"Stability Indicating RP-HPLC Method Development And Validation For Simultaneous Estimation Of Ethamsylate And Mefenamic Acid In Tablet"

Sharmila Devi.R* , Dr. S. Ravichandran

Department of pharmaceutical Analysis, PSG college of pharmacy, Peelamedu, Coimbatore – 641004, Tamilnadu, India.

ABSTRACT:
A simple, accurate, precise, sensitive, rapid and economical stability indicating RP-HPLC method was developed for simultaneous estimation of ethamsylate and mefenamic acid in bulk and tablet dosage form. A binary gradient RP-HPLC assay was used with PDA detection. Good chromatographic separation was attained using shimadzu model shim pack (solar) – C_8 (4.6*250mm, 5µm) column with mobile phase acetonitrile and buffer (potassium dihydrogen orthophosphate) in the ratio of 60:40 adjusted to a pH 4.5 with orthophosphoric acid, at flow rate of 1ml/min. Detection was carried out at 299nm. The retention time of ethamsylate and mefenamic acid was found to be 2.5min and 7.7min respectively. For stability studies, drugs were subjected to acid hydrolysis, alkaline hydrolysis, oxidative degradation, thermal degradation and photolytic degradation. The proposed method was validated for linearity, precision, system suitability, selectivity, LOD and LOQ as per the International conference on harmonization (ICH) guideline. It shows good linearity response in the concentration range of 3-7µg/ml for both ethamsylate and mefenamic acid. The limit of detection were 0.190µg/ml and 0.323µg/ml for ethamsylate and mefenamic acid respectively. The limit of quantification were 0.578µg/ml and 0.981µg/ml for ethamsylate and mefenamic acid respectively. Percentage RSD of ethamsylate and mefenamic acid was found to be 0.6 and 0.5 respectively. The proposed method can be applied successfully for the determination of ethamsylate, mefenamic acid and its degradation product.
KEYWORDS: Ethamsylate, Mefenamic acid, RP-HPLC, Simultaneous estimation, Forced degradation, Validation.

I. INTRODUCTION:

Ethamsylate (ETM) is chemically 2, 5-dihydroxybenzenesulphonic acid, diethyl amine[1]. It is a haemostatic agent that maintains the stability of the capillary walls. It is given for the prophylaxis and control of haemorrhages from small blood vessels[2] . Ethamsylste is official in british pharmacopoeia[3].
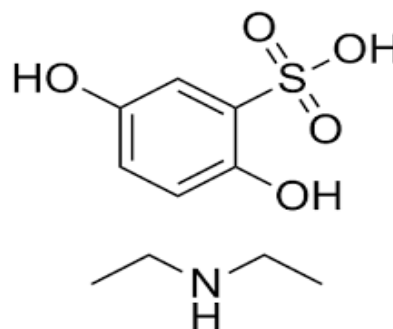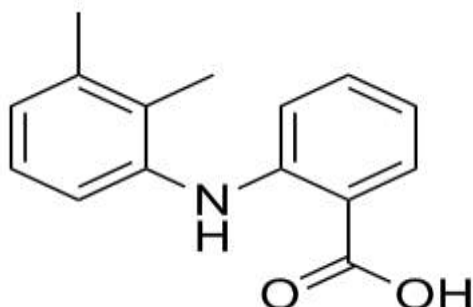

Fig-1: Chemical structure of ETM

Mefenamic acid (MFA) is chemically 2-(2,3- dimethylphenyl)aminobenzoic acid[4]. Mefenamic acid is commonly used for the management of pain, fever and menstrual pain. Mefenamic acid decreases inflammation and uterine contractions by the inhibition of prostaglandin synthesis by competitive blocking of the enzyme cyclo-oxygenase (COX)[5].The drug and the capsules are official in IP, EP, BPC, BP and USP[6-10].

Fig-2: Chemical structure of MFA

Recently, the combination of ETM and MFA has demonstrated a significant activity against painful menstruation. ETM and MFA are co-formulated in a medicinally recommended ratio of 1:1

A literature survey revealed the analytical methods for the determination of ETM and MFA either individually or in combination with other drug using spectrophotometric[11-17], HPLC[18-25], HPTLC[26, 27] and bioanalytical methods[28]. However, no study till date has performed the HPLC determination of ETM and MFA with the mobile phase employed in the present investigation. Moreover, the stability indicating method of the ETM and MFA with the adopted mobile phase in this investigation has not yet been performed. Therefore, the present study aims to develop a stability indicating method development for ETM and MFA using RP-HPLC technique. The method is validated as per the international conference on harmonization(ICH) guidelines[29].

II. MATERIALS AND METHODS:

Chemicals and reagents:

The standard drug of ETM and MFA was obtained from yarrow chem products, Mumbai, India. The combined dosage form SYLATE-M (Emcure pharmaceuticals Pvt.ltd) label claimed 500mg of ETM and 500mg of MFA was purchased from local market. The HPLC grade solvent Acetonitrile were procured from Thermo fisher scientific india pvt ltd., Mumbai and Pottasium dihydrogen orthophosphate were procured from HiMedia laboratories pvt ltd., Mumbai. Double Distilled water (HPLC grade) was prepared in the laboratory.

Equipment and chromatographic conditions

The HPLC system of Shimadzu, CBM-20A consisted Pump-LC-20 AR series, SPD-M2OA Diode array detector with a universal loop injector of injection capacity 20μl. Shimadzu LC lab solution software program was applied for data collecting and processing. The experimental conditions were optimized on Shimadzu, C_8 (250 x 4.6 mm, 5 μm) model shim pack-solar column at room temperature.

