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A sensitive method for the determination of entecavir at picogram per milliliter level in human plasma by solid phase extraction and high-pH LC–MS/MS

Duxi Zhang*, Yunlin Fu, Jane P. Gale, Anne F. Aubry, Mark E. Arnold

Bristol-Myers Squibb Company, Research & Development, Bioanalytical Sciences, Route 206 & Province Line Road, Princeton, NJ 08543, USA

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ABSTRACT

Entecavir is a guanine nucleoside analogue used in the treatment of hepatitis B virus (HBV) infection. In this paper, we describe an LC–MS/MS method that was developed and validated for the quantitation of entecavir in human EDTA plasma with both high sensitivity (lower limit of quantitation (LLOQ) of 5 pg/mL) and a wide concentration range (5000-fold) intended for low dose ascending clinical studies. High enrichment was achieved by taking advantage of the excellent loading capacity and reproducibility of Oasis HLB 96-well solid phase extraction plate, which allowed 1 mL of plasma samples to be processed in two equal sequential loading steps. Lobucavir, a structural analogue, was used as the internal standard. A filtration step following the reconstitution proved to be vital for the method robustness. The analyte and internal standard were separated on an Xterra MS C18 column with a gradient elution and high-pH mobile phases. Analytes were detected by positive ion electrospray tandem mass spectrometry. The high-pH mobile phase provided both excellent analyte on-column retention and peak shape, leading to the desired sensitivity. Validation results show good intra-assay (12.3%CV) and inter-assay (3.1%CV) precisions, and good assay accuracy (±7.6%Dev). Recovery was high (~80%), however, the large volume of plasma used did result in a considerable matrix effect (~0.45) which was well compensated by the analog internal standard. The method was applied to sample analysis of a Phase I clinical study.

1. Introduction

Hepatitis B virus (HBV) is a deoxyribonucleic acid (DNA) virus that produces both acute and chronic infection of the liver in humans. HBV infection is a major global health concern and two billion people worldwide show evidence of having been infected with HBV and more than 350 million of these are chronically infected [1,2]. Individuals with chronic hepatitis B are at high risk of hepatic cirrhosis and primary hepatocellular carcinoma, which together, are responsible for an estimated 1 million deaths annually worldwide. There are six approved drug therapies including small molecule nucleoside analogues lamivudine, entecavir (Baraclude™), and telbivudine, acyclic nucleotide analog adefovir dipivoxil, and injection proteins interferon alfa-2b (~14 kDa) and pegylated interferon alfa-2a (~40 kDa) for the treatment of chronic HBV infection. Among the nucleoside analogs, entecavir (Baraclude™, Fig. 1) is a novel guanine nucleoside analogue that inhibits HBV DNA polymerase at both the priming and elongation steps required for viral replication [3–5] and the excellent *in vitro* potency and resistance profile of entecavir addresses unmet medical needs [6,7].

Over the past decade or so, modern liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has greatly multiplied the sample analysis throughput. Moreover, technological advances in column technology and mass spectrometers have also driven ever lower the limit of quantitation of bioanalysis required to fully understand the pharmacokinetics of the low dose/high potency drug candidates and have made LC–MS/MS the choice for small molecule analyses. Assays for small molecule antiretroviral drugs, however, still present significant challenges in method development and assay validation because these compounds are typically small, very polar, exist as ionized species at physiological pH, and are not ideally suited for reverse-phase extraction and chromatography. A variety of extraction methods from protein precipitation [8], solid phase extraction [9,10], ultrafiltration [11] to the combination of more than one extraction methods [12] have been used in these assays to achieve the desired recovery efficiency to address the need for acceptable analytical methods for drug analysis or therapeutic monitoring. However, better sample clean-up and enrichment are often needed for very low dose drugs or when the sample volume is limited. In the case of entecavir, the high potency (the clinical efficacious dose is 0.5 or 1.0 mg/day) suggested that to effectively evaluate the pharmacokinetics of the drug in the early multiple ascending dose clinical studies, a very low (pg/mL) limit of quantitation would be required. Like other nucleoside analogues, entecavir is

* Corresponding author. Tel.: +1 609 252 4687.
E-mail address: duxi.zhang@bms.com (D. Zhang).

