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LC Method for the Determination of Abacavir in Human Plasma for Pharmacokinetic Studies

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A simple, rapid, sensitive, selective and high performance liquid chromatography method with MS/MS was developed and validated for determination of abacavir in human plasma. Extraction from the plasma was by liquid-liquid extraction. Tenofovir was used as the internal standard. The chromatographic separation was performed by Thermo C18, 4.6 × 50 mm, 5 µm column with mobile phase comprising of (phase pH 5 adjusted with acetic acid) ammonium acetate:acetonitrile (20:80 % v/v). The assay precision ranged from 2.1 to 4.3 and accuracy between 90.3 to 104.8 %, revealing that the method has good reproducibility over the concentration range 20 to 10000 ng mL^{-1}. The lower limit of quantitation is 20 ng/mL and the recovery of analyte and internal standard (IS) were found to be 62.86-63.62 % and 60.71-62.49 %. Frequently co administered drugs did not interfere with the described methodology. The proposed validated method is suitable to support a wide range of pharmacokinetic/ bioequivalence studies.

Key Words: Method validation, Abacavir, Human plasma, Tenofovir.

INTRODUCTION

Abacavir (Fig. 1) belongs to the class nucleoside reverse transcriptase inhibitors. The agent is anabolized by a unique intracellular mechanism form carbovir triphosphate, which potently and selectively inhibits human immunodeficiency virus (HIV) reverse transcriptase[1]. The nucleoside reverse transcriptase inhibitors (NRTIs) form the backbone of current antiretroviral treatment for HIV. The first drug approved for use, zidovudine (ZDV), is a member of this class of compounds. Lamivudine (3TC) and abacavir (ABC) are also NRTIs and are frequently given in combination with zidovudine in a formulation marketed as TRIZIVIR®. All members of the NRTI class of antiretroviral agents, while differing in pharmacokinetics, toxicity and efficacy, require conversion to the triphosphate form in order to inhibit viral replication. Efficacy of this class of compounds depends on many factors including parent drug

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pharmacokinetics (absorbance, clearance, *etc.*), intracellular metabolism factors such as uptake/transport into the cell, multi-step phosphorylation and de-phosphorylation or other enzymatic processes and the activation status of the cells[1,2]. This complex series of interactions and associated variability are the reasons for the relationship between plasma parent drug concentration and intracellular triphosphate concentration is difficult to predict. Since the triphosphate form is the active form of the NRTI class of compounds it is of interest to measure the triphosphate levels in the target cells, primarily peripheral blood mononuclear cells (PBMC). Previous studies have shown that intracellular concentrations of zidovudine triphosphate (ZDV-TP) correlate with antiviral activity and immunological response to therapy[2]. Intracellular ZDV-TP in human PBMC has been measured using several techniques.

Abacavir is classified by the FDA as a pregnancy category C drug, meaning that animal studies have shown an adverse effect on the fetus and there are no well-controlled studies in humans, but potential benefits may warrant use of this drug in pregnant women despite potential risks[3]. While the use of combinations of antiviral drugs is becoming increasingly common, the impact of such combination therapies on placental transport is largely unknown. A series of studies have reported the lack of interaction between several anti-HIV drugs, suggesting passive diffusion as the primary mechanism of placental transfer[4,5]. However, the combination of abacavir and azidothymidine (AZT) was not studied. Recent studies of other antiviral compounds found substantial interactions between antiviral compounds in placental transport[6,7]. Continued study of these compounds is necessary to gain further understanding of the mechanism of placental transport for this important class of therapeutic agents. Due to ethical concerns, pregnant women are excluded from clinical trials, making it difficult to study placental and fetal distribution in humans[8]. Therefore, an animal model must be utilized that will provide clinically useful information. The pregnant rat model has been proved successful for the investigation of the basic mechanisms involved in placental transfer of nucleoside analogs due to the structural similarities between rat and human placenta[9]. The large litter size allows for serial sampling, providing a complete concentration versus time profile. The pregnant rat model has been utilized in maternal-fetal drug transfer studies of a variety of compounds, including nucleoside analogs[7,10-16].

Several HPLC methods have been developed for abacavir and azidothymidine analysis[17-20]. However, none of these methods deal with the analysis of these compounds from complex matrices such as maternal plasma, amniotic fluid, placental and fetal homogenates. Also, some of the methods use long run times and large sample volumes.

Prompted by these findings, an attempt has been made to develop a simple, sensitive method for the quantifications of Abacavir (Fig. 1) in human plasma using tenofovir (Fig. 2) as an internal standard and same might be applied to complex matrices in future to overcome the existing problems in analysis.

