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LC–ESI-MS/MS method for the quantification of entecavir in human plasma and its application to bioequivalence study

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ABSTRACT

Liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) was used for a quantitative estimation of entecavir (EV) in human plasma using lamivudine (LM) as internal standard (IS). The method herein described is simple, sensitive, and specific. Chromatographic separation was performed on XBridge-C18, 4.6 mm × 50 mm, 5-μm column with an isocratic mobile phase composed of 10 mM ammonium hydrogen carbonate (pH 10.5):methanol (85:15 v/v), pumped at 0.3 ml/min. EV and LM were detected using proton adducts at m/z 278.1 → 152.1 and 230.2 → 112.0 in multiple reaction monitoring (MRM) positive mode. Solid phase extraction method was employed in the extraction of EV and LM from the biological matrix. This method was validated over a linear concentration range of 50.0–20000.0 pg/ml with a correlation coefficient (r) ≥0.9983. Intra and inter-day precision of EV was found within the range of 1.2–4.2 for EV and 4.4–4.5 for LM. EV was stable throughout three freeze/thaw cycles, bench top and postoperative studies. This method was successfully used in the analysis of plasma samples following oral administration of EV (0.5 mg) in 26 healthy human volunteers.

1. Introduction

Entecavir EV is 2-amino-9-[(1S, 3R, 4S)-4-hydroxy-3-(hydroxymethyl)-2-methylidenecyclopentyl]-3H-purin-6-one [1]. EV is an oral antiviral drug used in the treatment of hepatitis B infection. It is marketed under the trade name Baraclude (Bristol–Myers Squibb (BMS)). EV is a guanosine nucleoside analog with selective activity against the HBV. It is selectively inhibiting the HBV, blocking all three steps in the replication process. EV is more efficient than LM, which was previously used in the treatment of hepatitis B infection. By competing with the natural substrate deoxyguanosine triphosphate, it functionally inhibits all three activities of the HBV polymerase (reverse transcriptase (RT)), namely (1) base priming, (2) reverse transcription of the negative strand from the pregenomic messenger RNA, and (3) synthesis of the positive strand of the HBV DNA. On absorption following oral administration in healthy subjects, EV peak plasma concentrations occur between 0.5 and 1.5 h. The bioavailability of the tablet is 100% relative to the solution. Healthy subjects who received single EV dose up to 40 mg/day or multiple doses up to 20 mg/day for up to 14 days had no increase in or unexpected adverse events. If overdose occurred, the patient was monitored for evidence of toxicity and standard supportive treatment applied as necessary. Binding of EV to human serum proteins in vitro is approximately 13%. EV is not a substrate, inhibitor, or inducer of the cytochrome P450 (CYP450) enzyme system. It is efficiently phosphorylated to the active triphosphate form. After reaching peak concentration, EV plasma concentrations decrease in a bi-exponential manner with a terminal elimination half-life of approximately 128–149 h. The phosphorylated metabolite has a half-life of 15 h [2–7].

Only a few methods were reported to estimate EV in pharmaceutical [8,9] and biological matrices [10]. Zhang et al. [10] developed a method for determination of entecavir in human plasma by LC–MS/MS, the peak shape and baseline noise is not good, and they used gradient programme for mobile phase optimization. They developed the method with complicated regression model and used large volume (1 ml) of plasma for sample analysis.

Abbreviations: LC–ESI-MS/MS, liquid chromatography–electro spray ionization-tandem mass spectrometry; MRM, multiple reaction monitoring; EV, entecavir; LM, lamivudine; LLOQ, lower limit of quantification; LOQ, limit of quantification; LQC, low quality control; MQC, medium quality control; HQC, high quality control; IS, internal standard; HBV, hepatitis B virus; CAD, collisionally activated dissociation.

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In the present study, we have reported a simple, selective and reproducible analytical method for the determination of EV in plasma samples by liquid chromatography coupled to ESI-MS/MS.

2. Experimental

2.1. Materials and reagents

EV was purchased from Torronto research chemicals, Torronto, Canada and LM (IS) was obtained from USP. Formic acid was purchased from Merck (Darmstadt, Germany). HPLC grade methanol was purchased from Jt. Baker Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA. Ammonium hydrogen carbonate/ammonium bi carbonate (reagent grade) was purchased from Merck Limited, Worli, Mumbai. Ammonia solution (25% w/w) was purchased from Merck Specialty private limited, Worli, Mumbai. Human plasma was obtained from Dr. Pathological Lab, Hyderabad, India. Oasis HLB SPE cartridges, 1 cm^3, 30 mg was purchased from Waters Corporation USA. Milli Q water (from MilliQ-system Millipore, Bedford, MA, USA) was used throughout the study. All other chemicals in this study were of analytical grade.