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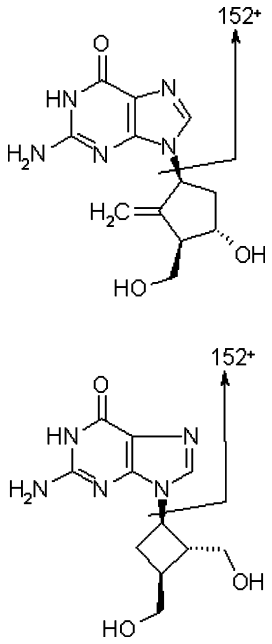

Fig. 1. Chemical structures of entecavir (top) and its internal standard (bottom).

small and very polar which presents chromatographic challenges. After extensive method parameter evaluation, an LC–MS/MS assay method was developed and validated which included the use of Oasis HLB extraction plate for large volume (1 mL) enrichment of plasma samples, filtration of reconstituted sample solutions to improve ruggedness, and high-pH mobile phases to improve on-column retention, peak shape and MS detection sensitivity. While there are very few assays reported in the literature with a limit of quantitation in the pg/mL level [13–17], this method allowed the quantitation of entecavir in human plasma in the concentration range of 5 pg/mL to 25 ng/mL. The existing pg/mL level assays all used relatively large sample volume (0.5–1 mL) coupled with either mixed-mode solid phase extraction (SPE) [13,16,17] or LLE [14,15] for matrix clean-up and enrichment before LC–MS/MS analysis. Typical reversed phase HPLC methods were used in all assays although the orthogonal mixed-mode extraction did provide a dimension for reducing matrix effect [16,17].

2. Experimental

2.1. Materials and reagents

Entecavir (Fig. 1) and lobucavir (internal standard, Fig. 1) were synthesized at Bristol-Myers Squibb Research & Development. Control human K_3EDTA plasma was purchased from Bioreclamation Inc. (Hicksville, NY). Ammonium bicarbonate (>99% purity) and ammonium hydroxide (28–30%) were purchased from Sigma (St. Louis, MO). Methanol and acetonitrile, ACS HPLC grade, were from EM Sciences (Gibbstown, NJ).

2.2. LC–MS/MS instrumentation

HPLC analytical column (Xterra MS C18, 2.1 mm × 50 mm, 5 μm) and 96-well SPE plates (Oasis HLB, 30 mg/well) were from Waters (Milford, MA). The 96-well collection plates and 0.2 μm CoStar Spin-X filters were from VWR (Bridgeport, NJ). Mobile phase delivery pumps (Model LC-10AD VP pump) were from Shimadzu Scientific Instruments, Inc. (Columbia, MD) and the autosampler (Series 200 LC) was from PerkinElmer (Norwalk, CT). Sciex API 3000 tandem mass spectrometer with TurboIon Spray interface was from Applied Biosystems (Foster City, CA).

2.3. Stock solutions and mobile phases

A stock solution of entecavir was prepared at 100 μg/mL in water and serially diluted to generate a series of secondary stock solutions at the concentrations of 0.5, 1.0, 10, 50, 400, 1000, 2000, and 2500 ng/mL in water. Plasma calibration standards of 0.005, 0.010, 0.100, 0.500, 4.00, 10.0, 20.0, and 25.0 ng/mL were prepared with each extraction set from the secondary stock solutions by diluting 30 μL of each solution with 2.97 mL of control human plasma on the day of preparation. QC samples were prepared at 0.015, 10.0 and 20.0 ng/mL in plasma and stored at −20 °C. The internal standard, lobucavir, was prepared at 200 μg/mL in water, serially diluted to 10.0 ng/mL and used as the working solution for daily use. Mobile phase A (pH ∼10.5) was a 5-mM ammonium bicarbonate aqueous solution with 0.15% of ammonium hydroxide; mobile phase B was a 5-mM ammonium bicarbonate with 0.15% ammonium hydroxide in 90% (v:v) methanol.

2.4. Sample preparation

Plasma samples were processed with a Tecan Genesis RSP150 robotic liquid handling system (Tecan US, Durham, NC) using Oasis HLB 96-well extraction plates for the analyte extraction. The extraction plate was first conditioned with 900 μL of methanol, then equilibrated with 900 μL of water. Subsequently, 500 μL of the samples were loaded to the SPE wells and followed by 250 μL of 10.0 ng/mL IS aqueous working solution (equivalent to 5.00 ng/mL in plasma) to allow mixing of analyte and the IS. Vacuum was then applied to the manifold to draw liquid mixture through while the analyte and the IS were retained. These two steps were repeated to allow the loading of 1-mL plasma samples to the extraction plate. The SPE wells were then washed with a total of 2.3 mL of water before elution. Each well was eluted under vacuum with two portions of 150 μL of methanol and the eluants were collected into a clean 96-well collection plate. The wells of the collection plate were subsequently dried under nitrogen flow at ∼40 °C. The resulting residues were reconstituted each with 100 μL of water, vortex mixed and the reconstituted samples were filtered through 1.7-mL spin filters (0.2 μm) using centrifugation. The sample solutions were then transferred to 0.2-mL glass vials for analysis.

