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METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF LAMIVUDINE AND ABACAVIR IN BULK SAMPLES AS WELL AS IN TABLET DOSAGE FORMS BY USING RP-HPLC

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ABSTRACT
In the study, Lamivudine and Abacavir in tablets and bulk were analyzed using reverse phase high performance liquid chromatography. Different trails were performed with different mobile phases and different columns, peaks were observed and optimized based on the retention time, the optimized conditions were: stationary phase - Dionex C18 column, mobile phase - Methanol (40% volume) and potassium dihydrogen phosphate (60% volume) with 0.1 M strength and 3.5 pH, flow rate – 1 ml per min and detecting wavelength 232 nm. The linearity range was 150 to 450 µg/ml (abacavir) and 75 to 225 µg/ml (lamivudine). The developed technique was applied to tablets. The retention time for Abacavir and Lamivudine were found to be 2.397 min and 3.296 min respectively, accuracy, sensitivity and precision were found significant. During stability studies, extra peaks were well segregated for Lamivudine and Abacavir and hence this method can be applied to study degradation of Lamivudine and Abacavir during quality control analysis.

KEYWORDS: Abacavir, Lamivudine, HPLC, LOQ.

INTRODUCTION

HIV: Human Immunodeficiency Virus enters the body, reaches several distinct cells and implements its genes into the human DNA resulting in synthesis of HIV by cell hijack.[1,2] AIDS: - Acquired Immuno Deficiency Syndrome. To combine both of the HIV infection and its related diseases and malignancies, many drugs have become accessible which are called HART (highly active antiretroviral therapy)[3] which are simply termed as ART. Even though these drugs do not heal HIV/AIDS, antiretroviral have decreased complications and fatalities associated with HIV significantly.

Two nucleoside/nucleotide inhibitors are chosen for reverse transcriptase, Abacavir and Lamivudine to create a stability showing the RP-HPLC technique for bulk and dosage tablet testing.

This technique is a laboratory analytical technique used to segregate and recognize compounds.[4,5] It is in the category of column chromatography. Column chromatography is based on different polarities in a blend solution shown by the compounds in order to segregate them. Pressure is used in HPLC to rapidly force the sample solution through the column, therefore generates outcomes faster and more precise. The mobile phase and stationary phase are reversed, the use of a stationary hydrophobic phase is intrinsically the reverse of normal phase chromatography– and thus the term reversed-phase chromatography.[6,7] The development and validation of Lamivudine and Abacavir assay in tablets and bulk are reported in this work by RP-HPLC at 232nm.

MATERIALS AND METHODS

Materials

Lamivudine, Abacavir, EPIZOM tablets, DIONEX column C18, Pottasium dihydrogen phosphate, methanol, hydrochloric acid, sodium hydroxide, hydrogen peroxide and phosphoric acid. All materials were within the pharmaceutical grade.

Mobile phase

Methanol (40% volume) and potassium dihydrogen phosphate (60% volume) with 0.1 M strength and 3.5 p^H are blended and used as mobile phase. Phosphoric acid is used to alter the p^H.

Diluent

This above mixture was utilized as diluent to made solutions (stock and working standards).

HPLC Conditions

- Temperature in column – ambient

- Injecting quantity - 10 µl
- Analyzing wavelength - 232 nm
- Stream rate of mobile phase – 1 ml/minute
- Total analyzing time – 6 minutes

Lamivudine and Abacavir solutions (stock and standard)

For preparing lamivudine and abacavir stock solution, 600 mg abacavir and 300 mg lamivudine were appropriately weighed to a volumetric flask (100 ml) and diluted with diluting solvent. Stock concentration (µg/ml) – 6000 µg/ml abacavir and 3000 µg/ml lamivudine.

Five conventional linearity solutions were produced using the above inventory solution with right dilution using the diluent solvent. Concentration of conventional linearity solutions were listed in table no 1.

Table No. 1: Concentration of linearity solutions.

S.No	Description	Abacavir Concentration (µg/ml)	Lamivudine Concentration (µg/ml)
1	Linearity Solution I	150	75
2	Linearity Solution II	225	112.5
3	Linearity Solution III	300	150
4	Linearity Solution IV	375	187.5
5	Linearity Solution V	450	225

For validation research, Lamivudine and Abacavir solution was performed by diluting 1 ml stock solution in 10 ml flask to 10 ml using diluent. For validation linearity solution III with concentration of 300 µg/ml of Abacavir and 150 µg/ml of lamivudine was taken.

Method validation

The method established to evaluate Lamivudine and Abacavir is validated for linearity, system fit, selectivity, precision, LOD, LOQ, accuracy, robustness.[10]

Evaluation of Lamivudine and Abacavir in EPIZOM tablet form

10 tablets were drawn; homogenized and mean weight of tablet in powdered form was calculated in order to analyze Lamivudine and Abacavir in EPIZOM tablets. An accurately measured tablet equivalent to 600 mg of Abacavir and 300 mg of lamivudine was disintegrated by sonication and tainted to 100 ml by a diluent which is a mixture of KH_2PO_4 buffer (0.1 M, pH – 3.5): methanol (60:40 v/v). Solution of stock tablet concentration – 6000 µg/ml Abacavir and 3000 µg/ml Lamivudine. For solution with concentration of 300 µg/ml of abacavir and 150 µg/ml of lamivudine, a suitable amount of prepared solution was diluted. Before the assessment, the solution got filtered by a membrane filter of 0.45 µm. The tablet solution was analyzed as HPLC conditions.

