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A High Sensitive Method for the Simultaneous Determination of Abacavir and Lamivudine in Human plasma by using Liquid chromatography-electro spray ionization tandem mass spectrometry and application to a pharmacokinetic study

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ABSTRACT

A rapid, specific and high sensitive liquid chromatography tandem mass spectrometry (LCMS/MS) method was developed for the simultaneous determination of Abacavir and Lamivudine in K_3EDTA human plasma. The method involves simple, solid phase extraction procedure and separation with a C18 column (5 µm, 150 × 4.6 mm) with Acetonitrile/10mM Ammonium Formate [80/20, V/V] isocratic elution at a flow-rate of 1.0 mL/min with a total run time of 3.0 minutes. Labeled isotopes were used as the internal standard. The protonate of analyte's were quantitated in positive ionization by multiple reaction monitoring with a mass spectrometer. The mass transitions 287.200/191.100 (m/z) and 230.100/112.100 (m/z) were used to measure Abacavir and Lamivudine, respectively. The method was developed and validated using 100 µL of plasma, over a concentration range of 5.005 – 4004.253 ng/mL for Abacavir and 2.506-2005.148 ng/mL for Lamivudine. The intra and inter day precision and accuracy values were found to be within the assay variability limits as per regulatory guidelines. The developed assay method was applied to a clinical pharmacokinetic study in human volunteers.

KEY WORDS: Abacavir, Lamivudine, Tandem mass spectrometry, HPLC, LC-MS/MS, Human plasma.

1. INTRODUCTION

The chemical name of the abacavir is (1S, cis)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol sulfate (salt) (2:1). The IUPAC name of the abacavir is [(1S, 4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl] cyclopent-2-en-1-yl] methanol. The molecular formula is ($C_{14}H_{18}N_6O$) and is soluble in water, methanol and buffers, fine crystal form with white in color. The mechanism of action by inhibiting the activity of HIV-1 reverse transcriptase (RT) (Rx list, Mallal, 2000; Rauch, 2006).

The chemical name of Lamivudine is 4-amino-1-[(2R, 5S)-2-(hydroxymethyl)-1,3- Oxathiolan -5-yl] -1, 2-dihydropyrimidin-2-one. Lamivudine is a synthetic nucleoside analogue and is phosphorylated intra cellularly to its active 5'-triphosphate metabolite, Lamivudine triphosphate (L-TP). This nucleoside analogue is incorporated into viral DNA by HIV reverse transcriptase and HBV polymerase, resulting in DNA chain termination (Veldkamp, 2001).

Few analytical methods have been reported individually and simultaneous for the estimation of Abacavir and Lamivudine in bulk drugs and plasma. These reported methods utilized techniques like individual method with high-performance liquid chromatography (HPLC) Abacavir (Ravitch and Moseley, 2001; Rezk, 2012; Donnerer, 2003; Lamivudine & Mandloi, 2013; Jayakar, 2012; Anantha kumar, 2010) and individual method of high-performance liquid chromatography (HPLC) with mass spectrometer (Fung, 2007; Compain, 2005) for Abacavir and individual method of high-performance liquid chromatography (HPLC) with mass spectrometer (MS/MS) (Hiren, 2009) for olmesetran. The only LC-MS method (Ashuthosh, 2008) with above combination but without labeled internal standards and lesser sensitivity. The HPLC methods have the issue of sensitivities. Remaining LC-MS/MS methods were developed individually. But no LCMS/MS method has so far been reported for the simultaneous determination of these drugs in pharmaceutical preparations as well as biological fluids with high sensitive and labeled internal standard. So this is only the method simultaneous estimation of both the analyte's in single run by using LC-ESI-MS/MS with major regulatory guidelines [Regulatory authorities, WHO, FDA, ANVISA, EMA and CDSCO].

