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LC Determination of Diastereomeric Impurities of Entecavir in Drug Substances and Drug Products.

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ABSTRACT

Effective chromatographic separation was achieved on a C18 stationary phase (150 x 4.6 mm, 3.5 microns particles) with the economical and simple mobile phase combination delivered in an isocratic mode at a flow rate of 1.0 ml min^{-1} at 254 nm. In the developed method, the resolution between Entecavir and its diastereomeric impurities was found to be greater than 2.0. Regression analysis shows an r^2 value (correlation coefficient) greater than 0.999 for Entecavir and for its diastereomeric impurities. This method was capable to detect Entecavir and its diastereomeric impurities at a level below 0.009% with respect to test concentration of 500 µg ml^{-1} for a 20 µL injection volume. The method has shown good, consistent recoveries for diastereomeric impurities (95–105%). The test solution was found to be stable in the diluent for 48 h. The drug was subjected to stress conditions. The mass balance was found close to 99.5%.

Keywords: Entecavir, HPLC, RP-LC, LC Development, Validation, Diastereomers

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INTRODUCTION

Several methods have been developed for the determination of Entecavir and its enantiomers by HPLC, LCMS techniques [1-12]. Entecavir is an oral antiviral drug used in the treatment of hepatitis B virus (HBV) infection. Entecavir is a reverse transcriptase inhibitor. It prevents the hepatitis B virus from multiplying and reduces the amount of virus in the body. More specifically, it is a deoxyguanosine analogue that inhibits reverse transcription, DNA replication and transcription in the viral replication process. Entecavir belonging to the chemical class of purine derivatives and chemically it is 2-amino-9-[(1S, 3R, 4S)-4-hydroxy-3-(hydroxymethyl)-2-methylidenecyclopentyl] - 3H-purin-6-one with molecular formula $C_{12}H_{15}N_5O_3$. Entecavir is a white to off-white powder. It is slightly soluble in water (2.4 mg/mL), and the pH of the saturated solution in water is 7.9 at 25° C ± 0.5° C. Baraclude film-coated tablets are available for oral administration in strengths of 0.5 mg and 1 mg of Entecavir.

The methods of [1-2] describes about the determination of Entecavir in tablet dosage form by LC. The LC method of [3-4] defines about the estimation of Entecavir in bulk as well as pharmaceutical dosage forms. The method of [5-7] explains about the determination of Entecavir using LC-MS techniques in drugs and plasma. The method of [8-9] clarifies the determination of Entecavir by spectrophotometric procedure. The approaches of [10] states that the optical isomer of Entecavir through enantiospecific HPLC. The tactics of [11] enlightens the determination of related compounds of Entecavir by LC. The method of [12] instructs about the determination of Entecavir by voltammetry in formulated dosage forms.

This research article describes a simple, sensitive and cost effective mobile phase method for determination/quantitation of diastereomeric impurities of Entecavir in drug substances as well as in drug products. Comparison of different techniques listed in Table 1. The work also includes the method development and the complete validation [13] as per ICH guidelines. Hitherto; there is no article for the quantification and determination of diastereomeric impurities of Entecavir in drug substances and drug products. This is a novel and sensitive method for the diastereomeric impurities in Entecavir using HPLC.

TABLE 1: COMPARISON OF DIFFERENT TECHNIQUES

References	Technique	Analyte / Impurity	LOD	LOQ	Recovery (%)	Linearity (r^2)
Present Method	HPLC	**Entecavir** **Imp-1** **Imp-2** **Imp-3**	0.008% 0.002% 0.002% 0.003%	0.025% 0.007% 0.006% 0.008%	101.3 to 105.1	0.999
[3]	HPLC	Entecavir	0.372 µg/mL	1.128 µg/mL	98.65-99.41	0.9991
[4]	HPLC	Entecavir	0.5 mg	0.49 mg	98.78-99.9	0.9927
[5]	LCMS	Entecavir	Not available	0.243 ng/mL	72.81-98.32	Not available
[8]	UV	Entecavir	0.06 µg/mL	0.123 µg/mL	99.0-102.5	0.9998

Sensitivity, Linearity and Recovery compared with previous publications.

