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HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF TENOFOVIR ALFENAMIDE HEMIFUMARATE IN BULK AND SIMULTANEOUS ESTIMATION OF TENOFOVIR ALFENAMIDE AND EMTRICITABINE IN COMBINED TABLET DOSAGE FORM

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ABSTRACT

A Simple, specific, precise and accurate chromatographic method for estimation of Tenofovir alafenamide fumarate and Emtricitabine in API and tablet dosage form was developed by C18 column having 250 mm length, 4.6 mm internal diameter, 5µ particle size. Peak was observed in mobile phase consist of Buffer (pH 3.5): Methanol (30:70). The flow rate was 1ml/min. The estimation was carried out at 262 nm. The retention time was found of Emtricitabine 3.6 and Tenofovir alfenamide5.3 minute. Linearity was found in range of 20-60mcg/ml for Emtricitabine Linearity was found in range of 2.5-7.5 mcg/ml for Tenofovir alafenamide The method was validated as per ICH guideline Q2R1. All validation parameters were found to be within accepted range specified in ICH guideline Q2R1.

KEYWORDS: Emtricitabine, Tenofovir alfenamide Hemifumarate, RP-HPLC, ICH guideline Q2R1.

INTRODUCTION

Emtricitabine is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV. Tenofovir alfenamide Hemifumarate is nucleotide reverse transcriptase inhibitor (NRTI) and a novel ester prodrug of the antiretroviral Tenofovir.[1-3] The IUPAC name of Emtricitabine 4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one with molecular formula $C_8H_{10}FN_3O_3S$ and IUPAC name of Tenofovir propan-2-

yl(2S)-2-{[(S)-({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)(phenoxy)phosphoryl]amino}propanoate with molecular formula$C_{21}H_{29}N_6O_5P$. It is White crystalline powder with melting point of 136-140 °C for Emtricitabine and melting point of Tenofovir 125ºc to 135ºc.[4-7] It is product of Gliend science brand name of Descovy strength of 200mg/5mg and 200 mg/25mg respectively.

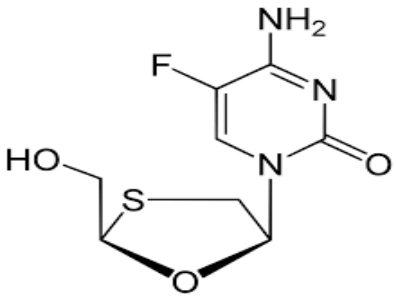

Figure 1: Structure of Emtricitabine.

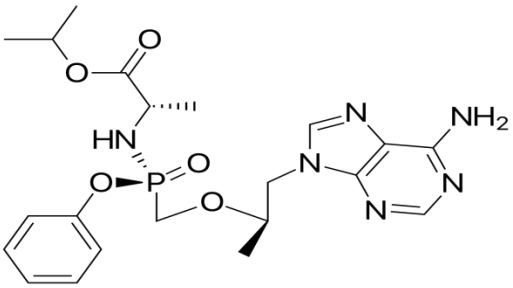

Figure 2: Structure of Tenofovir Alfenamide Hemifumarate.

MATERIAL AND METHODS

Instruments

Shimadzu model LC-20AT instrument, series RP-HPLC system with UV detector. Phenomex column C18 column having 250 mm length, 4.6 mm internal diameter,5µ particle size. Sonicator of Soltec-sonica ultrasonic cleaner (Spincotech Pvt Ltd) and analytical balance of Electronic analytical balance (AUX-200), Uni bloc- SHIMADZU.

Chemicals

Emtricitabine and Tenofovir alfenamide standard was supplied by Gifted by Emcure Pharmaceutical Company. Methanol, Potassium Dihydrogen Phosphate, Water HPLC grade was purchased from AR Grade, Merck.

HPLC condition

A chromatographic separation of drug was achieved using Phenomex column 250X 4.6mm, 5µ particle sizeC18 column with mobile phase of Buffer (pH 3.5): Methanol (30:70) Drug was monitored at detection wavelength of 262nm, the flow rate was 1ml/min and injection volume was 20µL. The retention time was found of Emtricitabine 3.6 and Tenofovir alfenamide 5.3 minute respectively.