The chromatographic separation of ETM, MFA and its degradation products were carried out on reverse phase C_8 column by using various mobile phase but the mobile phase containing mixture of acetonitrile : Potassium dihydrogen orthophosphate buffer in the ratio of 60:40 v/v at pH-4.5 adjusted by ortho phosphoric acid shows good resolution. The separation was achieved by binary gradient elution with a flow rate of 1.0ml/min and detection was carried out at 299nm as both the drugs showed reasonably good response with characteristic UV spectrum as exhibited in **Fig-3**.
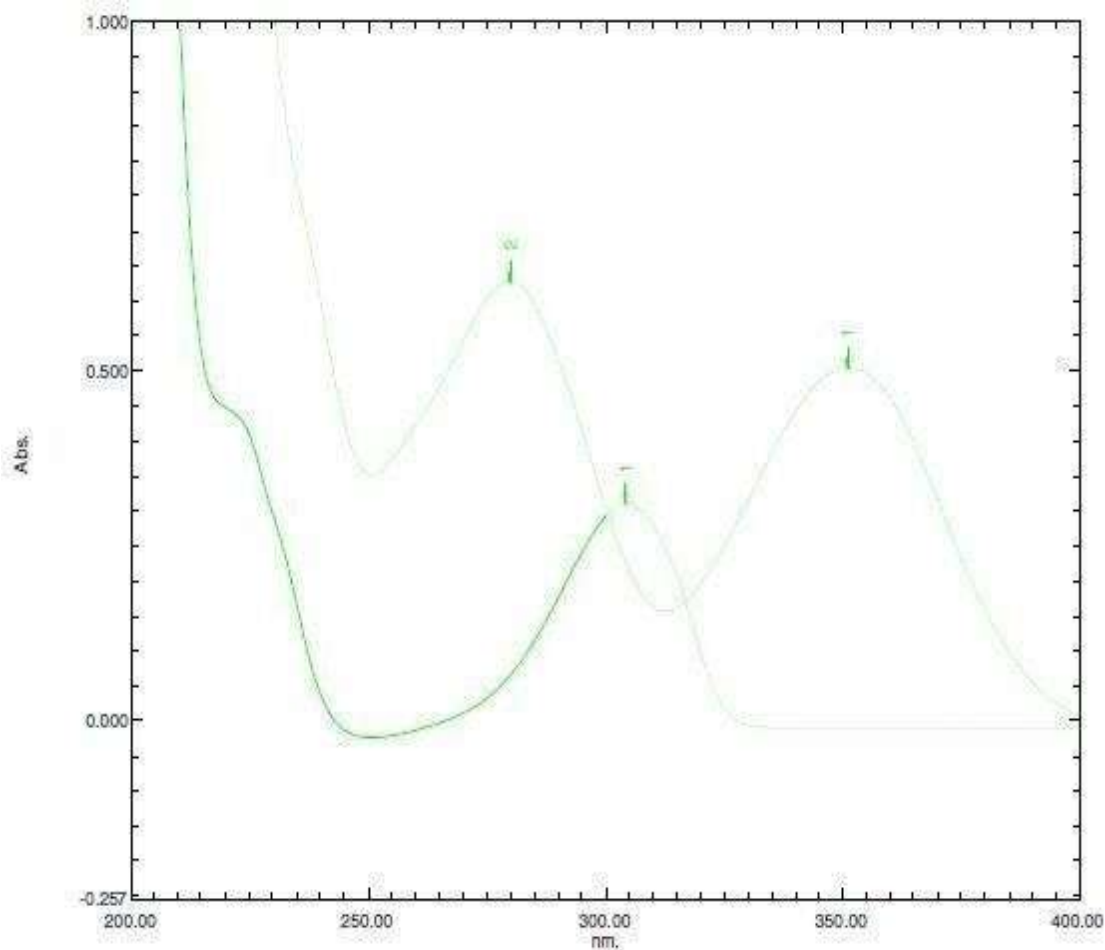
Fig-3: ISOBESTIC POINT OF ETM AND MFA

Preparation of mobile phase:

Mobile phase was prepared by dissolving 6.8 g of Potassium dihydrogen orthophospahte in 1000 ml water and pH adjusted to 4.5 with orthophosphoric acid. Obtained solution was filtered by 0.45µ filter, 40 ml of this was added to 60 ml of acetonitrile

Preparation of Standard Solutions:

The powder equivalent to 100 mg of ETM and MFA were weighed separately, taken in 100ml clean and dry volumetric flask and volume were made up with a mobile phase to give concentration 1000 µg/ml. The 10 ml of the stock solution of ETM and MFA separately was pipetted out in a 100 ml volumetric flask and made-up the volume with the mobile phase to give concentration 100 µg/ml. From this 5ml of each drug is diluted to 100ml separately with mobile phase to get a final concentration range of 5µg/ml for ETM and 5µg/ml for MFA. The chromatogram of the standard solution was exhibited in **Fig- 4**.
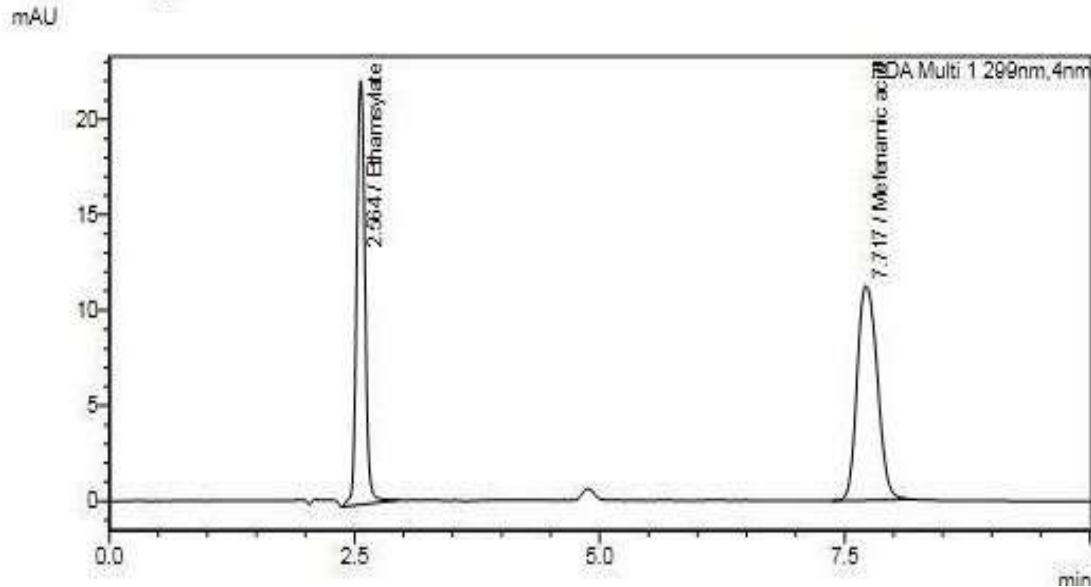
Fig-4: STANDARD CHROMATOGRAM OF ETM AND MFA

