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Quantitative Determination of Entecavir in Bulk and Tablet Formulation by a Validated Stability-indicating Reversed-phase HPLC Method

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Abstract

A simple and gradient RP- HPLC method has been validated and developed for Entecavir in bulk and tablet dosage form. The proposed method was validated to obtain official requirements including stability, accuracy, precision, linearity and selectivity. The method was developed on C18 column (250 x 4.6 mm ID) using the mobile phase composition as methanol: water (55:45 v/v). The flow rate was set as 1ml/minute and the maximum absorption was observed at 254 nm. The Entecavir drug showed a precise and good linearity at the concentration ranges of 5-25 µg/ml. The RP-HPLC, assay showed the highest purity ranging from 98.79 % to 99.91 % for Entecavir tablet formulation and 99.22 % was the mean percentage purity. The Entecavir retention time was found to be 3.5 minutes. The method accuracy was showed by statistical analysis. The developed RP-HPLC method can be utilized for the regular analysis of Entecavir in bulk and pharmaceutical dosage types in quality control laboratories. The proposed method was validated based on the ICH guidelines.

Keywords: Entecavir, methanol, water, HPLC and UV.

Introduction

Entecavir, chemically is 2-amino-1, 9 dihydro-9-[(1S, 3R, 4S)-4-hydroxy-3-(hydroxymethyl)-2smethylenecyclopentyl)]-6H-purine-6-one (O' Neil, 2006). It is a white to off-white crystalline powder, and freely soluble in water and methanol (Sweetman, 2007). Entecavir, an acyclic guanosine derivative, a nucleoside analogue with selective activity against hepatitis B Virus (HBV) polymerase, is efficiently phosphorylated to the active triphosphate form. Entecavir acts by reducing the production of the virus's DNA (Fung et al., 2011). Entecavir is generally safe for utilization in pregnancy with no harm having been reported (Sims and Woodland, 2006). It is considered safe during breastfeeding. There are many investigations published for the determination of Entecavir in biological fluids of different species. Several HPLC methods have been recorded for the determination of Entecavir in human serum using UV or fluorescence detection (Kalyana Chakravarthy et al., 2011; Dalmora et al., 2010; Reddy, Jampani and Suryadevara, 2014). Based on the literature review, several methods have been developed for Entecavir, like UV spectroscopy, fluorescence spectroscopy capillary electrophoresis, HPTLC, HPLC and voltammetry method (Reddy, Jampani and Suryadevara, 2014; Vijay Amirtharaj, Vinay Kumar and Senthil Kumar, 2011; Malipatil, Bharath and Mogal, 2012; Doredla et al., 2013; Vidyadhra et al., 2013). We present herein for the first time, a sensitive and selective HPLC method for the Entecavir. The method development and validation of a stability-indicating RP-HPLC method for the estimation of Entecavir is a new method, which will fulfill all ICH guidelines requirements of validation. Improving the speed, and reliability of analysis in pharmaceutical analytical laboratories, a new method for Entecavir determination in tablet formulation with a very short analysis time is described. The proposed method is very fast, quick and accurate in terms of chromatographic retention time and run time in comparison with other reported methods. The aim of this study was to develop simple, accurate, specific and precise RP-HPLC method for the Entecavir estimation in the bulk and pharmaceutical tablet formulation.

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Materials and Methods

Chemicals

The Entecavir reference standard (RS) was purchased from Sigma, Germany. The marketed Entecavir 1mg tablet brand name Entehep1 tablet manufactured and marketed by Zydus Heptiza, purchased from local Pharmacy from India. The HPLC grade acetonitrile, water and methanol were obtained from Sigma, Germany.

RP-HPLC instrumentation

Shimadzu LC-20 AT HPLC system, using SPD-10 detector (SPD- M20A, Japan) was used. The Chromatographic separation was performed on a C18 column [Agilent ODS UG 5 column, 250 mm x 4.5 mm]. The column temperature was kept at 27ºC and the flow rate was 1 ml/min. The sample injection volume is 20µl and the wavelength was set as 254nm, the HPLC run time was set for 10 minutes.

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 0.45µm membrane filter and sonicated for 15 minutes.

Preparation of Entecavir stock solution

Standard Entecavir solution

Entecavir (2 mg) weighed accurately and transferred to 100 ml volumetric flask and mixed with 100 ml of mobile phase solution. The resulting solution were kept in a sonicator for 5 minutes. The concentration of 5-25 µg/ml was achieved by diluting the standard stock solution with mobile phase. Entecavir powder was freely soluble in methanol.

Preparation of Entecavir tablet solution

1 gm of marketed sample of Entehep1 tablet was analyzed by this method. Twenty tablets were precisely weighed and their average weight was recorded. The tablets were then crushed to fine powder and powder equivalent to 10 mg was transferred into 25 ml volumetric flask, dissolved with 25 ml of mobile phase, and filtered through Whatman 1 filter paper. Further dilutions were made according to the required concentrations.