2.2. Instrumentation

HPLC system (1200 Series Agilent Technologies, Waldbronn, Germany) is equipped with triple quadrupole mass spectrometer API 4000 model (Toronto, Canada). Data processing was performed by the analyst 1.4.1 software package (SCIEX).

2.3. Mass spectrometry parameters

Electrospray source was operated in the positive ionization mode at 5500 V. The source temperature was maintained at 500 °C and the ion source consisted of gas (nitrogen) channels with nebulizer gas, heater gas, curtain gas 20 psi each and CAD gas 2 psi. Quantitative determination was performed in multiple reaction monitoring (MRM) mode with mass transitions from *m/z* 278.1/152 for EV and *m/z* 230.2/112.4 for LM.

2.4. Chromatographic conditions

Chromatography was performed on X Bridge-C18, 4.6 mm × 50 mm, 5-μm column. Separation and elution were achieved using 10 mM ammonium hydrogen carbonate (pH 10.5):methanol (85:15 v/v) as the mobile phase, at a flow-rate of 0.3 ml/min and 20 μl of injection volume. The column was maintained at room temperature (25–30 °C) and run time between injections was 6.0 min. Solid phase extraction was used to extract the drug and internal standard.

2.5. Preparation of standards and quality control (QC) samples

Standard stock solution of EV (50.0 μg/ml) was prepared in water. From this, 500.0, 20.0, and 1.0 ng/ml dilutions were prepared in plasma. From these dilution calibration curve standards at concentrations of 50.0, 100.0, 500.0, 1000.0, 2000.0, 4000.0, 8000.0, 12000.0, 16000.0, 20000.0 pg/ml and four levels of quality control standards at concentrations of 50.0, 150.0, 6000.0, 14000.0 pg/ml for corresponding LLOQ, LQC, MQC, HQC were prepared using the same plasma. According to the clinical protocol, spiked calibration curve standards and quality control standards were stored at −10 to −30 °C. Standard stock solution of LM (50.0 μg/ml) was prepared in methanol and further diluted to 50.0 ng/ml using water and stored in the refrigerator at 2–8 °C until analysis.

2.6. Sample preparation

A sample of volume 500 μl was taken in polypropylene tubes and mixed with 100 μl of IS (50.0 ng/ml). Samples were loaded into solid phase extraction cartridges (waters Oasis HLB 1 cm^3, 30 mg) which were preconditioned with 1 ml methanol followed by 1 ml water. Then the SPE cartridges were washed twice with 2 ml of water (1 ml each time) and dried. Followed by, cartridges were eluted with 1 ml methanol and the samples were collected in respective polypropylene tubes. All the samples were evaporated using nitrogen gas at less than 40 °C temperature. Subsequently, all the dried samples were reconstituted with 250 μl water. A sample of volume 20 μl was injected into the HPLC system connected to the mass spectrometer.

2.7. Recovery

The recovery of EV was evaluated by comparing the mean peak area of six extracted LQC, MQC and HQC samples of 150.0, 6000.0, and 14000.0 pg/ml to the mean peak area of six extracted, spiked drug-free plasma samples with the same amount of LQC, MQC, and HQC samples of EV. Similarly, the recovery of LM was evaluated by comparing the mean peak area of six extracted QC samples to that of LM in samples prepared by spiked, extracted drug-free plasma samples containing an equal amount of LM.

2.8. Selectivity and specificity

The selectivity of the method was determined by six different human blank plasma samples, which were pretreated and analyzed to test the potential interferences of endogenous compounds co-eluting with analyte and IS. Chromatographic peaks of analyte and IS were identified based on their retention times and MRM responses. The peak area of EV in blank samples should not be more than 20% as compared to its LOQ. Similarly, the peak area of LM in blank samples should not be more than 5% of the mean peak area of LOQ of LM.

2.9. Limits of detection and quantitation (LOD and LOQ)

The limit of detection (LOD) is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. LOD was determined using the signal-to-noise ratio (S/N) of 3:1 by comparing test results from samples with known concentrations of analytes with blank samples.

The limit of quantitation (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOQ was found by analyzing a set of mobile phase and plasma standards with a known concentration of EV.

2.10. Calibration curve standards, regression model, precision, and accuracy batches

Analytical curves for EV in human plasma were constructed for concentrations ranging from 50.0 to 20000.0 pg/ml. Calibration curves were obtained by weighted linear regression (weighing factor: $1/x^2$) with a correlation co-efficient of >0.9983. The ratio of the peak areas of EV and LM (instrument response) was plotted against the EV concentration in pg/ml. The fitness of the calibration curve was confirmed by back-calculating the concentrations of calibration standards.

