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Development and Validation of Stability Indicating RP-HPLC Method for Quantitative Estimation of Emtricitabine and Its Impurities in Pharmaceutical Dosage Form as Per ICH Guidelines

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ABSTRACT

The analysis of the improved RP-HPLC method for the separation and quantification of Emtricitabine and its impurities are described. Samples are analyzed utilizing reverse phase (RP-HPLC) using a Waters X-Bridge C18(250 x 4.6 mm, 5µm)and the mobile phase consists of two Channels A and B. Channel-A pH 4.0 buffer: Acetonitrile (960:40 %v/v) and Channel-B: Acetonitrile. The flow rate is 0.8 ml/min. The column temperature was maintained at 40°C and sample temperature was maintained at ambient (25°C)and wavelength fixed at 265nm UV-detection. It is found that the method of RP-HPLC with UV-detection system for the analysis of Emtricitabine impurities is straightforward and applied in qualitative and quantitative analysis. The developed LC method was validated concerning specificity, precision, linearity, ruggedness, and robustness. Validation study compared as per ICH guideline.

1. INTRODUCTION

Emtricitabine [1-3] is a novel drug used in combining fixed doses of the nucleoside reverse transcriptase inhibitor tenofovir disoproxil fumarate with the non-nucleoside reverse transcriptase inhibitor efavirenz represents the first once-daily, one-tablet antiretroviral regimen. Emtricitabine is chemically 5-fluoro-1-(2R, 5S)-[2-hydroxymethyl)-1,3-oxathiolan-5-ylcytosine [2]. The chemical structure of Emtricitabine is shown in **Figure: 1.1.**

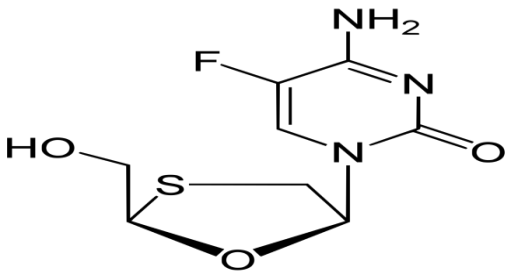

Figure: 1.1. Chemical Structure of Emtricitabine

Literature survey reveals few chromatographic methods [4-9] for the determination of Emtricitabine in biological fluids along with other antiretroviral drugs like tenofovir disoproxil and efavirenz. So far, only one HPLC procedure has been reported in the gradient mode for the estimation of tenofovir disoproxil, Emtricitabine, and efavirenz from the pharmaceutical dosage form. The availability of an HPLC method with high sensitivity and selectivity will be useful for the determination of Emtricitabine in pharmaceutical formulations. The study aims to develop a simple, precise, and accurate reversed-phase HPLC method for the estimation of Emtricitabine in bulk drug samples and pharmaceutical dosage form.

Impurity profiling of active pharmaceutical ingredients (API) in both bulk material and formulations is one of the most challenging tasks. The presence of unwanted or in certain cases unknown chemicals, even in small amounts, may influence not only the therapeutic efficacy but also the safety of the pharmaceutical products. For these reasons, all major international pharmacopeias have established maximum allowed limits for related compounds for both bulk and formulated APIs. As per the requirements of various regulatory authorities, the impurity profile study of drug substances and drug products has to be carried out using a suitable analytical method in the final product.

2.0 EXPERIMENTAL

2.1 Reagents and chemicals

Ammonium acetate, Methanol, and acetonitrile were procured from Merck. Milli-Q water. All chemicals were of an analytical grade and used as received.

2.2 Instrumentation

Chromatographic separation was achieved by using an Agilent-1200, Open-lab software using, Waters X-Bridge C18, 250 x 4.6 mm, 5µm column with Channel-A pH 4.0 buffer: Acetonitrile (960:40 %v/v) and Channel-B: Acetonitrile. The flow rate is 0.8 ml/min. The column temperature was maintained at 40°C and sample temperature was maintained at ambient (25°C) and wavelength fixed at 265nm UV-detection. The overall run time was 28 minutes. 20 µl of the sample was injected into the HPLC system. Retention times of impurities were 9.723 for PMPA anhydride, 13.412 for 5-Fluoro uracil, and 10.75 for Emtricitabine.

2.3 Preparation of mobile phase and standard and sample solution:

Preparation of Buffer:

Weighed accurately 3.85g of ammonium acetate transferred into a 1000mL beaker dissolved and diluted to volume with 1000mL milli-Q water. Adjusted the pH to 6.50 with dilute orthophosphoric acid.

