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Development and validation of analytical methods for anti-viral drugs

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ABSTRACT

BCS solubility study, 2% SLS in water was selected as a dissolution medium and dissolution parameter established. HPLC method for dissolution testing of Lamivudine and Efavirenz in tablet formulation was developed & validated. All system suitability parameters were passed in acceptable range. Linearity of the developed method was near to 1.0 within the specified range. % RSD was found to be less than 2 for repeatability. % Recovery for all three drugs was found to be within 98-102 % across all levels. These results indicate that the developed method is accurate, precise, specific, robust and less time consuming. It can be used in the routine quality control of marketed dosage form. The retention time of Lamivudine, & Efavirenz were about 2.883 min, 7.426 min & 8.572 min respectively. Resolution between all three drugs was more than 2.0. Total run time per sample analysis was 15 minutes. All system suitability parameters were passed in acceptable range. Peak purity of both active & all impurities was passed in sample preparation. Linearity of the developed method was near to 1.0 within the specified range. % RSD was found to be less than 5 % for repeatability. % Recovery of all impurities was found to be within 95-105% across all levels. These results indicate that the developed method is accurate, precise, specific & less time consuming. It can be used in the routine quality control of marketed dosage form. Resolution between both actives & impurities was more than 2.0. USP S/N achieved more than 3 in LOD and more than 10 in LOQ preparation.

Key words: Lamivudine, Efavirenz, Repeatability, Recovery, HPLC

INTRODUCTION TO DRUG COMBINATION [1-10]

Viral diseases are the infections due to different types of viruses and these viral diseases are widespread all over the world [1]. Several types of viral infections are most common in healthy people which are not dangerous like viral fever, malaria but certain types of viral infections are not self-limiting and cause serious complications such as HIV/AIDS and are eventually incurable[2,3]. Human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) is a spectrum of conditions caused by infection with the human immunodeficiency virus (HIV) [4]. The treatment of HIV/AIDS normally includes the use of multiple antiretroviral drugs in an effort to control HIV infection. There are number of antiretroviral medicines which act on different stages of the HIV lifecycle. The use of different fixed dose combination drugs that act on different viral targets is known as highly active antiretroviral therapy (HAART) [5,6]. HAART decreases the patient's total burden of HIV, maintains function of the immune system, and prevents opportunistic infections that often lead to death. There is no medicine available in the world which can completely cure the HIV/AIDS. The United States Department of Health and Human Services and the World Health Organization has recommended the use of antiretroviral medicines to all patients with HIV [7]. The World Health Organization has recommended a combination of antiretroviral drugs for people starting HIV treatment:

Tenofovir and Efavirenz [8-10]

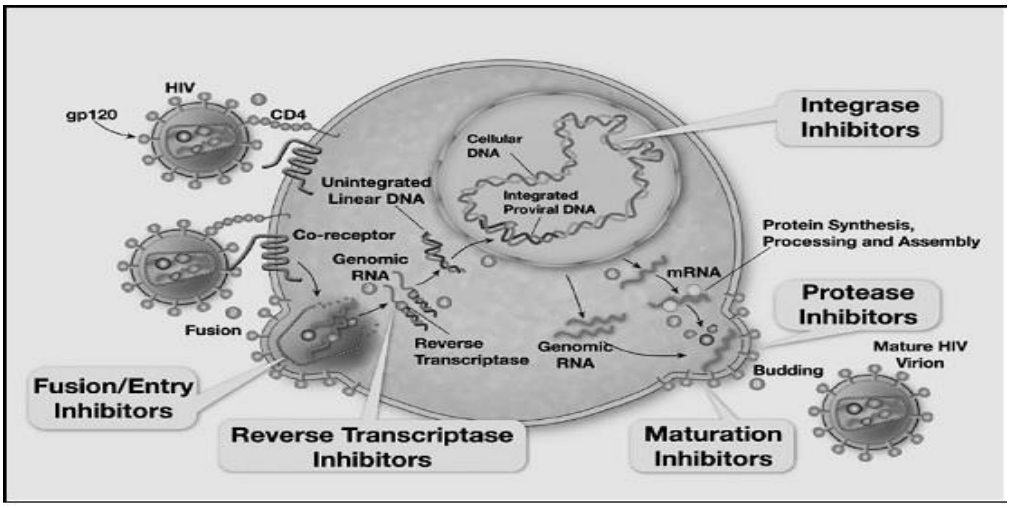


Figure 1: Targets of antiretroviral drugs in the HIV lifecycle

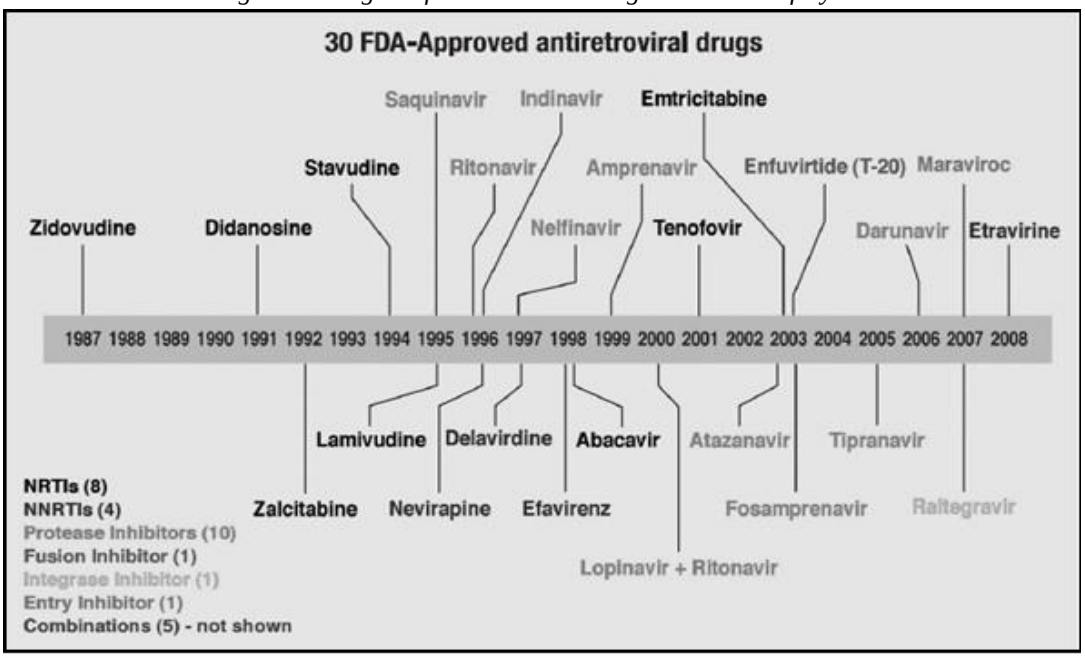


Figure 2: Antiretroviral drugs approved for HIV infection

Analytical chemistry is a branch which determines the nature & identity of a substance & its composition. In the early twentieth century there were only four accepted branches of chemistry namely, organic chemistry, inorganic chemistry, physical chemistry and biochemistry. Its importance grew and, in the process, adsorbed techniques and skills from all other four branchesso by the 1950s, analytical chemistry was finally accepted as a branch of chemistry in its own right.

