Development and Validation of a Selective and Rapid LC–MS–MS Method for the Quantification of Abacavir in Human Plasma

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Abstract

A simple, precise, and rapid liquid chromatography–tandem mass spectrometry method is developed and validated for the quantification of abacavir, a nucleoside reverse transcriptase inhibitor. Abacavir and granisetron (internal standard) were isolated from 100 µL human plasma by liquid–liquid extraction in ethyl acetate and dichloromethane (90:10, v/v). The chromatographic separation is achieved on Gemini C_{18} analytical column (150 mm × 4.6mm, 5-µm particle size) under isocratic conditions at a flow rate of 1.0 mL/min. The parent → product ion transitions for abacavir (*m/z* 287.2→191.2) and internal standard (*m/z* 313.1→138.2) were monitored on a triple quadrupole mass spectrometer operating in the multiple reaction monitoring (MRM) and positive ion mode. The linearity of the method for abacavir is established in the range of 29.8–9318 ng/mL with an analysis time of 2.0 min. Acceptable precision and accuracy were obtained for concentrations over the standard curves studied. The mean recovery and process efficiency of analyte obtained at three quality control levels was 86.8% and 87.9%, respectively. The application of this method for routine measurement of plasma abacavir concentration is demonstrated by a pharmacokinetic and/or bioequivalence study conducted in 28 healthy volunteers for a 300 mg tablet formulation under fasting condition.

Introduction

Abacavir is a carbocyclic 2'-deoxyguanosine nucleoside reverse transcriptase inhibitor that is used in the treatment of HIV infection. The antiviral effect of abacavir is due to its intracellular anabolite, carbovir-triphosphate (CBV-TP). It is intracellularly phosphorylated to its corresponding monophosphate, which is converted to carbovir monophosphate (CBV-MP) by cytosolic enzymes. CBV-TP is further phosphorylated to the biologically active moiety, which inhibits HIV reverse trancriptase by competing with the endogenous substrate deoxyguanosine-5-triphosphate (dGTP), leading to chain termination of the growing polynucleotide strand (1,2). Abacavir binding to plasma proteins is about 50% and is independent of the plasma abacavir concentration. It is rapidly absorbed after oral administration with peak concentrations occurring 0.63–1 h after dosing and has absolute bioavailability of about 83%. Abacavir pharmacokinetics is linear and dose-proportional over the range of 300–1200 mg/day. It is extensively metabolized by the liver; less than 2% is excreted as unchanged drug in urine (3). Abacavir is primarily metabolized via two pathways, uridine diphosphate glucuronyltransferase and alcohol dehydrogenase, resulting in the inactive glucuronide metabolite (361W94, ~36% of the dose recovered in the urine) and the inactive carboxylate metabolite (2269W93, approximately 30% of the dose recovered in the urine). The remaining 15% of abacavir equivalents found in the urine are minor metabolites, each less than 2% of the total dose. Fecal elimination accounts for about 16% of the dose. The terminal elimination half-life of abacavir is approximately 1.5 h. Abacavir is not significantly metabolized by cytochrome P450 (CYP) enzymes nor does it inhibit these enzymes. Therefore, clinically significant drug interactions between abacavir and drugs metabolized by CYP enzymes are unlikely (4,5). It is commercially available under the trade name Ziagen and in combination therapy with lamivudine (Epzicom), zidovudine, and lamivudine (Trizivir) with no clinically significant drug-drug interactions.

To meet the demands for clinical pharmacokinetic studies, a rapid, selective, and robust analytical method is highly desirable. Several analytical methods are presented to determine abacavir alone (6–10) or with metabolites (11–12) and also in combination with other drugs (13) and antiretrovirals (14–26). A reversed-phase liquid chromatographic method for the assay of abacavir and related impurities in pharmaceuticals has been presented by Seshachalam et al. (6). The method was validated over the range of 0.005–0.2 mg/mL. Uslu et al. (7) have used anodic voltammetry to determine abacavir in pharmaceuticals and serum samples. Two other methods have estimated abacavir in human plasma by high-performance liquid chromatography (HPLC)–UV with a sensitivity of 50 ng/mL (8) and 20 ng/mL (9), respectively. Both methods employed large plasma volume (≥ 300 µL) for sample preparation and long chromatographic run times (≥ 10 min). Clark and others (10) have determined abacavir in maternal plasma, amniotic fluid, and fetal and placental tissues by a polarity-switching liquid chromatography–tandem mass spectrometry (LC–MS–MS) method. The recovery of abacavir via protein precipitation with acetonitrile from different matrices ranged from 53 to 87%, and the limit of detection was

