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Research

Design Chromatographic method for Emtricitabine assay using Gradient Phase by RP-HPLC method

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Abstract

A stability indicating RP-HPLC method has been developed for quantitative determination of Emtricitabine, in Pharmaceutical dosage forms. Chromatographic separation was attained through gradient elution. Detection wavelength was monitored at 260 nm. The retention times of the Emtricitabine, Tenofovir disoproxil fumarate and Efavirenz was about 4.7min respectively. The developed method was validated as per ICH guidelines. This technique is found to be simple, fast and economical Hence this validated technique method can be used in routine quality testing of individual dosage forms and combination dosage forms of Emtricitabine, Tenofovir disoproxil fumarate and Efavirenz.

Keywords: RP-HPLC, Emtricitabine, Gradient chromatography, Validation

INTRODUCTION

Design chromatographic method for Emtricitabine assay by HPLC method using isocratic elution Scope of the study is to designed Isocratic chromatographic method for Emtricitabine assay by HPLC method and validates assay parameter by linearity, precision, accuracy and solution stability parameter by HPLC method. An estimate the drug content in these multicomponent formulations, several UV spectroscopy and HPLC methods have been advanced and validated as per the available literature. UV spectrophotometry and HPLC are among the most critical tools in the analysis of drugs in pharmaceutical manufacturing formulations.
The QbD based optimization approach applies to developed the simultaneous estimation method, which could be designed with predefined objectives that emphasize the product and process to maintain the desired quality. The QbD approach followed the guidelines mentioned in ICH Q8 (R2).

Above enhanced analytical method was exposed for validation to check its Specificity, Precision, Accuracy, Linearity and Robustness. The principal determination of analytical validation was to confirm that a selected analytical procedure will give reproducible and reliable results that are adequate for the intended purpose. Validation activity was strategic as per the International Conference on Harmonization (ICH) guidelines (Guideline I. H. T., 2010) Procedures, experimental plan and acceptance criteria were followed as per the general industrial practices

Experiment Requirements

Requirement: Chemical, Reagent, Placebo and Standards

Sr.No	Requirement		Mechanism Use
1.	Water	Reagent	**Solvent**
2.	Trifluoracacetic acid	Chemical	buffering agent- Lower the pH
3.	Ortho Phosphoric acid	Chemical	If the pH of the mobile phase needs to be increased to enhance LC separations then ammonium hydroxide (ammonia solution) is suitable.
4.	Triethlyamine	Chemical	a competing base for the retention control and peak shape improvement
5.	Methanol	Reagent	Lower boiling point, high solubility and low toxicity.
6.	Emtricitabine	Reference standard	Active material with potency
7.	Emtricitabine Placebo	Product Placebo	Mixture of excipient in product
8.	Efavirenz , Emtricitabine and Tinifocir sisoproxil tablets 600mg /200mg/245 mg tablets	Tablets	Finished product

Requirements: Instruments, Equipment, Glassware and Apparatus

Sr.No	Requirement		Identification number
1.	HPLC	Agilent	QC-HPLC-007
2.	Analytical Balance	Sartorius	QC-BAL-002
3.	pH Meter	Lab India	QC-pH-001
4.	Column	Inertsil	Inertsil ODS -3V 50 X 4.6 mm, 5 micron
5.	Detector	NA	UV, 260nm
6.	0.45 nylon membrane filter	Sartorius	membrane filter
7.	Glassware	Type A grade	Beaker, volumetric flask, pipette etc.

Design of Experiment –DOE by different trails by Reverse Phase -HPLC Method

Selection of Chromatographic System

Degradation studies were carried out on a system consisted of 1200 series HPLC (Agilent Technologies) comprising of an on-line degasser (G1322A), binary pump (G1312A), auto injector (G1367C), column oven (G1310B), DAD detector (G1315C) and E Z Crome Elite (software).