To provide timely, accurate, precise and reliable data is an essential requirement of the drug discovery, development and manufacturing of pharmaceuticals. Analytical data are used to screen potential drug candidates, support in the development of drug substance synthesis, design formulation studies, monitor the stability of bulk pharmaceuticals, in-process samples and finished products and test the final products for release. The need of the advanced analytical instruments and determination using them is almost a routine process for the modern analytical laboratories [11-15].

METHODOLOGY

Identification by UV

Identification by UV test was performed for evaluation of Lamivudine as per mentioned in IP- 2014. Solution of 0.001% w/v of lamivudine was prepared in a mixture of 50 volumes of water & 50 volumes of acetonitrile. This solution was scanned in the range of 200 to 400 nm.

Identification by IR

Identification by IR was carried out for all APIs. Accurately weighed 2 mg of individual API was mixed with 200 mg of previously dried potassium bromide at 105°C for 1 hr. and triturated to get homogenous mixture. This sample was scanned in the range of 400-4000cm^{-1}.

Melting point determination

Melting point determination was carried out for all APIs. Melting point was determined by using capillary method.

Development & validation of analytical method for dissolution testing of Lamivudine, & Efavirenz in fixed dose tablet formulation by RP- HPLC

Selection of Dissolution Media

As per USFDA guidance BCS solubility was performed in different pH buffers and Efavirenz is very less polar drug hence, surfactant was also used for dissolution media selection. For BCS solubility highest dose of API needs to be dissolved in 250mL of respective buffer media, but to avoid use of large amount of API, volume of buffer and API weights were scaled down to maintain same concentration as in 250 mL of buffer. Table-1 shows the actual amount and scaled down amount of API and volume of buffer medium. Samples were kept in thermomixer at 900RPM and 37°C temperature for 24hours. After 24 hours' samples were centrifuged and suitable dilution carried out with respective buffer. Samples were analysed using UV spectrophotometer. Analytical standards were prepared in methanol due to high solubility of all API in methanol. All API were dissolved in water, 0.1N HCl, pH-4.5 acetate buffer, pH- 6.8 phosphate buffer, 1% SLS in water, 1.5% SLS in water and 2% SLS in water.

Table 1: Actual amount and scaled down amount of API and buffer volume

Name of API	Actual amount of API (mg)	Actual volume of buffer (mL)	Scaled down amount of API (mg)	Scaled down volume of buffer (mL)
LAM	300	250	12	10
TDF	300	250	12	10
EFV	600	250	24	10

Maximum solubility of all three APIs were in 2% SLS in water to achieve sink condition. Based on these results 2% SLS in water selected as a dissolution medium.

Selection of Dissolution Parameters

Dissolution parameters selected as per guidance for Industry-Dissolution Testing of Immediate Release Solid Oral Dosage Forms. All dissolution parameter mentioned in Table- 6.10

Table 2: Dissolution conditions for LAM and EFV tablets

Apparatus	USP Apparatus –II (Paddle)
Stirrer speed	50
Dissolution medium	2% SLS in water
Bath temperature	37 °C±0.5 °C
Media volume	1000 mL
Sample volume	10 mL
Replenish volume	No replacement
Time points	45 minutes
Filters	10µm full flow PVDF filters

Analytical Method Development

Waters HPLC was used with PDA detector and auto injector module to perform analysis of samples. 20mM phosphate buffer pH-2.5±0.05 with 0.1% TEA was selected as a mobile phase-A and acetonitrile 100% as a mobile phase-B. Due to high difference in polarity of all three active gradient elution was performed for separation. Samples were injected in C18 column (Inertsil ODS-3V 100×4.6 mm; 3µm) which was eluted at 1 mL/min. Injection volume kept 5µL. HPLC column temperature was set to 35°C and auto sampler temperature kept ambient. Selected gradient was as follows: 0-4.0 min, isocratic 5% B 4.0-6. 0 min, linear gradient 5-80% B; 6.0-9.0 min, isocratic 80% B; 9.0-10.0 min, linear gradient 80-5%; 10.0-
13.0 min.

Procedure

Dissolution media preparation: (2% SLS in water)

Accurately weighed and transferred 200 gm of sodium lauryl sulphate into 10L of beaker containing 8L of water and sonicated to dissolve. Volume made up to 10L with water and mixed well.

Mobile phase-A preparation: (20 mM phosphate buffer pH-2.5±0.05)

Weighed and transferred about 2.72 g of potassium dihydrogen phosphate in 1000 mL of water and sonicated to dissolve. Transferred 1mL of trimethylamine into above buffer and mixed well. pH was adjusted to 2.5±0.05 with diluted ortho-phosphoric acid.

Mobile phase-B preparation:

Acetonitrile 100%

Standard preparation: (Prepared in duplicate)

Accurately weighed and transferred about 15 mg of Lamivudine and 30 mg Efavirenz into 25 mL of clean, dry volumetric flask and 5 mL of methanol added. Sonicated to dissolve and volume made up to the mark with dissolution media. Pipetted out 5.0 mL of this solution into a 20 mL volumetric flask. Volume was made up to the mark with dissolution media and mixed well

Sample preparation:

Individual tablets were weighed and transferred to each six individual dissolution bowl having 1000 mL of 2% SLS in water which was pre equilibrated at 37°C±0.5°C. RPM was set to 50 and dissolution was run. After 45 minutes (Q time point)' sample aliquots collected and filtered through 10µm PVDF filters after discarding 5mL of filtrate and transferred into HPLC vials.

Method validation

The performance characteristics considered for validation of the optimized method were: specificity, linearity and working range, accuracy, precision and robustness.