Stability evaluation of lamivudine and abacavir

This was accomplished using tablet solution following ICH criteria.[11]

Hydrolysis with acid

10 ml stock solution of tablet (concentration – 6000 µg/ml abacavir and 3000 µg/ml lamivudine) has been transmitted to 100 ml volumetric flask to which10 ml of 0.1 N HCl was added to the same flask and sonicated for over 30 min at room temperature. The solution was neutralized by adding 0.1 N NaOH. The subsequent mixture was diluted with buffer KH_2PO_4 of 0.1 M, pH-3.5: methanol (60:40, v/v) solvent system for final concentration of abacavir at 300 µg/ml and lamivudine at 150 µg/ml. The product solution was filtered with a membrane filter of 0.45 µm and analysed as per HPLC conditions. The sample stressed was quantified alongside the abacavir and lamivudine standard reference (300 µg/ml abacavir and 150 µg/ml lamivudine).

Hydrolysis with alkali

10 ml stock solution of tablet (concentration – 6000 µg/ml abacavir and 3000 µg/ml lamivudine) has been transmitted to 100 ml volumetric flask to which10 ml of 0.1 N NaOH was added to same flask and sonicated for over 30 min at room temperature. The solution obtained was neutralized by 0.1 N HCl. The subsequent mixture was diluted with buffer KH_2PO_4 of 0.1 M, pH-3.5: methanol (60:40, v/v) solvent system for final concentration of abacavir at 300 µg/ml and lamivudine at 150 µg/ml. The product solution was filtered with a membrane filter of 0.45 µm and analysed as per HPLC conditions. The sample stressed was quantified alongside the abacavir and lamivudine standard reference (300 µg/ml abacavir and 150 µg/ml lamivudine).

Oxidative hydrolysis with peroxide

10 ml stock solution of tablet (concentration – 6000 µg/ml abacavir and 3000 µg/ml lamivudine) has been transmitted to 100 ml volumetric flask to which 10 ml of peroxide of 30% strength was added to same flask and sonicated for over 30 min at. The subsequent mixture was diluted with buffer KH_2PO_4 of 0.1 M, pH-3.5: methanol (60:40, v/v) solvent system for final concentration of abacavir at 300 µg/ml and lamivudine at 150 µg/ml. The product solution was filtered with a membrane filter of 0.45 µm and analysed as per HPLC conditions. The sample stressed was quantified alongside the abacavir and lamivudine standard reference (300 µg/ml abacavir and 150 µg/ml lamivudine).

RESULTS AND DISCUSSION

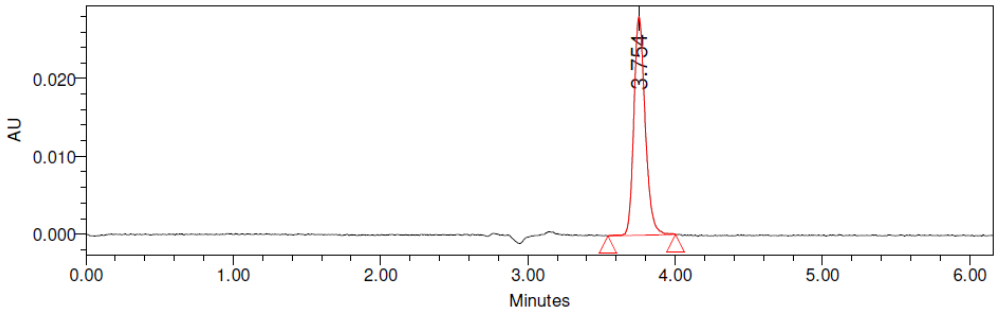
Trails for conditions optimization to assay of Lamivudine and Abacavir

Different trails were performed with different mobile phases and different columns, peaks were observed and

optimized based on the retention time.

Table No. 2: Trails for conditions optimization to assay of Lamivudine and Abacavir.

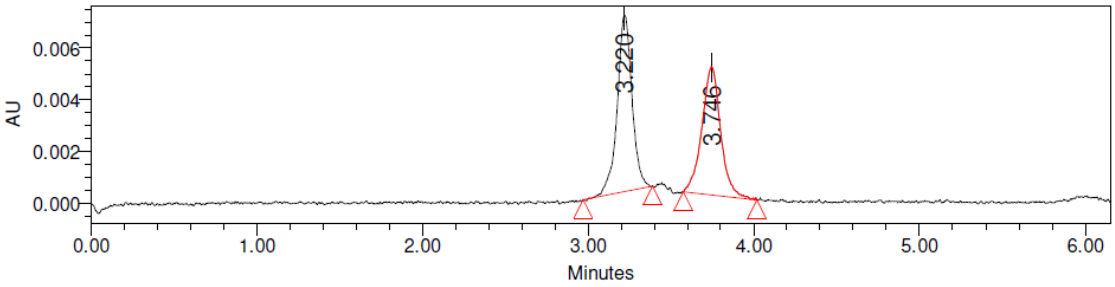
Description	Conditions				
	Trail-1	**Trail-2**	**Trail-3**	**Trail-4**	**Trail-5**
Mobile phase	Na_2HPo_4	Na_2HPo_4:Methanol (60:40)	KH_2Po_4:Methanol (70:30)	KH_2Po_4:Methanol (70:30)	KH_2Po_4:Methanol (60:40)
Column	WATERS,C18,250*4.6mm, 5 µm	Decelosil,C18,250*4.6 mm,5 µm	SUPELCO,C18, 250*4.6mm,5 µm	DIONEX,C18, 250*4.6MM,5 µm	DIONEX,C18, 250*4.6MM,5 µm
Flow rate	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min
Temperature	25	25	25	25	25
Volume	10 µl	10 µl	10 µl	10 µl	10 µl
Run time	10 min	10min	10min	10 min	10 min
Detector	PDA	PDA	PDA	PDA	PDA