2.5. Standards and QCs

Eight calibration standards were placed at the beginning and another eight at the end of each run so that a calibration curve was generated from the 16 standards, while QC samples were dispersed through the run. The accuracy and precision of the method were assessed by analyzing QC samples at concentrations of 0.015, 10.0, and 20.0 ng/mL. A dilution QC of 200 ng/mL was also analyzed after being diluted 20-fold with control human plasma. Five replicate at each concentration were analyzed in three (3) separate runs. The accuracy was determined by calculating the deviations of the predicted concentrations from their nominal values. The intra- and inter-assay precisions were determined by calculating the %CV values.

2.6. Specificity and lower limit of quantitation (LLOQ) test

Six different lots of control human plasma were analyzed with and without IS in order to determine whether any endogenous human plasma constituents interfered with the analyte or the IS. The degree of interference was assessed by inspection of the selected reaction monitoring (SRM) chromatograms. The LLOQ for

entecavir was assessed using human plasma sample at 5 pg/mL. Six different lots of control human plasma were spiked at 5 pg/mL to obtain the six LLOQ samples. The LLOQ samples were analyzed and their predicted concentrations determined.

2.7. Recovery and matrix effect assessment

The recovery of entecavir from human plasma during extraction was determined at 0.015, 10.0, and 20.0 ng/mL by comparing the response ratios of human plasma samples spiked with entecavir prior to extraction with those spiked post-extraction. The recovery of IS was determined similarly at 5.00 ng/mL. The matrix effect was assessed by comparing the responses in post-extraction spiked samples to that of neat solutions at the same concentration.

2.8. Freeze–thaw and long term storage stability in matrix

The freeze–thaw samples were prepared by completely thawing the samples at room temperature and freezing at −20 °C for at least 12 h for each cycle. Plasma QCs were also stored at −20 °C for long term stability assessment.

2.9. Analytical parameters

The samples were subjected to a linear gradient to separate the analyte from the matrix residues prior to mass spectrometric analysis. The gradient conditions were as follows: from 0 to 0.5 min, 0% B; from 0.5 to 2.5 min, linear to 60% B; from 2.5 to 2.51 min, linear to 0% B, from 2.51 to 5 min, 0% B. The analytical column was operated at ∼45 °C with a flow rate of 0.3 mL/min and injection volume of 20 μL. The retention times of both entecavir and IS were around 1.8 min under such conditions.

Samples were detected under positive ion electrospray ionization condition using SRM on an API 3000 instrument with typical settings as follows: mobilizing gas, 15 units; curtain gas, 8 units; collision gas, 7 units; spray voltage, 1600 V; turbo probe temperature, 425 °C; Q1 resolution, unit; Q3 resolution, low; dwell times, 400 ms for entecavir monitoring and 100 ms for IS monitoring.

The electrospray positive ion Q1 mass spectrum of each compound was dominated by the respective protonated molecular $[M+H]^+$ ions: m/z 278 for entecavir and m/z 266 for the IS. The MS/MS product ion spectra of $[M+H]^+$ for entecavir and the IS are shown in Fig. 2; both gave a major fragment ion at m/z 152. Hence, SRM transitions monitored were m/z 278 to m/z 152 for entecavir and m/z 266 to m/z 152 for the IS.

2.10. Data acquisition and processing

Peaks were integrated with Analyst 1.3 software and imported to Watson DMLIMS (V7.1, Thermo Fisher Scientific, Inc., PA) for standard regression using peak area ratios of the analyte to the IS. Calibration standards fit well into a quadratic regression model, weighted by reciprocals of the nominal concentrations ($1/X$) and the equation of this curve was then used to calculate the predicted concentrations in all samples within the run. Intra- and inter-assay assay CVs for QC samples were calculated within Watson using a one-way ANOVA analysis.

