplicate injections of the sample to be assayed. Therefore, a few hours of standard and sample solution stability can be required even for short (10-min) separation. When more than one sample is analyzed automated, overnight runs often are performed for better lab efficiency. Such practices add requirements for greater solution stability. Stability is defined as the capacity of a drug substance or drug product to remain within the established specifications to maintain its identify, strength, quality and Purity throughout the retest or expiration dating period.

The purpose of stability testing is to provide evidence on how the quality of active substances or pharmaceutical products varies with time under the influence of a variety of environmental factors such as temperature, humidity & light. In addition, product – related factors influence the stability.

Method Development Using HPLC:

It is important to pick the optimal chromatographic conditions for regular analysis of any medication in method development, such as the best column, the best mobile phase, and the detection wavelength. HPLC method creation requires a lot of sample information, such as the number of components, pKa values, UV-Visible spectra, solubility in various solvents, concentration ranges, and other characteristics of the sample. This information is required before developing a method, such as the choice of chromatography method according to the sample properties, a sample that is HPLC- analysed under conditions where all compounds elute quickly, and an optimization of HPLC method in terms of analysis times, resolution levels, selectivity and sensitivity.

AIM AND OBJECTIVE

AIM

To validate stability indicating RP-HPLC method for the estimation of Tranexamic Acid and Ethamsylate in bulk and pharmaceutical dosage forms.

Specific objectives:

- To develop a simple, rapid and specific RP-HPLC method for the estimation of Tranexamic acid and Ethamsylate in bulk and pharmaceutical dosage forms.
- To validate the proposed methods in accordance with the analytical parameters mentioned in the ICH guidelines, such as system suitability, accuracy, precision, specificity, linearity, robustness, LOD and LOQ.
- To develop the method under the forced conditions such as Acid, Alkali, Peroxide, Reduction, Thermal, Hydrolysis and Photo degradation.

PLAN OF WORK

To develop and validate an effective RP-HPLC method for the determination of Tranexamic acid and Ethamsylate in bulk and pharmaceutical dosage forms.

The plan of work for the HPLC method is as follows:

- Gathering physical chemical properties of drug
- Selection of chromatographic condition
  - Selection of stationary phase
  - Selection of mobile phase
  - Selection of flow rate
  - Selection of Initial separation condition
- Optimization of Chromatographic and Spectral conditions
- Validation of proposed method by ICH Guidelines
- Applying developed methods to the marketed formulation
- Summarizing methodology, and finalizing documentation.

Experimental and Analytical Methodology

Equipment:

Table 4: List of Apparatus used in HPLC

S.No	Name	Model	Manufacturer
1.	HPLC	ALLIANCE	Waters 2695-Empower Software 2.0 versions
2.	pH meter	-	Eutech
3.	Weighing balance	-	Sartouris
4.	Pipettes,beakers and Burettes	-	Borosil
5.	Ultra sonicator	UCA701	Unichrome
6.	Pump	Isocritic model	--

Reagents &Chemicals

Table .5: List of chemicals used in HPLC Method

S.No	Name	Grade	Manufacturer
1	Methanol	HPLC	Rankem
2	Water (Milli Q)	HPLC	In house production
3	Trifluoro acetic acid	HPLC	Analytical reagents
4	Formic acid	HPLC	Analytical reagents

Determination of Working Wavelength (λ_{max}):

In simultaneous estimation of two drugs isobestic wavelength was used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are inter convertible. So this wavelength was used in simultaneous estimation to estimate two drugs accurately.

The wave length of maximum absorption of the solution of the drugs in mixture of Methanol and 0.1% TFA (30:70) were scanned using PDA Detector within the wavelength region of 200–400 nm against Methanol and 0.1% TFA (30:70)as blank. The absorption curve shows isobestic point at 250nm. Thus 250 nm was selected as detector wavelength for the HPLC chromatographic method.

Preparation of solutions

Preparation of Tranexamic acid Stock Solution:

Accurately weigh and transfer 50 mg of Tranexamic acid working standard into 20 ml volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Ethamsylate Stock Solution:

Accurately weigh and transfer 25 mg of Ethamsylate working standard into 20 ml volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Standard Solution:

Further pipette 1 ml of the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent. (250ppm of Tranexamic acid, 125ppm of Ethamsylate).

Sample Solution Preparation:

Accurately weighed and transfer 39mg of Tranexamic acid and Ethamsylate sample into a 10mL clean dry volumetric flask add Diluent and sonicate it up to 30 minutes to dissolve, and centrifuge for 30min. to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through0.45 micron Injection filter (Stock solution).

Further pipette 1 ml of the above stock solutions into 10 ml volumetric flask and dilute up to the mark with diluents. (250ppm of Tranexamic acid, 125ppm a of Ethamsylate).

0.1% TFA Buffer Preparation: 1ml of Tri fluoro acetic acid is dissolved in 1 liter of HPLC water and filter through 0.45µ membrane filter paper.

Preparation of Mobile Phase: Mobile phase was prepared by mixing Methanol and 0.1% TFA taken in the ratio 30:70. It was filtered through 0.45µ membrane filter to remove the impurities which may interfere in the final chromatogram.

Diluent: Methanol.

Preparation of Tranexamic acid Stock Solution:

Accurately weigh and transfer 50mg of Tranexamic acid working standard into20ml volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Procedure:

Inject 10 □L of the standard, sample into the chromatographic system and measure the areas for Tranexamic acid and Ethamsylate peaks and calculate the %Assay by using the formulae.

HPLC method development

During the selection of chromatographic conditions, numbers of trails were carried out and the best trail was selected for optimized method.

Table.6: Trial-1 Chromatographic conditions

Parameter	Conditions
Column	Inertsil ODS(250x4.6mm,5µ)
Mobile phase ratio	Acetonitrile:0.1% Formic acid(80:20)
Detection wavelength	200-400nm
Flow rate	1ml/min
Injection volume	10µl
Runtime	10min
Observation:	System suitability conditions are not within the limit

Table.7: Trial-2 Chromatographic conditions

Parameter	Conditions
Column	Inertsil ODS(250x4.6mm,5µ)
Mobile phase ratio	Acetonitrile:0.1%Formic acid(70:30)
Detection wavelength	250nm
Flow rate	1ml/min
Injection volume	10µl
Runtime	10min
Observation	Splitting of the peaks are observed

Table.8:Trial-3 Chromatographic conditions

Parameter	Conditions
Column	Inertsil ODS(250x4.6mm,5µ)
Mobile phase ratio	Acetonitrile:0.1% Formic acid(60:40)
Detection wavelength	250nm
Flow rate	1ml/min
Injection volume	10µl
Runtime	10min
Observation	Retention time is not within the limit

Table.9:Trial-4 Chromatographic conditions

Parameter	Conditions
Column	X-Bridge Phenyl (150x4.6mm, 3.5µm)
Mobile phase ratio	Methanol:0.1%TFA(20:80)
Detection wavelength	250nm
Flow rate	1ml/min
Injection volume	10µl
Runtime	10min
Observation	Broad peaks are observed

Table 10:Trial-5 Chromatographic conditions

Parameter	Conditions
Column	X-Bridge Phenyl(150x4.6mm, 3.5µm)
Mobile phase ratio	Methanol:0.1%TFA(25:75)
Detection wavelength	250nm
Flow rate	1ml/min
Injection volume	10µl
Runtime	10min
Observation	Response of the peaks are very high

Table 11:Trial-6OptimizedChromatographic conditions

Parameter	Conditions
Column	X-Bridge Phenyl (150x4.6mm, 3.5µm)
Mobile phase ratio	Methanol:0.1%TFA(30:70)
Detection wavelength	250nm
Flow rate	1ml/min
Injection volume	10µl
Runtime	7min
Observation	This method is suitable for validation

The Tranexamic acid peak was observed at 2.522 min with peak area 2971647, tailing factor1.05,Ethamsylate peak was observed at 4.739min,with peak area 1457565, tailing factor 0.94 and resolution 10.48. This trial was optimized.

System Suitability:

Tailing factor for the peaks due to Tranexamic acid and Ethamsylate in Standard solution should not be more than 2.0. Theoretical plates for the Tranexamic acid and Ethamsylate peaks in Standard solution should not be less than 2000.
Resolution for the Tranexamic acid and Ethamsylate peaks in standard solution should not be less than 2.

HPLC Method Validation[5-16]
Specificity:
Specificity of an analytical method is ability to measure specifically the analyte of interest without interference from blank and known impurities. For this purpose blank chromatogram, standard chromatogram and sample chromatogram were recorded. The chromatogram of blank shows no response at the retention times of drugs which confirms the response of drugs was specific**.**

Linearity:

Preparation of stock solution:

Accurately weigh and transfer 50 mg of Tranexamic acid and 25 mg of Ethamsylate working standard into 20 ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level–I (62.5ppm of Tranexamic acid,31.25 ppm of Ethamsylate):

0.25 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level–II (125.0ppm of Tranexamic acid,62.50 ppm of Ethamsylate):

0.50 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level–III (187.5ppmof Tranexamic acid, 93.75ppm of Ethamsylate):
0.75 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluents.

Preparation of Level–IV (250.0ppm of Tranexamic acid, 125.00ppm of Ethamsylate):
1.00 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent**.**

Preparation of Level –V (312.5ppmof Tranexamic acid, 156.25ppm of Ethamsylate)
1.25 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluent**.**

Preparation of Level–VI (375.0 ppm of Tranexamicacid,187.50 ppm of Ethamsylate)
1.50 ml of above stock solutions has taken in different 10 ml of volumetric flasks, dilute up to the mark with diluent**.**
Procedure:

Inject each level in to the chromatographic system and measure the peak area.

Plot a graph of peak are a versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Range:

The Range of an analytical method is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated with precision, accuracy and linearity

Acceptance Criteria:

Correlation coefficient should be not less than 0.999.

Accuracy

Preparation Accuracy Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration):
Accurately weigh and transfer 19.5 mg of Tranexamic acid and Ethamsylate sample into a 10ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution)
Further pipette 1 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (125ppm of Tranexamic acid and 62.5ppm of Ethamsylate)

For preparation of 100% solution (With respect to target Assay concentration)

Accurately weigh and transfer 39mg of Tranexamic acid and Ethamsylate sample into a 10mlcleandryvolumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 1ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (250ppm of Tranexamic acid and 125ppm of Ethamsylate)

For preparation of 150% solution (With respect to target Assay concentration):
Accurately weigh and transfer 58.5mg of Tranexamic acid and Ethamsylate sample into a 10mlcleandryvolumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 1ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (375ppmofTranexamic acid and 187.5ppm of Ethamsylate)

Procedure:

Inject the standard solution,Accuracy -50%, Accuracy -100% and Accuracy -150% solutions.
Acceptance Criteria:

The % Recovery for each level should be between 98.0to 102.0%.

Precision

Precision is the degree of repeatability of an analytical method under normal operation conditions.

Precision is of 3 types

1. System precision
2. Method precision
3. Intermediate precision(a. Intraday precision, b. Interday precision)

System precision is checked by using standard chemical substance to ensure that the analytical system is working properly. In this peak area and % of drug of six determinations is measured and % RSD should be calculated.

In method precision, a homogenous sample of single batch should be analyzed 6 times. This indicates whether a method is giving constant results for a single batch. In this analyze the sample six times and calculate the % RSD.

The precision of the instrument was checked by repeatedly injecting (n=6) solutions of 250ppm of Tranexamic acid, 125ppm of Ethamsylate).

Acceptance Criteria:

The % RSD for the absorbance of six replicate injections results should not be more than 2%.

Robustness
As part of the Robustness,deliberate change in the Flow rate,Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A. The flow rate was varied at 0.9ml/min to 1.1ml/min.

Standard solution 250ppm of Tranexamic acid,125ppm of Ethamsylate was prepared and analysed using the varied flow rates along with method flow rate.
On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly.
Hence it indicates that the method is robust even by change in the flow rate ±10%.

B. The variation of Organic Phase ratio.

Standard solution of 250ppm of Tranexamic acid, 125ppm of Ethamsylate was prepared and analyzed using the varied in mobile phase ratio.

Limit of detection (LOD) and limit of quantification (LOQ)
The limit of detection (LOD) limit of quantification (LOQ) of the drug carry was calculated using the following equation as per international conference harmonization (ICH) guidelines.

$$LOD=3.3X\sigma/S \quad LOQ = 10 X \sigma /S$$

LOD for Tranexamic acid,was found to be 0.6µg/mL and LOQ for Tranexamic acid,was found to be 2.0 µg/ml, LOD for Ethamsylate was found to be 0.3g/ml and LOQ for Ethamsylate was found to be 1.0µg/ml.

Degradation Studies

Preparation of stock:

Accurately weigh and transfer 39mg of Tranexamic acid and Ethamsylate sample into a 10mlcleandryvolumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Acid degradation:

Pipette 1 ml of the aforementioned solution was added to a 10 ml vacuum flask, followed by 1 ml of 1N HCl. The vacuum flask was then maintained at 60°C for 1 hour before being neutralised with 1 N NaOH and diluted to 10ml with diluent. Filter the solution using 0.45 micron syringe filters and transfer to bottles.

Alkali degradation:

Pipette 1ml of above solution in to a10ml volumetric flask and add1ml of 1N NaOH was added. Then, the volumetric flask was kept at 60ºC for 1 hour and then neutralized with 1N HCl and make up to 10ml with diluent. Filter the solution with 0.45 microns syringe filters and place in vials.

Thermal degradation

Tranexamic acid and Ethamsylate Standard was taken in petridish and kept in Hot air oven at 105^0 C for 3 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

Peroxide degradation

Pipette 1 ml above stock solution was added to a 10 ml vacuum flask, 1 ml of 3 percent w/v hydrogen peroxide was added to the flask and the volume was built up to the mark using diluent. The vacuum flask was then maintained at 60ºC for 1hour.After that, the vacuum flask was left at room temperature for 15 minutes. Filter the solution using 0.45micron syringe filters and transfer to bottles.

Reduction degradation

Pipette 1ml of above-stock solution was added to a 10ml vacuum flask, 1ml of 10% Sodium bisulphite as added to a flask and the volume was built up to the required volume with diluent. The vacuum flask was then maintained at 60ºC for 1 hour. After that, the vacuum flask was left at room temperature for 15 minutes. Filter the solution using 0.45 micron syringe filters and transfer to bottles.

Photolytic degradation

Tranexamic acid and Ethamsylate sample was placed in Photo Stability Chamber for3hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

Hydrolysis degradation

Pipette 1mlofabove-stock solution was added to a 10ml vacuum flask, 1ml of HPLC grade water was added to a flask and the volume was built up to the required volume with diluent. The vacuum flask was then maintained at60°C for 1hour. After that,the vacuum flask was left at room temperature for 15 minutes. Filter the solution using 0.45micron syringe filters and transfer to bottles.

RESULTS AND DISCUSSION:

Determination of Working Wavelength(λ_{max})

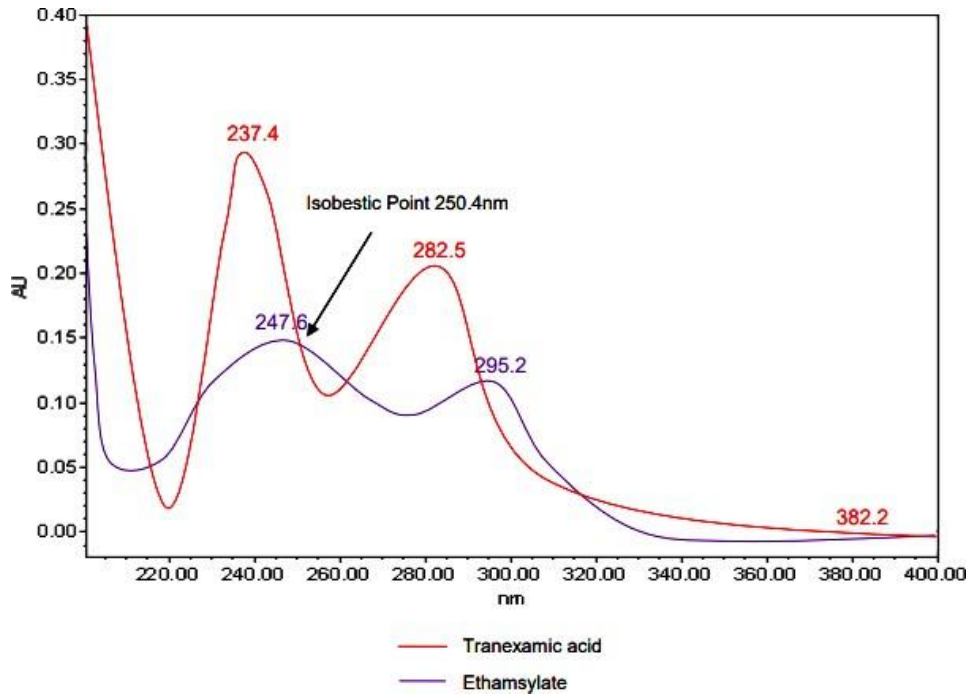


Figure 1: PDA –Spectrum of Tranexamic acid& Ethamsylate

Trial for Optimization of chromatographic conditions Trial-1

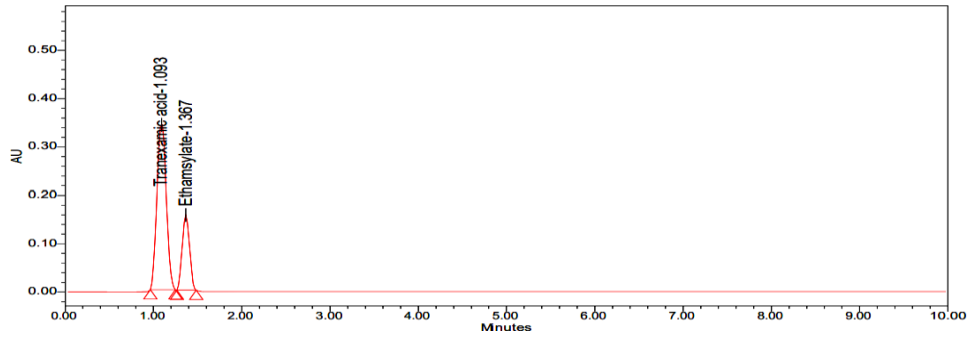


Figure 2 :Chromatogram of Trial-1

S.No	Name	RT	Area	USP Plate Count	USP Tailing	USP Resolution
1	Tranexamic acid	1.093	2517589	501	1.06	-
2	Ethamsylate	1.367	906817	1054	1.03	1.49

