



## Research Article

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## ESTIMATION AND VALIDATION OF ENTECAVIR IN BULK AND PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC

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**ABSTRACT**

A highly simple, sensitive, reliable, rapid and specific RP-HPLC method was developed for the determination of Entecavir in tablet dosage form. This method was carried out on a C<sub>18</sub> column (250 x 4.6 mm ID) maintained at 25°C. The mobile phase consisted of a methanol:water (1:1) was pumped at a flow rate of 0.8 ml/min. The chromatographic separation was obtained with a retention time of 9.36 minutes and the method was in linearity in the range of 5-25 µg/ml (r<sup>2</sup> = 0.9991). The accuracy of the method was found to be 98.65-99.41 %. The limit of detection and limit of quantitation were found to be 0.372 and 1.128 mg/ml, respectively. The method was validated and demonstrated acceptable results for precision and robustness. The system with methanol:water (50:50 v/v) with 0.8 ml/min flow rate was quite robust. The optimum wavelength for detection was 254 nm at which better detector response for the selected drug was obtained. The average retention time required for the elution of Entecavir was found to be 4.107 + 0.03 minutes. Hence, the developed RP-HPLC method can be adopted for the routine analysis of Entecavir in bulk and pharmaceutical dosage forms in quality control laboratories.

**Keywords:** RP-HPLC, Limit of detection, Limit of quantitation, Entecavir and C<sub>18</sub> column

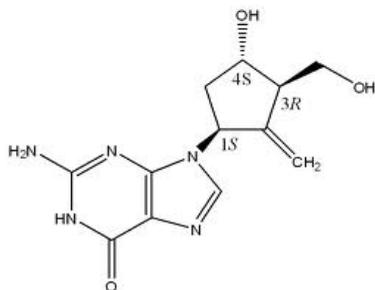
**INTRODUCTION**

Figure 1: Structure of Entecavir

Entecavir<sup>1-3</sup>, (Figure 1) chemically is 2-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2s-methylenecyclopentyl]-6H-purine-6-one. It is a white to off-white crystalline powder. Entecavir, a guanosine nucleoside analogue with selective activity against hepatitis B Virus (HBV) polymerase, is efficiently phosphorylated to the active triphosphate form, which has intracellular half-life of 15 hours. It is freely soluble in water, methanol, 0.1N NaOH, sparingly soluble in acetonitrile and glacial acetic acid and insoluble in 0.1N HCl. Literature survey reveals that only few analytical methods have been reported for the estimation of Entecavir in bulk and pharmaceutical dosage form. Several instrumental techniques HPLC<sup>4-8</sup>, Mass spectrometry<sup>9,10</sup>, NMR, UV<sup>11-15</sup> and Visible<sup>16,17</sup> regions, available for the assay of drugs. HPLC is a versatile tool for the qualitative and quantitative analysis of drugs and Pharmaceuticals. The analytical work was aimed to develop a simple, sensitive, precise, accurate and

economical RP-HPLC<sup>18-22</sup> method for the estimation of Entecavir in bulk and pharmaceutical dosage form and to validate according to ICH (Q2B) guidelines.

**MATERIALS AND METHODS****Materials and Instrument**

Entecavir was procured as a gift sample from Mylan Labs Pvt. Ltd, Hyderabad, India. Methanol, water and other reagents were of HPLC grade, purchased from Merck Specialties private limited, Mumbai, India. Agilent 1120 Compact LC system includes isocratic pump, manual injector, variable wavelength programmable UV detector, Ezchrome Elite Compact software, LMD software. The Chromatographic separation was carried out on a C<sub>18</sub> column [Agilent ODS UG 5 column, 250 mm x 4.5 mm], 1.5 LH Ultra-sonic bath sonicator and Axis AGN204-PO digital balance were used.