3. Results and discussion

3.1. Chromatography

Early method development using regular reversed phase columns with acidic to neutral mobile phases did not yield the desired chromatographic behavior and sensitivity for this polar analyte. Incidental observation that high-pH mobile phases improved the analyte response drove the method development effort toward the use of a pH tolerant stationary phase (Xterra MS C_{18}). The use of high-pH mobile phase was mostly avoided for silica-based column because it is generally believed that silica dissolves at pH > 7.5. Although studies were carried out to address the pH effect on silica-based columns and found the high pH separation for organic bases very attractive [18,19], very few applications of high-pH mobile phases in LC/MS analysis have been published until recently [20–23]. High-pH mobile phases maintain the basic analyte in a deprotonated (neutral) state which favors hydrophobic interactions with the C18 chains as well as minimize interactions with silanol groups, resulting in improved peak shape and retention. On the other hand, it is counterintuitive that a high-pH mobile phase (>10.5) would help positive ion electrospray ionization because the mobile phase environment does not promote the pre-formation of a protonated analyte for electrospray. This phenomenon of disparity between solution pH and ionization in electrospray, however, had been reported in the literature [24,25]. From the method development results and observation, we believe that the high-pH mobile phase served several advantageous purposes in the LC–MS/MS analysis of entecavir: (1) maintains the analyte (pK_a ∼9.7) in close to neutral form which facilitates the retention of the analyte on the stationary phase, (2) assists ionization probably by proton transfer from ammonium ion [24], and (3) improve specificity by separating out matrix components that are ionized at the mobile phase pH. The enhanced retention means that the analyte elutes in a part of the gradient with relatively higher organic content which also contributes to the better ionization efficiency.

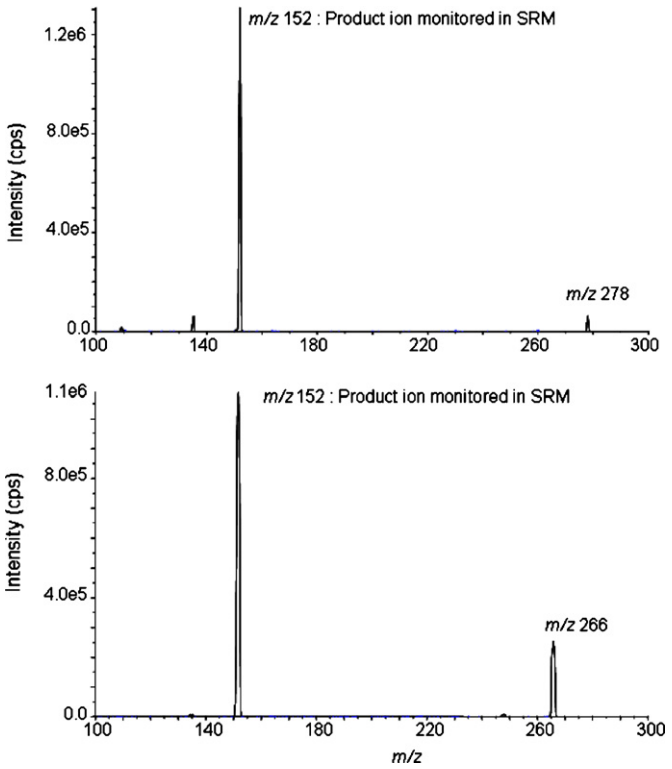

Fig. 2. Electrospray positive ion MS/MS product ion spectra of protonated entecavir (bottom) and its internal standard (top).

3.2. Solid phase extraction

Because of the low detection limit requirement of the assay, highly polar nature of entecavir, and the prevalence of endogenous interference due to the large sample volume, SPE became the choice

of sample treatment over protein precipitation and liquid–liquid extraction. Even with SPE, entecavir was poorly retained and matrix interference was still very severe with many C8, C18, phenyl and mixed-mode cation exchange SPE plates. Highly variable results and poor detection sensitivity were observed, probably due to low affinity of the drug to the packing materials with analyte loss observed in wash solutions to various degrees. Notably, the recovery was very sensitive to the pH and salt contents in the wash solutions, suggesting a weak interaction with the packing material. Also, endogenous compounds from plasmas, retained on and eluted from the SPE column, caused extensive matrix effects with electrospray ionization. Overall, the use of Oasis HLB 96-well SPE plate allowed higher loading capacity, better recovery and more uniform extraction reproducibility of the analyte than traditional C18 or other packed SPE plates. Oasis plates also allowed high volume washes for removal of water-soluble matrix components. However, even with this SPE plate, a small amount of acid, base or organic solvent in the SPE wash solutions can result in significant loss of the analyte; indicating that pH, ionic strength, and organic contents all contribute to weaken the interactions of the analyte with the SPE material. The final method employed water washes to obtain the desired recovery and avoid the potential impact of buffered solutions. The current procedure provides the most consistent extraction of entecavir from plasma. The high loading capacity combined with the high-pH mobile phase allows for the quantitation of entecavir at 5 pg/mL level in human plasma samples.