Filter compatibility study

Filter study was performed on sample preparation to select suitable filter. 10µm PVDF & 10µm Polyethylene inline filters were evaluated against centrifuged sample preparation.

Table 3: Sequence schedule for Filter compatibility study

Sr. No.	Sample Name	No. of Injections
1.	Blank (Dissolution media)	1
2.	Standard-1 preparation	5
3.	Standard-2 preparation	2
4.	Centrifuged sample preparation	1
5.	Sample preparation filtered through 10µm PVDF inline filter	1
6.	Sample preparation filtered through 10µm Polyethylene inline filter	1
7.	Standard-1 Bracketing	1

Specificity

Specificity was performed by checking interference from dissolution medium and placebo (excipients of formulation) at the retention time of all three active in standard preparation.

Placebo preparation:
Weighed and transferred about 400 mg of placebo powder into dissolution bowl having 1000 mL of 2% SLS in water which was pre equilibrated at 37°C±0.5°C. RPM was set to 50 and dissolution was run. After 45 minutes (Q time point)' sample aliquot collected and filtered through 10µm PVDF filters after discarding 5mL of filtrate and transferred into HPLC vials.

Sample preparation:
Weighed and transferred 1 tablet into dissolution bowl having 1000 mL of 2% SLS in water which was pre equilibrated at 37°C±0.5°C. RPM was set to 50 and dissolution was run. After 45 minutes (Q time point)' sample aliquot collected and filtered through 10µm PVDF filters after discarding 5mL of filtrate and transferred into HPLC vials.

Table 4: Sequence schedule for specificity study

Sr. No.	Sample Name	No. of Injections
1.	Blank (Dissolution media)	1
2.	Standard-1 preparation	5
3.	Standard-2 preparation	2
4.	Placebo preparation	1
5.	Sample preparation	1
6.	Standard-1 Bracketing	1

Linearity and working range
Linearity was assessed visually and by means of a lack-of-fit test [19]. The working range was defined as the interval between the upper and the lower levels of the analytes within the calibration curve. The Linearity was determined at five concentration levels from 20% to 120% levels of the sample preparation (i.e. 300 µg/mL of LAM, TDF and 600 µg/mL of EFV). Linearity plot – A graph of area response v/s concentration in µg/mL was plotted for all three actives.

Linearity stock solution preparation (L1):
Accurately weighed and transferred about 30 mg Tenofovir disoproxil fumarate, 30 mg of Lamivudine and 60 mg Efavirenz into 25 mL of clean, dry volumetric flask and 5 mL of methanol added. Sonicated to dissolve and volume made up to the mark with dissolution media.
Linearity solutions prepared as mentioned in below table

Table 5: Dilution scheme for linearity solutions

Level	Pipette volume (L1) mL	Volumetric flask mL	Conc. of LAM (µg/mL)	Conc. of TDF (µg/mL)	Conc. of EFV (µg/mL)
L1-20%	1.0	20	60.2	59.4	120.5
L2-40%	2.0	20	120.5	118.8	240.9
L3-80%	4.0	20	240.9	237.6	481.9
L4-100%	5.0	20	301.2	297.0	602.3
L5-120%	6.0	20	361.4	356.4	722.8

Table 6: Sequence schedule for Linearity

Sr. No.	Sample Name	No. of Injections
1.	Blank (Dissolution media)	1
2.	Standard-1 preparation	5
3.	Standard-2 preparation	2
4.	Linearity solutions L1 to L5	1 of each level
5.	Standard-1 Bracketing	1

Accuracy
Accuracy of analytical method was evaluated by recovery study. Known amount of API and placebo spiked in 1000 mL of dissolution medium at different level (20%, 100% and 120%).

Sample solution preparation for recovery: (Prepared in triplicate)

Weighed and transferred about 400 mg of placebo powder and known amounts of LAM, TDF and EFV into dissolution bowl having 1000 mL of 2% SLS in water which was pre equilibrated at 37°C±0.5°C. RPM was set to 50 and dissolution was run. After 45 minutes (Q time point), sample aliquot collected and filtered through 10µm PVDF filters after discarding 5mL of filtrate and transferred into HPLC vials.

Table 7: Recovery sample solution preparation

Level	Wt. of placebo (mg)	Wt. of LAM (mg)	Wt. of EFV (mg)	Media Volume mL	Conc. of LAM µg/mL	Conc. of EFV µg/mL
20%	400	60	120	1000	60.0	120.0
100%	400	300	600	1000	300.0	600.0
120%	400	360	720	1000	360.0	720.0

Table 8: Sequence schedule for Accuracy

Sr. No.	Sample Name	No. of Injections
1.	Blank (Dissolution media)	1
2.	Standard-1 preparation	5
3.	Standard-2 preparation	2
4.	Recovery sample preparation-20%_Set-1 to Set-3	1 of each set
5.	Recovery sample preparation-100%_Set-1 to Set-3	1 of each set
6.	Recovery sample preparation-120%_Set-1 to Set-3	1 of each set
7.	Standard-1 Bracketing	1

System precision

The five replicate injections of standard preparation were injected to determine the reproducibility of the instrument.

Method precision

Six sample sets were injected to determine repeatability of analytical method.

Table 9: Sequence schedule for System precision and Method precision

Sr. No.	Sample Name	No. of Injections
1.	Blank (Dissolution media)	1
2.	Standard-1 preparation	5
3.	Standard-2 preparation	2
4.	Sample preparation_Set-1 to Set-6	1 of each set
5.	Standard-1 Bracketing	1

Solution stability

Solution stability of standard preparation and sample preparation was performed; the stability was evaluated for a period of 3 days at room temperature (15-25°C). The stored solutions of standard preparation and sample preparation at room temperature (15-25°C) were analysed at day-1, day-2 and day-3 against freshly prepared standards.

Table 10: Sequence schedule for Solution stability (Initial)

Sr. No.	Sample Name	No. of Injections
1.	Blank (Dissolution media)	1
2.	Standard-1 preparation	5
3.	Standard-2 preparation	2
4.	Sample preparation	1
5.	Standard-1 Bracketing	1

Table 11: Sequence schedule for Solution stability (Day-1, Day-2 and Day-3)

Sr. No.	Sample Name	No. of Injections
1.	Blank (Dissolution media)	1
2.	Standard-1 preparation	5
3.	Standard-2 preparation	2
4.	Standard-1 preparation (prepared at zero time point)	1
5.	Sample preparation (prepared at zero time point)	1
6.	Standard-1 Bracketing	1

Robustness

Robustness of an analytical method was evaluated by changes in column oven temperature, detection wavelength and buffer pH. System suitability monitored during robustness study.

