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

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ABSTRACT
A simple, accurate, precise Stability Indicating Reverse Phase High Performance Liquid Chromatography (RP-HPLC) Method was developed and validated for the simultaneous estimation of Tranexamic acid and Ethamsylate tablet dosage form using C_{18} column Hypersil (250mm× 4.6mm, 5µm) with Mobile Phase Phosphate Buffer: Methanol (25:75 v/v) pH was adjusted to 3.5 by addition of Orthophosphoric acid at a Flow rate of 1mL/min. Detection was carried out at wavelength 215nm. Retention time for Tranexamic acid and Ethamsylate was found to be 6.58min and 3.27min respectively. The Forced Degradation was carried out using Acid hydrolysis, Alkaline hydrolysis, Oxidative degradation, Photolytic degradation and Thermal degradation. The Forced Degradation study indicates that Tranexamic acid significantly degrade under Photolytic degradation and Ethamsylate under Acid hydrolysis. Linearity of both the drugs was observed between 25- 75µg/mL. The LOD of Tranexamic acid and Ethamsylate is found to be 3.65µg/mL and 4.06µg/mL respectively. The LOQ of Tranexamic acid and Ethamsylate is found to be 11.08µg/mL and 12.31µg/mL respectively. The Accuracy was performed at 80% to 120% and was found to be in between 99.88% to 100.18% and 99.50% to 99.87% for TRX and ETM respectively. Assay percentage for Tranexmic acid and Ethamsylate was found to be 102.50% and 96.72% respectively.

KEY WORDS: Tranexamic acid, Ethamsylate, RP-HPLC Method, Validation.

1. INTRODUCTION
Menorrhagia is a menstrual period with abnormally heavy flow and falls under the larger category of abnormal uterine bleeding (AUB). A normal menstrual cycle is 21-35 days in duration, with bleeding lasting an average of 5 days and total blood flow between 25 and 80mL. Deviations in terms of frequency of menses, duration of menses, or volume of menses qualifies as abnormal uterine bleeding.

Tranexamic acid is chemically Trans- 4- (Aminomethyl) cyclohexane carboxylic acid. Tranexamic acid competitively inhibits activation of plasminogen, thereby reducing conversion of plasminogen to plasmin (fibrinolysis), and other plasma protiens, including the procoagulant factors V andVIII. Tranexamic acid also directly inhibits plasmin activity, but higher doses are required than are needed to reduce plasmin formation.

Ethamsylate is chemically 2, 5-Dihydroxybenzenesulfonic acid; N-ethylethanamine. It is Haemostatic agent; also promotes angioprotective and Proggregant action. It stimulates the process of thrombopoiesis and their release from bone marrow.

Literature survey reveals that there is no Stability indicating method for simultaneous estimation of Tranexamic acid and Ethamsylate in tablet dosage form.

The aim of this work is to develop a simple, accurate and precise Stability Indicating method and to validate the same.

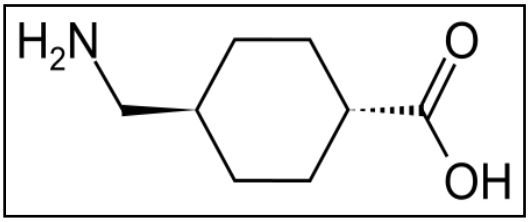


Figure 1: Structure of Tranexamic acid

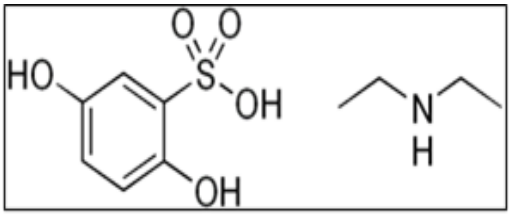


Figure 2: Structure of Ethamsylate

2. EXPERIMENTAL

2.1 Instrumentation: A Shimadzu HPLC, Model: LC-10 AVP (Shimadzu) with Auto Injector with Capacity Loop of 10µL, UV- Visible detector Model: SPD- 10A VP (Shimadzu) and LC Solution Software. C_{18} Column Hypersil (250mm× 4.6mm, 5µm).

2.2 Chemicals and Reagents: The pure drug form of Tranexamic acid and Ethamsylate is collected from Sun Pharmaceuticals Silvasa. HPLC grade Acetonitrile (Merck Ltd., Mumbai, India), HPLC grade Methanol (Merck Ltd., Mumbai, India), Ortho Phosphoric acid (S.d. fine chem. Pvt. Ltd.).

2.3 Chromatographic Conditions: HPLC method – The Mobile Phase has been used for separation consisting of Phosphate Buffer (pH 3.5): Methanol (25: 75 v/v, pH adjusted with1% Orthophosphoric acid) using C_{18} column Hypersil (250mm× 4.6mm, 5µm) with Flow Rate 1mL/min. The Detection Wavelength was 215nm, and the injection volume was 50µL.

2.4 Stress Studies: Acidic Conditions, For Acidic hydrolysis 1mL standard stock solution of both the drugs and stock solution of sample was treated with 2mL 0.1 N HCl at RT for 2hrs and neutralizing it with 2mL 0.1N NaOH then diluted up to 10mL with Mobile Phase and then analysed.

Alkaline Conditions, For Acidic hydrolysis 1mL standard stock solution of both the drugs and stock solution of sample was treated with 2mL 0.1 N NaOH at RT for 2hrs and neutralizing it with 2mL 0.1HCl then dilute up to 10mL with Mobile Phase and then analysed.

Oxidative Degradation, For Oxidative degradation 1mL standard stock solution of both the drugs and stock solution of sample was treated with 2mL 3% at RT for 2hrs then diluted up to 10mL with Mobile Phase and then analysed.

Photolytic Degradation, For Photolytic degradation 1mL standard stock solution of both the drugs and stock solution of sample was kept in sunlight for 2hrs then diluted up to 10mL with Mobile Phase and then analysed.

Thermal Degradation, For Thermal degradation 1mL standard stock solution of both the drugs and stock solution of sample was kept in oven at 105ºC for 2hrs then diluted up to 10mL with Mobile Phase and then analysed.

2.5 Preparation of standard stock solutions: Standard stock solution of TRX, 50mg of TRX was accurately weighed and transferred to 100mL volumetric flask and dissolved in Water and sonicated for about 10min. Volume was made up to the mark with water to give a solution containing 500µg/mL TRX solution.

Standard stock solution of ETM, 50mg of ETM was accurately weighed and transferred to 100mL volumetric flask and dissolved in Methanol and sonicated for about 10min. Volume was made up to the mark with methanol to give a solution containing 500µg/mL ETM solution.

2.6 Preparation of stock solution of Sample: 50 Tablets were weighed and powdered. Powder equivalent to 50mg TRX/ 50mg ETM was taken and transferred to 100mL volumetric and dissolved in 60mL diluent and sonicated for about 10min. Volume was made up to the mark with methanol to give a solution containing 500µg/mL Ethamsylate and 500µg/mL Tranexamic acid solution. Diluent for Sample Stock Solution was Water: Methanol 50: 50.

Preparation of working standard solution of sample: 1mL of Ethamsylate Standard Stock Solution was transferred in 10mL volumetric flask. Volume was made up to the mark with Mobile Phase to give a solution containing 50µg/mL Ethamsylate and Tranexamic acid solution.

2.7 System Suitability Test: System suitability test of chromatographic system was performed before Validation using 6 replicate injection of 50µg/mL of TRX and ETM solutions. Theoretical plates and tailing factor were determined.

3. METHOD VALIDATION

3.1 Linearity and Range: 0.5mL, 0.75mL, 1mL, 1.25mL, 1.75mL of Tranexamic acid and Ethamsylate Standard Stock Solution respectively in five different 10mL volumetric flask. Volume was made up to the mark with Mobile Phase to give a solution containing 25µg/mL, 37.5µg/mL, 50µg/mL, 62.5µg/mL, 75µg/mL Tranexamic acid and Ethamsylate solution respectively in a binary mixture and were injected to the system with stated chromatographic conditions.

3.2 Limit of Detection and Limit of Quantification: The L.O.D. and L.O.Q was estimated from calibration curves.

LOD = 3.3 × (S.D./Slope) LOQ = 10 × (S.D./Slope)

3.3 Accuracy (%Recovery): Accuracy was performed at 80%, 100%, and 120% levels of drug concentrations by standard addition method. Accuracy was performed on 50µg/mL of TRX and ETM solutions and Binary mixture containing TRX and ETM equivalent to 50µg/mL of both

Preparation of Recovery Solution: For 80% level: 0.5mL of Sample solution, 0.4mL of Stock solution of ETM and 0.4mL of Stock Solution of TRX were transferred in 10mL volumetric flask and volume was made up to the mark with Mobile Phase.

For 100% level: 0.5mL of Sample solution, 0.5mL of Stock solution of ETM and 0.5mL of Stock Solution of

TRX were transferred in 10mL volumetric flask and volume was made up to the mark with Mobile Phase.

For 120% level: 0.5mL of Sample solution, 0.6mL of Stock solution of ETM and 0.6mL of Stock Solution of TRX were transferred in 10mL volumetric flask and volume was made up to the mark with Mobile Phase.

3.4 Precision

Intraday Precision: Intraday Precision was performed at three different concentrations of binary mixture of ETM and TRX thrice on the same day.

Interday Precision: Interday Precision was performed at three different concentrations of binary mixture of ETM and TRX on three different days. The concentrations of the binary mixtures are given below.

Repeatability: Repeatability study was performed on only one binary mixture (Middle concentration) of TRX and ETM repeated for 6 times.

Lower concentration (80%) - TRX- 25 µg/mL + ETM-25 µg/mL Middle concentration (100%) - TRX- 50 µg/mL + ETM- 50 µg/mL Higher concentration (120%) - TRX- 75 µg/mL + ETM-75 µg/mL

3.5 Robustness: Robustness was checked by analysing the solutions at different Flow rate, Mobile phase and pH at concentration 50 µg/mL of TRX and ETM.

4. ASSAY

Binary mixture containing TRX and ETM 50µg/mL of both was prepared from API's and Sample solution was prepared from the formulation containing TRX and ETM equivalent to 50µg/mL of both and were analysed and % drug content was calculated.

