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Improved Rp- Hplc Method for the Simultaneous Estimation of Tranexamic Acid and Mefenamic Acid in Tablet Dosage Form

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Abstract

An improved derivatized RP- HPLC method with PDA detection has been developed and validated for the simultaneous estimation of tranexamic acid and mefenamic acid in combined tablet dosage form. The method employs precolumn derivatization using 0.2% methanolic ninhydrin at primary amino group of tranexamic acid to form ruhemann purple product. Mefenamic acid could not react with ninhydrin. The chromatographic estimation was achieved using phenomenex C- 18 (250 X 4.6 mm, 5 µm) analytical column and the mobile phase consisting of methanol and 20 $mmol^{-1}$ acetate buffer (75:25, v/v) pH adjusted to 4.0 using ortho phosphoric acid at a flow rate of 1.0 $mLmin^{-1}$. The UV detection was carried out at 370 nm using photodiode array detector. The retention time of tranexamic acid and mefenamic acid were found to be 3.9 and 12.4 min. Tranexamic acid and mefenamic acid calibration curves were linear with correlation coefficient of 0.9973 and 0.9985 at a concentration ranging from 5μgmL^{-1} to 25 μgmL^{-1}. Recovery was between 98.5%- 100.5% for tranexamic acid and 99.7%- 104.3% for mefenamic acid. Limit of detection and quantification were 54.0$ngmL^{-1}$ and 62.6 $ngmL^{-1}$ for tranexamic acid, 12.3$ngmL^{-1}$ and 37.1 $ngmL^{-1}$ for mefenamic acid. The developed method is very sensitive as both peaks were well separated from its derivatizing agent peak with a short analysis time of 15 minutes.

Keywords: Tranexamic acid; Mefenamic acid; RP-HPLC; Quantification; Tablet dosage form

Introduction

Tranexamic acid, chemically trans- 4- aminomethyl-cyclohexacarboxylic acid (Figure 1), is the most potent antifibrinolytic lysine analogue used as broad spectrum pre and postoperative interventions and adjuvant drug for site specific pharmaco laser therapy. It is used for the treatment of hemophilic patient during tooth extraction, bleeding disorders [1], competitively inhibits activation of plasminogen, there by reducing conversion of plasminogen to plasmin (fibrinolysin) an enzyme that degrades fibrin clot [2], reduces menstrual blood loss, alternative to surgery in menorrhagia and has been used to control bleeding during pregnancy [3]. As an amino acid tranexamic acid lacks the electronic configuration (ie π-electrons) to behave as a chromophore or fluorophore, hence it is essential to make derivatives of tranexamic acid to make it detectable [2] and it shares structural similarity to aminoacids with its amino and carboxylic acid groups. The chromophores or fluorophores were reported for derivatisation of tranexamic acid to increase sensitivity of detection in pharmaceutical dosage forms, human blood, plasma and serum [4,5].

Mefenamic acid, chemically N-[(2,3-dimethyl phenyl) amino] benzoic acid (Figure 2), official in USP, BP and IP [6] is a potent non-steroidal anti-inflammatory drug with analgesic and antipyretic properties. It shows preferential inhibition of cyclooxygenase-2 and there by inhibits the prostaglandin synthesis [7]. It is used in the treatment of osteoarthritis, nonarticular rheumatism, healing of wounds, sport injuries, antiphlogistic, rheumatoid arthritis and other painful musculoskeletal illnesses [8]. Its oral bioavailability is very low due to poor solubility in water and insufficient dissolution rate. Overdoses of mefenamic acid may produce toxic metabolite accumulation which causes acute hepatic necrosis, inducing morbidity and mortality in humans.

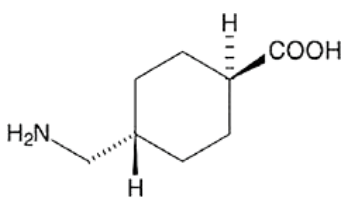

Figure 1: Chemical structure of Tranexamic acid.

