

DEVELOPMENT AND VALIDATION OF A NOVEL STABILITY INDICATING RP-HPLC METHOD FOR THE ESTIMATION OF ENTECAVIR IN TABLET FORMULATION

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ABSTRACT

A precise and selective RP-HPLC method has been developed for the estimation of entecavir in tablet dosage form. A Agilent HC-C18 (250 x 4.6 mm i.d., particle size 5 µm) column with, Buffer: Acetonitrile in the ratio of (95:5) was employed as mobile phase. The flow rate of 1 mL/min was used and the effluents were detected at 253 nm. The retention time was found to be 6.98 min. The method was further validated for linearity, accuracy, precision, and robustness where the method was found to be linear over the range concentration range of 2.05-15.53 µg/mL, accurate (recovery of about 99.9% with % RSD of <2), highly precise (% RSD of <2 in both intra-day and inter-day study), and robust enough to deliver accurate results thus, this simple method will help in determination of entecavir prove beneficial in quality control of drug.

KEYWORDS: entecavir, RP-HPLC, estimation, validation, forced degradation.

INTRODUCTION

Entecavir (ENT) chemically, 2-Amino-9-[(1*S*,3*R*,4*S*)-4-hydroxy-3-(hydroxymethyl)-2-methylidenecyclopentyl]-6,9-dihydro-3*H*-purin-6-one, (Figure 1) is an anti-viral drug analogous to deoxyguanosine, used for the treatment of hepatitis B viral infection and is also a prime candidate administered after liver transplant.^[1] It acts by inhibiting reverse transcriptase and DNA polymerase enzymes which inhibits DNA replication in the viral replication process.^[2] The literature revealed that not much effort have been put in developing a simple method for estimating ENT either in pharmaceutical formulations or its stability studies. ENT has been determined by RP-HPLC method in tablets using ammonium acetate in water and acetonitrile in the ratio of 75:25 v/v^[3] and by water and acetonitrile in the ratio of 80:20 v/v.^[4] ENT has been estimated in human plasma by liquid chromatography-electrospray ionization-tandem mass spectrometry method using ammonium hydrogen carbonate and methanol.^[5] Apart from determination of analyte content in marketed formulations, there is a need to develop a stability indicating analytical method that will possess the ability to detect the small but deliberate changes with respect to time. At present, no stability indicated RP-HPLC study has been reported so far. Several alterations in the chemical, physical and microbiological properties of the ENT occur with time and there is no method still

developed for specific quantification of active ingredient content, degradation product and other components of interest, without interference by using RP-HPLC. Therefore, this compelled us to develop a novel single separation method for the analysis of ENT and perform forced degradation study of the drug in tablet dosage form under diverse conditions to determine degradation patterns.

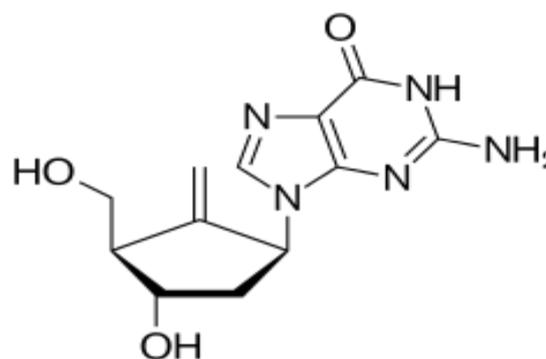


Figure 1: structure of Entecavir.

Experimental

Chemicals and reagents

Working standard of ENT was obtained as kind gift from RA CHEM PHARMA LIMITED, Hyderabad, India Fixed dose combination Enteca[®], Cipla Pharmaceutical Ltd. containing 2.5 mg of ENT was purchased from local

pharmacy hyderabad. The weight of each tablet was about mg and assay results according to pharmacopoeial method were found to be 99.45%. HPLC grade chemicals were purchased from Merck Chemicals Ltd., India. Double distilled water was used and was suitably filtered through 0.45 μ m filter.