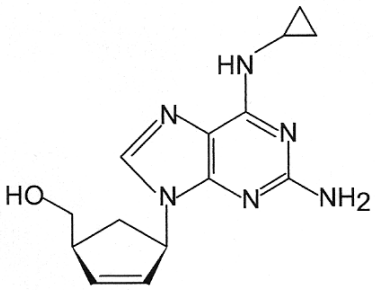


Fig. 1. Molecular structure of abacavir

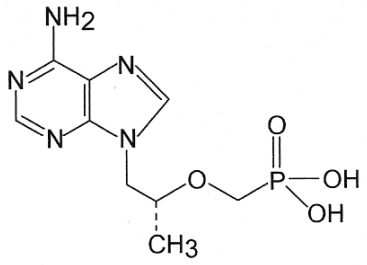


Fig. 2. Molecular structure of tenofovir

EXPERIMENTAL

All chemicals and reagents used in the study were LC grade. Acetonitrile, methanol and ethyl acetate obtained from Labscan. Formic acid was purchased from Merck (Darmstadt, Germany).Water was deionized and purified by a milli-Q water purification system from Millipore (Bedford, MA, USA). Working standards of abacovir sulphate and internal standard (IS) tenofovir disoproxil fumarate was obtained from Vardada Biotech Pvt. Ltd, (Bombay, India). Blank K_2EDTA plasma bags (six different lots) were obtained from Innovative Research, (Newark, USA) and they were stored at -20 °C until they were used for analysis.

LCMS/MS analysis was performed with prominence of Shimadzu and API 3200 of MDS Sciex. Analytical balance used AB265-S of Mettler Toledo, Micro balance MX-5 of Mettler Toledo, Filtration Setup Millipore, Shakers REAX top and Vibramax110 of Heidolph, micro pipettes and pipette tips Gilson and Axygen, Repetitive pipette and tips Handy Step Electronic of Brand, Sonicator Bandelin Sonorex (Zymark, Germani).

Chromatographic conditions

LC conditions: Chromatographic separation was performed on analytical column packed with C18, (4.6 × 50 mm, 5 μm, Thermo). The isocratic mobile phase was (phase pH 5 adjusted with acetic acid) ammonium acetate:acetonitril (20:80 % v/v) used. The injection volume was volume 5 μL and the flow rate was 0.1 mL/min.

MS/MS conditions:

Parameter	*Abacavir*	*Tenofovir*
Declustering potential (DP)	30	90
Entrance potential (EP)	10	10
Collision energy (CE)	27	40
Collision cell entrance potential (CEP)	13	18
Collision cell exit potential (CXP)	4	4
Polarity	Positive	Positive
Multiple reaction monitoring (MRM) pair (m/z)	287.10/191.20	288.10/176.20

Parameter	*Abacavir*	*Tenofovir*
Curtain gas (CUR)	25	
Collision associated dissociation (CAD)	6	
Ion spray voltage (ISV)	5000	
Heater temperature (TEM)	500	
Nebulizer gas (GS1)	40	
Heater gas (GS2)	45	
Dwell time	200 msec	
Run time	3.00 min	
Analytical column	Thermo C18, 4.6 × 50 mm, 5 µm	

Sample processing: Plasma sample specimens (50 µL) were pipetted into a glass tubes and spiked with 50 µL of internal standard solution (10 µg mL^{-1}). After adding of 50 µL of 10 % formic acid and vortexed for about 20 s in a shaker after adding 3 mL of ethyl acetate to the glass tubes, the plasma samples were then vortex-mixed for 10 min using Shakers (REAX top and Vibramax110 of Heidolph). Two phases were separated by centrifugation at 4000 rpm for 3 min. The upper organic layer was transferred into another glass tube and completely evaporated to dryness at a temperature at 40 ºC and 5 psi. The dry residue was reconstituted with 3 mL of mobile phase 20:80 (reconstitution solution), vortexed for about 30 s and transferred to auto injector vial 5 µL was chromatographed and analyzed by LC-MS/MS.

Bioanalytical method validation[21]

Calibration and quality control samples: Stock solution of abacavir was prepared in methanol. Spiking solution of different concentration for calibration curve and quality control samples were prepared from this stock solution by an adequate dilution using water/methanol (1/1 %). Calibration standards for control plasma were prepared by spiking this stock to obtain the concentration levels of 20.065, 40.129, 150.30, 500.99, 1802.1, 4505.3, 8002.4, 10003 ng mL^{-1} in human plasma. Quality control samples were prepared as bulk at a concentration of 20.163 ng mL^{-1} (LLOQ QC), 50.406 ng mL^{-1} (LQC), 3500.4 ng mL^{-1} (MQC) and 7000.9 ng mL^{-1} (HQC).

