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Short communication

Salting-out homogeneous liquid–liquid extraction approach applied in sample pre-processing for the quantitative determination of entecavir in human plasma by LC–MS

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ABSTRACT

A convenient, robust, economical and selective sample preparation method for the quantitative determination of entecavir in human plasma by LC–MS was developed and validated. Entecavir and the internal standard of acyclovir were extracted from 500 μL of human plasma by a salting-out homogeneous liquid–liquid extraction approach (SHLLE) with acetonitrile as the organic extractant and magnesium sulfate as the salting-out reagent. They were analyzed on a Hanbon® Lichrospher RP C18 HPLC column (150 mm × 2.0 mm; 5 μm) with gradient elution. The mobile phase comprised 0.1% acetic acid–0.2 mmol ammonium acetate in water (mobile phase A) and acetonitrile (mobile phase B). The flow rate is 0.2 mL/min. The analytes were detected by a LC–MS 2010 single quadrupole mass spectrometer instrument equipped with an electrospray ionization interface using selective ion monitoring positive mode. A "post cut" column switch technique was incorporated into the method to remove interferences of earlier and later eluting matrix components than entecavir and internal standard, including salting-out reagent used in sample pre-processing. The method was validated over the concentration range of 0.05–20 ng/mL. The intra-day and inter-day precision of the assay, as measured by the coefficient of variation (%CV), was within 3.59%, and the intra-day assay accuracy was found to be within 4.88%. The average recovery of entecavir was about 50% and the ion suppression was approximately 44% over the standard curve. Comparison of matrix effect between SHLLE and SPE by continuous post column infusion showed that these two methods got similar, slight ion suppression. The SHLLE method has been successfully utilized for the analysis of entecavir in post-dose samples from a clinical study.

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1. Introduction

Entecavir, an inhibitor of HBV DNA polymerase at both the priming and elongation steps required for viral replication, has potent and selective activity against HBV with few side effects or mitochondrial toxicity [1]. Entecavir suppresses HBV replication more rapidly and effectively than lamivudine or adefovir in compensated patients [2,3] and has less resistance in chronic therapy [4,5].

In clinical pharmacokinetic studies of entecavir, the recommended dose is 0.5 and 1.0 mg/day. When healthy subjects received a single dose of 0.5 or 1.0 mg/day, blood concentrations of the 24 h samples were in the range of 0.1–3.19 ng/mL or 0.1–6.8 ng/mL respectively [6]. According to the comments from the FDA, companies may wish to truncate the AUC at 72 h due to its relatively long half-life while conducting bioequivalence studies, suggesting that a much lower (pg/mL) limit of quantitation would be required. Concentrating and enrichment are, therefore, of the utmost importance in determining the concentration of entecavir in blood.

Recently, a LC–MS/MS assay method was developed and validated which included the use of a Oasis HLB extraction plate for large volume (1 mL) enrichment of plasma samples, which can get a limit of quantitation at pg/mL level [7]. Just using a single quad LC–MS, our department has got a limit of quantitation at 200 pg/mL of entecavir in human plasma by Oasis MCX extraction column cartridges for sample pre-processing [8]. However, the limit of quantitation at 200 pg/mL is not sufficient. Furthermore, the process of solid phase extraction (SPE) is relatively expensive. There

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is also a potential concern about batch-to-batch reproducibility of the SPE column cartridges. To a certain degree, precision and reliability of the method depends on the product quality of extraction column cartridges [9].

Entecavir is a weak alkaline, low-mass compound with high polarity, so it is very hard to extract it efficiently from blood using conventional liquid–liquid extraction (LLE). However, the new technique of salting-out homogeneous liquid–liquid extraction (SHLLE) provides a feasible alternative for sample preparation. SHLLE utilizes the salt-induced phase separation phenomenon whereby the organic phase is separated from a homogeneous solution and simultaneously the target solutes are extracted into the separated organic phase when the salting-out reagent is added. This method can be applied to inorganic elements, very polar drugs or hydrophobic drugs dissolving in organic solvent such as acetonitrile that can be homogenized with water [10–12]. Recently, this approach has attracted attention from bioanalysts [13] and has been used for bioanalysis of drugs such as simvastatin and simvastatin acid [12], polycyclic aromatic hydrocarbons [14], catechol and hydroquinone [15], sulfonamides [16], lopinavir and ritonavir [17]. The operational approach of SHLLE is very similar to conventional LLE, and high-throughput, automated salting-out assisted liquid–liquid extraction has been implemented recently [12]. The precision of sample pre-processing depends on the operational quality, which can be precisely controlled by the operator.