Trial-2:

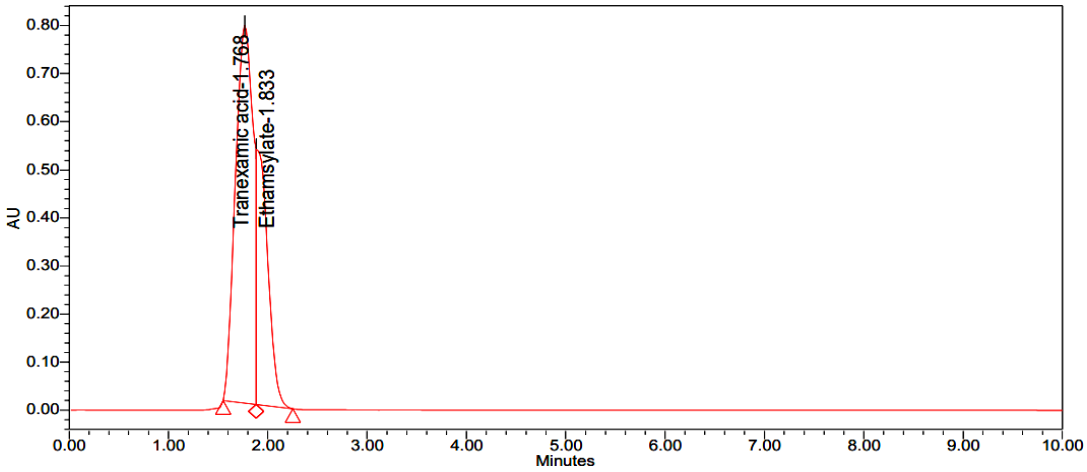
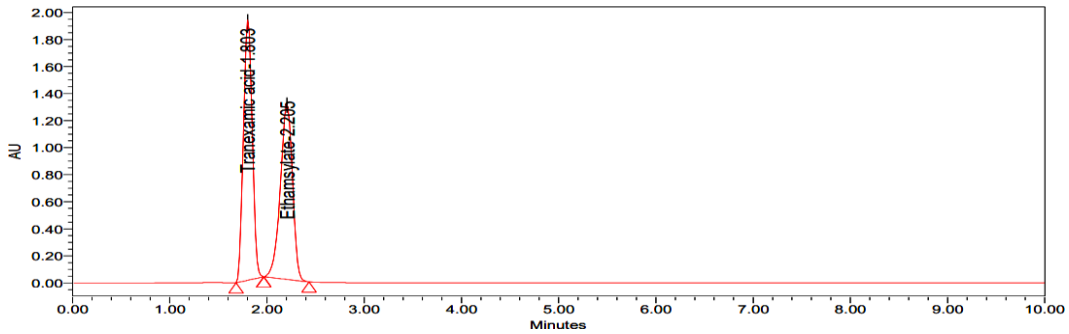


Figure 3:Chromatogram of Trial-2

S.No	Name	RT	Area	USP Plate Count	USP Tailing	USP Resolution
1	Tranexamic acid	1.768	9650660	2579	1.95	-
2	Ethamsylate	1.833	4260980	1632	2.12	0.31

Figure 4: Chromatogram of Trial Trial-3:

S.No	Name	RT	Area	USP Plate Count	USP Tailing	USP Resolution
1	Tranexamic acid	1.803	12226033	2681	1.03	-
2	Ethamsylate	2.205	11300104	3438	0.90	1.97



Trial-4:

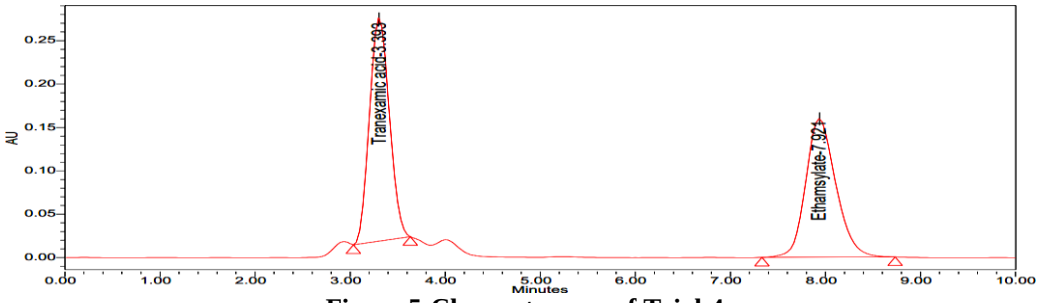


Figure 5:Chromatogram of Trial-4

S.No	Name	RT	Area	USP Plate Count	USP Tailing	USP Resolution
1	Tranexamic acid	3.393	1873516	2114	1.08	-
2	Ethamsylate	7.921	1758619	3038	1.17	9.42

Trial-5:

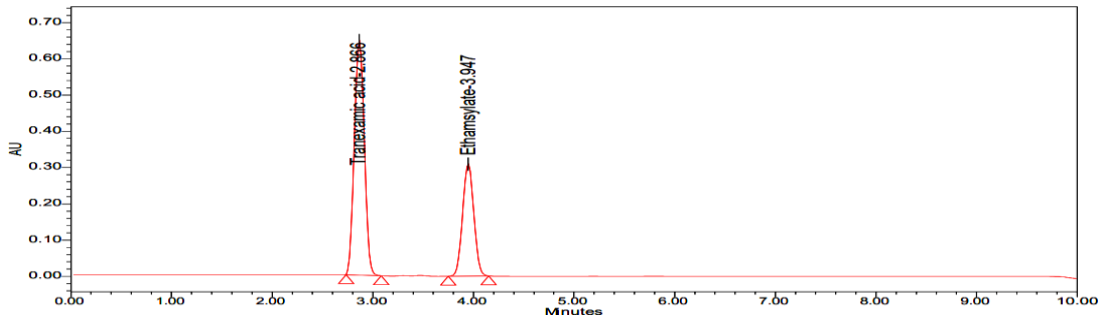


Figure 6:Chromatogram of Trial-5

S.No	Name	RT	Area	USP Plate Count	USP Tailing	USP Resolution
1	Tranexamic acid	2.866	6462192	3719	0.96	-
2	Ethamsylate	3.947	2993417	5768	1.04	5.43

Trial-6: Optimized chromatogram

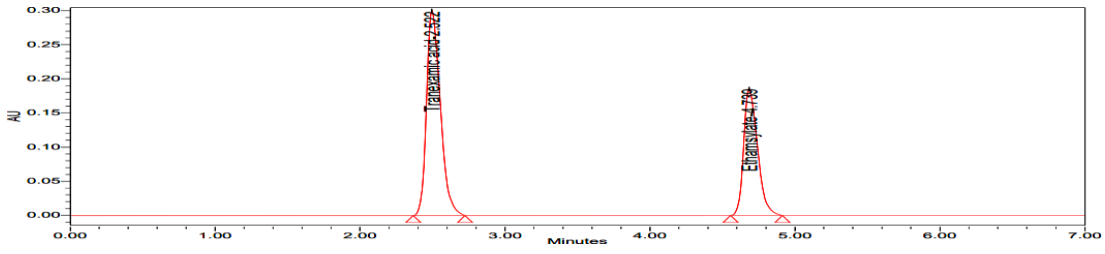


Figure 7:Chromatogram of Trial-6

S.No	Name	RT	Area	USP Plate Count	USP Tailing	USP Resolution
1	Tranexamic acid	2.522	2971647	9264	1.05	-
2	Ethamsylate	4.739	1457565	12365	0.94	10.48

Observation: All the parameters are within the limit this method is suitable for validation

Table 12:Optimized chromatographic conditions

PARAMETERS	OBSERVATION
Instrument used	Waters HPLC with auto sampler and PDA detector.
Injection volume	10µl
Mobile Phase	Methanol:0.1%TFA(30:70)
Column	X-Bridge Phenyl(150x4.6mm,3.5µ)
Detection Wave Length	250nm
Flow Rate	1 mL/min
Runtime	7min
Temperature	Ambient(25□C)
Mode of separation	Isocratic mode

System suitability: All the system suitability parameters were within the range and satisfactory as per ICH guidelines

Table 13: System suitability parameters for Tranexamic acid &Ethamsylate

S.no	Parameter	Tranexamic acid	Ethamsylate
1	Retention time	2.522	4.739
2	Plate count	9264	12365
3	Tailing factor	1.05	0.94
4	Resolution	----	10.48
5	%RSD	0.33	0.35

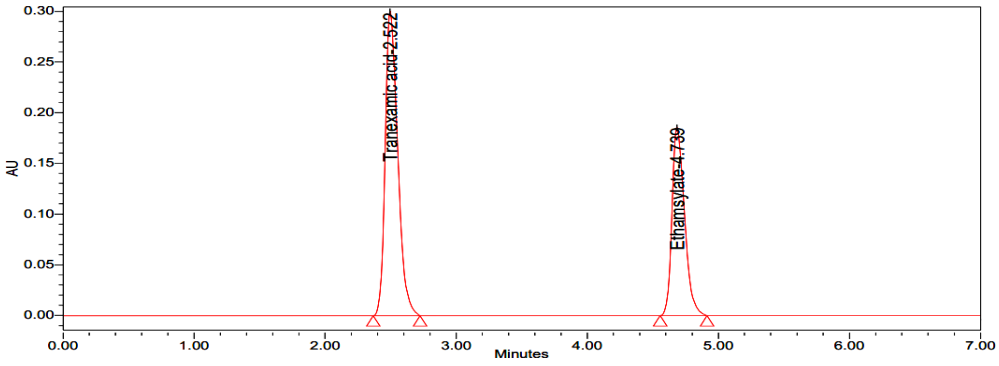


Figure 8: Chromatogram of standard

Acceptance Criteria: According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

Analytical Method Validation (HPLC)

The method was validated for its linearity range, accuracy, precision, and specificity. Method validation was carried out as per ICH guidelines.

Specificity:

The optimised conditions were used to detect the standard and sample solutions (in triplicates) of tranexamic acid and ethamsylate in relation to the blank and placebo samples in order to assess the selectivity and specificity of the devised analytical technique. It is discovered that the developed approach is selective for TXA and ETS. Figures 9, 10, and 11 display the findings of the selectivity/specificity analysis.

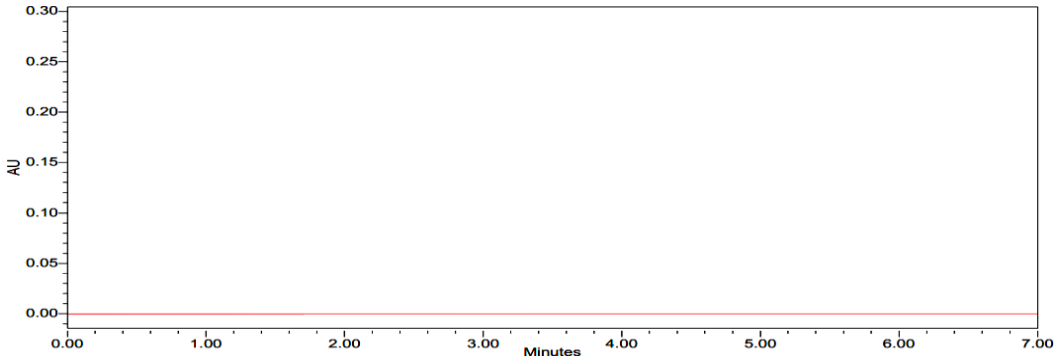


Figure 9: Chromatogram of blank

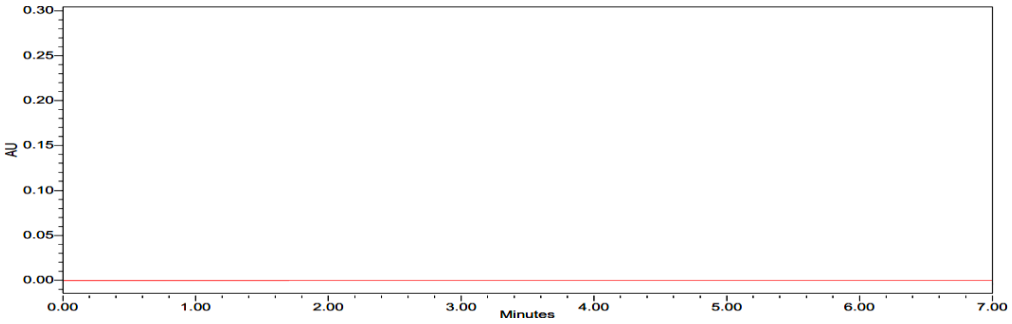


Figure 10: Chromatogram of placebo

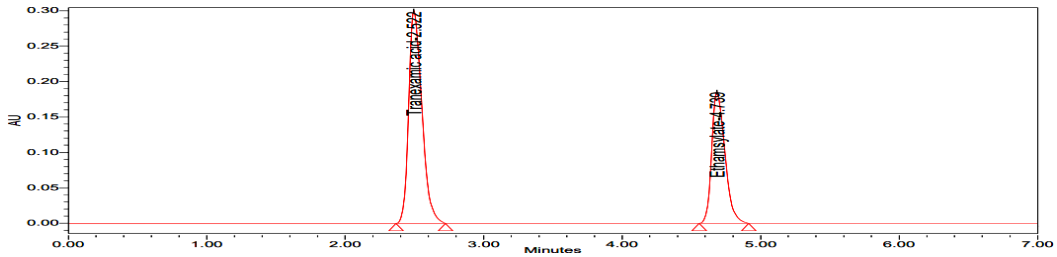


Figure 11: Standrad chromatogram

Discussion: Retention times of Tranexamic acid and Ethamsylate were 2.522 min and 4.739 min respectively. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Precision

Ruggedness (Intermediate Precision), System Precision, and Method Precision The peak regions of the six replicates of the injected Tranexamic acid & Ethamsylate mixture were assessed in order to assess the precision. System precision, method precision, and ruggedness (intermediate precision) data findings are computed as a percentage RSD. It is discovered that the developed procedure for estimating tranexamic acid and ethamsylate is accurate. Tables 14 and 15, as well as figures 12–14, display the values acquired for system precision, method precision, and ruggedness (intermediate precision).

Table 14:System precision of Tranexamic acid &Ethamsylate

	Concentration Tranexamic acid (µg/ml)	Area of Tranexamic acid	Concentration of Ethamsylate (µg/ml)	Area of Ethamsylate
1.	250	2971647	125	1457565
2.	250	2965482	125	1452268
3.	250	2988794	125	1446891
4.	250	2985133	125	1445449
5.	250	2974627	125	1447264
6.	250	2965479	125	1455870
Mean	2975194		1450885	
S.D	9852.42		5099	
%RSD	0.33		0.35	