Calibration curve: A calibration curve of abacavir was constructed from a blank sample (plasma sample processed without an internal standard), a zero sample (plasma processed with internal standard) and eight non-zero samples covering the total range of 20.065-10003 ng mL^{-1} excluding lower limit of quantitation. Three samples of each concentration were measured. Linearity was assessed by least squares regression analysis. The calibration curve had to have a correlation coefficient (r^2) of 0.98 or better. The acceptance criteria for each back calculated standard concentration was 15 % deviation from the nominal value except 20 % for LLOQ.

Precision and accuracy: The within-batch precision and accuracy was determined by analyzing six replicate of quality control samples in a batch. The between-

batch precision and accuracy was determined by analyzing six replicate of quality control samples in three different batches. the quality control samples were randomized daily, processed and analyzed in position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of batch. The acceptance criteria of within and between batch precession were 20 % or better for LLOQ and 15 % or better for the rest of the concentrations and accuracy was 100 ± 20 % or better for LLOQ and 100 ± 15 % or better for the rest of concentrations.

Recovery: Recovery of the drug and internal standard was evaluated by comparing by the mean responses of six replicate of extracted low, medium and high quality control samples to the respective aqueous quality control samples with IS.

Stability studies: Bench top stability (at room temperature) of low and high quality control samples were determined by comparing the mean of back-calculated freshly thawed quality control samples with those that were kept on bench top for about 10.0 h.

The freeze thaw stability of low and high quality control samples were tested with three freezing periods, where the first storage of 24 h at below -20 ºC was followed by two additional periods of 12-24 h. The percentage of degradation was determined by comparing the mean concentrations from the three freeze thaw cycles with that of a freshly thawed quality control sample.

The process stability was assessed by six replicate low and high quality control samples after 4 h of samples were processed.

Auto sampler stability was assessed by storing six replicate low and high quality control samples in an auto sampler (10 ºC) for 48 and 28 h by reinjecting the same samples and comparing the ratio of mean concentration.

RESULTS AND DISCUSSION

The aim of present work was to develop a rapid and sensitive method for detecting for abacavir in human plasma by LC-MS/MS for pharmacokinetic studies.

Method development: For the chromatographic analysis of abacavir an attempt has been made to develop a reverse phase chromatographic method with methanol or acetonitrile as a mobile phase. Acetonitrile was used instead of methanol, because it gave better sensitivity and resolution. The amount of acetonitrile in the mobile phase at 80 %, like wise the pH of the mobile phase was optimized at pH 5 by use of acetic acid (ammonium acetate) we obtain good chromatographic separation under these conditions. Abacavir and tenofovir (IS) were observed at retention at around 1.4 min.

Selection of internal standard: Several substances were investigated for IS. Finally tenofovir was chosen as the internal standard by virtue of its similarity in chromatographic behavior and good extraction efficiency.

Calibration curves: Calibration curve was linear over the concentration range of 20 to 10000 ng mL^{-1} of abacavir. The eight-point calibration curve gave acceptable results and was used for all the calculations. The mean correlation coefficient of the

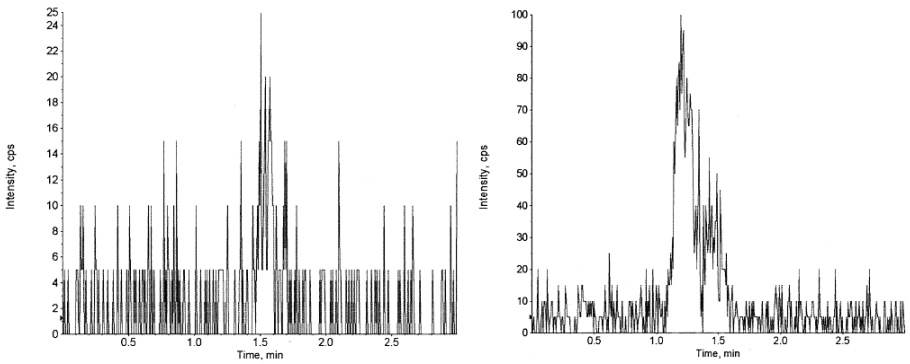
calibration curves generated during the validation was 0.999. Table-1 contains the measured precision and accuracy of back calculated concentrations of calibration samples for abacovir in human plasma. Precision of the method ranged from 2.1 to 4.3 and accuracy ranged in between 90.3 to 104.8 % and revealing that the method has good reproducibility over a wide concentration range. The calibration curve as described above was suitable for the generation of acceptable data for the concentration of abacavir validation during the validation.