The key points of developing a pre-processing method of SHLLE include organic extractant selection, salting-out reagent selection and optimization of their ratio. During the optimization process, two investigations of SHLLE, extraction recovery and matrix effect, are required. Extraction recovery may be low because of the analyte's high polarity and ion suppression may be strong because of the high concentration of the added salting-out reagent. As a successful method of SHLLE, extraction recovery and matrix effect at different concentrations should be constant with good precision and high sensitivity over the range of the standard curve. Many polar organic solvents, which can be homogenized with water, such as acetonitrile, isopropanol, methanol, ethanol, acetone and dimethyl sulfoxide can be used as extractant in SHLLE [13]. Acetonitrile is the most common extracting solvent in SHLLE. Three factors should be considered when evaluating a salt and its potential salting-out effect [13]. First, the solubility of the salt in extracting solvent must be negligible. Secondly, the solubility in water must be large in order to have maximum interaction with the water molecules. And thirdly, the ability of ions to precipitate hydrophilic substances (gels), according the lyotropic series, is ordered as Mg2+ > Ca2+ > Sr2+ > Ba2+ > Li+ > Na+ > K+ > Rb+ > Cs+. A potential concern of the method for the subsequent LC–MS analysis of biological samples is that a portion of the added salt (typically of high concentration) might be extracted to affect the chromatography separation and ionization of chromatography effluents in a mass spectrometer. So, a mass spectrometry friendly organic salt such as ammonium acetate or ammonium formate is the first choice as the salting-out reagent in SHLLE [18].

To date, no assay has been reported in the literature where entecavir concentrations in human plasma have been quantified using SHLLE for sample pre-processing. In this study, we developed a sensitive and reliable method for the quantitative determination of entecavir in human plasma using SHLLE and LC–ESI-MS. Acetonitrile was chosen as the organic solvent in SHLLE that entecavir would be extracted in acetonitrile separated from water phase when magnesium sulfate was added in. The layer of acetonitrile was dried and dissolved in water for concentration while back-extraction was operated following to remove organic interferences for further. A "post cut" column switch technique is incorporated into the method to remove interferences of earlier and later eluting matrix components than entecavir and internal standard, including magnesium sulfate. The method was validated over the analyte concentration range of 0.05–20 ng/mL and then was successfully utilized to analyze samples collected from Chinese healthy subjects after oral administration of entecavir capsule.

Matrix effect is a major problem in LC–MS analysis. Solid-phase extraction offers great extraction procedure selectivity from multiple chemistry packing commercially available, allowing the elimination of matrix interferences and sample concentration to reach desired sensitivity [19]. SPE conditions such as cation ion exchange have already been described to minimize the extraction of phospholipids prior to LC–MS/MS analysis [20]. So, we also compared matrix effect of two sample pro-processing methods, SHLLE and solid phase extraction by Oasis MCX extraction column cartridges, for further in API 3000 LC–MS/MS instrument by post-column infusion.

2. Materials and methods

2.1. Chemicals and reagents

Ultrapure water was obtained using a UPH Ultrapure Water System (UPH-II-5T, Chengdu, China), methanol and acetonitrile (Merck KGaA, Damstadt, Germany) were of gradient grade for liquid chromatography. Acetic acid, formic acid, ammonia water, magnesium sulfate, ammonium acetate and diethyl ether were purchased from Nanjing Chemical Reagent Co., Ltd (Nanjing, China). Entecavir was obtained from Hainan Zhonghe Pharmaceutical Co., Ltd (content = 100.2%). Acyclovir (internal standard, purity >99.5%) was purchased from the Jiangsu Institute for Food and Drug Control. Oasis MCX (3 cm^3, 60 mg, 30 μm) SPE columns were purchased from Waters (Milford, MA, USA).

2.2. Apparatus and conditions

LC–MS: An LC–MS 2010 system (Shimadzu, Kyoto Prefecture, Japan) comprising two LC-20AD pumps, a CBM-20A system controller, a CTO-20A column oven, a SIL-20AC auto sampler and a single quadrupole mass spectrometer equipped with an electrospray ionization interface was used for the analysis. Separation was carried out on a Hanbon® Lichrospher RP C18 column (150 mm × 2.0 mm; 5 μm) and the oven temperature was set at 35 °C. Gradient elution was performed with 0.1% acetic acid–0.2 mmol ammonium acetate in water as mobile phase A and acetonitrile as mobile phase B at a flow rate of 0.2 mL/min. One minute after sample injection, the percentage of mobile phase B was increased from 6% to 22% over 2.5 min and increased up to 80% in 0.01 min. This was retained at 80% for 2.5 min and then decreased to 6% within 0.01 min. It was retained at 6% for 5 min. A "post cut" column switch valve was set at 2.5–7 min flowing into mass analyzer for detection. Selected ion monitoring (SIM) was used to detect entecavir and acyclovir in positive mode at mass/charge ratio (m/z) 278.1 and 226.1, respectively. Operating parameters for electrospray ionization mass spectrometry included capillary voltage 1.5 kV, nebulizer nitrogen flow rate 1.5 L/min, drying gas temperature 280 °C and block temperature 220 °C. The injection volume was 5 μL.