Retention Time	Area	% Area	Height	USP Resolution	USP Tailing	USP Plate Count
3.754	150556	100	28068	-	1.21	11989

Figure 1: Obtained chromatogram in trail 1.

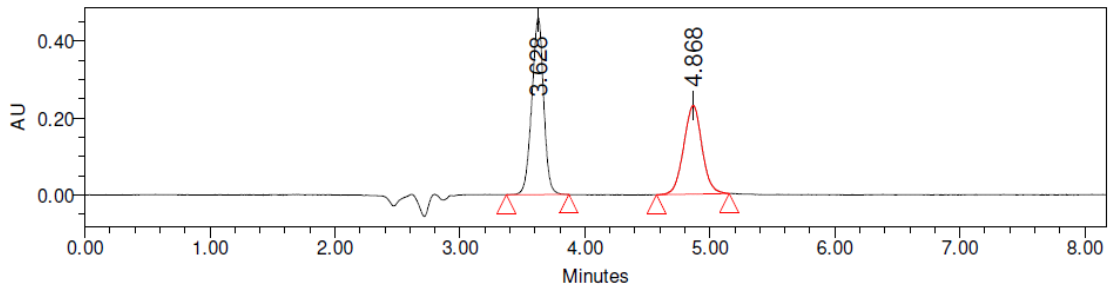
Remarks: Only one peak eluted. So the stated conditions are not preferred.



Retention Time	Area	% Area	Height	USP Resolution	USP Tailing	USP Plate Count
3.220	42510	52.15	6826	-	0.90	6949
3.746	39009	47.85	4958	2.85	1.07	5531

Figure 2: Obtained chromatogram in trail 2.

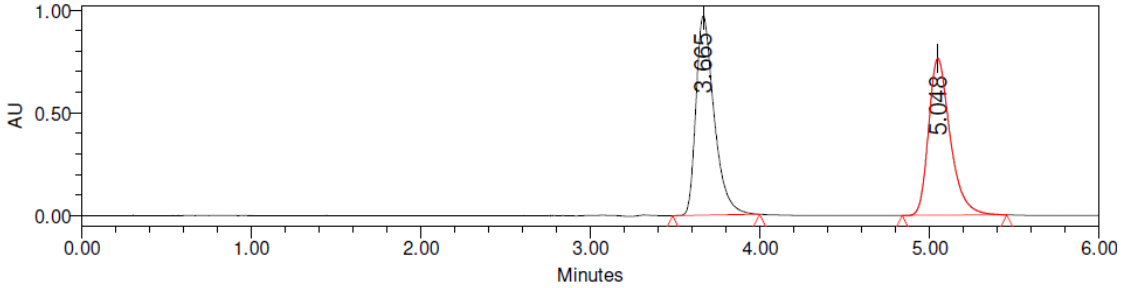
Remarks: Abacavir RT – 3.220 min, Lamivudine RT – 3.746 min. Base line noise is not good. So the stated conditions are not preferred.



Retention Time	Area	% Area	Height	USP Resolution	USP Tailing	USP Plate Count
3.628	3025152	56.31	459901	-	0.95	6975
4.868	2347609	43.69	230229	5.49	1.00	5373

Figure 3: Obtained chromatogram in trail 3.

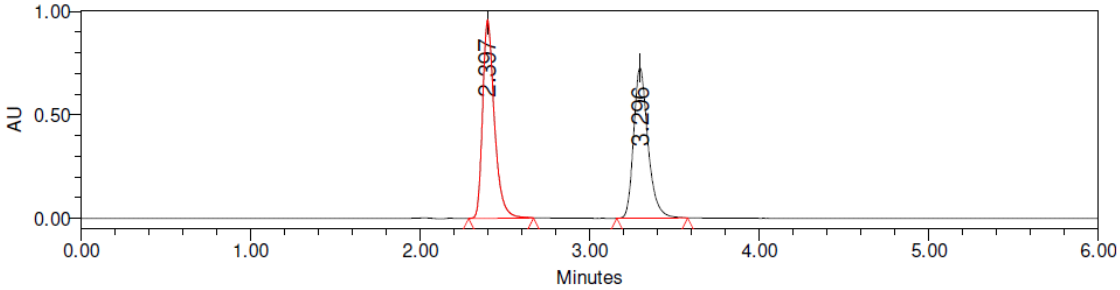
Remarks: Abacavir RT – 3.628 min, Lamivudine RT – 4.868 min. Good peak shape. Base line noise is not good. So the stated conditions are not preferred.



