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HPLC METHOD DEVELOPMENT AND VALIDATION OF LAMIVUDINE, DOLUTEGRAVIR AND TENFOVIR IN HUMAN PLASMA

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ABSTRACT

A simple, precised, accurate method was developed for the estimation of Lamivudine, Dolutegravir and Tenfovirdisoproxil fumarate in human plasma using the Cobicistat as internal standard by RP-HPLC (Reverse phase-High performance Liquid Chromatographic) technique. The chromatographic separation was achieved on discovery C18, 250mm x 4.6 mm, 5µ,Column at 30^0ctemperature.Separation was achieved employing a mobile phase consists of 0.1%v/v Orthophosphoric acid and Acetonitrile taken in the ratio of 65:35(v/v).The flow rate was maintained at 1.0ml/min, detection wave length was 277nm. Retention time of Lamivudine, Dolutegravir and Tenfovirwere found to be 2.994min, 4.350min and 5.688min. The peaks were found to be free of interference. The method was validated over a dynamic linear range of 60-2400ng/ml, 190-7600ng/ml, and 18-720ng/ml for Lamivudine, Dolutegravir and Tenfovir respectively, with a correlation coefficient of 0.998. The precision and accuracy of samples of six replicate measurements at lower limits of quantification level were within the limits. The analytes were found to be stable in human plasma at -28°C for 37 days.The stability, sensitivity, specificity, and reproducibility of this method make it appropriate for the determination of Lamivudine, Dolutegravir and Tenfovirin human plasma.The reported method was validated as per the US-Food and Drug Administration guidelines and found to be well within the acceptable range.

INTRODUCTION

The use of Lamivudine, Dolutegravir and Tenf ovir antiretroviral treatment has improved spect acularly in recent years.The approach works to improve the immune system and reduce pathog ens.This combination is aimed atlowering the h igh pill burden, medicinal interactions and adve rse effects on both short and long term[1].Lamivu dine is chemically known as 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one[2].Dolutegravir is chemically known as (3S, 7R)-N-[(2,4-difluorophenyl) methyl]- 11- hydroxy-7-methyl- 9, 12- dioxo- 4- oxa-1,8 diazatricyclo[8.4.0.0^{3,8}]tetradeca-10,13-diene-13-carboxamide[3].Tenofovir DF is chemically known as {[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)phosphonic acid[4].The above three drugs, antagonists of HIV / AIDS, nucleoside reverse transcriptase inhibitors and nucleotide reverse transcriptase inhibitors, were shown to be used for preventing the diagnosis of HIV / AIDS[5,6].A

review of the literature revealed that a few analytical methods like, HPLC,[7-11] methods are available for the estimation of these drugsin combination, either individually or in combination with other antiviral drugs like efavirenz.[12-15]but no method has been reported till now for the simultaneousquantitative determination of Dolutegravir, lamivudine andtenofovir disoproxil fumarate by LC-Mass spectrometry. The present work aimed to develop a simple, rapid, and accurate method for the estimation of dolutegravir, lamivudine andtenofovir disoproxil fumarate in human plasma, as per US-FDA guidelines.[16] Moreover and the present method is the first for the estimation of this combination in a biological matrix.

Fig no: 1- Structure of Lamivudine

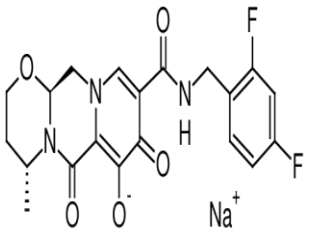


Fig no: 2 - Structure of Dolutegravir

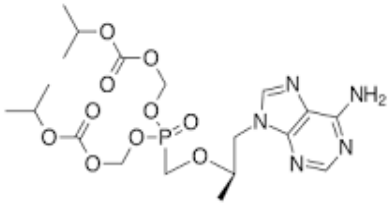


Fig no: 3- Structure of Tenofovir disoproxil fumarate

MATERIALS AND METHODS

Reagents and chemicals

The pure drug samples of Dolutegravir, lamivudine and tenofovir in were purchased from Selleckchem LLC supplied by Pro lab marketing. HPLC grade Acetonitrile, HPLC grade Methanol and all other chemicals were obtained from Merck chemical division, Mumbai. HPLC grade water obtained from Milli-Q water purification system was used throughout the study.