2. EXPERIMENTAL

Material and methods: Abacavir {chemical purity 100.00 %, C14H18N6O, MW= 286.37 g/mol} and its Internal standard Abacavir D4 {chemical purity 99.97 %, C14H14D4N6O, MW= 290.36 g/mol} ((Fig.1 A & B) were obtained from SimSonPharma (Mumbai, India). Lamivudine {chemical purity 99.90 %, C8H11N3O3S, MW=229.26 g/mol} and its Internal standard Lamivudine 13C15N2 {chemical purity 99.43 %, $C7^{13}CH9N^{15}N2O4S$, MW= 246.22 g/mol} ((Fig. 1 C & D) were obtained from Clearsynth (Mumbai, India). HPLC grade solvents Acetonitrile, Methanol, Formic acid, Ammonium formate and Water were Merck products (Merck, India). Human plasma with K_3EDTA as anticoagulant was obtained from in-house clinical facility of Aizant Drug Research Solutions Pvt Ltd.

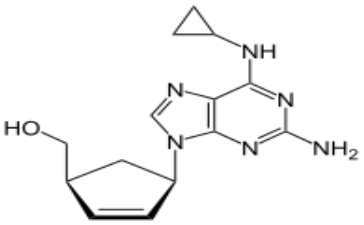
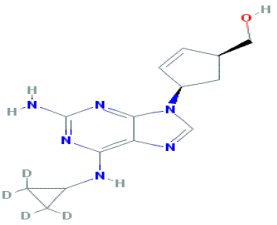
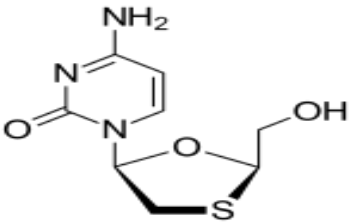


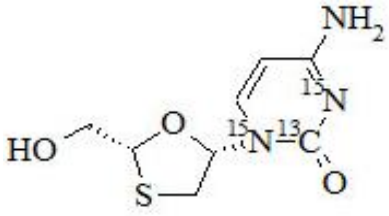
Figure.1. A. Structure of Abacavir



B. **Structure of Abacavir D4**



C. **Structure of Lamivudine**



D. Structure of Lamivudine $^{13}C^{15}N2$

Instrumentation: Liquid chromatography with tandem mass spectrometry detection was performed on a API Sciex 4000 (MS/MS) quadruple mass spectrometer equipped with an electro spray ionization (ESI) probe interfaced to a separation module Nexera XR UFLC system from Shimadzu.

Chromatographic condition: A shimadzu liquid chromatography Nexera XR system, consisting of an auto sampler, a multichannel mobile phase degasser, a column heater, two pumps and a Purospher® STAR, C18, 5µm, 150 × 4.6 mm (Merck, India), was used for the chromatographic separation of Abacavir (labeled internal standard Abacavir D4) and Lamivudine (labeled internal standard Lamivudine 13C15N2). The mobile phase used was Acetonitrile/10mM Ammonium formate [80/20, V/V]. Flow rate was set to 1.00 mL/min with 70 % flow splitting to the mass spectrometer. Column oven temperature was 40 ± 5 ºC and auto sampler temperature was 5 ± 3ºC. Volume of injection was 10 µL and runtime was 3.0 minutes.

Mass spectrometric conditions: Analyte's were detected by tandem mass spectrometry using multiple reaction monitoring (MRM) of precursor-product ion transitions with 200 ms dwell time, at 287.200/191.100 (m/z) for Abacavir, 230.100/112.100 (m/z) for Lamivudine and at 291.200/195.200 (m/z) for Abacavir D4, 233.100/115.100 (m/z) for Lamivudine 13C15N2. The instrument dependent parameters were optimized and maintained as follows: curtain gas, 30; Gas 1 and Gas 2, 55; Ion spray voltage, 5.5 kV; source temperature, 550ºC. Compound dependent parameters like Declustering Potential 80 for Abacavir and 42 for Lamivudine, collision energy 40 for Abacavir and 17 for Lamivudine, entrance potential 10 for Abacavir and Lamivudine. Data acquisition and processing were performed using Analyst software, Version 1.6.2.