LC–MS/MS: The system comprised an AB-Sciex (Concord, Ontario, USA) model API 3000 triple quadrupole mass spectrometer with vendor-supplied ESI sources, a CTC HTS PAL autosampler (LEAP Technologies, Carrboro, NC, USA), a set of LC-10ADvp pumps (Shimadzu, Columbia, MD, USA), a SCL-10AUP Cohensive controller, a CTO-10As vp column oven and a DGU-14A degasser. The chromatographic column and conditions were identical to those described above. The mass spectrometers coupled with their

standard ESI interface were operated in positive ion mode. The tandem mass transitions selected to monitor entecavir at m/z 278.3 → 152.1. The mass parameters of entecavir were optimized one by one including source gas parameters (NEB:10, CUR:10, CAD:4, IS:4500, TEM:500) and compound parameters (DP:68, FP:250, EPP:10, CE:23, CXP:10). Data acquisition software was Analyst version 1.4.2.

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

Analyte stock solutions containing entecavir or acyclovir at 1 mg/mL were prepared in purified water. The standard solutions of entecavir were prepared by diluting stock solution with purified water to 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 μg/mL. The internal standard solution was prepared as 0.4 μg/mL. The calibration standards and quality control samples were prepared by adding 5 μL of standard solutions of entecavir and 5 μL internal standard solution into 500 μL of pooled blank plasma and the nominal concentrations of the calibration standards were 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 ng/mL. In this way, QC samples at concentrations of 0.12, 2, 16 ng/mL were also prepared.

2.4. Sample extraction

2.4.1. SHLLE

Acetonitrile (1000 μL) was added to 500 μL plasma samples in 10 mL glass centrifuge tubes. After vortexing for 1 min, 500 μL of magnesium sulfate–water (37.5%) was added into the mixture and vortexed for 2 min. After centrifuging at 2130 × g for 5 min, the supernatant was collected into another 10 mL glass centrifuge tube and dried under air in a 60 °C water bath. The residue was redissolved in 50 μL mobile phase A and vortexed for 2 min. Then, the sample was transferred into a microcentrifuge tube and 150 μL diethyl ether added. After vortexing for 2 min and centrifuging at 20,627 × g for 5 min, 5 μL lower layer of liquid was injected into the LC–MS or LC–MS/MS system for analysis.

2.4.2. SPE

Six hundred μL of 10% perchloric acid was added to 1 mL of plasma. After vortexing for 1 min and centrifuging at 20,627 × g for 5 min, the supernatant was loaded on an Oasis MCX extraction column which was pretreated with 1 mL methanol and 1 mL water. After gravity elution, the column was washed with a 1 mL 2% formic acid and 1 mL methanol, and then the eluate was discarded. Subsequently, the column was washed with 1 mL mixture of 4% ammonia–methanol and the eluate was collected in a 10 mL glass centrifuge tube. The eluate was dried under air in a 60 °C water bath and the residue redissolved in 100 μL mobile phase A. After vortexed, the sample was transferred into a microcentrifuge tube and centrifuged at 20,627 × g for 5 min. Five μL of supernatant was injected into the LC–MS/MS system for analysis.

2.5. Post-column infusion

The post-column infusion method is a procedure to evaluate the matrix effect over the course of a chromatographic run and it is a powerful tool used during method development. The region where ionization suppression or enhancement occurs can be depicted on a chromatogram by post-column injection.

Post-column infusion experiments were conducted in which a 1 μg/mL solution of entecavir was constantly infused (10 μL/min) post-column through 'T-mixer' into the mass analyzer and blank plasma extract prepared by the sample pre-processing method of SHLLE or SPE was injected pre-column through 'T-mixer' into mass analyzer simultaneously. Matrix effect was performed on a triple quadrupole mass spectrometer equipped with an electrospray interface. The infusion was obtained as chromatograms for 11 min and any region of ionization suppression or enhancement was observed as a decrease or increase in the baseline.