The calibration curve standards and QC samples were prepared in replicates ($n = 6$) for analysis. Precision and accuracy of the inter-assay were evaluated for six days. Accuracy and precision for the back-calculated concentrations of the calibration points should be

within ±15% of their nominal values. However, for LLOQ the precision and accuracy should be within ±20%.

2.11. Stability

LQC and HQC samples ($n = 6$) were retrieved from a deep freezer after three freeze/thaw cycles according to clinical protocol. Samples were stored at −10 to −30 °C in three cycles of 24, 36, and 48 h. In addition, the long-term stability of EV in QC samples was also evaluated after 65 days of storage at −10 to −30 °C. Post spiked stability was studied following a 109 h storage period in the auto sampler tray. Bench top stability was studied for a 19 h period. Stability samples were processed and extracted along with the freshly spiked calibration curve standards. The precision and accuracy for the stability samples must be within 15 and ±15%.

2.12. Matrix effect

The matrix effect due to plasma was used to evaluate the ion suppression/enhancement in a signal when comparing the absolute response of QC samples after pretreatment (solid-phase extraction) with that of the reconstituted samples. Experiments were performed at MQC levels in triplicate with six different plasma lots. The acceptable precision (CV%) of ≤15% was maintained.

2.13. Analysis of human plasma samples

The method described above was used to determine EV concentrations in plasma following oral administration into healthy human volunteers. These volunteers were contracted in APL Research Pvt. Limited, India and one tablet of 0.5 mg dose was administered in 26 healthy volunteers by oral administration with 240 ml of drinking water. Reference product (Baraclude Tablets, 0.5 mg, Bristol–Myers Squibb) and test product (entecavir tablet, 0.5 mg, APL Research Pvt. Ltd) were used. Study protocol was approved by Institutional Ethical Committee (IEC) as per regulations of Indian Council of Medical Research (ICMR). Test and reference formulations were administered to the same human volunteers under fasting conditions separately with an interval of six days between two dosings as per approved protocol. Blood samples were collected as pre-dose (0 h) (5 min prior to dosing) followed by further samples at 0.167, 0.333, 0.5, 0.667, 0.833, 1.0, 1.25, 1.5, 2.0, 3, 4, 6, 8, 12, 18, 24, 36, 48 and 72 h. After dosing, a 5 ml blood was collected each time separately into vacutainer containing K_2EDTA. A total of 40 samples obtained containing test and reference samples collected from 20 time points each. All the samples were centrifuged at 3200 rpm at 10 °C for 10 min and stored at below −30 °C until sample analysis.

2.14. Pharmacokinetics and statistical analysis

Pharmacokinetic parameters from the human plasma samples were calculated by a noncompartmental model using WinNonlin 5.0 software (Pharsight, USA). Blood samples were taken for a period of 3–5 times the terminal elimination half-life ($t_{1/2}$). As per the FDA guidelines, the ratios of the area under concentration time curves (AUC) for test and reference in the range of 80–125% were considered to be bioequivalent. Plasma EV concentration–time profiles, C_{max} and T_{max} values were determined. The AUC_{0-t} was obtained by the trapezoidal method. $AUC_{0-\infty}$ was calculated up to the last measurable concentration. The slope of the terminal exponential phase obtained from the plasma of EV concentration–time curve by means of the linear regression method was utilized for the calculation of K_e. The terminal elimination half-life ($t_{1/2}$) was then calculated as $0.693/K_e$. Bioequivalence was assessed by means of the analysis of variance (ANOVA) and by calculating the standard 90% confidence intervals (CIs) of the test/reference ratios (logarithmically transformed data) by using AUC_{0-t}, $AUC_{0-\infty}$ and C_{max}. Bioequivalence was considered when the ratio of averages of log-transformed data is within 80–125% for AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} [11–13].

3. Results and discussion

3.1. Method development

In the way to develop a suitable method for EV assay in human plasma for pharmacokinetic study, HPLC with MS/MS detection was selected as the method of choice. Our main challenge is to develop a simple, reproducible, good peak shape with low baseline noise, high recovery method. For this, it was necessary to not only adjust the mass spectrometry parameters, chromatographic conditions but also develop an efficient extraction method that gives consistent and reproducible recovery of analyte from plasma.

The *MS optimization* was performed by direct infusion of solutions of both EV and LM into the ESI source of the mass spectrometer. Some vital parameter such as temperature, voltage, ionization mode, nebulizer gas, heater gas, declustering potential (DP), entrance potential (EP), focusing potential (FP), collision energy (CE) and collision cell exit potential (CXP) were optimized to obtain better ionization to form, protonated ionic EV and LM molecules (Fig. 1). A CAD product ion spectrum for EV and LM yielded high-abundance fragment ions of m/z 152.0 and m/z 112.4 (Figs. 2 and 3).