Mobile phase-A:

Transferred 960mL of ammonium acetate buffer and 400mL of acetonitrile into 1000mL beaker mixed well. Filtered through 0.45µ membrane filter and degas.

Mobile phase-B:

Acetonitrile

Preparation of diluent:

Mixed accurately ammonium acetate buffer and methanol in the ratio of (50:50 v/v).

Preparation of Standard stock solution:

Weighed accurately and transferred 50mg of Emtricitabine standard into a 100ml volumetric flask, added 50 ml of diluent sonicated to dissolved and diluted to the volume with diluent and mixed well.

Preparation of Standard solution:

Transferred 2.0 mL of the standard stock solution into a 250ml volumetric flask, added 100 ml of diluent and mixed well, and diluted to the volume with diluent and mixed well.

Preparation of PMPA Anhydride stock solution:

Weighed accurately and transferred 1mg of PMPA standard into a 25ml volumetric flask, added 15 ml of diluent sonicated to dissolved and diluted to the volume with diluent and mixed well.

Preparation of 5-Fluoro uracil analog stock solution:

Weighed accurately and transferred 1mg of 5-Fluoro uracil analog standard into a 10ml volumetric flask, added 5 ml of diluent sonicated to dissolved and diluted to the volume with diluent and mixed well.

Preparation of System suitability solution:

Weighed accurately and transferred 50mg of Emtricitabine standard into a 25ml volumetric flask, added to it 1.0 mL of each PMPA Anhydride stock solution and 5-Fluoro uracil analog stock solution mixed well and made up to the volume with diluent.

Preparation of Test solution:

Weighed 20 tablets to determined average weight and crushed the tablets to a fine powder using mortar and pastel. Accurately weighed and transferred powder equivalent to 50 mg of Emtricitabine into a 200 mL volumetric flask then added 100.0 mL of diluent. Sonicated for 20 minutes with intermediate shaking. Maintain the sonicate bath temperature below 25°C throughout the sonication and diluted to the volume with diluent and mixed well. Filtered the sample solution through 0.45µ PVDF filter and injected into HPLC system.

3.0 RESULTS AND DISCUSSION

3.1 Method optimization parameters

An understanding of the nature of API (functionality, acidity, or basicity), the synthetic process, related impurities, the possible degradation pathways, and their degradation products are needed for successful method development in reverse-phase HPLC. In addition, successful method development should result in a robust, simple, and time-efficient method that is capable of being utilized in a manufacturing setting.

3.2 Selection of wavelength

The sensitivity of the HPLC method depends upon the selection of detection wavelength. An ideal wavelength gives a good response for related substances and the drugs to be detected. The wavelength for measurement was selected as 265 nm from the absorption spectrum.

3.3. Selection of stationary phase

Proper selection of the stationary phase depends on the nature of the sample and chemical profile. The drug selected for the present study was a polar compound and could be separated either by normal phase chromatography or reverse-phase chromatography. From the literature survey, it was found that different C18columns could be appropriately used for the separation of related substances for Emtricitabine.

3.4. Selection of mobile phase

Different mobile phase and stationary phases were employed to develop a suitable LC method for the quantitative determination of impurities in Emtricitabine. Several column chemistries supplied by different manufacturers and different mobile phase compositions were tried to get good peak shapes and selectivity for the impurities present in Emtricitabine.

4.0 METHOD VALIDATION

4.1 Specificity

Blank and placebo interference

A study to establish the interference of blank was conducted. Diluent was injected as per the test method.