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1 ng/mL. The retention time of abacavir under gradient conditions was 5.1 min. Ravitch et al. (11) have developed an HPLC–UV assay for abacavir and its two major metabolites in human urine and cerebrospinal fluid. The lower limit of quantitation for all three analytes was ≥ 61 ng/mL in both the matrices with very long analysis time of 35 min. Similarly, Ziagen (abacavir) and its phosphorylated metabolites were determined by ion-pairing HPLC–MS–MS from human peripheral blood mononuclear cells by Fung and co-workers (12). Liquid chromatographic assay for the simultaneous determination of abacavir and mycophenolic acid from human plasma (13) and abacavir and zidovudine from rat tissues (14) have also been reported. Both these methods are less sensitive and have a long chromatographic run time of 20 min. Two other methods (15,16) describe simultaneous measurement of triphosphate metabolites of abacavir zidovudine, lamivudine, and tenofovir diphosphate in human peripheral blood mononuclear cells by LC–MS–MS. Other methods present simultaneous determination of abacavir with several antiretrovirals in human plasma by HPLC (17–20), LC–MS–MS (21,22), and MALDI-TOF/TOF (23). All four HPLC methods had a lower limit of quantitation between 10–25 ng/mL; however, the plasma volume (≥ 500 µL) used for sample preparation and the retention time (≥ 15min) for abacavir were very high. A promising method has been proposed by Jung et al. (21) to estimate abacavir and 16 other antiretroviral drugs in human plasma (50 µL) by polarity-switching LC–MS–MS. They employed a combination of liquid–liquid extraction and protein precipitation to extract all the drugs with at least 75% recovery. However, the linear dynamic range established for abacavir was very limited (1–500 ng/mL). Saux et al. (22) have quantified seven nucleoside/nucleotide reverse transcriptase inhibitors in human plasma by LC–MS. Plasma samples were precipitated by acetonitrile to obtain a mean recovery of 87.9% for abacavir. The limit of quantitation achieved was 20 ng/mL for abacavir, and the separation of all seven drugs was possible in a run time of 14 min. Notari et al. (23) have applied MALDI-TOF/TOF technology to quantify abacavir and five other antiretrovirals in the plasma (600 µL) of HIV-infected patients by standard additions analysis. The limit of quantification value was 0.01 pmol/µL for all the drugs.

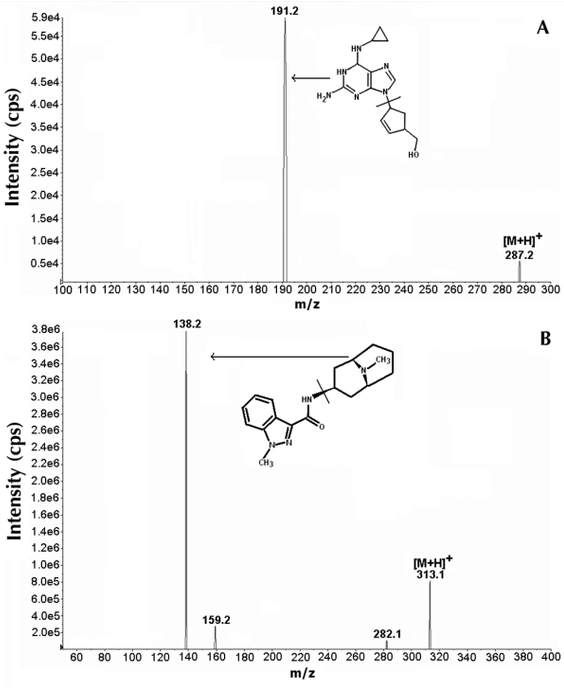

Figure 1. Product ion mass spectra of (A) abacavir (m/z 287.2 → 191.2, scan range 100–300 amu) and (B) internal standard granisetron (m/z 313.1 → 138.2, scan range 50–400 amu) in positive ionization mode.

In the present study, we have developed an accurate and rapid LC–electrospray ionization-MS–MS method for reliable measurement of abacavir in subject samples. The method is highly selective to quantify abacavir in the presence of 11 other antiretroviral agents. The validated method requires only 100 µL human plasma for liquid–liquid extraction and demonstrated excellent performance in terms of ruggedness and efficiency (2.0 min per sample). Interference due to matrix was ascertained by post-column infusion technique. It was successfully applied to a bioequivalence study in 28 healthy Indian males for 300 mg abacavir tablet formulation under fasting condition.

Experimental

Chemicals and materials

Reference standards of abacavir sulphate (99.04%) and granisetron hydrochloride (internal standard) (99.8%) were procured from Matrix Laboratories Limited (Secunderabad, India) and Samex Overseas (Ahmedabad, India), respectively. HPLC-grade methanol, acetonitrile, ethyl acetate, dichloromethane, ammonium formate, formic acid, and sodium hydroxide pellets were obtained from Merck (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at –20°C until use.

Liquid chromatographic conditions

A Shimadzu LC-VP HPLC system (Kyoto, Japan) consisting of an LC-10ADVP pump, SIL-HTc autosampler, CTO 10 ASvp column oven, and a DGU-14A degasser was used for setting the reverse-phase liquid chromatographic conditions. The separation of abacavir and granisetron was performed on a Phenomenex Gemini C_{18} analytical column (150 mm × 4.6 mm, 5-µm particle size) and was maintained at 40°C in a column oven. The mobile phase consisted of acetonitrile and 10 mm ammonium formate, pH 3.0 (70:30, v/v). For isocratic elution, the flow rate of the mobile phase was kept at 1.0 mL/min. The total chromatographic run time was 2.0 min. The autosampler temperature was maintained at 5°C. The total eluant from the column was split in 70:30 ratio; flow directed to the ISP interface was equivalent to 300 µL/min.

Mass spectrometric conditions

Ionization and detection of analyte and internal standard was carried out on a triple quadrupole mass spectrometer, MDS SCIEX API-3000 (Toronto, Canada), equipped with electrospray

ionization (TIS interface of the API 3000) and operating in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor parent → product ion (*m/z*) transitions for abacavir 287.2 → 191.2 and 313.1 →138.2 for internal standard (Figure 1).

The source dependent parameters maintained for both abacavir and granisetron were nebulizer gas: 6.0 psig; Gas 2 (heater gas flow): 7000 cc/min; ion spray voltage: 5000 V, turbo heater temperature: 350°C; collisional activation dissociation: 4 psig; and curtain gas, nitrogen: 7 psig. The optimum values for compound-dependent parameters like declustering potential, collision energy, entrance potential, focusing potential, and cell exit potential set were 80, 30, 10, 200, and 13 V for abacavir and 55, 35, 10, 3400, and 3 V for internal standard, respectively. Quadrupole 1 and 3 were maintained at unit mass resolution, and the dwell time was set at 200 ms. Analyst software version 1.4.1 was used to control all parameters of LC and MS.

