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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 Phenomenox C_{18} (250 x 4.6 mm i.d., particle size 5 µm) column with, water (pH 3.0 adjusted by glacial acetic acid) and acetonitrile (95:5) was employed as mobile phase. The flow rate of 1 mL/min was used and the effluents were detected at 254 nm. The retention time was found to be 7.724 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 5-30 µg/mL, accurate (recovery of about 99.2% with % RSD of <2), highly precise (% RSD of <2 in both intra-day and inter-day study), and robust enough to deliver accurate results. Forced degradation studies of entecavir were performed under neutral, acidic, alkaline conditions along with photolysis and oxidative conditions, where the drug exhibited degradation and various peaks were monitored. Highest degradation was observed under basic condition. Thus, this simple method will help in determination of entecavir and the result of degradation studies will help in both qualitative and quantitative determination of degraded products and may 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, 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 (**Figure 1**) [1]. It acts by inhibiting reverse

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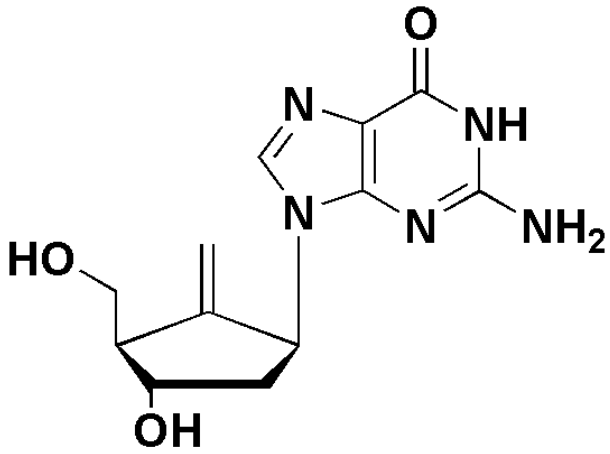

Figure 1. The structure of deoxyguanosine

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.

EXPERIMENTAL

Chemicals and reagents

Working standard of ENT was obtained as kind gift from Zim Laboratories Ltd., Nagpur, India. Fixed dose combination Enteca®, Cipla Pharmaceutical Ltd. containing 0.5 mg of ENT was purchased from local pharmacy of Nagpur, India. The weight of each tablet was about 185 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 µm 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 Phenomenox C_{18} (250 x 4.6 mm i.d., particle size 5 µm) 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 ±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

0.5 mg of ENT reference standard was transferred into 100 ml volumetric flask. 10 ml diluent (methanol) was added and sonicated to dissolve the content completely. The solution was cooled further to the room temperature and made up to volume with diluents to produce 5 µg/mL.

Preparation of standard solution

10 ml of above stock solution of ENT was pipette out and transferred to 50 ml volumetric flask, 5 ml of methanol was added and volume was made up to mark with mobile phase. Further, the solution was sonicated and filtered.

Preparation of mobile phase

A suitable filtered and degassed mixture of water (pH 3.0 previously adjusted by glacial acetic acid) and acetonitrile (95:5) was prepared.

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 254 nm.

Chromatographic conditions

The experiment was conducted using Phenomenex C_{18} column, which utilizes C_{18} stationary phase of 250 x 4.5 mm i.d., particle size 5 µm. The mobile phase was selected on the basis of 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, water (pH 3.0) and acetonitrile in ratio (95:5) was employed.

Force degradation studies

Forced degradation studies for ENT were conducted under acid/base hydrolysis, oxidative, thermal, and UV degradation conditions. In order to judge the effectiveness of the method, the sample was exposed to these harsh conditions and efficacy in separation of degradation products from the pure active ingredient were studied for the peak purity.

Alkaline degradation

Hydrolytic degradation of the drug was performed by exposing the molecule to acidic or basic conditions in due course of time to determine primary degradation products. Typically, under hydrolytic stress, the functional groups like ester, amide (prominently), etc. undergoes hydrolysis leading to change in characteristics of drug. The degradation of ENT under alkaline environment was studied by reacting with 2 N NaOH solution. Tablets were crushed and equivalent to 0.5 mg of ENT was transferred in 100 mL volumetric flask. 10 mL of methanol was added, sonicated for 15 min and volume was made up to 100 mL. The sample solution was further stirred for 30 min using magnetic stirrer, and centrifuged at 3000 rpm for 5 min. 5 mL of the resulting solution was pipette out in 50 mL volumetric flask and equal quantity of 2N NaOH was added with occasional shaking. The content was kept on boiling water bath for 1 hour, cooled to room temperature, neutralized with 5 mL of 2N HCl, sufficient mobile phase was added to make up the volume up and filtered through the 0.45 µm pore size nylon membrane.