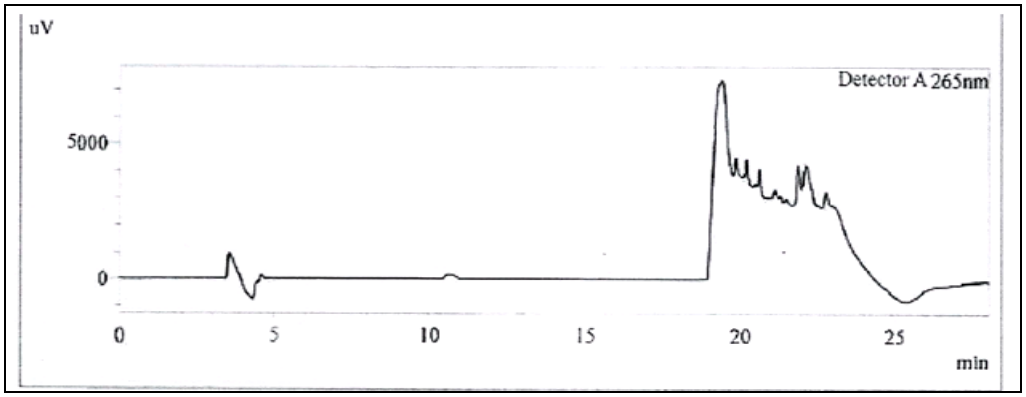

Figure: 1.2 typical chromatograms of Blank

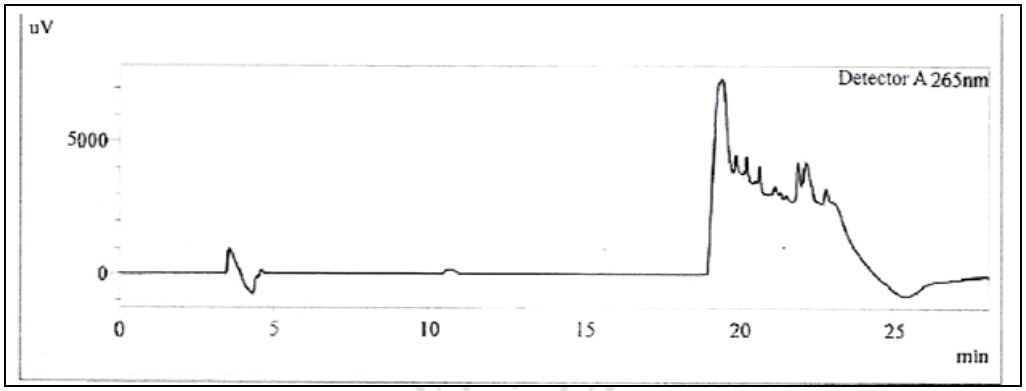

Figure: 1.3 typical chromatograms of Placebo

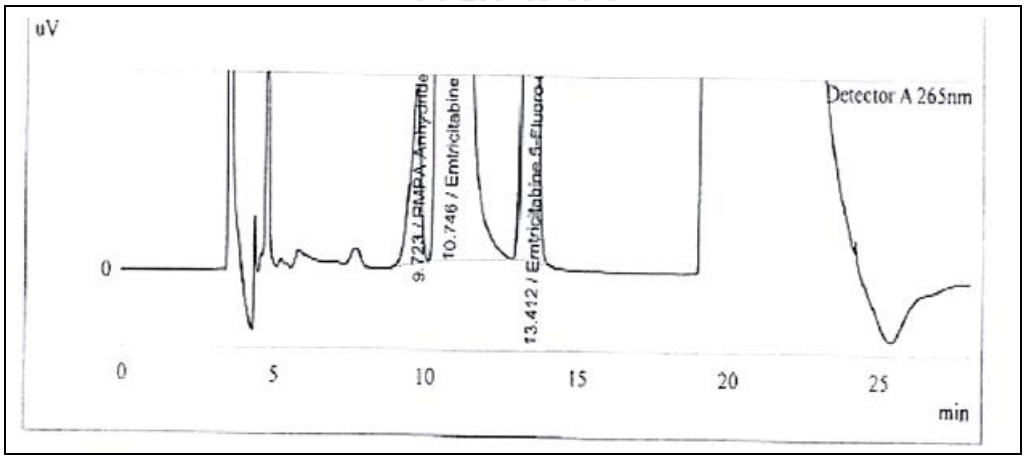

Figure: 1.4 typical chromatogram System suitability solution

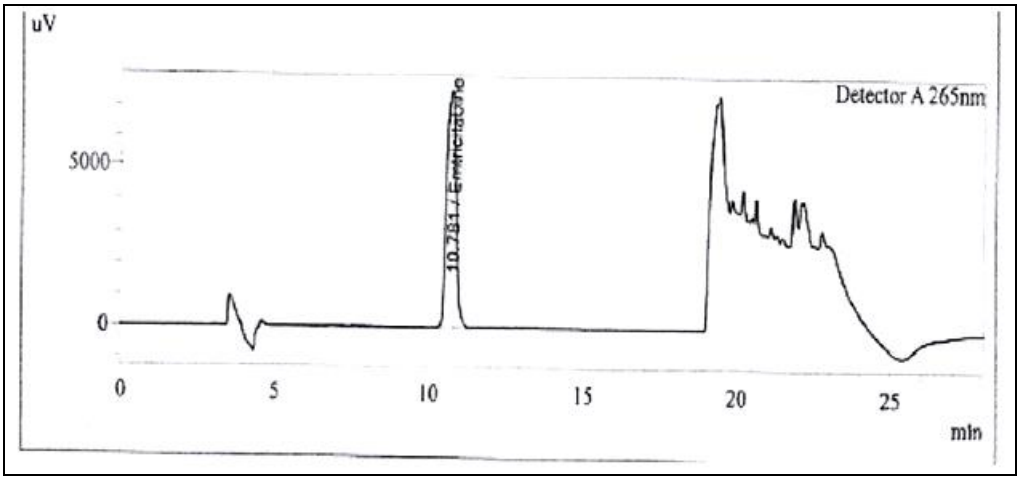

Figure: 1.5 typical chromatogram Standard solution

Table: 1.1 System suitability data of Emtricitabine

System suitability parameters	Results	Acceptance criteria
Resolution between PMPA Anhydride impurity and Emtricitabine peaks	1.92	Should be not less than 1.5
Resolution between Emtricitabine and 5-Fluoro uracil analog impurity peaks	4.47	Should be not less than 1.5
%RSD for peak areas of Emtricitabine from six replicate standard injections	0.04	Should be not more than 10
Tailing factor for Emtricitabine peak	1.0	Should be not more than 2.0

Table: 1.2 Impurity interference data

Peak Name	Blank	Placebo
PMPA Anhydride impurity	No	No
5-Fluoro uracil analog impurity	No	No
Emtricitabine	No	No

It was observed that known impurities are not co-eluting with each other and the main analyte peak. Emtricitabine standard solution preparation and spiked test preparation were calculated and found to be within the acceptable limit.

4.2 Precision

4.2.1 System precision

Perform the analysis of reference solution (Diluted standard) six times and determine the percentage relative standard deviation of peak area of replicate injections of Emtricitabine.

Table: 1.3 System Precision data for Emtricitabine

Injection No	Emtricitabine
1	159298
2	159285
3	159124
4	159187
5	159250
6	159286
Mean area	**159238**
SD	**69.07**
%RSD	**0.04**

The %RSD of peak area for Emtricitabine was found to be 0.04% which is below 10.0% indicates that the system gives precise results.

4.2.2 Method Precision

The precision of the impurities and degradants method was determined by injecting six sample solutions spiked with impurities (PMPA Anhydride impurity and 5-Fluoro uracil analog impurity) at the specification level. The samples were prepared as per the method and the result for the precision study is tabulated in **Table: 1.4.**