Preparation of Mobile Phase

Buffer preparation

6.8 gm Potassium dihydrogen phosphate buffer was transferred to 1000ml beaker and 800 ml water was added shacked to dissolve and volume was made up with water, pH 3.5 was adjusted with diluted o-Phosphoric acid.

Methanol, Phosphate buffer were sonicated for 2 min for degassing and filtered through 0.45 µ Millipore filter.

Preparation of Standard Solution

EMT Standard stock solution (400 µg/ml)

Standard solution 40mg EMT of drug was dissolved in 100ml Buffer (pH 3.5): Methanol (30:70).

Take 1ml of standard stock solution was transferred to 10ml volumetric flask and volume was made up with the Diluent **(**EMT 40µg/ml).

TEN Standard stock solutions (50 µg/ml)

Standard solution5mgTENof drug was dissolved in 100ml Buffer (pH 3.5): Methanol (30:70) Take 1ml of standard stock solution was transferred to 10ml volumetric flask and volume was made up with the Diluent **(**TEN 5µg/ml)

METHOD VALIDATION

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 50 µg/ml of TEN and 400 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.

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.

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 7.5µg/ml of Tenofovir Alfenamide.

An aliquot of 20µl of each solution was injected under operating chromatographic condition. Plot the calibration curve of area versus respective concentration and find out correlation co-efficient and regression line equation for EMT and. TEN Each response was an average of five determinations.

PRECISION

Intraday precision (n=3)

Intraday precision was determined by analyzing of EMT and TEN standard solution in the range EMT (20, 40, and 60 µg/ml) & TEN (2.5, 3.75 and 7.5 µg/ml) were analysed on three times on same day and % RSD was calculated.

Interday precision (n=3)

Interday precision was determined by analyzing of EMT and TEN standard solution in the range EMT (20, 40, and 60 µg/ml) & TEN (2.5, 3.75 and 7.5 µg/ml) were analysed on three different successive and % RSD was calculated.

Repeatability (n=6)

Repeatability 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.

ACCURACY (N=3)

The accuracy of the method was determined at 50%, 80% and 120% 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.

LIMIT OF DETECTION AND LIMIT OF QUANTITATION

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.

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)

Flow rate: 0.9ml/min and 1.1ml/min. Data of robustness are shown.

2) Different pH
3) Different Mobile phase

RESULT AND DISCUSSION

The analytical method was found to be specific as there was no interference of any excipients or impurities which can be shown from figure 3 -6. Overlay of linearity was shown in figure 7 and regression coefficient was found to be 0.9994 and 0.9992 which is shown in figure 8 and 9 calibration data are shown in table 1 and 2 and regression data is shown in table 3. The %RSD for repeatability was found to be 1.45 and 1.302% for Emtricitabine and Tenofovir Alfenamide as mentioned in table 4 and 5. The %RSD for intraday precision was found to be 0.80-0.95% of Emtricitabine mentioned in table 6.and 0.63-1.09% for Tenofovir Alfenamide

mentioned in table 7. The %RSD for Interday precision was found to be 1.11-1.46% of Emtricitabine mentioned in table 8 and 1.30-1.34% of Tenofovir Alfenamide mentioned in table 9.

Mean percentage recovery of Emtricitabine and Tenofovir Alfenamide were found to be in range of 100.02-101.43% and 99.32-100.83% mentioned in table 10 and table 11. The % RSD for robustness was found to be 0.17-0.78% and 0.44-1.78 % for Emtricitabine and Tenofovir Alfenamide as mentioned in table 12 and table 13.