MATERIALS AND METHODS

Materials

Samples and standard were gifted by Chemical Technology Company. Commercially available 0.5 mg Entecavir tablets (X-VIR) were purchased. LC grade acetonitrile was purchased from Merck, Darmstadt, Germany. Millipore water was used prepared from Milli-Q plus water purification system.

The LC system was a Waters 2695 quaternary pump plus auto sampler and a 2996 photo diode array detector. The output signal was monitored and processed by Empower 3 software on an Intel core i3 computer (Dell.), water baths equipped with MV controller (Julabo, Seelbach, Germany) were used for hydrolytic studies. Stability studies were carried out in a humidity chamber (Thermo lab humidity chamber, India) and photo stability studies were carried out in a photo stability chamber (Sanyo photo stability chamber, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (MACK Pharmatech, Hyderabad, India). The structures, chemical name of Entecavir and its diastereomeric impurities given in Fig.1.

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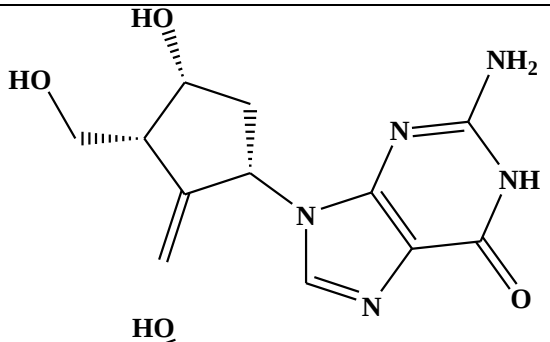
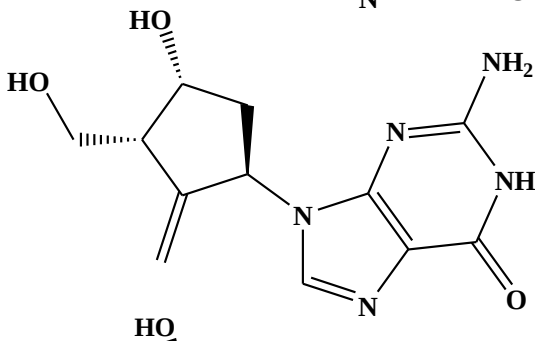
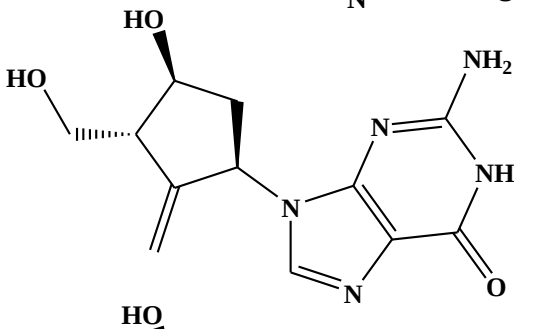
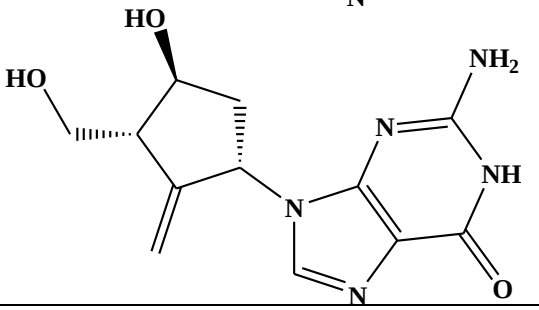
Imp-1 or ENE-III (RT ~ 6.8 min.)	2-amino-9-((1S,3R,4R)- 4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl)-1H-purin-6(9H)-one	
Imp-2 or ENE-IV (RT ~ 8.9 min.)	2-amino-9-((1R, 3R, 4R)- 4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl)-1H-purin-6(9H)-one	
Imp-3 or ENE-II (RT ~ 14.3 min.)	2-amino-9-((1R,3R,4S)- 4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl)-1H-purin-6(9H)-one	
Entecavir or ENE (RT ~ 7.9 min.)	2-amino-9-((1S, 3R, 4S)- 4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl)-1H-purin-6(9H)-one	