4. Instrumentation: Chromatography was performed with waters 2695 HPLC provided with a quaternary pump, high-speed auto sampler, column oven, degasser and 2996 PDA detector to provide a compact and with class Empower-2 software.

Chromatographic conditions: The separation was achieved by using Discovery C18 (250×4.6µ×5mm) column with a mobile phase consisting of 0.1% Ortho phosphoric acid pH (2.2): Acetonitrile in the ratio of 65:35(v/v) and flow rate was maintained at 1.0ml/min, detection wave length was 277nm. The sample dilution was carried by using water: acetonitrile (50:50) ratio as diluent

METHOD DEVELOPMENT

Preparation of internal standard: The working standard of internal standard was prepared by transferring 50 mg of Cobicistat to the 100 ml volumetric flask andthe volume was made by using diluent. From the resulting stock, 10µg/ml solution was prepared by further dilution.

Preparation of calibration and quality control solutions: The stock solutions of Lamivudine, Dolutegravir and Tenfovirwere prepared individual by dissolving 12 mg, 38mg and 3.6mg of the drug in 100 ml of diluent to obtain 120 µg/ml, 380µg/ml and 36µg/ml concentration each. The stock solutions were further diluted with diluent for spiking in plasma to obtain calibration curve standards. The spiking solutions for both analytes were prepared by transferring a varied amount to the 10mlvolumetric flasks and the volume was made by using diluent. The working concentration of Lamivudine (60 to 2400µg/ml), Dolutegravir (190-7600µg/ml) and Tenofovir(18µg/ml to 720µg/ml),Thecalibration and quality control samples were obtained by spiking 15µl of above-prepared solutions of each analyte to 750µl of plasma +500µl of internal standard, 250µl of Lamivudine, Dolutegravir and Tenfovir individually.

Sample preparation and extraction: The prepared spiking solution of analytes each

750µl of plasma +500µl of internal standard, 250µl of Lamivudine, Dolutegravir and Tenfovirindividually. To thespiked plasma, 1 ml of acetonitrile was added and vortexed for 2min. The resulting solutions were centrifuged at 3200 rpm for 5 min. The resultant organic layer was used for analysis.

Methodology: A thorough and complete method of validation was performed following the USFDA guidelines. The method was validated for system suitability, auto sampler carryover, specificity and screening of biological matrix, sensitivity, matrix effect, linearity, precision and accuracy, recovery of analyte and internal standard, ruggedness on Precision accuracy and linearity, reinjection reproducibility and stability on day zero, freeze-thaw stability, LT at-28 ℃ and LT at-80°C [15-17].

Specificity: Specificity and screening of biological matrix were assessed by usingsix blank standards and lower limit of quantification (LLOQ) levelsamples. All the samples were checked to determine the extent ofinterference contributed by plasma components with the analyte and internal standard.

Calibration curve

The Linearity of the method was determined by analysis of standardplots associated with an eight-point standard calibration curve. Theeight concentrations of the studied analytes range from Lamivudine 60 to 2400µg/ml,Dolutegravir 190µg/ml to 7600µg/ml and Tenfovir 18-720µg/ml. The calibration curve is constructed byplotting the peak area ratio of the analytes to the internal standardagainst standard concentrations.

Accuracy and precision: Intra-day precision and accuracy were evaluated at lower, middle,high and lower limit of quantification quality control samples LQC,MQC, HQC and LLOQ in six replicates for both the analytes, whileinter-day precision and accuracy were assessed for threeconsecutive days by using quality control samples. Mean valueswere obtained for calculated drug concentration over these batches.The accuracy and precision were calculated and expressed in terms of % mean accuracy and coefficient of variation (% CV), respectively

Recovery: Recovery of the analytes from the extraction procedure was performed at LQC, MQC, and HQC levels. It was evaluated by comparing peak area of extracted samples (spiked before extraction) to the peak area of un extracted samples (quality control working solutions spiked in extracted plasma).