Table 12: Sequence schedule for Robustness

Sr. No.	Sample Name	No. of Injections
1.	Blank (Dissolution media)	1
2.	Standard-1 preparation	5
3.	Standard-2 preparation	2
4.	Standard-1 Bracketing	1

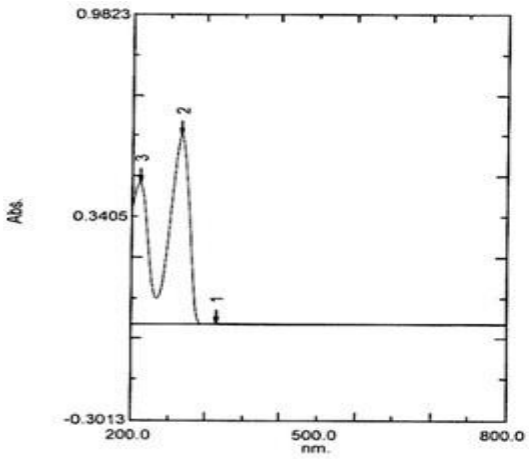
RESULTS & DISCUSSION

API Evaluation

API evaluation of all four drugs was carried out as per Pharmacopoeial requirement. Following tests were carried out for API evaluation

Identification by UV

Identification by UV test was performed for evaluation of Lamivudine as per mentioned in IP-2014. Solution of 0.001% w/v of lamivudine was prepared in a mixture of 50 volumes of water & 50 volumes of acetonitrile. This solution was scanned in the range of 200 to 400 nm.



No.	P/V	Wavelength	Abs.	Description
1		339.0	0.000	
2		270.0	0.597	
3		212.0	0.446	

Figure 3: UV Spectra of LAM

Acceptance criteria: Maximum absorption should be observed at about 270 nm.

Observation: Maximum absorption observed at 270 nm.

Identification by IR

Identification by IR was carried out for all APIs. Accurately weighed 2 mg of individual API was mixed with 200 mg of previously dried potassium bromide at 105°C for 1 hr. and triturated to get homogenous mixture.

This sample was scanned in the range of 400-4000cm^{-1}. Sample IR spectra of LAM, EFV were interpreted and compared with its reported interpretation. The referenced spectra of LAM and EFV in figures.

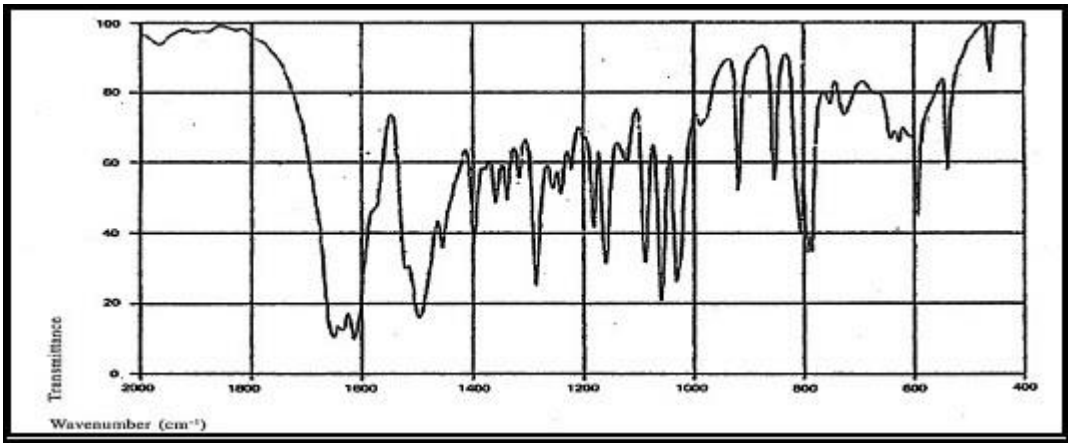


Figure 4: Reference FT-IR Spectra of LAM

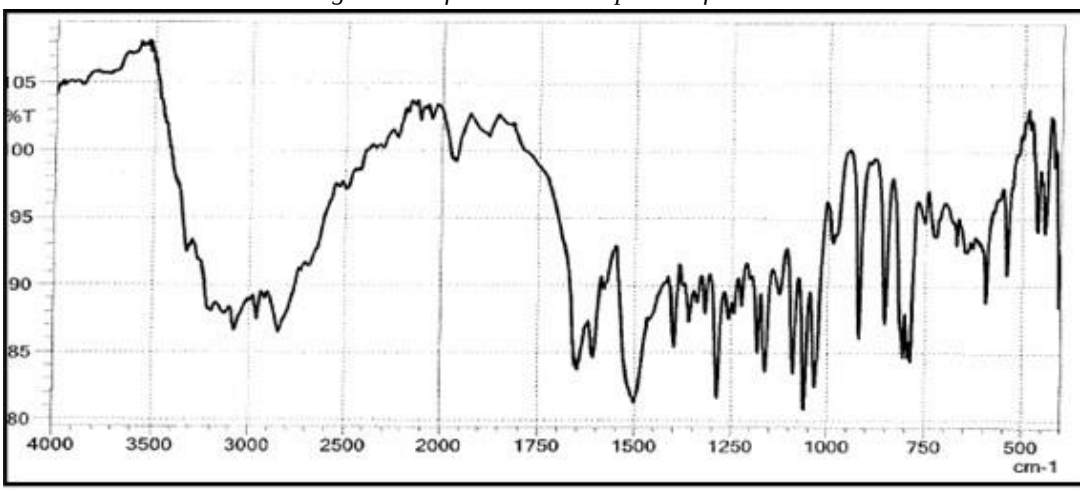


Figure 5: Sample FT-IR Spectra of LAM

Table 13: *Observed Wave number of LAM*

S. No	Peaks (cm^{-1})	Peak Assignment
1	1750-1700	C=O stretch
2	1650-1600	N-H bending
3	1600-1500	C=C Aromatic ring
4	1450-1400	CH_2 stretch
5	1360-1180	C-N stretch
6	950-800	=CH out of plane bend
7	700-600	C-S stretch