Preparation of stock solution, standard and quality control samples: Stock solutions of Abacavir, Abacavir D4 and Lamivudine, Lamivudine 13C15N2 were prepared by dissolving accurately weighed standard compounds in methanol to yield a concentration of 1 mg/mL. All subsequent dilutions were made with methanol/water 50/50 v/v. Standard working solutions at concentrations of 5.005, 10.011, 40.043, 80.085, 200.213, 400.425, 1001.063, 2002.126, 3203.402 and 4004.253 ng/mL for Abacavir and 2.506, 5.013, 20.051, 40.103, 100.257, 200.515, 501.287, 1002.574, 1604.118 and 2005.148 ng/mL for Lamivudine were prepared by serial dilutions. QC working solutions at concentrations of 3185.992, 1975.315, 395.063, 197.531, 13.827, 5.005 and 10001.595 ng/mL (DQC) for Abacavir and 1603.256, 994.019, 198.804, 99.402, 6.958, 2.506 and 5020.506 ng/mL (DQC) for Lamivudine were also prepared by successively diluting the 1 mg/mL QC stock solution. The linearity curve was built by 2% spiking of drug into the screened human plasma.

The internal standard stock solution was diluted to a working concentration of 100 ng/mL for Abacavir and 500 ng/mL for Lamivudine. These working solutions were stored at 2-8ºC.

Solid-Phase Extraction (SPE) procedure:

Extracted Sample Preparation: Plasma samples frozen at below -25ºC were thawed at room temperature followed by vortexing to ensure homogeneity. For the determination of Abacavir and Lamivudine, 50 µL of ISTD working solution was transferred to polypropylene tubes followed by 100 µL of spiked plasma and vortexed for 5 seconds. To this 100 µL of extraction buffer was added and vortexed for about 10 seconds, then loaded the sample into pre-labeled Hi-Purit HLB extraction cartridges (30 mg-1 cc). Washed the cartridges with 5% methanol in water followed by hplc water, dry the cartridge with high pressure for 1 minute, eluted the cartridge with 1.000 mL of methanol and evaporated to dryness under nitrogen gas at 40 ± 5°C using TurboVap (Caliper life sciences, United States). Finally 0.800 mL of reconstitution solution was added to all the tubes and vortexed for about 1 minute. An appropriate volume of the reconstituted solution was transferred into pre-labeled autosampler vials and injected 10µL into LC-MS/MS.

Aqueous Sample Preparation: About 500 µL of mixed ISTD working solution were added into pre-labeled tubes. To this 20µL of respective spiking solution was added and vortexed, followed by 7480 µL of reconstitution solution and vortexed. An appropriate volume of the reconstituted solution was transferred into pre-labeled autosampler vials and 10µL was injected into LC-MS/MS.

Method validation: A full Method validation was performed according to guidelines set by US FDA (33). The validation of this procedure was performed in order to evaluate the method in terms of selectivity, sensitivity, linearity, precision and accuracy, recovery, matrix effect, and matrix factor, ruggedness, reinjection reproducibility, effect of potential interfering drugs, stability of analyte's during both short-term sample processing and long-term storage.

3. RESULTS AND DISCUSSION

LC-MS/MS condition optimization: The product ion spectra of Abacavir, Lamivudine and it's internal standards were obtained (Fig. 2 A to D). Several fragment ions were observed in the product ion spectra for both Abacavir and Lamivudine. The parent and fragment ion at m/z 287.200/191.100 for Abacavir, m/z 291.200/195.200 for Abacavir D4 and parent and fragment ion at m/z 230.100/112.100 for Lamivudine, m/z 233.100/115.100 for Lamivudine 13C15N2. As these ions presented a higher abundance and stability with no cross-talk effect.