3. Results and discussion

3.1. Optimization of conditions in SHLLE

According to the references mentioned previously, we compared the extraction recoveries of four salting-out reagents, ammonium acetate, ammonium formate, zinc sulfate, magnesium sulfate, with acetonitrile as the extracting organic solvent. The extraction recovery using ammonium acetate or ammonium formate was too low to be useful. The extraction recovery using magnesium sulfate was higher than when using zinc sulfate. We also compared the extract recovery when using various concentrations of magnesium sulfate in mixed solution. When we compared the extraction recoveries of acetonitrile and methanol with magnesium sulfate as salting-out reagent, acetonitrile was chosen as the extracting organic solvent.

At last we chose acetonitrile as the extracting organic solvent and magnesium sulfate as salting-out reagent with the concentration of 1 mol/L in mixed solution, and the extraction recovery we obtained at last was about 50%. In order to diminish the damage to mass spectrometer from magnesium sulfate, a "post cut" column switch technique was incorporated into the method to remove magnesium sulfate eluting much earlier from the chromatographic column than entecavir and the internal standard under initial high aqueous phase conditions (94% A at first 1 min).

3.2. Method validation

3.2.1. Standard curve and linearity

Human plasma spiked with standard solutions of entecavir in concentrations of 0.005–2 μg/mL was used for preparing the standard curve. The standard curve used was 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 ng/mL entecavir. The peak area ratio of entecavir to the internal standard (f) was plotted against entecavir concentration (C) (Fig. 1). A linear regression with weighting at $1/C$ was used to determine slope, intercept, and correlation coefficient. The standard curve equation was $f = 0.28436 \times C - 0.0025448$ with the correlation coefficient $R = 0.99997$. The coefficient of variation (CV) was less than 15% for each concentration level of all calibration points over the curve.

3.2.2. Method specificity

The specificity of the method was examined by analyzing different blank human plasma extractions using the same methods as the sample preparation procedure. The retention time (min) of

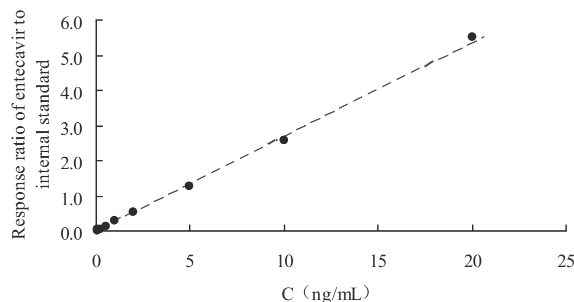

Fig. 1. Standard curve by weighting at $1/C$ with the range of 0.05–20 ng/mL of entecavir in human plasma.