Preparation of sample solution:

Twenty tablets of SYLATE M each containing 500mg of ETM and 500mg of MFA were finely powdered. A quantity of powder equivalent to 500mg of ETM and MFA was weighed and transferred to 100ml volumetric flask. The drugs were extracted with the mobile phase. The extracts were made up to the volume with mobile phase and further dilutions were made to get a concentration of 5μg/ml for both ETM and MFA. The contents were mixed thoroughly and filtered through 0.45μm membrane filter. An aliquot of 20μl of both standard and test solutions were injected separately and chromatograms were recorded up to 10mins. The chromatogram of the sample solution was exhibited in **Fig- 5**.
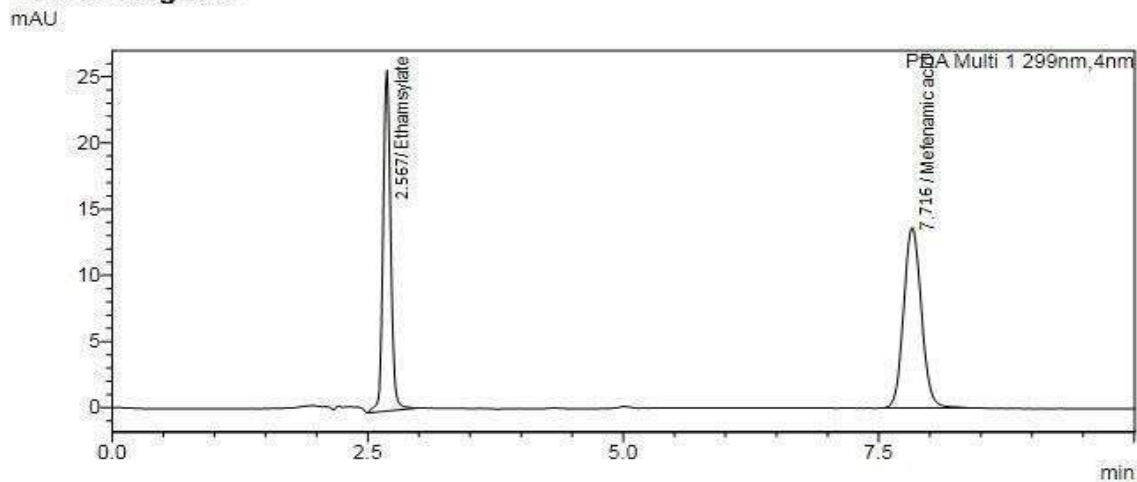

Fig-5 SAMPLE CHROMATOGRAM OF ETM AND MFA

Validation of proposed methods:
The developed method is validated as per ICH guidelines

Linearity:
Linearity was determined between the concentration range of 3-7µg/ml for both ETM and MFA. The injection was done twice for each concentration of ETM and MFA.

Precision:
The Precision was studied by taking the same concentration and results were observed by taking chromatograms for calculations. The concentration found for ETM and MFA was 5µg/ml. The results were obtained for 6 determinations.

Intraday and Interday Precision:
The Intraday precision was obtained by analyzing, the three different concentrations 4µg/ml, 5µg/ml and 6µg/ml of ETM and MFA for three determinations in the same day. The Interday precision was observed by analyzing day to day variability using the above mentioned concentrations on three different days, over a period of one week.

Repeatability:
It is measured by multiple injections of a homogenous sample of 5µg/ml of ETM and MFA. It performs the instrument under HPLC chromatographic conditions.

Limit of Detection and Limit of Quantitation:
Limit of Detection is the lowest amount of analyte in a sample which can be detected but not quantitated, and Limit of Quantitation is the lowest amount of analyte in the sample that can be quantitatively determined and were calculated by using the formula

$LOD = 3.3 \times \sigma / S$

$LOQ = 10 \times \sigma/S$

Where, σ = Standard deviation of the response
    S = Slope of the calibration curve.

System Suitability Test:
The system suitability testing parameters were observed for the chromatographic system and chromatographic conditions for developed methods.

III.   RESULT AND DISCUSSION:
Optimized chromatographic conditions:
Stationary phase : Shim pack solar C8 Column (4.6×250mm, 5µ)
Mobile phase : Acetonitrile : Buffer Mobile phase ratio : 60:40
pH : 4.5
Detection wavelength : 299nm
Flow rate : 1.0 ml/min
Sample volume   : 20µl
Temperature   : 25°C
The separation of ETM and MFA was observed with retention time of 2.5 and 7.7 min respectively.