Table: 1.4 Results of method precision

No. of Preparations	PMPA Anhydride impurity	5-Fluoro uracil analog impurity
1	0.23	3.80
2	0.24	3.83
3	0.23	3.84
4	0.25	3.84
5	0.23	3.84
6	0.22	3.82
Mean (%)	0.23	3.83
% RSD	**4.43**	**0.4**

The method precession was performed with spiked sample solutions six replicates and the results were found within the acceptance criteria.

4.3 Limit of Quantitation (LOQ)& Limit of detection (LOD)

A solution containing 1.0276 µg/ml of Emtricitabine standard, 0.1247 µg/ml of PMPA Anhydride impurity, and1.0410 µg/ml of 5-Fluoro uracil analog impurity was injected six times.

Table: 1.5 LOQ for Emtricitabine and impurities

Component Name	**Area**	**S/N**
Emtricitabine	40564	12.03
PMPA Anhydride impurity	4155	12.87
5-Fluoro uracil analog impurity	55076	13.23

Asolutioncontaining0.3114 µg/mL of Emtricitabine standard,0.0378µg/ml of PMPA Anhydride impurity, 0.3155 µg/mLof5-Fluoro uracil analog impurity was injected three times.

Table: 1.6 LOD for Emtricitabine and impurities

Component Name	Area	S/N
Emtricitabine	12290	4.01
PMPA Anhydride impurity	1289	3.90
5-Fluoro uracil analog impurity	16690	4.01

The limit of quantitation and limit of detection values obtained for each impurity and Emtricitabine are within the acceptance criteria.

4.4 Linearity and Range

The linearity is determined by injecting the solutions in duplicate containing known impurities and Emtricitabine and impurities ranging from LOQ to 200% of the specified limit. Perform the regression analysis and determine the correlation coefficient and residual sum of squares. Determine the response factor for each impurity concerning Emtricitabine. Report the linearity range as the range for determining the impurities. Results obtained are in the tables & figures show the line of best fit for peak area versus concentration for each impurity.