Chromatographic conditions, especially composition and nature of the mobile phase were optimized through several trials to achieve the best resolution and to increase the signal of EV and LM. Analysis of analyte and IS were initiated under isocratic conditions with an aim to develop a simple separation process with a short run time. Separation was tried using various combinations of mobile phases with different columns like X-terra and X-bridge as they are stable at high pH levels. Based on literature survey the analyte was soluble in methanol and water [1]. Initially, method development was performed with the standard stock solution of drug prepared in *methanol,* and further we continued with optimization of mobile phase and extraction of the method. We observed so many wonders by these trials.

Mobile phase optimization

Trial 1: Mobile phase combination with 10 mM ammonium formate:methanol with different ratios (90:10, 70:30, 60:40, 50:50, 30:70 v/v) by using *X-terra* column, *isocratic* mode with constant flow rate, we observed that peak was obtained at retention time 0.8 ± 0.2 min with total run time of 3 min for all combinations. Whereas peak shape, response was improved, without changing the retention time.

Trial 2: By replacing *X-bridge* column, *with similar conditions in trial* 1 *peaks were eluted at* 1.8 ± 0.2 min with total run time of 3.5 min.

Trial 3: Mobile phase combination with 10 mM ammonium formate:methanol with different ratios (gradient) by using *X-bridge* column, *gradient* mode with constant flow rate we observed that two peaks were obtained at retention time of 1.8 ± 0.2 min and 4.2 ± 0.2 min with total run time of 6 min.

Trial 4: By replacing *X-terra* column with same conditions in trial 3 peaks were eluted at 0.8 ± 0.2 min and 3.8 ± 0.2 min with total run time of 6 min.

Gradient program for trials 3, 4.

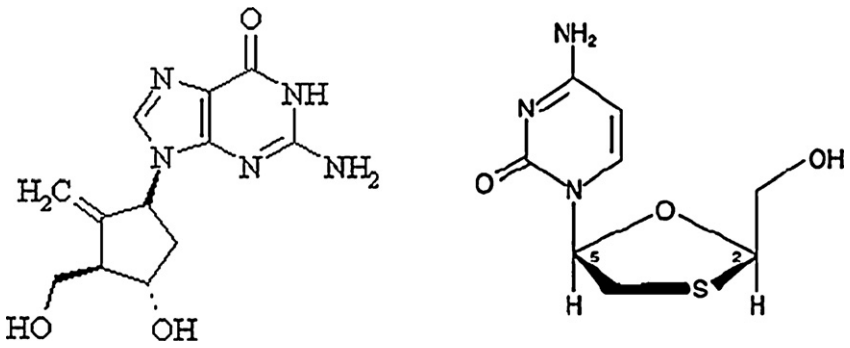

Fig. 1. Chemical structures of (A) EV and (B) LM.