Instrumentation

The HPLC system comprised of Shimadzu LC-2010 CHT (Japan) model with SPD 20-AD UV-Vis detector. The chromatographic separation was performed on Agilent HC-C18 (250 x 4.6 mm i.d., particle size 5 μ m) column. Spectroscopic analysis was carried out using double-beam Shimadzu[®] Ultraviolet-Visible Spectrophotometer (Kyoto, Japan) model UV-1800 connected with a computer having spectral bandwidth of 1 nm and wave length accuracy of \pm 0.3 nm with a pair of 10 mm path length matched quartz cells was used. All weighings were performed using Shimadzu[®] electronic balance (Kyoto, Japan) model AUW220D. Sonication was performed using Transonic Digital S (Sonicator), USA. Photo stability chamber (SVI equipments, Germany) was used during the experiment. The pH of solutions was measured using digital pH meter (Contech[®]).

Preparation of solutions

Preparation of stock solution

Accurately weigh and transfer 26.6 mg of Entecavir PRS into a 50 mL of volumetric flask, add 35 mL of diluent, dissolve in water bath at 60°C for 5 minutes, sonicate to dissolve the content then cool to room temperature and dilute to volume with diluent and mix. (This Solution Contains about: 500 μ g/mL Entecavir).

Preparation of standard solution

Transfer 2.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent. (This Solution Contains about: 10 μ g/mL Entecavir).

Preparation of mobile phase

Prepare a filtered and degassed mixture of Buffer and Acetonitrile in the ratio 95:50 V/V.

Preparation of sample solution

Tablets were crushed into fine powder in a glass mortar form where equivalent to 0.5 mg of ENT was taken and transferred in 100 mL volumetric flask. 10 mL methanol was added and sonicated for 30 minutes with intermittent shaking until the tablets were completely dissolved. The content was allowed to attain room temperature and shake well. The volume was made with mobile phase and filtered through 0.45 μ nylon filter.

Preparation of blank

In 100 mL volumetric flask, 10 mL of methanol was added and volume was made with mobile phase. The solution was sonicated, filtered and injected.

Detection of wavelength

The standard solution of ENT in methanol (10 ppm) was scanned in UV-spectrophotometer over the range of 400-200 nm. The λ_{max} of ENT was found to be 253 nm.

Chromatographic conditions

The experiment was conducted using Agilent HC-C18 column, which utilizes C18 stationary phase of 250 x 4.5 mm i.d., particle size 5 μ m. The mobile phase was selected on the basis of best separation, peak purity index, peak symmetry, theoretical plate etc. and a number of trials were taken for the selection of mobile phase. After number of trials, buffer (pH 3.0) and acetonitrile in ratio (95:5) was employed.

Method validation

Validation of analytical methods, in general, has been extensively covered in the ICH guidelines Q2A and Q2B, in the FDA guidance and by USP. The validation has been carried out as per ICH guidelines Q2A and Q2B.

Linearity and range

For determining the linearity of the proposed method, six concentrations were chosen ranging from 25-150% of the target analyte concentrations (25%, 50%, 75%, 100%, 125%, and 150%) in formulations. All the solutions were prepared by diluting in methanol. Equivalent volumes of each solution were injected under the chromatographic condition. Calibration graph was obtained by plotting average area versus concentration of standard drugs and the linearity was expressed in regression coefficient value (r^2).

Accuracy

The accuracy of an analytical method expresses the closeness of agreement between the value, which is accepted reference value, and the value found. Accuracy is determined by standard analysis method as percentage recovery of the standard spiked to previously analyzed test sample of drug. The accuracy/recovery was calculated by spiking the drug substance in placebo at three different concentrations of the standard drug viz. 80%, 100%, and 120% of target concentration of ENT tablets were used. The experiment was conducted in triplicate. The accuracy was reported as % recovery \pm (% confidence interval) with % relative error on the base of actual and estimated concentrations.

Precision

The precision of an analytical method is the closeness of replicate results obtained from analysis of the same homogeneous sample. Precision was determined through the estimate of the relative standard deviation (RSD) values. The studies of inter-day and intra-day variability were performed. Intra-day analysis was performed by injecting three concentrations (50%, 75%, and 150%) of standard solution of ENT six times in a single day. For inter-day, analysis was performed employing the similar protocol and recorded on three different days.

Robustness

Robustness of any analytical system demonstrates its ability to withstand small but deliberate changes without affecting the analysis. The experimental conditions were purposely altered and the chromatographic resolution of ENT was assessed. The effect of deliberate changes on system suitability parameters were studied by changing the flow rate by +0.2 mL/min; i.e. 1.2 mL/min, changing the pH of the mobile phase by +0.2 (i.e. at 3.2) and wavelength by +2 nm (256 nm), while keeping the other

chromatographic conditions constant. The impact of variations on system suitability parameters was recorded.