Fig 1: Structures, chemical name of Entecavir and its diastereomeric impurities

Methods

Chromatographic Conditions

The chromatographic column used for separation was a Zorbax SB-C18, 150 mm, 4.6 mm i.d with 3.5 µm particles. Mix 950 ml of Mill Q water and 50 ml of Acetonitrile is the Mobile phase. The flow rate of the mobile phase was 1.0 ml min^{-1}. The column temperature was maintained at 40°C and the detection was monitored at a wavelength of 254 nm. The injection volume was 20 µL. Mobile phase was used as sample diluent.

Preparation of stock solutions and system suitability

Stock solutions of Entecavir standard and sample (500 µg ml^{-1}) were prepared by dissolving appropriate amounts. Working solutions of 3.75 µg ml^{-1} were prepared from above stock solutions for diastereomeric impurities determination. A stock solution of diastereomeric impurities at 70 µg ml^{-1} was also prepared in sample with that of diluent and used as system suitability.

Preparation of Sample Solution

An amount of powdered tablets (X-VIR, 0.5 mg) equivalent to the active pharmaceutical ingredient (Entecavir) were transferred to a 100 **ml** volumetric flask, 30 ml of diluent were added and kept on a rotary shaker for 10 min to disperse the material completely, then sonicated for 10 min and diluted to 100 **ml** (500 µg ml^{-1}). The resulting solution was centrifuged at 3,000 rpm for 25 min (supernatant solution was used for purity evaluation).

Analytical Method Development

As all the diastereomeric impurities and Entecavir were having UV maxima at around 254 nm, detection at 254 nm was selected for the method development purpose. In order to develop a selective and sensitive method, primary concern during development was to achieve resolution between imp-1, Entecavir, imp-2 and imp-3 peaks and symmetry of the Entecavir peak. When pH moved from basic to acidic, there is no improvement in symmetry of the Entecavir peak was observed. At buffer pH 3.0, tailing of the Entecavir peak was 2.4, resolution between Entecavir and imp-3 was less (Rs < 1) which may be due to the ionization of the amine group in both Entecavir and imp-3. Upon study at different pHs, improvement in resolution (Rs > 1.8) between imp-1, Entecavir, imp-2 and imp-3 was observed and symmetry of the Entecavir (tailing < 2) peak was obtained. Various ion pairing agents such as Trifluoroacetic acid and Triethylamine were tested to improve peak properties such as peak height, peak symmetry. Addition of 2 ml of Triethyl amine to buffer (0.02 M) at pH 4.5 improved the symmetry of Entecavir (tailing factor was improved to 1.6).

To further improve the symmetry of the Entecavir peak column oven temperature was increased to 40°C. The symmetry of the Entecavir peak was improved to 1.4. The effect of various organic modifiers such as acetonitrile, methanol, and different gradient programs were tried to optimize the retention time of Entecavir and resolution between the impurities. Satisfactory results (retention time of Entecavir was ~ 7.5 min and the resolution between all the impurities was >2.0) were obtained with the above mentioned chromatographic conditions. Buffer pH and % acetonitrile were not played a major role in achieving the separation between diastereomeric impurities and Entecavir. In the optimized conditions Entecavir, imp-1, imp-2 and imp-3 were well separated with a resolution greater than 2.0. Analysis was performed for different batches of bulk drug samples (n = 3) and for pharmaceutical dosage forms (n = 3). Results were within specification. Stability study results as per ICH Q1A (R2) for Entecavir were generated (long term stability 12 months and accelerated stability 6 months) and the results were well within the limits.