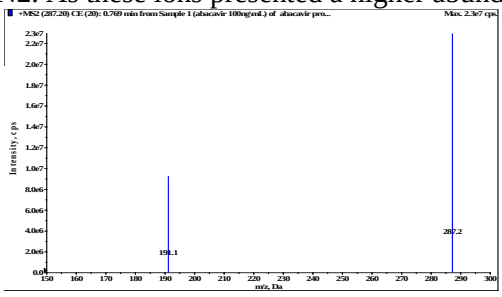
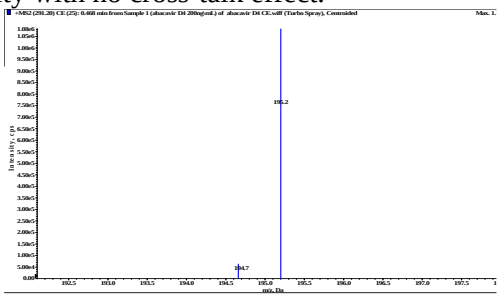
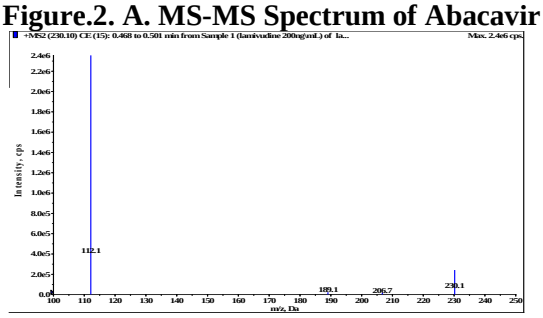


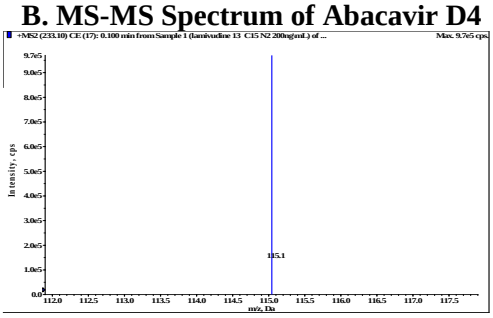
Figure.2. A. MS-MS Spectrum of Abacavir



B. MS-MS Spectrum of Abacavir D4



C. MS-MS Spectrum of Lamivudine



D. MS-MS Spectrum of Lamivudine 13 C15N2

The composition of mobile phase includes Acetonitrile/ 10mM ammonium formate. Compare to other volatile buffers ammonium formate shown better sensitivity and for better peak shape. The retention time of Abacavir was 1.61 and Lamivudine was 1.52 minutes. A representative chromatogram of double blank (A & B), standard Zero (C & D), and lower limit of quantitation (LLOQ) (E & F) and upper limit of quantitation (ULOQ) (G & H) samples were summarized in (Fig. 3)

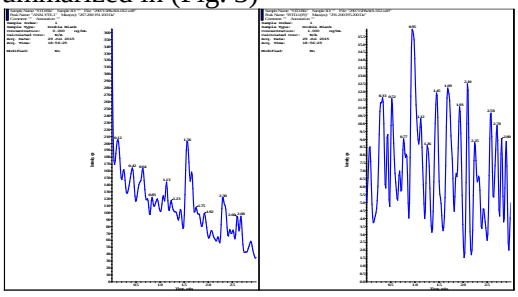
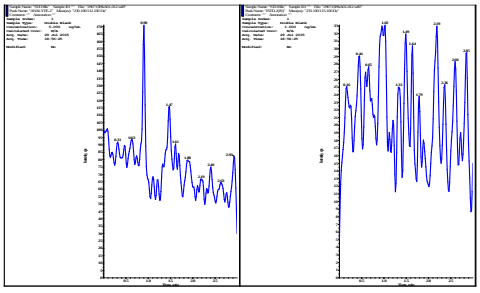
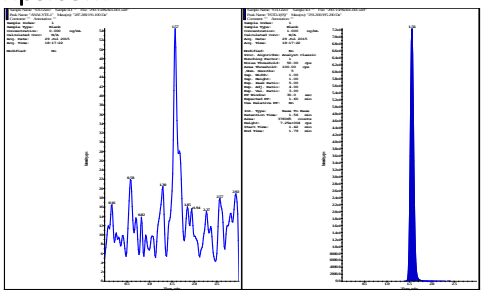