Sensitivity: Sensitivity is defined as the lowest analyte concentrations that can be measured with acceptable accuracy and precision (13). Sensitivity was done by LLOQ level sample in six replicates to know the lowest limit of detection, the % mean accuracy and % coefficient of variation was calculated.

Stability

Stability studies were performed as zero-hour, freeze-thaw, and longterm stability at-28 °C and at-80 °C. Day zero, Long-term stability at-28°C and at-80 °C stability was carried out by using six replicates of HQCand LQC level of samples. The long-term stability of at-28 °C□}5 °C wascarried out by storing samples for 37d. The samples stored at-80 °C are thawed and analyzed immediately. The results obtained are comparedwith those obtained by freshly prepared samples. Whereas free-thaw stability was assessed by using LQC and HQC level of samples, the %mean accuracy and % coefficient of variation was calculated.

RESULTS AND DISCUSSION

Method optimization: Chromatographic conditions used are stationary phase Discovery c18 (250 x 4.6 mm, 5 µ), Mobile phase 0.1% orthophosphoric acid (pH: 2.2) : Acetonitrile in the ratio of 65:35(v/v) and flow rate was maintained at 1.0ml/min, detection wave length was 277nm,The total chromatographic runtime is 10.0 min with Retention time of Lamivudine, Dolutegravir

and Tenfovirand were found to be 2.994min, 4.350min and 5.688min.

METHOD VALIDATION

System suitability

System suitability was assessed using the MQC level sample as six homogenous injections. The % CV for retention time and response was calculated. The results are presented in Table 1. The values obtained were lower than 1%, which shows the suitability of the system for the analysis of the selected combination in human plasma. Auto-sample carryover was assessed by ULOQ and LLOQ levels to ensure that it did not affect the accuracy and precision. No carryover was observed.

Selectivity/Specificity: To establish the selectivity of the method, possible interference at the retention time of Lamivudine, Dolutegravir and Tenfovirand Internal standard due to endogenous plasma components were checked during the validation. Selectivity was performed by testing six batches of K_2EDTA blank plasma and the mass detection of extracted blank plasma gave good selectivity of both drug and internal standard. No interferences were found at the retention times of analytes and internal standard. Representative chromatograms of standard blank and blank with internal standard sample using pooled plasma. This result was shown in Fig no: 4,5,6.

Linearity:

The ratio of peak area of analyte to internal standard was used for construction of the calibration curve. The linearity of Lamivudine, Dolutegravir and Tenfovir as established by an eight-point calibration curve. The most variable regression equation of the calibration curve for Lamivudine, Dolutegravir and Tenfovir were y = 0.1179x + 0.0011,y =0.0949 + 0.0011 and y =0.169x + 0.001. The coefficient correlation (r^2) value was found consistently greater than 0.999 in all the cases.This indicating linearity of results and an excellent correlation between peak area ratios for each concentration of analytes. A representative calibration curve is shown in Figure 7-9.

Precision and Accuracy:

The precision and accuracy of the methods were assessed by analyzing six replicates of LLOQ, LQC, MQC, and HQC levels. The accuracy of the method was determined by calculating the % mean accuracy and the precision by calculating relative standard deviation. The data regarding precision and accuracy are summarized in Table 2. The chromatogram of quality control samples is shown in Figure 10-13. The % mean accuracy of Lamivudine, Dolutegravir and Tenfovirrange varied from 99.36%-100.65%, 100.04%-100.24% and 99.51%-100.00% for intraday and 98.42%-100.31, 100.12%-100.85and 98.15%-99.87 for inter day respectively. The precision (%CV) of the analytes and plasma samples were calculated and found to be 0.62%-8.74%, 0.12%-5.52% and 0.8%-10.75% for intraday and 0.82%-10.55%, 0.14%-2.72% and 0.91%-10.64% for inter day respectively.