Acidic degradation

ENT was forced degraded under acidic condition by reacting with 2N HCl solution. Tablets were crushed and equivalent to 0.5 mg of ENT was transferred in 100 mL volumetric flask. 10 mL of methanol was added, sonicated for 15 min and volume was made up to 100 mL. The sample solution was further stirred for 30 min using magnetic stirrer, and centrifuged at 3000 rpm for 5 min. 5 mL of the resulting solution was pipette out in 50 mL volumetric flask and equal quantity of 2N HCl was added with occasional shaking. The content was kept on boiling water bath for 1 hour, cooled to room temperature, neutralized with 5 mL of 2N NaOH,

sufficient mobile phase was added to make up the volume up to 50 mL and filtered through the 0.45 µm pore size nylon membrane.

Oxidative degradation

Oxidative degradation of drug molecules are well known phenomenon, where reaction occurs between drug substance and molecular oxygen leading to commencement of auto-oxidation process. Generally, dilute peroxide solutions (3% H_2O_2) are used for testing oxidative stress of bulk drugs. Tablets were crushed and equivalent to 0.5 mg of ENT was transferred in volumetric flask. The content of flask was dissolved in 5 mL of H_2O_2 and boiled for 1 hr. The drug content was placed at room temperature for degradation. Further, the content was transferred in 100 mL volumetric flask. 10 mL of methanol was added, sonicated for 15 min and volume was made up to 100 mL. The sample solution was further stirred for 30 min using magnetic stirrer, and centrifuged at 3000 rpm for 5 min. It was then filtered through a 0.45 µm filter and injected into the HPLC. The obtained chromatogram was studied for any degradation underwent during the time given.

Thermal degradation

To determine the thermolytic processes which give rise to degradation products, elevated temperatures (>70°C) in the solid or solution states are employed. Compounds commence degradation through diverse mechanisms, ultimately leading to formation of degradation products. The study was performed by taking ENT content equivalent to 0.5 mg of tablets and exposed to heat at 80±1°C for 1 hour and evaluated for stability under the stress conditions. 10 mL of methanol was added, sonicated for 15 min and volume was made up to 100 mL with mobile phase. The sample solution was further stirred for 30 min using magnetic stirrer, and filtered through a 0.45 µm pore size nylon membrane. Twenty microliters of drug solution was injected into HPLC.

Photolytic degradation

In UV-degradation studies, molecules absorb light which leads to absorption band overlapping and a valence electron reaches excited state. For UV degradation, ENT equivalent to 0.5 mg was taken from tablet mixture and transferred into a petridish. The drug material was forced degraded under UV radiations for three consecutive days at 254 nm in UV chamber and primary degradation products were determined. The exposed material was transferred in volumetric flask. 10 mL of methanol was added, sonicated for 15 min and volume was made up to 100 mL with mobile phase. The sample solution was further stirred for 30 min using magnetic stirrer, and filtered through a 0.45 µm pore size nylon membrane. The drug solution (20 µL) was injected into HPLC.

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 ± (% 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.