**Selection of Mobile Phase**

The pure drug of Entecavir was injected in to the isocratic HPLC system and run at different solvent systems. Different mobile phases like methanol and water, acetonitrile and water, methanol and acetonitrile, methanol and ammonium acetate buffer were tried in order to find the best conditions for the elution of entecavir. It was found that methanol and water give satisfactory results as compared to other mobile phases. The mobile phase system was tried with different proportions and using different flow rates. Finally, the optimal composition of the mobile phase was determined to be methanol: water in the ratio of 50:50 at a flow rate of 1.0 ml/min.

## Method Development

### Preparation of Mobile Phase

Equal volumes of HPLC grade methanol and double distilled water were mixed in the ratio of (50:50 v/v), filtered through a 0.45  $\mu$  membrane filter and sonicated for 20 minutes.

### Preparation of Standard Solution

Entecavir (25 mg) weighed accurately and transferred to 25 ml volumetric flask and dissolved in 10 ml of solvent; further, made up to the mark with solvent to obtain a final concentration of 1000  $\mu$ g/ml (standard stock solution A). From the standard stock solution A 2.5 ml of aliquot was pipette in to 25 ml volumetric flask and dissolved in 10 ml solvent; further made up to the mark with solvent to obtain a final concentration of 100  $\mu$ g/ml (standard stock solution B).

### Chromatographic Conditions

|                     |                         |
|---------------------|-------------------------|
| Mode of Operation   | : Isocratic             |
| Mobile Phase        | : Methanol: water (1:1) |
| Column Temperature  | : Ambient               |
| Detector Wavelength | : 254 nm                |
| Injection Volume    | : 20 $\mu$ L            |
| Flow Rate           | : 0.8 ml/min            |
| Run Time            | : 10 min                |

### Selection of Analytical Concentration Range

Appropriate aliquots were pipette out from the working stock solution B to a series of 10 ml volumetric flasks. The volume was made up to the mark with the mobile phase to get a set of solutions having the concentration ranging of 5, 10, 15, 20, 25  $\mu$ g/ml. These solutions were filtered through a 0.45  $\mu$  membrane filter and sonicated for 20 minutes.

### Preparation of Sample Solution

Marketed tablet formulation (ENTAVIR) containing 1.0 mg of entecavir was analyzed by this method. Twenty tablets were accurately weighed and their average weight was determined. Tablets were then crushed to fine powder and powder equivalent to 10 mg was taken in 100 ml volumetric flask then dissolved in 50 ml of mobile phase. This solution was sonicated for 15 minutes and volume was made up to the mark with mobile phase and filtered through 0.45  $\mu$  membrane filter to get the concentration of 100  $\mu$ g/ml (sample stock solution A). From the above sample stock solution A, 1.0 ml of aliquot was transferred to a 10 ml volumetric flask and diluted to the mark with the mobile phase to obtain a concentration of 10  $\mu$ g/ml (sample stock solution B).

### Method Validation

This developed RP-HPLC method was validated as per ICH (Q2 B) guidelines, to ensure the performance characteristics and to meet the requirements of the intended analytical application.

### System Suitability and Specificity

System suitability test was carried out on each day of validation to evaluate the components of analytical system to meet the standards required by the method. The

HPLC system was equilibrated using the initial mobile phase composition, followed by six replicate injections of 100 % concentration containing 25  $\mu$ g/ml. The peak asymmetry, tailing factor and number of theoretical plates were calculated. Specificity of this method was determined using 100 % test concentration, injected to the chromatographic system.

### Linearity

To establish the linearity of proposed method, aliquots of standard stock solution B (0.5, 1.0, 1.5, 2.0 and 2.5 ml) were transferred in a series of 10 ml volumetric flasks and the volume was made up to the mark with mobile phase to obtain final concentration of 5, 10, 15, 20 and 25  $\mu$ g/ml. Three replicates per each concentration were injected into HPLC system and peak areas were reported to develop calibration curve. Further, regression equation was established and correlation coefficient was determined.

### Accuracy

Accuracy of the proposed method was determined by standard addition, in which a known amount of standard solution of pure drug was added to a pre-analyzed sample solution, at three recovery levels 80, 100 and 120 % of label claim.

### Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

### Limit of Quantitation and Limit of Detection (LOD)

It is the lowest concentration of analyte that can be determined with acceptable accuracy and precision by the analytical method. LOD was expressed as concentration of analyte generating an instrument response equivalent to ten times the noise (S/N ratio~ 10). Limit of detection (LOD) and limit of quantitation (LOQ) were manually calculated from the slope of the calibration curve and standard deviation. The LOD and LOQ values were calculated using the formula given below

$$\text{LOD} = 3.3 \times \sigma / S$$

$$\text{LOQ} = 10 \times \sigma / S$$

Where,  $\sigma$  = standard deviation of intercepts of calibration curve,  
S = slope of the calibration curve.

The statistical validation data of LOD and LOQ were reported in results and discussion.

### Robustness and Ruggedness

The robustness of a method is its ability to remain unaffected by the deliberate variations in method parameters such as column temperature, analytical wavelength and flow rate. Small changes in the operational conditions were allowed and the extent to which the robustness of the method was estimated. Deviations of  $\pm 2$  nm in the detection wavelength and  $\pm 0.1$  ml/min in the flow rate were tried individually. Ruggedness is a measure of reproducibility of the results under instrument to instrument and analyst to analyst variations.

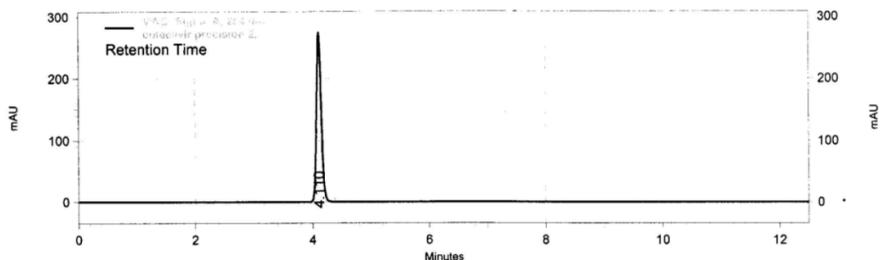


Figure 2: Chromatogram of entecavir

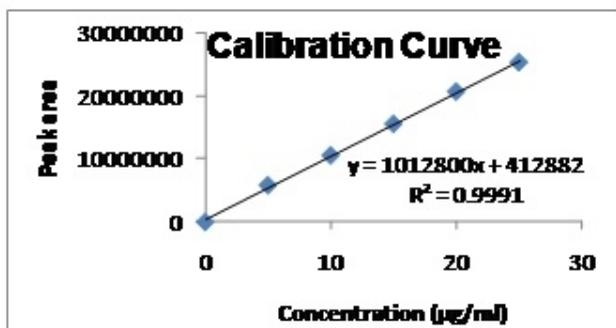


Figure 3: Calibration Curve of Entecavir

Table 1: Statistical Validation Data of System Suitability

| S. No | Parameters                    | Results    |
|-------|-------------------------------|------------|
| 1     | No. of Theoretical Plates (N) | 11594      |
| 2     | Tailing Factor (T)            | 0.8        |
| 3     | Retention Time (Min)          | 4.1 ± 0.01 |
| 4     | % RSD                         | 0.556      |

N is number theoretical plates, T is tailing factor, % RSD is regression standard deviation

Table 2: Statistical Validation Data of Linearity

| S. No                       | Concentration (µg/ml) | Retention Time (Minutes) | Peak Area |
|-----------------------------|-----------------------|--------------------------|-----------|
| 1                           | 5                     | 4.100                    | 5868457   |
| 2                           | 10                    | 4.106                    | 10630750  |
| 3                           | 15                    | 4.107                    | 15653557  |
| 4                           | 20                    | 4.103                    | 20800040  |
| 5                           | 25                    | 4.103                    | 25484490  |
| Slope                       |                       | 1012800                  |           |
| Intercept                   |                       | 412882                   |           |
| Correlation coefficient (R) |                       | 0.9991                   |           |

