HPLC Determination of Entecavir in Pure, Tablet Dosage Form and Spiked Plasma

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Summary: Entecavir is an analogue with selective activity against hepatitis B virus. In this study, an HPLC method for the determination of entecavir was developed. Entecavir was eluted through C_{18} ODS Hypersil column of 150 × 4.6 mm id with 5 µm particle size using simple mobile phase of acetonitrile: 10 mM phosphate buffer (80:20) at a flow rate of 1.0 mL min^{-1} and eluate was detected at 218 nm. Etoposide was used as an internal standard. The accuracy of the developed method was 97-99% for both with-in-batch and between-batches studies and CV (%) of < 3. The new method is highly sensitive upto 0.0097 µg mL^{-1}. The validation results and statistical data demonstrate that the method is more sensitive, reliable and reproducible and has an importance in quality assurance of entecavir analysis and in bioequivalence studies.

Keywords: Entecavir, Etoposide, HPLC, Spiked plasma, Tablet dosage form.

Introduction

There are six approved drug therapies including small molecule nucleoside analogues lamivudine, entecavir, and telbivudine, acyclic nucleotide analog adefovir dipivoxil, and injection proteins interferon alfa-2b and pegylated interferon alfa-2a for the treatment of chronic hepatitis B virus infection [1]. Among the novel guanine nucleoside analogs, entecavir (Fig. 1) that inhibits hepatitis B virus, DNA polymerase at both the priming and elongation steps required for viral replication [2]. The chemical name of drug is 2-amino-1,9-dihydro-9-[(*1S,3R,4S*)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6*H*-purin-6 one monohydrate. Its molecular formula is $C_{12}H_{15}N_5O_3 \bullet H_2O$, which corresponds to a molecular weight of 295.3 [3].

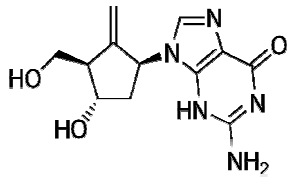

Fig. 1: Structure of entecavir.

The reported analytical methods for the determination of entecavir include capillary zone electrophoretic [4], LC-MS/MS [5, 6], LC-ESI-MS/MS [7], spectrophometry method [8, 9] and HPLC methods [10, 11]. The present paper describes a new and sensitive HPLC method for the determination of entecavir in the pure, tablet dosage form and in spike plasma which is several times more sensitive than the published methods regarding plasma studies.

Experimental

Chromatographic system and HPLC conditions

An HPLC Sykam Series chromatographic system having Solvent delivery system (S-2100), Injection Port (S-5111) and UV/Vis Detector (S-3210) with software Clarity Version 2.5. The column C_{18} ODS Hypersil column of 150 mm × 4.6 mm id with 5 µm particle size and simple mobile phase of acetonitrile: 10 mM phosphate buffer (80:20) was used at a flow rate of 1.0 mL min^{-1}. The pH of the mobile phase was adjusted to 3.5 with 0.1N phosphoric acid and analyte was detected at 218 nm.

Chemicals and reagents

Entecavir (pure) was gifted by NovaMed Pharmaceutical Private Ltd. Lahore, Pakistan, and etoposide was kind gift by Pharmedic Lab Private Ltd. Lahore, Pakistan. Methanol and acetonitrile of HPLC grade were purchased from Merck, Darmstadt, Germany. Hydrochloric acid, sodium chloride, phosphoric acid, de-ionized double distilled water and all kinds of chemicals and reagents were of analytical grade.

Preparation of phosphate buffer

The solution of potassium dihydrogen phosphate 10 mM was prepared by mixing 0.34 g in

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250 mL deionized double distilled water, Likewise, 10 mM stock solution of KOH was prepared by dissolving 0.14 g in 250 mL water. The pH was adjusted at 6.5 with KOH.

Preparation of mobile phase

The mobile phase was prepared by mixing of HPLC grade acetonitrile and 10 mM phosphate buffer at a ratio of (80:20) and then filtered with 0.45 µm membrane filter. The pH was set to 3.50 with 0.1N phosphoric acid. Mobile phase was prepared fresh daily, used after filtration and sonication.

Stock solutions

Stock solution of entecavir (1 mg mL^{-1}) was prepared in the methanol. Further dilutions were made upto 0.0097 µg mL^{-1}. Fresh solutions were made daily, filtered and degassed by sonication.