Table: 1.7 Linearity of detector response Emtricitabine

Level	Concentration (ppm)	Mean Area
LOQ	1.0276	40564
50	2.1409	82741
100	4.2818	169747
150	6.4226	251762
200	8.5635	333732
Correlation coefficient		0.9999
R^2 Value		0.9998
% Y-intercept		0.35
Slope		39026.3406
Intercept		587.0824

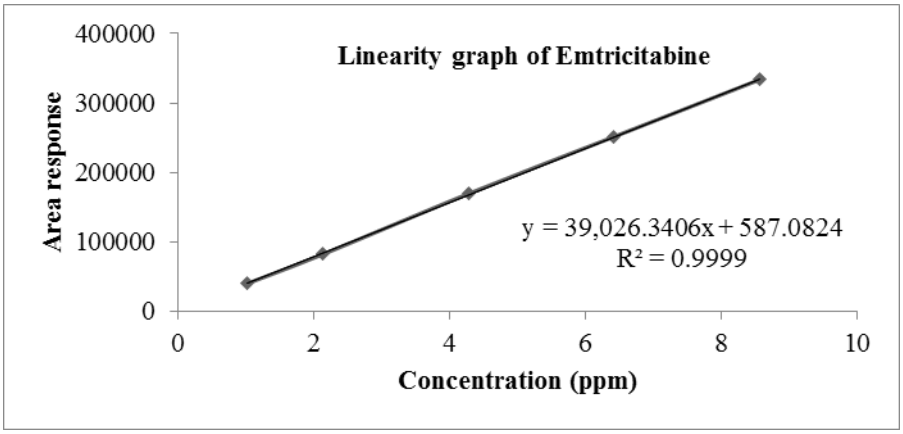

Figure: 1.6 linearity of detector response for Emtricitabine

Table: 1.8 Linearity of detector response PMPA Anhydride impurity

Level	Concentration (ppm)	Mean Area
LOQ	0.1247	4155
50	3.8966	134542
100	7.7932	281173
150	11.6898	444168
200	15.5864	567886
Correlation coefficient		0.9990
R^2 Value		0.9985
% Y-intercept		-1.400
Slope		37120.9108
Intercept		-3831.6773

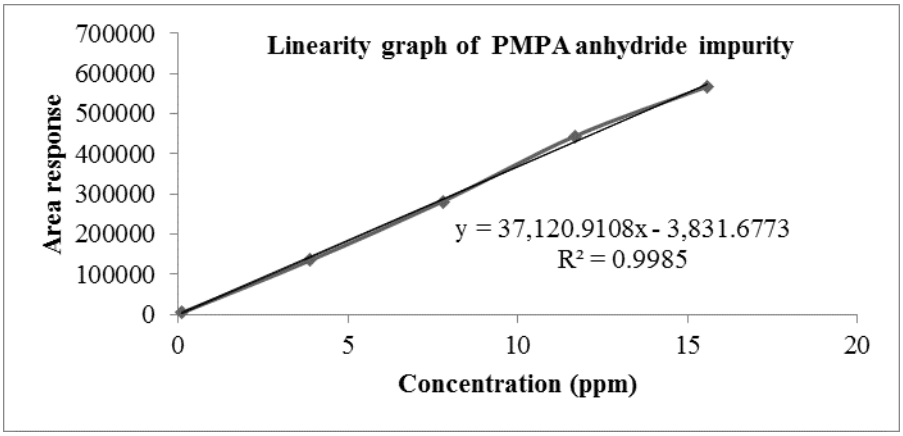

Figure: 1.7 linearity of detector response for PMPA anhydride impurity

Table: 1.9 Linearity of detector response 5-Fluoro uracil analog impurity

Level	Concentration (ppm)	Mean Area
LOQ	1.0410	55076
50	2.0332	107372
100	4.0663	216141
150	6.0995	318755
200	8.1327	428316
Correlation coefficient		1.000
R^2 Value		0.9999
Intercept		821.0157
% Y-intercept		0.38
Slope		52476.0522