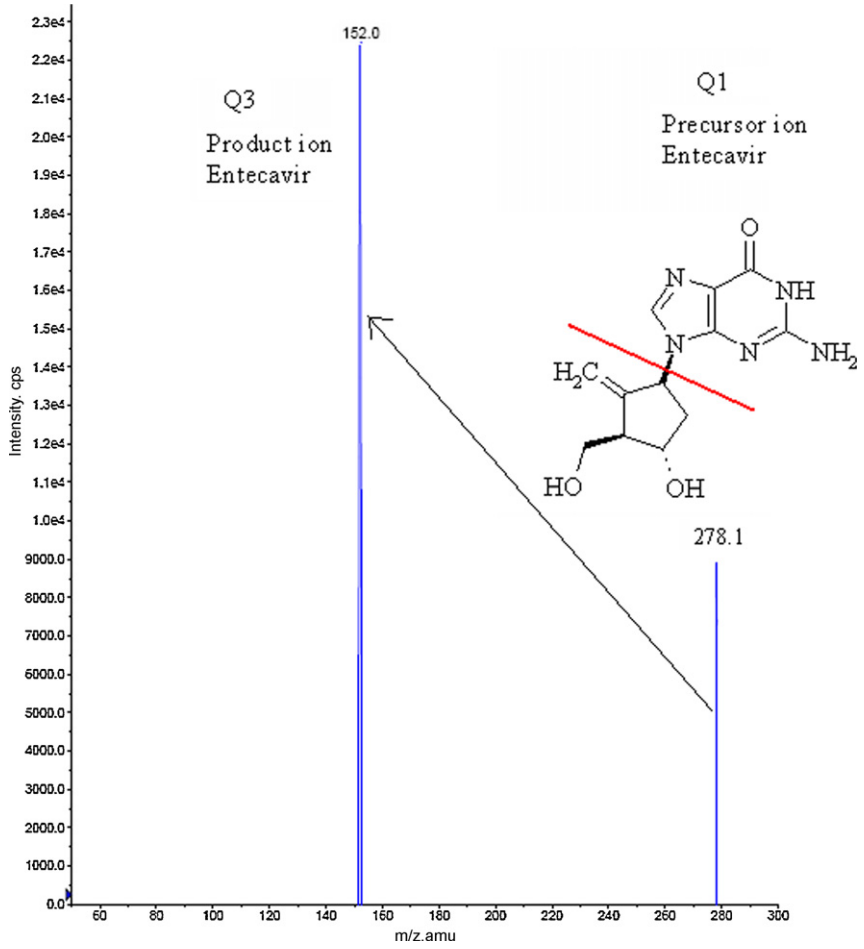

Fig. 2. Mass spectra of EV Q1 → Q3.

Step	Time (min)	Flow rate (μl/min)	A	B	C	D
0	0.00	300	0.0	100.0	0.0	0.0
1	0.50	300	0.0	100.0	0.0	0.0
2	0.51	300	100	0.0	0.0	0.0
3	1.50	300	100	0.0	0.0	0.0
4	1.51	300	0.0	100.0	0.0	0.0
5	5.00	300	0.0	100.0	0.0	0.0

A = 10 mm ammonium formate (pH 10.0); B = methanol.

Trial 5: Based on trials 1–4 we concluded that the solubility of the drug to be changed further we proceeded by dissolving the drug in water. We have optimized the mobile phase as similar to trials 1–4. It was observed that peak was disappeared at 0.8 min ± 0.2 (by using X-terra column) and 1.8 ± 0.2 min (by using X-bridge column). The peak was observed at 3.8 ± 0.2 (by using X-terra column) and 4.2 ± 0.2 min (by using X-bridge column) with moving retention time of drug as the mobile phase ratios change.

Trial 6: The mobile phase was optimized with various ratios (90:10, 85:15, 60:40, 50:50, 40:60, 20:00, 50:50) of 10 mM ammonium hydrogen carbonate pH (10.5):methanol in simple isocratic mode by dissolving the drug in water and injected into LC–MS/MS system with X-Bridge C18, 5 μm, 4.6 × 50 mm column.

Based on all the trials we have optimized 10 mM ammonium hydrogen carbonate pH (10.5):methanol (85:15 v/v) as mobile phase for the study.

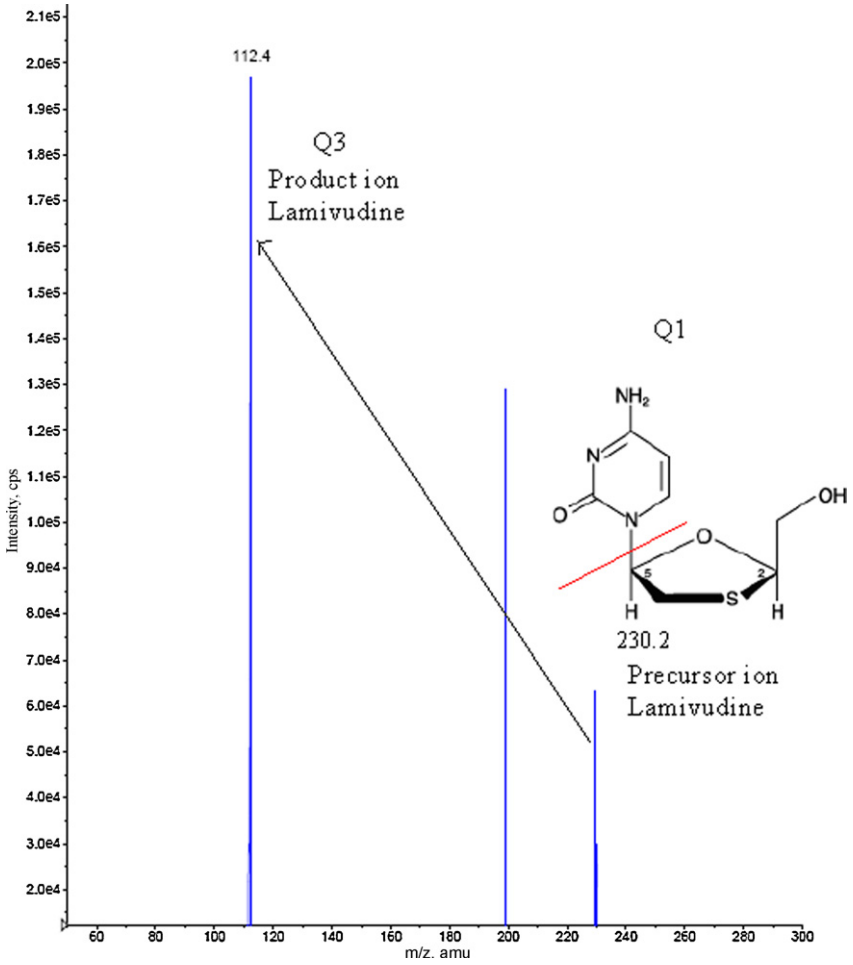

Fig. 3. Mass spectra of LM Q1 → Q3.