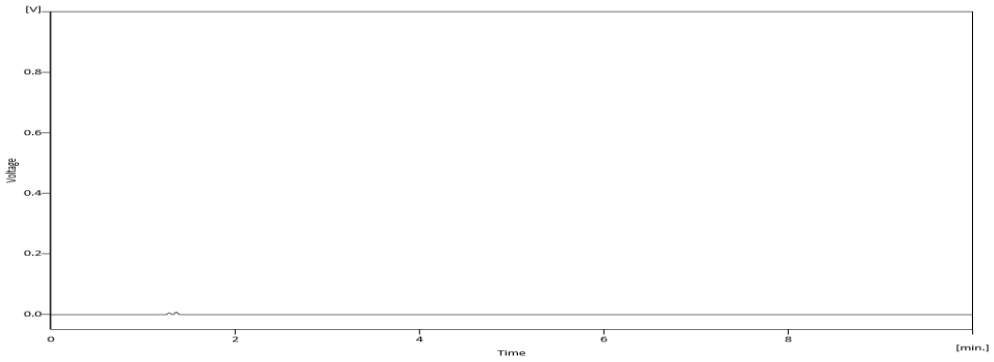

Figure 3: Chromatogram of blank.

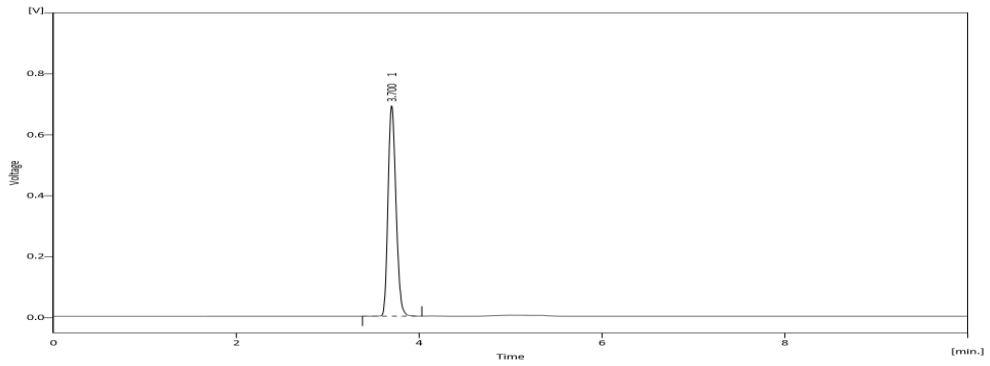

Figure 4: Chromatogram of standard solution of EMT.

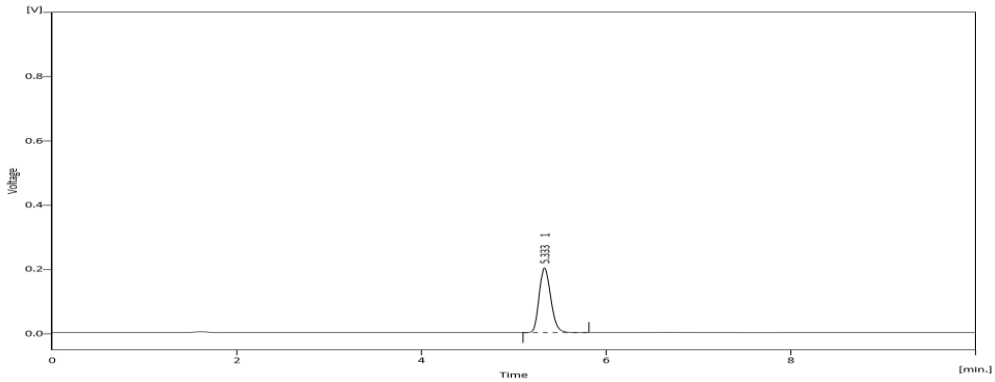

Figure 5 Chromatogram of standard solution of TEN.