Retention Time	Area	% Area	Height	USP Resolution	USP Tailing	USP Plate Count
3.665	7209560	51.65	970837	-	1.46	5848
5.048	6749100	48.35	763947	6.39	1.35	7746

Figure 4: Obtained chromatogram in trail 4.

Remarks: Abacavir RT – 3.665 min, lamivudine RT – 5.048 min. Good peak shape. Still want to reduce the RT. So tried another trail.



Retention Time	Area	% Area	Height	USP Resolution	USP Tailing	USP Plate Count
2.397	4625651	51.94	955911	-	1.38	5993
3.296	4280827	48.06	725621	6.33	1.29	7584

Figure 5: Obtained chromatogram in trail 5.

Remarks: Abacavir RT – 2.397 min, Lamivudine RT – 3.296 min. Good peak shape. RT was less. System suitability values are reasonable. So these conditions are preferred.

Validating the method

Linearity

During linearity test, defined volumes were pipetted from the blended stock solution (abacavir - 3000 µg/ml and lamivudine - 1500 µg/ml) to produce solutions with concentrations varying from 150 to 450 µg/ml (abacavir) and 75 to 225 µg/ml (lamivudine) using KH_2PO_4 buffer: methanol (60:40, v/v) solvent system. The suggested technique has registered solutions' peak area. Regression analysis affirmed linearity.

Regression equation

Lamivudine: y = 21641 c - 3792; R^2 = 0.9994

Abacavir: y = 22047 c + -6292; R^2 = 0.9997

In equation, 'y' = peak area and 'c' = concentration of lamivudine/abacavir

Table 3: Lamivudine and abacavir linearity information.

µg/ml amount of lamivudine	Lamivudine peak area	µg/ml amount of abacavir	Abacavir peak area
75	1615489	150	3293564
112.5	2423659	225	4951412
150	3247841	300	6603232
187.5	4056931	375	8269696
225	4864140	450	9914563

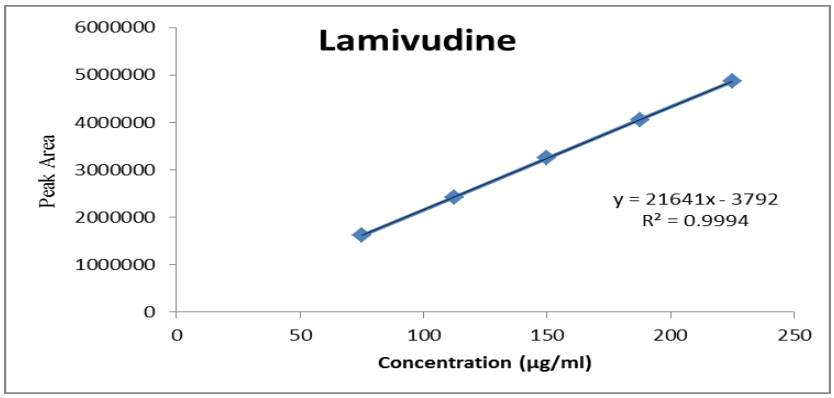


Figure 6: Linearity graph of Lamivudine.

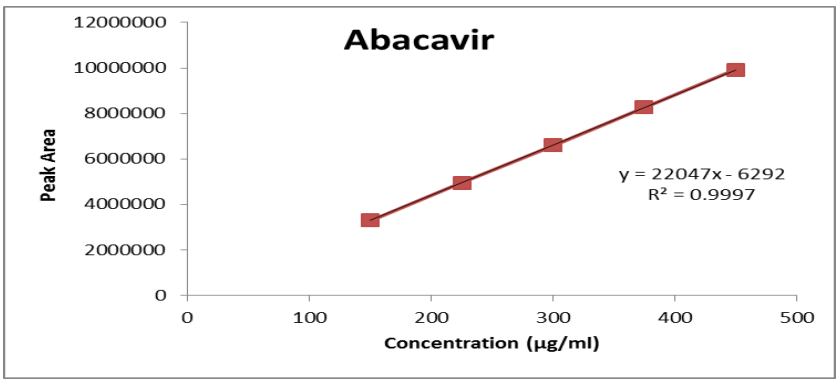


Figure 7: Linearity graph of Abacavir.

LOD – limit of detection & LOQ – limit of quantitation[12]

The formula given beneath was used to calculate the above said values for lamivudine and abacavir:

- LOD = Ratio of 3.3 times standard deviation of intercept to the slope of calibration plot
- LOQ = Ratio of 10 times standard deviation of intercept to the slope of calibration plot

The LOD and LOQ values for lamivudine were 0.228 µg/ml and 0.762 µg/ml and 0.347 µg/ml and 1.158 µg/ml for abacavir. This exhibited the suitable sensitivity of method.