Standard stock, calibration standards, and quality control sample preparation

The standard stock solution of 1000 µg/mL abacavir was prepared by dissolving its requisite amount in deionized water. Calibration standards and quality control (QC) samples were prepared separately by spiking blank plasma (2% of total volume of blank plasma) with stock solutions. Calibration curve standards were made at 29.8, 59.6, 149, 373, 932, 2330, 4659, and 9318 ng/mL concentrations, whereas quality control samples were prepared at four levels, viz. 7495 ng/mL (high quality control, HQC), 4197 ng/mL (middle quality control, MQC), 82.3 ng/mL (low quality control, LQC), and 30.4 ng/mL (lower limit of quantification quality control, LLOQ QC). Stock solution (1000 µg/mL) of the internal standard was prepared by dissolving 25 mg of granisetron hydrochloride (equivalent to granisetron) in 25 mL of methanol. An aliquot of 100 µL of this solution was further diluted to 50 mL in the same diluent to obtain a solution of 2.0 µg/mL. All the solutions (standard stock, calibration standards, and quality control samples) were stored at 2–8°C until use.

Sample extraction protocol

Prior to analysis, all frozen subject samples, calibration standards, and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 100 µL of spiked plasma sample, 50 µL of internal standard was added and vortexed for 10 s. Subsequently, 50 µL of 0.5N sodium hydroxide solution was added and vortexed for another 10 s. Further, 2.5 mL of ethyl acetate–dichloromethane mixture (90:10, v/v) was added and extracted on rotospin (rotary mixer) for 5 min at 32 × *g*. Samples were then centrifuged at 3204 × *g* for 5 min at 10°C. After centrifugation, 2.0 mL of the supernatant organic layer was transferred to an evaporation tube. The supernatant was evaporated to dryness in a thermostatically controlled waterbath maintained at 40°C under a gentle stream of nitrogen. After drying, the residue was reconstituted in 300 µL of mobile phase, and 2 µL was used for injection in the chromatographic system.

Bioanalytical method validation

A thorough and complete method validation of abacavir in human plasma was done following the U.S. FDA guidelines (24).

System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of abacavir (9318 ng/mL) and internal standard (2 µg/mL) at the start of each batch during method validation. Also, the carryover effect of the autosampler was evaluated by sequentially injecting solutions of aqueous standard (abacavir), reconstitution solution (mobile phase), standard blank, and extracted standard (abacavir) equivalent to highest standard in the calibration range.

The selectivity of the method towards endogenous plasma matrix components was assessed in twelve different batches (6 normal of K3 EDTA, 2 haemolyzed, 2 lipemic, and 2 heparinized) of blank plasma. After liquid–liquid extraction, plasma samples were chromatographed to determine the extent to which endogenous plasma components may contribute towards interference at the retention time of analyte and internal standard. The effect of potential concomitant antiretroviral drugs (nucleoside reverse transcriptase inhibitors- zidovudine, didanosine, stavudine, lamivudine, emtricitabine; non-nucleoside reverse transcriptase inhibitors- efavirenz, nevirapine; protease inhibitors- amprenavir, indinavir, ritonavir, lopinavir) was studied under the same conditions and their possible interference at the elution time of abacavir and internal standard was observed. Check for interference due to commonly used medications in human volunteers was done for paracetamol, chlorpheniramine maleate, caffeine, acetylsalicylic acid, and ibuprofen. Their stock solutions (100 µg/mL) were prepared by dissolving requisite amount in methanol. Further, working solutions (100 ng/mL) were prepared in the mobile phase, and 2 µL was injected to check any possible interference at the retention time of abacavir and internal standard.

The linearity of the method was determined by analysis of five linearity curves containing eight non-zero concentrations. The ratio of area response for abacavir to internal standard obtained from MRM was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression which was finalized during pre-method validation. Back-calculations were made from these curves to determine the concentration of abacavir in each calibration standard. A correlation coefficient (r^2) value > 0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was accepted as the LLOQ, if the analyte response was at least five times more than that of drug-free (blank) extracted plasma. In addition, the analyte peak of LLOQ sample should be identifiable, discrete, and reproducible with a precision (percent coefficient variation, %CV) not greater than 20 and accuracy within 80–120%. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±20%.

For determining the intra-assay accuracy and precision, replicate analysis of plasma samples of abacavir was performed on the same day. The run consisted of a calibration curve and six replicates of LLOQ QC, LQC, MQC, and HQC samples. The inter-assay accuracy and precision were assessed by analyzing five precision and accuracy batches on five consecutive validation days. The deviation at each concentration level from the nominal concentration was expected to be within ±15%, except for LLOQ which it should be within ±20%. Similarly, the mean accuracy should not deviate by ±15%, except for LLOQ where it can be ±20% of the nominal concentration.

Ion suppression/enhancement effects on the MRM LC–MS–MS sensitivity were evaluated by the post-column analyte infusion experiment. A standard solution containing abacavir (at MQC level) and granisetron (internal standard) was infused post-column via a 'T' connector into the mobile phase at 10 µL/min employing in-built infusion pump. Aliquots of 2 µL of extracted control plasma were then injected into the column by the autosampler, and MRM LC–MS–MS chromatogram was acquired for abacavir. Any dip in the baseline upon injection of double blank plasma (without internal standard) would indicate ion suppression, whereas a peak at the retention time of abacavir and internal standard indicates ion enhancement.