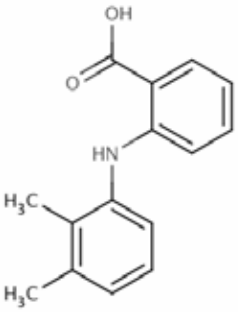

Figure 2: Chemical structure of Mefenamic acid.

New tablet formulation in combination of tranexamic acid 500 mg and mefenamic acid 250 mg is commercially available in Indian market for treatment of menorrhagia during menstruation. Literature

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survey revealed that few analytical methods have been reported for individual estimation of tranexamic acid and mefenamic acid. The different methods for the estimation of tranexamic acid such as liquid chromatography- tandem mass spectrometry [9], liquid chromatography- fluorescence method [1], HPLC coupled electrospray ionization mass spectrometry [10], colorimetric determination with p- dimethyaminobenzaldehyde, ninhydrin and ferric chloride [3] and spectrofluorimetric method [11] have been described. Gas Liquid chromatography [12], potentiometric [7], liquid chromatographic [13], voltammetric method [14] and spectrophotometric methods [5] have been described for the determination of mefenamic acid. However, no report has been found for simultaneous determination of tranexamic acid and mefenamic acid in pharmaceutical preparation up to best of our knowledge. The present manuscript describes a reproducible and selective isocratic reverse-phase HPLC method for the simultaneous determination of tranexamic acid and mefenamic acid in combined tablet dosage form.

Materials and Methods

Chemicals

Tranexamic acid was obtained from Zota Pharmaceuticals, Chennai; mefenamic acid was obtained from Fourrts India Pvt. Ltd, Chennai. Ninhydrin was purchased from Merck, Mumbai. Tablets were purchased from Indian market, containing 500 mg of tranexamic acid and 250 mg of mefenamic acid per tablet. Methanol (HPLC grade) was purchased from SD Fine Chemicals Ltd, Mumbai.

Apparatus

Analysis was performed on a chromatographic system of Shimadzu prominence consisting of LC 20 AD liquid pump equipped with manual 20 µl sample injection loop. Chromatographic separation was achieved on phenomenex C18 (250 X 4.6 mm, 5 µm) analytical column. Data acquisition was made with LC solution v.1.24 Spinchrome-1 soft ware.

Standard preparation and derivatization

Standard stock solution of tranexamic acid (25 μgmL^{-1}) and mefenamic acid (12.5 μgmL^{-1}) were prepared by transferring 40 mg of tranexamic acid and 20 mg of mefenamic acid in to a 100 mL volumetric flask containing 20 mL diluent (methanol: pH 7.2 phosphate buffer, 70:30, v/v). It was then sonicated for 15 minutes. The solution was diluted up to volume with diluent. From this 1 mL of stock solution is mixed with 2 mL of ninhydrin solution (0.2% in methanol) [3]. The mixture was warmed for 20 minutes at 80° C using constant temperature bath [15] and then cooled to room temperature finally volume was made up to the mark with diluent. Each working solution was individually derivatized prior to HPLC so as to attest the validity of method is retrospect.

Sample preparation

Twenty tablets were accurately weighed, crushed in mortar and finely powdered. The average weight of tablets was determined with the help of weight of 20 tablets. An accurately weighed portion of powder (45.0 mg) was transferred to 100 mL volumetric flask containing 70 mL diluent, sonicated with intermittent shaking for 20 minutes, the solutions were then made up to volume with diluent and filtered through 0.22 µm membrane filter. Aliquot of the filtrate was derivatized using 0.2% ninhydrin and further diluted to get final working concentration. 20 µL of sample was injected and chromatogram was recorded.

Method validation

The chromatographic conditions were validated by evaluating linearity, recovery, method and system precision, limit of detection (LOD), limit of quantification (LOQ), robustness, intra- day, inter- day variability and solution stability in accordance with ICH guideline Q2 (R1). Preliminary tests were performed with the purpose to select best and optimum conditions. The conditions affecting the efficiency and reproducibility of the derivatization process like reaction temperature, reaction time and concentration of ninhydrin were identified and optimized. The LOD and LOQ for analytes were estimated by injecting a series of dilute solutions with known concentration. Robustness of the method was determined by purposely altering the final experimental conditions like flow rate and pH of mobile phase. The nominal concentrations of standard and test solutions for tranexamic acid and mefenamic acid were 5 and 25μgmL^{-1}. Response function was determined by preparing standard solution at five different concentration levels using different analyst on two different days.