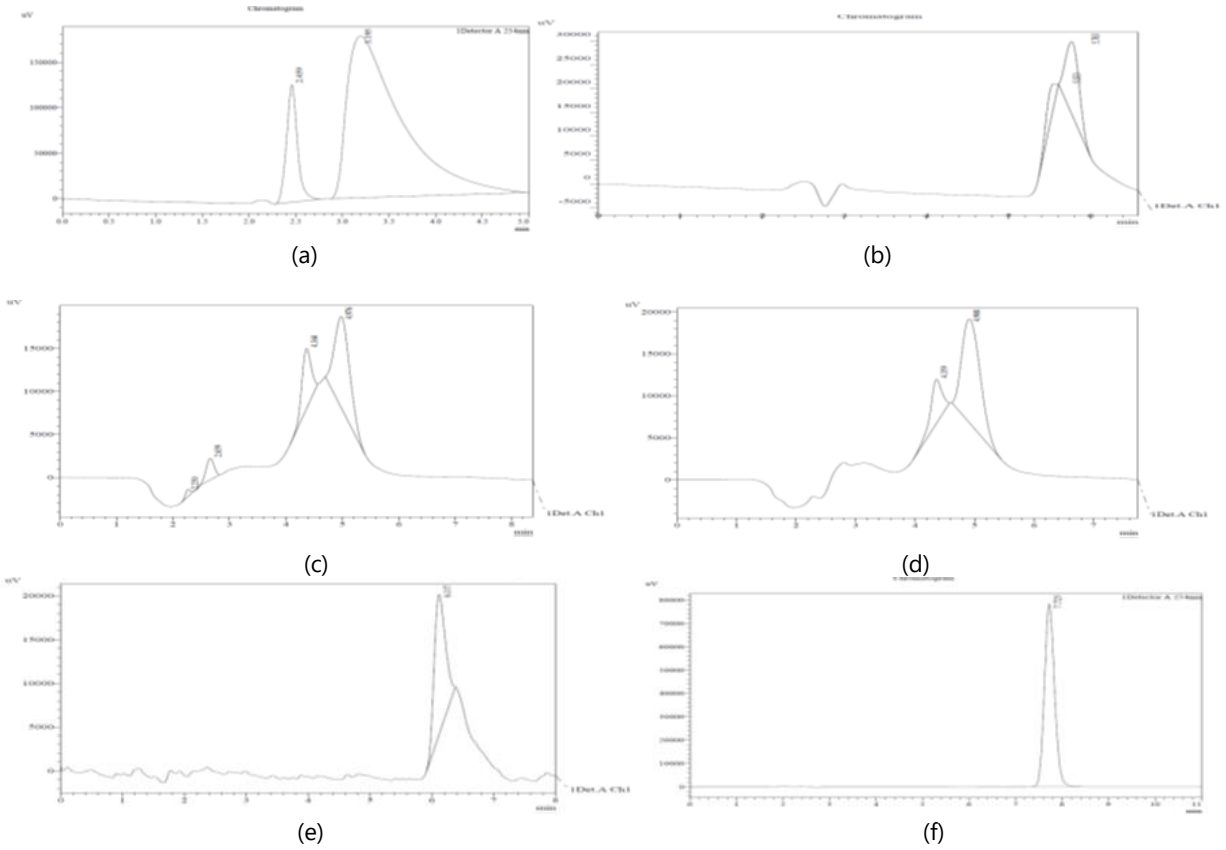

Figure 2. Chromatograms obtained during various trial runs in the new method development. **(a)** Water: Methanol (30:70) **(b)** Water: ACN (60:40) **(c)** Phosphate buffer (pH 4.0): ACN (40:60) **(d)** Phosphate buffer (pH 4.5): ACN (40:60) **(e)** Phosphate buffer (pH 5.0): Methanol (20:80) **(f)** Water (pH 3.0): Acetonitrile (95:5)

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.

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 C_{18} stationary phases of 250 x 4.5 mm i.d., particle size 5 µm, therefore, Phenomenex C_{18} 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

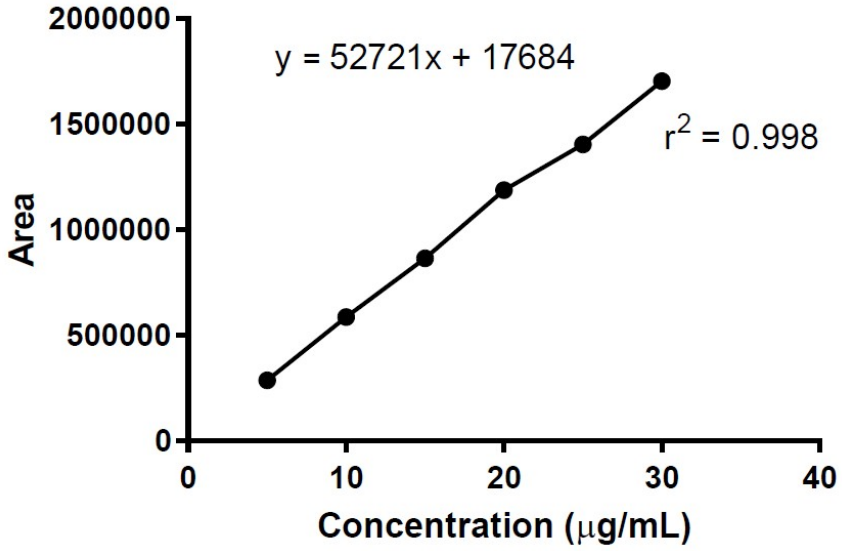

Figure 3. The linear regression equation for ENT

acetonitrile, methanol, water 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. Additionally, ENT gets degraded at basic pH, thus the operation was conducted at low pH. Water: methanol (30:70) demonstrated low intensity and high tailing (**Figure 2a**), Water: ACN (60:40) displayed distorted peaks with very poor resolution (**Figure 2b**) and phosphate buffer (pH 4.0): ACN (20:80) (**Figure 2c**), phosphate buffer (pH 4.5): ACN (40:60) (**Figure 2d**) and phosphate buffer (pH 5.0): methanol (20:80) (**Figure 2e**) represented highest tailing and was 'misfit' for experimentation. Water (pH 3.0): acetonitile (95:5) showed sharp peak and good theoretical plate (**Figure 2f**), therefore, it was chosen for analytical purpose. The experiment was performed using Phenomenex C_{18} 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 254 nm. The retention time of ENT was found to be 7.724 min.