Statistical Method

The robustness study were performed using the Minitab 17 software using the 6 as number factors viz., flow, column oven temperature, mobile phase organic ratio, particle size of the packing material, length of the column and wavelength.

RESULTS AND DISCUSSION

Analytical Method Validation

The developed chromatographic method was validated for selectivity, linearity, range, precision, accuracy, sensitivity, robustness and system suitability.

Specificity

Specificity of the developed method was assessed by performing forced degradation studies. The specificity of the developed LC method for Entecavir was determined in the presence of diastereomeric impurities namely imp-1, imp-2, imp-3. Forced degradation studies were performed on Entecavir to provide an indication of the stability-indicating property and specificity of the proposed method. All stress decomposition studies were performed at an initial drug concentration of 500 µg ml^{-1}.The chromatograms of blank, system suitability, standard and sample shown in Fig.2 – 5.

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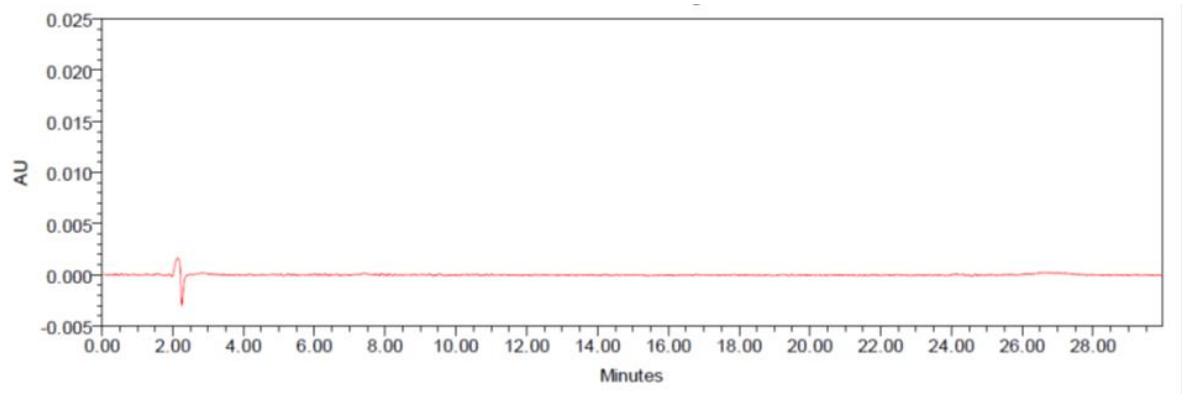