Method validation:
Linearity:
The correlation coefficient value was found to be 0.999 for ETM and MFA. Linearity results were shown in **Table 1**, and Calibration graphs were shown in **Fig. 6 and 7**.

S.No	ETM		MFA	
	Concentration(µg/ml)	Peak Area	Concentration(µg/ml)	Peak Area
1	3	76577	3	146923
2	4	86464	4	154460
3	5	96533	5	164518

4	6	107344	6	172282
5	7	118808	7	182770

TABLE 1: LINEARITY VALUES OF ETM AND MFA

$$y = 8951.x + 11943$$
$$r^2 = 0.9997$$
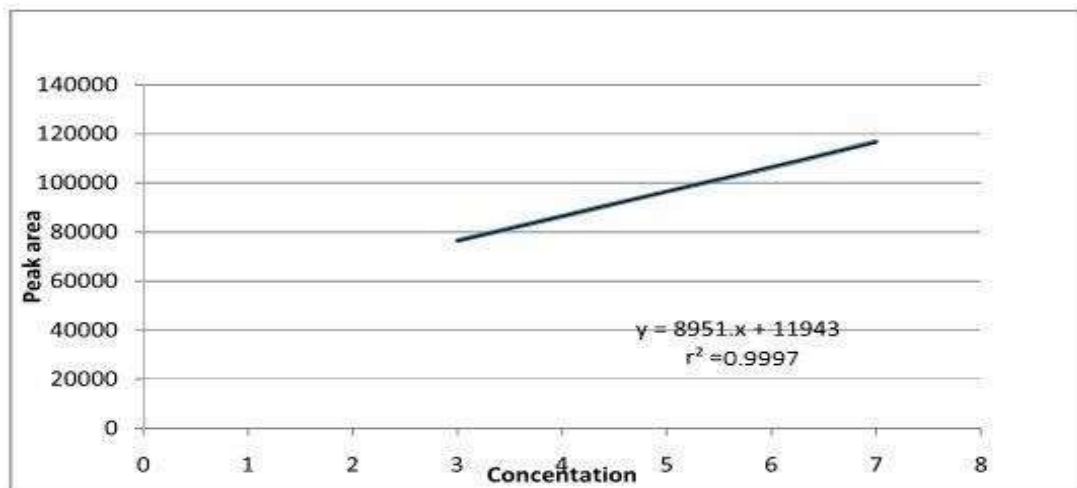
Fig-6: CALIBRATION CURVE OF ETM

$$y = 8951.x + 11943$$
$$r^2 = 0.9992$$
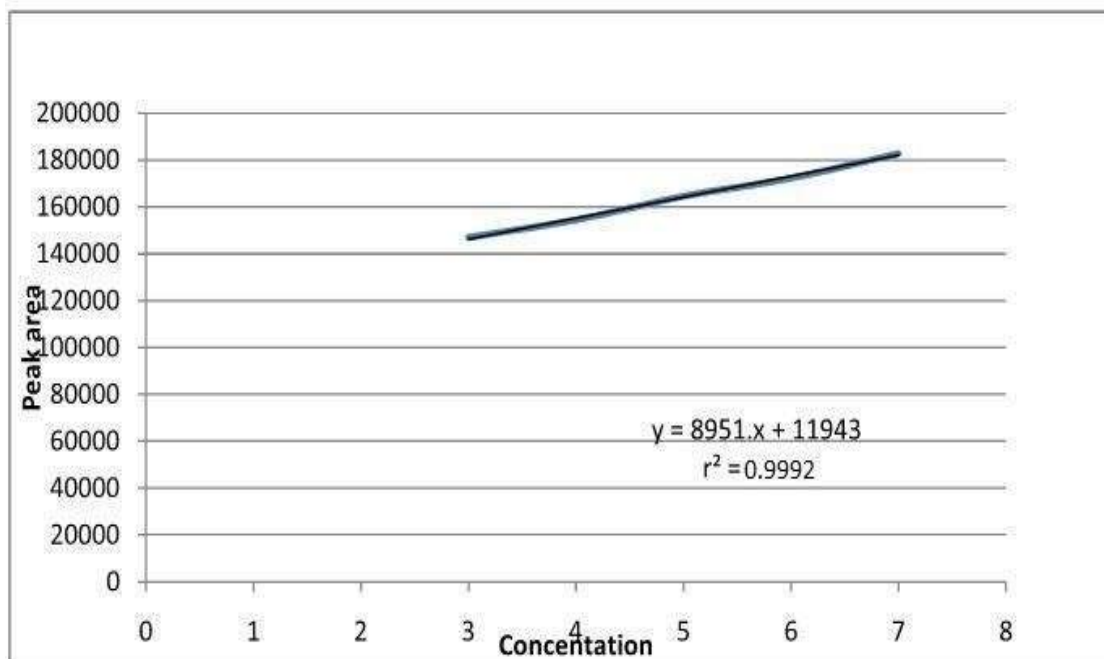
Fig-7: CALIBRATION CURVE OF MFA

Precision:

Percentage relative standard deviation (% RSD) was found to be less than 2% that proves the method is precisely shown in Tables 2, 3 and 4.