The relative recovery, matrix effect, and process efficiency (%PE) were assessed as recommended by Matuszewski et al. (25). All three parameters were evaluated at HQC, MQC, and LQC levels in triplicate. Relative recovery (RE) was calculated by comparing the mean area response of extracted samples (spiked before extraction) to that of unextracted samples (spiked after extraction) at each QC level. The recovery of internal standard was similarly estimated. As per the acceptance criteria, the recovery of the analyte need not be 100% but should be consistent, precise, and reproducible. Absolute matrix effect (ME) was assessed by comparing the mean area response of unextracted samples (spiked after extraction) with mean area of neat standard solutions. The overall %PE was calculated as (ME × RE)/100. Further, the effect of plasma matrix on analyte quantification was also checked in five different batches/lots of plasma. From each batch, five samples at HQC and LQC levels were prepared (spiked before extraction) and checked for the %accuracy and precision (%CV). The deviation of the standards should not be more than ±15%, and at least 90% of the lots at each QC level should be within the aforementioned criteria.

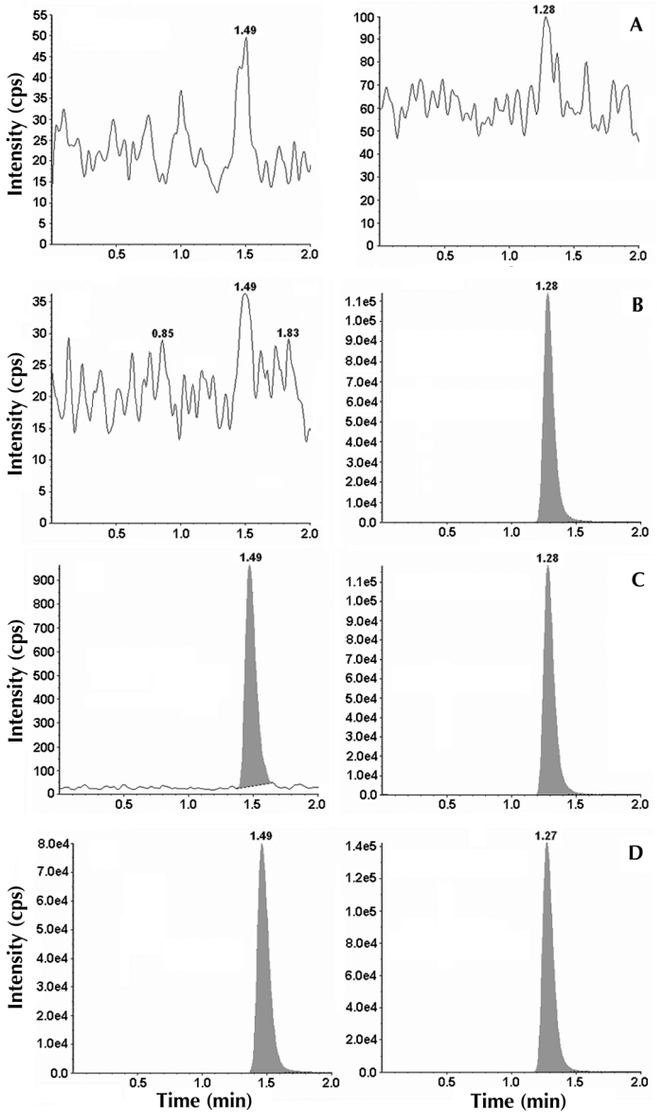

Figure 2. MRM ion chromatograms of (A) double blank plasma (without internal standard), (B) blank plasma with internal standard, (C) abacavir at LLOQ (*m/z* 287.2 → 191.2) and internal standard, and (D) real subject sample at 0.7 h after administration of 300 mg dose of abacavir.

Stability experiments were carried out to examine the analyte stability in stock solutions and in plasma samples under different conditions. Short-term stability at room temperature and long-term stock solution stability at –20°C and –50°C was assessed by comparing the area response of stability sample of analyte and internal standard with the area response of sample prepared from fresh stock solutions. The solutions were considered stable if the deviation from nominal value was within ±10%. Autosampler (wet extract) stability, bench top stability, dry extract stability, and freeze-thaw stability were performed at LQC and HQC, using six replicates at each level. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ±15%.

To authenticate the ruggedness of the proposed method, it was performed on two precision and accuracy batches. The first batch was analyzed by different analysts while the second batch was studied on two different columns. Dilution integrity experiment was evaluated by diluting the stock solution prepared as spiked standard at concentration 20,255 ng/mL in the screened plasma. The precision and accuracy for dilution integrity standards at 1/5 (4051 ng/mL) and 1/10 (2025.5 ng/mL) dilution were determined by analyzing the samples against calibration curve standards.