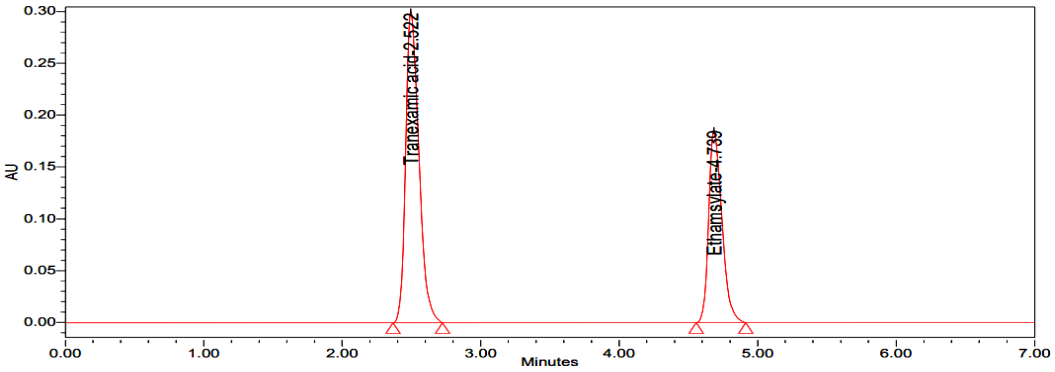


Figure 12:System precision chromatogram-1

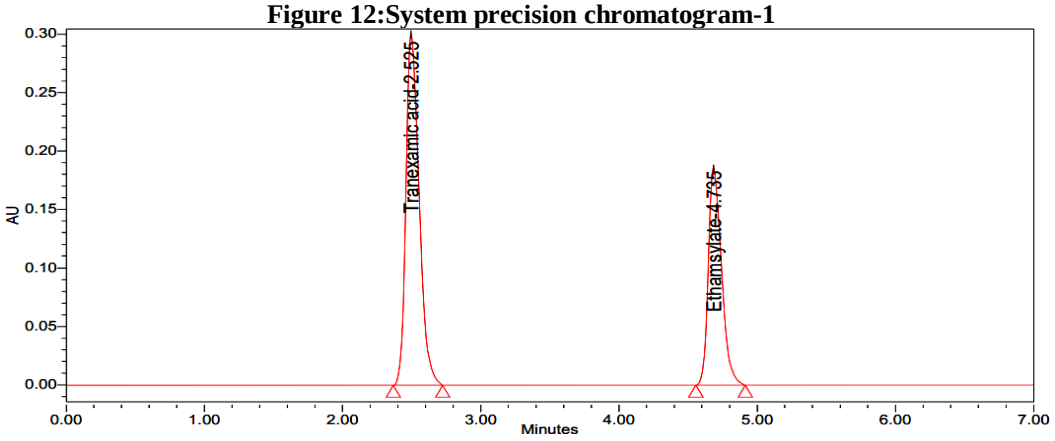


Figure 13:System precision chromatogram-2

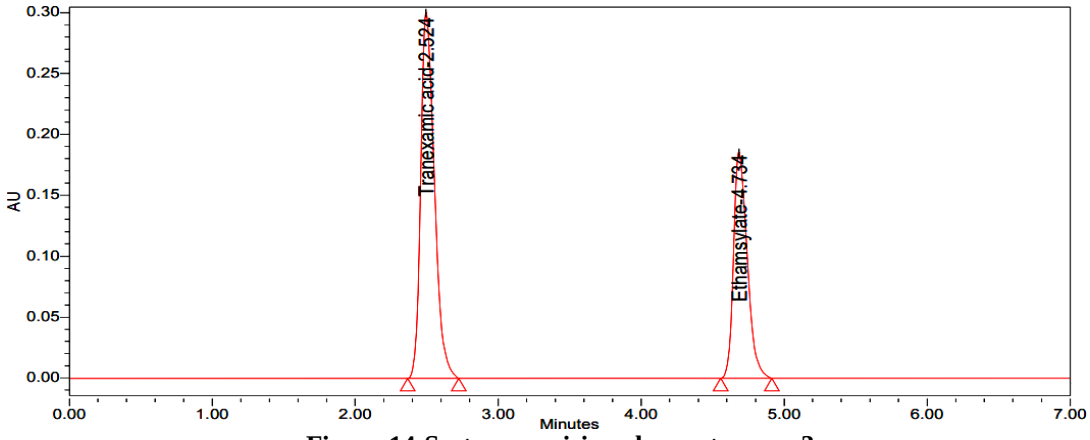


Figure 14:System precision chromatogram-3

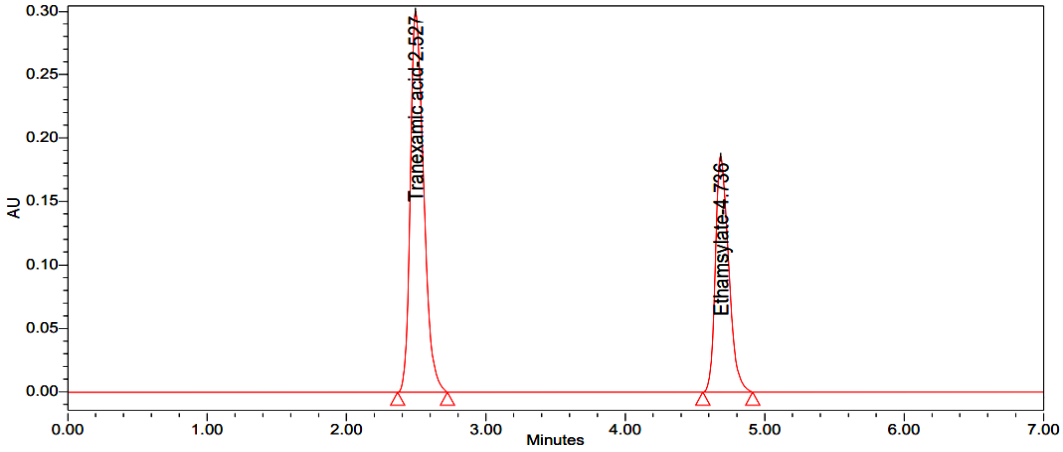


Figure 15:System precisionchromatogram-4

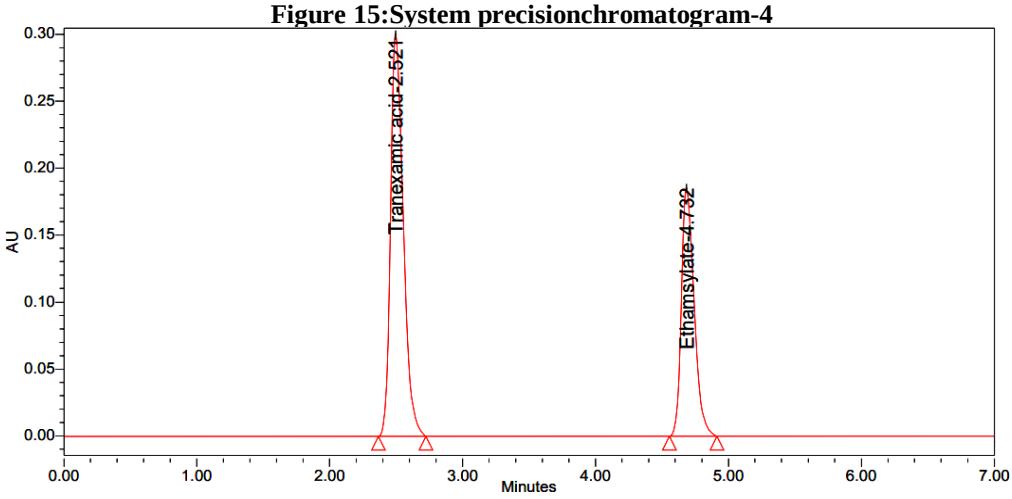


Figure 16:System precision chromatogram-5

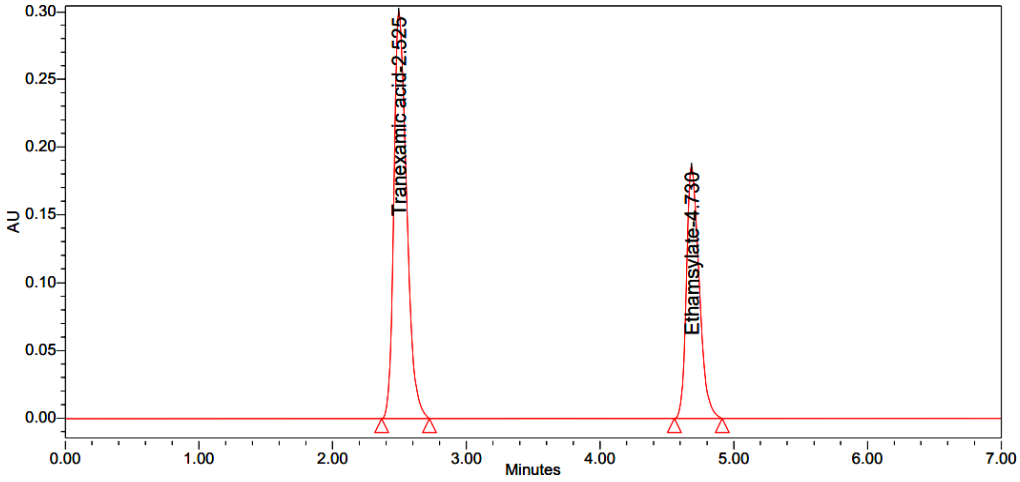


Figure 17:System precision chromatogram-6

Table 15:Method Precision for Tranexamic acid &Ethamsylate

S.No.	Area for Tranexamic acid	Area for Ethamsylate
1	2985466	1452718
2	2988874	1491265
3	2964359	1470527
4	2988887	1456634
5	2996778	1446796
6	2973195	1478790
Average	2982927	1466122
Standard Deviation	11905.189	17060.107
%RSD	0.40	1.16

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2%**.**

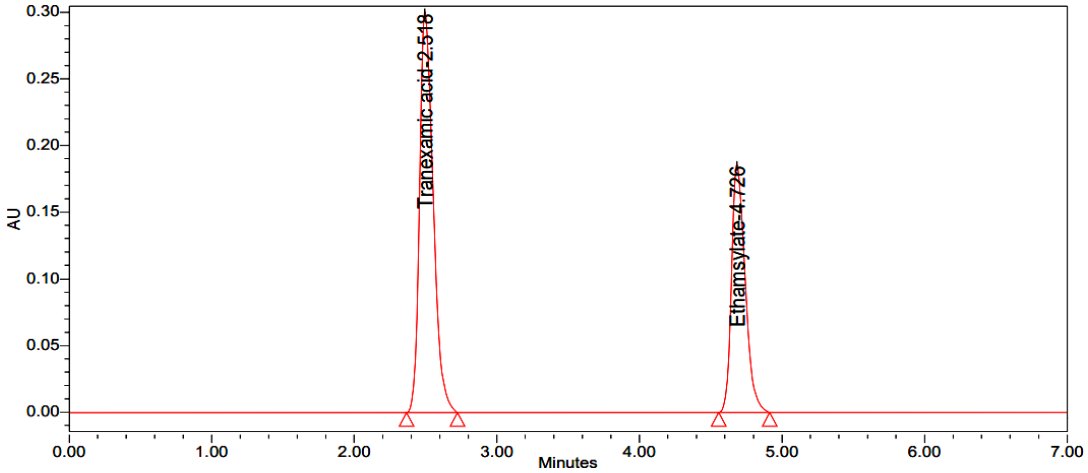


Figure 18:Repeatability chromatogram-1

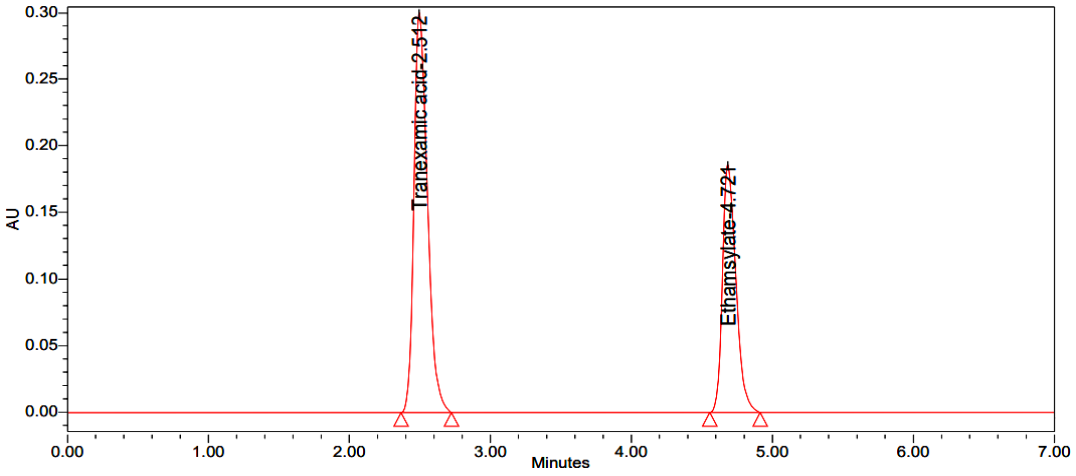


Figure 19:Repeatability chromatogram-2

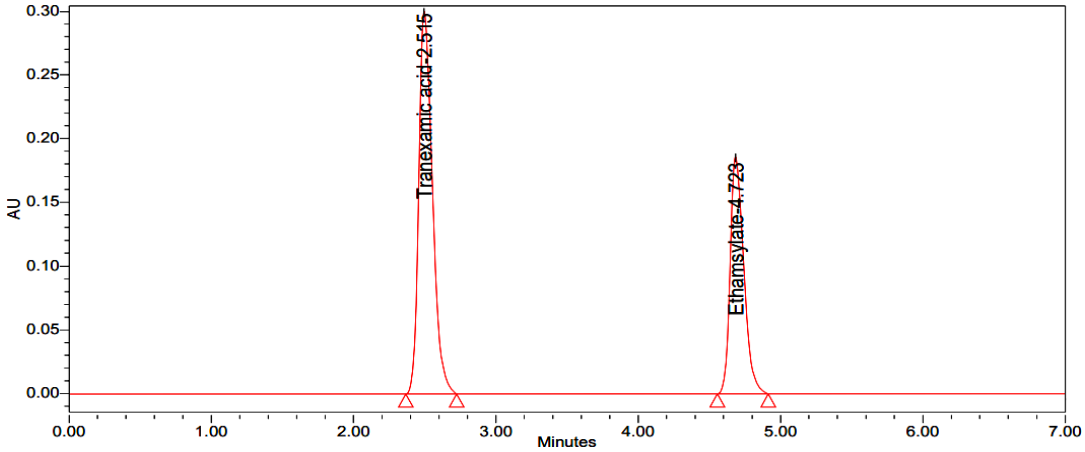


Figure 20:Repeatability chromatogram-3

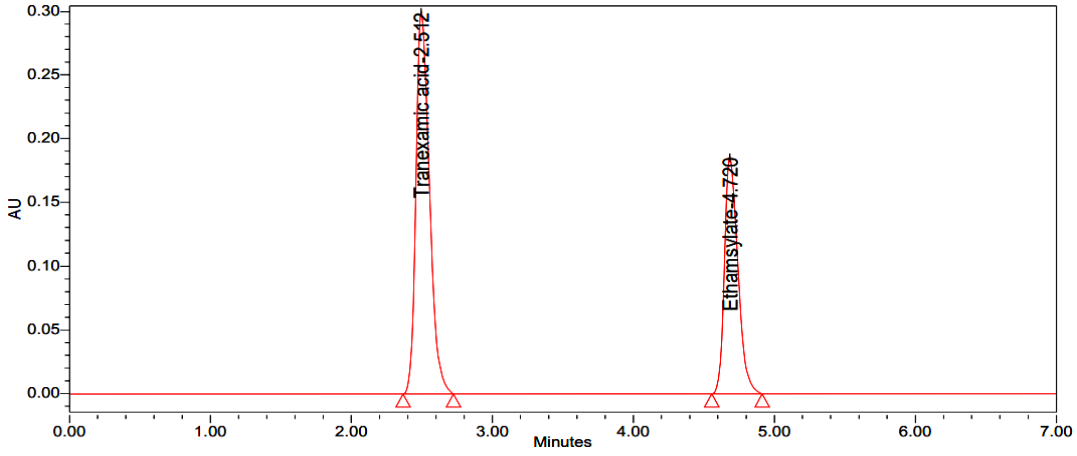


Figure 21:Repeatability chromatogram-4

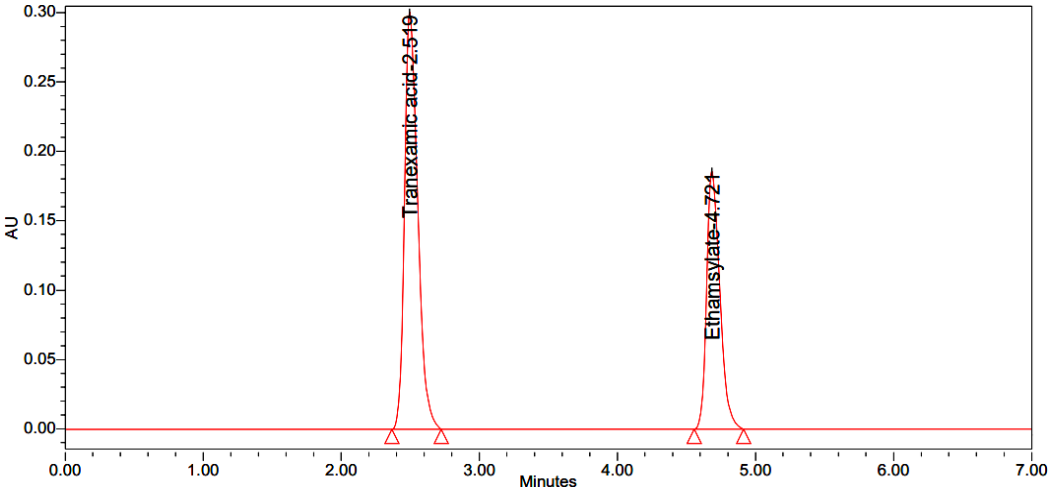


Figure 22:Repeatability chromatogram-5

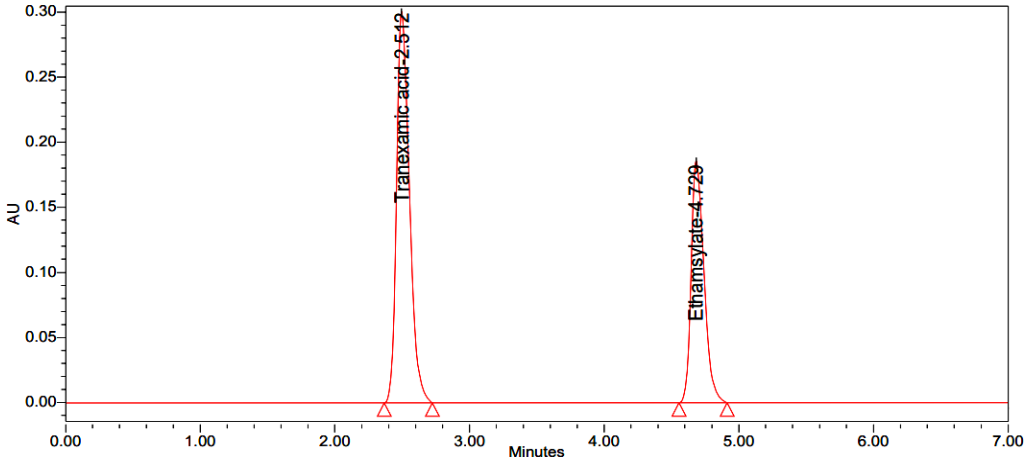


Figure 23:Repeatability chromatogram-6

Table 16: Intermediate Precision for Tranexamic acid and Ethamsylate

S.No.	Area for Tranexamic acid		Area for Ethamsylate	
	Day-1	Day-2	Day-1	Day-2
1	2994796	3014967	1482714	1447981
2	3023548	2975213	1469023	1460253
3	3016647	2984630	1464977	1482625
4	2996538	3028115	1458326	1470629
5	2987873	2999743	1484605	1463557
6	2984761	2963014	1461328	1459616
Average	3000694	2994280	1470162	1464110
Standard Deviation	15793.041	24644.834	11067.655	11668.980
%RSD	0.53	0.82	0.75	0.80

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2%.