Sample solutions and extraction of plasma

Plasma (0.5 mL) was taken in a centrifuge tube, vortexed for 5 min, and then sonicated for 5 min, 5 µL of 5%TCA was added, content were vortex mixed and then sample was centrifuged at 4000 rpm for 5 min. The supernatant was taken and filtered through the 0.45µm membrane. Now, 20 µL of samples were injected in the column.

Standard plasma sample preparations

Spiked plasma containing 20 µL entecavir and etoposide were vortexed for 5 min and sonicated for 5 min, followed by the addition of 15 µL of TCA, contents were vortexed and centrifuged at 4000 rpm for 5 min. The supernatant was taken after passing through the filter paper of 0.45µ. Ten µL samples were injected.

Method development

An HPLC method was developed and standardized for the analysis of entecavir in tablet dosage form and spiked plasma. Entecavir and etoposide were eluted through column C_{18} ODS Hypersil column of 150 mm × 4.6 mm id with 5 µm particle size using simple mobile phase of acetonitrile:10 mM phosphate buffer (80:20) at a flow rate of 1.0 mL min^{-1}. The pH of the mobile phase was adjusted to 3.5 with 0.1N phosphoric acid and run time of every elution was 10 min at 218 nm.

Results and Discussion

Peak identification and retention time of entecavir

The peak of entecavir was identified by the evaluation of retention times of sample and pure drug standard solution. The increase or decrease in size of the peak with a time and variations in the concentration of standard solution was determined. The retention time of entecavir was 3.3 min (Fig. 2). The peak of internal standard etoposide was identified by comparison of retention times of entecavir and etoposide. During application of this method, etoposide was eluted at 6.6 min (Fig. 3).

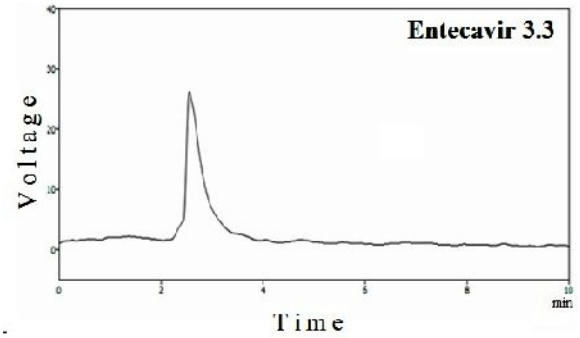

Fig. 2: A representative chromatogram of entecavir.

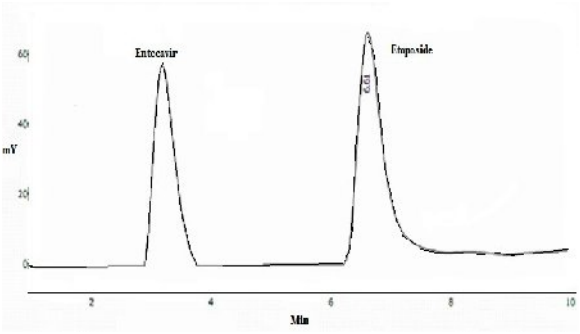

Fig. 3: A representative chromatogram of entecavir with etoposide.

Validation of HPLC method the determination of entecavir

The above developed method was validated step wise according to good analytical practice with FDA guidelines.

Linearity and range

Linearity was determined by the construction of a calibration curve (Fig. 4) to find out the affiliation between instrumental response i.e, peak area and known concentrations of the drug. The standard stock solution (1.0 mg mL^{-1}) was diluted with mobile phase to prepare a set of solutions of different concentrations ranging from 0.039 to 100 µg mL^{-1} (Table-1). The three calibration curves were constructed for three batches of pure drug and the

parameters like slope, intercept and r^2 were determined (Table-1).