5. RESULT AND DISCUSSION

Table 1: System suitability result

Parameters		Ret. Time (min)	Theoretical Plate (N)	Tailing Factor (T)	Resolution (R)
Specifications		-	NLT 2000	NMT 2	MT 2
ETM		3.26	4343	1.57	-
TRX		6.58	6003	1.55	-
Std.	**ETM**	3.28	4379	1.68	12.20
	TRX	6.56	5966	1.57	
Sample	**ETM**	3.27	4370	1.61	12.27
	TRX	6.58	5997	1.51	

Table 2: Finalized chromatographic conditions

Parameters	Specifications
Column	C18 column Hypersil (250mm× 4.6mm, 5µm)
Mobile Phase	Phosphate Buffer: Methanol (25:75 v/v) pH was adjusted to 3.5 by addition of Orthophosphoric acid
Flow rate	1mL/min
Volume of Injection(µL)	50µL
Run time	10min
Detection wavelength (nm)	215nm
Retention time	ETM-3.27, TRX- 6.58 min

Table 3: Result of Forced degradation studies

Degradation type	Std. Area: ETM: 4637.109 ,TRX: 4130.606				%Degradation of Single drug		%Degradation of Sample	
	Area of Single drug		Area of sample					
	ETM	TRX	ETM	TRX	ETM	TRX	ETM	TRX
Acidic	4065.32	3769.35	4028.55	3801.42	12.33	8.74	13.12	7.96
Alkaline	3932.85	3582.41	3930.91	3612.51	15.19	13.27	15.23	12.54
Oxidative	3796.65	3478.13	3800.36	3427.53	18.12	15.79	18.04	17.02
Photolysis	4135.84	3356.56	4204.62	3341.66	10.81	18.73	9.33	19.09
Thermal	4164.12	3568.59	4211.32	3525.72	10.20	13.60	9.18	14.64

Table 4: Linearity of TRX

Sr. No.	Conc. (µg/mL)	Mean Area ± SD	% RSD
1	25	2000.337± 31.24	1.56
2	37.5	3016.36± 29.43	0.97
3	50	4050.77± 45.57	1.12
4	62.5	4860.294± 79.22	1.63
5	75	6082.623± 60.03	0.98

Table 5: Linearity of ETM

Sr. No.	Conc. (µg/mL)	Mean Area ± SD	% RSD
1	25	2263.755± 23.22	1.02
2	37.5	3410.475± 39.42	1.15
3	50	4577.802± 45.09	0.98
4	62.5	5510.737± 95.06	1.72
5	75	6864.27± 68.36	0.99

Table 6: Limit of Detection and Limit of Quantification

Drugs	LOD (µg/mL)	LOQ (µg/mL)
Ethamsylate	3.65	11.08
Tranexamic acid	4.06	12.31

Table 7: Accuracy data of TRX

Level	Amount of sample taken (µg/mL)	Amount of standard spiked (µg/mL)	Total amount (µg/mL)	Std. Amount recovered (µg/mL)	% Recovery	Mean %Recovery ± SD n=3	% RSD
80%	25	20	45	19.79	98.96	100.18±1.127	1.125
	25	20	45	20.23	101.18		
	25	20	45	20.08	100.40		
100%	25	25	50	24.81	99.25	99.88±0.651	0.652
	25	25	50	25.13	100.55		
	25	25	50	24.96	99.85		
120%	25	30	55	30.17	100.58	99.95±0.603	0.603
	25	30	55	29.81	99.38		
	25	30	55	29.96	99.89		

Table 8: Accuracy data of ETM

Level	Amount of sample taken (µg/mL)	Amount of standard spiked (µg/mL)	Total amount (µg/mL)	Std. Amount recovered (µg/mL)	% Recovery	Mean %Recovery ± SD n=3	% RSD
80%	25	20	45	19.74	98.70	99.87±1.061	1.062
	25	20	45	20.15	100.77		
	25	20	45	20.02	100.14		
100%	25	25	50	25.74	98.99	99.55±0.663	0.666
	25	25	50	24.07	100.29		
	25	25	50	25.84	99.50		
120%	25	30	55	30.03	100.11	99.50±0.532	0.535
	25	30	55	29.74	99.13		
	25	30	55	29.78	99.27		

Table 9: Interday Precision of both the drugs

Tranexamic acid			Ethamsylate		
Conc. (µg/mL)	Area Mean ± S.D. (n=3)	%RSD	Conc. (µg/mL)	Area Mean ± S.D. (n=3)	%RSD
25	1990.363±4.022	0.202	25	2251.009±2.662	0.118
50	4030.555±8.060	0.199	50	4553.359±6.687	0.151
75	6040.088±12.113	0.200	75	6812.243±8.272	0.121

Table 10: Intraday Precision of both the drugs

Tranexamic acid			Ethamsylate		
Conc. (µg/mL)	Area Mean ± S.D. (n=3)	%RSD	Conc. (µg/mL)	Area Mean ± S.D. (n=3)	%RSD
25	1987.712±4.192	0.210	25	2248.453±3.058	0.136
50	4026.534±8.060	0.200	50	4548.969±7.081	0.155
75	6041.056±16.746	0.277	75	6817.609±8.739	0.128

Table 11: Repeatability Study

Sr. No.	Tranexamic acid Conc. 50 µg/mL	Ethamsylate Conc. 50 µg/mL
	Area	Area
1	4559.521	4034.591
2	4568.662	4042.68
3	4564.741	4050.77
4	4564.082	4038.63
5	4573.218	4046.71
6	4581.133	4054.803
Avg	4568.559	4044.697
SD	7.6958	7.5640
% RSD	0.1684	0.1870

Table 12: Result of Robustness of Tranexamic acid

Sr. No.	Tranexamic acid 50 µg/mL					
	pH		Flow rate		Mobile Phase	
	+ 0.2 units	− 0.2 units	+ 0.2 units	− 0.2 units	+ 0.2 %	− 0.2 %
1	3852.296	4127.457	3933.336	4176.314	3925.448	4131.598
2	3876.42	4151.794	3957.545	4200.735	3949.616	4155.959
3	3900.674	4176.314	3981.774	4225.232	3977.783	4180.502
Avg	3876.463	4151.588	3957.552	4200.760	3950.949	4156.020
SD	24.189	24.428	24.219	24.459	26.192	24.452
% RSD	0.623	0.588	0.611	0.582	0.662	0.588

Table 13: Result of Robustness of Ethamsylate

Sr. No.	Ethamsylate 50 µg/mL					
	pH		Flow rate		Mobile Phase	
	+ 0.2 units	− 0.2 units	+ 0.2 units	− 0.2 units	+ 0.2 %	− 0.2 %
1	4353.197	4664.684	4444.819	4719.762	4435.906	4669.372
2	4380.534	4692.115	4472.269	4747.479	4459.962	4696.825
3	4403.825	4713.435	4495.727	4768.105	4489.469	4714.34
Avg	4379.185	4690.078	4470.938	4745.115	4461.779	4693.512
SD	25.340	24.439	25.480	24.258	26.826	22.666
% RSD	0.578	0.521	0.569	0.511	0.601	0.578

Table 14: Result of Assay

Drug	**Actual conc. of Drug (µg/ml)**	Conc. of Drug Found (µg/ml)	% of Drug found	**Avg. of % Drug found**	**SD**	**%RSD**
Tranexamic acid	**50**	102.782	51.391	**102.504**	**1.108**	**1.081**
		101.283	50.641			
		103.447	51.723			
Ethamsylate	50	98.084	49.042	96.721	1.183	1.22
		96.130	48.065			
		95.949	47.974			

Table 15: Summary of RP-HPLC Method Development AND Validation

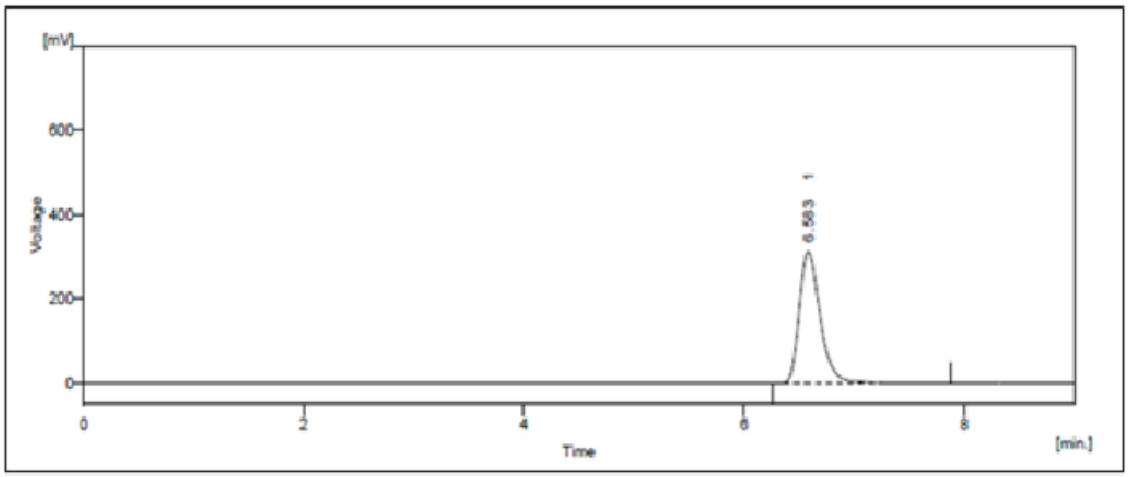
Parameters	**TRX**	**ETM**
Detection wavelength (nm)	215 nm	
Mobile Phase	Phosphate Buffer: Methanol (25:75 v/v) pH was adjusted to 3.5 by addition of Orthophosphoric acid	
Run time	10min	
Retention time of Standard	6.58min	3.27min
Resolution of Standard	12.277	
Concentration range (µg/mL)	25-75	25-75
Slope	80.06	90.41
Intercept	1.325	4.891
Correlation coefficient	0.997	0.997