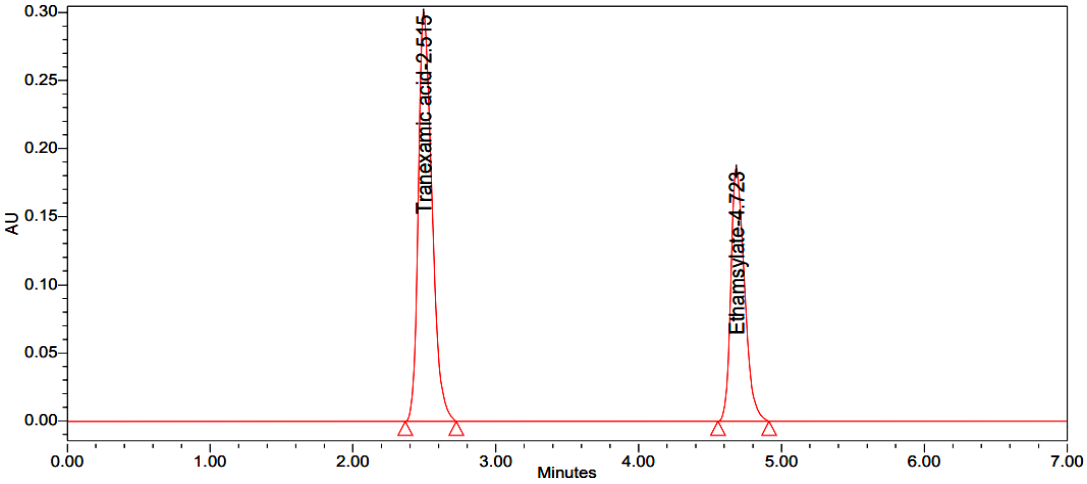


Figure 24:Interday precision chromatogram-1 (Day-1)

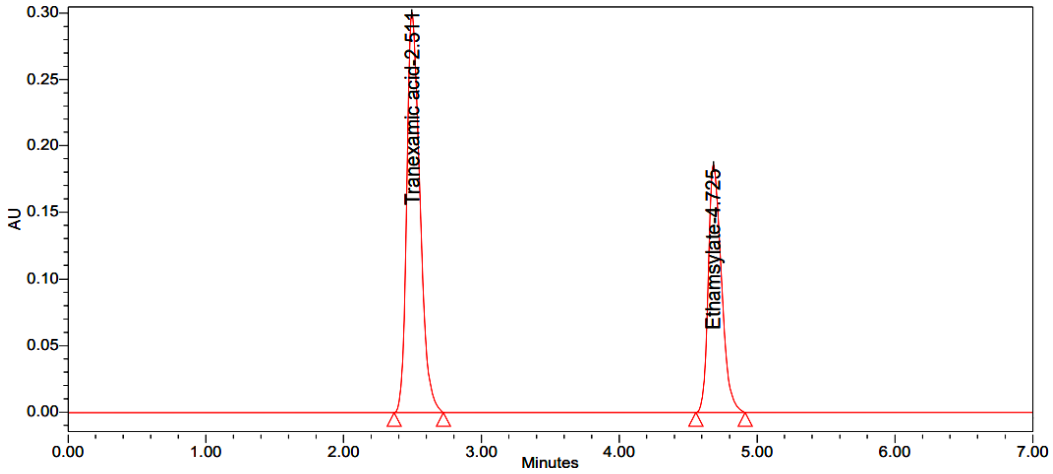


Figure 25:Interday precision chromatogram-2 (Day-1)

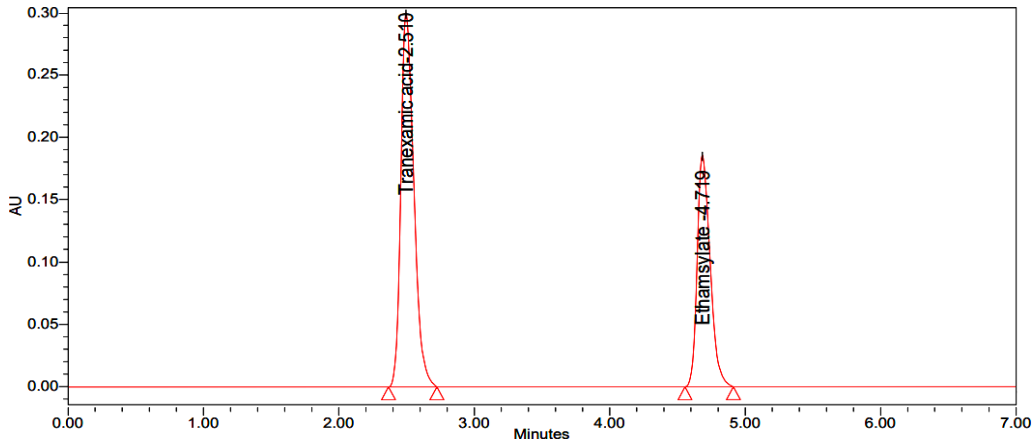


Figure 26:Interday precision chromatogram-3 (Day-1)

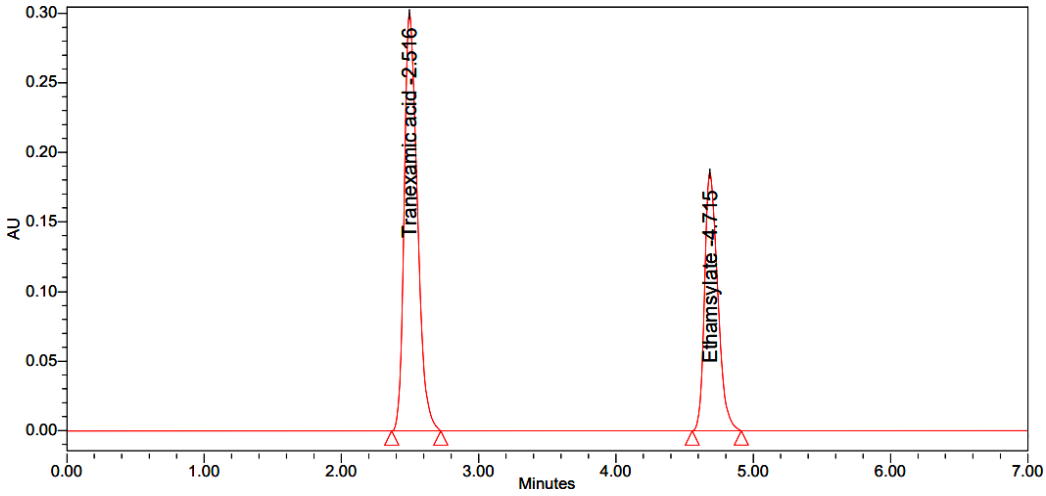


Figure 27:Interday precision chromatogram-4 (Day-1)

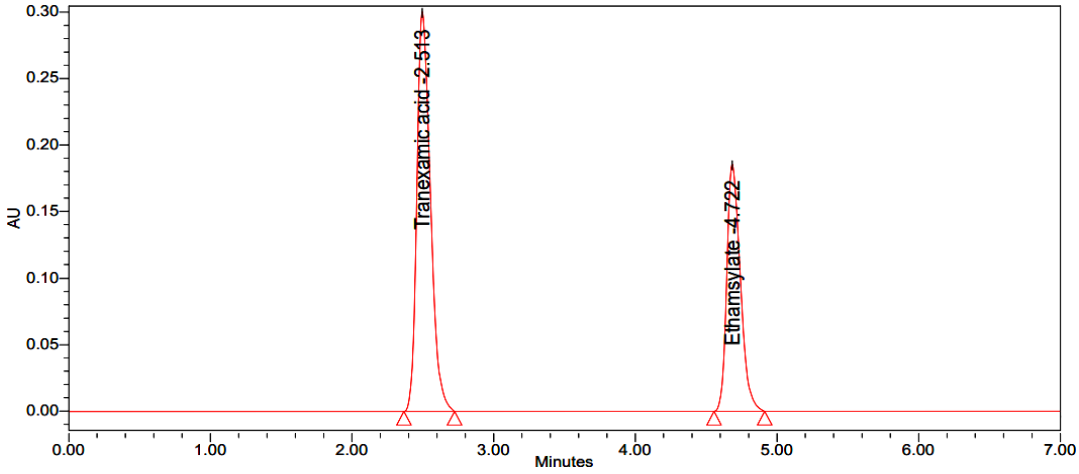


Figure 28:Interday precision chromatogram-5 (Day-1)

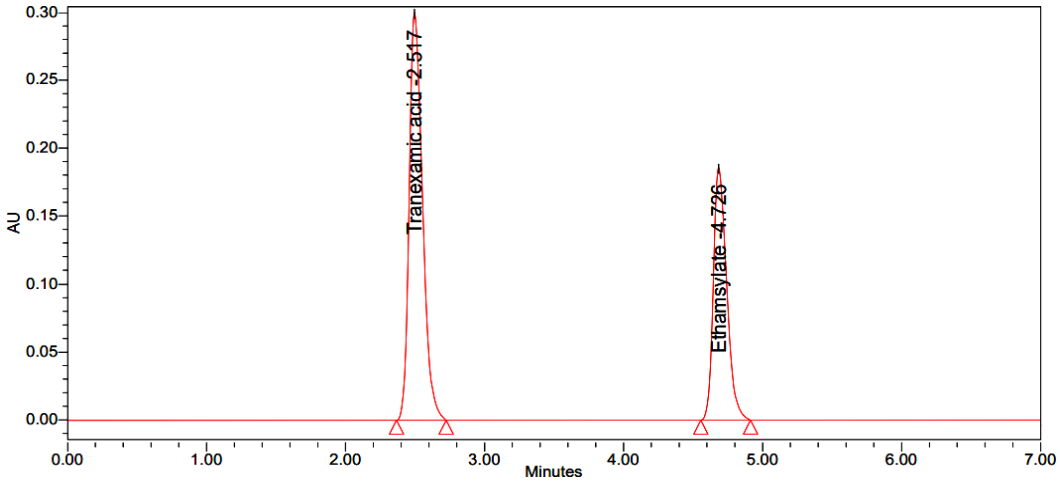


Figure 29:Interday precision chromatogram-6 (Day-1)

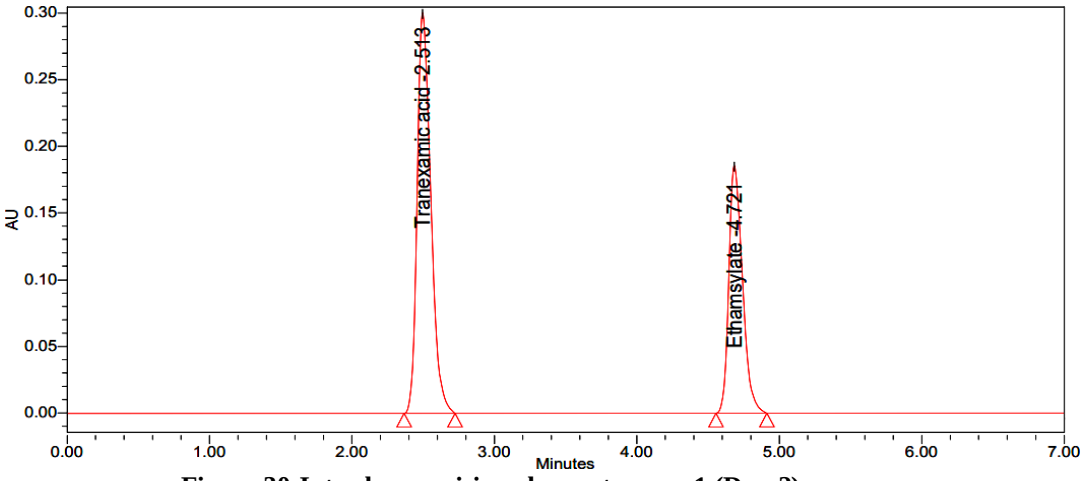


Figure 30:Interday precision chromatogram-1 (Day-2)

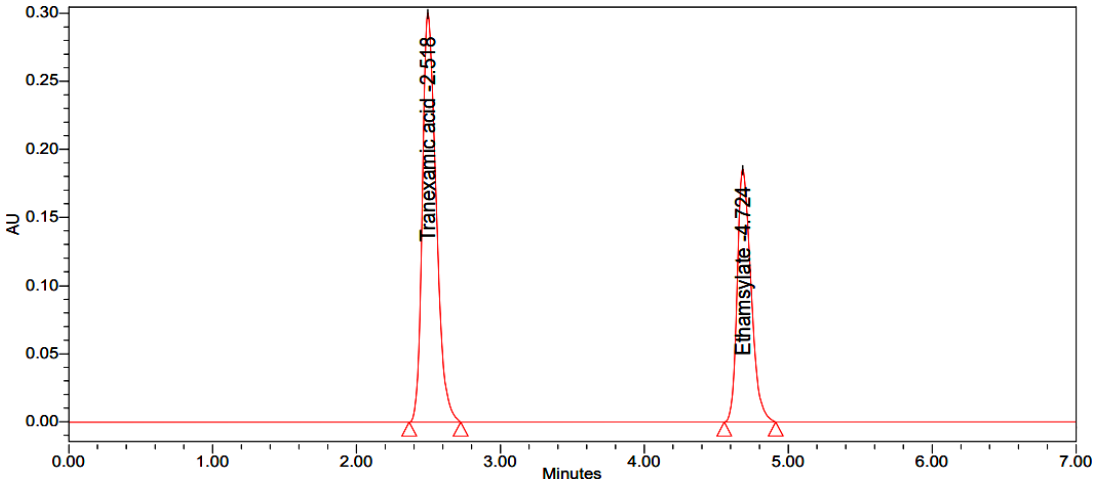


Figure 31:Interday precision chromatogram-2 (Day-2)

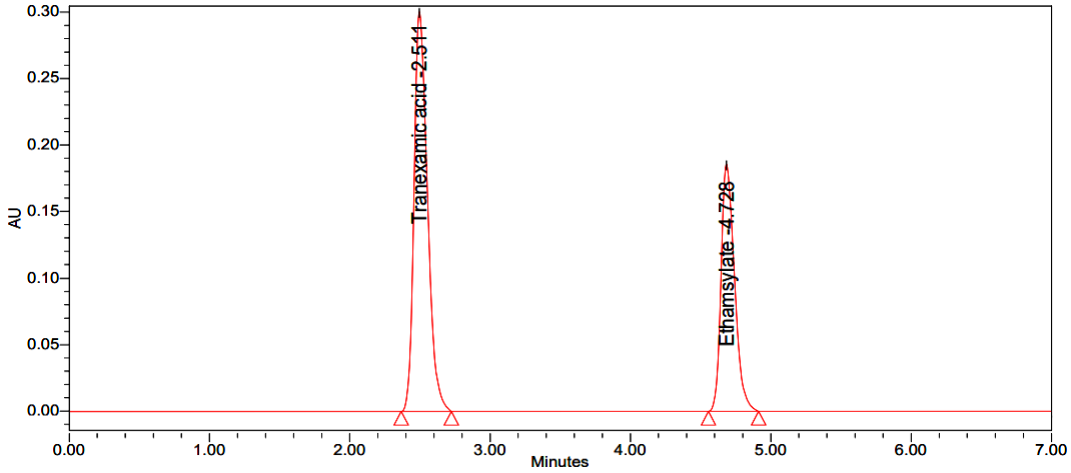


Figure 32:Interday precision chromatogram-3 (Day-2)

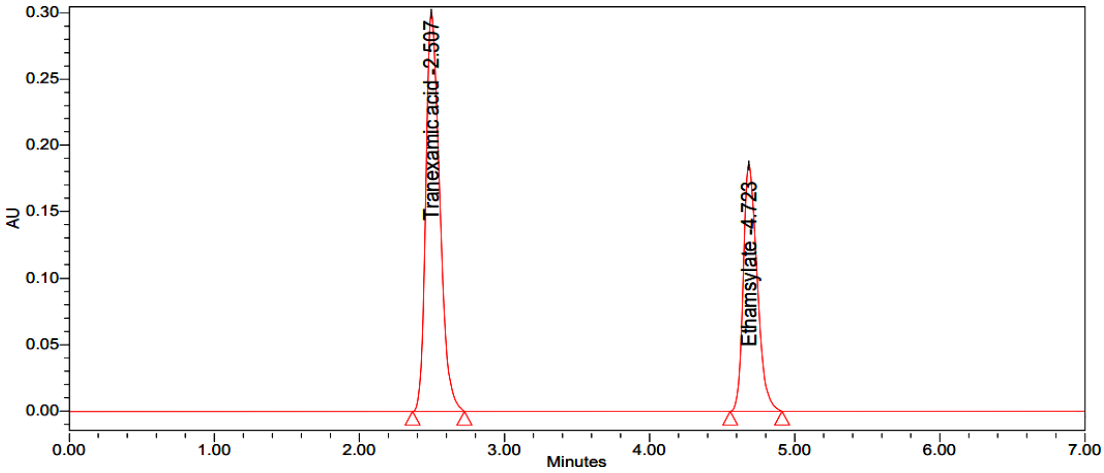


Figure 33:Interday precision chromatogram-4 (Day-2)