Method validation

Linearity and range

The linear regression equation for ENT was found to be $y = 52721x + 17684$ (**Figure 3**). The regression coefficient value was 0.998, indicating an acceptable degree of linearity (**Table 1**).

Table 1. Linearity levels of entecavir

Linearity Levels	Conc. of ENT (µg/mL)	Average Area
Level 1	5	285477
Level 2	10	585230
Level 3	15	863639
Level 4	20	1186673
Level 5	25	1404712
Level 6	30	1703585

Table 2. Recovery for accuracy studies of ENT

Level	Powered tablet taken (mg)	Eq. amount added to placebo (mg)	Amount recovered (mg)	% RSD	Average Area	% Recovery
80%	148	0.4	0.379	**0.92**	583540	94.75
	148	0.4	0.383		583689	95.75
	148	0.4	0.386		583797	96.50
100%	185	0.5	0.496	**0.82**	1172713	99.20
	185	0.5	0.488		1172228	97.60
	185	0.5	0.491		1172584	98.20
120%	222	0.6	0.587	**0.77**	1693577	97.83
	222	0.6	0.592		1693859	98.66
	222	0.6	0.583		1693513	97.16

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 2**. The measured % RSD values for the proposed method was found to 0.92, 0.88 and 0.77 at three different concentrations. All the values were within the acceptance limit of ±2% which indicated good accuracy of the developed method.

Precision

The intra- and inter-day variability or precision data are given in **Table 3**. 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.

Robustness

With the change in chromatographic conditions, minor but deliberate changes were observed. The actual peak observed at 7.724 min gets shifted to 7.516 min when the flow rate was changed to 1.2 mL/min (**Figure 4a**). When the detecting wavelength was intentionally changed to 256 nm, the peak was retained at 7.514 min (**Figure 4b**). At pH 3.2, it was observed that the retention time gets changed drastically to 7.350 (**Figure 4c**). Therefore, this method is robust enough to detect the ENT content even at small change in chromatographic conditions;

Table 3. Precision data of inter- and intra-day variability

			Intra-day variability		Inter-day variability	
Level	**Powered tablet taken (mg)**	**Equivalent amount of drug (mg)**	**Amount determined (mg)**	**% RSD**	**Amount recovered (mg)**	**% RSD**
	91.0	25	24.67		24.61	
	91.0	25	24.62		24.69	
	91.0	25	24.93	**0.47**	24.85	**0.46**
50%	91.0	25	24.85		24.90	
	91.0	25	24.73		24.66	
	91.0	25	24.70		24.72	
	136.5	37.5	37.53		37.58	
	136.5	37.5	37.45		37.40	
	136.5	37.5	37.60	**0.16**	37.48	**0.19**
75%	136.5	37.5	37.51		37.51	
	136.5	37.5	37.44		37.55	
	136.5	37.5	37.48		37.59	
	273.0	75	74.64		74.80	
	273.0	75	74.94		75.11	
	273.0	75	74.75	**0.19**	74.67	**0.23**
150%	273.0	75	74.87		75.05	
	273.0	75	74.90		74.96	
	273.0	75	74.59		74.77	

however, pH must be kept in concern. This indicated that the proposed method has desired precision and will be suitable for analysis.

System suitability parameters

The system suitability parameters described that the method exhibited the competency as per the minimum requirements of monographs of United States Pharmacopoeia (USP). The system demonstrated a mean of 5796 theoretical plates, which is more than the pharmacopoeial limit of 2000, thereby describing high column efficacy with better resolution. The peak areas were measured to be 1189014 averagely with % RSD of 0.032. The % RSD of less than 2 indicates high column efficacy and reproducibility on every injection. The tailing factor (TF) on an average remained 1.115, which is indicative of the fact that the peak exhibit symmetry. The value of nearly 1 represents that the asymmetric factor (AF) is nearly or equal to 1, since, with an increase in AF, the tailing becomes pronounced. The TF remained below the limit of 2, indicating almost the shape of an ideal Gaussian peak. A similar retention time of 7.72 min was observed in all the cases signifying that the system affirms a very high reproducibility. Taken together into account, the developed method system is highly robust, reproducible, accurate, and is of great interest in pharmaceutical analysis. The system suitability parameters are represented in **Table 4**.