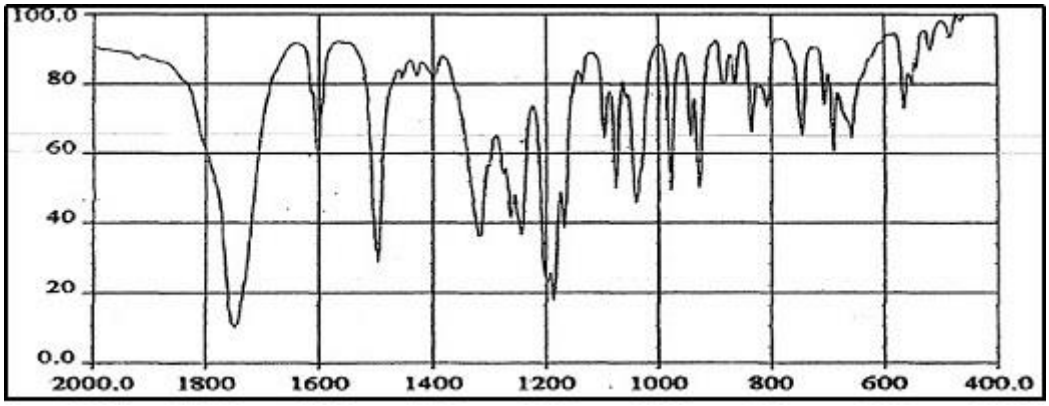


Figure 6: Reference FT-IR Spectra of EFV

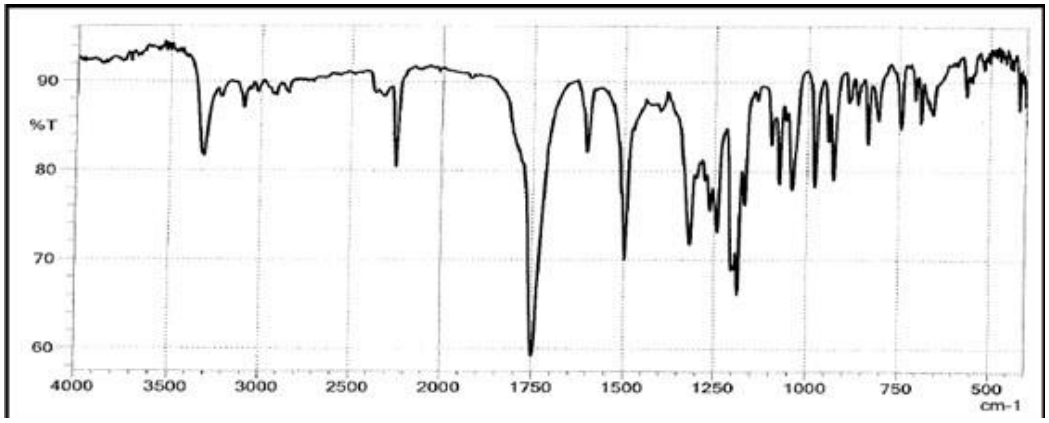


Figure 17: Sample FT-IR Spectra of EFV

Table 14: Observed Wave number of EFV

S. No	Peaks (cm^{-1})	Peak Assignment
1	1250	C-O-C stretch
2	1715	-C=O stretch
3	2100	C≡C stretch
4	1350, 1475	CH stretch
5	850-820	C-Cl stretch

Acceptance criteria:

The IR spectrum obtained with the sample should be concordant with that of Referencespectra.

Observation:

The IR spectrum obtained with the sample concordant with the reference spectra for LAM, EFV.

Conclusion:

Based on above observation it was concluded that IR spectra of sample matched with reference spectra, so LAM and EFV, API were found to be authentic.

Melting point determination

Melting point determination was carried out for all APIs. Melting point was determined by using capillary method.

Table 15: Melting point Determination

API Name	Observation			Specification
	ST*	ET*	MP*	
LAM	160.5°C	162.4°C	161.3°C	160°C-162°C
EFV	137.4˚C	137.7˚C	137.4˚C	137˚C-141˚C

**ST: Start Temperature, *ET: End Temperature, *MP: Melting point*

Conclusion:
Based on observation of above data it was concluded that the melting point results of LAM, TDF, EFV and EMT met specification so, all three API were found to be authentic.

Development & validation of analytical method for dissolution testing of Lamivudine & Efavirenz in fixed dose tablet formulation by RP- HPLC

Selection of Dissolution Media:
As per USFDA guidance BCS solubility was performed in different pH buffers and Efavirenz is very less polar drug hence, surfactant was also used for dissolution media selection. Solubility study of LAM and EFV was performed as per procedure whiv shows the results of solubility study.

Table 16: BCS Solubility data of LAM and EFV

Buffer media	**Solubility (mg/mL)**	
	LAM	**EFV**
0.1N HCl	276.08	0.12
pH-4.5 Acetate Buffer	230.50	0.05
Water	140.01	0.10
pH-6.8 Phosphate Buffer	92.76	0.05
2% SLS in water	**186.05**	**4.24**

Observation:
Maximum solubility of all three APIs were in 2% SLS in water to achieve sink condition.

Conclusion:
Based on these results 2% SLS in water selected as a dissolution medium.

Selection of Dissolution Parameters
Dissolution parameters selected as per guidance for Industry-Dissolution Testing of Immediate Release Solid Oral Dosage Forms.

Analytical Method Development

Experiment-1:

Background: -

- Two different analytical methods have been reported for dissolution testing of LAM, TDF and EFV. For dissolution testing of LAM and TDF, chromatographic condition of assay methods was used and for EFV isocratic method was used.
- In both analytical methods, ion pairing reagent (0.1% of sodium octane sulphonate) was used in mobile phase preparation.
- As a starting point Assay method for lamivudine, TDF and Efavirenz tablets as per IP monograph was evaluated by modifying mobile phase and gradient and all remaining parameters were kept same

Objective: -
To evaluate chromatographic parameters of assay method of drug product available in IP monograph of tablet formulation without use of ion pair reagent.

Experiment conditions

Column	Inertsil ODS-3V (100 x 4.6) mm, 3 µm
Mobile Phase-A	0.1% TFA in water
Mobile Phase-B	Methanol
Mobile Phase program	Gradient
Column temperature	35°C
Injection volume	5 µL
Flow rate	1.0 mL/minute
Detection	260 nm, UV
Run time	13 minutes

Gradient	Time (min)	Mobile Phase-A	Mobile Phase-B
	0	60	40
	4	60	40
	6	25	75
	9	25	75
	10	60	40
	13	60	40

LAM peak eluted in void and bad peak shape observed peak.
Good symmetrical peak observed for EFV.