The published methods of analysis for determination and separation of Emtricitabine in their formulation were not evaluated for specificity and degradation study. Therefore, method having specificity for degradation products and formulation excipients is considered as a prime requirement. Degraded samples, prepared by systematic forced degradation study, were used for method development trials to optimize the method as a stability indicating Emtricitabine method for determination of

Selection of Buffer in Mobile Phase

0.05M Phosphate buffer pH2.0 with orthophosphoric acid was used to optimize the peak shape retention time and to proper separation of impurities peaks from main drugs peaks. The ratio of (Buffer: Methanol: Acetonitrile) was selected on the basis of resolution between the major degradation peaks and main peaks, and it was finalized as (50:30:20) v/v after analyzing all the degraded samples and evaluating the Peak purity, resolution, specificity and stability indicating nature of the method.

Selection of Mobile Phase

Different ratios of Acetonitrile and Methanol was used to optimize the retention time of late eluting impurities and Methanol to proper separation of impurities peaks from main drugs peaks. The ratio of (Buffer: Methanol: Acetonitrile) was selected on the basis of resolution between the major degradation peaks and main peaks, and it was finalized as (Buffer: Methanol: Acetonitrile) [50:30:20 v/v] after analyzing all the degraded samples and evaluating the peak purity, resolution, specificity and stability indicating nature of the method.

Selection of HPLC Column

For HPLC, various columns are available, but as the main aim of the method to resolve the compound in the presence of polar and non-polar degradation products and impurities, a C_{18} column was preferred over other columns O ODS MG, 50 X 4.6mm, 5 µm or equivalent column was chosen to give good peak shape, good lifetime and high resolution on compared to other C_{18} columns.

Selection of Diluent / Solvent for extraction:-

Different solvents were tried including single solvent and combination of solvents like ACN: Water, Methanol: Water, in different concentrations, But Emtricitabine tablet gets dissolved in Methanol. Hence first stock was prepared in methanol and followed by second dilution done in diluents as [Methanol: Acetonitrile: Buffer 30:20:50] same as that of mobile phase to reduce the peak shape related problems.

The results of all validation parameters are given in following tables and all lie well within the limit of acceptance criteria.

Chromatographic Method

The chromatographic methods for the determination of assay tablet for validate the parameter Specificity and System suitability, linearity, precision, precision, intermediate precision, accuracy, range stability of solution and Robustness.

Equipment - Instrument –Glassware-Standard –Solvent-Chemicals Requirement

Equipment and Instrument

All equipment and instrument used during method validation shall be qualify, validate and within calibration and preventive maintenances validity.

Glassware

All Class A types' glassware shall be used. Before used it shall be cleaned and dried as per validated and approved procedure.

Standards

Reference standards of Emtricitabine which has purity 99.99 %

Solvent and Chemicals Used during analysis

Sr.No	Chemical	Source	Grade	Batch No	Purity
1.	Water	Merck	HPLC grade	PE2561	NA
2.	Potassium Di hydrogen phosphate	Merck	GR Grade	HRU232436	NA
3.	Orthophosphoric acid	Merck	GR Grade	QD25648	NA
4.	Triethlymine	Merck	AR Grade	REI24362	NA
5.	Methanol	Finer	HPLC grade	MEL230823	NA
6.	Acetonitrile	Finer	HPLC grade	OA234531	NA
7.	Emtricitabine standard	USP	Pharmacopeia	RS/24/003	99.3%
8.	Emtricitabine placebo	In-house	In-house	----	NA
9.	Emtricitabine 200mg	In-house	In-house	TELMI24015	NA

Methodology to be adopted for design

A. Specificity and System Suitability
   - Identification
   - Blank interference of the Experiment
   - System Suitability

B. Linearity and Range

C. Precision
   - System Precision
   - Method Precision

D. Accuracy

E. Solution Stability
   - System Stability
   - Solution stability

F. Robustness
   - Change in wave length

- Filter variability

- Linearity will be performed from about 50% to 150 % of target concentration
- Accuracy will be performed from about 50% to 150 % of stock solution.
- The following experimental design is drawn in order to prove test method is capable to yield consistent, reproduction results within the pre-determine acceptance limits.
- Acceptance criteria design is drawn validation parameters are specified in individual experimental design.
- Observations and results are recorded individual method validation data sheet