Level	Concentration (µg/ml)		Peak area		%RSD	
	ETM	MEF	ETM	MEF	ETM	MEF
1	4	4	103243	144460	0.5	0.7
			103694	145652		
			102653	143453		
2	5	5	113991	169518	0.8	0.4
			113721	167865		
			112299	168802		
3	6	6	132458	172282	0.3	0.5
			133240	172358		
			132568	173894		

Table 2: PRECISION STUDY OF INTRADAY

Level	Concentration (µg/ml)		Peak area		%RSD	
	ETM	MEF	ETM	MEF	ETM	MEF
1	4	4	103243	144460	0.5	0.7
			103694	145652		
			102653	143453		
2	5	5	103991	149934	0.8	0.6
			103778	149518		
			102305	141561		
3	6	6	122458	165328	0.2	0.9
			122549	162497		
			123021	162282		

Table 3: PRECISION STUDY OF INTERDAY

Concentration (µg/ml)	Peak area	%RSD

ETM	MEF	ETM	MEF	ETM	MEF
5	5	113721	169518	0.6	0.5
		112975	169165		
		112299	168802		
		113788	167898		
		113991	167037		
		112305	167605		

Table 4: PRECISION STUDY OF REPEATABILITY

LOD and LOQ:

Limit of detection of ETM and MFA was determined 0.190 and 0.323, respectively. Limit of quantitation of ETM and MFA was determined 0.578 and 0.981, respectively **Table 5**.

Drugs	Parameters	
	LOD	LOQ
Ethamsylate	0.190	0.578
Mefenamic acid	0.323	0.981

Table 5: LOD and LOQ VALUES OF ETM and MFA

System suitability:

System suitability parameters were observed like tailing factor, capacity factor, and theoretical plates for AML and CHL were in the acceptance criteria as per the ICH guidelines. **Table 6**.

Parameters	ETM	MEF
Linearity range µg/ml	3-7 µg/ml	3-7 µg/ml
Retention time	2.56 min	7.71 min
No. of theoretical plates	8543	2387
Correlation Co-efficient	0.9997	0.992
Tailing factor	1.076	1.178

![IJPRA Journal logo]

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 5 Sep-Oct 2023, pp: 1067-1080 www.ijprajournal.com ISSN: 2249-7781

|		
LOD	0.190	0.323
LOQ	0.578	0.981

Table 6: SYSTEM SUITABILTY STUDY

FORCED DEGREDATION STUDIES:

The stress studies of ETM and MFA were performed by various stress conditions to study the effect over wide range of acid, alkali, oxidation, thermal and photo degradation as per the ICH guidelines.

Acid degradation:

Accurately weigh and transferred 5mg of both ETM and MFA drug in 10ml volumetric flask to add 1ml of 0.1M hydrochloric acid and make up the volume with mobile phase then refluxed in round bottom flask on boiling water bath for 30min at 40ºC. After completion of 30min, about 0.1 ml of the above solution was transferred into 10ml volumetric flask and diluted with 10ml using mobile phase. The solution was injected into HPLC and chromatogram was recorded **Fig 8.**
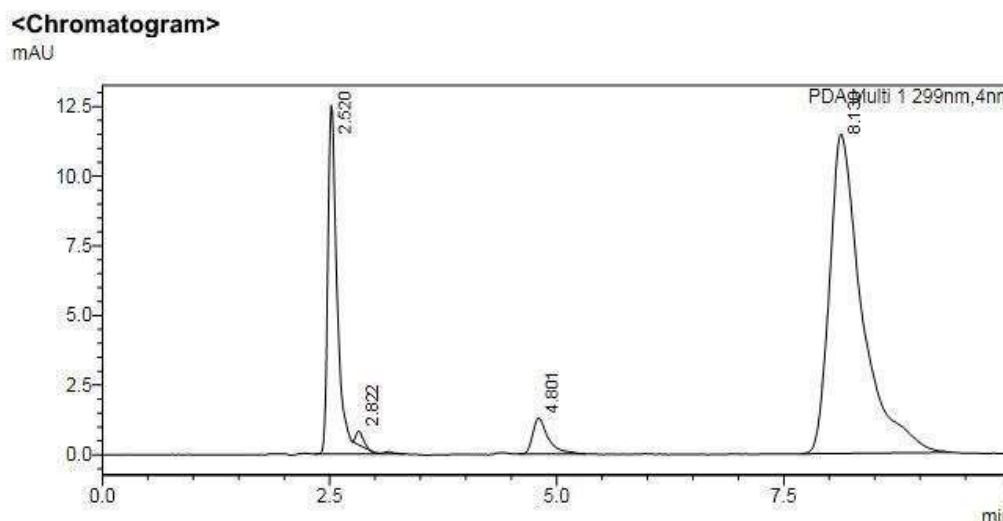
Fig 8: HPLC CHROMATOGRAM OF ACIDIC DEGRADATION

Alkali degradation:

Accurately weigh and transferred 5mg of ETM and MFA drug in 10ml volumetric flask to add 1ml of 0.1M sodium hydroxide and make up the volume with mobile phase then refluxed in round bottom flask on boiling water bath for 30min at 40ºC. After completion of 30min, about 0.1 ml of the above solution was transferred into 10ml volumetric flask and diluted with 10ml using mobile phase. The solution was injected into HPLC and chromatogram was recorded **Fig 9.**
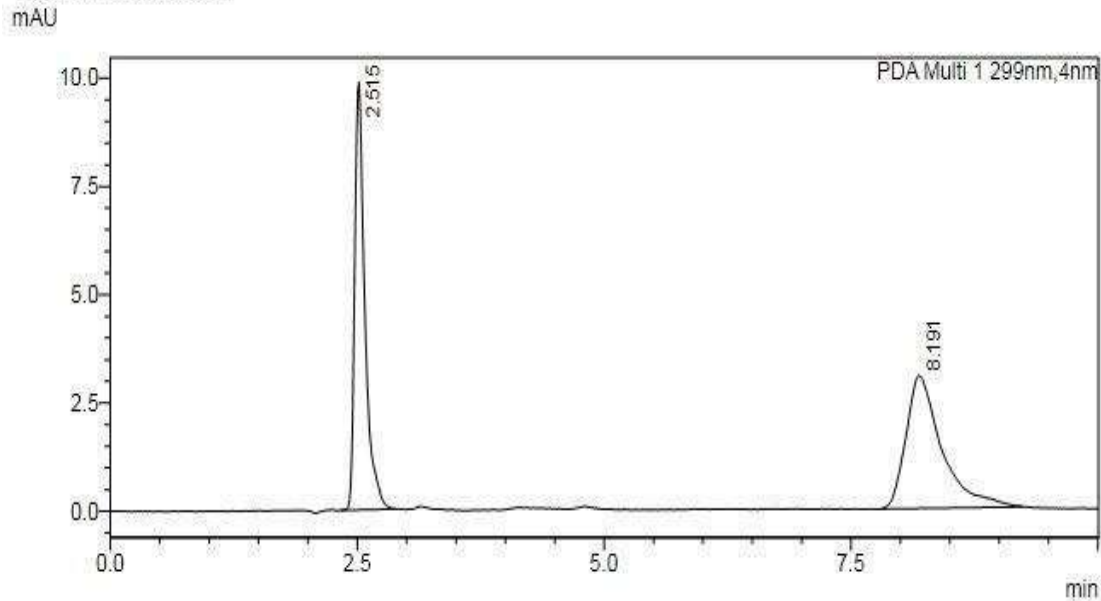
<Chromatogram>
mAU