Bioequivalence study design

The design of the study comprised of "an open-label, balanced, randomized, two-treatment, two-period, two-sequence, single dose, crossover bioequivalence study of test formulation of abacavir sulphate (300 mg tablets of Matrix Laboratories Limited) and a reference formulation Ziagen® (300m g tablets of GlaxoSmithKline, Durham, NC) in 28 healthy adult human subjects under fasting conditions." Each subject was judged to be in good health through medical history, physical examination, and routine laboratory tests. Written consent was taken from all the subjects after informing them about the

objectives and possible risks involved in the study. An independent ethics committee constituted as per Indian Council of Medical Research (ICMR) approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and U.S. FDA (26). The subjects were orally administered a single dose of test and reference formulations after a recommended wash-out period of three days with 240 mL of water. Blood samples were collected at 0.0 (pre-dose), 0.18, 0.33, 0.5, 0.67, 0.83, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, and 12.0 h after oral administration of the dose for test and reference formulation in labeled EDTA-vacuettes. The maximum volume of blood drawn during the entire study was 287 mL, which included (other than for measurement) up to 10 mL for screening, about 10 mL for post-study safety assessment (hematology and biochemical tests) while 0.5 mL of heparinized blood was discarded prior to each sampling through venous cannula. Plasma was separated by centrifugation and kept frozen at –20°C until the completion of period and then at –50°C until analysis. During the study, subjects had a standard diet, and water intake was free. The samples were processed based on the proposed extraction protocol for quantification of abacavir. The pharmacokinetic parameters were compared by using general linear model ANOVA. The value of $p \leq 0.05$ was considered statistically significant for all the tests. An incurred sample re-analysis (assay reproducibility test) was also conducted by computerized random selection of 20 subject samples. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the value should not be more than ±20% (27).

Results

Assay performance and validation

Throughout the method validation, the %CV of system suitability test was observed in the range of 0.07–0.28 for the retention time of analyte and the internal standard, whereas 0.33–3.5% for the response of drug and its internal standard, which is not more than 4% as per the acceptance criteria.

Carryover evaluation was performed in each analytical run to ensure that it does not affect the accuracy and the precision of the proposed method. There was practically no carryover observed during autosampler carryover experiment. No enhancement in the response was observed in double blank after subsequent injection of highest calibration standard (aqueous and extracted) at the retention time of abacavir and internal standard, respectively. Moreover, no ghost peaks appear during the analysis of blank samples.

The calibration curves were linear over the concentration range of 29.8–9318 ng/mL. A straight-line fit was made through the data points by least square regression analysis, and a constant proportionality was observed. The mean and standard deviation value for slope, intercept, and correlation coefficient (r^2) observed were 0.0002 and 0.0000; –0.0002 and 0.0001; 0.9983 and 0.0006, respectively. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 91.0 to 105.4% and 0.9 to 3.7, respectively. The LLOQ of 29.8 ng/mL was measured at a signal-to-noise ratio (S/N) of ≥ 100. Thus, it was possible to lower the LLOQ ten-fold; however, the limit was adequate to quantify clinical concentrations of abacavir. The detailed results are presented in Table I.

Representative MRM ion chromatograms in Figure 2 of extracted blank human plasma (double blank), blank

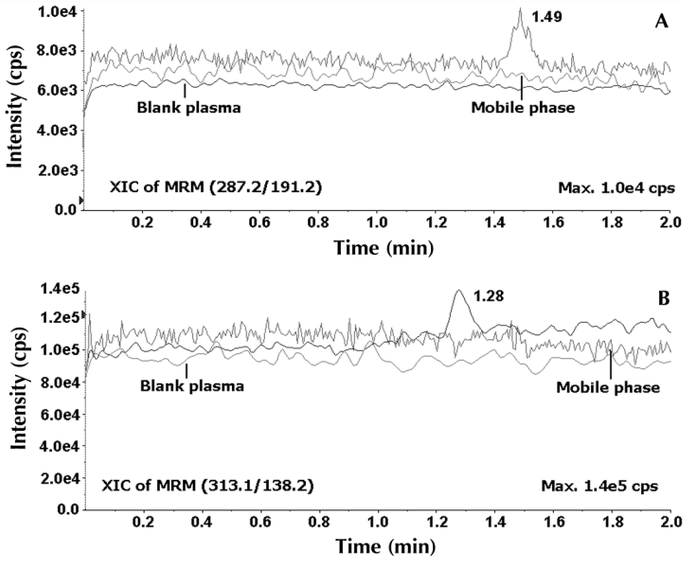

Figure 3. Post-column analyte infusion experiment with overlaid MRM LC–MS–MS chromatograms for (A) abacavir at LLOQ and (B) granisetron.

Table I. Summary of Calibration Curve with Back-Calculated Concentration for Abacavir

ID	STD-1	STD-2	STD-3	STD-4	STD-5	STD-6	STD-7	STD-8	Regression Parameters		
	Nominal concentration (ng/mL)										
No.	29.8	59.6	149	373	932	2330	4659	9318	Slope	Intercept	r^{2*}
1	29.1	60.8	156	388	997	2355	4397	8334	0.0003	–0.0001	0.9978
2	29.4	59.7	156	386	967	2347	4477	8641	0.0003	–0.0001	0.9989
3	29.9	58.6	151	383	956	2317	4837	8518	0.0002	–0.0003	0.9990
4	29.5	59.7	152	393	970	2397	4626	8549	0.0003	–0.0004	0.9980
5	29.6	59.0	153	414	954	2327	4510	8320	0.0002	–0.0002	0.9976
Mean	29.5	59.6	154	393	969	2349	4569	8472	0.0002	–0.0002	0.9983
SD*	0.3	0.9	2.0	12.6	17.1	30.9	171	191	0.0000	0.0001	0.0006
% CV*	0.9	1.6	1.3	3.2	1.8	1.3	3.7	2.3			
% Nom.	99.0	99.9	103.3	105.4	104.0	100.8	98.1	91.0			

* CV = coefficient of variance; SD = standard deviation; r^2 = correlation coefficient.

plasma fortified with internal standard (*m/z* 313.1 →138.2), abacavir at LLOQ (*m/z* 287.2 → 191.2), and an actual subject sample at 0.7 h demonstrates the selectivity of the method. The extraction procedure together with mass detection gave very good selectivity for the analysis of abacavir and internal standard in the blank plasma. No endogenous interferences were found at the retention times of abacavir (1.49 min) and internal standard (1.28 min) in the blank plasma. None of the concomitant antiretroviral drugs studied or the commonly used medications by human volunteers showed interfering signals at the retention time of abacavir or the internal standard.