Results and Discussion

Optimization of the chromatographic conditions

The goal of this study was to develop a single isocratic reverse phase HPLC method for the simultaneous determination of tranexamic acid and mefenamic acid. During optimizing the method two organic solvents (methanol, acetonitrile) were tested. The chromatographic conditions were also optimized by using different buffers like phosphate, acetate, citrate for mobile phase preparation. After a series of screening experiments, it was concluded that acetate buffer gave better peak shapes than their phosphate, citrate counterparts. The resolution of chromatogram obtained with methanol is better than acetonitrile. The cost of acetonitrile also favoured to choose methanol as solvent for further studies. The chromatographic separation was achieved on a Shimadzu Prominence consisting of LC 20 AD liquid pump, Phenomenex C- 18 column (250 X 4.6 mm, 5 µm), using methanol-acetate buffer 20 $mmol^{-1}$ (75:25, v/v) as mobile phase. The pH of buffer was adjusted with orthophosporic acid to pH 4.0; pH of buffer was selected according to Pka value of tranexamic acid and mefenamic acid. Iso-absorptive point (ie the specific wavelength at which two chemical species having same molar absorptivity) was selected from multi wavelength overlain spectra for both drugs which meet at 370 nm [16,17]. So this wavelength was fixed for our study and at this point the peak response was maxima. During derivatization using 0.2% ninhydrin warming temperature of 80°C and warming time of 20 minutes, should be carefully monitored since variations in the above will causes deviations in result indicating that the time may not be sufficient for complete derivatization [18,19]. Tranexamic acid contains primary amino group which reacts with ninhydrin in the presence of phosphate buffer via oxidation deamination followed by condensation to form ruhemann purple product.

Linearity and carry over

Linearity was determined for tranexamic acid and mefenamic acid in the range of 5- 25 μgmL^{-1}. The correlation coefficient (r^2) values for both drugs were >0.999 and no significant variation of slope and intercept over the concentration range studied was observed. Overlaid chromatogram for linearity of tranexamic acid and mefenamic acid is shown in (Figure 3).

Recovery

In order to judge the quality and applicability of method the recovery analysis was performed at three levels like low, middle and high concentrations (80%, 100% and 120%) by standard addition method and the overlaid chromatogram obtained are shown in (Figure 4). These

mixtures were determined by the proposed method in triplicates. The results of recovery (%) and R.S.D (%) are shown in (Table 1).

Method and system precision

Precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of same homogenous sample under prescribed conditions. Method precision was determined by injecting the standard solution of the analytes five times [20-22]. The system precision (injection repeatability) is a measure of the method variability that can be expected for a given analyst performing the analysis for five repeated analysis of the same sample working solution. Results of method and system precision are shown in (Table 2). The RSD% of peak area for five replicates was found to be less than 2.0% for both method precision and system precision. Method precision chromatogram is shown in (Figure 5).

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ of tranexamic acid and mefenamic acid were determined by calibration curve method which includes signal-to-noise ratio, use of standard deviation of the response and slope of the calibration curve. Solutions of both tranexamic acid and mefenamic acid were prepared and injected in triplicate. Average peak area of three analyses was plotted against concentration. LOD and LOQ were found