System suitability parameters

This test is an essential element of an analytical method which validates competence of reproducibility of system. The study was performed by injecting the standard solution five repetitive times. Parameters like peak area, retention time, tailing factor and theoretical plates of the peaks were calculated. Tabulated in Table 1.

Table 1: system suitability parameters

Peak No	Name	Ret.Time	Area	Tailing Factor	Theoretical Plate
1	Entecavir	6.99	509627	1.2	12065

RESULTS AND DISCUSSION**Method development and optimization of chromatographic conditions**

The development of this method was based on the HPLC method developed previously for the analysis of ENT^[3, 4] which suggested the use of C18 stationary phases of 250 x 4.5 mm i.d., particle size 5 µm, therefore, Agilent C18 column was utilized. For achieving a reasonable degree of separation of ENT, the composition of mobile phase was exhaustively studied. The mobile phase was selected on the basis of peak purity index, peak symmetry, and theoretical plate. Lots of trials were taken employing number of binary eluants like acetonitrile, and phosphate buffer at different pH (3.0-6.0) conditions for the selection of mobile phase. A change in pH alters the retention within 2 units of pKa. Therefore, it is judicious to regulate the pH of mobile phase 2.0 units higher or

lower the pKa to assure unionization of analyte. pH greater than 7 may result in dissolution of silica of columns, in contrast, low pH produces a milieu in which peak tailing is reduced and method ruggedness is maximized.. The experiment was performed using Agilent C18 column maintained at ambient temperature with mobile phase optimized in isocratic mode at flow-rate of 1 mL/min in 10 min run-time, keeping detector at 253 nm. The retention time of ENT was found to be 6.684 min.

Method validation**Linearity and range**

The linear regression equation for ENT was found to be $y = 49919x + 20018$ (Figure 3). The regression coefficient value was 0.999, indicating an acceptable degree of linearity (Table 2).

Table 2: linearity.

Actual Conc. (%)	Conc. (µg/mL)	Area	Avg Area	%RSD
20	2.07	99273	99347	0.2
		99544		
		99224		
50	5.18	249570	249624	0.0
		249676		
		249627		
80	8.28	401220	401015	0.1
		400841		
		400983		
100	10.36	500944	501267	0.1
		501419		
		501438		
120	12.43	599340	599201	0.0
		599278		
		598985		
150	15.53	742886	742713	0.1
		742962		
		742291		

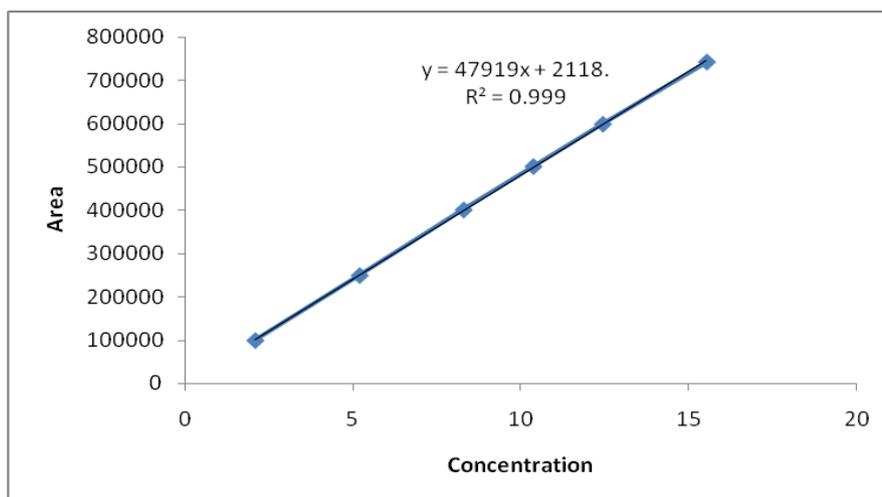


Fig 2: linearity Graph for entecavir.

Accuracy

The recovery was determined using calibration curve, where the slope and Y-intercept of the graph was employed to estimate the % recovery. The recovery data for accuracy studies are given in Table 3. The measured

% RSD values for the proposed method was found to 0.3-0.1 at three different concentrations. All the values were within the acceptance limit of $\pm 2\%$ which indicated good accuracy of the developed method.