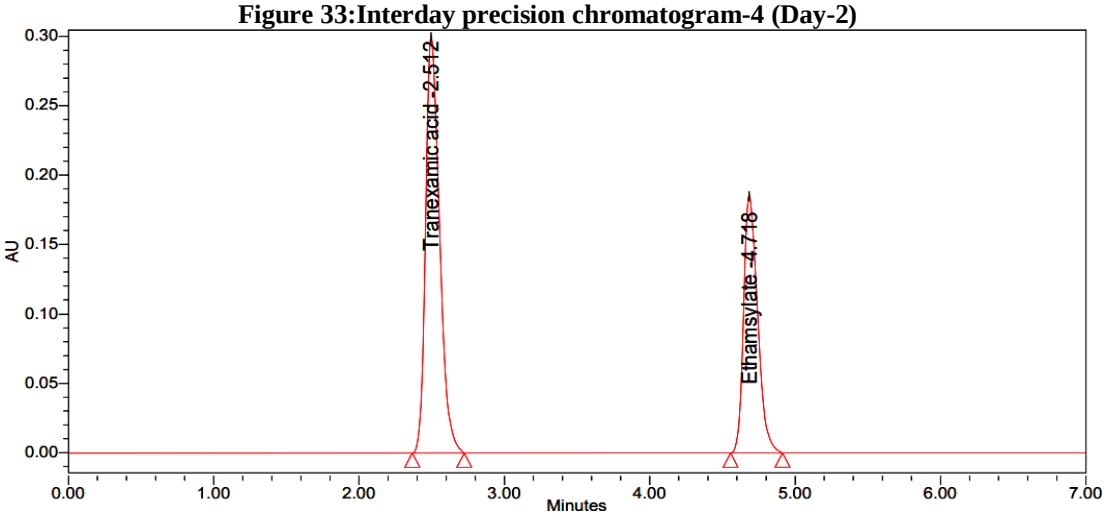


Figure 34:Interday precisionchromatogram-5 (Day-2)

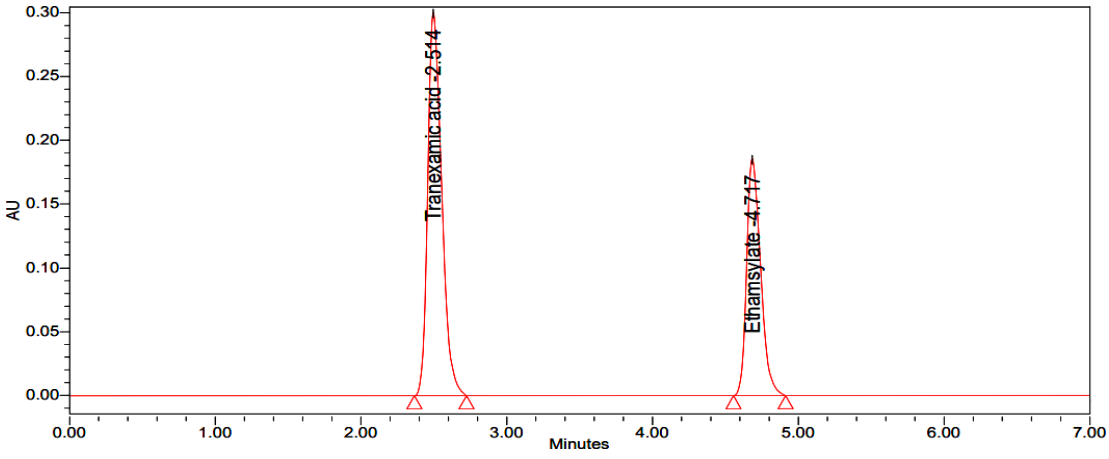


Figure 35:Interday precision chromatogram-6(Day-2)

Discussion: From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 0.33% and 0.35% respectively for Tranexamic acid and Ethamsylate. As the limit of

Precision was less than “2” the system precision was passed in this method.

Linearity

Tranexamic acid and ethamsylate calibration curves were obtained by injecting samples into the HPLC system in triplicate, ranging in concentration from low to high, in order to assess linearity. Tranexamic acid's range of drug concentrations was 62.50 µg/ml to 375.00 µg/ml, whereas Ethamsylate's range was 31.25 µg/ml to 156.25 µg/ml. To determine the correlation coefficient, the peak area of each concentration was examined; for tranexamic acid and ethamsylate, the results were 0.99967 and 0.99964, respectively. The developed approach is shown to be linear and falls within the range of ethamsylate and tranexamic acid. The figures are displayed in Figure 18-23 and Table 17.

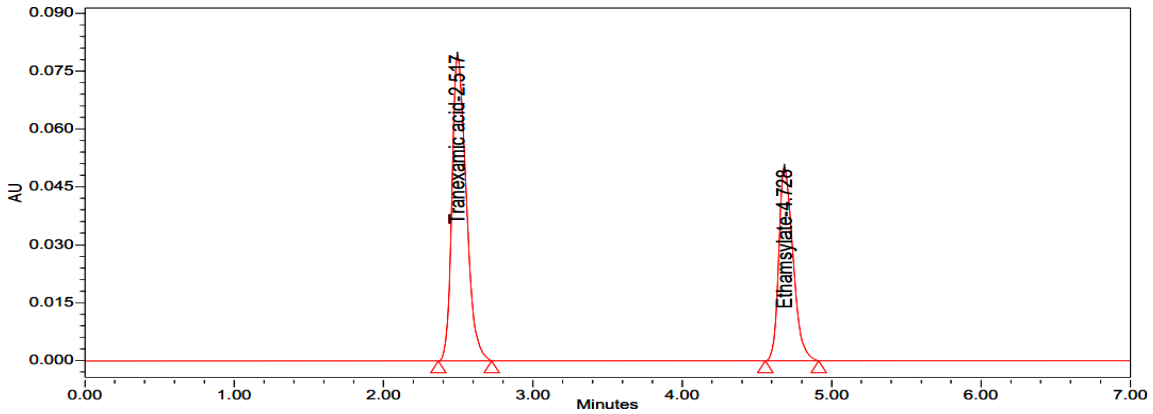


Figure 36:Chromatogram of Linearity-25%

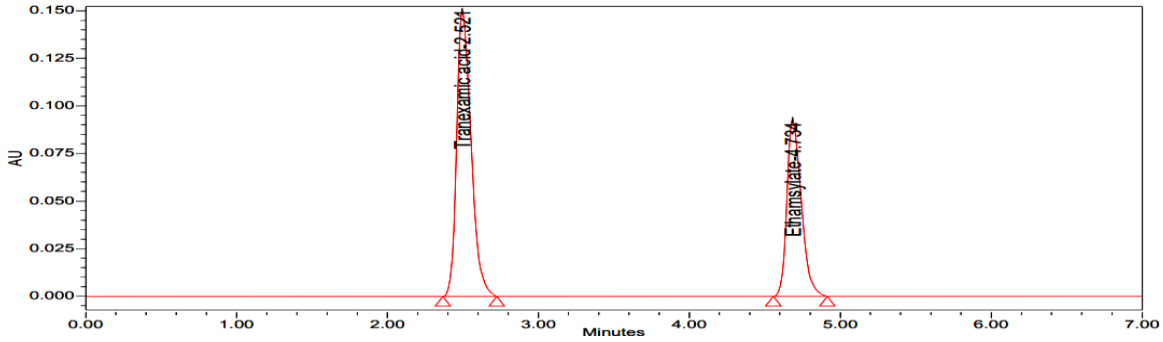


Figure 37:Chromatogram of Linearity-50%

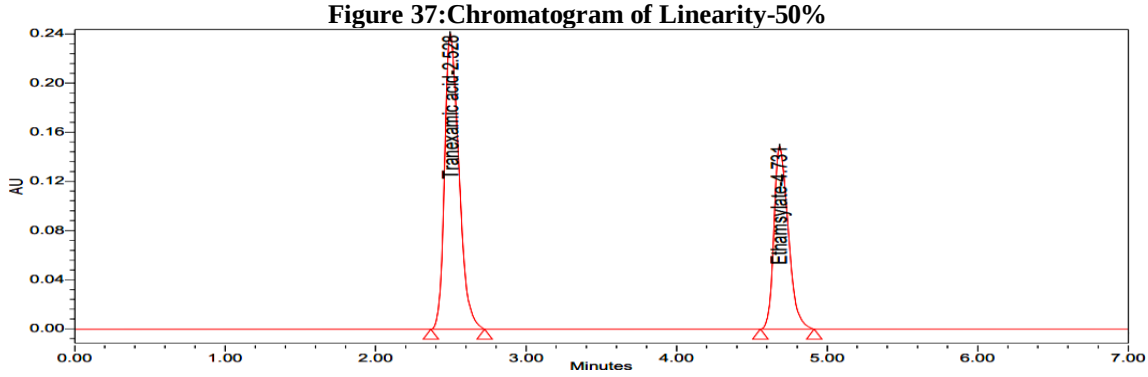


Figure 38:Chromatogram of Linearity-75%

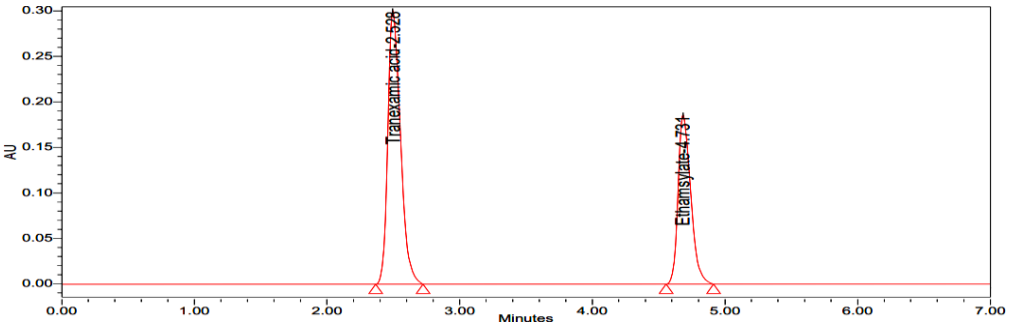


Figure 39:Chromatogram of Linearity-100%

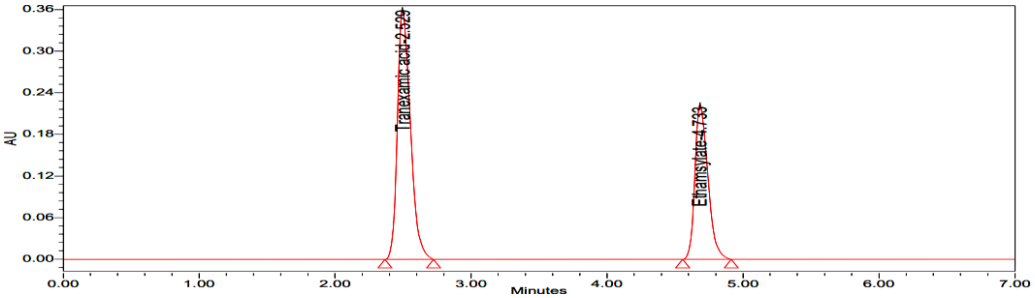


Figure 40:Chromatogram of Linearity-125%

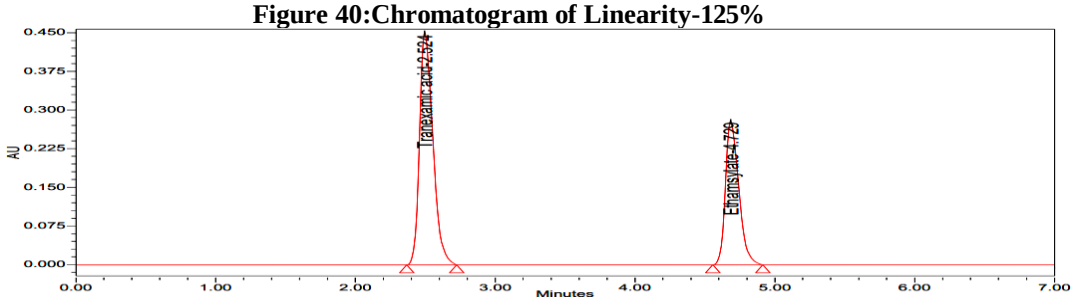


Figure 41:Chromatogram of Linearity-150%

Table 17: Results of linearity for Tranexamic acid& Ethamsylate

S.NO	Tranexamic acid		Ethamsylate	
	Conc.(µg/ml)	Peak area	Conc.(µg/ml)	Peak area
1	62.50	794860	31.25	366125
2	125.00	1464681	62.50	720714
3	187.50	2244870	93.75	1150226
4	250.00	2986315	125.00	1456718
5	312.50	3615432	156.25	1829447
6	375.00	4352174	187.50	2205619
Regression equation	y=11553.89x +41979.57		y=11748.01x+2745.75	
Slope	11553.89		11748.01	
Intercept	41979.57		2745.75	
R^2	0.99967		0.99964	

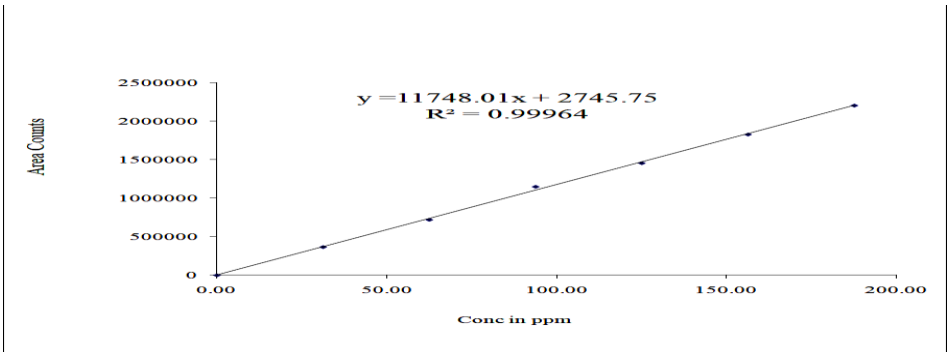


Figure 42: Calibration curve for Ethamsylate

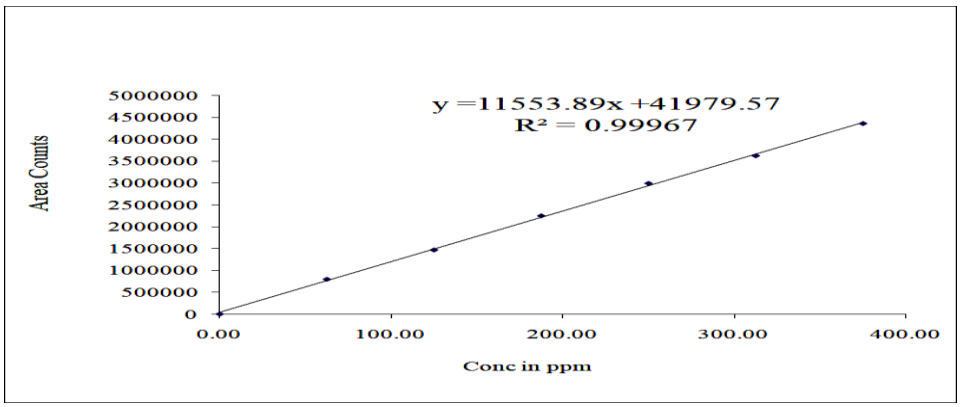


Figure 43:Calibration curve for Tranexamic acid

Assay

The assay of sample was determined and results were tabulated in table 18and 44-45.

Table 18: Assay of Tranexamic acid &Ethamsylate

Brand	Drug	Avg sample area (n=2)	Std purity	% assay
Tranlok-E	Tranexamic acid	2988467	99.9	100.4
	Ethamsylate	1472761	99.8	101.5

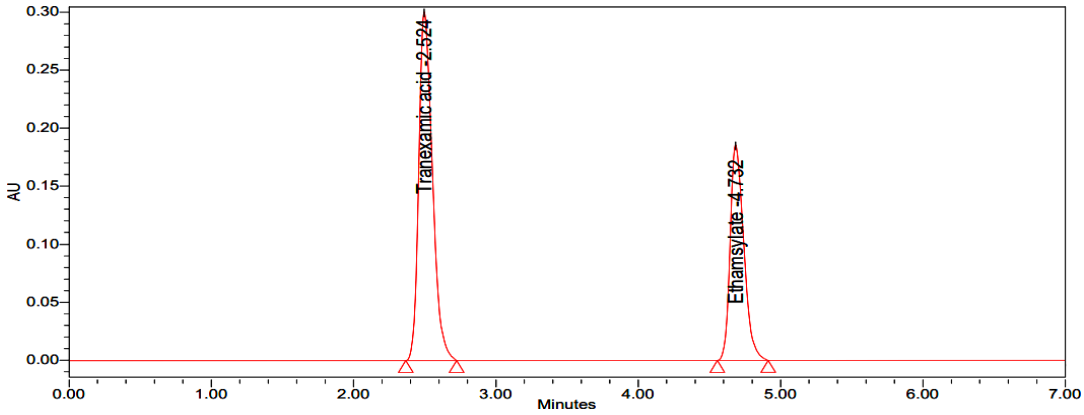


Figure 44:Chromatogram ofAssay-1

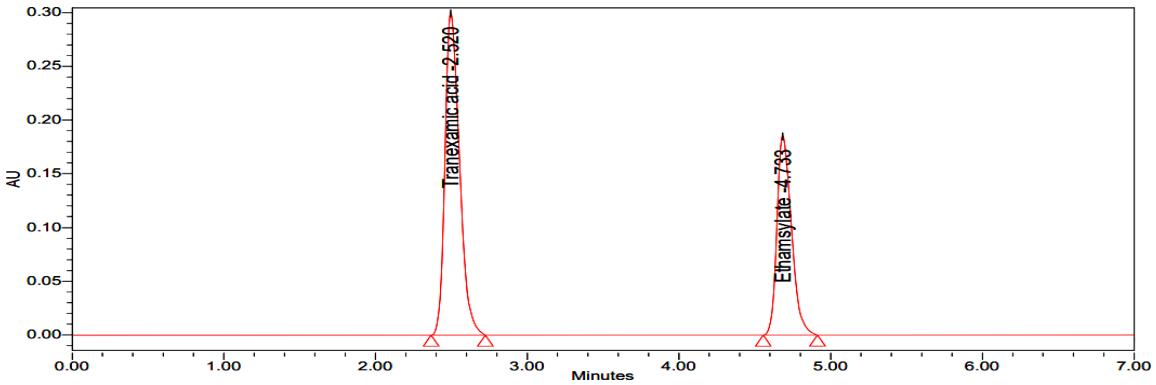


Figure 45: Chromatogram ofAssay-2

Accuracy

Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean% Recovery was Obtained as 99.7% and100.1% for Tranexamic acid and Ethamsylate respectively and results were tabulated in table 6.20, 22 and figures 46- 54.