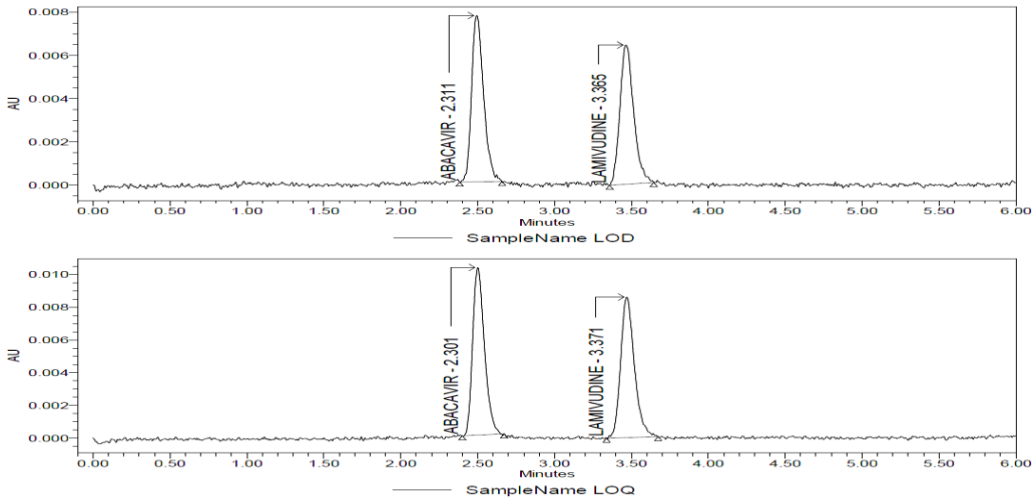


Figure 8: Obtained chromatograms in LOD & LOQ.

Selectivity[13]

Working (300 µg/ml — abacavir and 150 µg/ml — lamivudine), solvent system blank (KH_2PO_4 buffer: 60:40 ratio methanol, v/v), tablet (300 µg/ml — abacavir and 150 µg/ml — lamivudine) and placebo (only with tablet excipients) solutions were incorporated in system separately. By equating the chromatograms obtained, the specificity was evaluated. The non-interference of almost any extra peak at abacavir (3.306 min) and lamivudine (2.206 min) elution times showed specificity.

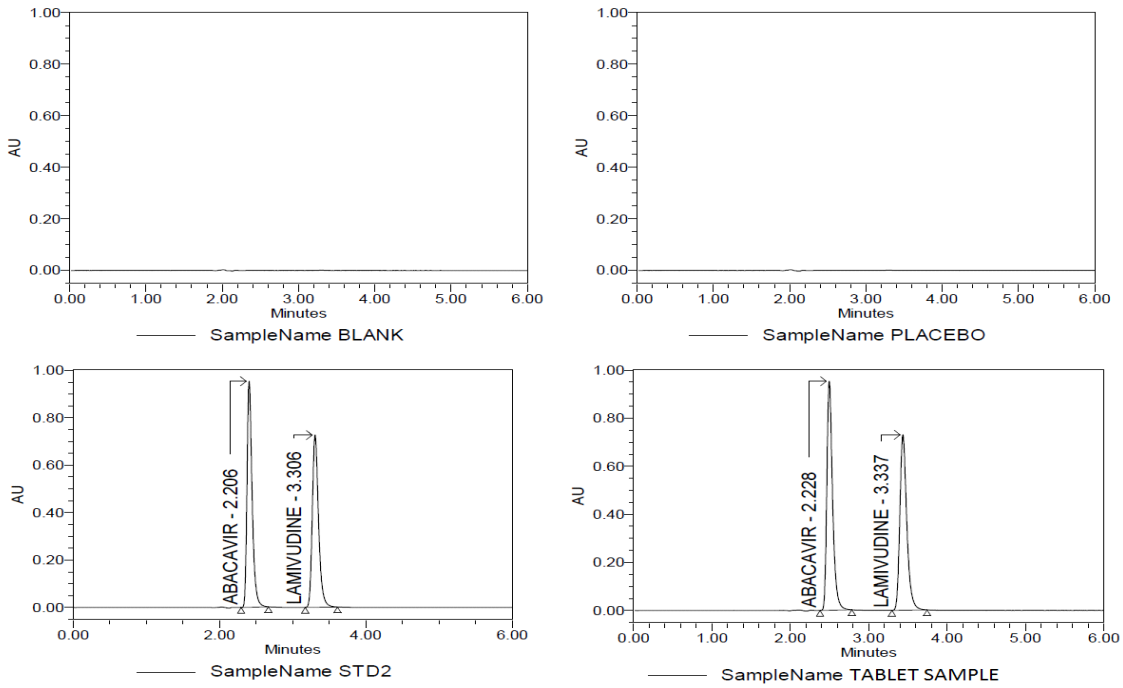


Figure 9: Obtained chromatograms in selectivity.

Precision[14]

Precision was proved through intraday research of variation. Six replicate peak region measurements of blended working solution were evaluated in this research (abacavir - 300 µg/ml and lamivudine - 150 µg/ml). By assessing the mean peak region and relative standard deviation, the outcomes of the replicate assessments were evaluated. The mean peak area of lamivudine was 3249922 and that of abacavir was 6609065. The RSD values for lamivudine and abacavir were respectively 0.175 percent and 0.105 percent. A lesser RSD < 2.0% showed precision.

Table 4: Lamivudine and abacavir precision information.