3.3. Contamination and carryover

Working in the low pg/mL range, contamination by casual exposure of samples to surfaces or atmosphere in a laboratory where reference materials are handled represents a significant challenge. Extra precaution in cleaning of work area, use of balance enclosures, and separation of reference standards from work area helped to avoid contamination problems. The post-SPE evaporation step under nitrogen did not appear to cause any contamination. We were also able to control cross-contamination in the 96-well SPE processing and sample handling by programming high volume tip washes in the SPE procedures after each plasma sample transfer for all tips, as evidenced by the results in validation and the subsequent sample analysis work. Carryover from the autosampler was determined to be less than 0.05%. To ensure that carryover did not interfere in sample analysis, blank samples were also strategically added after the high concentration standards and QC samples to minimize the effect. There was no apparent carryover from the liquid handlers since all blank samples and pre-dose samples were free from observed analyte peaks in assay applications.

3.4. Filtration of reconstituted samples

One additional step required in the method was the filtration of reconstituted samples before sample injection. This became evident during the initial method validation work when responses of lower standards began to deteriorate in later portion of the injection sequence; leading to failed runs. To assess the effect of filtration, a comparison was made between the filtered and non-filtered samples at the LLOQ level. Such an effect is shown in Fig. 3 with a comparison of the chromatograms of an injection of a filtered LLOQ sample (open circle) and a non-filtered LLOQ sample after consecutive injections of 16 non-filtered LLOQ samples. Peak broadening and signal to noise decrease are clearly visible in the chromatogram of an unfiltered sample. To better assess the effect, the signal to noise ratios of the IS peaks of the two samples were calculated. The signal to noise ratios are 2134 vs. 1056, i.e., 2-fold better for the filtered sample although the peak height of the filtered sample was only ~20% higher. If the signals of the analyte were compared, the effect was even more pronounced. We suspect that the filter membrane (made of nylon) selectively traps some matrix components from the sample that would otherwise accumulate on the column and affect the analyte response. Filtration may also remove fine SPE packing materials washed into samples that could accumulate at the column head and affect column and assay performance. The effect of sample filtration was confirmed by more than one laboratory that supported the clinical studies. Although the underlining mechanism is not well understood, it is nonetheless a critical consideration in the success of the assay implementation with this low concentration assay.

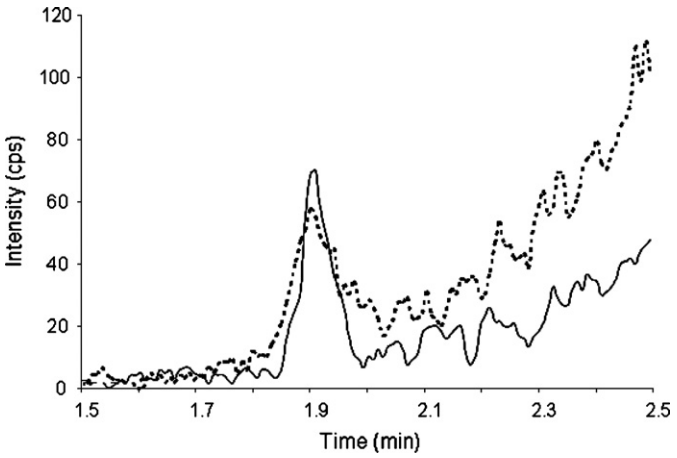

Fig. 3. LLOQ sample chromatograms comparison between a filtered sample (solid trace) and a non-filtered sample (dashed trace).

3.5. Linearity

A quadratic regression model was selected to cover the wide dynamic range of the assay taking into consideration the limited dynamic range of the instrument. The quadratic term is very small but can provide adequate correction on days when the MS response is particularly strong. The quadratic behavior observed in validation runs was likely due to detector response non-linearity at high concentrations and the quadratic behavior became less obvious when signal responses were lower. However, the regression model did not impact the analysis results.