Regression Coefficient Equation			y = 80.068x - 1.325	y = 90.41x + 4.891
LOD(µg/mL)			11.08	3.65
LOQ(µg/mL)			12.31	4.06
% Recovery	**80%**		100.18± 1.127	99.87± 1.061
	100%		99.88± 0.651	99.55± 0.663
	120%		99.95± 0.6035	99.50± 0.5324
Interday Precision (n=3)			0.118-0.121	0.136-0.155
Intraday Precision (n=3)			0.199-0.202	0.200-0.277
Repeatability (n=6; 50 µg/ml)			0.187	0.168
Robustness	**pH**	**+ 0.2 units**	0.623	0.578
		– 0.2 units	0.588	0.521
	F.R	**+ 0.2 units**	0.611	0.569
		– 0.2 units	0.582	0.511
	M.P	**+ 0.2 %**	0.662	0.601
		– 0.2 %	0.588	0.578
Assay			102.50	96.72

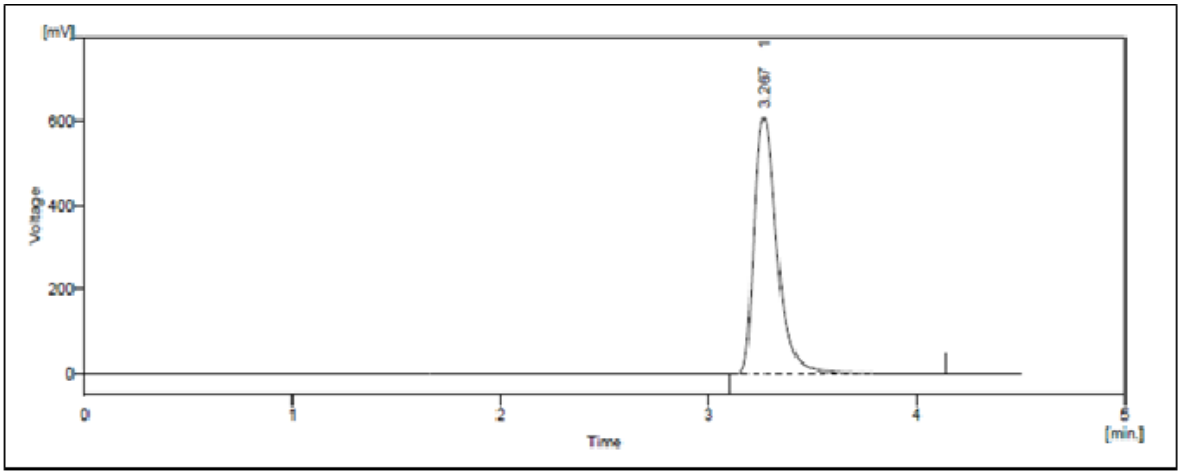
Table 16: Summary of Forced Degradation study

Degradation type	Condition	Volume of Stock Solution (mL)	Time hours	Final Dilution up to (mL)	%Degradation of Standard		%Degradation of Sample	
					TRX	ETM	TRX	ETM
Acidic	2mL 0.1N HCl	1	2	10	8.74	12.33	7.96	13.12
Alkaline	2mL 0.1N NaOH	1	2	10	13.27	15.19	12.54	15.23
Oxidative	2mL 3%H2O2	1	2	10	15.79	18.12	17.02	18.04
Photolysis	Sunlight	1	2	10	18.73	10.81	19.09	9.33
Thermal	105ºC in Oven	1	2	10	13.60	10.20	14.64	9.18

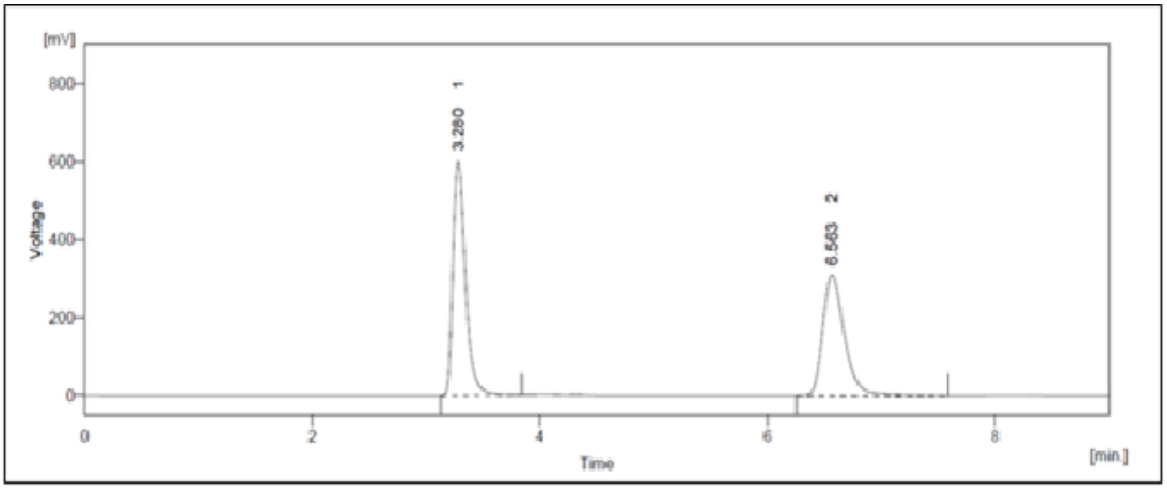
5. FIGURES AND CHROMATOGRAM



Chromatogram of Tranexamic acid



Chromatogram of Ethamsylate



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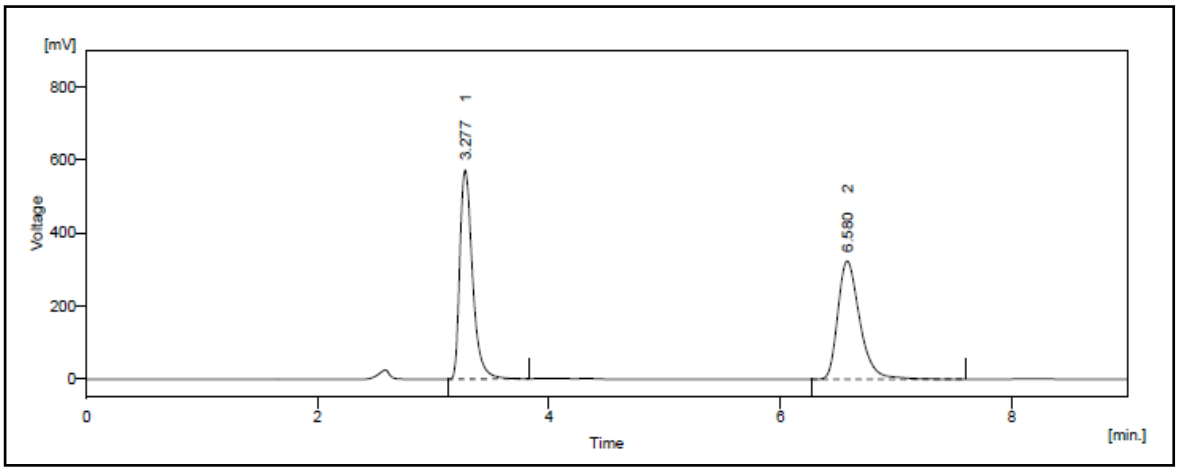
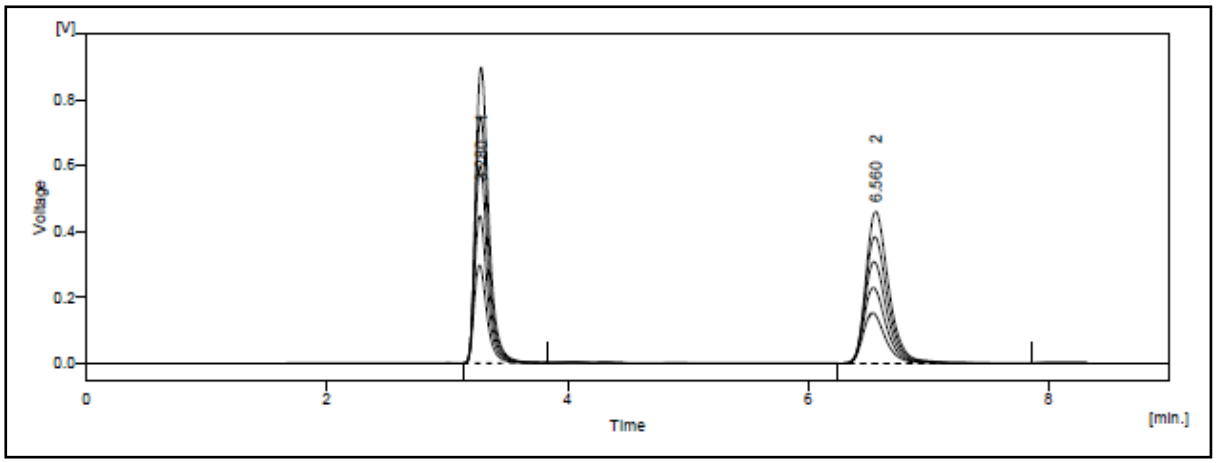
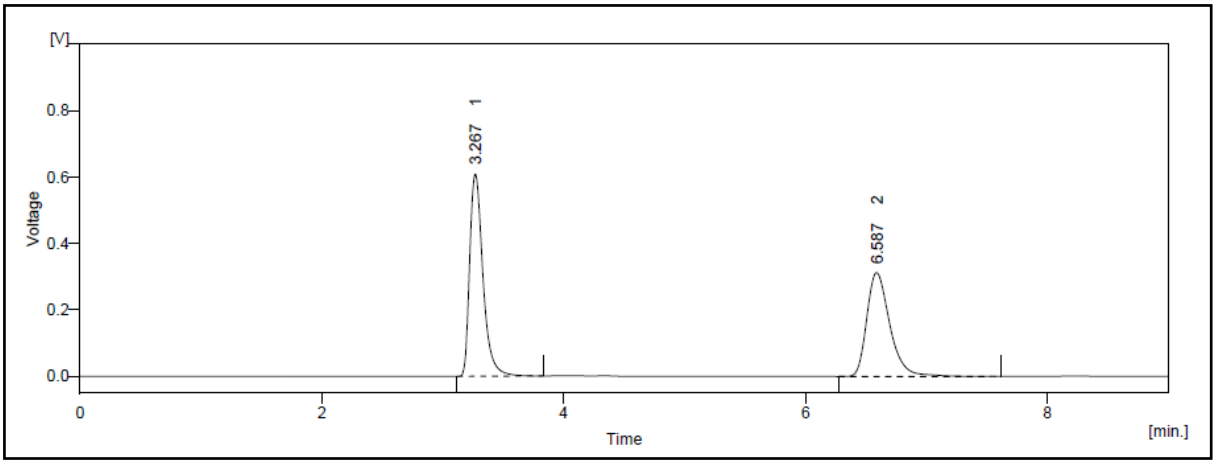