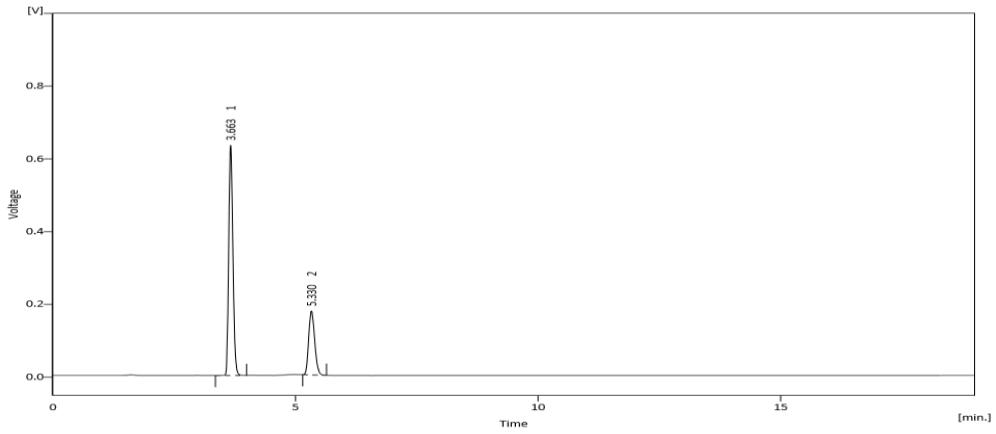

Figure 6: Chromatogram of standard solution of EMT and TEN.

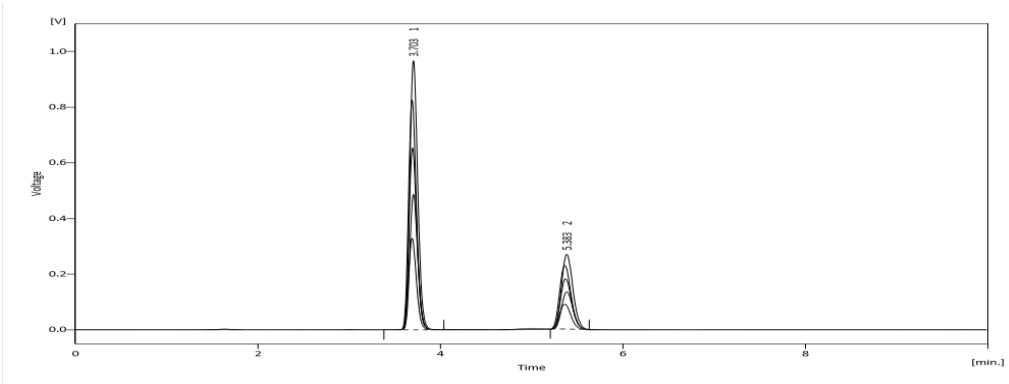

Figure 7: Linearity overlay spectra of EMT and TEN.

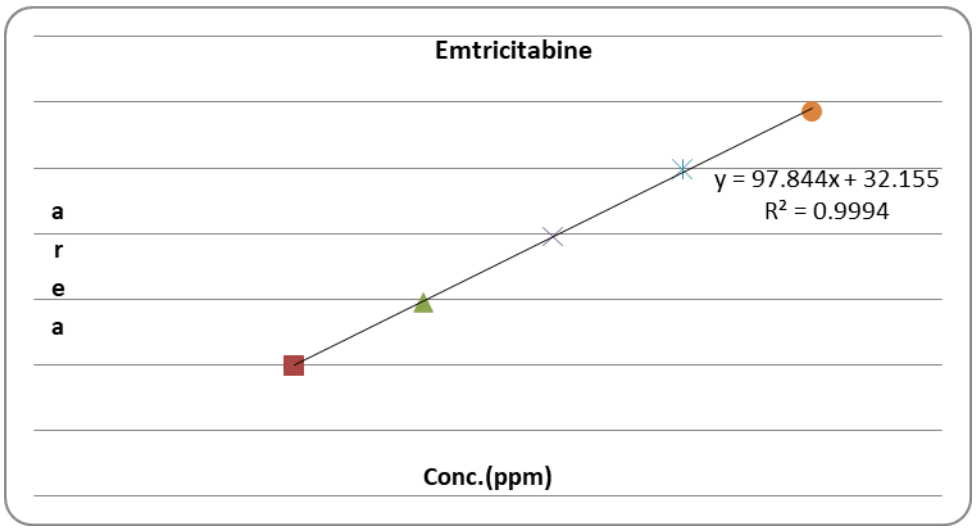

Figure 8: Calibration curve of EMT for HPLC.