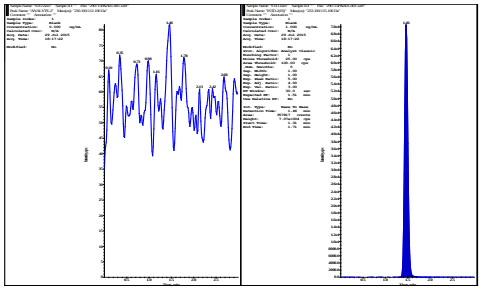
Figure.3. A. Chromatograms of STD Blk for (Abacavir, Abacavir D4)



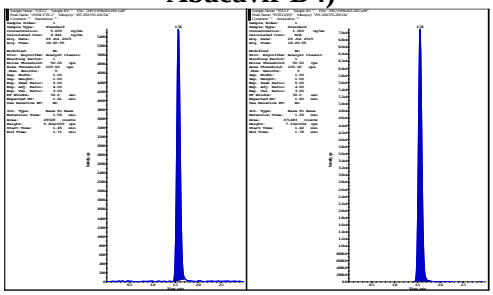
B. Chromatograms of STD Blk for (Lamivudine, Lamivudine $^{13}C^{15}N_2$)



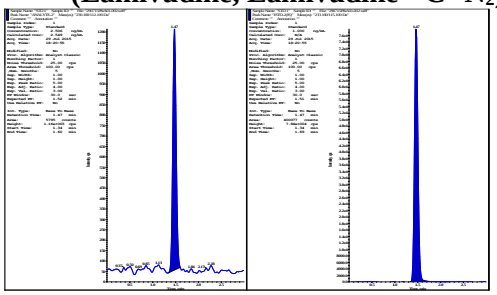
C. Chromatograms of STD Zero for (Abacavir, Abacavir D4)



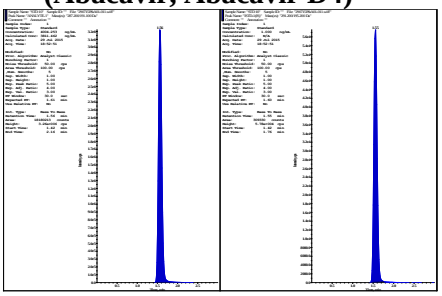
D. Chromatograms of STD Zero for (Lamivudine, Lamivudine $^{13}C^{15}N_2$)



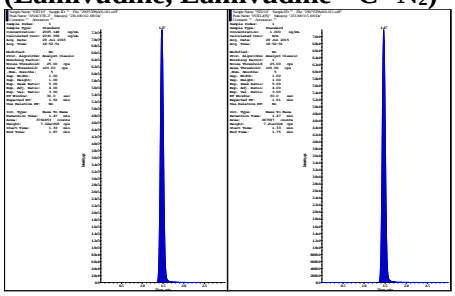
E. Chromatograms of LLOQ for (Abacavir, Abacavir D4)



F. Chromatograms of LLOQ for (Lamivudine, Lamivudine $^{13}C^{15}N_2$)



G. Chromatograms of ULOQ for (Abacavir, Abacavir D4)



H. Chromatograms of ULOQ for (Lamivudine, Lamivudine $^{13}C^{15}N_2$)

Sample preparation optimization: Solid phase extraction and liquid-liquid extraction are often used in preparation of biological samples due to their ability to improve the sensitivity and robustness of assay. Due to less recovery and matrix effect observed in liquid-liquid extraction method was developed in solid-phase extraction technique. In SPE technique extraction buffer and washing solvents plays a major role for their selectivity and extraction issues. For extraction purpose we tried ammonium acetate p^H 2.50, 0.1% Formic acid, 0.1% Acetic acid. Finally 0.1%Formic acid was chosen because of better recovery. For eluting the neutrals different washing solutions were tried and finally choosen lesser organic solutions. The Optimization of these parameters in the SPE technique made the method more sensitive, rugged, no matrix interferences and good recoveries.