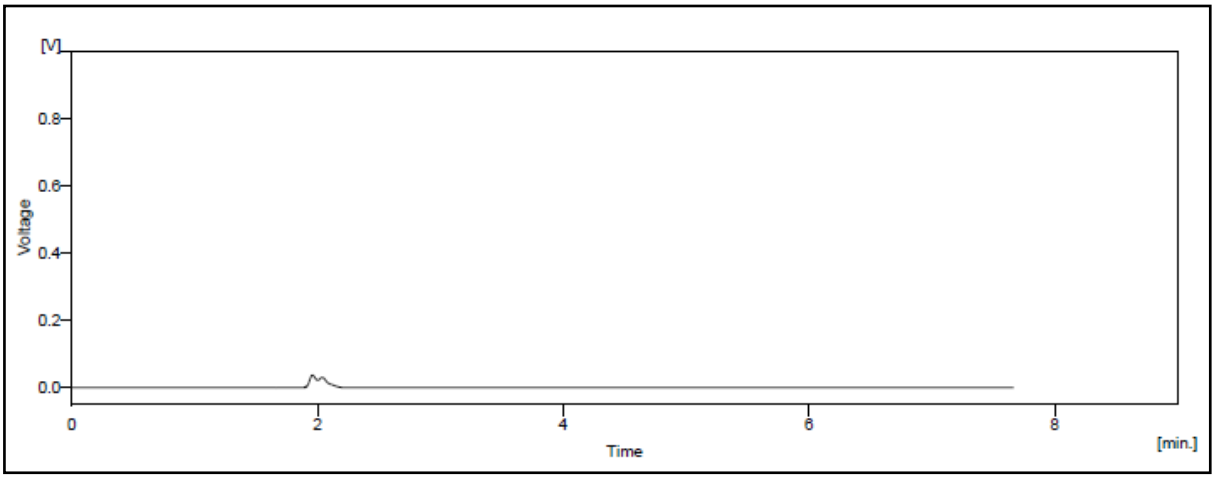
Chromatogram of Sample Solution



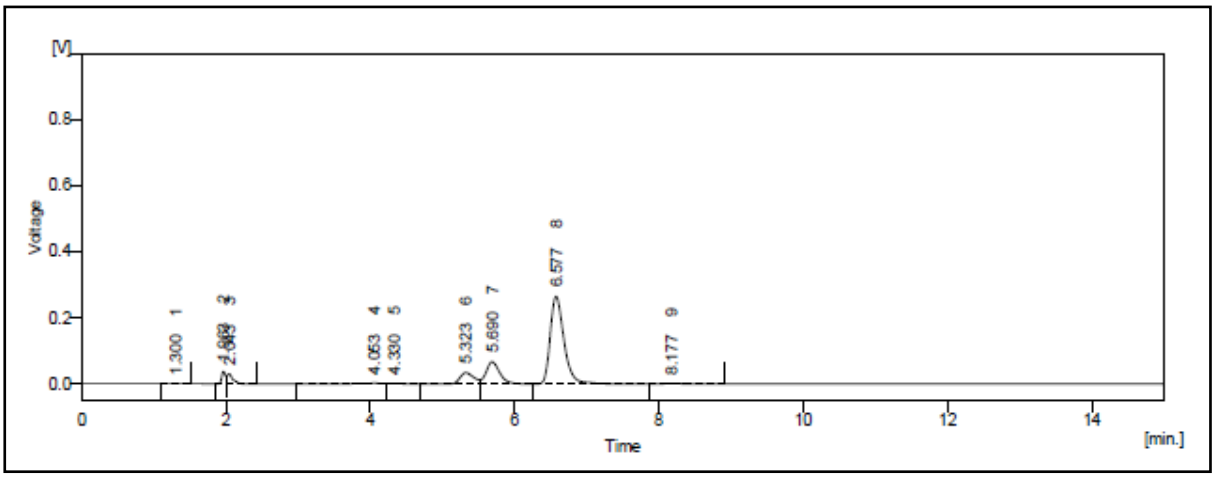
Chromatogram of Linearity and Range



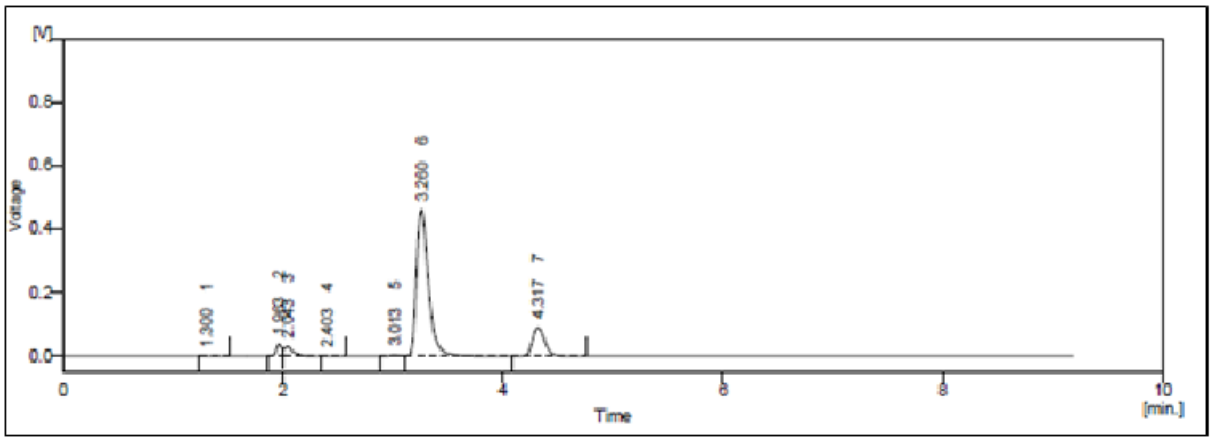
Chromatogram of Standard for Forced Degradation



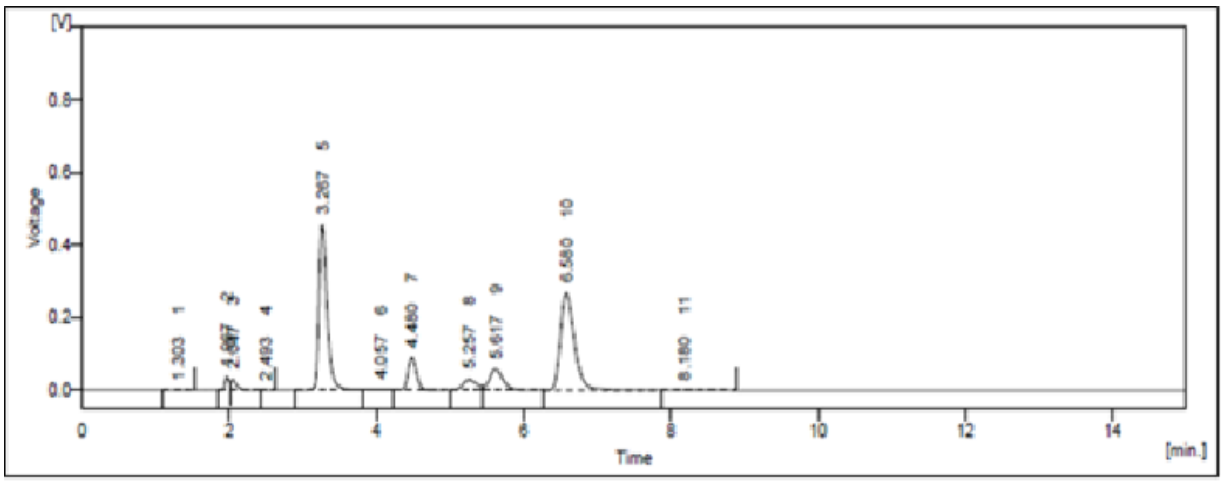
Chromatogram of Acid Blank



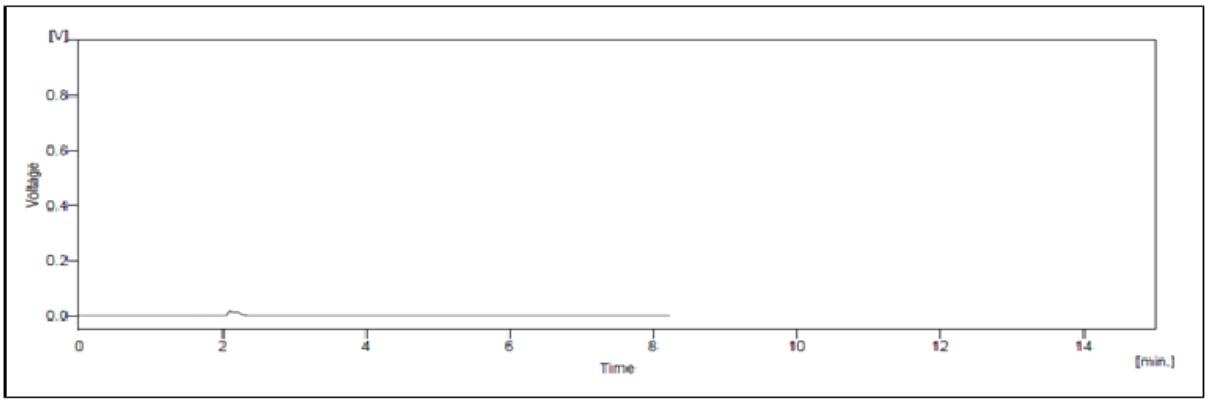
Chromatogram of TRX under Acid hydrolysis