Solution stability

The prepared drug solution stability was analyzed during the time of analysis and also repeated the same analysis method on same day with different time intervals. The same analysis was repeated after 24 hrs by keeping the drug solution under laboratory temperature (37 ± 1°C) and in refrigeration (5 ± 1°C).

Method validation

The proposed method was preceded to achieve a new, sensitive and easy method for estimation of Entecavir by RP-HPLC. The experimental analysis was validated according to the ICH (Q2 B) guidelines, recommendations and USP-30.

System suitability

The resolution, retention time, tailing factor and column theoretical plates parameters were performed by six replicates of standards and three replicates of sample preparation.

Results and Discussion

Method optimization

Chromatogram with good shape peaks and good retention time shows good resolution for Entecavir. The typical RP-HPLC conditions are presented in Table 1. The good separation of Entecavir shows the success of the method. The HPLC chromatogram of Entecavir standard and Entecavir tablet is presented in figure 1 and 2.

Table 1. RP-HPLC conditions for estimation of Entecavir.

Parameters	Description
Column	Agilent ODS UG 5 column, (250 mm x 4.5 mm)
Mode of operation	Isocratic
Column temperature	27 ± 1°C
Mobile phase	Methanol: water (55:45 **v/v**).
Detection	Photodiode array detection at 254 nm
Injection volume	20 µl
Flow rate	1 ml min^{-1}

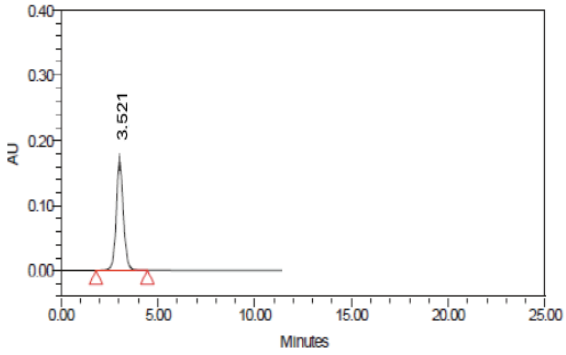

Fig.1. A Chromatogram of Entecavir Standard

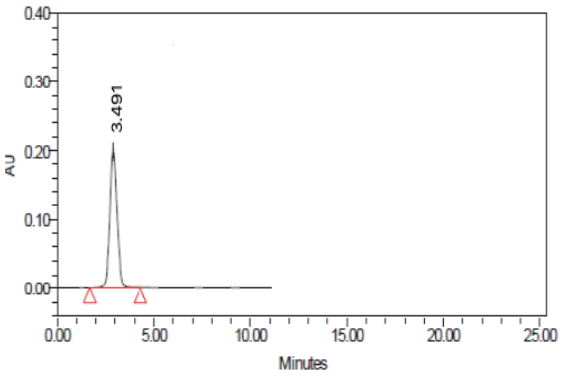

Fig.2. A Chromatogram of Entecavir tablet formulation

Linearity

The proposed method linearity was examined for five concentrations. The concentration ranges from 5-25 µg/ml. The Entecavir standard linearity was determined by the plotting graph concentration vs absorbance. By absorbance as a functional of

analyte concentration linearity was evaluated for Entecavir. The linearity graph presented in figure 3, and data presented in Table 2. The system suitability is demonstrated by the linearity analysis.

Table 2. RP- HPLC linearity for Entecavir

Concentration (µg/ml)	Peak area
5	20567.90
10	41321.77
15	62435.57
20	80097.55
25	1027649.43

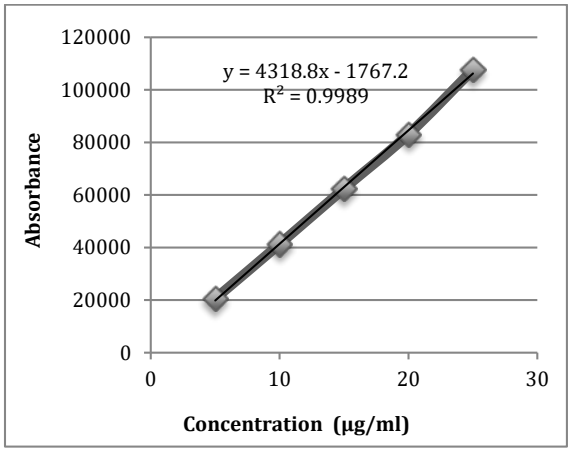

Fig. 3. Calibration graph of Entecavir 5-25 µg/ml precision

Accuracy

The recovery experiment shows the accuracy of the method. The good recovery reveals that the method was accurate. The analysis for recovery was carried out by known amount of Entecavir working standard added to pre-analyzed solution of formulation in the test concentration ranges of 80%, 100%, and 120 %. For each recovery level, three samples were prepared and repeated for 3 consecutive days. The statistical results for recovery study are well within the range S.D. < 2.0. The Entecavir tablet formulation recovery results are presented in Table 3.