The intra-batch and inter-batch precision and accuracy were established from validation runs performed at HQC, MQC, LQC, and LLOQ QC levels (Table II). The intra-batch precision was 2.9% and the accuracy 98.0% for abacavir at the LLOQ QC level. For the inter-batch experiments, the precision ranged from 3.8 to 10.8%, and the accuracy was within 95.8 to 103.4%.

Result of post-column infusion experiment in Figure 3 indicates no ion suppression or enhancement at the retention time of abacavir and internal standard, as evidenced from the flat baseline. The relative recovery, absolute matrix effect and process efficiency data at LQC, MQC, and HQC levels is presented in Table III. The recovery for internal standard in human plasma was 98.7%. Further, the extent of matrix effect in different lots of plasma (spiked before extraction) was within the acceptable limit as evident from the precision (%CV) values in Table IV.

The stability of the abacavir and internal standard in human plasma and stock solutions was examined under different storage conditions. For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time, exceeding that encountered during the routine sample preparation (6 h). The drug and internal standard stock solutions were stable for a minimum of 7 days at refrigerated temperature below 8°C. Abacavir in control human plasma (bench top) at room temperature was stable at least for 6 h at 25°C and for a minimum of three freeze-and-thaw cycles at –20°C. Spiked plasma samples stored at –20°C and –50°C for the long-term stability experiment were found stable for a minimum period of 65 days. The dry extract of the spiked quality control samples stored at –20°C was stable up to 24 h. Autosampler stability (wet extract) of the spiked quality control samples maintained at 5°C was determined up to 27 h without significant drug loss. Different stability experiments in plasma at two QC levels with the values for %change is shown in Table V.

The dilution integrity experiment was performed with an aim to validate the dilution test carried out on higher analyte concentration above the ULOQ, which may be encountered during real subject sample analysis. The precision for dilution integrity of 1/5 and 1/10 dilution were 1.4 and 3.3%, while the accuracy results were 107.8 and 103.7%, respectively, which is within the acceptance limit of 15% for precision (%CV) and 85 to 115% for accuracy.

Method ruggedness was evaluated using re-injection of analyzed samples on two different columns of the same make and also with different analysts. The precision (%CV) and accuracy values for two different columns ranged from 2.1 to 5.1% and 100.8 to 104.9%, respectively, at all four quality control levels. For the experiment with different analysts, the results for precision and accuracy were within 1.97–7.7% and 93.8–107.0%, respectively, at the four levels.

Table II. Intra- and Inter-batch Precision/Accuracy for Abacavir

QC ID	Nominal conc. (ng/mL)	n*	Mean conc. observed (ng/mL)†,‡	%CV*	%Accuracy
Intra-batch					
HQC	7495	6	7015	2.0	93.6
MQC	4197	6	4040	1.4	96.3
LQC	82.3	6	83.1	2.2	101.0
LLOQ QC	30.4	6	29.8	2.9	98.0
Inter-batch					
HQC	7495	30	7180	10.8	95.8
MQC	4197	30	4061	4.7	96.8
LQC	82.3	30	85.1	3.8	103.4
LLOQ QC	30.4	30	30.6	5.5	100.6

* n = total number of observations and CV = coefficient of variance.
† Intra-batch = Mean of six replicates at each concentration;
‡ Inter-batch = Mean of six replicates for five precision and accuracy batches.

Table IV. Matrix Effect Data of Abacavir in Five Different Lots of Human Plasma at HQC and LQC Level

	HQC 7495*		LQC 82.3*	
Lot no.	Calculated conc. (ng/mL)	% Accuracy	Calculated conc. (ng/mL)	% Accuracy
1	6877	91.8	83.0	100.9
2	7291	97.3	84.6	102.8
3	7127	95.1	86.0	104.6
4	7843	104.6	85.9	104.5
5	7723	103.0	90.5	110.0
Mean		98.4		104.5
SD		5.4		3.4
%CV		5.5		3.2

* Nominal concentration (ng/mL).

Table III. Relative Recovery, Process Efficiency, and Absolute Matrix Effect for Abacavir (n = 3)

Abacavir	A* (%CV)	B† (%CV)	C‡ (%CV)	Absolute matrix effect (%ME)§	Relative recovery (% RE)**	Process efficiency (% PE)††
LQC	71521 (0.89)	73585 (4.83)	61660 (1.37)	103.0	84.0	86.2
MQC	3496745 (1.32)	3366337 (1.20)	3198380 (2.81)	96.3	95.0	91.5
HQC	6043872 (3.04)	6239357 (0.77)	5195498 (2.10)	103.2	83.3	86.0

* Mean area response of three samples prepared in mobile phase (neat sample).
† Mean area response of three samples prepared by spiking in extracted blank plasma.
‡ Mean area response of three samples prepared by spiking before extraction.
§ B/A × 100. ** C/B × 100. †† C/A × 100 = (ME × RE)/100.