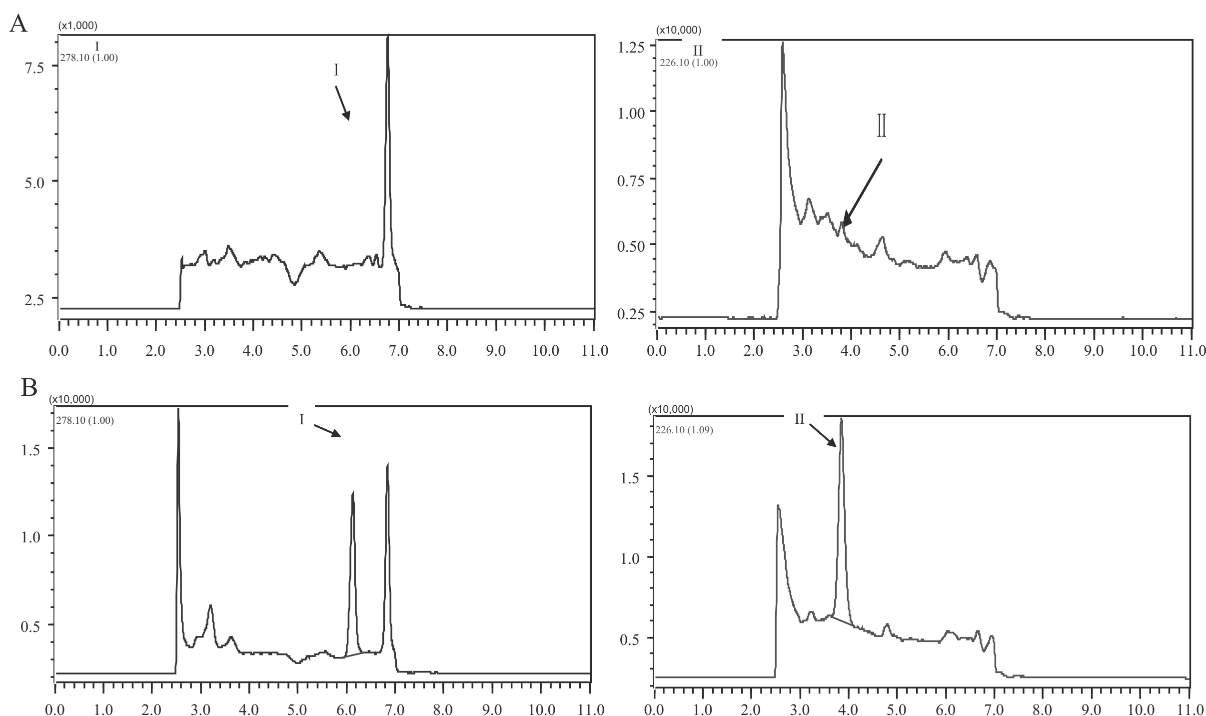

Fig. 2. Chromatograms of method specificity (A) blank plasma and (B) blank plasma with entecavir at 2 ng/mL and its internal standard (I: entecavir, II: acyclovir).

Table 1
Accuracy data for the determination of entecavir in five lots of human plasma.

Actual conc. (ng/mL)	0.12	2	16
Measured conc./actual conc.Mean (%, $n = 5$)	103.01	102.29	99.71
CV (%)	3.71	1.62	4.88

entecavir is 6.1 min and internal standard is 3.8 min. We got smooth baseline and sharp peaks without any endogenous interference. No peak eluting at the retention times of entecavir or the internal standard was detected in the blank samples prepared from six different plasma sources. The relative chromatograms are shown in Fig. 2.

3.2.3. Lower limit of quantification

The LLOQ was determined as the lowest concentration of entecavir giving a response that was five times compared to blank response and quantified with the inter assay variation of 5.17%. Representative chromatograms from the standard at LLOQ (lower limit of quantization) are shown in Fig. 3.

3.2.4. Accuracy and precision

Accuracy was measured as the percentage of measured concentration to theoretical concentration by injecting five standard samples of each concentration of QC sample. The accuracy of the QC samples is presented in Table 1, demonstrating that the measured concentrations are within 90–110% of the actual concentration.

Table 2
Intra-day and inter-day precision data for the determination of entecavir in five lots of human plasma.

Actual conc. (ng/mL)		0.12	2	16
Mean of measured conc. (ng/mL, $n = 5$)		0.12	2.04	16.03
CV (%)	Intra-day	2.67	0.5	2.1
	Inter-day	2.9	3.59	3.59

Table 3
Assessment data of stability of entecavir under various storage conditions in human plasma based on the analysis of quality control samples ($n = 3$).

QCs	Low	Middle	High
Actual conc. (ng/mL)	0.12	2	16
[a] Instant conc. (ng/mL)	0.12	1.96	15.65
[b] Benchtop conc. (ng/mL)	0.12	1.9	15.63
[c] Freeze/thaw conc. (ng/mL)	0.12	1.97	15.74
[d] Long-term storage conc. (ng/mL)	0.13	2.1	16.86
[e] Remained on autosampler conc. (ng/mL)	0.12	1.92	15.53

[a] Injected instantly after preparation.
[b] Benchtop at room temperature for 24 h.
[c] Three freeze (−20 °C)/thaw cycles.
[d] Long-term storage (−20 °C) for 50 d.
[e] Samples remained on the autosampler for 24 h.

Injecting five standard samples at each concentration of QC sample the same day assessed intra-day variation of the assay. Inter-day variation was assessed by injecting another five samples of each concentration on 3 subsequent days. The precision of the method was expressed in coefficients of variation (%CV). The %CV of inter-day and intra-day at each concentration of QC sample was less than 5%. This was within the acceptable limits to meet the bioanalytical method guidelines for bioanalytical validation. Data for precision is shown in Table 2.

Table 4
Data of extraction recovery.

Conc. (ng/mL)		0.12	2	16
Mean ($n = 5$)	$f1^a = A_s/A_i$	0.0162	0.221	1.481
	$f2^b = A_s/A_i$	0.418	0.546	3.557
$R^c = f1/f2$ (%)		48.46	50.61	52.06

[a] The response ratio of extracted plasma samples spiked with entecavir before extraction and internal standard after extraction.
[b] The response ratio of extracted plasma samples spiked with entecavir and internal standard after extraction.
[c] Extraction recovery.