3.6. Matrix effects

The magnitude of absolute matrix effect of 0.45 was considered to be moderate. Attempts to reduce matrix effects by modifying extraction and chromatographic conditions did not yield apparent improvement. Considering the highly polar nature of the analyte and IS and the presence of matrix components of similar properties in plasma, it would be very difficult to completely eliminate the matrix effect without significant loss of analyte. The relative matrix effect, considering the matrix effects across multiple matrix lots, was minimal as indicated by the very precise results from LLOQ samples prepared in individual plasma lots and had minimal impact on the results. During the analysis of incurred samples in a clinical study, the responses of the IS from both incurred samples and QCs were monitored and found to be similar and consistent, indicating that the IS adequately correct for matrix interferences. Moreover, the successful implementation of this assay to support clinical studies confirmed its ruggedness.

Table 1
Back-calculated entecavir concentrations in human plasma standards from the assay validation[a].

Concentration (ng/mL)	0.00500	0.01000	0.10000	0.50000	4.00000	10.00000	20.00000	25.00000
Run 1	0.00562	0.01121	0.09780	0.47238	3.94661	10.13031	19.58153	24.59455
	0.00495	0.00941	0.09567	0.47782	3.85270	10.45708	19.78815	25.70982
Run 2	0.00524	0.00984	0.09117	0.43075	3.57543	9.25875	18.54177	24.02823
	0.00478	0.01081	0.10800	0.55593	4.23490	10.63068	22.77061	10.30639[b]
Run 3	0.00466	0.00855	0.08387	0.45777	3.71586	9.15580	17.22062	22.93398
	0.00524	0.01153	0.11018	0.58039	4.23011	11.14859	21.93669	27.64500
Run 4	0.00516	0.01121	0.10152	0.49440	3.98849	10.11668	20.24577	25.56501
	0.00434	0.01012	0.09934	0.49650	3.83369	10.08922	19.93311	24.23636
Overall mean	0.00500	0.01034	0.09844	0.49574	3.92222	10.12339	20.00228	24.95899
%CV	8.0	10.1	8.7	10.0	5.9	6.6	8.8	6.1
%Dev	0.0	3.4	−1.6	−0.9	−1.9	1.2	0.0	−0.2
N	8	8	8	8	8	8	8	7

[a] The curve parameters were calculated in Watson DMLIMS based on peak area ratio of the analyte vs. internal standard.
[b] This sample was deactivated due to abnormally lower IS response.

4. Validation results

Acceptance criteria used for the establishment of the validation runs were in compliance with the FDA Guidance for Industry—Bioanalytical Method Validation (2001). Overall, only one standard was statistically excluded from regression and only three additional of the 64 total standards failed to meet acceptance criteria. Individual standard results were shown in Table 1. The average equation for the $1/X$ weighted quadratic was $Y = -0.00058 + 0.26873X + 0.00017X^2$ with a correlation factor greater than 0.99.

In each run, the deviations of the predicted concentrations from their nominal values were within ±15.0% for at least two-thirds of the QC samples. Overall, only 4 of 45 QC samples in the three accu-