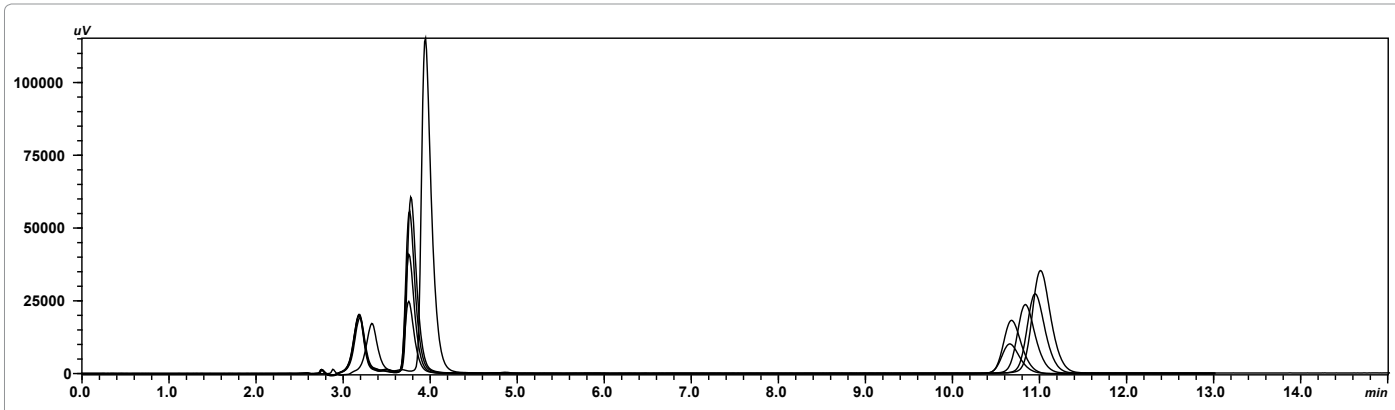

Figure 3: A typical overlaid chromatogram for linearity in simultaneous estimation of tranexamic acid and mefenamic acid.

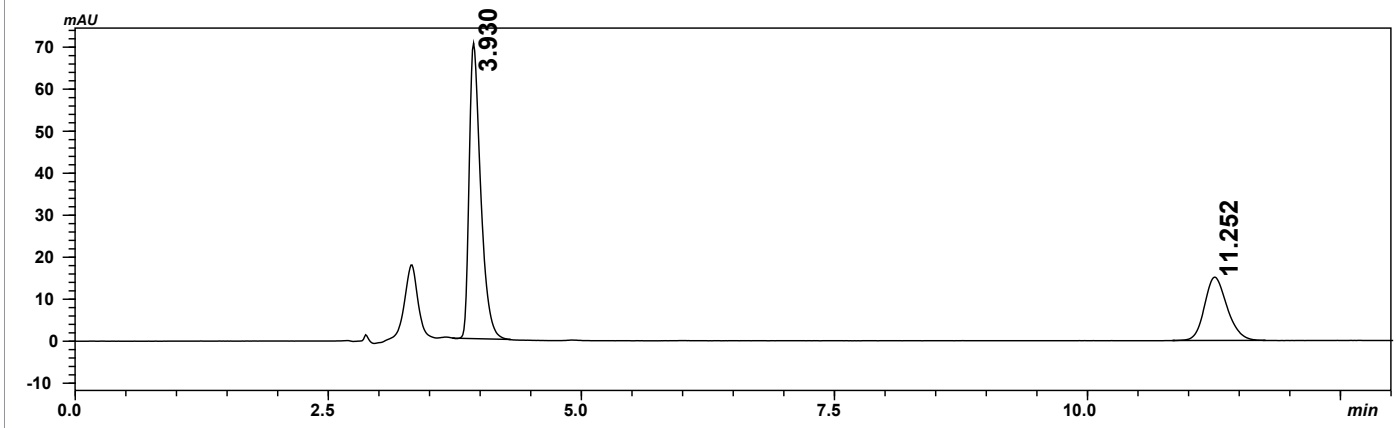

Figure 4: A sample recovery 80% level chromatogram for simultaneous estimation of tranexamic acid and mefenamic acid.

	Tranexamic acid			Mefenamic acid	
S.No	Recovery (%)	RSD (%) of area	Recovered %	RSD (%) of area	Recovered %
1	80	1.1	100.5	0.5	101.5
2	100	1.3	98.5	1.0	99.7
3	120	0.7	99.8	0.1	104.3

Table 1: Recoveries obtained in the analysis of Tranexamic acid and Mefenamic acid.

S.No	**Parameters**	**% RSD of peak area**	
		Tranexamic acid	**Mefenamic acid**
1	**Method precision**	**0.3**	**0.3**
2	**System precision**	**0.3**	**1.3**

Table 2: Method and System precision of Tranexamic acid and Mefenamic acid.

to be 17.8ngmL^{-1} and 54.0 ngmL^{-1} for tranexamic acid, 20.6ngmL^{-1} and 62.6ngmL^{-1} for mefenamic acid.