Table 19: Accuracy results of Tranexamic acid

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	%Recovery	Mean Recovery
50%	1467407	12.5	12.33	98.6	99.6
	1490469	12.5	12.52	100.2	
	1486724	12.5	12.49	99.9	
100%	2982163	25.0	25.06	100.2	100.4
	2998790	25.0	25.20	100.8	
	2979643	25.0	25.04	100.2	
150%	4397985	37.5	36.96	98.6	99.0
	4426017	37.5	37.19	99.2	
	4435412	37.5	37.27	99.4	

Table 20: The Accuracy results for Ethamsylate

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	%Recovery	Mean Recovery
50%	719452	6.25	6.20	99.2	99.4
	724218	6.25	6.24	99.8	
	718636	6.25	6.19	99.0	
100%	1458247	12.50	12.56	100.5	100.3
	1466981	12.50	12.64	101.1	
	1442761	12.50	12.43	99.4	
150%	2187608	18.75	18.85	100.5	100.5
	2178761	18.75	18.77	100.1	
	2192482	18.75	18.89	100.7	

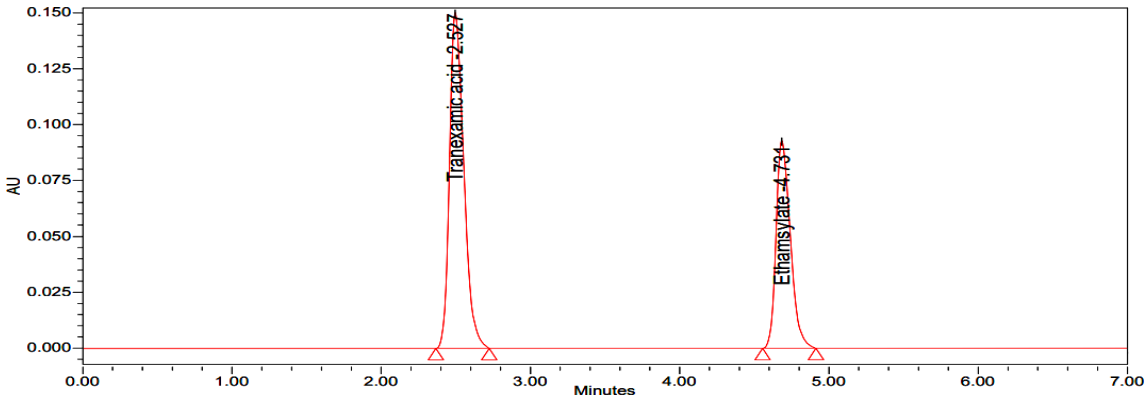


Figure 46:Chromatogram of Accuracy 50%-1

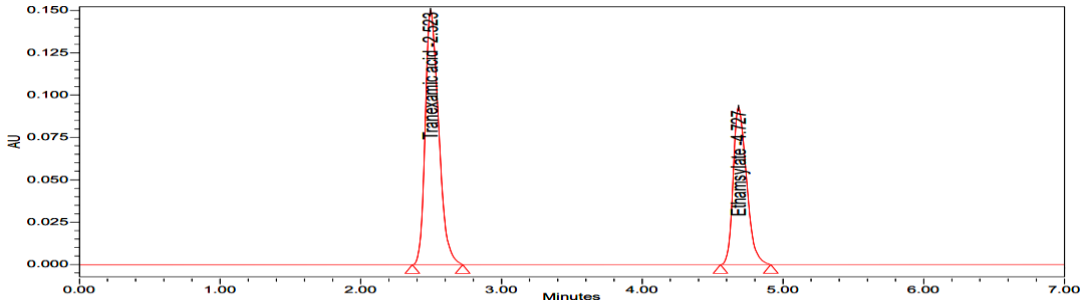


Figure 47:Chromatogram of Accuracy 50%-2

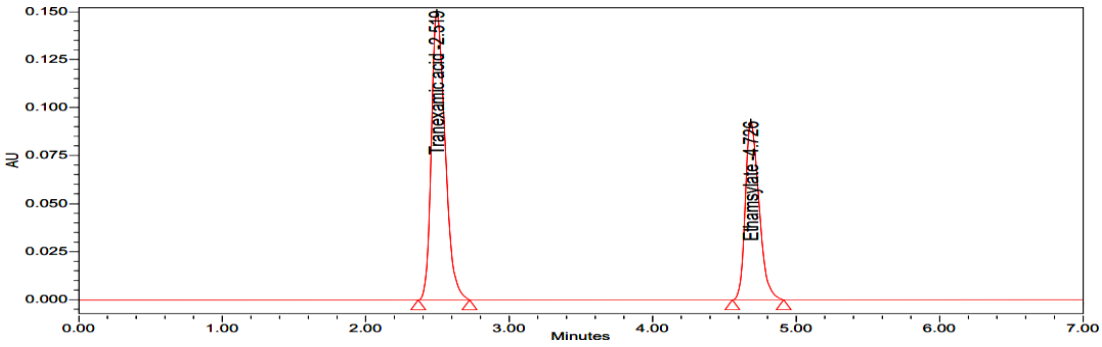


Figure 48:Chromatogram of Accuracy50%-3

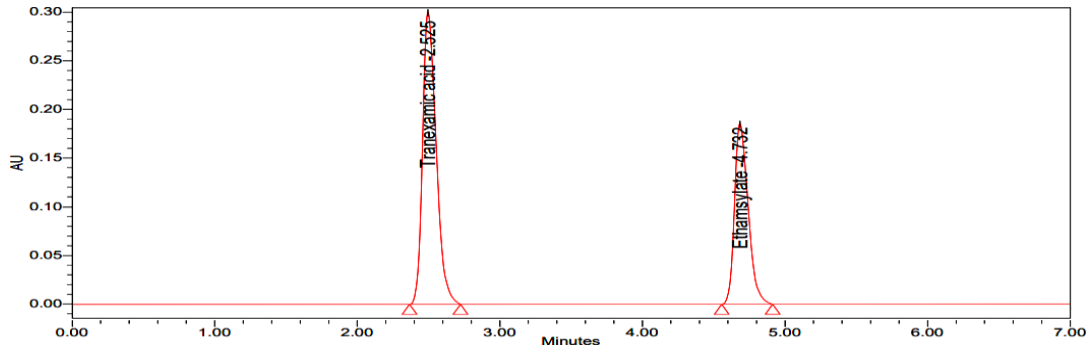


Figure 49:Chromatogram ofAccuracy 100%-1

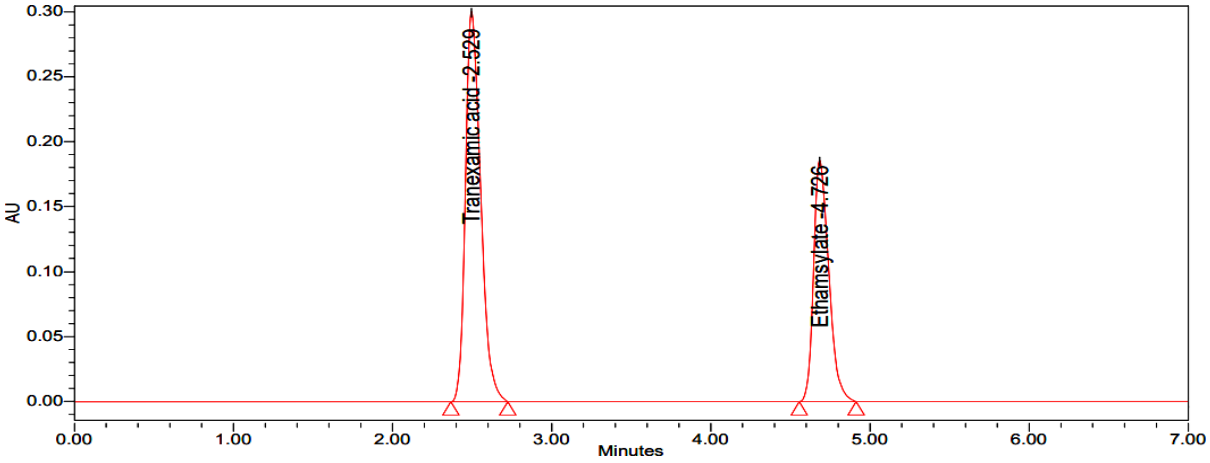


Figure 50:Chromatogram ofAccuracy 100%-2

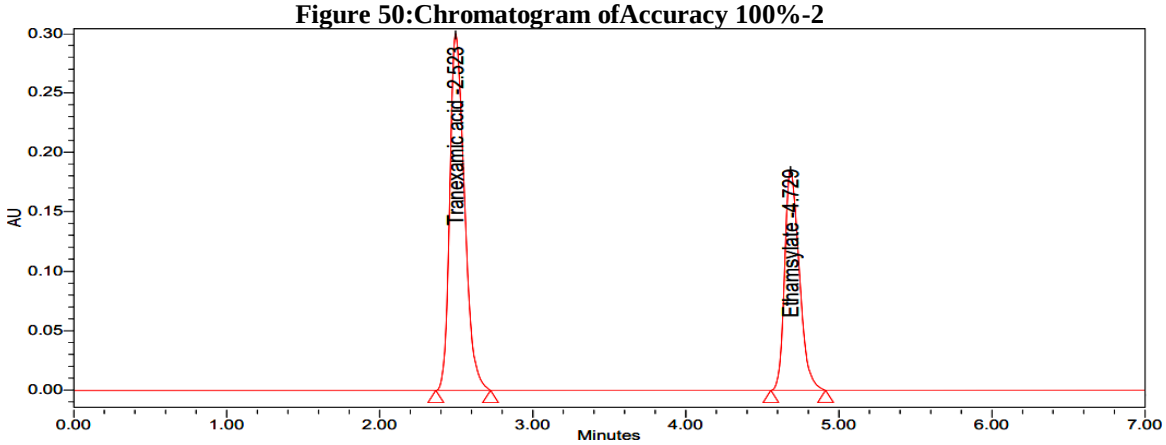


Figure 51:Chromatogram ofAccuracy100%-3

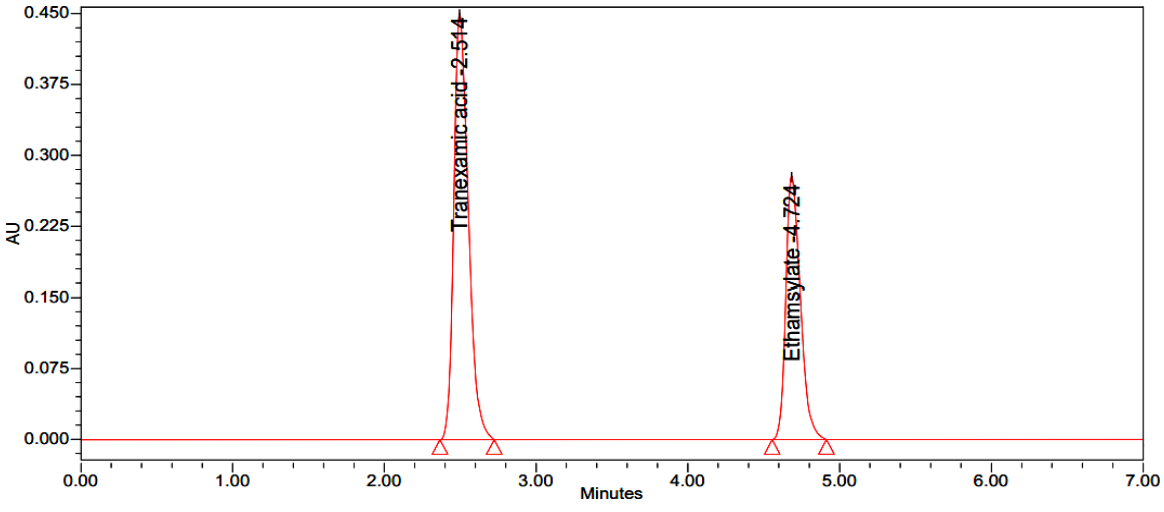


Figure 52:Chromatogram ofAccuracy150%-1

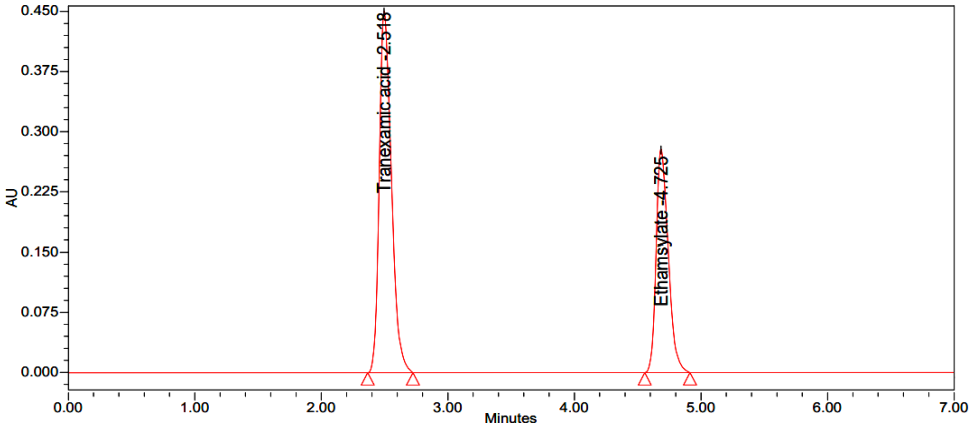


Figure 53:Chromatogram of Accuracy 150%-2

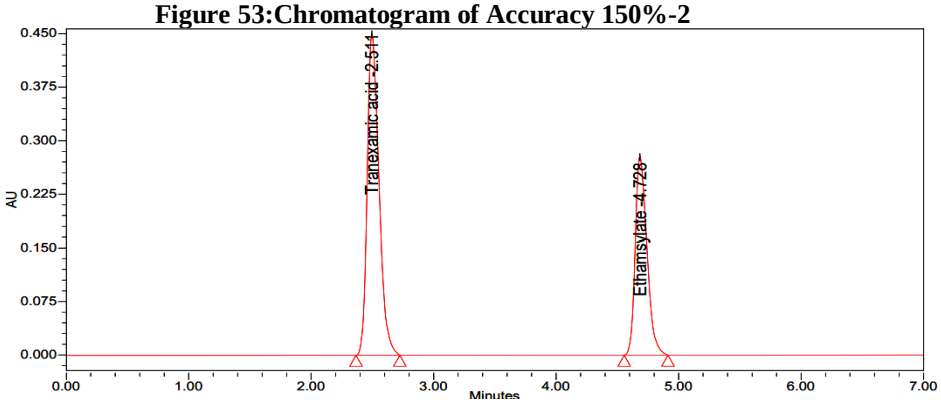


Figure 54:Chromatogram of Accuracy 150%-3

Robustness

The method was found to be robust when subjected to minor changes in the chromatographic condition such as oven temperature (±1°C), mobile phase flow rate (±0.3 ml/min), and wavelength nm (±1 nm). It was observed that there was no marked change in analytical method which indicates good reliability during normal usage. The results are shown in Table 21, 22 & figure 55 - 58.