Recovery:

Recovery was determined by measuring the peak areas obtained from prepared plasma samples with those extracted blank plasma spiked with standards containing the same area with known amount of Lamivudine, Dolutegravir and Tenfovir. The overall % mean recovery for Lamivudine, Dolutegravir and Tenfovir was found to be 98.14%,98.23% and 98.48%. The recoveries obtained for Lamivudine, Dolutegravir and Tenfovir. The overall % mean recovery for Cobicistat was found to be 98.02%. The results of the recovery study are given in Table 3. The results are within the acceptance limits.

Stabilities

The stability of the analytes in human plasma was assessed by analysis of six replicates of quality control samples at low and high concentration levels at room temperature over 24 h (bench-top stability). The measured concentrations were

compared with those of freshly prepared and processed samples. The results obtained indicated that the two drugs Lamivudine, Dolutegravir and Tenfovir were stable for at least 24 h in human plasma when retained at room temperature. On the other hand, the results obtained for quality control samples subjected to long-term storage at -28°C for 37 days and at -80°C indicate the stability of analytes in human plasma. In contrast, the freeze-thaw stability determined by using LLOQ, LQC, MQC, and HQC level of samples also indicated the stability of analytes in human plasma. The results obtained are compiled in Table 6.

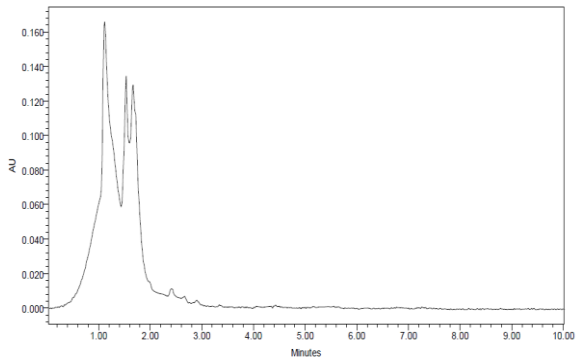


Fig. no 4: Extracted standard blank sample

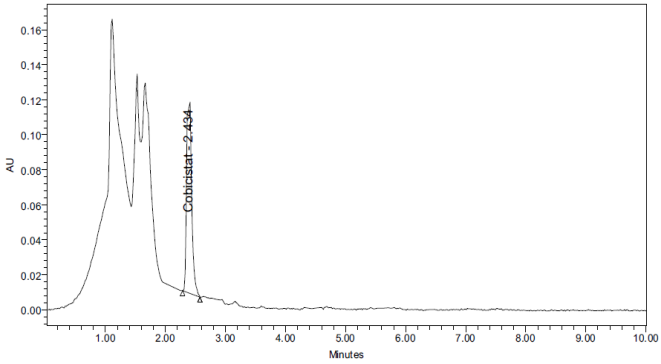


Fig no 5: Chromatogram of pure drug (Cobicistat)