Discussion

The present study was conducted using electrospray ionization for MRM LC–MS–MS analyses as it gave high intensity for abacavir and internal standard and a good linearity in regression curves. As abacavir and internal

standard have primary and secondary amine groups that can be protonated in solution under the experimental conditions, the intensity found was much higher in the positive mode. The protonated precursor $(M+H)^+$ ions at *m/z* 287.2 and 313.1 were observed in the Q1 MS full-scan spectra for abacavir and internal standard, respectively. The most abundant product ions found in Q3 MS spectra for abacavir and internal standard were at *m/z* 191.2 (22) and 138.2 at 30 V and 35 V collision energy, respectively. The source-dependent and compound-dependent parameters were suitably optimized to obtain a consistent and stable response for the analyte. A dwell time of 200 ms for abacavir and internal standard was adequate, and no crosstalk was observed between their MRMs.

All three extraction techniques, namely protein precipitation (9,10,13,14,21), liquid–liquid extraction (8,21), and solid-phase extraction (19,20), have been reported for the determination of abacavir in human plasma. Veldkamp et al. (9) have employed protein precipitation with perchloric acid to obtain a recovery of 88.1% using 0.3 mL plasma. Liquid–liquid extraction with ethyl acetate-diethyl ether has been reported by Ferrer and co-workers (8) to achieve quantitative recovery (94.3%) of abacavir. A solid-phase extraction procedure on Oasis MAX cartridges has been described by Verweij-van Wissen et al. (19) to isolate abacavir and other antiretrovirals from human plasma. In the present study, reproducibility and recovery data for both the drugs (abacavir and granisetron) supported liquid–liquid extraction to be used as the extraction technique. Initially, protein precipitation was tried with solvents like acetonitrile and methanol in acidic or alkaline medium. However, specificity and ion suppression were significantly affected with frequent clogging of the column. Solid-phase extraction under alkaline condition gave very good recovery (> 90%) but showed a non linear behavior for highest calibration standard on calibration curve. Thus, liquid–liquid extraction technique was tested to isolate the drug from very low plasma volume (0.1 mL) using diethyl ether, methyl *tert* butyl ether, dichloromethane (alone and in combination with isopropyl alcohol), *n*-hexane, and ethyl acetate as extracting solvents. The recovery obtained in all these solvents was inconsistent with some ion suppression (greater than 15% CV). However, addition of a strong base like sodium hydroxide helped in minimizing matrix interference as well as giving ≥ 86% process efficiency at all three quality control levels in ethyl acetate and dichloromethane (90:10, v/v).

For isocratic separation of abacavir and internal standard, three reversed-phase columns of different dimensions were tested, namely Chromolith RP_{18} (100 × 4.6 mm, 5-µm particle size), Kromasil (50 and 100 × 4.6 mm, 5-µm particle size), and Gemini C_{18} (50 and 150 × 4.6mm, 5-µm particle size), to ensure

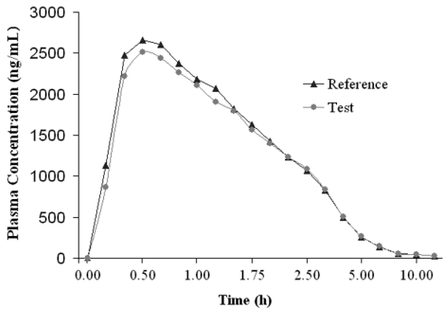

Figure 4. Mean plasma concentration-time profile of abacavir after oral administration of 300 mg (test and reference) tablet formulation to 28 healthy volunteers.

Table V. Stability of Abacavir Under Different Conditions (*n* = 6)

Storage conditions	Mean comparison samples	Calculated conc. (ng/mL) Mean stability samples	%Change*
Bench top stability			
HQC	7015 ± 139.7	7056 ± 136.7	0.58
LQC	83.1 ± 1.9	86.2 ± 3.8	3.77
Autosampler stability			
HQC	6575 ± 104.6	6977 ± 139.0	6.11
LQC	85.7 ± 3.9	84.2 ± 2.3	–1.81
Dry extract stability			
HQC	8288 ± 1192	7414 ± 892	–10.55
LQC	83.2 ± 1.5	90.6 ± 16.7	8.91
Freeze and thaw stability			
HQC	6994 ± 91.4	7156 ± 375.6	2.31
LQC	85.6 ± 3.5	84.8 ± 1.9	–0.97
Long term matrix stability at –20°C			
HQC	7390 ± 148.4	7457 ± 214.1	0.91
LQC	84.8 ± 3.1	88.1 ± 3.6	3.9
Long term matrix stability at –50°C			
HQC	6533 ± 156	7276 ± 243	11.37
LQC	79.8 ± 5.9	86.8 ± 3.9	8.73

* % Change = Mean stability sample – Mean comparison sample × 100.

Table VI. Mean Pharmacokinetic Parameters Following Oral Administration of 300 mg Tablet Formulation (Test and Reference) of Abacavir in 28 Healthy Human Subjects

Parameter	Test* Mean ± %SD	Reference† Mean ± %SD
C_{max} (ng/mL)	2512 ± 742	2754 ± 786
T_{max} (h)	0.7 ± 0.4	0.7 ± 0.4
$t_{1/2}$ (h)	1.2 ± 0.2	1.2 ± 0.6
$AUC_{0-12\,h}$ (h.µg/mL)	6206 ± 1371	6401 ± 1592
AUC_{0-inf} (h.µg/mL)	6304 ± 1370	6487 ± 1592

* 300 mg abacavir sulphate tablets from Matrix Laboratories Limited.
† 300 mg abacavir tablets (Ziagen) from GlaxoSmithKline.