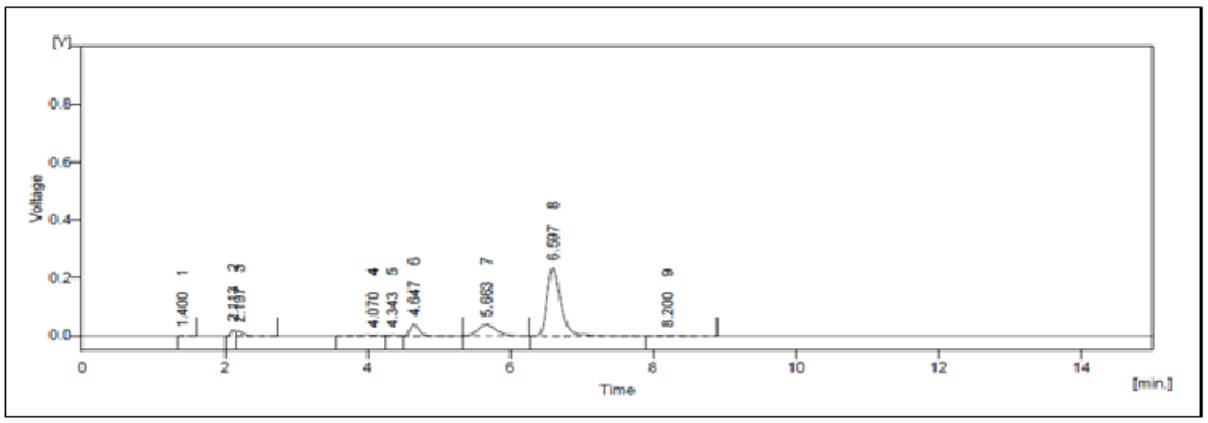
Chromatogram of ETM under Acid hydrolysis



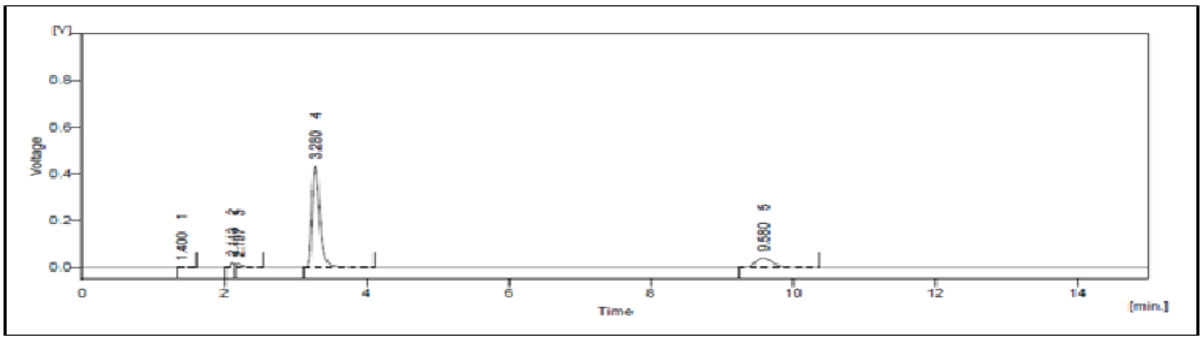
Chromatogram of Formulation under Acid hydrolysis



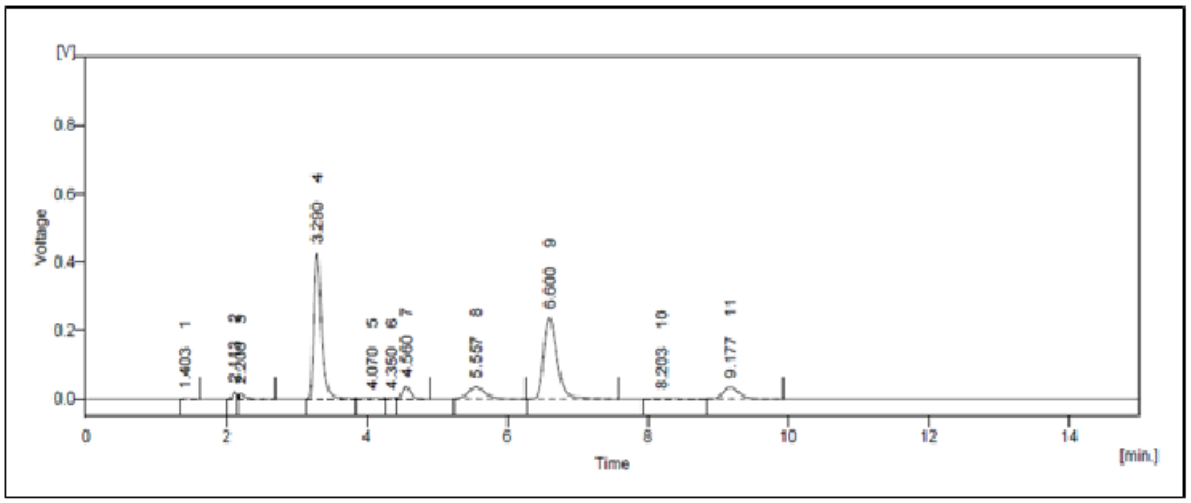
Chromatogram of Alkaline Blank



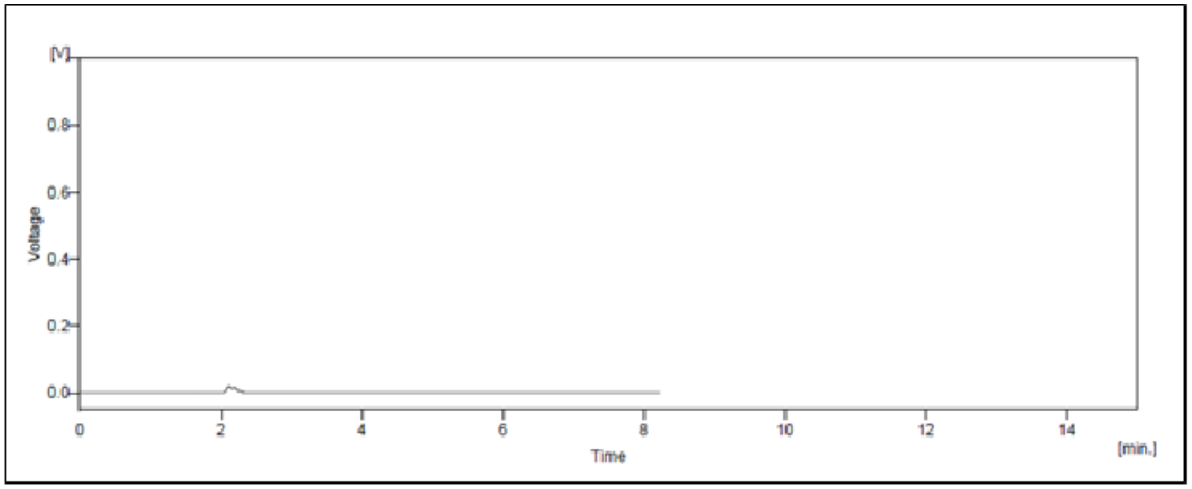
Chromatogram of TRX under Alkaline hydrolysis



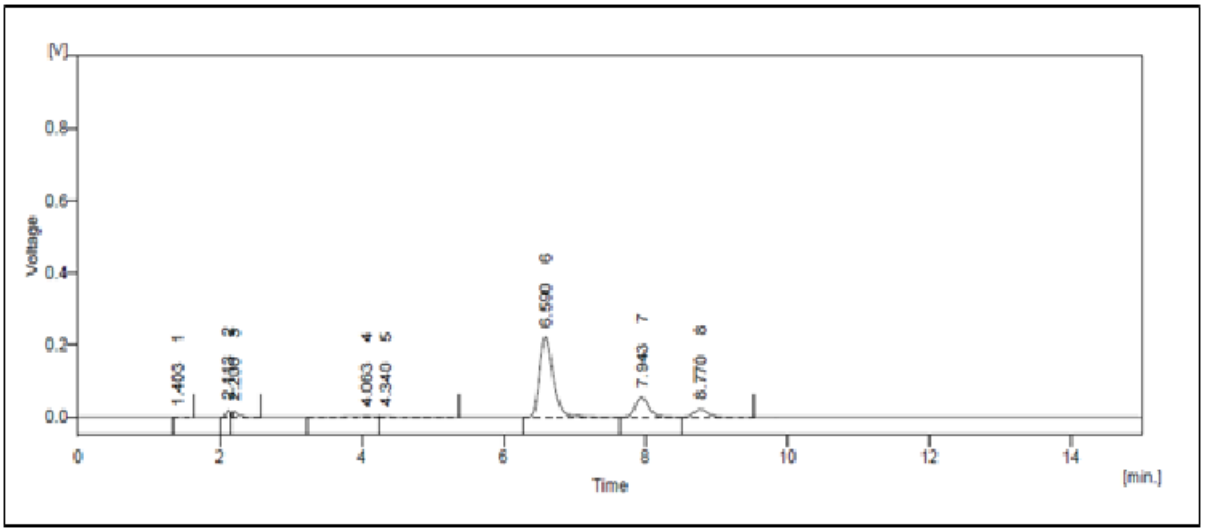
Chromatogram of ETM under Alkaline hydrolysis



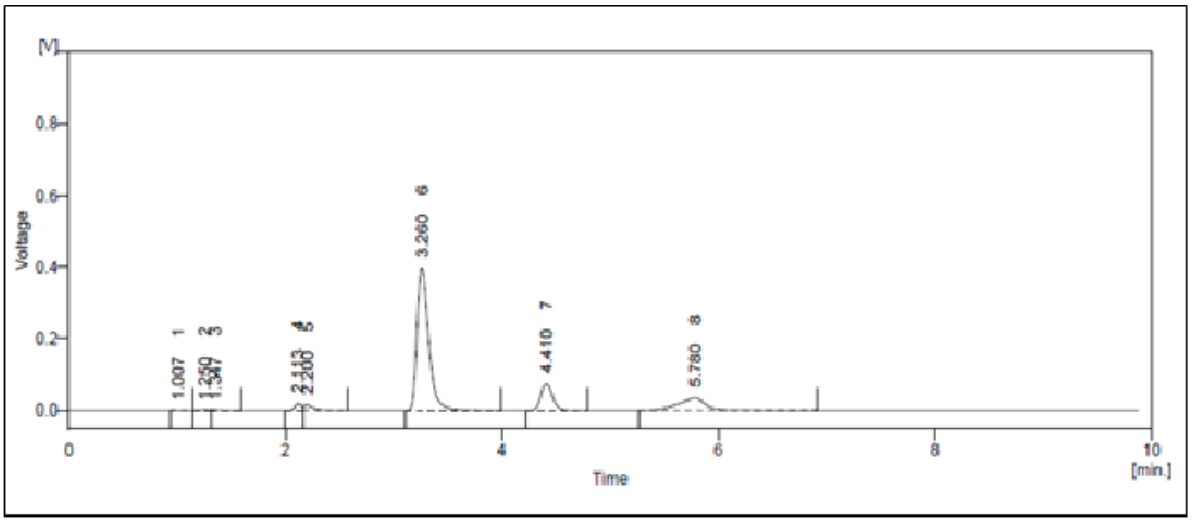
Chromatogram of Formulation under Alkaline hydrolysis



