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Development, Validation and Forced Degradation Study of Emtricitabine and Tenofovir Alafenamide in its Pharmaceutical Dosage Form Using RP-HPLC

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Authors' contributions

This work was carried out in collaboration among all authors. Author KP designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors US and HJ managed the analyses of the study. Authors JKP and TBP managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The present research was aimed to develop and validate a reverse phase high performance liquid chromatographic (RP-HPLC) method for the quantification of Emtricitabine (EMT) and Tenofovir Alafenamide (TEN) in combination.
Methodology: Separation was achieved under optimized chromatographic condition on an Inertsil C18, 250 x 4.6 mm, 5µm column. Various composition of mobile phase was tried. Separation of EMT and TEN was started with Methanol: Buffer and Methanol finally using solvent system of Buffer (pH 3.5) and Methanol in ratio of (30:70) and flow rate adjust at 1.0 ml/min was used as solvent system, the detection was carried out at 262nm using Shimazdu UV-visible detector. The mobile phase run time for the developed analytical method was 10 minutes.
Results: The standard curve was found linear in the concentration range of 20-60 µg/ ml (r^2-0.9994) and 2.5-7.5 µg/ ml (r^2-0.9992) for EMT and TEN respectively. The %RSD was found to be 0.80-0.95% and 0.63-1.09 for EMT and TEN respectively. Percentage (%) recoveries for EMT and

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TEN to be in range of 100%-100.6% and 99.32%-100.83% respectively. The limit of detection and the limit of quantification were found to be 4.80 µg/ ml and 14.7 µg/ ml respectively for EMT and 0.11 µg/ ml and 0.33µg/ ml respectively for TEN. Results of forced degradation study showed EMT degradation in acid and base medium while TEN was showed degradation in oxidative stress. The proposed developed RP-HPLC method was validated statistically and the values were found to be within the acceptable limits.

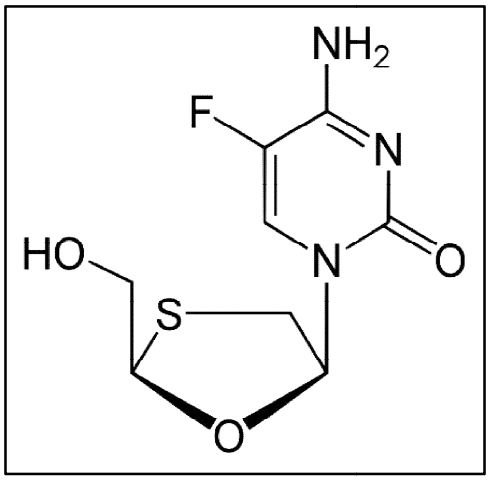
Conclusion: In conclusion, the developed RP-HPLC method was found to be simple, specific, and rugged for simultaneous estimation of EMT and TEN. Validation results of method was found within the acceptable limits. Hence it can be used for analysis of EMT and TEN.

Keywords: Degradation; RP-HPLC; EMT; TEN; validation.

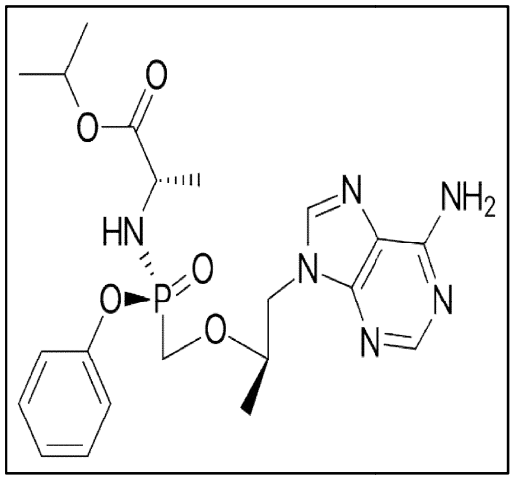
1. INTRODUCTION

Human immunodeficiency virus (HIV) is a virus that damages the immune system. The immune system helps the body fight off infections. Untreated HIV infects and kills CD4 cells, which are a type of immune cell called also known as T cells. Over time, as HIV kills more CD4 cells, the body is more likely to get various types of infections and may lead to cancer. Emtricitabine (EMT) is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults. Emtricitabine (EMT) is an analogue of cytidine (Fig. 1). Tenofovir Alafenamide (TEN) is a nucleotide reverse transcriptase inhibitor (NRTI) and a novel ester prodrug of the antiretroviral tenofovir (Fig. 2).