TABLE-1
PRECISION AND ACCURACY DATA OF BACK-CALCULATED CONCENTRATIONS

Concentrations added (ng mL^{-1})	Concentration found (mean ± SD, n = 3) ng mL^{-1}	Precision (%)	Accuracy (%)
20.065	18.125 ± 0.69878	4.3	90.3
40.129	41.895 ± 0.98162	2.2	104.4
150.300	157.460 ± 4.19380	2.5	104.8
500.990	516.520 ± 11.17400	2.1	103.1
1802.100	1784.700 ± 38.37400	2.2	99.0
4505.300	4455.700 ± 170.31000	3.9	98.9
8002.400	7671.600 ± 185.45000	2.5	95.8
10003.000	10426.000 ± 293.12000	2.7	104.2

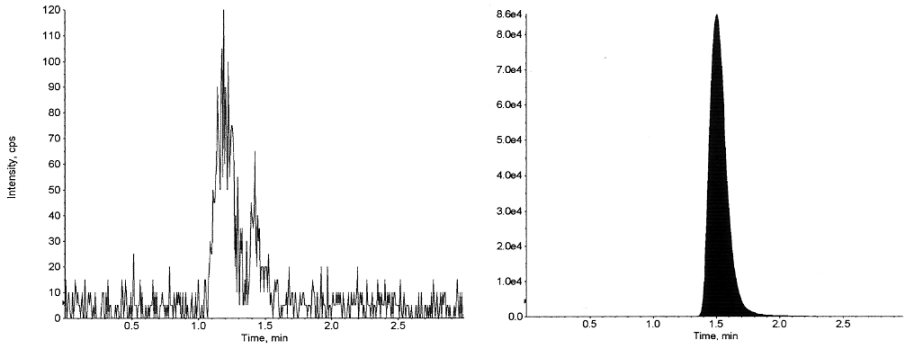
Averaged for three individual measurements at each concentration levels (n =3)
Accuracy = (Mean observed concentration) (Spiked concentration)$^{-1}$ × 100

Specificity: LCMS/MS analysis of the blank plasma and human plasma samples showed the separation of abacavir and tenofovir and no interference with either of this were observed. Hence the specificity was established by comparison with human plasma (control). Representative chromatograms of extracted blank plasma (Fig. 3), blank plasma fortified with IS (Fig. 4) are shown indicating no interference in the blank plasma and in drug free human plasma at the retention time of analyte and the IS.



Extracted blank-IS Extracted blank-Analyte

Fig. 3. Representative chromatograms of extracted blank plasma



Extracted zero standard-Analyte Extracted zero standard-IS

Fig. 4. Representative chromatograms of blank plasma and IS

Matrix effect: The matrix affects in the LC/MS/MS method was evaluated by spiking human plasma with low and high quality control samples. Six independent plasma lots are used with six samples from each lot. The percentage of nominal concentrations estimated was well within the acceptable limits. Hence the effect of matrix on estimation of drug is negligible.

Extraction recovery: Analyte recovery from a sample matrix (extraction efficiency) is a comparison of analytical response from an amount of analyte added to that determined from sample matrix. Because of basic properties of abacavir, extraction was carried out using ethyl acetate as organic solvent. Experiments with spiked compounds resulted in recoveries of analyte 62.86-63.62 % and for IS 60.26-62.49 %, as summarized in Table-2.

TABLE-2
PER CENT RECOVERY OF MEASUREMENT

Nominal concentrations ($ng\ mL^{-1}$)	%Recovery*,**	
	Drug	IS
50.406	63.62	62.49
3500.400	63.53	60.26
7000.900	62.86	60.71

*Averaged for six measurements at each concentrations (n = 6)
**Recovery = (response of aqueous) (response of plasma spiked concentration)$^{-1}$ × 100.

LLOQ QC and LQC: On the basis of signal to noise ratio (S/N) for 10, the limit of quantitation (LOQ) for abacavir was found to be 0.20 ng mL^{-1} on injection of 5 µL of sample into LCMS system as shown in Table-3. Within batch precision of LLOQ QC and LQC were 4.4 and 2.5 %, respectively. Between batch precision was 16.9 and 4.3 %, respectively. The within batch accuracy of LLOQ QC and LQC are 93.9 and 103.8 %, respectively where as between batch accuracy was found to be 99.0 and 103.7, respectively.