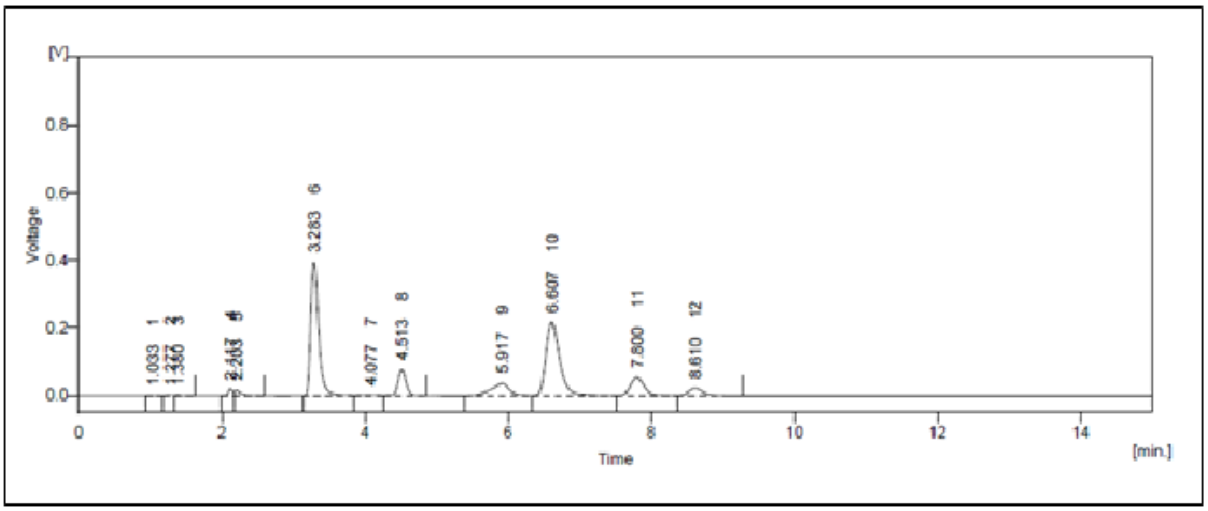
Chromatogram of Oxidative Degradation Blank



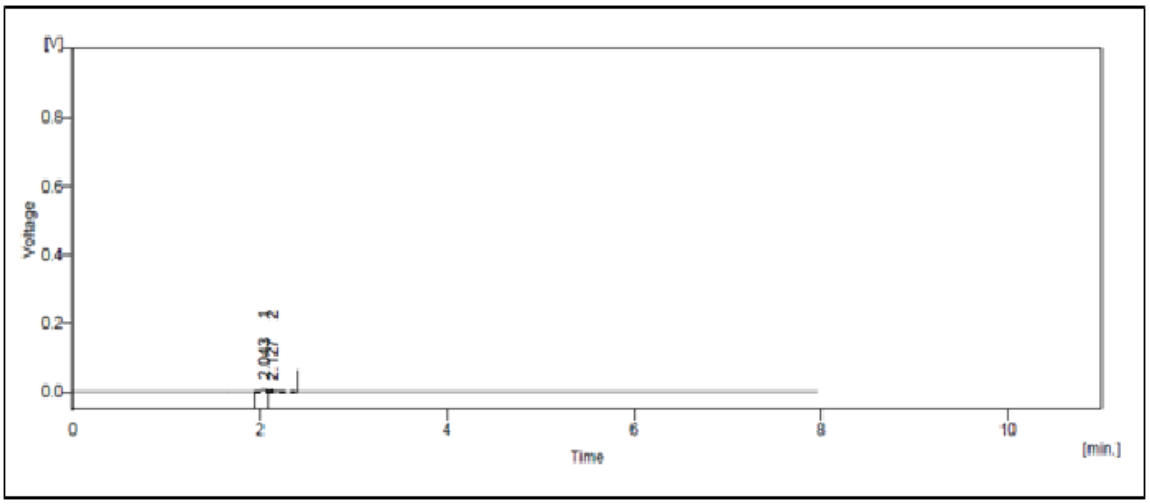
Chromatogram of TRX under Oxidative Degradation



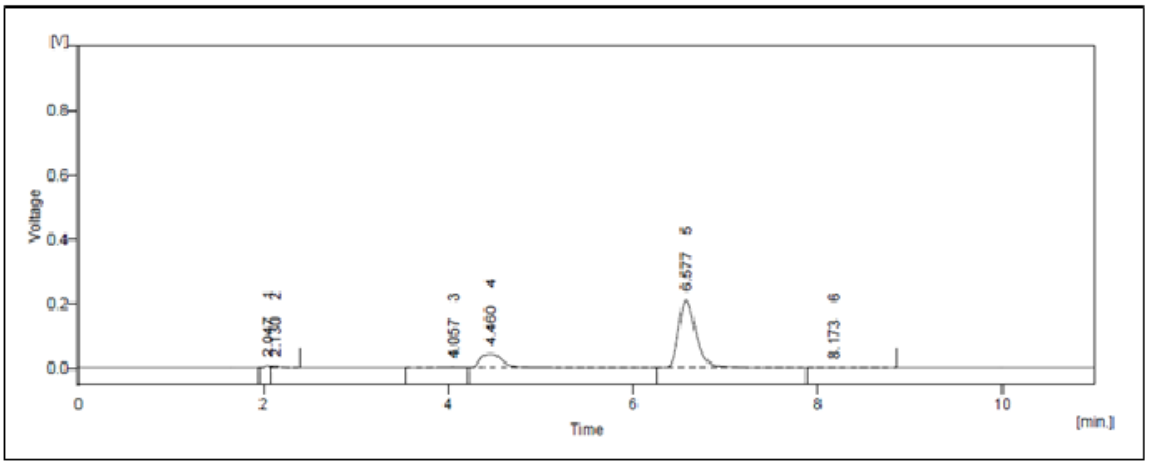
Chromatogram of ETM under Oxidative Degradation



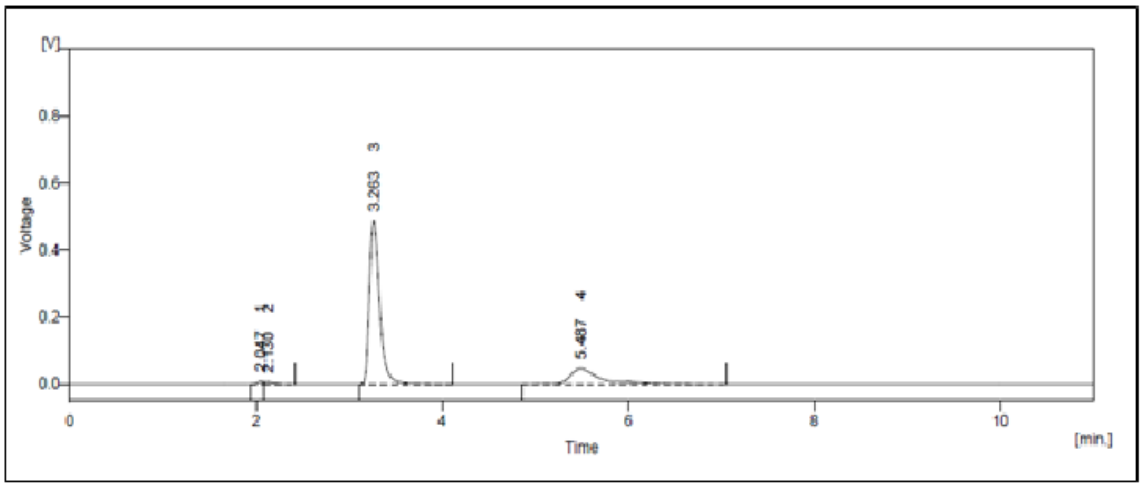
Chromatogram of Formulation under Oxidative Degradation



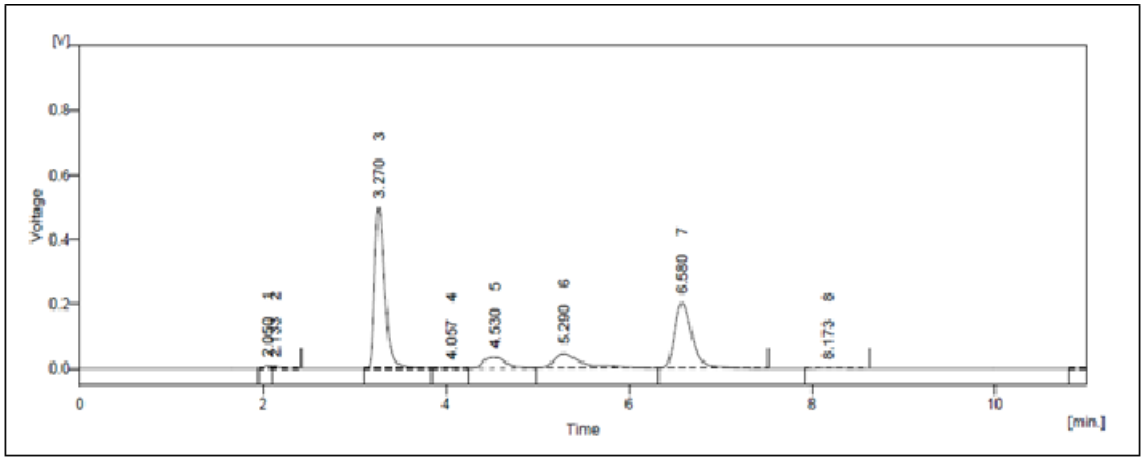
Chromatogram of Photolytic Degradation Blank