The fixed dose of the EMT and TEN is approved by FDA. The formulation of EMT and TEN is commercially available in the Indian market for treatment of HIV [1,2]. So, as per FDA, validation is an essential requirement in any pharmaceutical industry to build and designed quality, safety and efficiency of product. The literature survey reveals that these drugs have been analyzed along with many other Anti-HIV agents by analytical methods like spectrophotometric [3], HPTLC [4], RP-HPLC [5], Stability Indicating RP-HPLC [6], Bioanalytical LC-MS [7] etc. but no any RP-HPLC method have been reported for the estimation of specifically EMT and TEN. Before commercial production, the company need to perform the process validation of TEN and EMT dosage forms for assurance of the quality. Quantitative analysis of any drug is an important tool in industry, so it was thought of interest to develop and validate chromatographic method for TEN and EMT. The number of the drug and drug formulation introduced into the market has been increasing at alarming rate, so at the end of the project comparison of all the marketed TEN and EMT will be carried out. In the present research work, an attempt was made to Develop and validate of chromatographic Method for Estimation of TEN and EMT in bulk and tablet dosage form.



Chemical Structure of EMT



Chemical Structure of TEN

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Potassium Dihydrogen Phosphate was used in the method development was of HPLC grade. Other solvents like methanol and water was also of HPLC grade. Emtricitabine and Tenofovir Alafenamide was gifted by Emcure Pharmaceuticals Ltd, Ahmedabad. % purity of both EMT and TEN was 101.21% and 100.21% respectively, as per Certificate of Analysis (CoA) supplied by Emcure Pharmaceuticals Ltd, Ahmedabad.

2.2 Instrumentation

Shimadzu HPLC system containing LC-20AT pump and SPD-20AT UV-visible detector was used for the development of analytical method and forced degradation study. HPLC system was used an isocratic elution technique at a flow rate of 1ml/min on an Inertsil C18 (GL Sciences, Japan) 250 x 4.6 mm x 5µm column at ambient temperature. A Rheodyne injector (20 µl) was used for injecting the sample. Detection wavelength for EMT and Ten was 381nm and 272nm, respectively.

2.3 Preparation of Sample Solution

Standard stock solution of EMT (400 µg/ml) was prepared by adding accurately weighed quantity of EMT (40 mg) to 100 ml volumetric flask, dissolved and diluted up to the mark with Buffer (pH 3.5): Methanol (30:70) to give a stock solution of 400µg/ml. Standard stock solution of TEN (50 µg/ml) was prepared by adding accurately weighed quantity of TEN (5 mg) to 100 ml volumetric flask, dissolved and diluted up to the mark with Buffer (pH 3.5): Methanol (30:70) to give a stock solution of 50µg/ml. Transfer 1 ml of standard stock solution of EMT and TEN to 10 ml volumetric flask and dilute up to mark with Buffer (pH 3.5): Methanol (30:70). (Final Sample concentration 40µg/ml(EMT) & 5µg/ml (TEN). Each solution was scanned between 200-400 nm and the spectrum was recorded. The point at which drug shows absorbance was selected as wavelength for determination. Sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected.

2.4 Method Validation of RP-HPLC Method

2.4.1 System suitability test parameters

System suitability testing is an internal part of a liquid chromatographic method, and it is used to verify that the chromatographic method is able to produce good resolution between the peaks of interest with high reproducibility. The system suitability was determined by making six replicate injections from a freshly prepared standard solution of 5 µg/ml of TEN and 40 µg/ml of EMT and analyzing each solute for its retention time (Rt), Number of theoretical plates (N), resolution (RS) and tailing factor (T). The system suitability method acceptance criteria set in each validation run were- a %RSD<2% , Capacity factor > 2.0, tailing factor ≤ 2.0, and theoretical plates>2000 [8-11].

2.4.2 Selectivity

It is ability of the method to measure specifically the analyte of interest, in the presence of other components, such as impurities, degradation products, excipients that be expected to be present in the sample preparation.