Extraction method optimization

For extraction of analyte and IS initially we tried with precipitation method and liquid–liquid extraction method and observed that both are unsuitable to serve this purpose. Finally, we selected Solid phase extraction by using Waters Oasis, HLB 1 cm^3, 30 mg cartridges to extract the drug and internal standard and achieved best results.

After all the trials, the mobile phase containing 10 mM ammonium hydrogen carbonate (pH 10.5):methanol (85:15 v/v) at a flow-rate of 0.3 ml/min and injection volume of 20 μL was finalized. Solid phase extraction (SPE) was selected as extraction method to isolate the drug and IS.

3.2. Selectivity and specificity

The selectivity of the method was assessed by comparing the chromatograms of a blank plasma (Fig. 4A) and a spiked plasma (Fig. 4B). The retention time was 2.8 min for EV and 3.7 min for LM (Fig. 4B). As shown in the Fig. 4A, there were no significant endogenous peaks interfering with EV and LM. The results indicate that the method exhibited good specificity and selectivity.

3.3. Limits of detection and quantitation

The LOQ was found for this method is 50.0 pg/ml with signal-to-noise (S/N) of 11.93 and LOD was found as 0.1 pg.

3.4. Matrix effect

The ion suppression/enhancement in the signal was found as CV% 5.67 at MQC level for EV, indicating that the matrix effect on the ionization of analyte is not obvious under these conditions.

3.5. Calibration curve, precision and accuracy

The calibration curves were plotted as the instrument response (peak area ratio (EV/LM)) versus EV concentration. Calibration was found linear over the concentration range of 50.0–20000.0 pg/ml. The precision was less than 4.2% and the accuracy of the mean of measured concentrations ranged from 96.7 to 101.5%. The correlation coefficient (r^2) was greater than 0.9983 for all curves (Table 1). Precision and accuracy for this method were controlled by calculating the intra- and inter-batch variations at three concentrations (150.0, 6000.0, and 14000.0 pg/ml) of QC samples in six replicates. As shown in Table 2, the intra-batch precision and accuracy were between 1.2 to 4.2 and 97.0 and 97.5%. Similarly, the corresponding inter-batch precision and accuracy were between 4.4 to 4.5 and 97.2 to 98.7%. These results indicate the adequate reliability and reproducibility of this method within the analytical range.

3.6. Stability

EV in plasma was subjected to three freeze/thaw (−10 to −30 °C to room temperature) cycles. The accuracy of EV obtained was between 86.8 and 99.2%. No significant degradation of EV was