Chromatogram of TRX under Photolytic degradation



Chromatogram of ETM under Photolytic degradation



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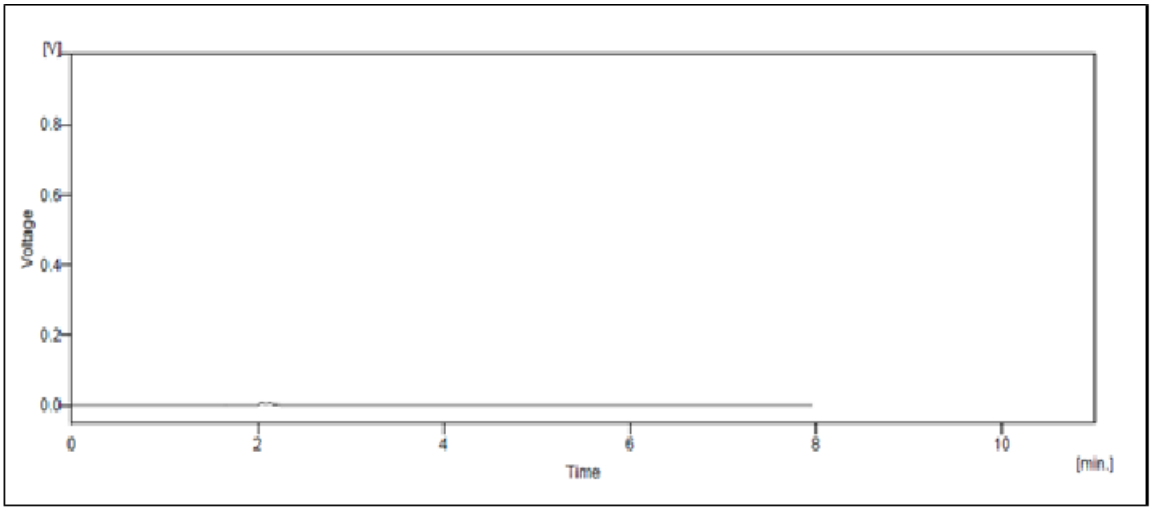
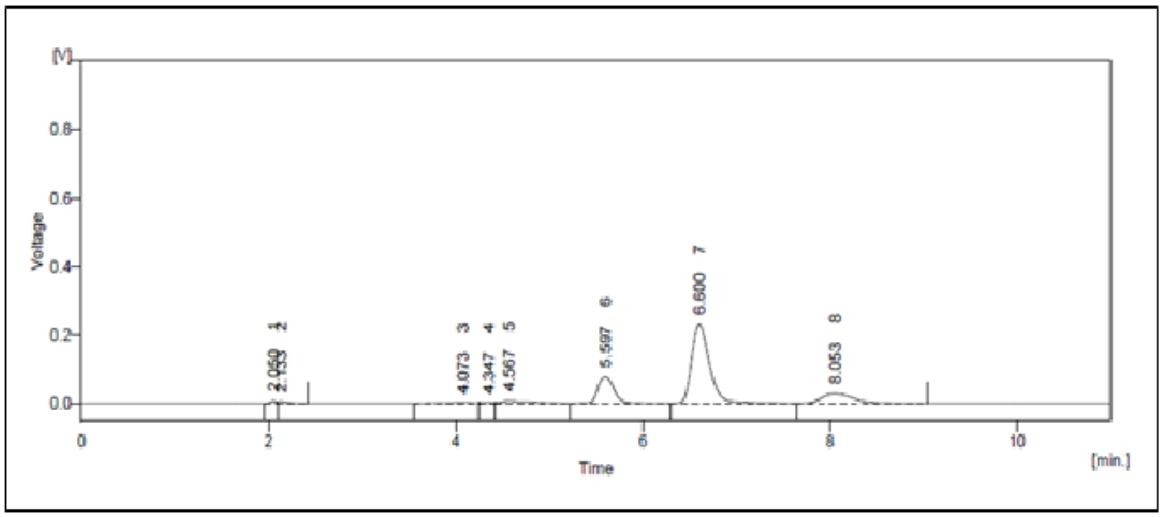
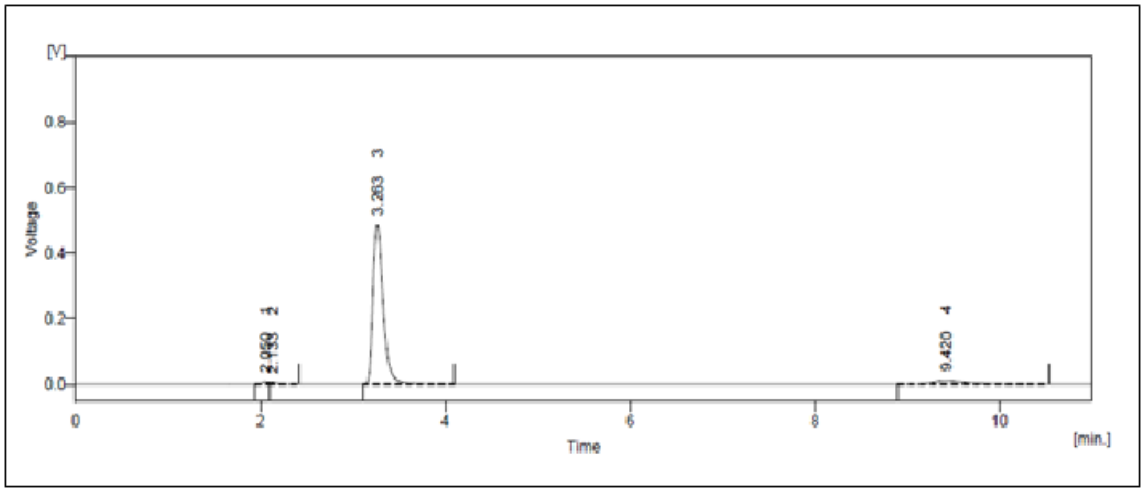


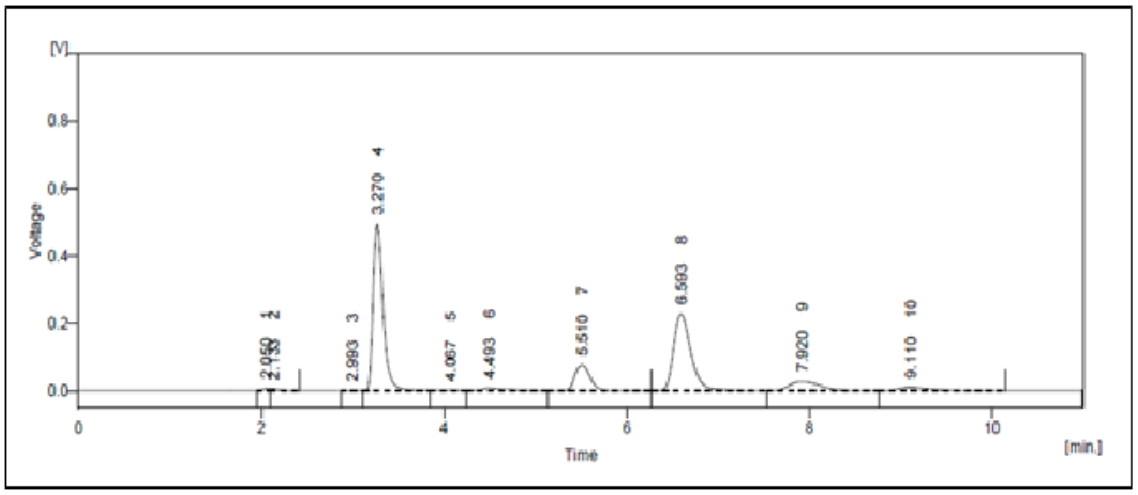
Chromatogram of Thermal Degradation Blank



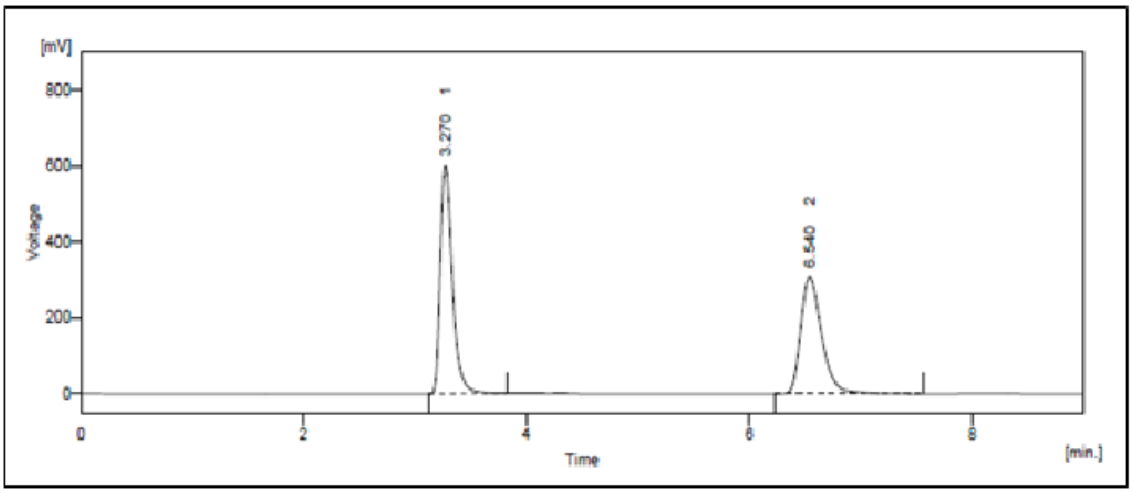
Chromatogram of TRX under Thermal Degradation



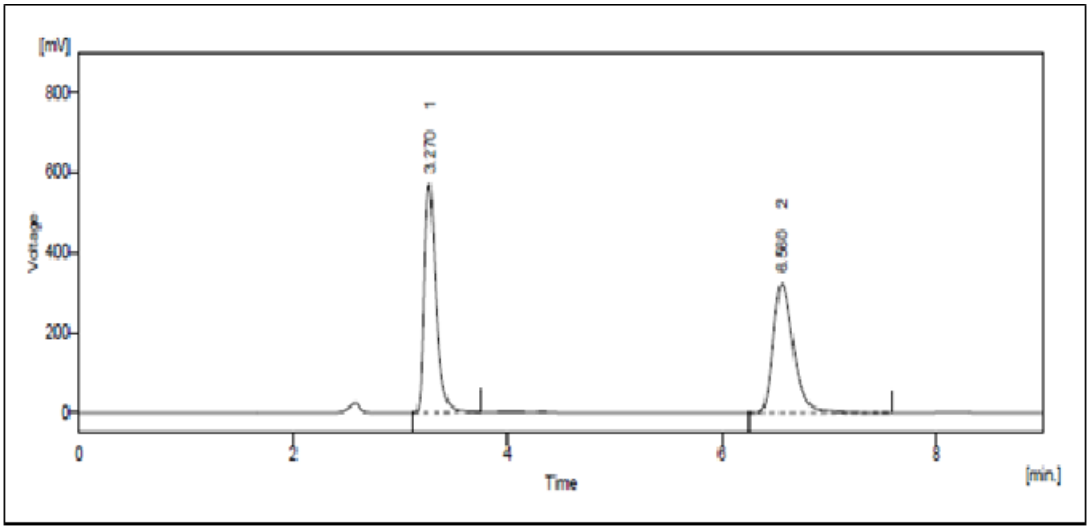
Chromatogram of ETM under Thermal Degradation