Fig 9: HPLC CHROMATOGRAM OF ALKALINE DEGRADATION

Oxidation degradation:

Accurately weigh and transferred 5mg of ETM and MFA drug in 10ml volumetric flask to add 1ml of 30% hydrogen peroxide and make up the volume with mobile phase then refluxed in round bottom flask on boiling water bath for 30min at 40°C. After completion of 30min, about 0.1 ml of the above solution was transferred into 10ml volumetric flask and diluted with 10ml using mobile phase. The solution was injected into HPLC and chromatogram was recorded **Fig 10.**
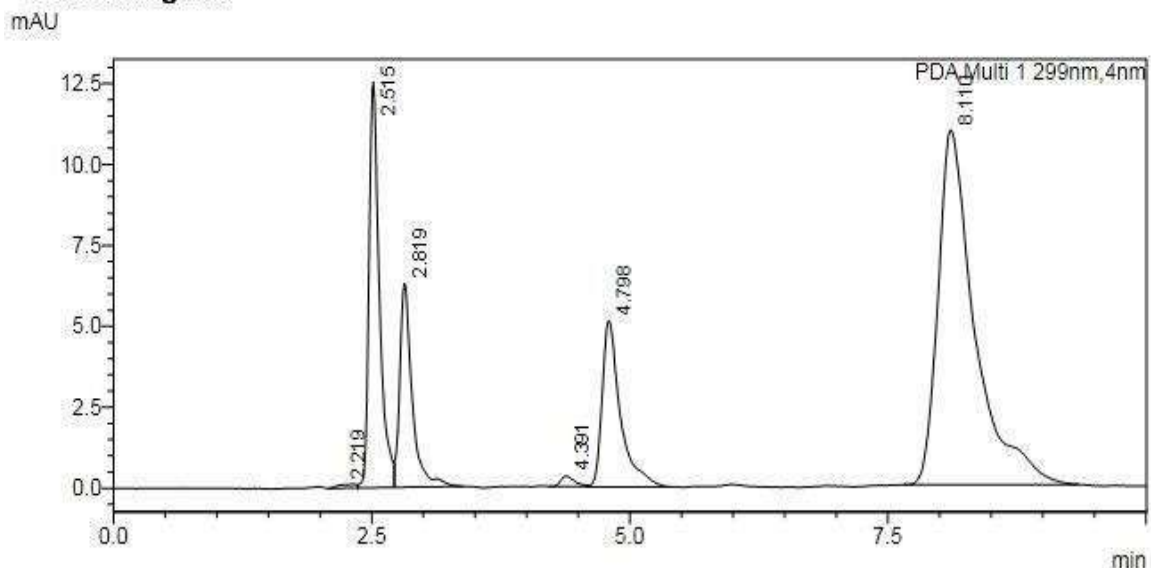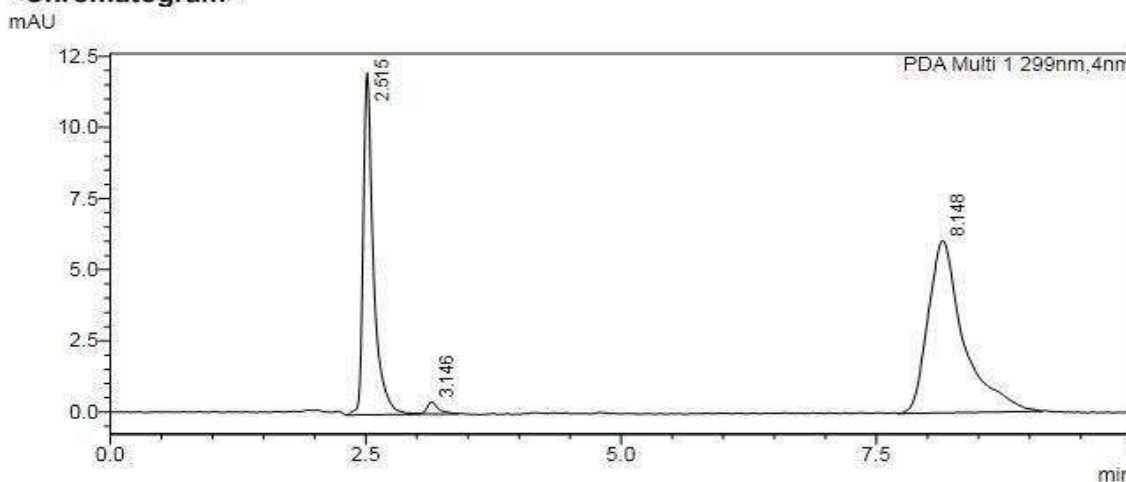
<Chromatogram>
mAU