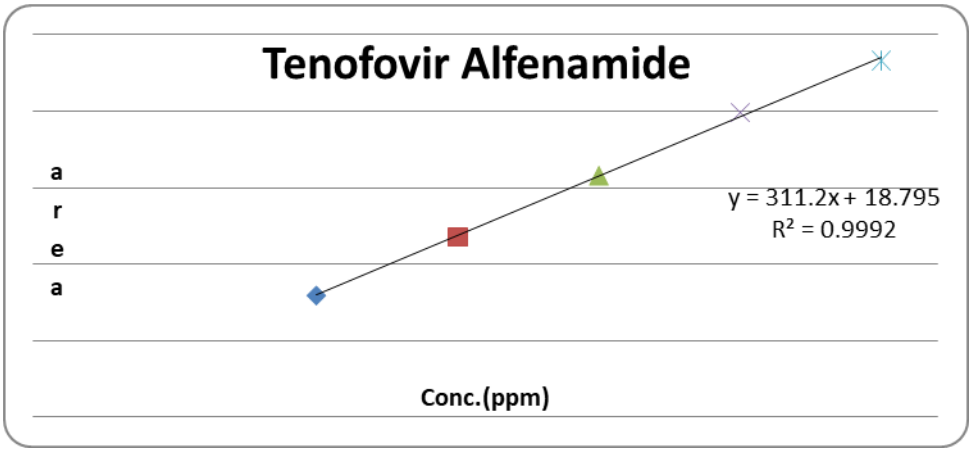

Figure 9: Calibration curve of TEN for HPLC.

Table 1: Calibration data for EMT at 262 nm.

Conc. (mcg/ml)	Mean Response
20	1986.60
30	2948.48
40	3949.97
50	4982.79
60	5861.63

Table 2: Calibration data for TEN at 262 nm.

Conc. (mcg/ml)	Mean Response
2.5	793.88
3.75	1178.23
5	1578.42
6.25	1991.01
7.5	2332.51

Table 3: Data of regression analysis of EMT & TEN.

Drug	Straight line equation of Calibration curve	Correlation Coefficient
Emtricitabine	Y = 97.844x+ 32.155	0.9994
Tenofovir	Y = 311.2x+ 18.795	0.9992

OBSERVATION

A method is linear is a range of 20-60 mcg/ml and 2.5-7.5 mcg/ml of Emtricitabine and Tenofovir Alfenamide of standard solution.

A correlation coefficient for Emtricitabine is 0.9994 and Tenofovir Alfenamide is 0.9992. the areas obtained were directly proportional to the concentration of analyte in the sample. The method can, therefore be termed as linear in the specified range.

Precision

Repeatability

The repeatability studies carried out by measuring response for a single concentration for 6 times a day.

Intraday precision

Intraday precision was performed by analyzing three different concentrations within linearity range, three times in a day (3*3 determination).

Table 4: Repeatability data for Emtricitabine.

Conc of Emtricitabine (mcg/ml)	Area (n=6)
40	3890.104
	3838.764
	3881.034
	3927.668
	3966.982
	3990.65
Mean	3915.867
SD	56.8620199
% RSD	1.45

Table 5: Repeatability data for Tenofovir Alfenamide.

Conc of Tenofovir Alfenamide (mcg/ml)	Area (n=6)
5	1554.488
	1533.86
	1550.72
	1569.501
	1585.177
	1585.919
Mean	1563.278
SD	20.64542
% RSD	1.32

Table 6: Data for Intraday Precision for Emtricitabine.

Emtricitabine		
Conc. (mcg/ml)	Mean response	% RSD
20	1989.802	0.80
40	3922.067	0.95
60	5970.083	0.85

Table 7: Data for Intraday Precision for Tenofovir.