TABLE-3
PRECISION AND ACCURACY OF THE METHOD FOR DETERMINING ABACAVIR CONCENTRATIONS IN PLASMA SAMPLES

Concentration added (ng mL^{-1})	Within-batch precision (n = 6)			Between-batch precision (n = 6)		
	Concentration found (mean ± SD, n = 6) ng mL^{-1}	Precision (%)	Accuracy (%)	Concentration found (mean ± SD, n = 6) ng mL^{-1}	Precision (%)	Accuracy (%)
20.163	18.933 ± 0.77821	4.4	93.9	19.957 ± 3.3355	16.9	99.0
50.406	52.321 ± 1.34620	2.5	103.8	52.282 ± 2.3572	4.3	103.7
3500.4	3577.4 ± 105.870	2.9	102.2	3582.1 ± 266.34	7.3	102.3
7000.9	6846.8 ± 192.270	2.9	97.8	7012.6 ± 316.31	4.5	100.2

Averaged for six measurements at each concentrations (n = 6)
Accuracy = (mean observed concentration) (spiked concentration)$^{-1}$ × 100

Middle and upper concentrations: Middle and upper quantification levels of abacavir were 3500.4 and 7000.9 ng mL^{-1}. For within batch and between batch experiments, the precision ranged from 2.9 to 7.8, whereas accuracy ranged in between 97.8 to 102.3.

Stability: The stability of stock solutions were determined by comparing the mean of the area responses obtained from triplicate analysis of aqueous standard (1000.2 ng mL^{-1}) at 0 and 25 h. Ratio of means of area was 97.1 % for the drug and 103.4 % for the IS which is within the acceptable range of 90-110 %.

The bench top stability (at room temperature) was determined by comparing the ratio of means of the concentrations for the low and high QCs and was found to be 105.2 and 97.8 % as shown in Table-4. This was within the acceptable range of 85-115 %.

TABLE-4
STABILITY OF ABACAVIR IN HUMAN PLASMA SAMPLES

Sample concentrations (ng mL^{-1}) (n = 6)	Concentration found (mean ± SD, n = 6) ng mL^{-1}	Precision (%)	Accuracy (%)
Bench top stability (10 h)			
50.406	53.035 ± 1.6386	2.9	105.2
7000.9	6843.4 ± 193.50	2.9	97.8
Freeze thaw stability (after 4 cycles)			
50.406	49.440 ± 7.1730	14.8	98.1
7000.9	7481.6 ± 395.32	4.9	106.9
Auto sampler stability (9 h at 10 ºC)			
50.406	45.349 ± 0.47533	1.2	90.0
7000.9	6575.0 ± 467.990	7.6	93.9

The freeze-thaw stability was determined by measuring the assay and precision and accuracy of the LQC and HQC samples, which underwent four freeze thaw cycles. The stability data was used to support the repeatability of the analysis. In each freeze thaw cycle, the frozen plasma samples were thawed at room temperature

for 2-3 h and refrozen for 12-24 h. After completion of each cycle the samples were analyzed and the results were compared with that of zero cycle. The results showed that the analyte was stable in human plasma through freeze thaw cycles as shown in Table-4. The ratio of means of concentrations for the low and high QC was 98.1 and 106.9 %. This was within the acceptable range of 85-115 %. The results demonstrated that human plasma samples could be thawed and refrozen without compromising the integrity of the samples.

Stability of low and high quality control samples, after processing and its internal standard in the auto sampler provide advantage for determining a large number of plasma samples. Six sets of quality control samples (low and high) were placed into auto sampler at 10 °C. They were analyzed at once and three sets 9 h later. The ratios of means of concentrations for the low and high QC were 90.0 and 93.9 %. This was within the acceptable range of 90-110 %. A difference in response was found to be in between 0 and 9 h. For abacavir signifies the per cent change. Stability of extracted dry residues was also established over 48 h.

Conclusion

A sensitive and specific method for determination of abacavir in human plasma has been developed. The development was validated and found that the assay gave good precision and accuracy over a wide concentration range and no interference caused by endogenous compounds was observed. The limit of quantification of abacavir was 20 ng mL^{-1}. This simple, rapid and robust assay will be helpful in processing of large number of samples with minimal run time for pharmacokinetic studies of abacacir in human plasma. This method can also be extended to complex matrices such as maternal plasma, amniotic fluid, placental and fetal homogenates in future to overcome the existing problems in analysis of abacavir.

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