Table 3: Statistical Validation Data of Accuracy

| Recovery Level (%)     | Amount Added(µg/ml) |      | Amount Found (µg/ml) | Percentage Recovery (% w/w) | % RSD |
|------------------------|---------------------|------|----------------------|-----------------------------|-------|
|                        | Standard            | Test |                      |                             |       |
| 80                     | 12                  | 5    | 17.78                | 98.82                       | 0.761 |
| 100                    | 15                  | 5    | 19.88                | 99.41                       | 0.417 |
| 120                    | 18                  | 5    | 22.68                | 98.65                       | 0.697 |
| *Mean Recovery (% w/w) |                     |      | 98.65-99.41          |                             |       |

% RSD: regression standard deviation, \*nine determination of three concentrations

Table 4: Statistical Validation Data of Precision

| Parameters | Precision |           |          |          |
|------------|-----------|-----------|----------|----------|
|            | Intra-Day | Inter-Day |          |          |
|            |           | Day 1     | Day 2    | Day 3    |
| SD         | 144005.8  | 200751    | 219351.7 | 152465.2 |
| Mean       | 25615819  | 25593567  | 25591091 | 25907351 |
| %RSD       | 0.562     | 0.784     | 0.857    | 0.588    |

**Table 5: Statistical Validation Data of LOD and LOQ**

| Parameters                  | Results ( $\mu\text{g/ml}$ ) |
|-----------------------------|------------------------------|
| Limit of detection (LOD)    | 0.372                        |
| Limit of quantitation (LOQ) | 1.128                        |

**Table 6: Statistical Validation Data of Robustness**

| Parameters             |     | % RSD |
|------------------------|-----|-------|
| Wave length + 2 nm     | 252 | 0.273 |
|                        | 256 | 0.750 |
| Flow rate + 0.1 ml/min | 0.7 | 0.836 |
|                        | 0.9 | 0.748 |

% RSD is percent regression standard deviation

**Table 7: Statistical Validation Data of Ruggedness**

| S. No               | Peak Area |           |
|---------------------|-----------|-----------|
|                     | Analyst 1 | Analyst 2 |
| 1                   | 25694286  | 25894624  |
| 2                   | 25427342  | 25768424  |
| 3                   | 25542683  | 25698426  |
| 4                   | 25427869  | 25749241  |
| 5                   | 25687212  | 25654826  |
| 6                   | 25227654  | 25547251  |
| *Standard Deviation | 178473.2  | 116824.7  |
| **Mean              | 25501174  | 25718799  |
| % RSD               | 0.699     | 0.454     |

% RSD is percent regression standard deviation, \*mean of six results, \*\*average of six determinations

**Table 8: Assay Data of Entecavir**

| Brand Name | Label Claim | Amount Recovered | Percentage Purity<br>(% w/w) |
|------------|-------------|------------------|------------------------------|
|            |             | (mg)             |                              |
| Entavir    | 1.0         | 0.987            | 98.0                         |

**Table 9: Summary of Validation Parameters of RP-HPLC**

| Parameters                                 | Results                 |
|--|-------------------------|
| Detection Wave Length (nm)                 | 254                     |
| Rt (min)                                   | 4.1 + 0.01              |
| Beer's Law Range ( $\mu\text{g/ml}$ )      | 5-25                    |
| Regression Equation                        | $Y = 1012800x + 412882$ |
| Correlation Coefficient                    | 0.9991                  |
| Accuracy (% w/w)                           | 98.65-99.41             |
| <b>Precision (% RSD)</b>                   |                         |
| Intra-Day                                  | 0.562                   |
| Inter-Day                                  | 0.743                   |
| Limit of Detection ( $\mu\text{g/ml}$ )    | 0.372                   |
| Limit of Quantitation ( $\mu\text{g/ml}$ ) | 1.128                   |
| <b>Robustness (% RSD)</b>                  |                         |
| Detection Wave length (nm)                 | 252                     |
|  | 256                     |
| Flow Rate (ml/min)                         | 0.7                     |
|  | 0.9                     |
| <b>Ruggedness (% RSD)</b>                  |                         |
| Analyst 1                                  | 0.699                   |
| Analyst 2                                  | 0.454                   |
| Assay (% w/w)                              | 98.0                    |