Table3: Accuracy and Recovery Data.

Conc. (%)	Added conc. (mg/mL)	Area	Recovered conc. (mg/mL)	Recovered (%)	Avg	%RSD
20	0.00216	108554	0.00211	97.5	97.8	0.3
	0.00216	108927	0.00211	97.9		
	0.00216	109038	0.00212	98.0		
50	0.00539	271748	0.00527	97.8	97.8	0.0
	0.00539	271643	0.00527	97.8		
	0.00539	271679	0.00527	97.8		
80	0.00862	437264	0.00848	98.4	98.5	0.1
	0.00862	437857	0.00850	98.6		
	0.00862	438230	0.00850	98.6		
100	0.01078	545798	0.01059	98.2	98.2	0.2
	0.01078	544504	0.01057	98.0		
	0.01078	546368	0.01060	98.3		
120	0.01293	655503	0.01272	98.4	98.4	0.1
	0.01293	655152	0.01271	98.3		
	0.01293	655715	0.01272	98.4		
150	0.01617	818380	0.01588	98.2	98.2	0.1
	0.01617	817991	0.01587	98.2		
	0.01617	818881	0.01589	98.3		
AVG				98.2		
SD				0.3		
%RSD				0.3		

Precision

The intra- and inter-day variability or precision data are given in Table 4. The % RSD values were found to be less than 2% in each case (0.2–0.89%), which revealed that the method is precise enough to determine the drug. The difference between inter- and intra-day variability was found to be minimal and within range.

Table 4: Precision data for ere.

Prep #	Area	% Assay
1	517341	101.1
2	517549	101.1
3	517793	101.1
4	517406	101.0
5	517245	101.1
6	517769	101.2
Average		101.1
%RSD		0.0

Robustness

With the change in chromatographic conditions, minor but deliberate changes were observed. The actual peak observed at 6.68 min gets shifted to 6.54 min when the flow rate was changed to 1.2 mL/min. When the detecting wavelength was intentionally changed to 253 nm, the peak was retained at 6.45 min At pH 3.2, it was

observed that the retention time gets changed drastically to 6.84 min Therefore, this method is robust enough to detect the ENT content even at small change in chromatographic conditions; however, pH must be kept in concern. This indicated that the proposed method has desired precision and will be suitable for analysis.

Table 5: Robustness.

Parameter	Theoretical Plates	Tailing Factor	%RSD	System Performance (%)	Pass/Fail
Method Specified	12006	1.2	0.0	0.3	Pass
Low flow (0.9 mL/min)	13224	1.2	0.0	0.0	Pass
High flow (1.1 mL/min)	11889	1.2	0.1	0.1	Pass
Low Temperature (30°C)	12499	1.2	0.1	0.2	Pass
High Temperature (40°C)	12501	1.2	0.1	0.3	Pass
97% Buffer : 3% CAN	14519	1.1	0.0	0.0	Pass
93% Buffer : 7% CAN	12694	1.1	0.0	0.1	Pass

CONCLUSION

The new method developed using the mobile phase buffer (pH 3.0) and acetonitrile at ratio 95:5 on a Agilent C18 column at a flow rate of 1 mL/min demonstrated superior and sharp peak. As compared to other methods, which have displayed shorter (2-3 min) or longer (8.5-9.5 min) retention time, this method has an optimum retention time of 6.98 min. HPLC method is preferred in several pharmaceutical analyses in industrial scale as compare to other methods, owing to its simplicity, accuracy, and precision. With a minor change observed in the robustness studies in terms of pH, wavelength and flow rate, the developed method bear all such attributes in exhibiting greater reproducibility and can be adopted for routine quality control analysis of ENT in pharmaceutical dosage form. Additionally, after successful validation, this method with slight modifications might be employed in determining the drugs having similar scaffold-like ENT, active pharmaceutical ingredients, diluents, etc. and may have perspectives in estimating non-pharmaceutical products also. No interference of degradation product and diluents was encountered in this method. Following the ICH Guidelines, the forced degradation studies have revealed the possible ways of degradation of ENT under various conditions. It was observed that the drug underwent the highest degradation in basic medium followed by acidic medium and oxidation. Thus, the degradation studies will help in both qualitative and quantitative determination of degraded products and may prove beneficial in the quality control of the drug. Further studies on other pharmaceutical formulation would throw mere light on these studies.

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