2.4.3 Linearity and range (n=5)

Aliquots of working standard solution (0.5, 0.75, 1.0, 1.25, and 1.5 ml) of EMT (400 µg/ml) were transferred to a series of 10 ml volumetric flask. The volume was adjusted up to the mark with Diluent to obtain 20, 30, 40, 50, and 60 µg/ml of Emtricitabine. Aliquots of working standard solution (0.5, 0.75, 1.0, 1.25 and 1.5 ml) of TEN (50 µg/ml) were transferred to a series of 10 ml volumetric flask. The volume was adjusted up to the mark with Diluent to obtain 2.5, 3.75, 5, 6.25 and 7.5 µg/ml of TEN. An aliquot of 20µl of each solution was injected under operating chromatographic conditions. Plot the calibration curve of area versus respective concentration and find out correlation coefficient and regression line equation for EMT and. TEN Each response was an average of five determinations.

2.4.4 Precision

Intraday precision (n=3) was determined by analyzing of EMT and TEN standard solution in the range EMT (20, 40, and 60 µg/ml) & TEN (2.5, 5 and 7.5 µg/ml) were analyzed on three times on same day and % RSD was calculated. Interday precision (n=3) was determined by

analyzing of EMT and TEN standard solution in the range EMT (20, 40, and 60 µg/ml) & TEN (2.5, 5 and 7.5 µg/ml) were analyzed on three different successive and % RSD was calculated. Repeatability (n=6) was determined by analyzing EMT and TEN test solution having the concentration 40µg/ml & 5µg/ml of EMT and TEN Measure six times. Calculate %RSD for EMT and TEN [12,13,14].

2.4.5 Accuracy (n=3)

The accuracy of the method was determined at 50%, 100% and 150% by calculating recoveries of EMT and TEN by the standard addition method. Known amount of standard solutions of EMT and TEN were added to pre-quantified sample solution of EMT and TEN. Each solution was injected in triplicated and the percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equation of the respective calibration curves [13].

2.4.6Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ of the drug were calculated using following equations according to ICH guideline. LOD = 3.3 σ/s and LOQ = 10 σ/s Where σ is the SD of the response and S is the slope of the calibration curve.

2.4.7 Robustness

The robustness study was performed to evaluate the influence of small but deliberate variation in the chromatographic condition. The robustness was checked by changing three small changes. (1) Different flow rate (1±0.2ml/min) (2) Flow rate: 0.9ml/min and 1.1ml/min. Data of robustness are shown. (3) Different pH (4) Different Mobile phase

2.5 Forced Degradation Study

Stress degradation study was carried out under the acid catalysis and base catalysis. Study was also performed under oxidative stress, thermal degradation and photolytic degradations [15,16,17]. Standard stock solutions of EMT (400µg/ml) and TEN (50 µg/ml) was prepared by dissolving accurately weighed 40mg of EMT Reference Standard and 5mg of TEN Reference Standard to 100ml volumetric flask respectively. The final volume was made up with the Diluent.

2.5.1 Preparation of sample for acid degradation

1 ml of EMT Standard Stock solution (400 µg/ml) and TEN Standard Stock solutions (50 µg/ml) was transferred to 10ml volumetric flask, respectively. 2 ml of HCl of different concentration was added. Keep the solution for particular time period [18].

2.5.2 Preparation of sample for base degradation

1 ml of EMT Standard Stock solution (400 µg/ml) and TEN Standard Stock solutions (50 µg/ml) was transferred to 10ml volumetric flask, respectively. 2 ml of NaOH of different concentration was added. Keep the solution for particular time period [19,20].

2.5.3 Preparation of sample for oxidation degradation

1 ml of EMT Standard Stock solution (400 µg/ml) and TEN Standard Stock solutions (50 µg/ml) was transferred to 10ml Volumetric flask and 2ml different concentration of H_2O_2 of was added, respectively. Keep for particular time period.

2.5.4 Sample preparation for photo degradation

Accurately weighed 40mg of EMT (40 mg) and TEN (5mg) Reference Standard was transferred to 100ml volumetric flask, respectively. The solutions were kept it into UV chamber for time period specified as per ICH. After specific time period the volume was made up with mobile phase.

2.5.5 Sample preparation for thermal degradation

Accurately weighed 40mg of EMT (40mg) and TEN (5mg) Reference Standard was transferred to 100ml volumetric flask, respectively. The solution was kept it into Oven at 80^0C temperature for time period specified as per ICH, after time period the volume was made up with mobile phase.