Table 21: Robustness results of Tranexamica cid

Parameter	Tranexamic acid				
	Condition	Retention Time (min)	Tailing	Plate count	%RSD
Flow rate Change (mL/min)	Less flow (0.9ml)	2.758	1.10	9180	0.20
	Actual (1.0ml)	2.522	1.05	9264	0.33
	More flow (1.1ml)	2.413	1.01	9371	0.50
Organic Phase change	Less Org (27:73)	2.874	1.13	9042	0.32
	Actual (30:70)	2.525	1.08	9253	0.33
	More Org (33:67)	2.309	1.04	9488	0.61

Table .22:Robustness results of Ethamsylate

Parameter	Ethamsylate				
	Condition	Resolution	Tailing	Plate count	%RSD
Flowrate Change (mL/min)	Less flow (0.9ml)	10.59	0.99	12258	0.87
	Actual (1.0ml)	10.48	0.94	12365	0.35
	More flow (1.1ml)	11.04	0.90	12474	0.40
Organic Phasechange	Less Org (27:73)	9.98	1.03	12139	0.97
	Actual (30:70)	10.42	0.98	12350	0.35
	More Org (33:67)	10.63	0.95	12566	1.05

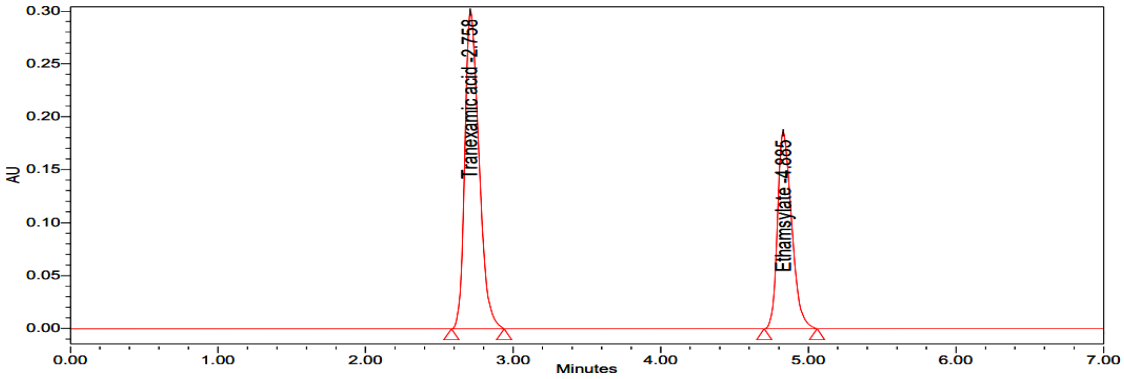


Figure 55:Chromatogram for less flow rate(0.9 ml)

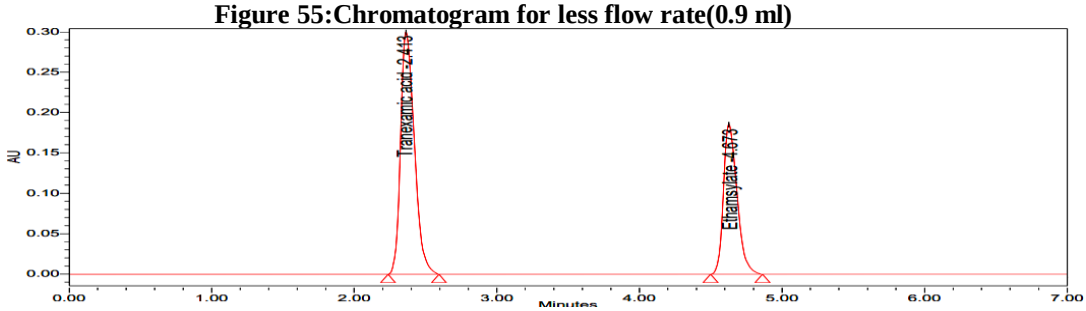


Figure 56: Chromatogram for more flow rate (1.1mL)

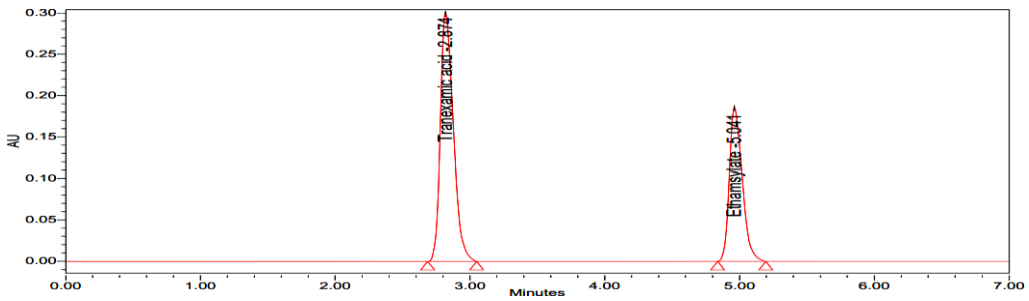


Figure 57:Chromatogram for less Organic Phase (27:73)

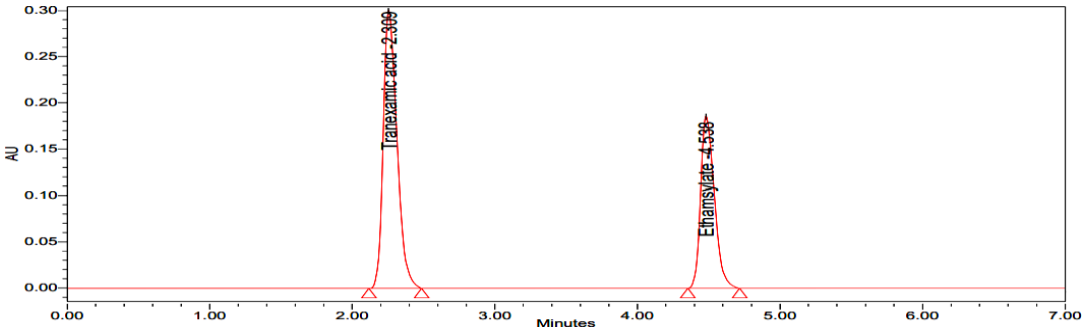


Figure 58:Chromatogram for more Organic Phase (33:67)

LOD and LOQ

The values for tranexamic acid and ethamsylate's LOD and LOQ were calculated and are displayed in Table 21 and Figure 59, 60, respectively.

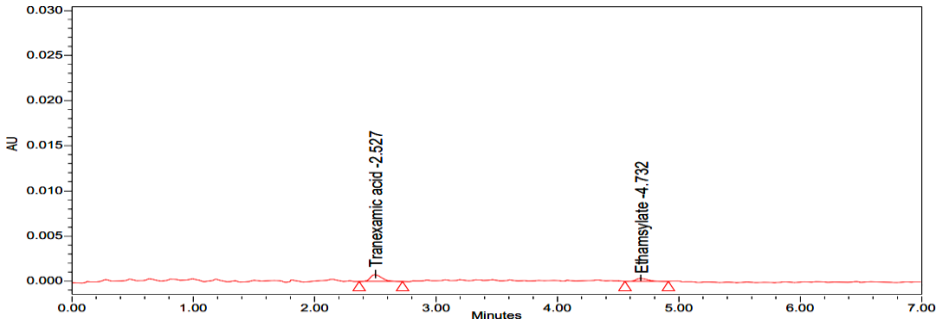


Figure 59: Chromatogram for LOD

Table .21: Sensitivity parameters (LOD&LOQ)

Name of drug	LOD (µg/ml)	s/n	LOQ (µg/ml)	s/n
Tranexamic acid	0.6	3	2.0	10
Ethamsylate	0.3	3	1.0	10

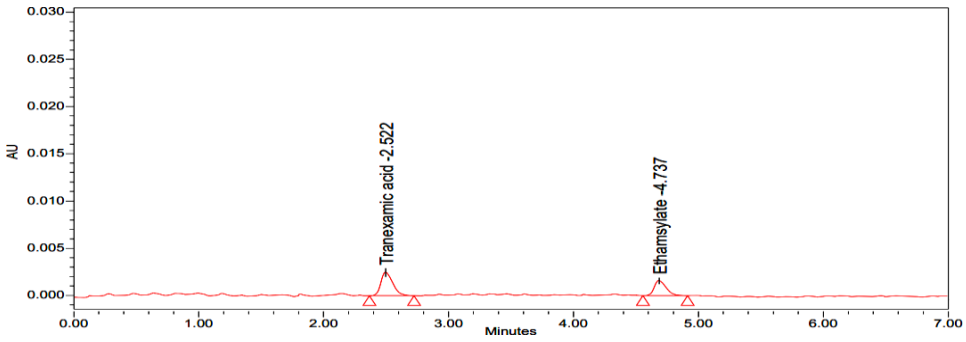


Figure 60: Chromatogram for LOQ

Degradation Studies

Samples were injected to assess the linearity. The following force degradation conditions were applied to both tranexamic acid and ethamsylate:acidic, alkaline, neutral, oxidation, and photolytic. The percentages of degradation and recovery are computed and displayed in Table 22 & 23 and Figure 61, respectively.

Table.22:Forced Degradation results for Tranexamic acid

Conditions	Tranexamic acid				
	Area	%Assay	%Deg	Purity Angle	Purity Threshold
Control	3012098	100	0	1.955	14.331
Acid	2614107	86.8	13.2	1.963	14.386
Alkali	2624876	87.2	12.8	1.955	14.352
Peroxide	2554864	84.8	15.2	1.936	14.397
Reduction	2716760	90.2	9.8	1.954	14.322
Thermal	2992876	99.4	0.6	1.988	14.310
Photolytic	2945671	97.8	2.2	1.947	14.305
Hydrolysis	2927963	97.2	2.8	1.921	14.377

Table 23:Forced Degradation results for Ethamsylate

Conditions	Ethamsylate				
	Area	%Assay	%Deg	Purity Angle	Purity Threshold
Acid	1466718	100	0	5.627	9.234
Alkali	1277389	87.1	12.9	5.659	9.265
Peroxide	1305967	89.0	11.0	5.611	9.218
Reduction	1253123	85.4	14.6	5.674	9.277
Thermal	1437921	98.0	2.0	5.656	9.258
Photolytic	1411330	96.2	3.8	5.622	9.219
Hydrolysis	1457833	99.4	0.6	5.609	9.206