Table 3. Recovery studies of Entecavir tablet formulation

Recovery Level (%)	Amount added (µg/ml)		Amount Found (µg/ml)	% Recovery	Mean recovery
	Standard	Test			
80	12	5	17.98	99.51	99.4
100	15	5	19.91	99.45	
120	18	5	22.98	99.15	

Precision

The proposed method precision (repeatability) experiment results are shown in Table 4. In the proposed method intraday and interday precision was examined by analyzing the responses of the sample on the same day for 4 repetitions and 3 alternate days for 20µg/ml concentration range of Entecavir. The obtained results are represented in % RSD. The % CV of the proposed method was precise as the values < 1.0 % for the repeatability study. The precision data are presented in Table 5.

Table 4. Method precision data of Entecavir by RP-HPLC method

Entecavir 20µg/ml (n=4)	Retention time	Area
1	3.51	80497.45
2	3.59	79897.75
3	3.56	79197.48
4	3.54	81297.40
Mean	3.55	80222.22
S.D [a]	0.0254	0.1232
% CV[b]	0.77	2.43

n=4 observations

Table 5. Intermediate precision data of Entecavir by RP-HPLC method

Entecavir µg/ml	Inter-day measured mean area ± S.D.[a]	%CV[b] (n^c=4)	Intra-day measured mean area ± S.D.[a]	%CV[b] (n^c=4)
15	59457.25± 3.09	0.0562	60197.44± 4.65	0.0155
20	81091.75±1.22	0.0517	79672.75±2.15	0.0117
25	109613.12±1.71	0.0712	106545.12±4.26	0.1036

n^c = 4 observations

Specificity

The standard reference and the drug formulation reveal specificity of the method. The RP-HPLC chromatogram of Entecavir both bulk and the tablet formulation are presented in figures 1 and 2. The bulk and tablet formulation retention time was 3.5 minutes. For the tablet formulation no excipient interference was observed, which reveals the specificity of the method. The proposed method shows the potential to determine the analyte in presence of excipients.

Limit of detection and quantitation

The limit of detection and quantification for Entecavir is shown in table 6. Limit of detection (LOD) and limit of quantification (LOQ) were studied by minimum detectable peak area by injecting known concentration of drug solution. As per the International Conference on Harmonization guidelines, the results are multiplied thrice to get LOD and 10 times to get LOQ. LOD and LOQ were recorded at concentrations of 0. 357 µg/mL and 1.125 µg/mL, respectively. The limit of detection and quantification for Entecavir is shown in table 6.

Table 6. Limit of detection and quantification

Parameters	Results (µg/ml)
Limit of detection (LOD)	0.357
Limit of quantification (LOQ)	1.125

System suitability

For the system suitability parameters five repeats of standards and two repeats of sample preparation are injected, the data is shown in table 7. The Assay data of Entecavir is shown in table 8.

Table 7. Results of system suitability parameters

SNo	Parameters	Entecavir
1.	Theoretical plates	12100
2.	Tailing factor	0.876
3.	Resolution factor	1.15
4.	Retention time	3.51± 0.1
5.	Calibration range or Linear dynamic range	5-25µg/ml

Table 8. Quantitative estimation (Assay) data of Entecavir

Drug	Label claim (mg)	Amount found (mg)	Mean amount found (mg /ml)	Percentage purity (% w/w)	Mean purity (%w/w)	% Deviation
Entecavir	1.0	0.987 0.977 0.989 0.981 0.979	0.982	99.91 98.79 99.21 98.97 99.24	99.22	+ 0.1 +0.2 +0.1 +0.1 +0.2

n= 4 observations

Statistical parameters

The obtained assay results are subjected to the coefficient of variation; statistical analysis, regression equation and standard deviation are presented in table 9.

Table 9. Results of statistical parameters

SNo	Parameters	Entecavir
1.	Standard deviation (SD)	1.27
2.	Relative standard deviation (RSD)	0.0576
3.	% RSD	0.316
4.	Standard error (SE)	0.02321
5.	Correlation Coefficient (r)	0.9988
6.	Slope (a)	4318.8
7.	Intercept (b)	1767.2

Conclusion

The suggested and validated RP-HPLC method was carried out based on the guidelines of International Conference on Harmonization (ICH). The developed RP-HPLC method indicates the accuracy, sensitive and stability. It is rapid, reproducible, and can be utilized for the routine analysis for Entecavir tablet formulations.

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