System suitability and specificity

System suitability test are an integral part of a liquid chromatographic method and they were used to verify whether the proposed method was able to produce good resolution between the peaks of interest with high reproducibility [18,19]. It was determined by making five replicate injections from freshly prepared standard solutions and analyzing the peaks for theoretical plates (*N*) and tailing factors (*T*). The system suitability parameters are shown in (Table 3). The blank chromatogram for specificity showing ninhydrin peak at retention time 3.2 was shown in (Figure 6). The USP theoretical plate was found to be greater than 6000 for both peaks and tailing factor was found to be less than 1.4 for tranexamic acid and less than 1.2 for mefenamic acid and the resolution between two peaks was found to be 31.76.

Intra- inter day and analyst variability

The intra-day (repeatability), inter-day (intermediate precision) variability and analyst to analyst variations were determined using standard solutions. These experiments were repeated on second day to evaluate day- day variability [20-22]. The %RSD of peak area for intra-day, inter- day variability and analyst to analyst variations as shown in Table 4 was found to be less than 4.0%. The observed results of day to day, inter day and analyst variation are within the acceptable limit of repeatability.

Robustness

The robustness was conducted by deliberate variations in the chromatographic conditions [18]. The conditions studied were flow rate altered flow rate (1 ± 0.1 mLminute^{-1}) and altered buffer pH (pH 4.0 ± 0.1) and the results obtained are shown in (Table 5). The standard deviation of peak areas was calculated for each parameter and %R.S.D was found to be less than 7.0% for tranexamic acid and 6.0% for mefenamic acid for purposely altered changes [23-24].

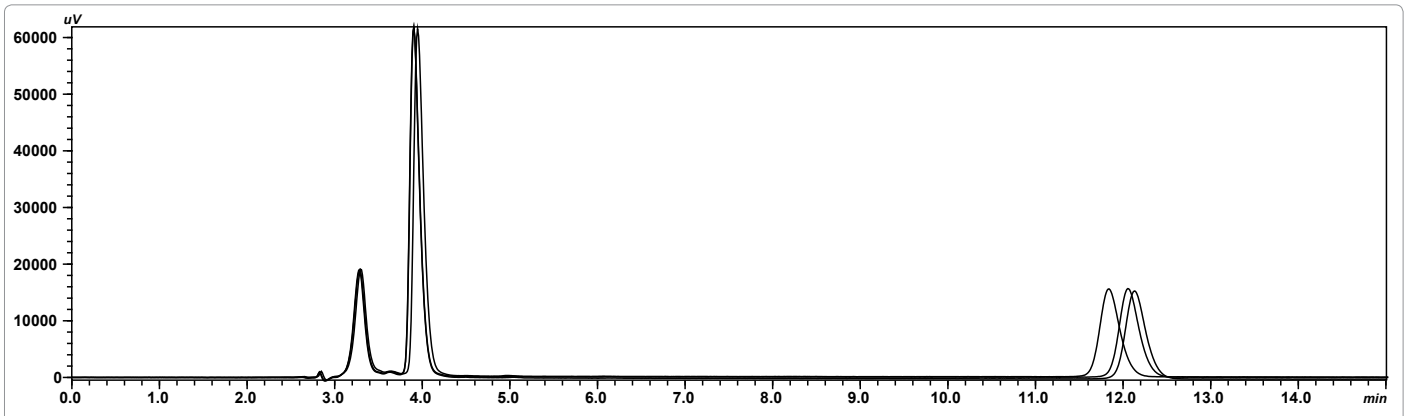

Figure 5: A typical method precision chromatogram for simultaneous estimation of tranexamic acid and mefenamic acid.

S.No	System suitability parameters	Tranexamicacid	Mefenamic acid
1	Retention time (min)	3.9	12.4
2	Tailing factor	1.4	1.2
3	Theoretical plates	6587	12552
4	Resolution	-	39.1
5	Area	671978	256359

Table 3: System suitability parameters of Tranexamic acid and Mefenamic acid.