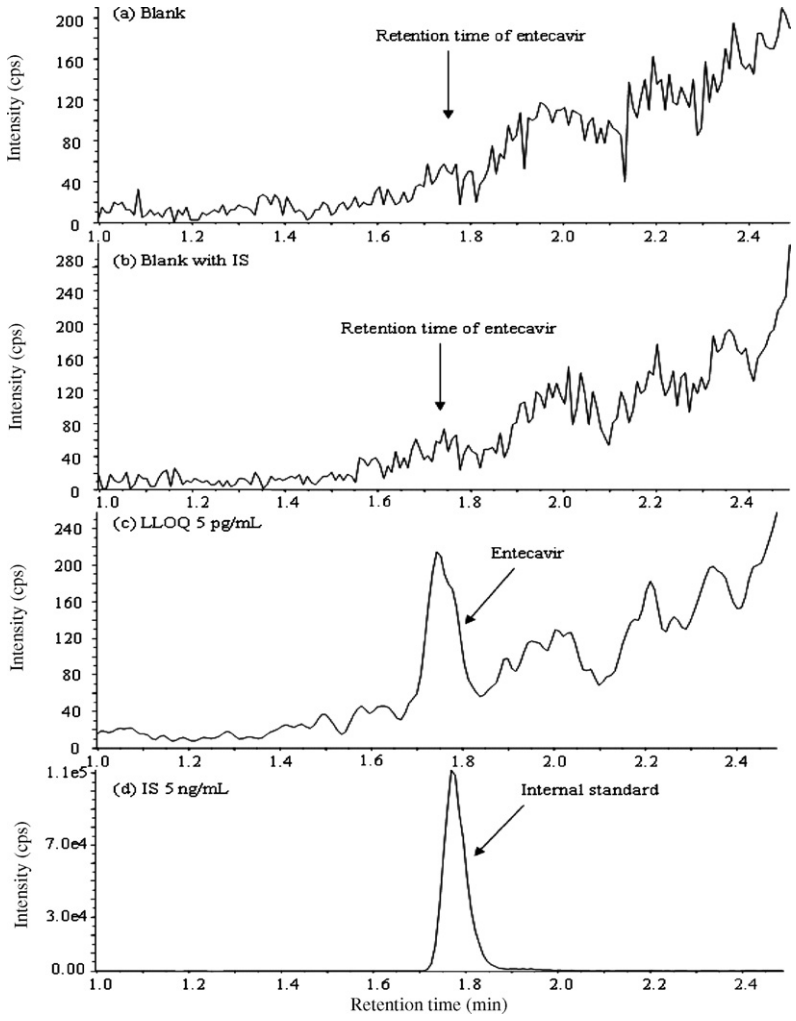

Fig. 4. Selected reaction monitoring chromatograms for entecavir obtained from: (a) blank plasma; (b) blank plasma with internal standard at 5 ng/mL; (c) plasma containing entecavir at 5 pg/mL and its internal standard; (d) internal standard of LLOQ sample (c).

Table 2
Accuracy and precision[a] for entecavir determination in human plasma from the assay validation[a].

Nominal conc. (ng/mL)	0.0150	10.0	20.0
Mean observed conc. (ng/mL)	0.0160	9.41	19.4
%Dev	7.6	−5.9	−3.0
Between run precision (%CV)	0.0[a]	0.0[a]	3.1
Within run precision (%CV)	12.3	8.5	6.9
Total variation (%CV)	11.0	8.4	7.5
N	15	15	15
Number of runs	3	3	3

[a] The accuracy and precision values were calculated in Watson DMLIMS using a one-way ANOVA calculation and 0.0%CV indicates that no additional variability observed as a result of analyzing samples in separate runs.

racy and precision runs failed and just 5 of 54 within all four runs. The results of one-way ANOVA for the three runs used to determine the accuracy and precision of the method are shown in Table 2. The intra-assay precision was within 12.3% and inter-assay precision was within 3.1%. The assay accuracy was within ±7.6% of the nominal values.

No significant interfering peaks from the human plasma were found at the retention time and in the ion channels of either the analyte or the IS. The deviations of the predicted concentrations from the nominal value were within ±10.3% for five of the six LLOQ samples. A SRM chromatogram at the LLOQ concentration is shown in Fig. 4.

The recoveries of entecavir were 86.8% at 0.015 ng/mL, 82.7% at 10.0 ng/mL and 78.9% at 20.0 ng/mL. The IS had an average recovery of 82.8% with a range of 81.2–85.0%.

The calculated absolute matrix effect was around 0.45 at 0.015, 10.0, and 20.0 ng/mL for the analyte, and at 5 ng/mL for the IS. Lobucavir served to compensate for matrix effect variations as indicated by the agreement between standards and QCs.

The storage stability of entecavir in human EDTA plasma at −20 °C was evaluated in triplicate using QC samples stored for a period of 14 weeks. The deviations of the mean predicted concentrations of the QC samples from the nominal concentrations were used as indicators of the −20 °C stability of entecavir in human plasma and ranged from +5.9% at 0.0150 ng/mL to −6.3% at 10.0 ng/mL. In a subsequent validation, storage stability in human plasma at −20 °C over one-year-period has been demonstrated (data not shown).

The deviations of the mean predicted concentrations of the test freeze–thaw QC samples from the nominal concentrations ranged from +6.3% at 0.0150 ng/mL to −11.7% at 20.0 ng/mL. Autosampler stability over 120 h was demonstrated at room temperature, and stock solution stability for over a year was also demonstrated. The compound appears very stable under various stability conditions tested.

5. Application to study sample analysis

The assay was used to support study sample analysis for a low dose Phase I study in humans [26]. Overall analytical figures of merit of the analysis, summarized in Table 3, were very similar to those of the validation, indicating excellent applicability of the assay to study sample analysis. Plasma drug concentration vs. time after an oral dosing of 0.5 mg entecavir in a healthy subject is shown in Fig. 5. SRM chromatograms of two incurred samples, a pre-dose and 0.25 h samples from a selected individual were exhibited in Fig. 6. Although there is considerable background noise at later times, there is no endogenous interference at the retention time of entecavir indicating specificity of the assay.

Table 3
Accuracy and precision[a] for entecavir analysis in human plasma from a low-dose human study.

