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Application News

No. AD-0106

Quantitative Bioanalysis / LCMS-8060

LC/MS/MS High Sensitivity Bioanalytical Method: Entecavir in Human Plasma

❑ Introduction

Hepatitis B virus (HBV) is a deoxyribonucleic acid (DNA) virus that causes both acute and chronic infection of the liver in human [1]. Entecavir (Figure 1) used as an oral antiviral drug is one of the FDA approved drugs for treatment of HBV infection. Entecavir is a reverse transcriptase inhibitor which prevents the hepatitis B virus from multiplying and reduces the amount of virus in the body by acting like a deoxyguanosine analogue that inhibit the activities of HBV polymerase with reverse transcription, DNA replication and transcription in the viral replication process [1,2]. Bioanalyses of entecavir for pharmacokinetics and bioequivalence studies have been reported, which are carried out mostly on LC/MS/MS with liquid-liquid extraction (LLE) [3] or solid phase extraction (SPE) [1, 2] method for high sensitivity and selectivity. The reliability of a bioassay depends on the performance of LC/MS/MS system employed and the method of sample pre-treatment. With rapid progress of interface and triple quadrupole mass spectrometry techniques, simpler sample pre-treatment without SPE may be adopted to achieve not only reliable quantitative results, but also higher throughput and lower running cost. Here we describe a simple, fast and high sensitivity bioanalytical method for quantitative determination of entecavir in human plasma on Shimadzu LCMS-8060 system with a heated ESI and coupled with UHPLC. The high sensitivity and robust interface design of the system allow the use of protein-precipitation only in plasma pre-treatment and achieve a LLOQ of 40 pg/mL in plasma or 10 pg/mL in the injection solution after pre-treatment of plasma sample with 1uL injection volume.

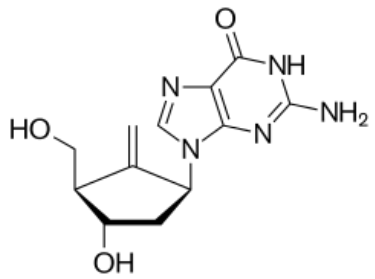

Figure 1: Chemical structure of Entecavir

❑ Experimental

A LCMS-8060 triple quadrupole coupled with Nexera X2 UHPLC (Shimadzu Corporation) was employed. The details of column, mobile phases and gradient programs of LC separation and MS conditions are compiled into Table 1. Pooled human plasma was obtained from i-DNA Biotechnology Pte Ltd. Entecavir was obtained from Sigma Aldrich. A stock solution of entecavir was prepared using high purity chemicals and solvents. A calibrant series was prepared from the stock using a mixed solvent as diluent (water/methanol/acetonitrile, 2:3:3, vol/vol/vol). The sample pre-treatment of plasma, without use of SPE or LLE cleanup method, involved only protein precipitation, which details are shown in Figure 1. Cooled acetonitrile/methanol (1:1 volume ratio) was added to the plasma for protein precipitation followed by vortex and centrifuge at high speed. The supernatant was collected and filtered, then was injected directly into the LC/MS/MS for analysis.

Table 1: LC/MS/MS analytical conditions of Entecavir on LCMS-8060

LC condition:

Column	Kinetex 2.6u Phenyl-Hexyl 100A (150 mmL. x 2.10mm I.D.)
Flow Rate	0.3 mL/min
Mobile Phase	A: 5mM Ammonium Formate + 0.01% formic acid in Water B: 5mM Ammonium Formate + 0.01% formic acid in Methanol
Elution Mode	Gradient elution, LC program 10 minute: 5% (0.0 to 0.5min) ➔ 90% (5.0 to 7.0min) ➔ 5% (7.5 to 10.0min)
Oven Temperature	40°C
Injection Volume	1μL

MS Interface condition:

Interface	ESI
MS mode	Positive
Block Temperature	400°C
Interface Temp.	300°C
DL Temperature	250°C
CID Gas	Ar (270kPa)
Nebulizing Gas Flow	N2, 2.0L/min
Drying Gas Flow	N2, 10.0L/min
Heating Gas Flow	Zero Air, 10.0L/min

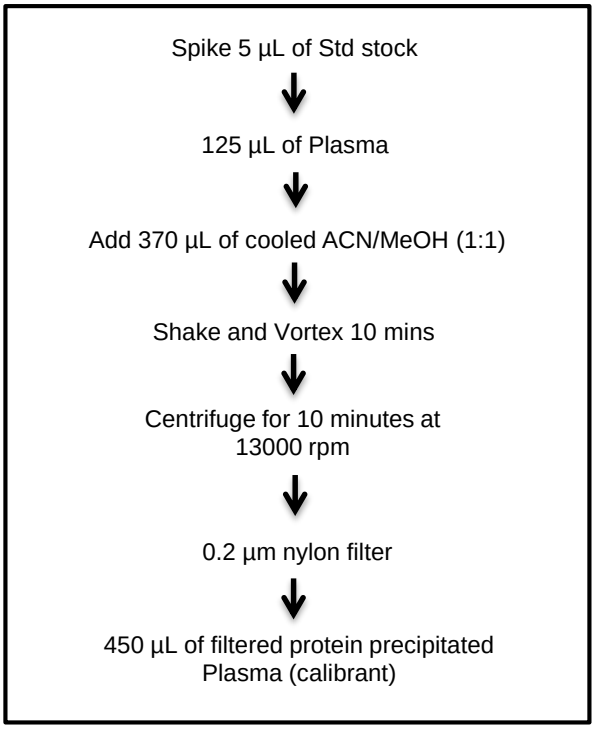

Figure 2: Flow chart of sample pre-treatment of human plasma with pre-spiked atorvastatin. Post-spiked samples are prepared in similar procedure by adding stock standard at last step.