Lamivudine peak area	Verification by statistics	Abacavir peak area	Verification by statistics
3254519	**Mean:**	6601254	**Mean:**
3245648	3249922	6610203	6609065
3241254	**SD:**	6611236	**SD:**
3251477	5700.449	6601548	6961.015
3249965	**RSD:**	6610248	**RSD:**
3256669	0.175	6619898	0.105

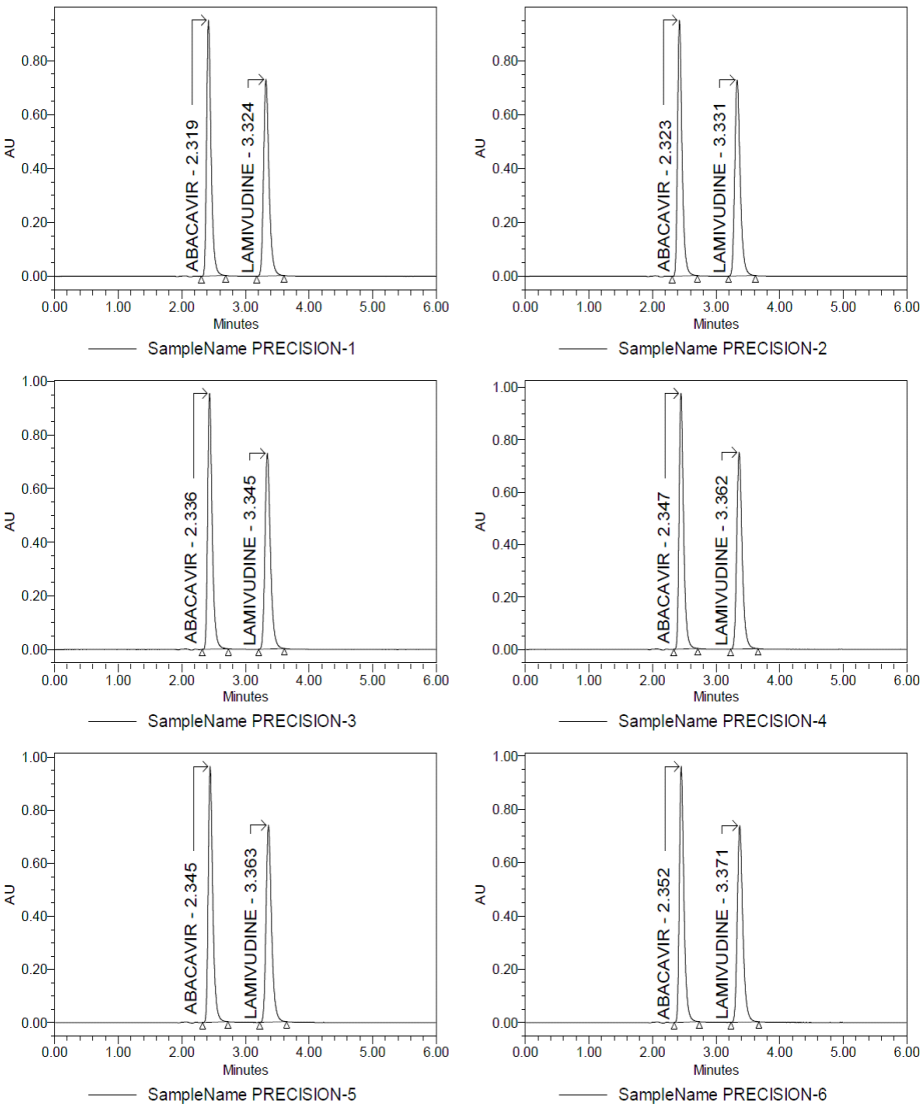


Figure 10: Obtained chromatograms in precision.

Accuracy[14]

Accuracy was appraised by assessing lamivudine and abacavir recoveries in triplicate, since spiking pure lamivudine and abacavir to the tablet sample at 50% (abacavir – 148.5 µg/ml and lamivudine – 74.25 µg/ml), 100% (abacavir - 297 µg/ml and lamivudine – 148.50 µg/ml) and 150% (abacavir – 445.50 µg/ml and lamivudine – 222.75 µg/ml) concentration levels. The excellent mean lamivudine and abacavir recoveries proved accuracy.

Table 5: Lamivudine and Abacavir accuracy information.

% Added point	Lamivudine			Abacavir		
	µg/ml amount of lamivudine appended	µg/ml amount of lamivudine determined	% Lamivudine recovered	µg/ml amount of abacavir appended	µg/ml amount of abacavir determined	% Abacavir recovered
50	74.25	74.50	100.34	148.50	148.60	100.06
50	74.25	73.91	99.54	148.50	149.04	100.36
50	74.25	74.00	99.66	148.50	148.79	100.20
100	148.50	148.76	100.17	297.00	298.04	100.35
100	148.50	148.94	100.30	297.00	298.79	100.60
100	148.50	148.10	99.73	297.00	298.13	100.38
150	222.75	223.19	100.20	445.50	447.58	100.47
150	222.75	222.89	100.06	445.50	447.94	100.55
150	222.75	222.90	100.07	445.50	447.78	100.51