Method validation parameters Carryover Effect: The carryover effect due to the auto sampler was investigated by injecting a sequence of un extracted samples consisting of RS, AQ ULOQ, RS, AQ LLOQ and extracted samples containing STD Blk, ULOQ, STD Blk and LLOQ. No significant carry over observed during this experiment.

Linearity and Sensitivity: The linearity of the method was determined (in K_3EDTA) by using a $1/x^2$ weighted least square regression analysis of standard plots associated with an Ten-point standard curve. All the three calibration curves analyzed during the course of validation were found to be linear. The correlation coefficient (r) was observed to be ≥ 0.9995 for Abacavir and 0.9996 for Lamivudine during the course of validation. The Sensitivity of the method was evaluated by analyzing six LLOQ samples. The % CV and % mean accuracy at LLOQ level were found to be 1.93 and 1016.4 for Abacavir and 3.17 and 94.41 for Lamivudine. The S/N ratio Calculated for sensitivity experiment was found to be more than 503 for Abacavir and 76 for Lamivudine.

Precision and accuracy: The precision (% CV) of the LC-MS/MS method was evaluated in K_3EDTA by analyzing 6 replicates at different concentration levels corresponding to HQC, MQC1, MQC2, MQC3, LQC, DQC and LLOQ during the course of validation.

Within Batch Precision and accuracy: The % CV of back calculated concentrations for all quality control samples concentration levels were ranged from 0.80 to 2.12. The % CV of back calculated concentration for all the samples of LLOQ was found to be 1.93. The % mean accuracy of back calculated concentrations for all quality control

samples concentration levels were ranged from 96.49 to 103.96. The % mean accuracy of back calculated concentration for all the samples of LLOQ was found to be 101.64 for Abacavir.

The % CV of back calculated concentrations for all quality control samples concentration levels were ranged from 1.04 to 4.22. The % CV of back calculated concentration for all the samples of LLOQ was found to be 3.17. The % mean accuracy of back calculated concentrations for all quality control samples concentration levels were ranged from 96.34 to 100.71. The % mean accuracy of back calculated concentration for all the samples of LLOQ was found to be 94.41 for Lamivudine.

Between Batch Precision and accuracy: The % CV of back calculated concentrations for all quality control samples concentration levels were ranged from 1.39 to 2.67. The % CV of back calculated concentration for all the samples of LLOQ was found to be 5.97. The % mean accuracy of back calculated concentrations for all quality control samples concentration levels were ranged from 96.69 to 102.99. The % mean accuracy of back calculated concentration for all the samples of LLOQ was found to be 100.77 for Abacavir.

The % CV of back calculated concentrations for all quality control samples concentration levels were ranged from 1.52 to 3.90. The % CV of back calculated concentration for all the samples of LLOQ was found to be 9.21. The % mean accuracy of back calculated concentrations for all quality control samples concentration levels were ranged from 97.11 to 101.86. The % mean accuracy of back calculated concentration for all the samples of LLOQ was found to be 99.96 for Lamivudine. The results were summarized in table 1.

Table.1. Precision and accuracy of Abacavir

Nominal concentration of Abacavir (ng/mL)	Intra-day (n=6)			Inter-day (n=6)		
	Mean	CV (%)	Accuracy (%)	Mean	CV (%)	Accuracy (%)
HQC (3185.992)	3082.2845	1.43	96.74	3091.9953	9.15	97.05
MQC1 (1975.315)	1971.5545	1.79	99.81	1967.6840	2.35	99.61
MQC2 (395.063)	401.9933	0.54	101.75	411.2550	3.07	104.10
MQC3 (197.531)	197.8865	1.22	100.18	211.2920	1.43	106.97
LQC (13.827)	14.2342	1.58	102.94	14.5410	3.38	105.16
DQC (10001.595)	10052.6273	0.92	100.51	10588.8840	1.02	105.87
LLOQ (5.005)	4.9770	3.67	99.44	4.9533	2.89	98.97