❑ Results and Discussion

Method Development

A MRM method was established on the LCMS-8060 with the optimized MRM parameters as shown in Table 2. The first MRM transition (278 → 152) was used to establish calibration method. The second MRM (278 → 135) was used as reference ion for identification. Entecavir was eluted as a sharp peak under the gradient conditions as shown in Figures 3a and 3b.

Table 2: MRM transitions and CID parameters of entecavir on LCMS-8060

Name	RT (min)	MRM Transition (m/z)		CID Voltage (V)		
		Precursor [M+H]+	Product	Q1 Pre Bias	CE	Q3 Pre Bias
Entecavir	4.398	278.10	152.00	-24	-19	-29
			135.05	-23	-36	-26

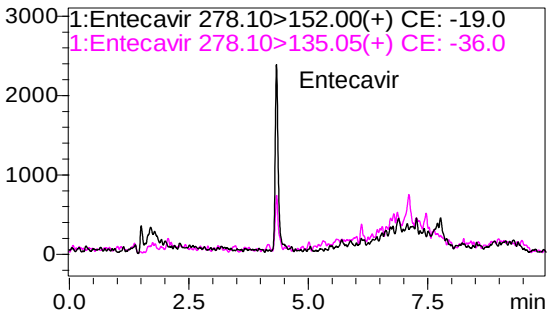

Figure 3a: MRM chromatograms of entecavir (50 pg/mL) post-spiked in plasma

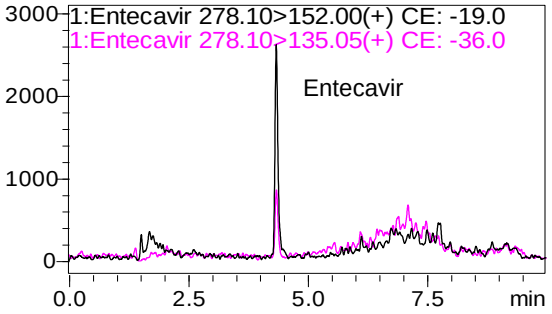

Figure 3b: MRM chromatograms of entecavir (50 pg/mL) pre-spiked in plasma

Calibration curve and linearity

A quantification method for entecavir in plasma was set up based on the first MRM transition of entecavir in Table 2. A linear calibration curve was established by external standard method for plasma samples prepared by post-spiked procedures (see Figure 4).

Excellent linearity was obtained with an R2 coefficient greater than 0.999 across the range from 10 pg/mL to 10,000 pg/mL. The details of the calibrant series, RSD% and accuracy in triplicate measurements are shown in Table 3.

Table 3: Performance of calibration method for quantitation of entecavir in plasma (post spiked)

Level[1]	Conc. Cal. (pg/mL)	Conc. Mea. (pg/mL)	RSD% (n=3)	Average Acc. (%)
1	10	10.0	7.6	100.1
2	20	19.2	5.9	95.9
3	50	49.0	4.4	98.0
4	100	103.2	4.3	103.2
5	500	524.7	2.7	104.9
6	1000	955.5	1.0	95.6
7	5000	5209.1	1.1	104.2
8	10000	9809.3	0.3	98.1

[1] Triplicate injections of each level

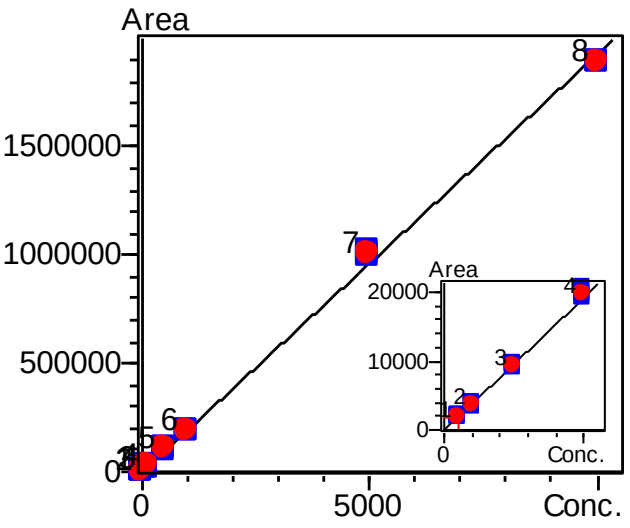

Figure 4: Calibration curve of entecavir post-spiked in plasma, from 10 pg/mL ~ 10000 pg/mL

Evaluation of method performance

The MRM chromatograms of blank and spiked with 10 pg/mL are shown in Figures 5 and 6. The LLOQ of the method was determined to be 10 pg/mL (S/N~10) with post-spiked plasma sample (Figure 6). The LLOQ of entecavir for the method is at 10 pg/mL in plasma solution and 40 pg/mL in plasma before adding three volumes of acetonitrile for protein precipitation (dilution factor of sample preparation: 4.0).