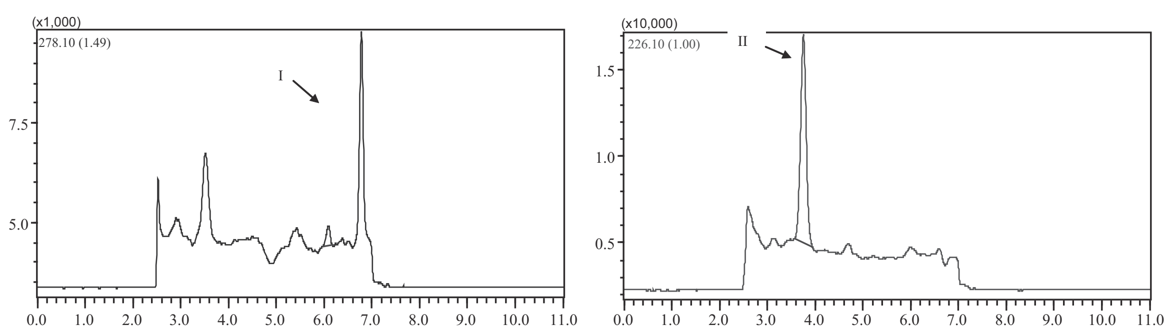

Fig. 3. Chromatogram at LLOQ containing target analyte of entecavir (0.05 ng/mL) and internal standard of acyclovir (I: entecavir, II: acyclovir).

Table 5
Post-extraction addition for assessment of matrix effect in LC/MS with SHLLE and back-extract in sample pre-processing.

Conc. (ng/mL)	0.12		2		16	
Area of the chromatography peek (A)	[c] A_s	[d] A_i	[c] A_s	[d] A_i	[c] A_s	[d] A_i
[a] A_{plasma} (mean, n = 6)	11,757	346,579	159,490	375,508	903,465	357,728
[b] A_{water} (mean, n = 3)	21,100	359,794	293,725	409,672	1,569,782	380,503
A_{plasma}/A_{water} (%)	55.72	96.33	54.30	91.66	57.55	94.01

[a] Entecavir and internal standard added in blank plasma extraction.
[b] Entecavir and internal standard added in water extraction.
[c] Chromatography peek area of entecavir.
[d] Chromatography peek area of internal standard.

3.2.5. Analyte stability

Stability of entecavir under various storage conditions was investigated. Room temperature (RT) benchtop, freeze/thaw and long-term storage stability were assessed by the analysis of quality control samples that were left on the benchtop at room temperature for 24 h, or subjected to three freeze/thaw (RT) cycles, or left under frozen condition for 50 days prior to the sample extraction. The results indicated that entecavir is stable in plasma under these conditions. Processed sample stability was evaluated by the re-injection of a group of samples consisting of QC samples that were remained on the autosampler for 24 h later (Table 3).

3.2.6. Extraction recovery and assessment of matrix effect

3.2.6.1. Extraction recovery. Extraction recoveries from human plasma were determined by comparing the response ratio of extracted plasma samples spiked with known amounts of entecavir (0.12, 2 or 16 ng/mL, n = 5) before extraction, and fixed concentration of internal standard after extraction with the response ratio of extracted blank plasma samples to which analyte and internal standard have been added at the same nominal concentration prior to injection. The extraction recoveries obtained for each concentration were within 48.46–52.06% (Table 4). The average recovery of entecavir over the standard curve range of the assay was approximately 50%.

3.2.6.2. Post-extraction addition for assessment of matrix effect. Post-extraction addition experiments were conducted in which human blank plasma from different people (n = 6) were aliquoted and extracted as described in Section 2.4.1, reconstituted with a standard solution of entecavir at concentrations of 0.12, 2, or 16 ng/mL, mixed, then injected into LC–MS system for the response (A_{plasma}). The same process previously used was repeated for the response (A_{water}) with human blank plasma replaced by purified water (n = 3). The response ratio (A_{plasma}/A_{water}) was used as the matrix effect by the common outflow. The response ratio of entecavir at different analyte concentrations was within 54.30–57.55% and the response ratio of internal standard at the concentration in the analysis was within 91.66–96.33% with the chromatographic condition described in Section 3.2. The result of matrix effect in LC–MS by SHLLE combined with back-extraction is presented in Table 5. The result shows relatively constant ion suppression (~44%) of entecavir and slight ion suppression (~6%) of internal standard with low coefficient of variation (CV < 1.5%).

3.3. Comparison of matrix effect between SHLLE and SPE by post-column infusion

In order to directly compare the location and the level of ionization suppression or enhancement of entecavir between SHLLE and SPE pretreatment methods, we used a LC–MS/MS system which was equipped with a post-column device. The chromatographic behavior of entecavir under the condition of LC–MS/MS described in Section 3.2 is different from that described previously. The retention time of entecavir is 4.57 min. Blank plasma extract was prepared by two sample pre-processing methods of SHLLE and SPE as described in Section 3.4, and injected pre-column through a 'T-mixer' into the mass analyzer. Fig. 4 shows that the distribution of suppression or enhancement regions of the chromatogram is very similar between these two pretreatment methods. There was only slight ion suppression at the retention time of entecavir.