Methodology

Chemical and Reagent

Sr.No	Chemical	Source	Grade	Batch No	Purity
1.	Water	Merck	HPLC grade	PE2561	NA
2.	Potassium Di hydrogen phosphate	Merck	GR Grade	kRU232436	NA
3.	Orthophosphoric acid	Merck	GR Grade	ND25648	NA
4.	Triethlymine	Merck	AR Grade	REI24362	NA
5.	Methanol	Finar	HPLC grade	MEL230823	NA
6.	Acetonitrile	Finar	HPLC grade	O5234531	NA
7.	Emtricitabine standard	USP	Pharmocopiea	RS/24/003	99.3%
8.	Emtricitabine placebo	In-house	In-house	----	NA
9.	Emtricitabine 80mg	In-house	In-house	TELMI24015	NA

Design Analytical method validation

G. Specificity and System Suitability
   - Identification
   - Blank interference of the Experiment
   - System Suitability

H. Linearity and Range

I. Precision
   - System Precision
   - Method Precision

J. Accuracy

K. Solution Stability
   - System Stability
   - Solution stability

L. Robustness
   - Change in wave length
   - Filter variability

Methodology of the Experiment

Chemical and Equipment: Acetonitrile HPLC grade, Water HPLC grade, Dehydrate alcohol

Method for the assay Preparation of mobile phase A : Mix water, Methanol and Trifluroacetic acid in the ratio of 90:10:0.05(%v/v/v)

Method for the assay Preparation of mobile phase B : Mix water, Methanol and Trifluroacetic acid in the ratio of 10: 90:0.05 (%v/v/v)

Preparation of Diluent: Prepare a mixture of dehydrated alcohol and water in the ratio 20:80 v/v

Chromatographic system: Analytical column: Inertsil ODS -3V, 150 mm X 4.6, 5 µm Flow rate 1.0 ml/min Detector 260 nm, Injection volume 20 µl, Run time: 10 minute Needle wash: Degasses mixture of methanol and water (800:200) Seal wash: Degasses mixture of methanol and water (200:800) Gradient Method.

Time (min)	% Mobile Phase A	% Mobile Phase A
0.01	95.0	5.0
4.00	95.0	5.0
6.00	55.0	45.0
15.00	40.0	60.0
16.00	0.0	100.0
20.00	0.0	100.0
22.00	95.0	5.0

25.00	95.0	5.0

Preparation of standard stock solution 1
Accurately weigh and transfer about 25 mg of Emtricitabine standard into 250 ml volumetric flask, dissolve in and dilute to volume with diluent.
Preparation of standard stock solution 2
Accurately weigh and transfer about 5 mg of Emtricitabine related compound a standard into 50 ml volumetric flask, dissolve in and dilute to volume with diluent.
Preparation of standard solution:
Transfer 5 ml of standard stock solution into a 20 ml volumetric flask, dilute to volume with dilute and mix
Preparation of the resolution solution
Transfer 5 ml of standard stock solution 1 and 5 ml of standard stock solution 2 into a 20 ml volumetric flask, dilute to volume with dilute to volume with diluent and mix
Chromatographic procedure
Inject 20 µl of diluent as a blank into the chromatographic system
- Inject 20 µl of resolution solution into the chromatographic system
- Inject 20 µl of five times standard into the chromatographic system
- Inject 20 µl of sample solution into the chromatographic system

Evaluation of System Suitability parameters
- The resolution between Emtricitabine and Emtricitabine related component A is not less than 3.0.
- The relative standard deviation for Emtricitabine peak area obtained from five replicate injections of standard solution is not more than 2.0%

Preparation of the sample solution
- Weight powder not less than 20 tablets. Transfer an accurately weighed portion of the tablets powder equivalent to 200mg of Emtricitabine into a 200 ml of volumetric flask, add about 150 ml diluent.
- Sonicate the solution for about 20 minute and then shake for about 20 minute
- Allow the solution cool to room temperature, dilute to volume with diluent and mix. Centrifuge a portion of the resulting solution at about 1500 rpm for about 5 minutes
- Dilute 5 ml of the supernatant into 200 ml volumetric flask and dilute to volume with diluent and mix.
- Inject 20 µl of sample solution into the chromatographic system

Method Parameter for the Validation
The main objective of method validation process is to prove that an analytical method is acceptable for its intended purpose. The necessity for laboratories to use fully validated methods is now universally accepted as a way to obtain reliable results. There are diverse documents for method validation including information about different performance parameters. The classical performance characteristics are accuracy limit of detection, precision, recovery, robustness, ruggedness, selectivity, specificity and trueness. Unfortunately, contradictory information is normally present among the method validation documents used by laboratories.