3. RESULTS AND DISCUSSION

3.1 Method Development of RP-HPLC Method for EMT and TEN

For development of method development various chromatographic condition trail was carried out in preliminary experimental work. Chromatographic

condition of the HPLC system was consisted of Inertsil C18 column (250 x 4.6 mm x 5µm) as stationary phase. The mobile phase composition was set as Buffer (pH 3.5): Methanol (30:70). Flow rate of mobile phase was set to 1ml/min. volume of the HPLC injection was set to 20µL. The wavelength for detection was set to 262nm.

The final chromatographic condition for the method development was depicted in Table 1. Chromatogram of the mobile phase run was showed in Fig. 1.

3.1.1 Linearity

A method was found linear in a range of 20-60 mcg/ml & 2.5–7.5mcg/ml of EMT & TEN of standard concentration respectively were found linear as shown in Fig. 2. A correlation coefficient for EMT and TEN was 0.9994 & 0.9992 respectively. The areas obtained were directly proportional to the concentration of analyte in the sample. The method can, therefor be termed as linear in the specified range.

3.1.2 Precision

The Intraday precision was assessed by analyzing samples of pharmaceutical formulation (n=3) The %RSD was found to be 0.80-0.95 % and 0.63-1.09 for EMT and TEN respectively. The Interday precision was determined using mean values and the % RSD for the analysis of

Table 1. Chromatographic condition for method development

Name	Retention Time(min)	Area	Asymmetry	Theoretical plates
EMT	3.6	3792.81	1.36	7956
TEN	5.3	1515.59	1.27	8030

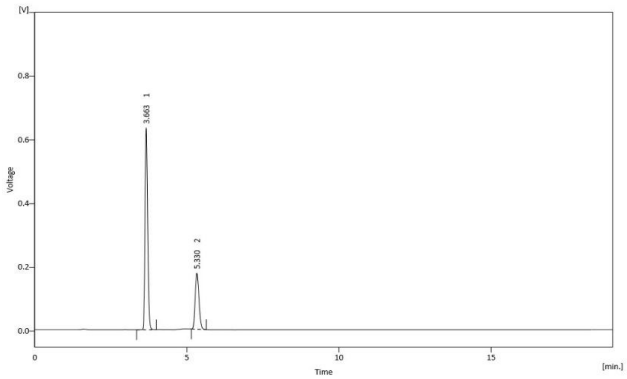


Fig. 1. Chromatogram of EMT and TEN

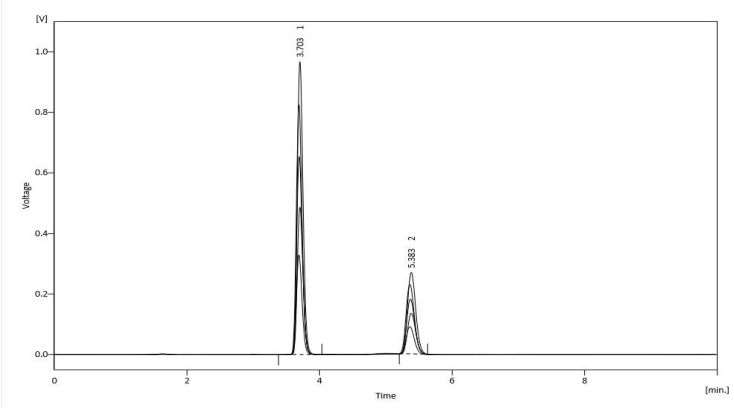


Fig. 2. Linearity overlay chromatogram of EMT and TEN

three samples of the pharmaceutical formulation on different days. The % RSD was found to be 1.11-1.46 % and 1.30-1.34 for EMT and TEN respectively. The precision evaluated as the repeatability in th % RSD was found to be 1.45% and 1.32% for EMT and TEN respectively. The results are shown in Table 2 and Table 3. The results obtained were well within the acceptance criteria. The method at selected chromatographic condition can therefore be termed as precise.

3.1.3 Accuracy

The accuracy of the method was determined by standard addition method at three different concentration level i.e 80%, 100% and 120%. The results of the accuracy were presented in Table 4 for EMT and TEN. It showed % recoveries for EMT and TEN to be in range of 100%-100.6% and 99.32%-100.83% respectively. The % recovery at each level, mean% recovery, % RSD met the established acceptance criteria.

3.1.4 Robustness

The robustness data for the method was given tin Table 5. The %RSD is below 2% which is within the given limit of acceptance criteria. The method at selected chromatographic condition is therefore robust.