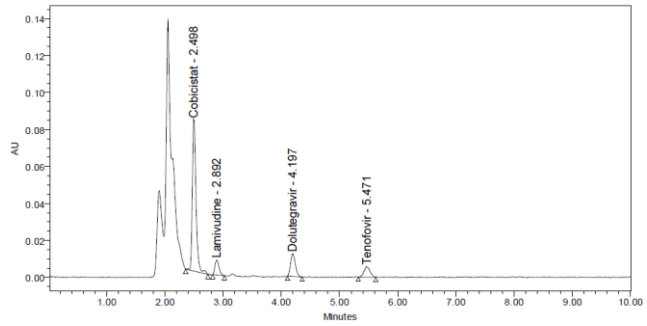


Fig no 6: Chromatogram of **Lamivudine**

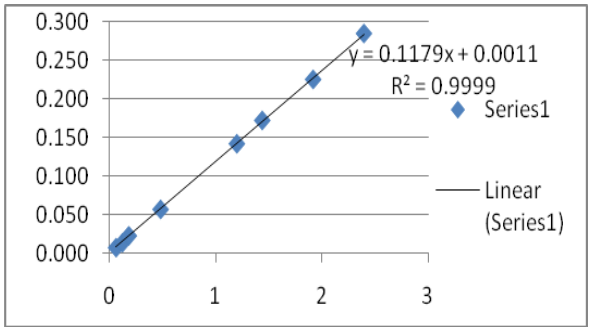


Fig no7: calibration curve of Lamivudine

Dolutegravir and Tenfovir

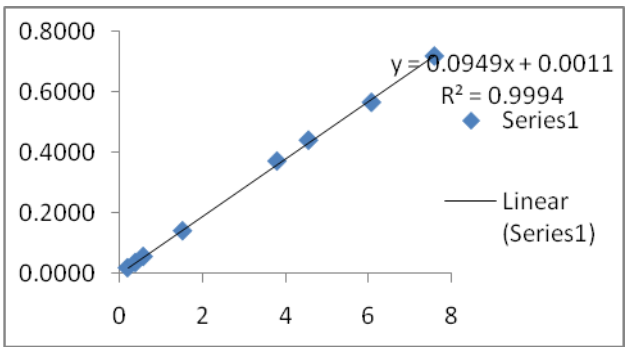


Fig no8: Calibration curve of dolutegravir

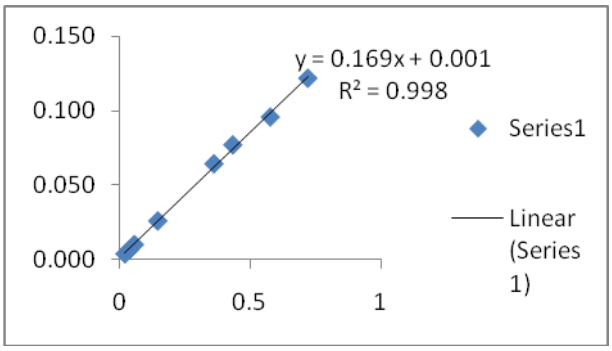


Fig no 9: Calibration curve of Tenofovir

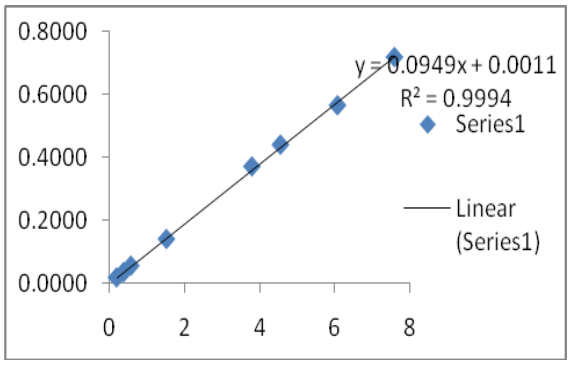
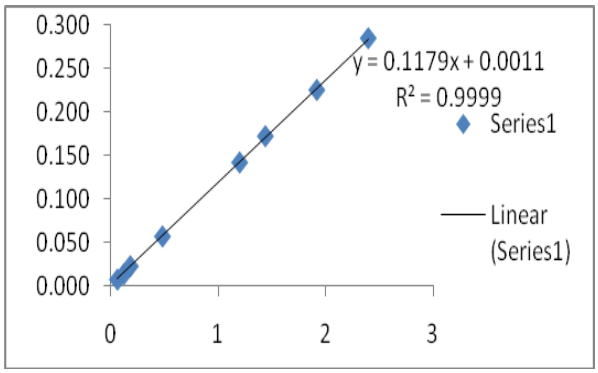
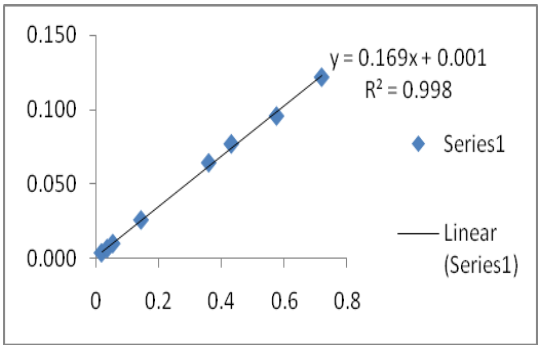