Table VII. ANOVA of Abacavir Pharmacokinetics in Treatments Test and Reference*

Parameter	Factors	Df*	F*	PS*	Conclusion
C_{max}	Sequence	1	0.02	0.881	P > 0.05 (NS)
	Period	1	5.53	0.027	P > 0.05 (NS)
	Formulation	1	0.83	0.371	P > 0.05 (NS)
$AUC_{0-12\,h}$	Sequence	1	0.01	0.912	P > 0.05 (NS)
	Period	1	0.20	0.661	P > 0.05 (NS)
	Formulation	1	1.48	0.235	P > 0.05 (NS)
AUC_{0-inf}	Sequence	1	0.01	0.922	P > 0.05 (NS)
	Period	1	0.21	0.654	P > 0.05 (NS)
	Formulation	1	1.19	0.285	P > 0.05 (NS)

* df = degree of freedom; NS = not significant; PS = probability signifiation.

high throughput with minimum matrix interference. Separation was tried using various combinations of methanol/acetonitrile, acidic buffers, and additives like formic acid, ammonium formate on these columns. Best results were obtained in terms of superior retention and better peak shapes on the Gemini C_{18} column using acetonitrile and 10mm ammonium formate, pH 3.0 (70:30, v/v) as the mobile phase. This may be attributed to the large surface area (396 m^2/g) compared to other columns used. The total chromatographic run time of 2 min with a retention time of 1.49 min for abacavir was the shortest compared to previous assays (9,11,14,15,22).

A general internal standard was used to minimize any analytical variation due to solvent evaporation, integrity of the column, and ionization efficiency of abacavir. Granisetron hydrochloride used as an internal standard in the present study had similar chromatographic behavior and was easily extracted with ethyl acetate–dichloromethane (90:10, v/v) mixture. There was no effect of internal standard on analyte recovery, sensitivity, or ion suppression.

Table VIII. Multiple Comparisons ANOVA of Log-Transformed C_{max} and AUC_{0-t} for Abacavir Between Test and Reference

Parameter		90% confidence interval Lower Bound	90% confidence interval Upper Bound	BE* Conclusion
LN† (AUC_{0-t})‡	Test vs. Ref.	94.4	101.3	Equivalent
LN (AUC_{0-inf})§	Test vs. Ref.	86.5	104.5	Equivalent
LN (C_{max})**	Test vs. Ref.	94.3	101.0	Equivalent

* BE = bioequivalent. † LN = natural logarithm.
‡ AUC_{0-t} = area-under-the-plasma conc.-time curve from 0 h to the last measurable conc.
§ AUC_{0-inf} = area-under-the-plasma conc.-time curve from 0 h to infinity.
** C_{max} = maximum plasma conc.

Table IX. Incurred Sample Reproducibility Data

Sr. No.	Initial Value (ng/mL)	Repeat Value (ng/mL)	% Change*
1	2486	2477	–0.3
2	320	323	0.8
3	1810	1758	–2.9
4	230	224	–2.8
5	1080	978	–9.9
6	88.8	89.0	0.2
7	1569	1417	–10.2
8	99.8	99.6	–0.2
9	1495	1334	–11.4
10	720	691	–4.2
11	3026	2888	–4.7
12	428	424	–1.0
13	1613	1723	6.6
14	280	313	11.4
15	1916	1975	3.0
16	237	258	8.3
17	1730	1632	–5.8
18	2539	2225	–13.2
19	253	233	–8.2
20	251	247	–1.8

*% Change = (Repeat value – Initial value) × 100.

Application to a pharmacokinetic/bioequivalence study

The validated method has been successfully used to quantify abacavir concentration in the human plasma samples after the administration of a single 300 mg oral dose of abacavir. Figure 4 shows the plasma concentration versus time profile of abacavir in human subjects under fasting condition. The method was sensitive enough to monitor the abacavir plasma concentration up to 12 h. In all approximately 1178 samples including the calibration, QC, and volunteer samples were run and analyzed during a period of six days, and the precision and accuracy were well within the acceptable limits. The mean pharmacokinetic parameters obtained for the test and reference formulation are presented in Table VI. Comparison of ANOVA for C_{max}, AUC_{0-t}, and AUC_{0-inf} shown in Table VII indicates no significant difference in abacavir pharmacokinetics between test and reference. Multiple comparison ANOVA of the natural log-transformed C_{max}, AUC_{0-t}, and AUC_{0-inf} for abacavir (Table VIII) shows that the test and reference formulations are bioequivalent in terms of rate and extent of absorption. The 90% confidence interval of individual ratio geometric mean for test/reference was within 80–125% for AUC_{0-t}, AUC_{0-inf}, and C_{max}. The %change in the randomly selected subject samples for incurred samples (assay reproducibility) analysis was within ±13% (Table IX). This authenticates the reproducibility and ruggedness of the proposed method. Further, there was no adverse event during the course of the study.

Conclusions

To summarize, the LC–MS–MS method for the quantitation of abacavir in human plasma was developed and fully validated as per U.S. FDA guidelines. The method offers significant advantages over those previously reported in terms of lower sample requirements, simplicity of extraction procedure, and overall analysis time. Also, the in-source loading (5 pg per injection) of sample at the LLOQ level was much lower compared to other reported procedures. The efficiency of liquid–liquid extraction and a chromatographic run time of 2.0 min per sample make it an attractive procedure in high-throughput bioanalysis of abacavir. The method can be applied to quantify abacavir in combination therapy as there was no interference in presence of 11 other antiretrovirals. A wide linear dynamic range ensures application of the method for higher dose strength with acceptable precision and accuracy. With dilution integrity up to 10-fold, it is possible to extended the ULOQ to 20,255 ng/mL. The current method has shown adequate sensitivity and selectivity for the quantification of abacavir in human plasma in a clinical study.

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