Precision and accuracy of Lamivudine

Nominal concentration of Lamivudine (ng/mL)	Intra-day (n=6)			Inter-day (n=6)		
	Mean	CV (%)	Accuracy (%)	Mean	CV (%)	Accuracy (%)
HQC(1603.256)	1565.4785	1.53	97.64	1533.1210	4.48	95.63
MQC1(994.019)	1000.2822	2.59	100.63	976.6293	4.18	98.25
MQC2(198.804)	199.4248	0.98	100.31	194.2735	3.30	97.72
MQC3(99.402)	99.1117	2.87	99.71	97.6363	2.07	98.22
LQC(6.958)	7.2075	4.41	103.59	6.6275	9.79	95.25
DQC(5020.506)	5009.6560	1.94	99.78	4918.2358	2.05	97.96
LLOQ(2.506)	2.3860	1.64	95.21	2.3268	16.02	92.85

Table.2. Recovery of analyte and ISTD

Analyte	HQC	MQC1	LQC	Over all mean accuracy	CV (%)
Abacavir	74.32	66.73	64.36	68.47	7.60
Abacavir-D4	91.19	89.77	80.14	87.03	6.91
Lamivudine	57.41	32.69	33.72	54.61	7.17
Lamivudine 13 C15N2	55.85	55.06	50.59	53.83	6.47

Table.3. Matrix effect of Abacavir

	HQC	LQC
Overall mean (%)	1.014	1.008
CV (%)	0.73	1.66

Matrix effect of Lamivudine

	HQC	LQC
Overall mean (%)	1.009	1.011
CV (%)	1.24	2.39

Recovery:

Recovery for Analyte: The % mean recoveries were determined by measuring the responses of the extracted plasma quality control samples against unextracted quality control samples at HQC, MQC1 and LQC levels. The overall %

mean recovery at HQC, MQC1 and LQC levels were found to be 74.32, 66.73 and 64.36 for Abacavir and 57.41, 52.69 and 53.72 for Lamivudine respectively. Over all recovery and % CV at all QC levels was 68.47 and 7.60 for Abacavir and 54.61 and 7.17 for Lamivudine respectively, which is within the acceptance limit of 20.00 %.

Recovery for Internal Standard: The % mean recoveries were determined by measuring the responses of internal standard in the extracted samples against unextracted samples at HQC and LQC levels respectively. The overall % mean recovery at HQC and LQC levels were found to be 91.19, 89.77 and 80.14 for Abacavir and 45.85, 45.06 and 40.59 for Lamivudine respectively. Over all recovery and % CV at all QC levels was 87.03 and 6.91 for Abacavir and 53.83 and 6.47 for Lamivudine respectively, which is within the acceptance limit of 20.00 %..

Matrix factor: Matrix factor was assessed by using six different lots (4 normal plasma, 2 hemolytic plasma and 1 lipemic plasma) of previously screened plasma. Blank samples in duplicate for each lot in each level were processed and after extraction evaporation the samples were spiked in aqueous to achieve the concentration equivalent to HQC and LQC and injected.

Unextracted samples concentration equivalent to HQC and LQC were prepared and injected. The % CV of ISTD normalized matrix factor at HQC and LQC samples was found to be 0.73 and 1.66 for Abacavir and 1.24 and 2.39 for Lamivudine. The overall % CV of ISTD normalized matrix factor from two levels (HQC and LQC) was found to be 1.05.

Stability of Analytes: Concomitant drugs was performed using 1 STD Blk by spiking concomitant spiking solution separately for each concomitant drug (Paracetamol, Caffeine, Diclofenac, Nicotine, Ondansetron, Pantoprazole and Hyoscine) and 3 samples equivalent to LLOQ using screened blank plasma and analyzed. There was no effect observed by all the above drugs on Abacavir and Lamivudine.

Stability of Analytes: The stabilities of Abacavir were investigated at two concentrations of QC samples [Low (LQC) and High (HQC) concentrations] to cover expected conditions during analysis, storage and processing of all samples. Stability was assessed by comparing the stability samples against the comparison samples with freshly prepared calibration curve. Which include the stability data from various stability exercise like auto sampler, dry extract, wet extract, bench-top, freeze thaw, blood, short term and long-term stability tests. These data were summarized in table 4.