Conclusion:

Further trial to be carried out with gradient modification to retain LAM peak.

Experiment-2

Background:

LAM peak was eluted in void in Experiment-1 which may be due to high organic ratio in mobile phase gradient at initial step.

Objective:

To retain LAM peak by changing organic ratio from 40 % to 5 % of mobile phase-B at 0-4 minute.

Experiment conditions:

Column	Inertsil ODS-3V (100 x 4.6) mm, 3 µm		
Mobile Phase-A	0.1% TFA in water		
Mobile Phase-B	Methanol		
Mobile Phase program	Gradient		
Column temperature	35°C		
Injection volume	5 µL		
Flow rate	1.0 mL/minute		
Detection	260 nm, UV		
Run time	13 minutes		
Gradient	**Time (min)**	**Mobile Phase-A**	**Mobile Phase-B**
	0	95	5
	4	95	5
	6	20	80
	9	20	80
	10	95	5
	13	95	5

LAM peak retained but broad peak observed.

Conclusion:

Further trial to be carried out with buffer as mobile phase-A to improve symmetry ofLAM peak.

Experiment-3

Background:

LAM peak was retained but broad peak observed in Experiment-2 which may be dueto low buffer capacity of 0.1% TFA in water as mobile phase-A.

Objective:

To get symmetrical peak of LAM by using 20mM Phosphate buffer pH-2.5 with 0.1 % TEA as mobile phase-A.

- Phosphate buffer used to retain LAM peak.
- TEA-triethylamine used as a peak modifier for peak sharpness

Experiment conditions:

Column	Inertsil ODS-3V (100 x 4.6) mm, 3 µm
Mobile Phase-A	20mM Phosphate buffer pH-2.5 + 0.1% TEA
Mobile Phase-B	Methanol
Mobile Phase program	Gradient
Column temperature	35°C
Injection volume	5 µL
Flow rate	1.0 mL/minute
Detection	260 nm, UV
Run time	13 minutes

Gradient

Time (min)	Mobile Phase-A	Mobile Phase-B
0	95	5
4	95	5
6	20	80
9	20	80
10	95	5
13	95	5

Peak symmetry improved but broad peak observed for LAM.

Conclusion:

Further trial to be carried out with different organic solvent as mobile phase-B to improve symmetry of LAM peak.

Experiment-4

Background:

Peak symmetry improved but broad peak observed for LAM in Experiment-3 which may be due to methanol as organic solvent in mobile phase-B.

Objective:

- To get symmetrical peak of LAM by using acetonitrile as organic solvent mobilephase-B and by changing organic ratio from 5-3% of mobile phase-B at 0-4 minute.
- Acetonitrile is less viscous than methanol which helps to improve peak symmetry by reducing band broadening in HPLC column.
- To retain LAM peak along with the usage of acetonitrile less organic ratio at initial in mobile phase gradient is required.

Experiment conditions:

Column	Inertsil ODS-3V (100 x 4.6) mm, 3 µm
Mobile Phase-A	20mM Phosphate buffer pH-2.5 + 0.1% TEA
Mobile Phase-B	Acetonitrile
Mobile Phase program	Gradient
Column temperature	35°C
Injection volume	5 µL
Flow rate	1.0 mL/minute
Detection	260 nm, UV
Run time	13 minutes

Gradient

Time (min)	Mobile Phase-A	Mobile Phase-B
0	97	3
4	97	3
6	20	80
9	20	80
10	97	3
13	97	3

- Good symmetrical peaks observe for LAM, TDF and EFV.
- All three peaks are well resolved from each other.
- Retention time variation observed for LAM in 5 replicate injections of standardpreparation.

Conclusion:

Run time to be increased to avoid retention time variation of LAM peak.

Experiment-5

Background:

Good symmetrical peaks observe for LAM and EFV but retention time variationobserved for LAM in 5 replicate injections of standard preparation in Experiment-4.

Objective:

- To resolve retention time variation of LAM peak by changing run time from 13minutes to 15 minutes.
- Variation in retention time of LAM peak may be due to less re-equilibration of HPLC column to get initial gradient condition.

Experiment conditions:

Column	Inertsil ODS-3V (100 x 4.6) mm, 3 µm		
Mobile Phase-A	20mM Phosphate buffer pH-2.5 + 0.1% TEA		
Mobile Phase-B	Acetonitrile		
Mobile Phase program	Gradient		
Column temperature	35°C		
Injection volume	5 µL		
Flow rate	1.0 mL/minute		
Detection	260 nm, UV		
Run time	15 minutes		
Gradient	**Time (min)**	**Mobile Phase-A**	**Mobile Phase-B**
	0	97	3
	4	97	3
	6	20	80
	9	20	80
	10	97	3
	15	97	3

Observation:

Number of Injections	**Retention Time (minutes)**	
	LAM	**EFV**
1	2.885	8.570
2	2.882	8.572
3	2.900	8.573
4	2.889	8.571
5	2.881	8.572
Mean	2.883	8.572
% RSD	0.2	0.0

No variation observed in retention time for LAM and EFV peak.

Conclusion:

Analytical method can be used for method validation.

Method validation

The performance characteristics considered for validation of the optimized method were: specificity, linearity and working range, accuracy, precision and robustness.

Filter compatibility study

Filter study was performed on sample preparation to select suitable filter. 10µm PVDF & 10 µm Polyethylene inline filters were evaluated against centrifuged sample preparation.

Observation

Table 17: Results of filter compatibility study

	% Release				
Drug Name	**Control**	**10 µm PVDF Filter**	**Abs. difference**	**10 µm Nylon Filter**	**Abs. difference**
LAM	98.4	98.5	0.1	97.8	0.6
EFV	99.8	99.6	0.2	99.0	0.8

Acceptance criteria:

Absolute difference of filtered solution against control solution should not be more than 2.0 %.

Conclusion:

Results of filter study met acceptance criteria and based on above results; 10µm PVDF inlinefilter was found to be more suitable.

Specificity

Specificity was performed by checking interference from dissolution medium and placebo (excipients of formulation) at the retention time of all three active in standard preparation.

No interference observed from blank and placebo preparation at the retention time ofLAMand EFV peaks.