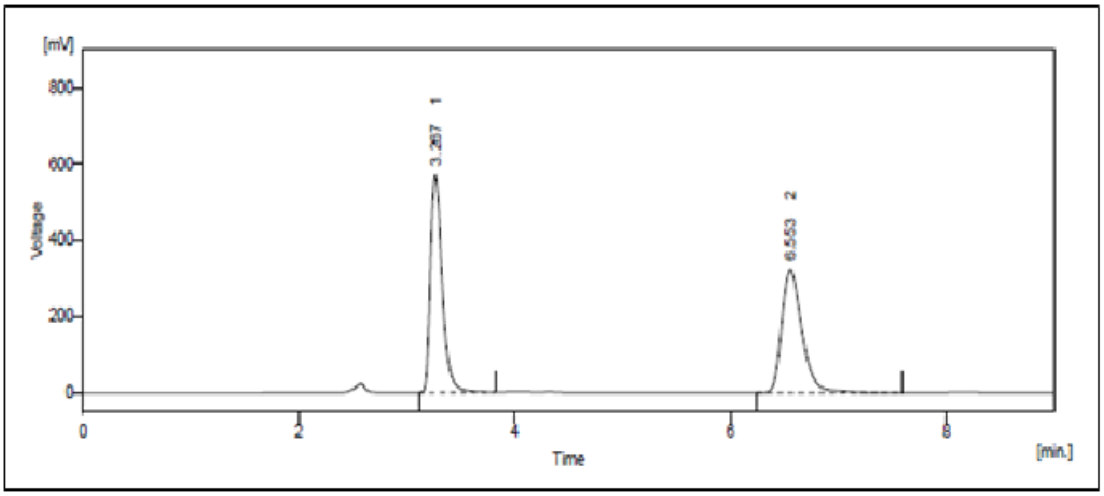
Chromatogram of Formulation under Thermal Degradation



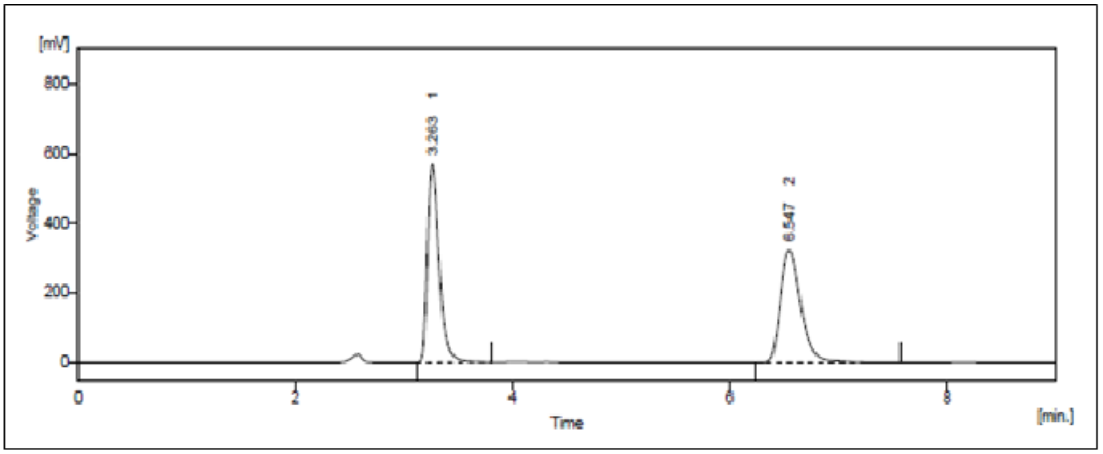
Chromatogram of Standard for Assay



Chromatogram of Sample (1) for Assay



Chromatogram of Sample (2) for Assay



Chromatogram of Sample (3) for Assay

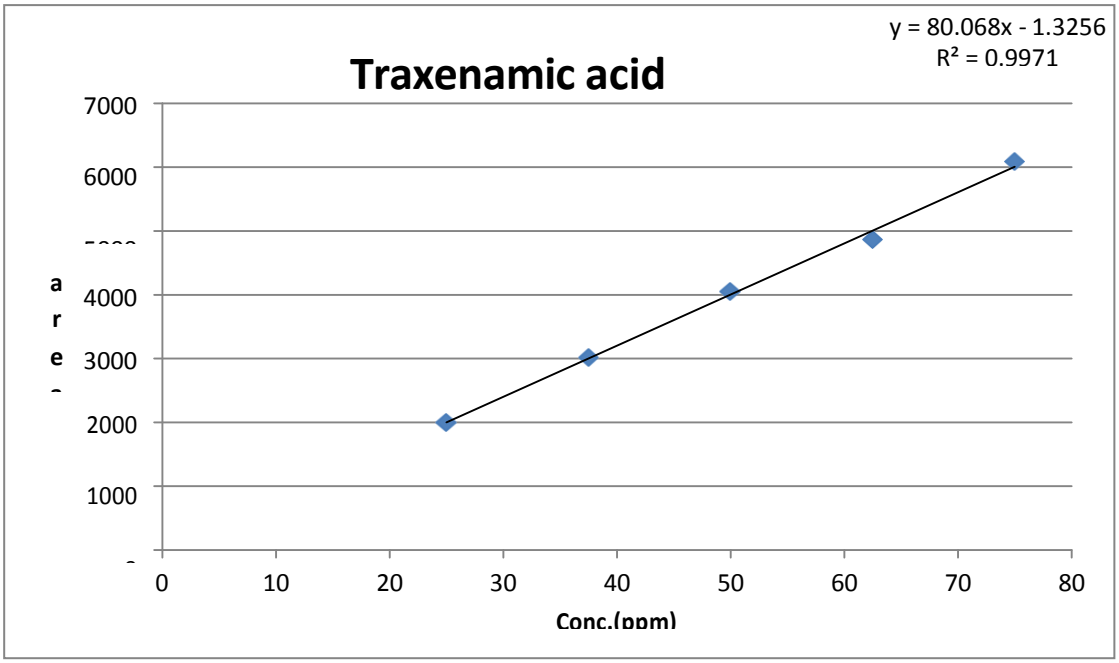


Figure 3: Calibration Curve of Tranexamic acid

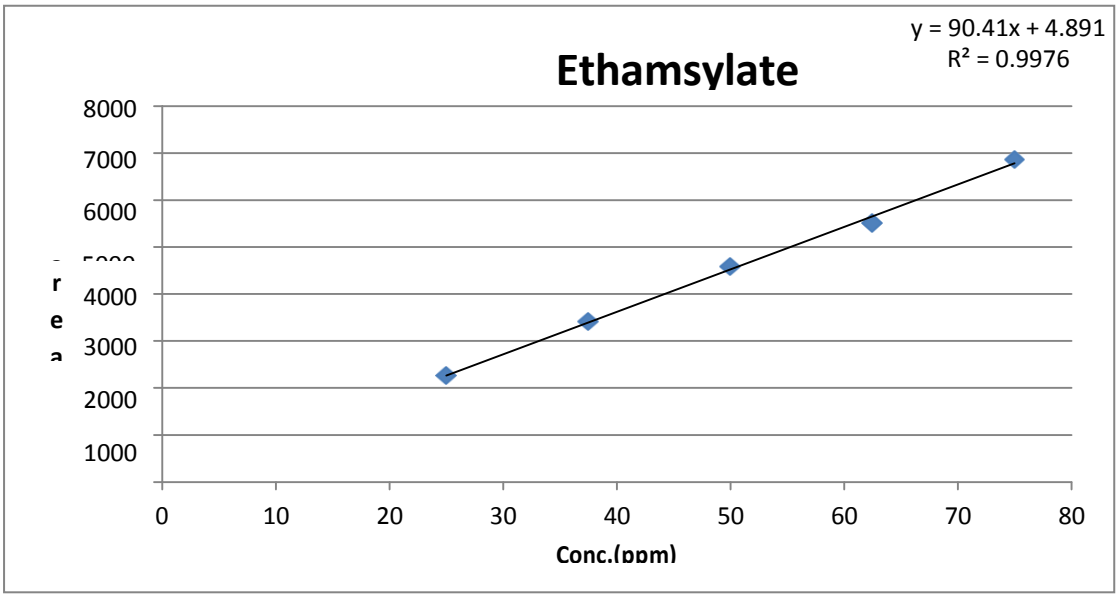


Figure 4: Calibration Curve of Ethamsylate

6. CONCLUSION

A simple, economic, accurate and robust RP-HPLC method has been developed and validated for the estimation of. The detection was done at 215nm. The linearity range was found in range of **25-75µg/ml** for Tranexamic acid and **25-75µg/mL** Ethamsylate. Limit of detection for Tranexamic acid and Ethamsylate was found to be and **3.65µg/mL** and **11.08µg/mL** respectively. Limit of quantitation for Tranexamic acid and Ethamsylate was found to be **12.31µg/mL** and **4.06µg/mL** respectively. All method validation parameters lie within its acceptance criteria as per ICH Q2 (R1) guideline so we can conclude that method is simple, linear, accurate and precise.

The Forced Degradation Study was carried out and it was found that ETM is most stable in Photolytic Degradation and Thermal Degradation and least stable in Oxidative Degradation whereas, TRX is most stable in Acid hydrolysis and least stable in Photolytic Degradation and both the drugs are equally stable in Alkaline hydrolysis.

7. ACKNOWLWDGEMENTS

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