Specificity and System Suitability
- **Specificity** is the ability of the analytical method to distinguish between the analyte(s) and the other components in the sample matrix [13]. In case of an HPLC method, it is assured by complete separation of peak(s) of analyte(s) from other peaks originated from the sample matrix
- **The System Suitability Testing (SST)** is used to verify that an analytical method was suitable for its intended purpose the day the analysis was done. It is an essential parameter to ensure the quality of the method for correct measurements
  - This test is performed for the identification of analyte and placebo interference and peak purity of Analyte
  - For reagent, diluent preparation, buffer preparation, chromatographic condition, resolution solution, standard solution and sample proceed as per Methodology defined

Acceptance Criteria
Identification: The retention time of standard solution and sample solution should be comparable with respect to retention time
Placebo and Blank Interference: There should not be any interfering peak in the chromatogram obtained from blank solution and placebo solution at the retention time of analyte peak in the chromatogram obtained with the standard.
System Suitability: The resolution between Emtricitabine related compounds A is NLT 3.0.
The relative standard deviation for Emtricitabine peak areas obtained from five replica injection of standard solution is not more than2.0%

Identification

Results of identification	
Solution	Retention T
Standard solution- Emtricitabine	4.819 min
Test solution- Emtricitabine	4.819 min
Conclusion: RT of Emtricitabine obtained with standard and test sample are comparable. Hence method is specific	
Acceptance Criteria: The retention time of standard solution and sample solution should be comparable with respect to retention time	

Placebo Interference

Results of Placebo Interference
There are no interference peak observed due to place to at the retention time of Nevitapine peak. 3Hence method is specific
Acceptance Criteria: There should not be any interfering peak in the chromatogram obtained from blank solution and placebo solution at the retention time of analyte peak in the chromatogram obtained with the standard

System Suitability- Emtricitabine

Results of System Suitability	
Injection	**Peak Area**
1	2788665
2	2770412
3	2831567
4	2789371
5	2789130
Mean	2788629
% RSD	0.336
The resolution between Emtricitabine related compound A is 11.3Hence method is specific	
Acceptance Criteria: Resolution: The resolution between Emtricitabine related compounds A is NLT 3.0.	

Linearity

Linearity of a method is its ability to obtain test results that are directly proportional to the sample concentration over a given range. For HPLC methods, the linear relationship between detector response (peak area and height) and sample concentration is determined. The relationship can be demonstrated directly on drug substance by dilution of standard stock or by separate weighing of the sample components, using the proposed procedures
To demonstrate the linearity of analyst response from 50 % to 150 %of target concentration. Evaluate the range of method using the data from linearity, precision and accuracy studies

- For reagent, diluent preparation,, buffer preparation, chromatographic condition, resolution solution, standard solution and sample proceed as per Methodology defined
- Accurately weight and transfer about 25 mg of Emtricitabine anhydrous standard into a 250 ml volumetric flask, dissolved to volume diluent.

Linearity level	**Take stock solution**	**Dilute up to ml diluent**	**Concentration in ppm**
L1 (50%)	2.55	20	12.50
L2 (80%)	4.05	20	20.00
L3 (100%)	5.05	20	25.00
L4 (120%)	6.05	20	30.00
L5 (150%)	7.55	20	37.50

- Inject 20 µl of diluent as a blank into the chromatographic system
- Inject 20 µl of 50 % and 150 % concentration in 6 times and other levels in duplicate

Instrumental Procedure

- into the chromatographic system
- Plot a graph of concentration against the peak Reponses and calculate the linearity regression coefficient ,% Y – intercept and % RSD peak area response for 50 % level and 150 % level

Acceptance criteria

- The co-relation is not less than 0.999
- The % Y intercept is between -2 % to +2 %
- % RSD of peak Reponses of 50 % level and 150% level should be NMT 2.0