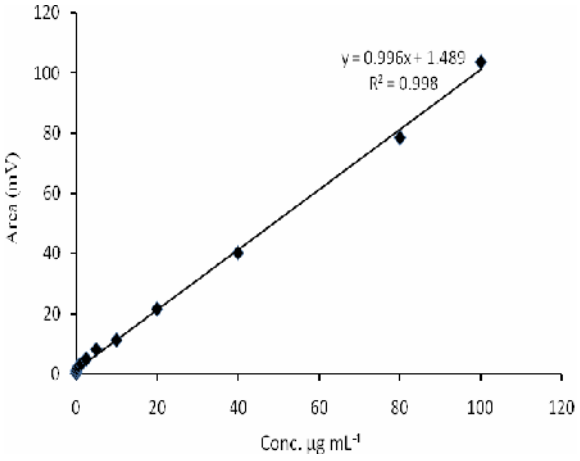

Fig. 4: Calibration curve of entecavir.

Table-1: Standard curve parameters for entecavir batches.

Batches	Slope	Intercept	r-square
Enta-01	0.996	1.489	0.998
Enta-02	1.002	1.437	0.998
Enta-03	0.997	1.414	0.996
Mean	0.998	1.4	0.997
S.D.	0.0	0.0	0.0012
N	3	3	3
%CV	0.3	2.7	0.1158

Accuracy

Accuracy of developed method was determined at three different concentrations (low, medium and high) of entecavir in triplicate in three different batches. The values (low, medium and high) of accuracy (%) for pure entecavir were 99.47, 97.72 and 99.09, respectively (Table-2).

Table-2: Validation parameter calculation for inter-day accuracy (n=9) for three batches.

Nominal value (µg mL^{-1})	Recovered conc. (µg mL^{-1})	SD	CV (%)	Accuracy (%)
5	4.95	0.09	1.72	99.09
20	19.54	0.37	1.90	97.72
80	79.57	0.48	0.60	99.47

Precision

Precision of this analytical assay was carried out by drug elution at three different concentrations (low, medium and high) in triplicates. The CV (%) with-in-batch for low, medium and high concentrations of entecavir were 0.67, 1.73, 2.04 (Table-3) and between-batches CV (%) were 0.60, 1.90 and 1.72 (Table-2) respectively, which are 3%, and within the range of FDA guidelines.

Table-3: Validation parameter calculation for intra-day precision (n=6) for single batch.

Nominal value (µg mL^{-1})	Recovered conc. (µg mL^{-1})	SD	CV (%)	Accuracy (%)
5	4.96	0.10	2.04	99.20
20	19.61	0.34	1.73	98.06
80	79.71	0.54	0.67	99.63

Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) values of entecavir were 0.0097 µg mL $^{-1}$ and 0.039 µg mL $^{-1}$, respectively (Table-4).

Robustness

The technique was found robust under different conditions of flow rate (± 0.1 mL min^{-1}), mobile phase formations (± 1-5% of organic component) and wavelength (± 1-5 nm) and no clear changes were seen in evaluated statistical data (Table 5).

System suitability

The system suitability was also done to ensure that the chromatographic system is adequate for specific analysis and kept in consideration the factors like temperature, pH of mobile phase, symmetry factor, column dimensions and specific surface area etc. The signal-to-noise ratio is a useful system suitability parameter (data not given).

Application of method in tablet dosage form

This developed system was applied on two different brands of tablet dosage form of entecavir (Enta-B tablets by NovaMed Pharmaceutical Pvt. Limited, Lahore, Pakistan and tablets Etava by SJ & G Fazal Ellahi Pharma, Karachi, Pakistan) available in the market. The results are given in Table-6.

Table-4: Different parameters regarding HPLC analysis of entecavir.

LOD	LOQ	Retention Time	Flow Rate	Wave length	Mobile phase
0.0097 µg mL^{-1}	0.039 µg mL^{-1}	3.3 ± 0.1 min	1.0 mL min^{-1}	218 nm	acetonitrile : 10mM phosphate Buffer (80:20)

Table-5: Study of robustness of the proposed method.

S. No.	Flow rate (mL min^{-1})	Wavelength (nm)	Mobil Phase	Retention Time (minutes)
1	1.1	220	Acetonitrile : 10 mM phosphate buffer (78:22)	3.3
2	1.0	218	Acetonitrile : 10 mM phosphate buffer (80:20)	3.3
3	0.9	225	acetonitrile : 10 mM phosphate buffer (85:15)	3.3

Table-6: Determination the concentration of two different tablet brands of entecavir by Forecast Formula (y = ax + b).