Table 4. System suitability parameters

S. No.	Area Reproducibility	Retention Time	Tailing Factor	Theoretical Plates
1.	1189316	7.72	1.114	5775
2.	1189499	7.72	1.115	5804
3.	1188838	7.72	1.114	5796
4.	1188831	7.72	1.118	5800
5.	1188587	7.72	1.116	5804
Mean	1189014	7.72	1.115	5796
% RSD	0.032			

Forced degradation studies

The **Figure 5** described the degraded products of ENT under various conditions. The neutral hydrolysis study of ENT revealed that no degradation occurs over 4h under neutral environment. In contrast, under acidic hydrolysis condition, the chromatogram showed degradation peaks at 1.66, 1.91, and 6.7 min along with the drug peak. The peak area demonstrated that more than 50% of drug degradation occurred when the drug was kept in 0.1M HCl at 80°C up to 4h. At lower pH, ionization of amine group takes place. ENT underwent deplorable degradation in 0.01 M NaOH at 80°C up to 4h, showing degradation peak at 1.62, 1.93, 2.51, 2.91, and 6.2, along with drug peak at 7.6 min, respectively. ENT exhibit the character of a weak acid (pKa value of 8.0), in completely ionized state results in good solubility which subsequently initiate faster degradation. The probable reason for highest degradation in alkaline media is mechanistically, the degradation progression involved abstraction of the proton by the base *OH radical. Moreover, it may be believed that the ENT stability is dependent on pH, temperature, buffer concentration, and environment impact. In the oxidative degradation study, ENT, under the influence of H_2O_2 showed degradation peak at 1.72 min along with the drug peak (~7.5 min), after 4 h of exposure. The thermal degradation study showed that ENT gets degraded when kept at 80°C for 24 hr. The degradant was retained at 1.9 min in the chromatogram along with the drug peak. ENT gets degraded after it was kept in the UV chamber for 48 h and displayed characteristic degradation peak at 1.9 min, quite similar to the thermal degradation study. On comparing acidic, basic, thermal and photolytic degradation chromatograms, few similarities were observed. In 1.9 min, a small peak was observed in all the conditions with similar intensity. Likewise, a nearly identical retention time of 1.6 min was detected in both the acidic and basic conditions, however with different intensities. On the contrary, between 6-8 min, a noticeable change was identified where in alkaline media prominent peak was observed at 6.25 min and in the presence of acid, ENT degraded product displayed retention at 7.37. The results of force degradation studies are represented in **Table 5**.

Table 5. Forced degradation studies of entecavir tablet

S. No.	Retention time (min)	Area	Area %	Height	Height %	f theoretical
			Alkaline degradation			
1.	1.623	181013	12.717	34518	44.91	1928
2.	1.939	11931	0.838	1843	2.39	1722
3.	2.515	12287	0.863	1517	1.97	2400
4.	2.910	32165	2.260	2063	2.68	978
5.	6.258	1086250	76.315	33290	43.34	827
6.	7.603	99733	7.007	3622	4.71	2705
		1423378	**100.00**	**76853**	**100.00**	
			Acidic degradation			
1.	1.663	55792	5.815	11971	20.36	2395
2.	1.910	12863	1.341	1510	2.56	951
3.	6.709	46788	4.876	1058	1.87	805
4.	7.379	844056	87.968	44216	75.21	3306
		960943	**100.00**	**58783**	**100.00**	
			Oxidative degradation			
1.	1.728	12385486	91.869	2485758	97.27	2378
2.	7.498	1096268	8.131	69512	2.73	5085
		13481754	**100.00**	**2555271**	**100.00**	
			Thermal degradation			
1.	1.905	26870	2.263	1884	2.55	982
2.	7.492	1160376	97.737	72280	97.45	4894
		1187246	**100.00**	**74164**	**100.00**	
			Photolytic degradation			
1.	1.906	32418	2.729	2638	3.56	1020
2.	7.477	1155527	97.271	71496	96.44	4820
		1187945	**100.00**	**74134**	**100.00**	

CONCLUSION

The new method developed using the mobile phase water (pH 3.0) and acetonitrile at ratio 95:5 on a phenomenox C_{18} 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 7.72 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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