The accuracy and precision of the method were evaluated with the LLOQ, QC1 (low), QC2 (mid) and QC3 (high) samples. The results are compiled into Table 4. The accuracy and RSD% obtained for the QC samples are within the acceptable range of ±20% and <15% respectively. Six MRM chromatograms of consecutive injections of QC1 are plotted in Figure 7, which shows the excellent reproducibility of the method on LCMS-8060.

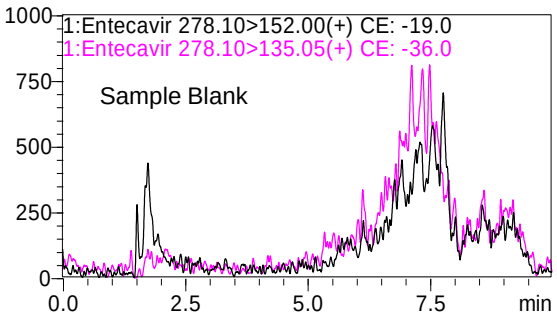

Figure 5: MRM chromatograms of blank sample (plasma)

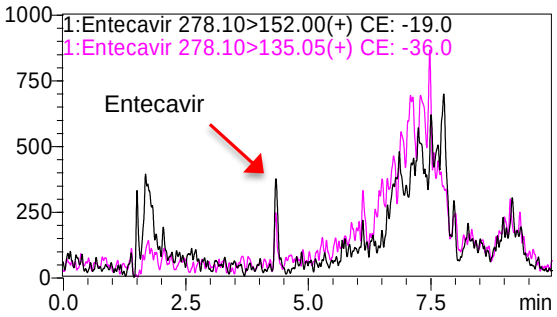

Figure 6: MRM chromatograms of entecavir (10 pg/mL, S/N ~10) post-spiked in plasma

Table 4: Performance evaluation of quantitation method for Entecavir in plasma with QC samples (n=6)

Sample	Conc. Cal. (pg/mL)	Ave. Conc. (pg/mL)	Accuracy (%)	RSD (%)
LLOQ	10	10.3	103.0	9.1
QC1 (low)	20	21.3	106.3	5.6
QC2 (mid)	100	101.0	101.0	3.2
QC3 (high)	5000	5187.6	103.7	1.5

Table 5: Evaluation of Recovery (R.E.) and Matrix effect (M.E)

Conc. (pg/mL)	10	20	50	100	500	1000	5000	10000
R.E. %	113.8	97.8	112.4	100.2	97.2	111.1	97.2	95.4
M. E %	153.8	169.2	163.6	179.8	162.6	168.2	173.1	167.1

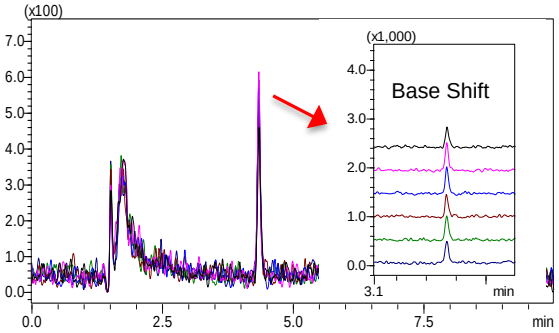

Figure 7: Overlay of six chromatograms of LLOQ (10.0 pg/mL entecavir post-spiked in plasma)

The recovery efficiency and matrix effect were investigated. The results were tabulated in Table 5. The recovery percentage was within the ±20%. However, there was ion amplification found in the matrix with an average matrix effect of 167.2% (±13%). .

❑ Conclusions

A high sensitivity bioanalytical method for determination of entecavir in human plasma was established on LCMS-8060 with a heated ESI. The method with using simple sample pre-treatment achieves a high sensitivity (LLOQ: 10pg/mL, 1uL injection volume) and reliable performance required for bioanalysis.

❑ References

1. Duxi Zhang, Yunlin Fu, Jane P. Gale, Anne F. Aubry, Mark E. Arnold. A sensitive method for the determination of entecavir at pictogram per milliliter level in human plasma by solid phase extraction and high-pH LC-MS/MS. Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 1027-1033
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3. Suraj sythana, Lavnya, Sankar. A.S.K., Shanmugasundaram. P., Ravichandiran. V., Determination Of Entecavir In Human Plasma By LC-MS/MS And Method Validation. International Journal of PharmTech Research Vol.4, No.4, pp 1721-1729, Oct-Dec 2012

Note: The method and data shown in this application notes are for research use only. Not for Use in Diagnostic Procedures.

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