## RESULTS AND DISCUSSION

The determination of entecavir and its validation for the development of method was carried out according to the ICH (Q2B) guidelines. The system with methanol:water (50:50 v/v) as mobile phase at 0.8 ml/min flow rate was quite robust. The optimum wavelength for detection was 254 nm at which better detector response for the selected drug was obtained. The average retention time required for the elution of entecavir was found to be 4.107 + 0.03 minutes (Figure 2). The system suitability (Table 1) was

carried out on freshly prepared stock solutions. The calibration curve (Figure 3) was linear in the concentration range of 5-25  $\mu\text{g/ml}$  with correlation coefficient 0.9991 (Table 2). The accuracy of the method was examined by performing recovery studies by standard addition method, by adding a known amount of standard solution of pure drug to a pre-analyzed sample solution, at three levels 80, 100 and 120 % of the label claim. The accuracy was evaluated using a minimum of 9 determinations over 3 concentration levels covering the

specified range. The mean recoveries were found in the range of 98-102 % (Table 3). Precision was evaluated using six samples of six different concentrations, which were prepared and analyzed on same day. Day to day variability was also assessed using six concentrations analyzed on three different days in a week (Table 4). The limit of detection and limit of quantitation were found to be 0.372 µg/ml and 1.128 µg/ml respectively (Table 5). Robustness of the proposed method was determined by variation in the flow rate (+ 0.1 ml/min) and variation in the wave length (+ 2 nm). It was evaluated by using three determinations over 100 % concentrations for each varied operational conditions and % RSD was found to be less than 2 (Table 6). Ruggedness of the proposed method was determined by analysis of aliquots from homogeneous slot by different analysts using similar operational conditions and the % RSD was found to be less than 2 % (Table 7). Results obtained with the proposed method confirm the suitability for pharmaceutical dosage form. The excipients present in the marketed formulation did not interfered in the estimation, when analyzed by these methods (Table 8). Hence, the developed RP-HPLC methods can be adopted for the routine analysis of Entecavir in bulk and pharmaceutical dosage forms in quality control laboratories.

## CONCLUSION

The proposed and validated RP-HPLC method was highly simple, sensitive, reliable, rapid and specific. The developed method was found to be economical with low solvent consumption (0.8 mL/min), short analytical run time of less than 10.0 minutes lead to an environmental friendly chromatographic procedure that allows the analysis of a large number of samples in a short period of time. This method has been found to be better than previously reported methods, due to its wider range of linearity, use of economical mobile phase and lack of extraction procedures. Hence above method can be used in quality control for routine analysis of finished products of Entecavir without any interference.

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## REFERENCES

1. www.drugbank.ca/drugs/ DBO0442.
2. Bertan G Katzung. Basic and clinical pharmacology. 9<sup>th</sup>ed; 2004.
3. Sweetman SC, Martindale: The Complete Drug Reference. 35<sup>th</sup>ed. London: Pharmaceutical press; 2007. p. 779-86.
4. Elena Katz Roy Eksteen, Peter Schoenmaker. Hand Book of HPLC. Vol 78. Mumbai: Pearson Education Limited; 1998. p. 143-7.
5. Kalyana Chakravarthy V, Gowri Sankar D. LC Determination of Entecavir in Pharmaceutical Formulation: Int. J. Pharm Res and Dev 2011; 2 suppl 12: 233-41.
6. Synder LR and Kirkland JJ. Introduction to Modern Liquid Chromatography. 2<sup>nd</sup>ed. Mumbai: Wiley International Publication; 1979. p. 205-7.