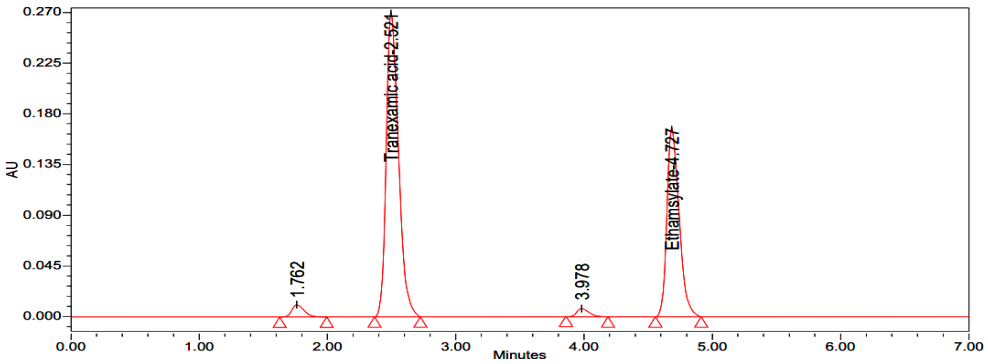


Figure 61:Chromatogram of Acid degradation

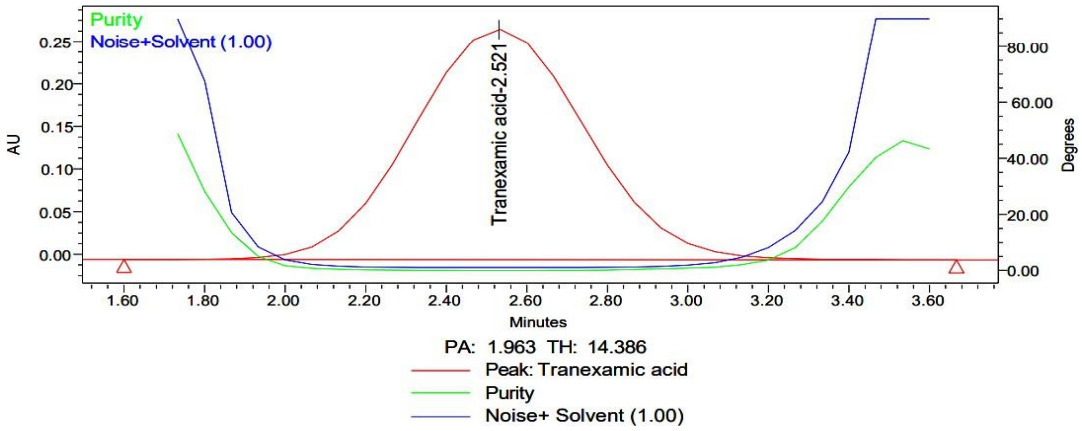


Figure 62:Purity Plot of Tranexamica cid

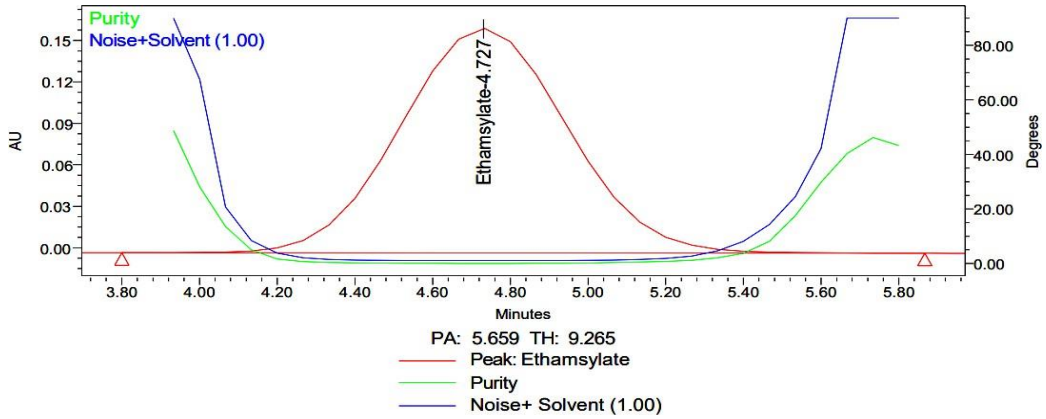


Figure 63: Purity Plot of Ethamsylate

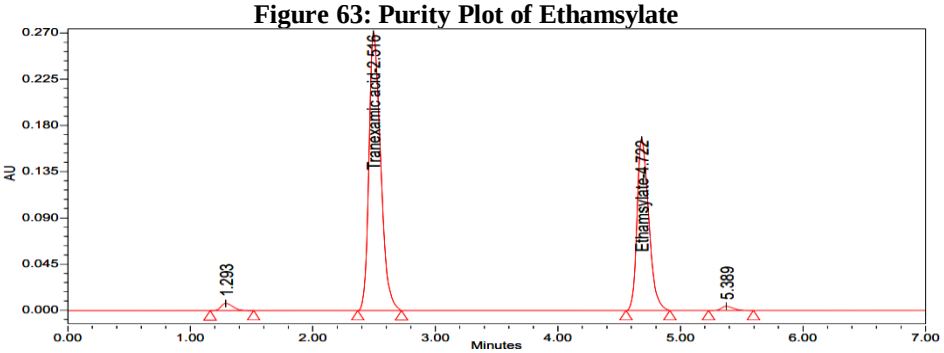


Figure 64:Chromatogram of Alkali degradation

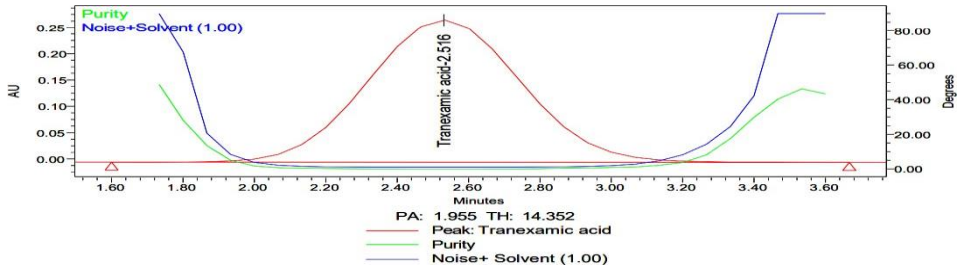


Figure 65: Purity Plot of Tranexamic acid

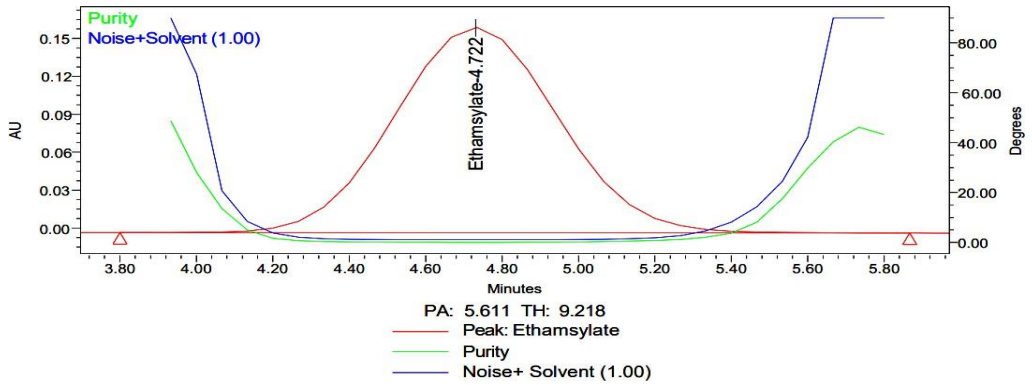


Figure 66: Purity Plot of Ethamsylate

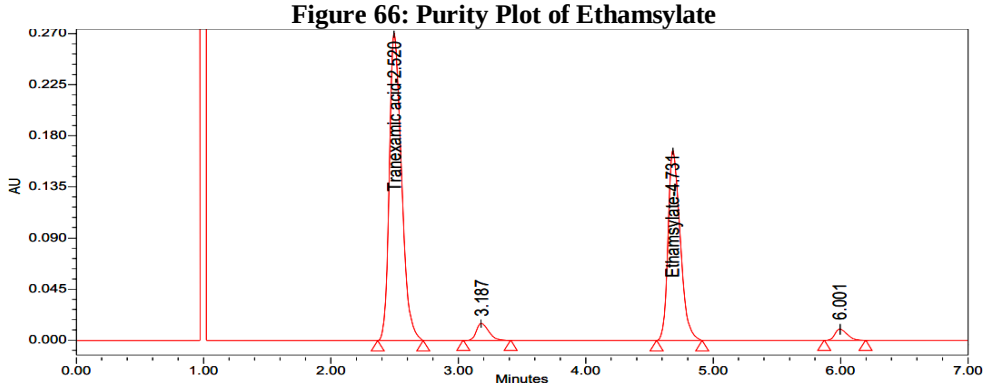


Figure 67:Chromatogram of Peroxide degradation

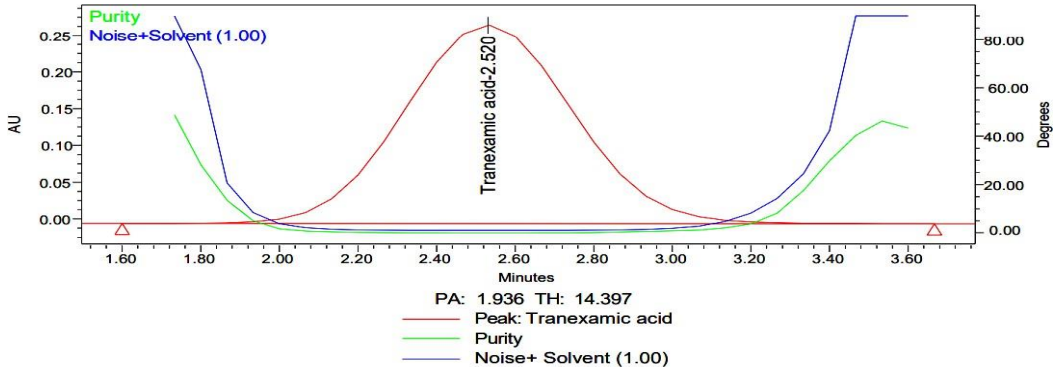


Figure 68:Purity Plot of Tranexamica cid

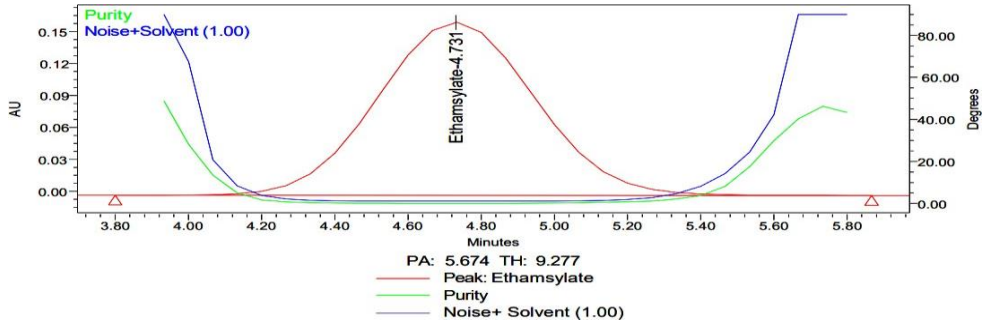


Figure 69: Purity Plot of Ethamsylate

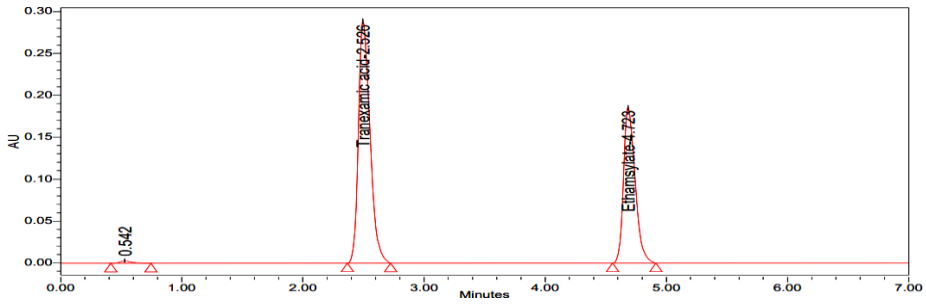


Figure 70: Chromatogram of Reduction degradation

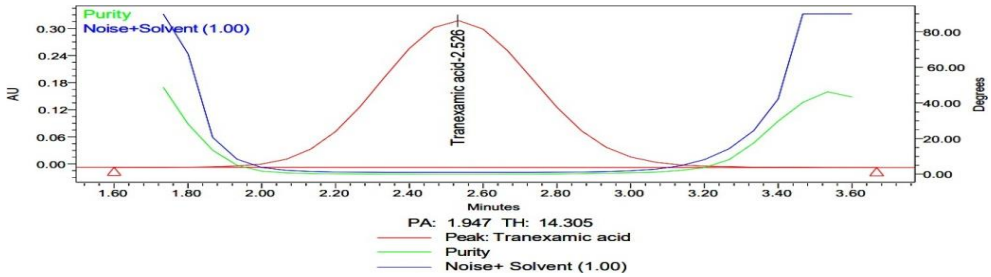


Figure 71:Purity Plot of Tranexamica cid

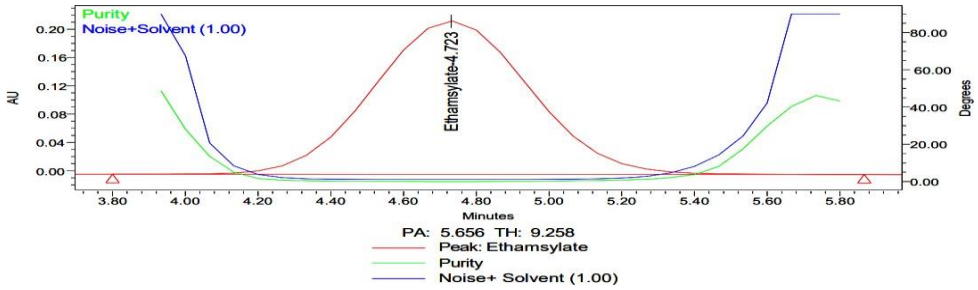


Figure 72: Purity Plot of Ethamsylate

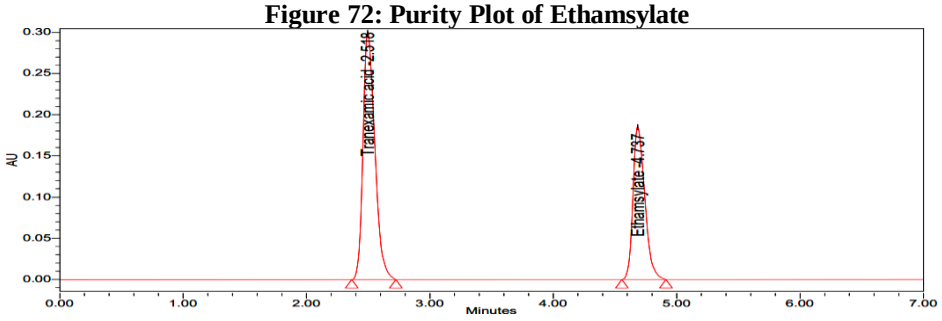


Figure 73:Chromatogram of Thermal degradation

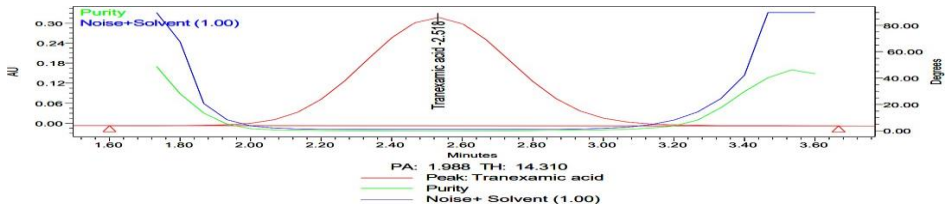


Figure 74:Purity Plot of Tranexamic acid

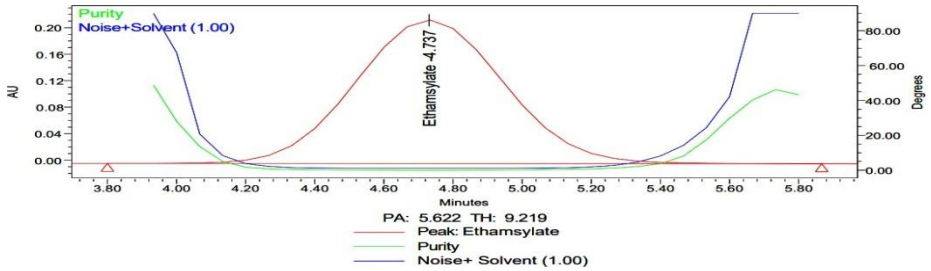


Figure 75: Purity Plot of Ethamsylate

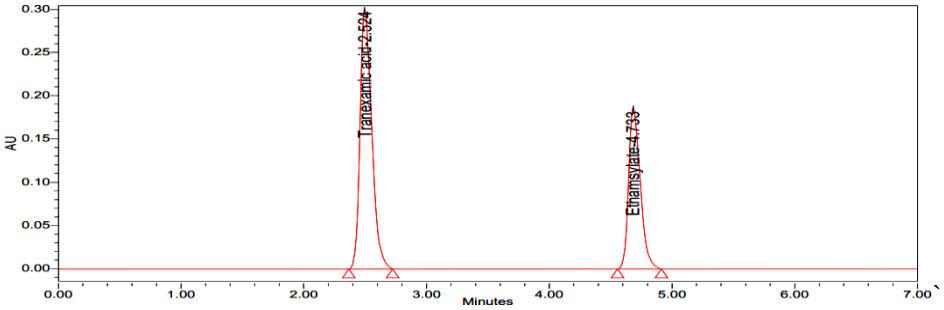


Figure 76: Chromatogram of Photolytic degradation

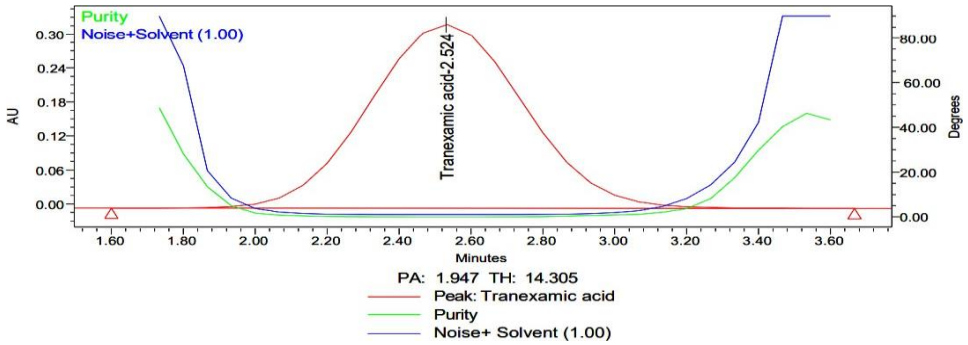


Figure 77:Purity Plot of Tranexamic acid

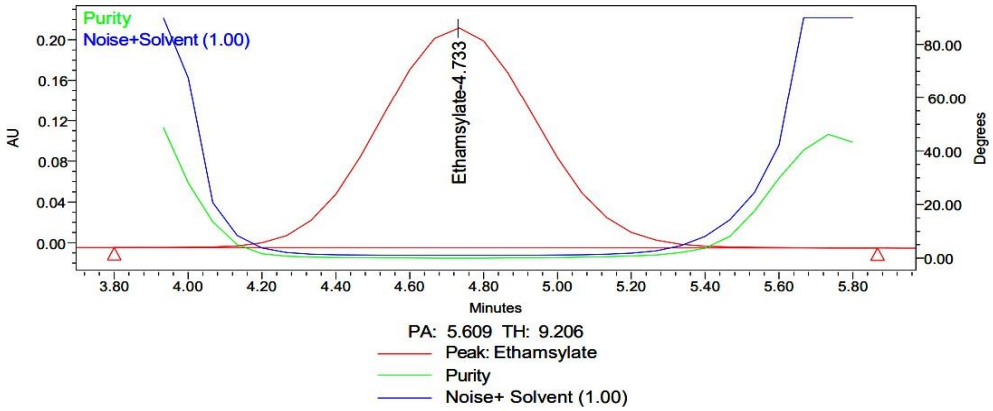


Figure 78: Purity Plot of Ethamsylate

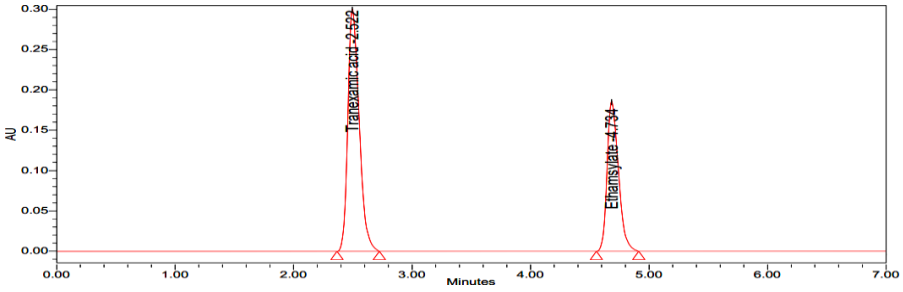


Figure 79: Chromatogram of Hydrolysis degradation

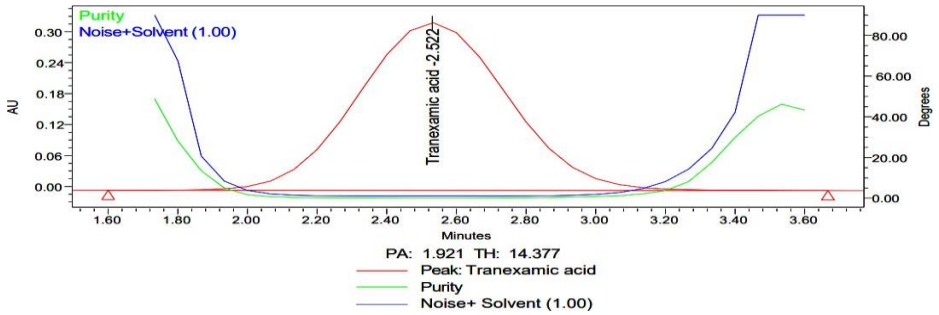


Figure 80:Purity Plot of Tranexamic acid

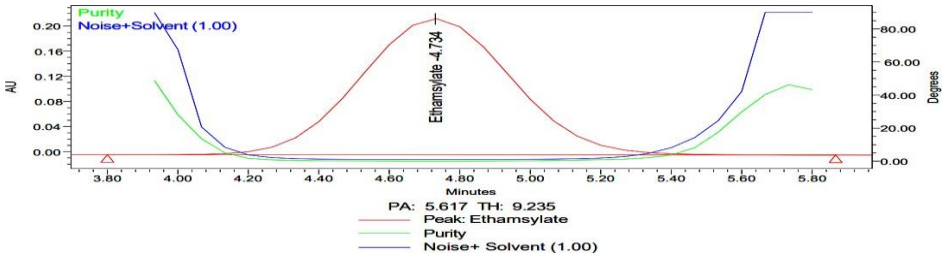


Figure 81: Purity Plot of Ethamsylate

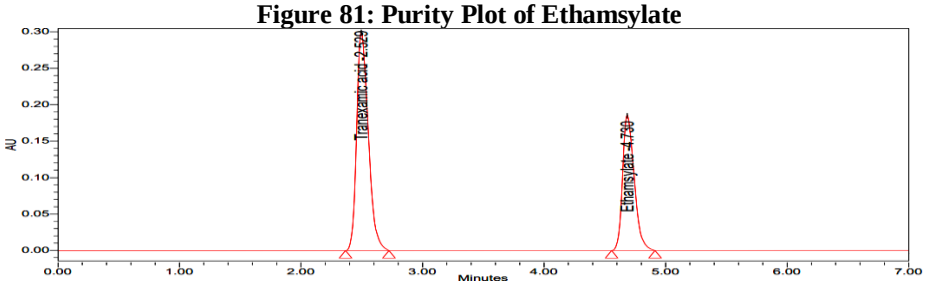


Figure 82:Chromatogram of Control degradation

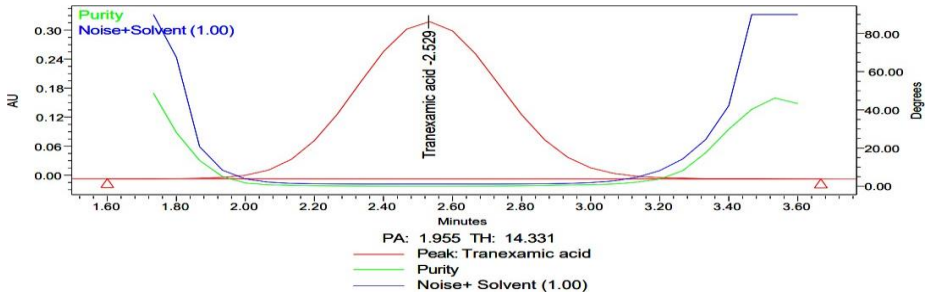


Figure 83:Purity Plot of Tranexamic acid

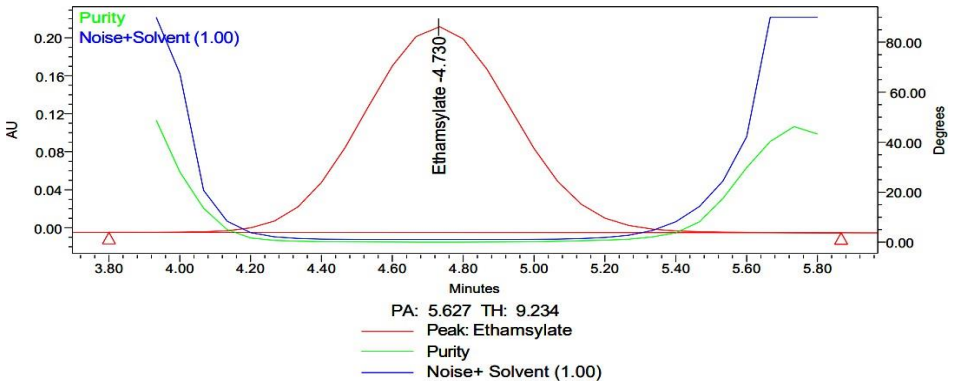


Figure 84: Purity Plot of Ethamsylate

SUMMERY AND CONCLUSION:

In the drug discovery and development process, and in turn, in the manufacture of drugs and pharmaceuticals, the development and validation of analytical methods plays an important role. A holistic approach is recommended in order to be efficient in method development and validation. The purpose of the current work was to evaluate the applicability of High-Performance Liquid Chromatography (HPLC) to the study of different groups of drugs and to apply the developed method of analytical analysis to the analyte in its dosage forms and biological matrices.

The technique for the simultaneous estimation of bulk tranexamic acid and ethamsylate and its pharmaceutical formulation is discussed and validated by RP- HPLC method. The procedure was successfully validated in compliance with ICH guidelines and found that the results of all validation parameters were within the accepted limits.

CONCLUSION:

For the determination of some selected drugs, and RP- HPLC methods were developed and validated. The developed methods were very simple and results obtained by them confirm appropriate specificity, accuracy and precision. For HPLC methods, validation has been done according to ICH requirements. Validation parameters like specificity, linearity, accuracy, precision, robustness etc., were evaluated. Results of validation study proves that newly developed methods have advantage over previously reported methods, especially with respect to very less runtime, low limit of detection and quantification with high accuracy and wide range of linearity.

The developed RP- HPLC methods have therefore proven to be appropriate for the assessment of routine quality control analysis as well as for research and development. The preparation of samples was simple. No excipient interference was observed in the products examined, so no additional extraction or separation procedures were required. The methods showed the speed, precision and accuracy necessary for the determination of dosage forms.

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