Fig 10: HPLC CHROMATOGRAM OF OXIDATION DEGRADATION

Photolytic degradation:

Accurately weighed 5mg of ETM and MFA was transferred into a clean, dry petridish. The petridish was placed in direct sunlight for 5 days. In this study the drug substance were exposed to direct sunlight for 5 days to determine the effect of irradiation on the stability of the drug in solid state. Afterwards drug was transferred into 10ml volumetric flask and make up the volume with mobile phase. Take 0.1ml of above solution and transferred into 10ml volumetric flask and diluted with10ml using mobile phase. The solution was injected into HPLC and chromatogram was recorded **Fig 11.**
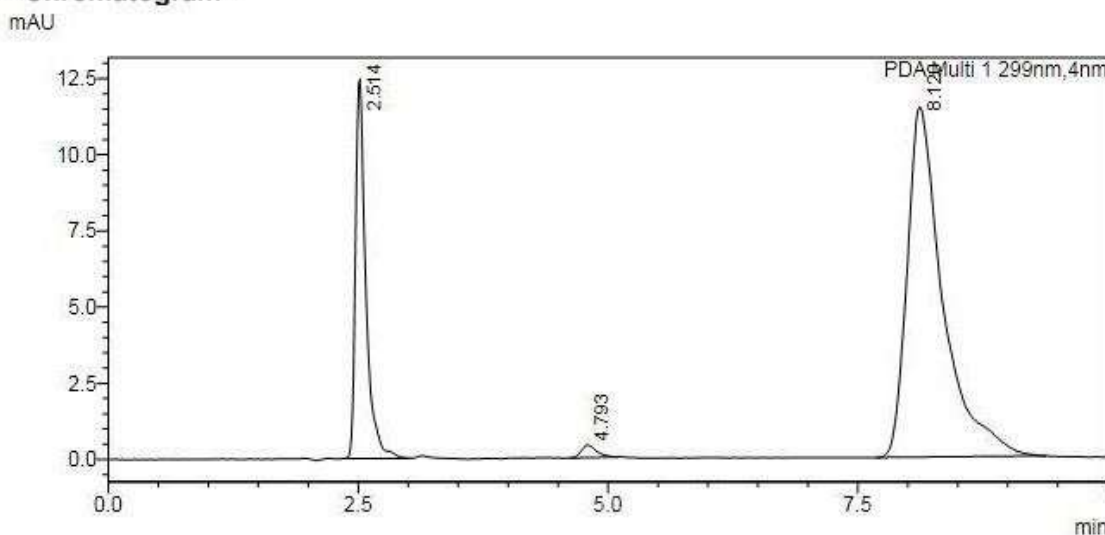

Fig 11: HPLC CHROMATOGRAM OF PHOTOLYTIC DEGRADATION

Thermal degradation:

Accurately weighed 5mg of ETM and MFA was transferred into clean and dry petridish. The petridish was placed in a oven at 50ºC for 3 hrs. The drug transferred into 10ml volumetric flask and make up the volume with mobile phase. Take 0.1ml of above solution and transferred into 10ml volumetric flask and diluted with10ml using mobile phase. The solution was injected into HPLC and chromatogram was recorded **Fig 12.**

Fig 11: HPLC CHROMATOGRAM OF THERMAL DEGRADATION

S.No	Analyte	Retention time of degradation product
1	Acid degradation	2.8, 4.8
2	Alkali degradation	No degradation product
3	Oxidation degradation	2.2, 2.8, 4.3, 4.7
4	Photolytic degradation	3.1
5	Thermal degradation	4.7

Table 7: FORCED DEGRADATION STUDY OF ETM AND MFA

IV. CONCLUSION:

Here, an attempt was made to develop the stability-indicating method development for the estimation of ETM and MFA in combined dosage form by using RP-HPLC. The mobile phase was optimized for better separation of the ETM anf MFA with good resolution at flow rate of 1.0 ml per minute. The separation was obtained by scanning at 299 nm using a PDA detector. The linearity was obtained with a good correlation coefficient at their respective retention time. The developed method was validated as per ICH guidelines by using statistical parameters.

This was followed by stress degradation of both the drugs under different conditions like acid, base, oxidative, thermal and photolytic study. The reliable, specific, reproducible and efficient method is developed and validated which can be easily acceptable for laboratory applications.

ACKNOWLEDGEMENT:

CONFLICTS OF INTEREST:
The authors declare that they have no conflict of interest.

BIBLIOGRAPHY:

[1]. The Merck Index –An encyclopedia of chemicals, drugs and biologicals, 13th edn., Merck and company, USA, 1989, p 3757, 9648.

[2]. Sweetman, S.C.; Martindale: the complete drug reference; 1064, 36th ed. The Pharmaceutical Press, London, (2009), 80.

[3]. The British Pharmacopoeia; Her Majesty's Stationary Office, London, (2013), Electronic Version.

[4]. Martindale. 2007. The Complete Drug Reference, 35th Edition. p 210-220

[5]. MHRA Drug Safety Update October. 2007: 1 (3), p 3-4

[6]. Indian Pharmacopoeia; Government of India, Ministry of Health and Family Welfare, Controller of Publications: Delhi, 1996; Vol. I, 459– 460.

[7]. British Pharmaceutical Codex; Department of Pharmaceutical Sciences of the Pharmaceutical Society of Great Britain, The Pharmaceutical Press: London, 1973; 285.

[8]. USP 23, NF 19, Asian Ed.; United States Pharmacopoeial Convention: Rockville, M.D., 1982; 1105.

[9]. USP 27, NF 22, Asian Ed.; United States Pharmacopoeial Convention: Rockville, M.D., 2004; 1151– 1152.