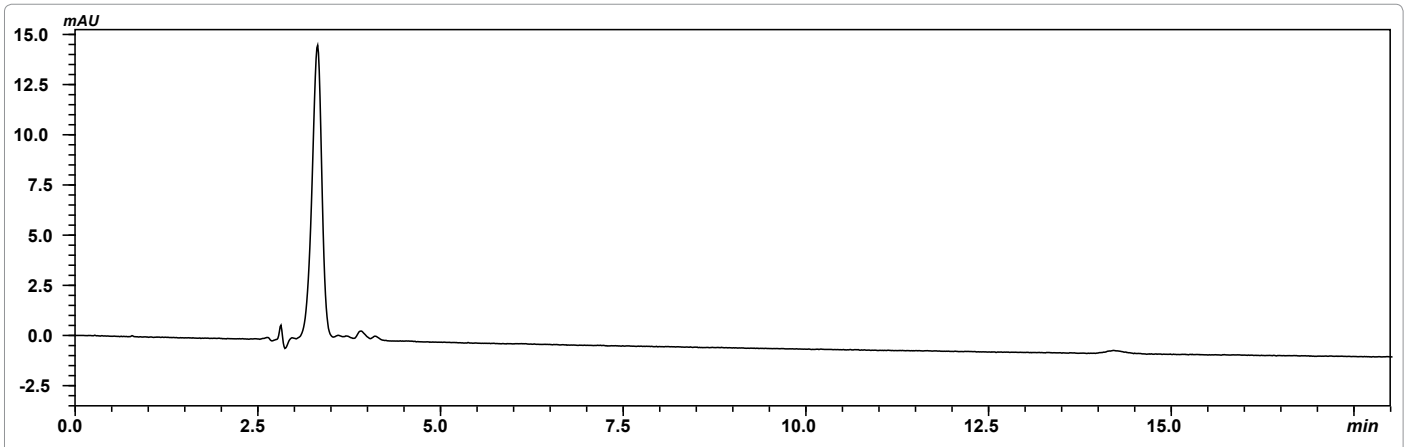

Figure 6: A typical blank chromatogram indicating specificity of tranexamic acid and mefenamic acid.

Solution stability

In anticipation of unexpected delays during analysis it is important to have information about the stability of all solutions and stability of derivatized product which have to be examined thoroughly for signal intensity of derivatized tranexamic acid during long sequence, standard solutions and sample solutions. The solutions are stored at room temperature around 25°C and another set stored at refrigerator condition were separately injected at 0, 12, 24 hours. The chromatogram of sample solution stored at refrigerated conditions after 24 hours are shown in (Figure 7).

Previously published works focused on analyzing either tranexamic acid or mefenamic acid individually. [25] reported a HPLC method of derivatizing tranexamic acid using phenyl iso thiocyanate at 60°C in C18 column consisting 65:35 (v/v) mixture of 10 $mmol^{-1}$ phosphate buffer, pH 3.6, and acetonitrile as mobile phase, but in this method we reported combination of tranexamic acid and mefenamic acid estimation using a simple derivatizing reagent ninhydrin, temperature maintained at 80°C using constant temperature bath. Delyle et al [10] reported a HPLC validated assay for the quantitative analysis of tranexamic acid in serum using formate buffer and acetonitrile as mobile phase. [13] determined mefenamic acid by HPLC on C8 column using acetonitrile- water (50: 50, v/v, pH 3) as mobile phase. Our current approach focuses on a stability indicating method consisting of methanol: acetate buffer (75:25, v/v) pH 4.0 at short analysis time. The linearity curves obtained for each drug were linear over a wide range of concentrations. No interferences from constituents of ninhydrin at the retention times of major peaks were observed. When a blank sample was analyzed immediately after the highest calibration standard, mean carry over was lower than 2.0 % for both tranexamic acid and mefenamic acid. The mean percentage recoveries obtained for tranexamic acid and mefenamic acid were between 98.0% and 104.3%. In peak purity analysis with photodiode array detector, purity angle was less than purity threshold for both tranexamic acid and mefenamic acid, which indicates the peak of analytes was pure and excipients in formulation did not interfere the analytes. The peak area and retention time remained almost unchanged and no significant degradation was observed within the given period, indicating the standard and sample solutions were stable for at least 24 hours. The derivatization procedure applied in this work yields good recovery as well as high repeatability for both drugs hence this method can be adopted for routine analysis.