Acceptance criteria:

Interference from blank and placebo should not be more than 2.0%.

Conclusion:

No interference observed from blank and placebo at the retention time of LAM andEFV peaks. So, it can be concluded that method is specific.

Linearity and working range

The Linearity was determined at five concentration levels from 20% to 120% levels of the sample preparation (i.e., 300 µg/mL of LAM and 600 µg/mL of EFV). Linearity plot –A graph of area response v/s concentration in µg/mL was plotted for all three actives.

Observation Linearity of LAM

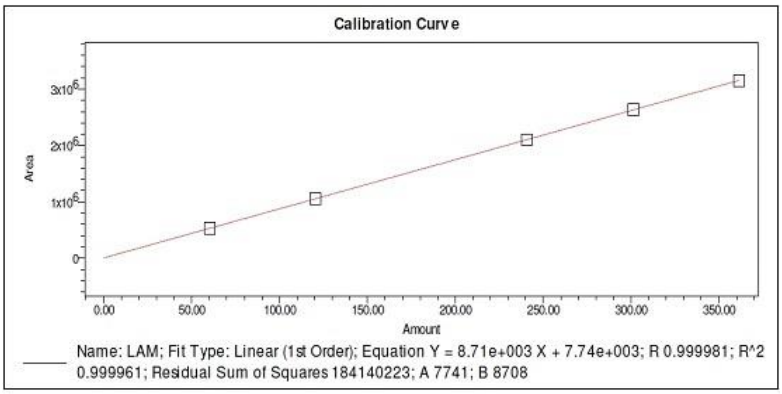


Figure 17: Linearity graph of LAM

Level	Concentration (µg/mL)	Area	Response Factor
20%	60.2	527850	8768
40%	120.5	1061316	8808
80%	240.9	2102393	8727
100%	301.2	2640270	8766
120%	361.4	3148049	8711
	Correlation co-efficient (r)		1.000
	Y-intercept		7741
	Slope		8708
	Plot (Visual)		Linear

Linearity of EFV

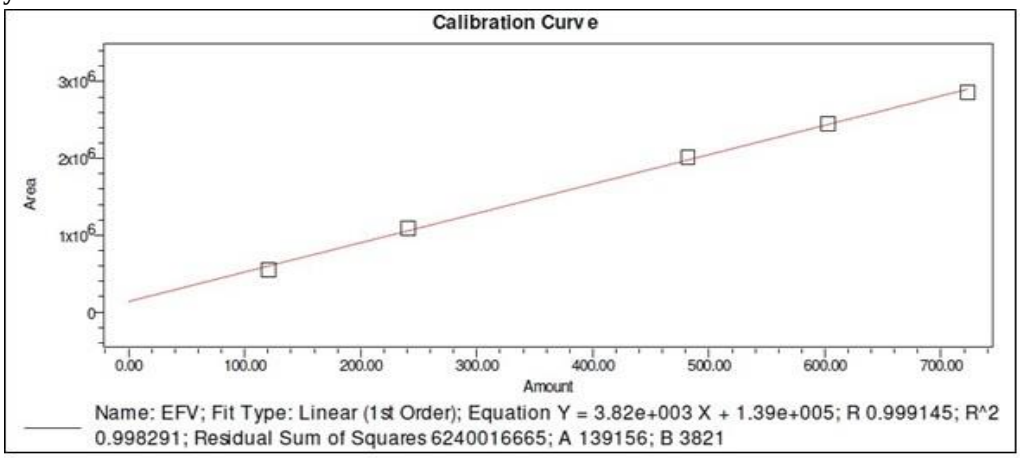


Figure 18: Linearity graph of EFV

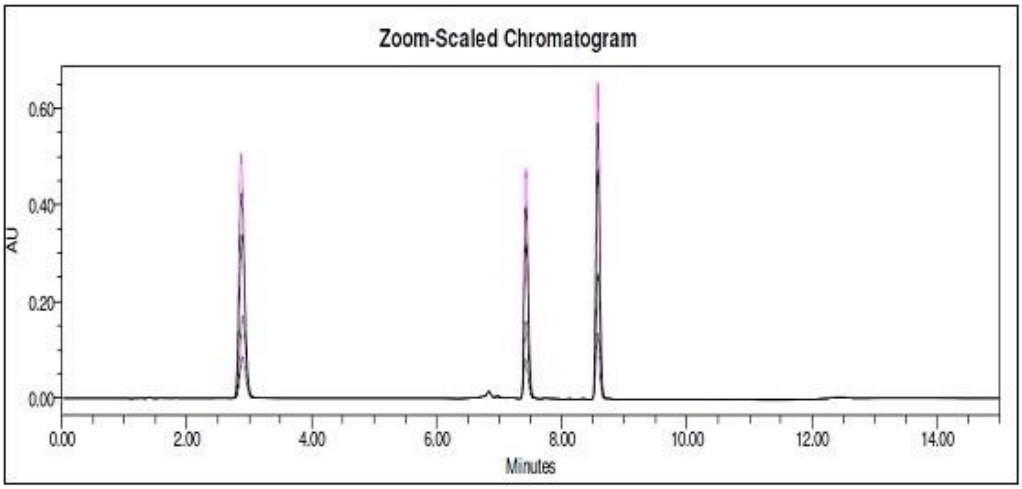


Figure 19: *Overlay chromatograph of Linearity Acceptance criteria*

The correlation coefficient should be more than 0.95.

Conclusion:

The correlation coefficient of LAM and EFV were found to be 1.000. So it can beconcluded that method is linear.

Accuracy

Accuracy of analytical method was evaluated by recovery study. Known amount of API and placebo spiked in 1000 mL of dissolution medium at different level (20%, 100% and 120%).

Table 18: Accuracy results of LAM

Level	Sample ID	Amount Added (µg)	Amount Recovered (µg)	% Recovery	Mean	% RSD
	Set-1	59.68	60.02	100.57		
	Set-2	59.91	60.11	100.34		
20%	Set-3	59.69	59.99	100.49	100.5	0.1
	Set-1	296.07	294.44	99.45		
	Set-2	295.63	294.69	99.68		
100%	Set-3	296.40	294.87	99.48	99.5	0.1
	Set-1	356.33	355.31	99.71		
	Set-2	356.12	355.96	99.95		
120%	Set-3	356.22	355.28	99.74	99.8	0.1

Table 19: Accuracy results of EFV

Level	SampleID	Amount Added (µg)	Amount Recovered (µg)	% Recovery	Mean	% RSD
	Set-1	120.56	120.22	99.72		
	Set-2	121.89	121.97	100.06		
20%	Set-3	119.76	118.71	99.12	99.6	0.5
	Set-1	600.56	596.29	99.29		
	Set-2	601.23	599.29	99.68		
100%	Set-3	601.23	599.51	99.71	99.6	0.2
	Set-1	719.80	713.23	99.09		
	Set-2	721.48	715.71	99.20		
120%	Set-3	719.56	713.08	99.10	99.1	0.1

Acceptance criteria:

Mean recovery across all levels should be within the range of 95-105%.