Nominal conc. (ng/mL)	0.0150	10.0	20.0
Mean observed conc. (ng/mL)	0.0149	9.96	19.0
%Dev	−0.7	0.4	−4.8
Between run precision (%CV)	4.3	1.9	4.6
Within run precision (%CV)	9.7	5.3	5.3
Total variation (%CV)	10.6	5.6	7.0
N	59	60	60
Number of runs	20	20	20

[a] The accuracy and precision values were calculated in Watson DMLIMS using a one-way ANOVA calculation.

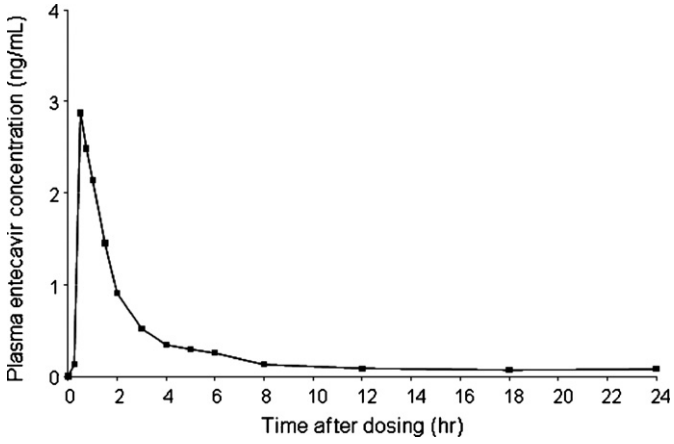

Fig. 5. Concentration of entecavir in plasma vs. time curve after oral dosing of 0.5 mg entecavir in a healthy subject.

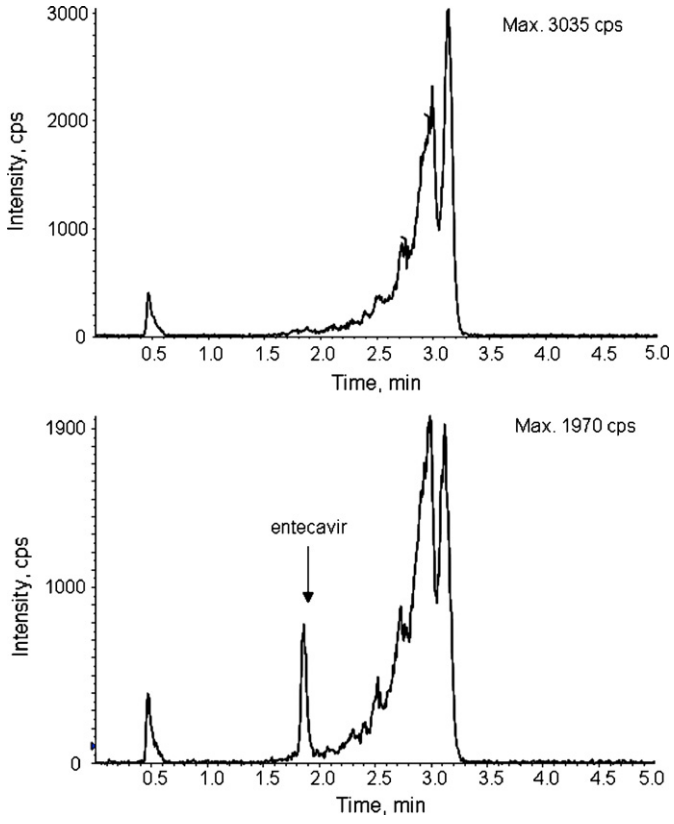

Fig. 6. Selected reaction monitoring chromatograms of plasma samples from a subject dosed with 0.5 mg entecavir (top: pre-dose; bottom: 0.25 h post-dose sample with a concentration of ~14 pg/mL).

6. Conclusions

A sensitive LC–MS/MS method for the quantitation of entecavir in 1 mL of human EDTA plasma was developed and validated over the concentration range of 5 pg/mL to 25 ng/mL owing to a high enrichment of analyte using Oasis HLB SPE plate and a sensitivity enhancement under high-pH mobile phase conditions. The method was successfully applied to the sample analysis of a Phase I low dose MAD study and analytical figures of merit from the sample analysis runs suggest that the assay was precise and accurate. The methodology has been successfully transferred to several CRO laboratories to support clinical studies for entecavir. The observations and methodologies employed in the assay, while unique, should benefit broadly the researchers in developing methods for low-level small molecular weight, polar analytes or drugs.

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