Linearity level	Concentration in ppm	Area-Average	% of RSD	Statistic Analysis	
L1 (50%)	12.50	1352892	0.04	Y Intercept	2265.7
L2 (80%)	20.00	2112529	--		
L3 (100%)	25.00	2632630	--	% Y Intercept	0.85
L4 (120%)	30.00	3159809	--	Correlation coefficient	0.99998
L5 (150%)	37.50	3961236	0.22	R^2	0.9999

Response of Emtricitabine is linear overt the concentration range 50% to 150% target concentration

Precision

System precision

For resolution solution and standard solution into the chromatic graphic system,

Instrumental Procedure

- Inject 20 µl of resolution solution into the chromatographic system
- Inject 20 µl of standard solution in 5 replica into the chromatographic system

Acceptance criteria

- The resolution between Emtricitabine and Emtricitabine related compound A is NLT 3.0
- The relative standard deviation for Emtricitabine peak area obtained from five replica injection of standards solution is not more than 2.0 %

Results of System Precision

Results of System Precision	
Injection	**Peak Area**
1	2665770
2	2675632
3	2672525
4	2673231
5	2671511
Mean	2671814
% RSD	0.05
The resolution between Emtricitabine related compound A is 10.9 Hence method is specific	
Acceptance Criteria: Resolution: The resolution between Emtricitabine related compounds A is NLT 3.0.	

Method Precision

Weight powder not less than 20 tablets. Transfer an accurately weighed portion of the tablets powder equivalent to 200mg of Emtricitabine into a 200 ml of volumetric flask, add about 150 ml diluent.
Sonicate the solution for about 20 minute and then shake for about 20 minute
Allow the solution cool to room temperature, dilute to volume with diluent and mix. Centrifuge a portion of the resulting solution at about 1500 rpm for about 5 minutes
Dilute 5 ml of the supernatant into 200 ml volumetric flask and dilute to volume with diluent and mix.

Results of Method Precision			
Sample	**% Assay**	**Statistical Analysis**	
1	100.2	Mean	100.0
2	99.7		
3	99.9	SD	0.16
4	100.2		
5	99.7	% RSD	0.16
6	99.8		

Test results are showing that the test method is precise.

Accuracy: To demonstrate the accuracy by assessment of placebo sample spiked with known amount of Drug substance by using at least 3 replicates of 3 test concentration levels
For reagent, diluent preparation, buffer preparation, chromatographic condition, resolution solution, standard solution and sample proceed as per Methodology defined

Preparation of resolution solution: For resolution solution and standard solution proceed as per methodology.

Preparation of sample solution: The recovery of Emtricitabine in table is asses by adding a series of known amount of drug substance to placebo from 50 % to 150 % of test concentration in separate volumetric flask add about 150 ml diluent.
Sonicate the solution for about 20 minute and then shake for about 20 minute
Allow the solution cool to room temperature, dilute to volume with diluent and mix. Centrifuge a portion of the resulting solution at about 1500 rpm for about 5 minutes
Dilute 5 ml of the supernatant into 200 ml volumetric flask and dilute to volume with diluent and mix.

Preparation of the accuracy solution

Accuracy level	Weight of drug added in mg	Placebo of added in mg	diluent solution	dilute ml	diluent solution
L1 (50 %)	100 mg	160 mg	200 ml	5.0 ml	200 ml
	100 mg	160 mg	200 ml	5.0 ml	200 ml
	100 mg	160 mg	200 ml	5.0 ml	200 ml
L2 (100 %)	200 mg	160 mg	200 ml	5.0 ml	200 ml
	200 mg	160 mg	200 ml	5.0 ml	200 ml
	200 mg	160 mg	200 ml	5.0 ml	200 ml
L3 (150 %)	300 mg	160 mg	200 ml	5.0 ml	200 ml
	L1 (50 %)	160 mg	200 ml	5.0 ml	200 ml
	300 mg	160 mg	200 ml	5.0 ml	200 ml

Instrumental Procedure
Inject 20 µl of blank, accuracy level samples into the chromatographic system and check the % recovery
Acceptance criteria
System suitability
The resolution between Emtricitabine and Emtricitabine related compound A is NLT 3.0
The relative standard deviation for Emtricitabine peak area obtained from five replica injection of standards solution is not more than 2.0 %
% Recovery
The % recovery of accuracy levels should be not less than 98.0 and not more than 102.0

Results of System Precision	
Injection	**Peak Area**
1	2669979
2	2671733
4	2673636
5	2671617
Mean	2671718
% RSD	0.04
The resolution between Emtricitabine related compound A is 10.9 Hence method is specific	
Acceptance Criteria: Resolution: The resolution between Emtricitabine related compounds A is NLT 3.0.	