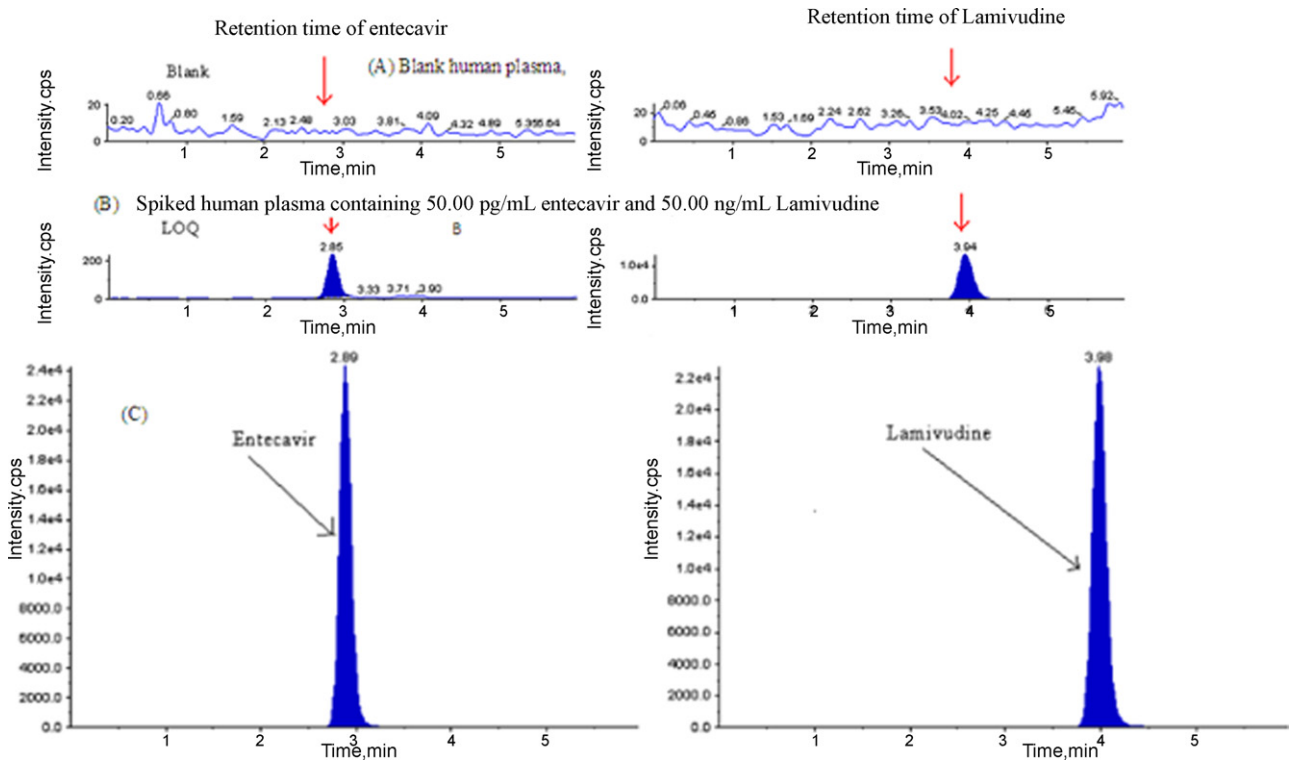

Fig. 4. Chromatograms of EV and LM in human plasma (A), blank human plasma (B), spiked human plasma containing 50.0 pg/ml EV and 50.00 ng/ml LM (C) chromatograms of plasma samples from a subject administered with 0.5 mg EV (1 h post dose sample).

Table 1
Calibration curve details of the validation section.

Spiked plasma concentration (pg/ml)	Concentration measured (mean ± sd) (pg/ml)	Precision (CV%) or RSD[a] ($n = 5$)	Accuracy (%)
50.0	50.0 ± 0.7	1.4	100.0
100.0	100.0 ± 3.1	3.1	100.0
500.0	503.8 ± 21.0	4.2	100.8
1000.0	1007.7 ± 40.6	4.0	100.8
2000.0	1933.4 ± 60.9	3.1	96.7
4000.0	3985.3 ± 145.9	3.7	99.6
8000.0	8017.8 ± 288.4	3.6	100.2
12000.0	12010.7 ± 167.2	1.4	100.1
16000.0	16058.6 ± 541.5	3.4	100.4
20000.0	20304.1 ± 451.2	2.2	101.5

[a] [Standard deviation/mean concentration measured] × 100.
n = number of curves.

observed even after a 109.0 h storage period in the auto sampler tray and the final concentrations of EV was between 85.1 and 87.9%. In addition, the long-term stability of EV in QC samples after 65 days of storage at −10 to −30 °C and bench top stability for 19 h was also evaluated. The concentrations ranged from 85.3 to 100.5% for long term stability and 88.1 to 98.4% for bench top stability studies. These results confirmed the stability of EV in human plasma for at least 65 days at −10 to −30 °C (Table 3).

Table 2
Precision and accuracy (analysis with spiked plasma samples at three different concentrations).

Spiked plasma concentration (pg/ml)	Within-run ($n = 6$)			Between-run ($n = 30$)		
	Concentration measured (pg/ml) (mean ± sd)	Precision (CV%) or RSD[a]	Accuracy (%)	Concentration measured (pg/ml) (mean ± sd)	Precision (CV%) or RSD[a]	Accuracy (%)
150.0	146.3 ± 1.7	1.2	97.5	147.0 ± 6.6	4.5	98.0
6000.0	5822.5 ± 244.1	4.2	97.0	5919.4 ± 264.7	4.5	98.7
14000.0	14409.7 ± 579.6	4.0	97.1	14396.8 ± 633.6	4.4	97.2

[a] [Standard deviation/mean concentration measured] × 100.

Table 3
Stability of EV in human plasma samples.