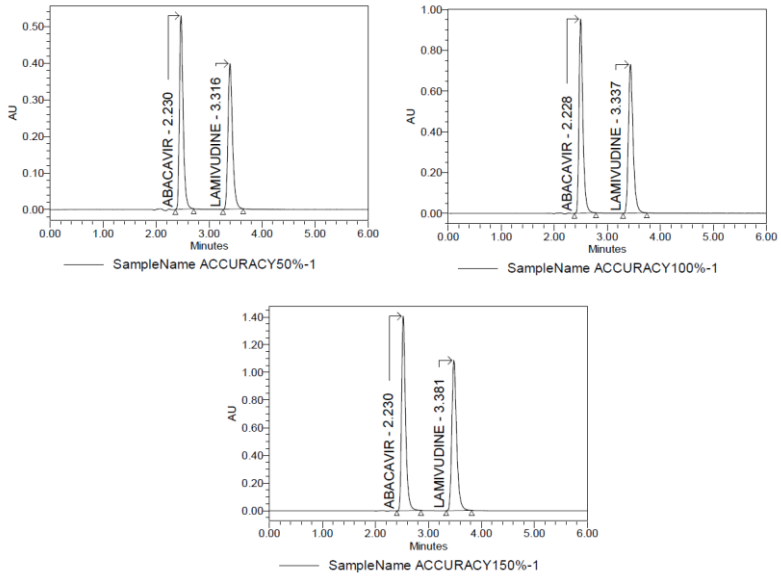


Figure 11: Obtained chromatograms in accuracy.

Robustness[14]

Robustness was assessed by examining the modification of certain HPLC circumstances (flow rate ± 0.1 ml/min; pH ± 0.1 unit; temperature ± 2°C; wavelength ± 2 nm and organic solvent ratio ±5%) on peak characteristics (resolution, plate count and tailing factor). No important changes in peak characteristics were detected in the diverse HPLC circumstances.

Table 7: Abacavir and Lamivudine robustness information.

Condition Altered	Abacavir			Lamivudine		
	Area response	Asymmetry in peak tail	Plate theoretical count	Area response	Asymmetry in peak tail	Plate theoretical count
pH - 3.3 unit	6025979	1.38	9984	3010928	1.29	8576
pH - 3.7 unit	7122748	1.38	9507	3523868	1.28	8520
Temperature - 23 (˚C)	7684856	1.33	8030	4211254	1.32	5834
Temperature - 27 (˚C)	8214578	1.38	7828	5741214	1.35	8738
Methanol fraction - 45 (%)	5421369	1.28	8446	2358746	1.30	7725
Methanol fraction - 35 (%)	7512364	1.33	9030	4201256	1.32	8834
Flow value - 0.9 (ml/min)	5612549	1.28	8446	2354581	1.30	5725
Flow value - 1.1 (ml/min)	6145891	1.29	7698	2894125	1.29	8153

Degradation study

The lamivudine and abacavir tablet solution was subjected to compelled degradation with 0.1 N NaOH in alkaline hydrolysis, 0.1N HCl in acid hydrolysis, H_2O for neutral hydrolysis, H_2O_2 - oxidation. The extra peaks were well segregated from lamivudine

and abacavir peaks throughout degradation process. There were no vital alterations in lamivudine and abacavir retention times, but variations in lamivudine and abacavir peak regions were identified. This decrease in peak area diminishment was a result of degradation product due eluted at specific retention times. The outcomes of the degradation research demonstrated the specificity and stability of the technique devised to assess lamivudine and abacavir in the environment of compounds of degradation.

Table 9: Lamivudine and abacavir stability information.

Condition	% of Lamivudine determined	% of Lamivudine degraded	% of Abacavir determined	% of Abacavir degraded
Alkali – 0.1N NaOH	95.37	4.63	96.7	3.3
Acid - 0.1N HCl	94.73	5.27	93.05	6.95
Oxidation – H_2O_2	96.46	3.54	97.2	2.8

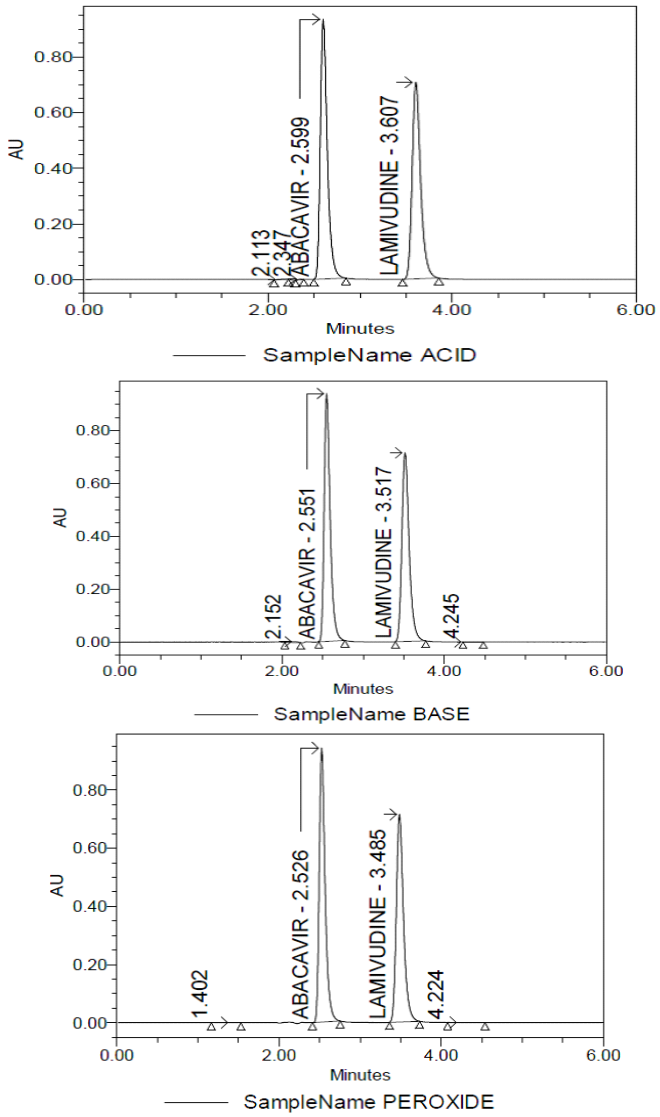


Figure 13: Obtained chromatograms in degradation.