Result of Linearity					
Linearity level	**Amount added (mg)**	**Amount found (mg)**	**% Recovery**	**Statistic Analysis**	
L1 (50%) Sample-1	100.07	101.06	101.0	Mean	100.6
L1 (50%) Sample-2	100.06	100.82	100.8	SD	0.26
L1 (50%) Sample-3	100.07	100.54	100.8	% RSD	0.26

Result of Linearity					
Linearity level	**Amount added (mg)**	**Amount found (mg)**	**% Recovery**	**Statistic Analysis**	
L3 (100%) Sample-1	200.05	197.90	98.9	Mean	98.7
L3 (100%) Sample-2	200.09	197.62	98.9	SD	98.12
L3 (100%) Sample-3	200.05	197.46	98.7		
L5 (150%) Sample-1	300.12	297.71	99.2	Mean	99.24
L5 (150%) Sample-2	300.19	297.63	99.2		
L5 (150%) Sample-3	300.18	297.13	99.2		
Over all Statistical Analysis					
Mean	99.6	SD	0.90	%RSD	0.90

The recovery results indicating that the test methods has an acceptable level of accuracy

Solution stability

Demonstrate the solution stability of the standard and sample solution by injecting in regular interval. For reagent, diluent preparation, buffer preparation, chromatographic condition, resolution solution, standard solution and sample proceed as per Methodology defined.

Preparation of resolution solution : For resolution solution and standard solution proceed as per methodology.

Preparation of sample solution : The recovery of Emtricitabine in table is asses by adding a series of known amount of drug substance to placebo from 50 % to 150 % of test concentration in separate volumetric flask add about 150 ml diluent.

Sonicate the solution for about 20 minute and then shake for about 20 minute

Allow the solution cool to room temperature, dilute to volume with diluent and mix. Centrifuge a portion of the resulting solution at about 1500 rpm for about 5 minutes. Dilute 5 ml of the supernatant into 200 ml volumetric flask and dilute to volume with diluent and mix

Instrumental Procedure

Inject 20 µl of resolution into the chromatographic system.

Inject 20 µl of standard solution 5 replicate and sample solution at regular interval for initial, 6, 12, 24 hours along with freshly prepared standard, into chromatographic system.

Calculate the % assay of standard and sample Monitored pattern of chromatogram of standard and sample for chromatographic system.

The % RSD and % Assay from six sampled should be NMT 2.0

Acceptance criteria

System suitability

- The resolution between Emtricitabine and Emtricitabine related compound A is NLT 3.0
- The relative standard deviation for Emtricitabine peak area obtained from five replica injection of standards solution is not more than 2.0 %

Solution Stability

- There should be no extraneous peak or degradants are observed in chromatogram of sample and standard for chromatographic method.
- For standard the % difference of Assay of initial standard to standard at regular interval should be NMT 2.0
- The % difference of assay of the sample solution at initial to that at regular interval should be more than 2.0

Results

Standard Solution

The % difference for the peak area response from 5 replica injection standard solution .From precision study and one from solution stability should be not more than 2.0

Time in Hours	**% Assay**	**% Difference**
Initial	99.9	-
Standard about 6 hours	99.8	0.1
Standard about 12 hours	99.7	0.1
Standard about 24 hours	99.2	0.6

Sample Solution

The % difference of % assay of sample solution from initial to the % assay at regular intervals should be not more than 2.0

Time in Hours	% Assay	% Difference
Initial	99.8	-
Standard about 6 hours	99.8	0.2
Standard about 12 hours	99.8	0.2
Standard about 24 hours	99.3	0.6

Standard and sample are stable for 24 hours

CONCLUSION

This study, isocratic chromatography by HPLC method was developed for Emtricitabine and the developed method was validated for parameters like linearity, precision, accuracy, ruggedness and robustness. This method is advantageous over the reported method in terms of cost effectiveness and do not involve any tedious procedures.

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