Table.4. Stability data of Abacavir and Lamivudine

Name of the Experiment	Condition	Stability Period
Freeze Thaw Stability	-28 ± 5°C	5 Cycles
	-70 ± 10°C	5 Cycles
Bench Top Stability	Room Temperature	08 hours 04 minutes
Auto sampler Stability	5±3°C	72 hours 42 minutes
Wet Extract Stability	Room Temperature	05 hours 57 minute
	2-8°C	73 hours 11 minutes
Dry Extract Stability	Room Temperature	06 hours 04 minutes
Blood Stability (Analyte)	Room Temperature	03 hours 58 minutes
	2-8°C	03 hours 28 minutes
Short Term Stock Solution Stability for Analyte's & ISTD's	Room Temperature	06 hours 40 minutes
Short Term Spiking/Working Solution Stability for Analyte's & ISTD's	Room Temperature	06 hours 38 minutes
Long Term Stock Solution Stability for Analyte & ISTD	2-8°C	12 days 15 hours
Long Term Spiking/Working Solution for Analyte & ISTD	2-8°C	12 days 15 hours
Long Term stability in Matrix	-28 ± 5°C	36 days
	-70 ± 10°C	36 Days

Drug-Drug Reactivity: DDR experiment was performed to evaluate the effect of Abacavir in presence of Lamivudine and the effect of Lamivudine in presence of Abacavir. Screened blank sample of abacavir was spiked with Lamivudine Cmax concentration and lamivudine was spiked with Abacavir Cmax concentration for interference check. There was no drug-drug reaction observed.

Application to a pharmacokinetic study: An open-label, balanced, randomized, two-treatment, two-period, two-sequence, single dose, crossover, oral bioequivalence study of Abacavir 600mg and Lamivudine 300 mg Tablets in normal healthy adult human subjects under fed conditions. Mean plasma concentration of Abacavir and Lamivudine with corresponding pharmacokinetic parameters listed in Table.5.

Table.5. Pharmacokinetic data of Abacavir and Lamivudine

Parameters (Units)	Abacavir		Lamivudine	
	Test Product (T) n=16	**Reference Product (R) n=16**	**Reference Product (R) n=16**	**Reference Product (R) n=16**
*T_{max} (hr)	2.151	2.172	2.938	2.969
C_{max} (ng/mL)	4545.792±1341.3751	4968.67±1337.1228	2589.167±613.4258	2268.375±371.5533
AUC_{0-t} (ng.hr/mL)	15198.386±2455.6119	15490.16±3084.7168	16064.101±2316.0159	14700.7±2038.1122
AUC_{0-inf} (ng.hr/mL)	15603.209±2662.6277	15909.82±3084.7168	16314.384±2329.8377	14974.135±2057.6084
K_{el} (1/hr)	0.349±0.0794	0.336±0.075	0.133±0.00279	0.134±0.0318
$t_{½}$ (hr)	2.095±0.5038	2.157±0.4504	5.631±2.2687	5.687±2.641
AUC Ratio (%)	92.12±7.215	90.42±8.483	95.14±8.012	93.44±6.452

4. CONCLUSION

A high sensitive, simple, accurate and reproducible LC-MS/MS method has been developed and validated for the simultaneous estimation of Abacavir and Lamivudine in human plasma. This method was developed and validated using 100 µL of plasma, over a concentration range of 5.005 – 4004.253 ng/mL for Abacavir and 2.506 – 2005.148 ng/mL for Lamivudine. This is a more sensitive and less runtime than previously reported techniques with individual methods. To extract Abacavir and Lamivudine from plasma, simple SPE extraction was used. Total run time was 3.0 min only for each sample. This method provided a very simple simultaneous procedure with much better sensitivity for the determination of Abacavir and Lamivudine in human plasma. The method was successfully applied to a human pharmacokinetic study and has the potential to be useful for bioequivalence studies and routine therapeutic drug monitoring.

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