System suitability

The thresholds of system suitability were assessed by infusing the blended working solution – abacavir 300 µg/ml and lamivudine 150 µg/ml, successively for five times. The theoretical plates, analyte resolution, peak asymmetry, relative standard peak region deviation and relative standard retention time deviation were assessed. It was confirmed that every value are inside of permitted boundaries.

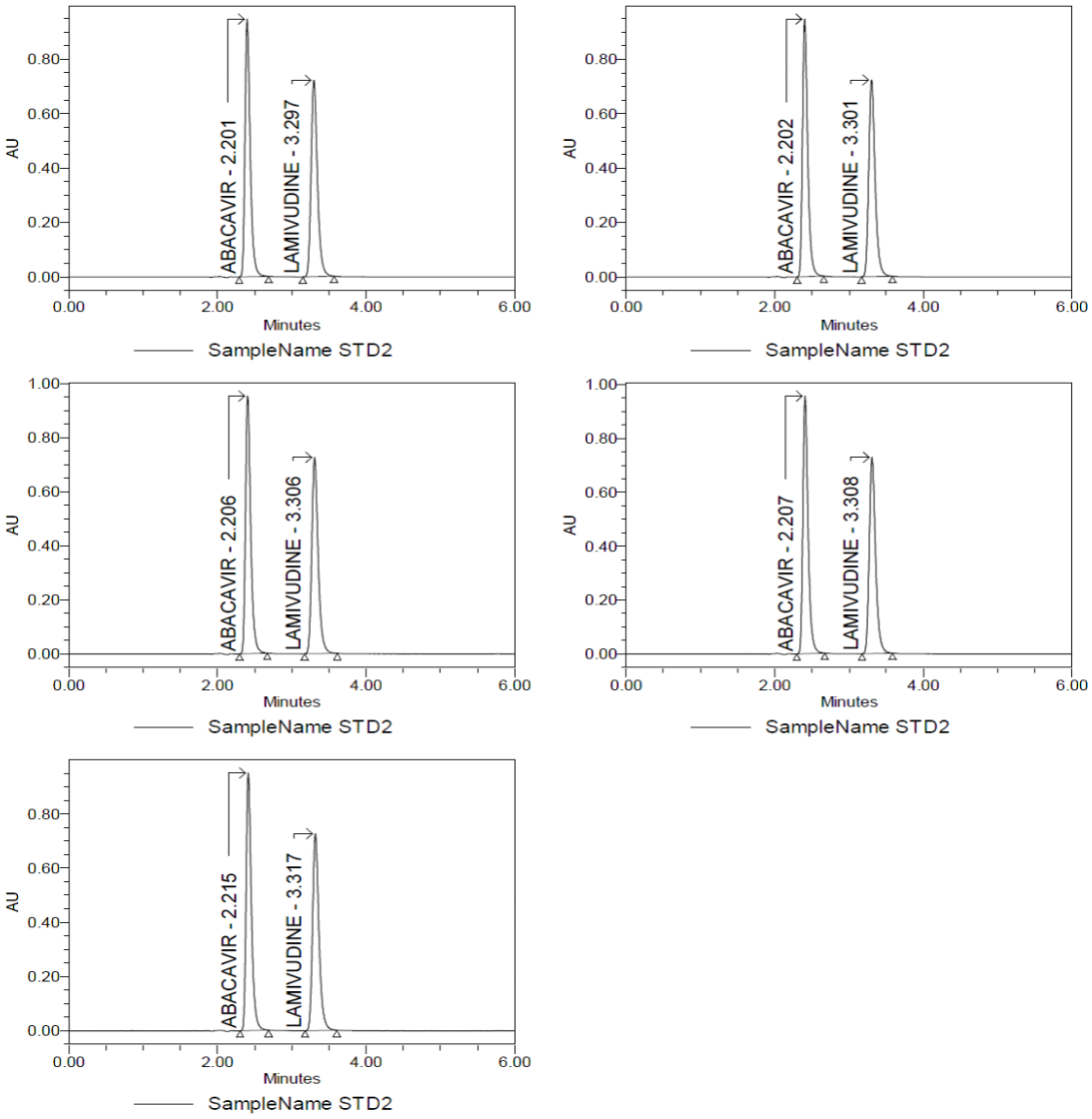


Figure 14: Obtained chromatograms in system suitability.

CONCLUSION

An easy, quick and sensitive stability demonstrating the HPLC technique was developed to quantify abacavir and lamivudine concurrently in tablet forms. The method validation outcomes are discovered to be sufficient and suitable to quantify abacavir and lamivudine simultaneously. The results of degradation research mandated the specificity and stability of the method, as well as data on abacavir and lamivudine stability. The mentioned technique was appropriate for regular assessment in quality control laboratories of abacavir and lamivudine.

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