Fig no7 (a) : Linearity of Lamivudine



linearity of dolutegravir



Linearity of Tenofovir

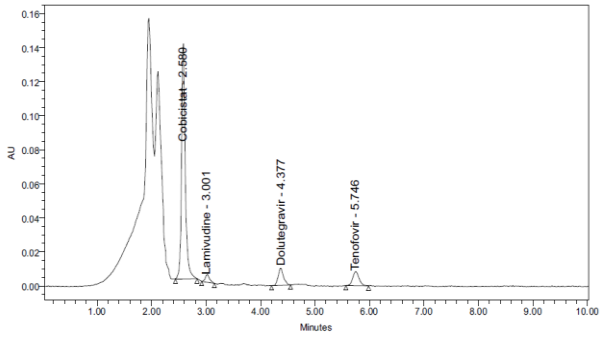


Fig 10: Optimized Chromatogram

Standard zero sample:QC-LLOQ

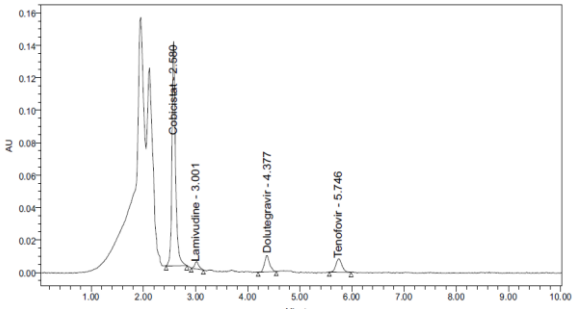


Fig no 11: chromatogram of standard Zero sample

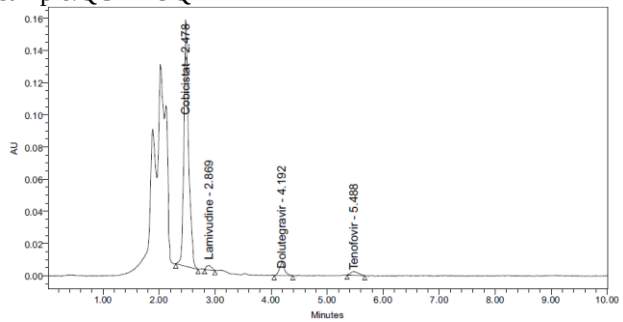
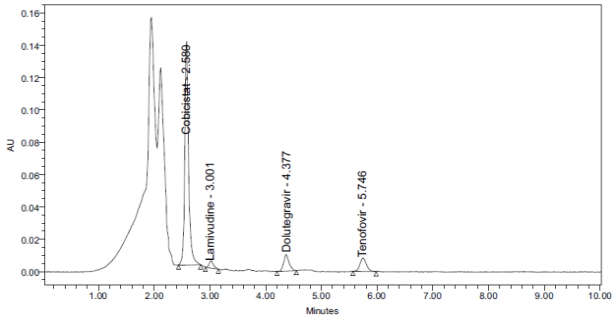


Fig no 12: chromatogram of QC-LLOQ sample Lamivudine, Tenfovir and dolutegravir

QC-LQC, QC-MQC



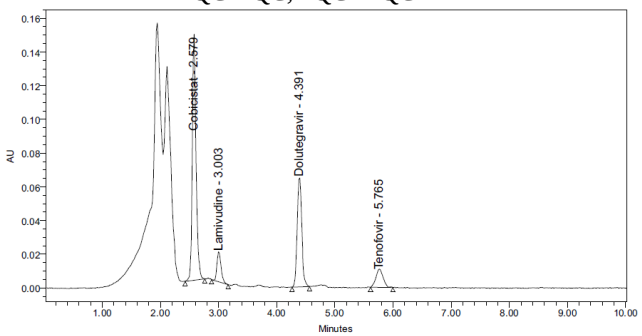


Fig no 13: chromatogram of QC-LQC sample Lamivudine, Tenfovir and dolutegravirTenfovir and dolutegravir