3.1.5 LOD and LOQ

The LOD and LOQ were obtained by using the mean of the slope and the standard deviation of the intercept of the independent curves. The limit of detection and the limit of quantification were found to be 4.80 µg/ ml and 14.7 µg/ ml respectively for EMT and 0.11 µg/ ml and 0.33µg/ ml respectively for TEN.

3.1.6 System suitability and specificity

The % RSD values calculated in the system suitability test for the different parameters tested were within the acceptable range (RSD < 2.0%) as presented in Table 6. This result showed that the system is suitable for the analysis of the EMT and TEN. These results indicate that the analytical method was found to be specific as there was no interference of any excipients or impurities.

Table 2. Results of precision determination for EMT and TEN

Intraday precision data for EMT and TEN					
EMT			**TEN**		
Conc. (mcg/ml)	**Mean response**	**% RSD**	**Conc. (mcg/ml)**	**Mean response**	**% RSD**
20	1989.802	0.80	2.5	794.21	0.65
40	3922.067	0.95	5	1565.78	1.09
60	5970.083	0.85	7.5	2382.10	0.63
Interday precision data for EMT and TEN					
EMT			**TEN**		
Conc. (mcg/ml)	**Mean response**	**% RSD**	**Conc. (mcg/ml)**	**Mean response**	**% RSD**
20	1920.774	1.41	2.5	768.099	1.31
40	3908.014	1.46	5	1562.963	1.34
60	5872.962	1.11	7.5	2343.988	1.30

Table 3. Repeatability data for EMT and TEN

Concentration of EMT (mcg/ml)	**Area (n=6)**	**Concentration of TEN (mcg/ml)**	**Area (n=6)**
40	3890.104	**5**	1554.488
	3838.764		1533.86
	3881.034		1550.72
	3927.668		1569.501
	3966.982		1585.177
	3990.65		1585.919
Mean	3915.867	**Mean**	1563.278
SD	56.8620199	**SD**	20.64542
% RSD	1.45	**% RSD**	1.32

Table 4. Determination of accuracy of EMT and TEN

Results for Accuracy of EMT						
% Level Spiked	**Sample No**	**Sample Concentration (µg/ml)**	**Concentration Recovered (µg/ml)**	**% Recovery**	**% Recovery Mean**	**%RSD**
50	1	20	19.996	99.98	100.09	0.15
	2	20	20.062	100.31		
	3	20	19.996	99.98		
100	1	40	40.092	100.23	100.07	0.11
	2	40	39.992	99.98		
	3	40	40.004	100.01		
150	1	60	60.120	100.2	100.39	0.43
	2	60	60.600	101		
	3	60	59.994	99.99		
Results for Accuracy of TEN						
50	1	2.5	2.497	99.86	99.11	0.54
	2	2.5	2.470	98.80		
	3	2.5	2.467	98.66		
100	1	5	4.995	99.90	100.96	0.84
	2	5	5.050	101		
	3	5	5.100	102		
150	1	7.5	7.266	96.88	98.80	1.38
	2	7.5	7.470	99.60		
	3	7.5	7.497	99.92		

Table 5. Data of Robustness for EMT and TEN

Robustness data for EMT				
			Mean	**% RSD**
	Flow Rate	0.9ml/min	4032.572	0.41
		1.1ml/min	3851.567	0.51
EMT (40mcg/ml)	**Mobile phase**	32:68	4024.366	0.17
		28:2	3795.889	0.64
	pH	3.3	3851.725	0.78
		3.7	3954.017	0.50
Robustness data for TEN				
			Mean	**% RSD**
	Flow Rate	0.9ml/min	1603.228	0.59
		1.1ml/min	1539.024	0.50
TEN (5mcg/ml)	**Mobile phase**	32:68	1611.095	0.44
		28:72	1519.001	0.87
	pH	3.3	1527.075	1.78
		3.7	1578.209	0.44

Table 6. System suitability parameters

Parameters	**EMT**	**TEN**	**Specification**
Retention Time (min)	3.6	5.3	
Theoretical Plate (N)	7956	8030	≥ 2000
Tailing Factor (T)	1.3	1.2	T ≤ 2

3.2 Forced Degradation Studies

Stress degradation studies were performed as per the ICH guideline. Table 7 shows results of degradation studies performed on EMT and TEN. It was observed that both the drugs EMT and TEN have significant degradation in acidic, basic, oxidation, photo degradation and thermal degradation. Results indicated, in acid and base EMT found more degradation and TEN showed more degradation with oxidative stress. As per ICH guidelines peak, purity angle should be less than peak purity threshold. Hence, degradation products of EMT and TEN was not interfere in the analysis of EMT and TEN using proposed method. So proposed method was also used for determination of stability of EMT and TEN in pharmaceutical dosage form. Chromatogram resulted in the various stress study was presented in Fig. 3.