% RSD of recovery values should not be more than 5%.

Conclusion:

Results met acceptance criteria across all levels. Hence, the method is accurate over the rangeconsidered.

System precision

The five replicate injections of standard preparation were injected to determine the reproducibility of the instrument.

Table 20: Results of System precision

Parameters	Specification	Observation	
		LAM	EFV
%RSD	NMT 2.0%	0.1%	0.1%

Acceptance criteria:

% RSD of five replicate injections of standard preparation should not be more than 2%.

Conclusion:

Results met acceptance criteria. Hence, the method is precise.

Method precision

Six sample sets were injected to determine repeatability of analytical method.Chromatographic

Table 21: Results of Method precision

Sample Preparation	Method Precision	
	LAM	EFV
1	98	99
2	98	99
3	99	100
4	98	100
5	99	100
6	98	100
Mean	98	100
% RSD	0.1	0.4

Acceptance criteria:

% RSD of six set of sample preparation should not be more than 5%.

Conclusion:

Results met acceptance criteria. Hence, the method is precise

Solution stability

Solution stability of standard preparation and sample preparation was performed; the stabilitywas evaluated for a period of 3 days at room temperature (15-25°C). The stored solutions of standard preparation and sample preparation at room temperature (15-25°C) were analyzed at day-1, day-2 and day-3 against freshly prepared standards.

Table 22: Solution stability results of LAM

Solution stability of LAM Standard preparation			
Time interval	**Storage condition**	**% Assay**	**% Recovery against control**
Initial	NA	100.0	NA
Day-1		100.2	100.2
Day-2	Room temperature (15to 25°C)	100.2	100.2
Day-3		100.3	100.3
Solution stability of LAM Sample preparation			
Initial	NA	98	NA
Day-1		98	100
Day-2	Room temperature (15to 25°C)	98	100
Day-3		99	101

Table 23: Solution stability results of EFV

Solution stability of EFV Standard preparation			
Time interval	**Storage condition**	**% Assay**	**% Recovery against control**
Initial	NA	100.0	NA
Day-1		99.9	99.9
Day-2	Room temperature (15to 25°C)	99.9	99.9
Day-3		99.8	99.8
Solution stability of EFV Sample preparation			
Initial	NA	99	NA
Day-1		98	99
Day-2	Room temperature (15to 25°C)	98	99
Day-3		98	99

Acceptance criteria:

% recovery value against control solution should be within the range of 98-102%.

Conclusion:

% Recovery values for standard preparation and sample preparation were found to be within the range of 98-102%.

Standard preparation and Sample preparation were found to be stable up to 3 days at room temperature (15-25°C).

Robustness

Robustness of an analytical method was evaluated by changes in column oven temperature, detection wavelength and buffer pH. System suitability monitored during robustness study.

Table 24: Robustness results of LAM

Robustness study for LAM					
Parameters	**Condition**	**% RSD**	**Tailing Factor**	**Theoretical Plates**	**USP Resolution**
Change in Column oven (35°C ± 5°C)	30°C	0.1%	1.2	4726	
	40°C	0.1%	1.1	4887	NA
Change in Wavelength (260nm ± 2 nm)	258	0.1%	1.2	4864	
	262	0.1%	1.2	4823	NA
Change of pH in buffer (2.5 ± 0.2)	2.3	0.1%	1.2	4869	
	2.7	0.1%	1.2	4871	NA

Table 25: Robustness results of EFV

Robustness study for EFV					
Parameters	**Condition**	**% RSD**	**Tailing Factor**	**Theoretical Plates**	**USP Resolution**
Change in Column oven (35°C ± 5°C)	30°C	0.1%	1.2	90647	9.3
	40°C	0.1%	1.1	91785	9.4
Change in Wavelength (260nm ± 2 nm)	258	0.1%	1.1	91264	9.4
	262	0.1%	1.1	91911	9.4
	2.3	0.2%	1.1	90764	9.3
Change of pH inbuffer (2.5 ± 0.2)	2.7	0.1%	1.1	89983	9.4

Acceptance criteria:

- The % RSD of area responses of five replicate injections of standard-1 preparation should not be more than 2.0%.
- Average USP tailing factor (T) of five replicate injections of standard-1 preparation for LAM and EFV peak should not be more than 2.0.
- Average USP plate counts of five replicate injections of standard-1 preparation for LAM and EFV peak should not be less than 2000.
- Resolution between EFV peaks should not be less than 2.0.

Conclusion:

All results met acceptance criteria for robustness study. Hence, method is robust.

Discussion

From BCS solubility study, 2% SLS in water was selected as a dissolution medium and dissolution parameter established. HPLC method for dissolution testing of Lamivudine and Efavirenz in tablet formulation was developed & validated. All system suitability parameters were passed in acceptable range. Linearity of the developed method was near to 1.0 within the specified range. % RSD was found to be less than 2 for repeatability. % Recovery for all three drugs was found to be within 98-102% across all levels. These results indicate that the developed method is accurate, precise, specific, robust and less time consuming. It can be used in the routine quality control of marketed dosage form. The retention time of Lamivudine, & Efavirenz were about 2.883 min, 7.426 min & 8.572 min respectively. Resolution between all three drugs was more than 2.0. Total run time per sample analysis was 15 minute. All system suitability parameters were passed in acceptable range. Peak purity of both active & all impurities was passed in sample preparation. Linearity of the developed method was near to 1.0 within the specified range. %RSD was found to be less than 5% for repeatability. %Recovery of all impurities was found to be within 95-105% across all levels. These results indicate that the developed method is accurate, precise, specific& less time consuming. It can be used in the routine quality control of marketed dosage form. Resolution between both actives & impurities was more than 2.0. USP S/N achieved more than 3 in LOD and more than 10 in LOQ preparation.

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