Conclusion

The developed and validated HPLC method outlined is very obvious, affordable, dynamic, low cost, rapid and easy to perform with small sample volume and good repeatability. It can be adapted for the routine quality control analysis of simultaneous determination of tranexamic

S.No	Variabilities	% RSD of peak area	
		Tranexamic acid	Mefenamic acid
1	Inter day	3.6	3.5
2	Intra day	0.8	1.2
3	Analyst-1	0.3	0.6
4	Analyst-2	0.2	0.8

Table 4: Intra - inter day and analyst variability of Tranexamic acid and Mefenamic acid.

			% RSD of peak area	
S. No	Parameters	Chromatographic conditions	Tranexamic acid	Mefenamic acid
1	Altered flow rate	0.9 $mLmin^{-1}$	6.6	5.8
2		1.0 $mLmin^{-1}$	1.2	0.9
3		1.1 $mLmin^{-1}$	0.7	0.5
4	Altered pH	pH 3.4	2.3	1.9
5		pH 3.5	1.2	0.9
6		pH 3.6	1.3	3.1

Table 5: Robustness study of Tranexamic acid and Mefenamic acid.

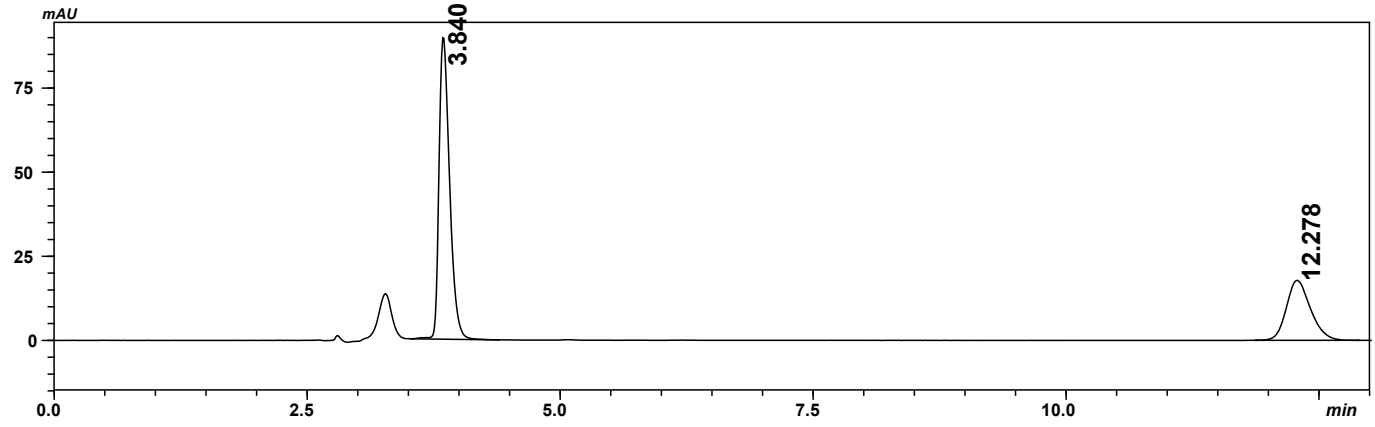

Figure 7: A typical chromatogram of tranexamic acid and mefenamic acid sample solution stored at refrigerated temperature for 24 hours.

acid and mefenamic acid at therapeutic doses because of good separation and resolution of the chromatographic peaks. The method is very specific as the derivatized tranexamic acid gives major peak at retention time 3.9 and which was not observed when derivatizing blank with ninhydrin. The developed method relatively requires one step derivatization before chromatography and the method is very sensitive as both peaks were well separated from its derivatizing agent peak with a short analysis time of 15 minutes using simple extraction technique. The signal intensity of derivatized tranexamic acid was unchanged after 24 hours.

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