Serial No.	Sample	File name	Peak area of entecavir	Entecavir stated quantity (µg mL^{-1})	Conc of entecavir (µg mL^{-1})
1		Enta-1	470.12	500.00	487.15
2		Enta-2	240.3	250.00	248.25
3	Brand 1	Enta-3	119.21	125.00	122.38
4		Enta-4	55.31	62.50	55.96
5		Enta-5	30.12	31.25	29.77
1		Etva-1	461.12	500.00	477.80
2		Etva-2	237.3	250.00	245.14
3	Brand 2	Etva-3	118.21	125.00	121.34
4		Etva-4	56.1	62.50	56.78
5		Etva-5	29.02	31.25	28.63

Table-7: Data for recovery of entecavir in plasma.

Conc. Of entecavir (µg mL^{-1})	Area of chromatogram	Entecavir recovered (µg mL^{-1})	Percentage recovery (%)
5	5.57	4.10	82.0
40	34.41	33.05	82.62
80	73.16	71.96	89.95

Application of method in plasma

The standard curve of entecavir along with internal standard in extracted plasma was made and percentage recovery in plasma was monitored (Fig. 5). Three concentrations, lower (1.56 µg mL^{-1}) medium (25 µg mL^{-1}) and high (100 µg mL^{-1}) were spiked for recovery studies. The percentage recovery for high concentration was 89.95, for medium it was 82.62 and for lower concentration it was 82.0 as given in Table-7.

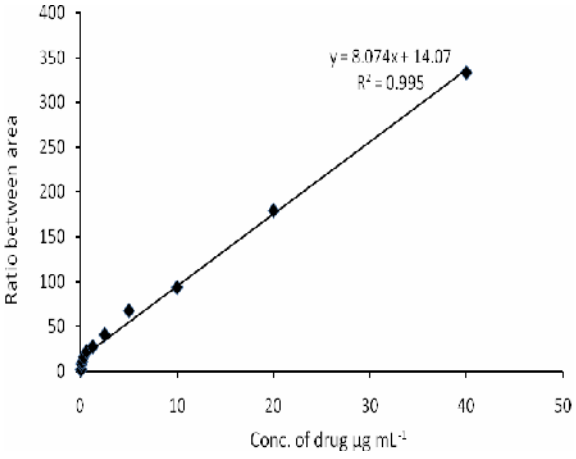

Fig. 5: Graph between concentration and ratio of entecavir and etoposide in plasma.

The aim of the study was the development and validation of an analytical method for entecavir and application of this method on formulation brands and spiked plasma using HPLC. FDA guidelines were followed for the development and validation of the method in pure and dosage forms. Calibration curves were constructed and assayed in triplicates to evaluate linearity, accuracy, precision, sensitivity (LOD and LOQ) and stability of the method.

In the development process, different compositions of mobile phase with several combinations of organic and aqueous phase were tested and selected to a final mobile phase acetonitrile: phosphate buffer in ratio of 80: 20 (%, v/v). The pH of 3.5 was adjusted with phosphoric acid and selected after many trials to avoid peak tailing because silica gel particles were unstable at low pH <2. After much experimentation, for individual analysis of entecavir and etoposide as an internal standard, the flow rate was adjusted to 1.0 mL min^{-1}. The retention time for entecavir was 3.6 min and for etoposide it was 6.6 min when analyzed individually at flow rate of 1.0 mL min^{-1}. The standardization and validation of analytic methods was evaluated by the parameters, *i.e.,* linearity, accuracy, precision, stability, sensitivity and robustness as given in results section. The developed method was successfully applied for the assay of brands of entecavir and recovery from plasma was excellent. The method was found linear ranging from 0.039 to 100 µg mL^{-1}. The values of slope, intercept and r^2 for entecavir were 0.996, 1.489 and 0.998, respectively. The LOQ and LOD values of entecavir were 0.039 µg mL^{-1} and 0.0098 µg mL^{-1}, respectively.

Conclusions

The new HPLC method for the estimation of entecavir was more sensitive than the published methods in plasma using etoposide as an internal standard and validated according to FDA guidelines and system suitability. The method was successfully applied in the quantification of entecavir of two brands of tablet dosage form and in spiked plasma. This method therefore can be used in the determination of entecavir in quality assurance and bioequivalence studies.

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