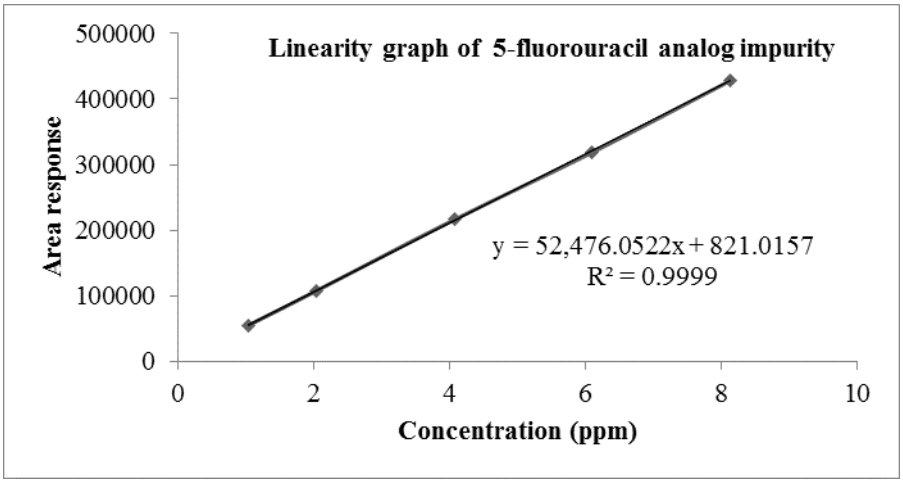

Figure: 1.8 linearities of detector response for 5-Fluoro uracil analog impurity

The linearity results for Emtricitabine and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.

4.5 Accuracy

Recovery of Emtricitabine impurities in Emtricitabine was performed. The sample was taken and varying amounts of Emtricitabine impurities representing 50 to 150 % of the specification level were added to the flasks. The spiked samples were prepared as per the method and the results are tabulated in **Table 1.10.**

Table: 1.10 Accuracy study of Emtricitabine

S.No.	% spike level	% Mean Recovery	
		PMPA anhydride impurity	5-Fluoro uracil analog impurity
1	LOQ	100.4	96.5
2	50	97.1	105.8
3	100	98.4	113.4
4	150	100.5	103.8

5.0 RESULTS AND DISCUSSION:

A simple, economic, accurate, and precise HPLC method was successfully developed. This method was carried out by using Waters X-Bridge C18, 250 x 4.6 mm, 5µm**.** An injection volume of 20µl is injected and eluted with the mobile phase-A: pH 4.0 buffer: Acetonitrile (960:40 %v/v) and mobile phase-B: Acetonitrile, which is pumped at a flow rate of 0.8 ml/min. Column temperature 40°C and sample temperature 25°C. Detection was carried out at 265 nm. The results obtained were accurate and reproducible. The method developed was statistically validated in terms of Selectivity, accuracy, linearity, precision, and stability of the solution.

For Selectivity, the chromatograms were recorded for standard and sample solutions of Emtricitabineand its related substances. Selectivity studies reveal that the peak is well separated from each other. Therefore, the method is selective for the determination of related substances in Emtricitabine. There is no interference of diluent at Emtricitabine and impurities peaks. The elution order and the retention times of Impurities and Emtricitabine obtained from individual standard preparations and mixed standard Preparations are comparable.

The limit of detection (LOD) and limit of quantitation (LOQ) for Emtricitabinestandard0.3114&1.0276µg/mL, PMPA anhydride impurity 0.0378 & 0.1247µg/mL and 5-Fluoro uracil analog impurity 0.3155 & 1.0410 µg/mL respectively.

The linearity results for Emtricitabineand all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99. Calibration curve was plotted and correlation co-efficient for Emtricitabineand its impurities were found to be 0.9999, 0.9990, and 1.000 respectively.

The accuracy studies were shown as % recovery for Emtricitabineand its impurities at the specification level. The limit of % recovered shown is in the range of 80 and 120% and the results obtained were found to be within the limits. Hence the method was found to be accurate.

For Precision studies six (6) replicate injections were performed. %RSD was determined from the peak areas of Emtricitabineand its impurities. The acceptance limit should be no more than 10, and the results were found to be within the acceptable limits.

Hence, the chromatographic method developed for Emtricitabineand its related substances is rapid, simple, sensitive, precise, and accurate. Therefore, the proposed method can be successfully applied for the routine analysis of the active pharmaceutical ingredients for assurance of its quality during its formulation.

6.0. CONCLUSION

The new HPLC method was developed and validated for the determination of Emtricitabinepharmaceutical dosage forms and assured the satisfactory precision and accuracy and also determining the lower concentration of drug in its solid dosage form by RP-HPLC method. The method was found to be simple, accurate, economical, and rapid and they can be applied for routine analysis in laboratories and is suitable for the quality control of the raw materials.

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