Fig no 14: chromatogram of QC-MQC sample Lamivudine,

QC-HQC

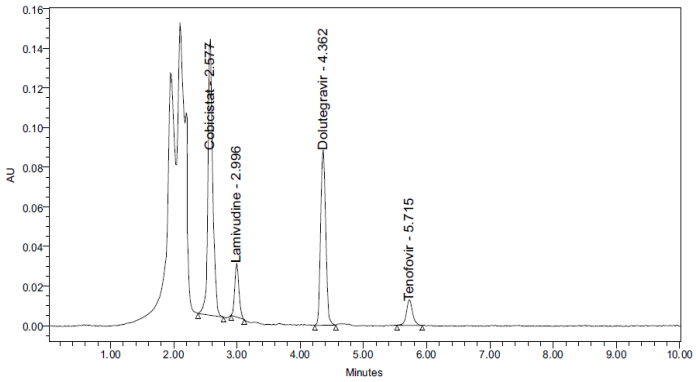


Fig no 15: chromatogram of QC-HQC sample Lamivudine,

Tenfovir and dolutegravir

METHOD VALIDATION

Table 1: System suitability of Lamivudine,Dolutegravir and tenofovir

Sample Name	Analyte RT (min)	Area Ratio	USP plate count	USP tailing	USP Resolution
Cobicistat	2.498		7952.1	1.4	
Lamivudine	4.197	0.14	7630.4	1.3	3.2
Dolutegravir	360086	0.35	11837.7	1.3	9.0
Tenofovir	69838	0.063	12831.1	1.1	7.2

Table 2:Precision&Accuracy (intra-day and inter day runs of Lamivudine,Dolutegravir and Tenfovir)

	Lamivudine				**Dolutegravir**				**Tenfovir**			
	HQC	MQC	LQC	LLOQQ	HQC	MQC	LQC	LLOQ	HQC	MQC	LQC	LLOQ
Between Batch (n=18)												
Mean	1902.0	1198.2	179.	60.2	6088.	3791.	602.3	190.2	571.	359.	52.9	17.8
SD	28.84	10.36	6.46	5.65	149.5	34.75	19.04	6.16	6.41	3.43	4.1	1.95
%CV	1.52	0.86	3.60	9.39	2.46	0.92	3.16	3.24	1.12	0.96	7.8	10.9
%MeanAccuracy	99.0	99.8	99.8	100.	100.1	99.7	100.3	100.1	99.2	99.8	98.0	99.3
Day-1(n=6)												
Mean	1907.7	1200.8	178.	60.3	6094.	3801.	603.8	190.1	573.	359.	52.8	18.0
SD	11.74	12.2	5.97	5.28	199.8	4.53	33.3	3.32	4.60	3.20	4.4	1.93
%CV	0.62	1.02	3.35	8.74	3.28	0.12	5.52	1.75	0.80	0.89	8.3	10.7
% Mean Accuracy	99.3	100.0	99.1	100.	100.2	100.0	100.6	100.0	99.5	99.8	97.9	100.
Day-2(n=6)												
Mean	1908.7	1198.3	182.	60.0	6131.	3804.	604.2	191.30	570.	359.	52.6	17.6
SD	17.74	9.806	6.04	6.33	166.8	5.38	7.44	4.92	9.27	3.48	3.82	2.3
%CV	0.93	0.82	3.32	10.5	2.72	0.14	1.23	2.58	1.63	0.97	7.27	13.2
% Mean Accuracy	99.41	99.8	101.	100.	100.8	100.1	100.7	100.68	98.9	99.8	97.4	98.1
Day-3(n=6)												
Mean	1889.7	1195.5	178.	60.1	6039.	3767.	598.9	189.3	571.	358.	53.4	18.0
SD	45.83	10.1	7.69	6.36	55.81	55.04	6.87	9.56	5.17	4.17	4.92	1.9
%CV	2.43	0.85	4.31	10.5	0.92	1.46	1.15	5.05	0.91	1.16	9.22	10.6
% Mean Accuracy	98.4	99.6	99.2	100.	99.3	99.1	99.8	99.6	99.1	99.7	98.9	100.