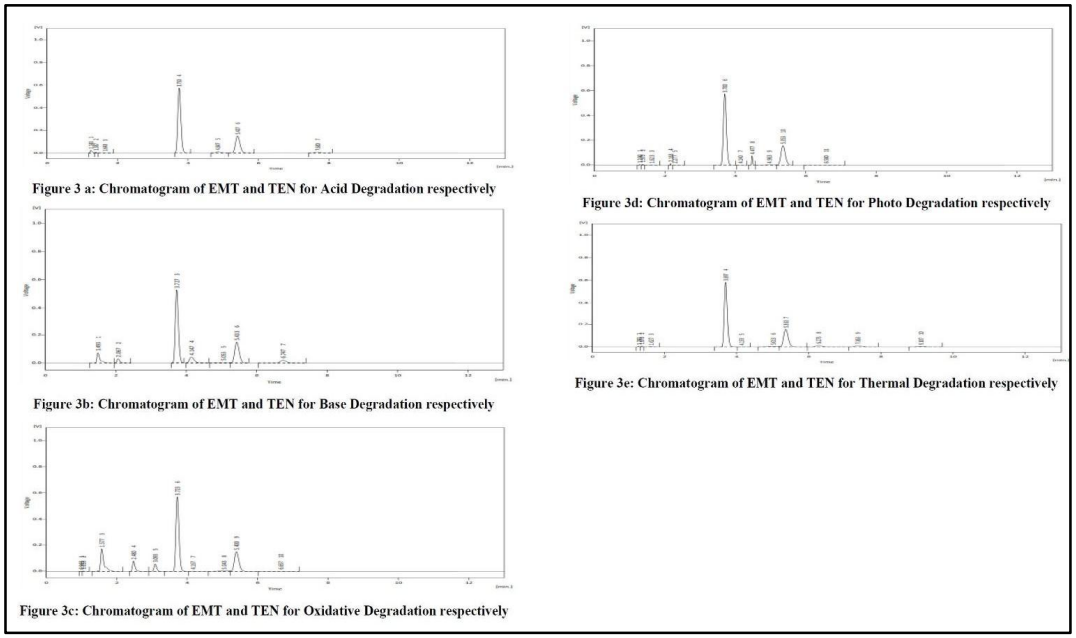


Fig. 3. Chromatograms of various stress degradation condition for EMT and TEN Respectively

Table 7. Results of forced degradation studies on EMT and TEN

EMT % Degradation					
Parameter		**Standard**		**Sample**	
	Condition	**Area**	**% Degradation**	**Area**	**% Degradation**
Acid	1N HCl 3hrs	3514.749	10.390	3506.776	10.59
Base	1N NaOH 3hrs	3272.773	16.559	3201.417	18.38
Oxidation	3% H_2O_2 4hrs	3504.401	10.654	3480.093	11.27
Photo	UV light 48hrs	3418.988	12.831	3476.611	11.36
Thermal	80 degree 10hrs	3612.987	7.885	3526.228	10.10
TEN% Degradation					
Parameter		**Standard**		**Sample**	
	Condition	**Area**	**% Degradation**	**Area**	**% Degradation**
Acid	1N HCl 3hrs	1363.827	12.985	1353.861	13.62
Base	1N NaOH 3hrs	1356.492	13.453	1328.135	13.62
Oxidation	3% H_2O_2 4hrs	1311.971	16.294	1340.393	14.48
Photo	UV light 48hrs	1318.022	15.908	1368.934	12.66
Thermal	80 degree 10hrs	1383.434	11.734	1386.652	11.53

4. CONCLUSION

In the present research, EMT and TEN was simultaneously estimated by RP-HPLC. The results of the validation studies in the present research work indicated that the proposed method was specific, robust, selective, linear and high precision characteristics without any interference from the excipients and degradation products. RP-HPLC method for selected combination of drug was not till reported. Therefore, the developed method was successfully used for quantitative analysis of EMT and TEN in synthetic mixture of EMT and TEN.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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