[10]. British Pharmacopoeia, International Ed.; HMSO: Cambridge, 2004; Vol. II, 1244– 1255.

[11]. Anju Goyal and I.Singhvi "Spectrometric estimation of ethamsylate and mefenamic acid from a binary mixture by dual

wavelength and simultaneous equation methods" IndianJ.Pharm.sci.,2008;70(1);108-111

[12]. G.Garg, SwarnlataSaraf and S.Saraf "Simultaneous etimation ofmefenamic acid and ethamsylate in tablets" Indian J.Pharmsci.,2007;69(2);279-28

[13]. Vinit D Patil, Ajay S Bhadoriya, Kunal D Ingale, Aniruddh R Chabukswar, Vishnu PChoudhari, Bhanudas S kuchekar "Development and validation of ratio spectra derivative spectrophotometric method for determination of mefenamic acid and ethamsylate in combiined formulation" Research journal of pharmacy and technology,2010;3(3)921-923. 28.

[14]. Anju Goya and I.singhvi "Spectrophotometric estimation of ethamsylate and mefenamic acid from a binary miture by dual wavelength and simultaneous equation method" Indian journal of pharmaceutical science,2008;70(1);108-111.

[15]. Arshad, MD, karadi,A rvindB, Raju, S.Appala, Darak, Venugopal "Spectrophotometric estimation of ethamsylte in bulk drug and its pharmaceutical formulations" Pharma ScienceMonitor,2014;2(4);36-40.

[16]. Priti. H. Patel, Vaishali v. Kharkhanis "Development and validation of uv spectrophotometric method for estimation of ethamsylate in bulk and pharmaceutical dosage forms" Asian Journal of Research in Chemistry,2013;6(2);166-168.

[17]. Azad Raza "Spectrophotometric determination of mefenamic acid in pharmaceutical preparations" Journal of Analytical Chemistry,2008;63(3);244-247

[18]. Yogini Jaiswal, Gokul Talele and Sanjay Surana "Application of hplc for the simultaneous estimationn of ethamsylate and mefenamic acid in bulk drugs and tablets", Journal of liquid chromatography and related technologies, 2007;30(8);1115-1124.

[19]. Fawzia Ibrahim, MohieK.Sharaf El-Din, Asmaa Kamal El-Deen and KuniyoshiShimizu "Misceller hplc for simultaneous determination of ethamsylate and mefenamic acid in presence of their main impurities and degradation product"

journal of chromatographic science,2017;55(1);23-29.

[20]. Asma Ahmed El-Zaher, MaiannealphonseMahrose, Ahmed Mohammed al-Ghani "Validated chromatogrphic method for the simultaneous estimation of ethamsylate and mefenamic acid in presence of their main impurities" Current pharmaceutical analysis,2019;15(6);624-631.

[21]. A.R. Rote, Durgehwari Chudaman Nerkar, SiddeshC. Thakare "Rp-hplcmethod development and validation for simultaneous estimation of ethamsylate and mefenamic acid in tablet formulation" International journal institutional pharmacy and lifesciences,2014;4(6).

[22]. P. Nagaraju, K Krishnachaitanya, VDN Srinivas, SVN Padma "Reverse phase high performance liquid chromatograpic method for the determination of ethamsylate in bulk and tablet dosageform" Asian Journal of Research in Chemistry, 2008;1(2);88-90

[23]. Prashil Nanotkar, Prashant Zurao and Avinash Kasture"Simultaneous estimation of tranexamic acid and ethamsylate in combined dosage form by rp-hplc" Open Access Scientific Reports,2012;1(5).

[24]. VenkaataNanda Kumar Kopparapu, Poonima Kasula, Ajay Kumar Reddy Ankinapalli, M Venkateswarlu "Rp-hplc method development and validation for simultaneous estimation of paracetamol and mefenamic acid in pharmaceutical suspension" Interntional Journal of Pharmaceutical Quality Assurance,2019;10(4);583-587.

[25]. BhagyashreeR.Dhumal, Kishore P. Bhusari, Madhukar R. Tajne, Mahavir H. Ghante, NishantS.Jain "Stability indicating method for the determination of mefenamic acid in pharmaceutical formulations by hplc" Journal of applied pharmaceutical science,2014;4(12),60-64.

[26]. Yogini s. Jaiswal, GokulTalele, Sanjay surana "Quantitative analysis of ethamsylate and mefenamic acid in tablets by use of planar chromatography"Journal of planar chromatography-modern TLC, 18(106).

[27]. Yogini S. Jaiswal, GokulTalele, Sanjay Surana "A simple and sensitive hptlc method for quantitative analysis of

ethamsylate in tablets" Journal of Planar Chromatography-Modern TLC. 2015;18(105);380-383.

[28]. Mona karia, BhargavGohel, BinaThanki, DarshanMadiya, ShitalFaldu "Development and validation of bioanalytical method for simultaneous estimation of ethamsylate and mefenamicacid in human plasma by rp-hplc" Pharmatutor-ART-1874.

[29]. ICH guidelines.ICH Harmonized Tripartite Guideline Q2 (R1), Validation of analytical procedure: text and methodology, Current step 4 version, 2005.

[30]. V. Pavan Kumar , Narayanaswamy Harikrishnan and Gejalakshmi Subramanian "rp-hplc method development and validation for the simultaneous estimation of ledipasvir and sofosbuvir in fixed dosage form" International journal of pharmaceutical science and research., 2021; Vol. 12(7): 3852-3857

[31]. P. H. Sakpal * 1 and A. R. Chabukswar "stability indicating rp - hplc method development and validation for simultaneous estimation of amlodipine and chlorthalidone in bulk and tablet dosage form", International journal of pharmaceutical science and research., 2020; Vol. 11(5): 2161-2168.