Spiked plasma concentration (pg/ml)	Concentration measured ($n = 6$) (pg/ml) (mean ± sd)	Precision (CV%) or RSD[a] ($n = 6$) (%)	Accuracy (%)
Bench top stability at room temperature for 19 h			
150.0	147.6 ± 7.3	5.0	98.4
16000.0	14093.4 ± 619.2	4.4	88.1
Auto sampler stability at refrigerated condition for 109 h			
150.0	131.8 ± 7.7	5.8	87.9
16000.0	13506.1 ± 432.6	3.2	85.1
Lang term stability at −10 °C to −30 °C for 65 days			
150.0	150.8 ± 4.9	3.3	100.5
16000.0	13648.8 ± 766.4	5.6	85.3
Freeze/thaw stability for 3 Cycles (−10 °C to −30 °C)			
150.0	148.9 ± 7.3	4.9	99.2
16000.0	13887.5 ± 738.2	5.3	86.8

[a] [Standard deviation/mean concentration measured] × 100.

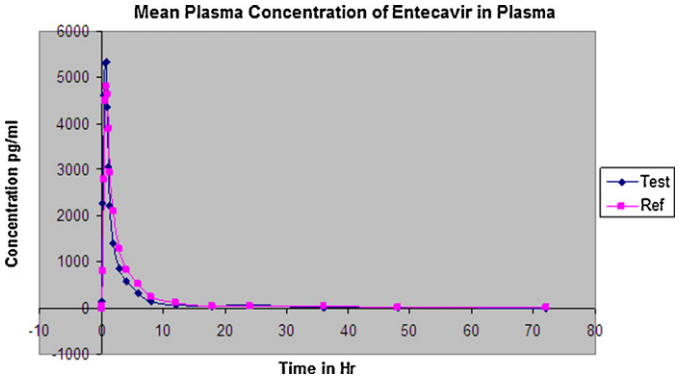

Fig. 5. Mean plasma concentrations of test vs. reference after a 0.5 mg (one 0.5 mg tablet) single oral dose (26 healthy volunteers).

3.7. Recovery

The extraction recoveries of EV determined at three different concentrations 150.0, 6000.0 and 14000.0 pg/ml were found as 85.6 ± 4.7%, 89.9 ± 6.8% and 89.70 ± 5.4%. The overall average recoveries were found as 88.42 ± 5.8% for EV and 93.9 ± 3.8% for LM. Recoveries of the analyte and IS were high, consistently precise and reproducible.

Table 4
Pharmacokinetic parameters of EV in 26 healthy human volunteers after oral administration of 0.5 mg test and reference products.

Entocavir pharmacokinetic details		
Pharmacokinetic parameter	Reference	Test
	Mean ± sd	Mean ± sd
C_{max} (pg/ml)	4817.5 ± 767.4	5352.5 ± 928.4
AUC_{0-t} (pg h/ml)	12953.5 ± 469.1	10773.1 ± 340.2
$AUC_{0-\infty}$ (pg h/ml)	13184.2 ± 492.4	10778.21 ± 374
T_{max} (h)	8.4	8.1
$t_{1/2}$	0.833	0.833

Test/ref C_{max} (T/R)	AUC_{0-t} (T/R)	$AUC_{0-\infty}$ (T/R)
111.1	83.17	81.75

$AUC_{0-\infty}$: area under the curve extrapolated to infinity; AUC_{0-t}: area under the curve up to the last sampling time; C_{max}: the maximum plasma concentration; T_{max}: the time to reach peak concentration.

3.8. Application to biological samples

The above-validated method was used in the determination of EV in plasma samples for establishing the bioequivalence of a single tablet of 0.5 mg dose in 26 healthy volunteers. MRM chromatograms of plasma samples from subject 1.0 h post dose sampling (Fig. 4B) were represented. Typical plasma concentration versus time profiles is shown in Fig. 5. All the plasma concentrations of EV were in the standard curve region and remained above LOQ (50.0 pg/ml) for the entire sampling period. Pharmacokinetic parameter details are represented in Table 4. Therefore, it is concluded that the two EV formulations (reference and test) analyzed are bioequivalent.

4. Conclusion

The developed LC–ESI-MS/MS method was successfully employed for quantitative determination of entecavir (EV) in the bioequivalence study following oral administration of EV (0.5 mg) in healthy human volunteers. The proposed method is affordable, reliable, simple, selective, rugged and reproducible. We have developed the method with good peak shape and low base line noise. Mobile phase optimization was done with simple isocratic mode. One of the major advantages of this method is that only a 0.5 ml of plasma is needed for analysis, which greatly facilitates the collection of blood samples. This method was determined by simple, linear, regression analysis ($1/C^2$ weighing factor) with linearity range of 50.0–20000.0 pg/ml. The validation data demonstrated good precision and accuracy. More over recoveries for analyte and IS from plasma was found 88.42 ± 5.8% and 93.9 ± 3.8%. This method can be successfully applied in bioequivalence study to evaluate the plasma concentrations of EV in human subjects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.02.023.

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