3.4. Method application

The described SHLLE assay has been successfully utilized for the analysis of entecavir by LC–MS in post-dose samples in a bioequivalence study of entecavir in Chinese healthy subjects. This bioequivalence study got official approval of hospital ethics committees and was performed in Jiangsu province hospital of TCM. Representative chromatograms from post-dose samples are

Table 6
Accuracy and variation of QCs in the analytic process of determining all the post-dose plasma samples in bioequivalence study of entecavir in Chinese healthy subjects.

QCs	Low (n = 14)	Middle (n = 14)	High (n = 12)
Mean of measured conc./actual conc. (%)	103.4	104.8	103.6
CV (%)	5.61	3.18	3.23

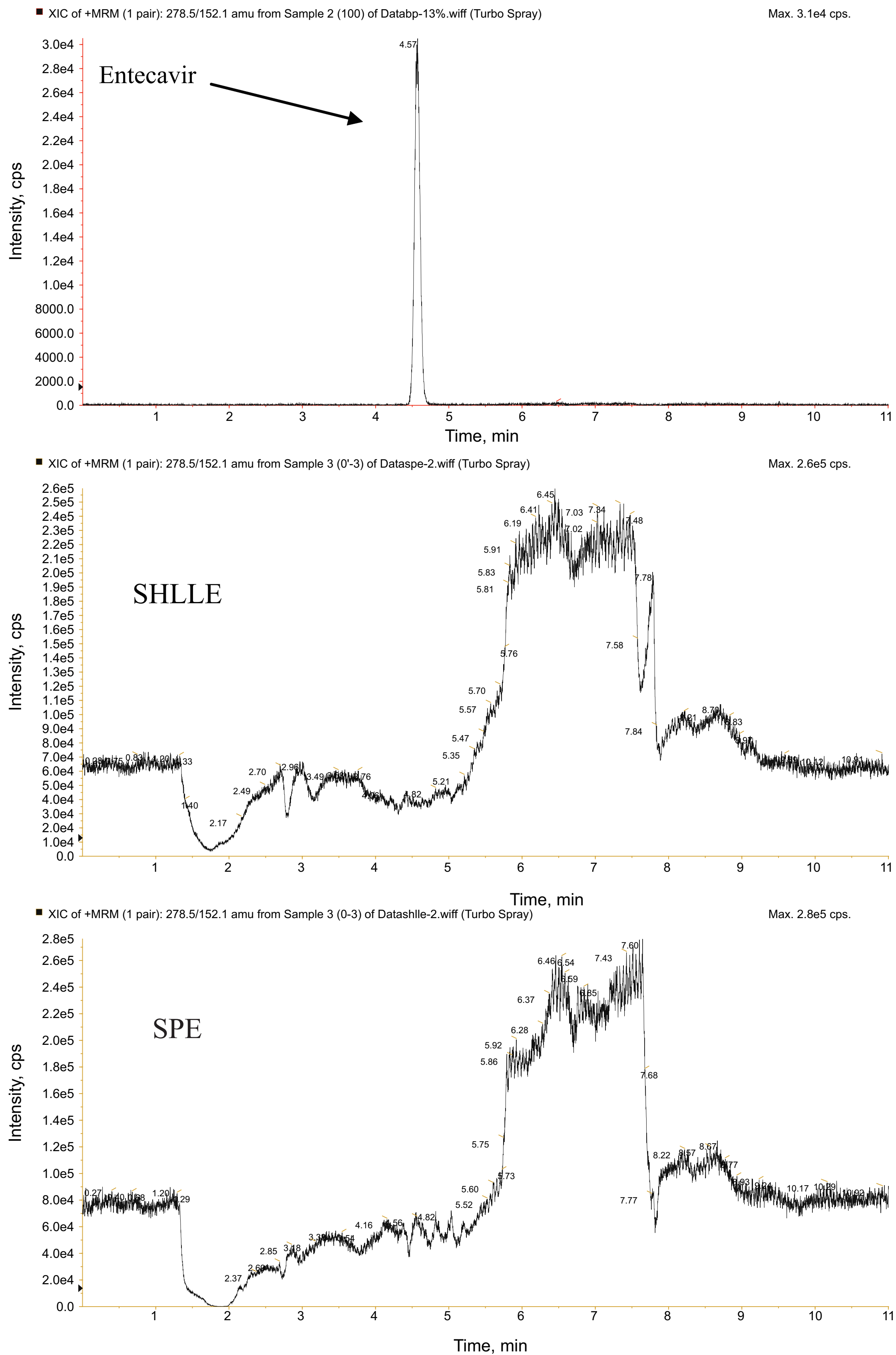

Fig. 4. Comparison of matrix effect between SPE and SHLLE pretreatment methods by continuous post-column entecavir infusion in a HPLC–MS/MS analysis system. The distribution of suppression or enhancement regions of the chromatogram is very similar between these two methods. There was only slight ion suppression at the retention time of entecavir.

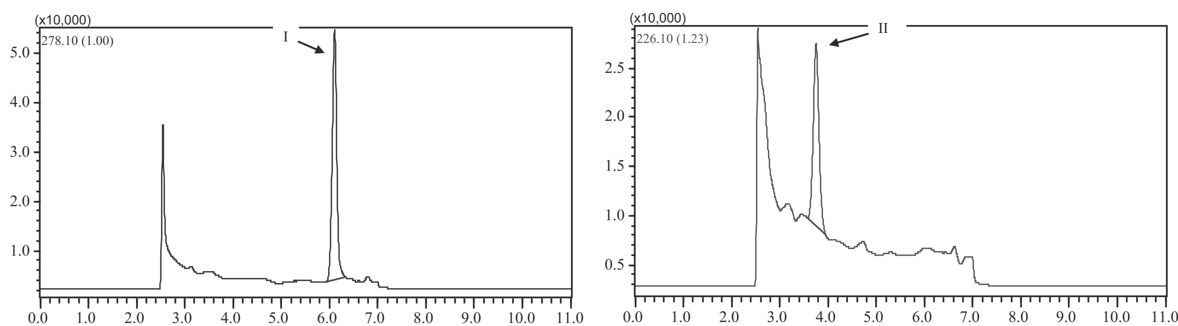

Fig. 5. Representative chromatogram of entecavir post-dose (subject A was given oral entecavir 1 mg 0.5 h later) plasma sample with internal standard 5 μL from 4 μg/mL (conc. of entecavir = 9.22 ng/mL) (I: entecavir, II: acyclovir).

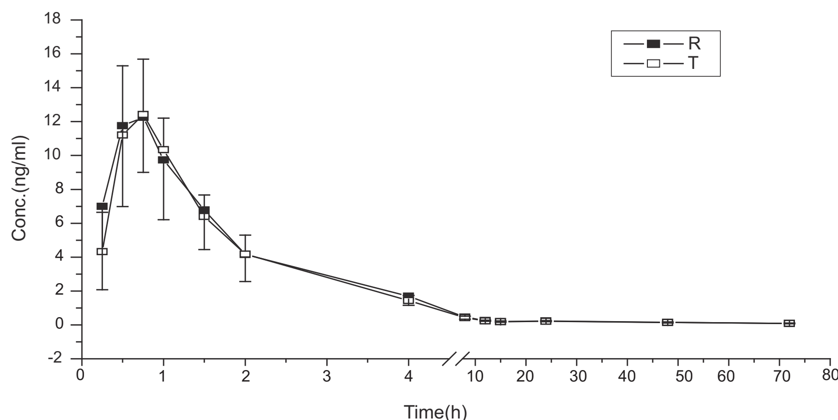

Fig. 6. Average concentration–time curve of twenty Chinese healthy subjects after an oral administration of entecavir capsule (T) or entecavir tablet (R).

presented in Fig. 5. Average concentration–time curve of twenty Chinese healthy subjects after an oral administration of entecavir capsule (T) or entecavir tablet (R) is depicted in Fig. 6. The accuracy was found to be within 90–110% of the nominal concentration for all QC samples in the determination process of all the post-dose plasma samples. Inter-day variation of QC samples assay was within 6% (Table 6).

4. Conclusion

A robust, selective, and sensitive LC–MS method with SHLLE as sample pre-processing for the quantitative determination of entecavir in human plasma has been developed and validated. Acetonitrile was chosen as organic solvent and magnesium sulfate as salting-out reagent. A “post cut” column switch technique is incorporated into the method to remove magnesium sulfate avoiding damage to mass spectrometer. There is no obvious matrix effect in LC–MS/MS with SHLLE as sample preparation method.

This study has shown that SHLLE may be applicable for bioanalytical assays of polar, acid or basic organic molecules, which provides an alternative method of biological sample pretreatment for bioanalysis, especially without automatic SPE device.

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