Table 3:Recovery – Internal standard (Cobicistat)

Acquisition Batch ID	Date	
S.No.	Un extracted Area Ratio	Extracted Area Ratio
1	552924	555317
2	557866	545537
3	568364	564914

4	552577	557846
5	564590	545219
6	556620	550566
n	6	6
Mean	558823.5	553233.2
SD	6385.43	7650.28
% CV	1.14	1.38
% Mean Recovery	99.00	

Table 4: Stability at Zero day,- 28±5^{0}C-80±5 ^{0}C for 37days (Lamivudine, Dolutegravir and Tenfovir)

	Lamivudine		Dolutegravir		Tenfovir	
	HQC	LQC	HQC	LQC	HQC	LQC
Zero day stability						
Mean	1901.01	177.1833	6132.9220	600.4012	570.2133	53.1517
SD	19.35515	6.77212	237.96174	10.17781	5.06802	4.42272
% CV	1.02	3.82	3.88	1.70	0.89	8.32
% Mean Accuracy	99.01	98.44	100.87	100.07	99.00	98.43
Stability at -28±5 °C (Long-term stability)						
Mean	1929.5000	1907.7167	5999.2450	6090.9700	569.2783	568.5817
SD	32.26608	18.83225	41.38361	187.05839	8.72013	6.37271
% CV	1.67	0.99	0.69	3.07	1.53	1.12
% Mean Accuracy	100.49	99.36	98.67	100.18	98.83	98.71
Stability at -80±5 ^{0}C (Long-term stability)						
Mean	1910.0167	1908.8500	6079.0122	6079.4100	572.5283	571.9767
SD	10.58592	11.38749	198.04827	164.70148	6.59444	7.56653
% CV	0.55	0.60	3.26	2.71	1.15	1.32
% Mean Accuracy	99.48	99.42	99.98	99.99	99.40	99.30

DISCUSSION

Since there is no reported sensitive method for the estimation Lamivudine, Dolutegravir and Tenfovir in combination, the validated LC-UV method was developed for routine analysis in a biological matrix. Moreover, the available methods were developed to assess drugs either individually or in combination. Therefore, there is a need to develop an analytical method for the estimation of this combination. The current method aims to develop a simple, accurate, and reliable method for the simultaneous estimation of Lamivudine, Dolutegravir and Tenfovir in human plasma. Good resolution and minimum tailing were achieved using this method. The method used simple single-step protein precipitation with acetonitrile and provided good selectivity when tested for peak interference from endogenous sources by comparing the blank chromatogram with quality control samples. The retention times of the internal standard, Lamivudine, Dolutegravir and Tenfovir were found to be 2.994min, 4.350min and 5.688min, respectively. The developed method proved to be rugged and had adequate recovery and no matrix effect. The recovery was determined by comparing the extracted sample with the unextracted samples at three quality control sample levels, i.e., LQC, MQC, and LLOQ. The results were found to be within acceptable limits. The linearity of the method was tested by developing an eight-point calibration curve that included all quality control sample concentrations. The linear range for Lamivudine, Dolutegravir and Tenfovir was found to be 60 to 2400 ng/mL,190 to 7600ng/ml and 18 to 720 ng/mL, respectively. The regression

coefficient for saxagliptin and dapagliflozin was 0.996 and 0.999, respectively. The linear range and statistical parameters prove that the developed method is more sensitive than the reported LC coupled with a PDA detector. Using the stability studies, it was found that the analytes were stable in plasma throughout the analysis period. The stability data were built by comparing the stability samples with freshly prepared samples. On the other hand, long-term stability was established by subjecting quality control samples to -28℃ for 37 days and to -80°C. The results obtained indicate that the method is sensitive, reliable, and cost-effective. Furthermore, the method can be made applicable to pharmacokinetic estimation.

CONCLUSION:

The proposed method for the estimation of a Lamivudine, Dolutegravir and Tenfovirbinary mixture in human plasma is simple, accurate, and reliable. The single-step protein precipitation, short runtime of 10 min, and isocratic elution make the method economical and suitable for the analysis of a large number of samples. The method has been validated as per the requirements of the US-FDA. It can therefore be concluded that the method is suitable for the routine quantification of Lamivudine, Dolutegravir and Tenfovirin human plasma.

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