7. Skoog DA, Holler FJ Nieman. Principles of Instrumental Analysis. 5<sup>th</sup>ed. New Delhi: CBS Publishers; 2005. p. 733-6.
8. Feng Juan Zhao, Hong Tang, Qing Hua Zhang, Jin Yang, Andrew Davey K, Ji Ping Wang. Salting Out homogenous Liquid Liquid Extraction approach applied in Sample Pre-Processing for the Quantitative Determination of Entecavir in Human Plasma by LC-MS: Journal of Chromatography B 2012; 881: 119-25. <http://dx.doi.org/10.1016/j.jchromb.2011.12.003>
9. Jung BH, Naser L Rezk, Bridges AS, Corbett AH, Angela D. Simultaneous Determination of 17 Antiretroviral drugs in Human Plasma for Quantitative Analysis with Liquid Chromatography-Tandem Mass Spectrometry: Biomed Chromatography B 2007; 10: 1095-1104. <http://dx.doi.org/10.1002/bmc.865>
10. Zhang D Fuy, Gale JP, Aubry AF, Arnold ME. 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: J Pharma Biomed Analysis 2009; 49 suppl4: 1027-33. <http://dx.doi.org/10.1016/j.jpba.2009.02.003>
11. KPR Chowdary, Girish Kumar K, Devala Rao G. Spectrophotometric determination of Nimesulide using 3-methyl-2-benzothiazolinone hydrazone hydrochloride reagent: Indian Drugs 1999; 36 suppl 3: 185.
12. Vijay Amirtharaj R, Vinay Kumar Ch, N Senthil Kumar. Development and Validation of UV- Spectrophotometric for the Estimation of Entecavir in Tablet Dosage Form: Journal of Pharmacy Research 2011; 4 suppl 4: 1145-46.
13. Malipatil SM, Bharath S Athanikar, Mogal Dipali. UV-spectrophotometric estimation of Entecavir in tablet dosage form: Int J Pharm Sciences 2012; 3 suppl 3: 55-67.
14. Silverstein RM, Bassler GC and Morrill TC. Spectrophotometric Identification of Organic Compounds. 4<sup>th</sup>ed. New Delhi: Academic press; 1981. p. 87-90.
15. Murali D, Venkatarao SV, Rambabu C. Spectrophotometric determination of etravirin in bulk and pharmaceutical formulations: American Journal of Analytical Chemistry 2014; 5 suppl 2: 77-80. <http://dx.doi.org/10.4236/ajac.2014.52012>
16. KPR Chowdary, G Devalarao. Visible Spectrophotometric methods for the determination of Salmeterol xinafoate: Indian J Pharm Sci 1999; 61 suppl 4: 246-47.
17. Vijay Amirtharaj R, Vinay Kumar Ch, Senthil Kumar N. Development and Validation of RP-HPLC For the Estimation of Entecavir in Tablet Dosage Form: International Journal of Reserch in Pharmaceutical and Biomedical Sciences 2011; 2 suppl 3: 1033-40.
18. Dalmora SL, Sangoi Mda S, Dr Nogueira, Da Silva LM. Validation of a Stability indicating RP-HPLC Method for the Determination of Entecavir in Tablet Dosage Form: Journal AOAC; Journal of the Association of Official Agricultural Chemists International 2010; 93 suppl 2: 523-30.
19. Fang Yu Chun, Yang Xi Hong. RP-HPLC determination of entecavir sodium tablets: J Pharma Analysis 2007; 0 suppl 8: 1267-68.
20. Jiang Yin Mei, Luo Xiu Qin, Xu Yan, Bi Kai Shun. RP-HPLC determination of related substances in entecavir: Chinese J Pharm Analysis 2009; 04.
21. Doredla Narasimharao, Prasadarao M, Nagahussain J, S Lakshmi Sumanoja, V Rajeswara Rao. Method development and validation of forced degradation studies of metformin hydrochloride by using UV spectroscopy: International Journal of Pharmaceutical, Chemical and Biological Sciences 2013; 3 suppl 3: 546-53.
22. Vidyadhra S, Srinivasarao Y, Ramu A, Sasidhar RLC, Jayaramya A. Method development and validation for the simultaneous estimation of cinitapride and pantaprazole in solid dosage forms by RP-HPLC: Orient J Chem 2013; 29 suppl 3: 1213-20. <http://dx.doi.org/10.13005/ojc/290355>

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