Tenofovir Alfenamide		
Conc. (mcg/ml)	**Mean response**	**% RSD**
2.5	794.21	0.65
5	1565.78	1.09
7.5	2382.10	0.63

Interday precision (n=3)

Interday precision was performed by analyzing three different concentrations within linearity range on different days.

Table 8: Data for Interday Precision for Emtricitabine.

Emtricitabine		
Conc. (mcg/ml)	**Mean response**	**% RSD**
20	1920.774	1.41
40	3908.014	1.46
60	5872.962	1.11

Table 9: Data for Interday Precision for Tenofovir.

Tenofovir		
Conc. (mcg/ml)	**Mean response**	**% RSD**
2.5	768.099	1.31
5	1562.963	1.34
7.5	2343.988	1.30

Observation

Repeatability -The % RSD was found to be 1.45%for Emtricitabine and 1.32% for Tenofovir Alfenamide. % RSD value was found to be less than 2.0 indicate that the method is precise.

Intraday- The % RSD was found to be 0.80-0.95% for Emtricitabine and 0.63-1.09% for Tenofovir Alfenamide. % RSD value was found to be less than 2.0 indicate that the method is precise.

Interday- The % RSD was found to be 1.11-1.46 % for Emtricitabine and 1.30-1.34 % for Tenofovir Alfenamide. % RSD value was found to be less than 2.0 indicate that the method is precise.

Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation at three

levels (80%, 100%, and 120%) of standard addition.

Table 10: Determination of accuracy of Emtricitabine.

Amount of EMT present(mcg/ml)	% Amount of std EMT added	Total amount of EMT present(mcg/ml)	Amount recovered mean (mcg/ml)	SD n=3	% Recovery
40	80	20	15.99	1.2243	100.02
	100	20	20.28	1.1146	101.43
	120	20	24.14	0.9952	100.61

Table 11: Determination of accuracy of Tenofovir Alfenamide.

Amount of TEN present(mcg/ml)	% Amount of std TEN added	Total amount of TEN present (mcg/ml)	Amount recovered mean (mcg/ml)	SD n=3	% Recovery
5	80	2.5	1.98	1.25	99.32
	100	2.5	2.51	1.89	100.80
	120	2.5	3.02	1.38	100.83

To develop method and validation for estimation of Tenofovir Alfenamide Hemifumarate in bulk and Simultaneous estimation of Tenofovir Alfenamide and Emtricitabine in combined tablet dosage form and to validate the developed method as per ICH guideline Q2 (R1).

Robustness

Change in flow rate, pH and Mobile phase.

Table 12: Data of Robustness for EMT.

			Mean	% RSD
EMT (40mcg/ml)	**Flow Rate**	0.9ml/min	4032.572	0.41
		1.1ml/min	3851.567	0.51
	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

Table 13: Data of Robustness for TEN.

			Mean	% RSD
TEN (5mcg/ml)	**Flow Rate**	0.9ml/min	1603.228	0.59
		1.1ml/min	1539.024	0.50
	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

SUMMARY OF VALIDATION PARAMETER

Table 14: Summary of validation parameters.

Parameter	EMT	TEN
Linearity range (n=5)	20-60mcg/ml	2.5-7.5mcg/ml
Accuracy (%)	100.02-100.61	99.32-100.83
LOD (mcg/ml)	4.80	0.11
LOQ (mcg/ml)	14.57	0.33
Repeatability (n=6) %RSD	1.45	1.32
Intraday (n=3) %RSD	0.80-0.95	0.63-1.09
Interday (n=3) %RSD	1.11-1.41	1.30-1.34
Robustness(% RSD)	0.17-0.78	0.44-1.78

CONCLUSION

The method was found to be simple, specific, accurate, economic and responsible. Method can be successfully applied for routine QC analysis. It reveals that RP-HPLC method was validated as per ICH guideline Q2R1 as all validation parameters were found within range.

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