Fig 2: Blank chromatogram

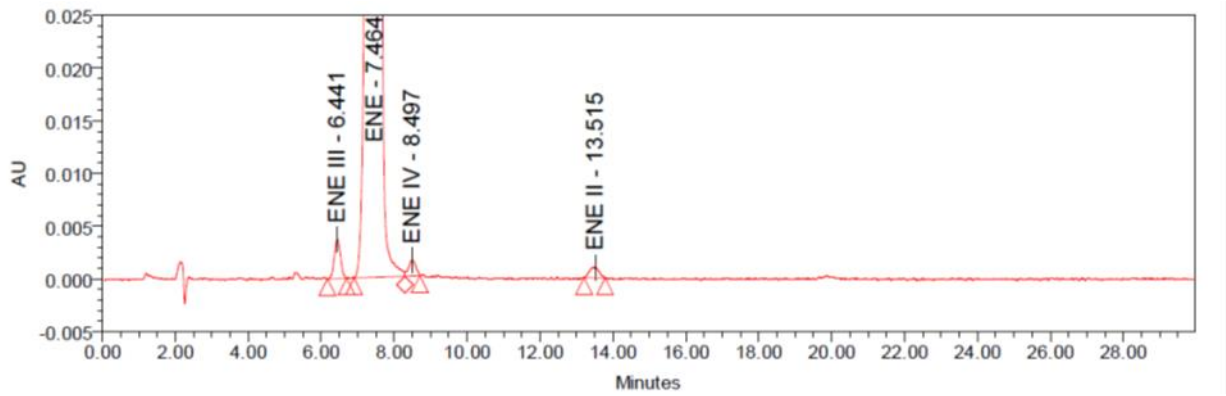

Fig 3: System suitability chromatogram

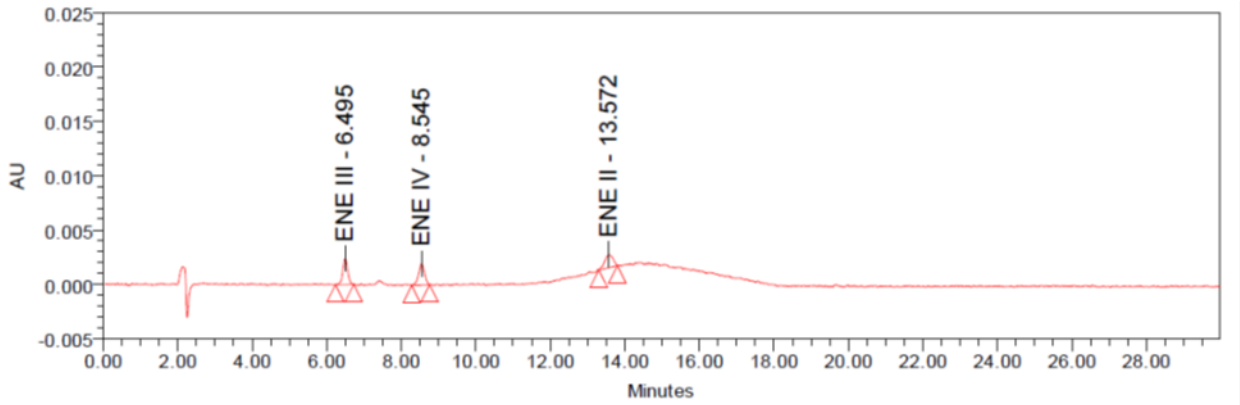

Fig 4: Standard chromatogram

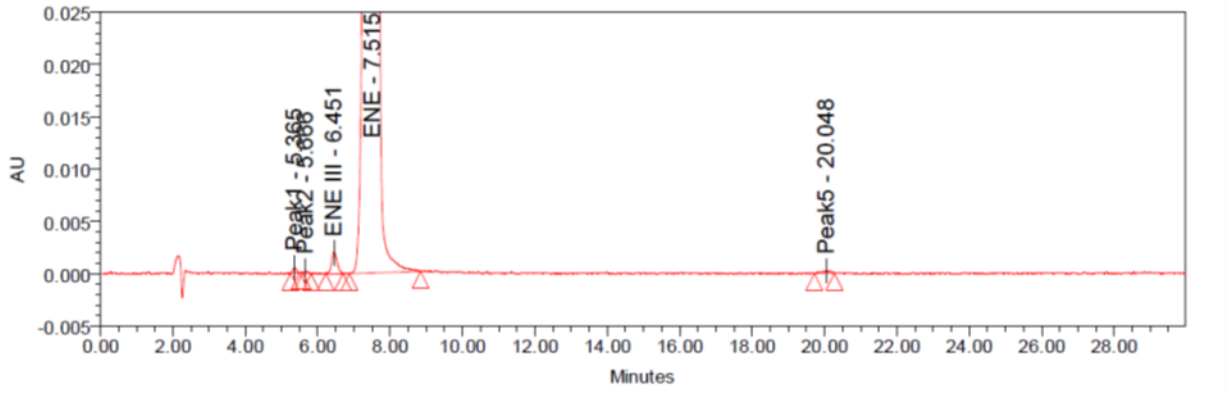

Fig 5: Sample chromatogram

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Results of Forced Degradation Studies

Stress studies on Entecavir under different stress conditions (carried out asper ICH Q1B), suggested the following degradation behavior. The drug was exposed to base hydrolysis under reflux conditions. Entecavir showed very slight sensitivity towards the treatment of base hydrolysis. When the drug was exposed to 0.1 N NaOH (24 h reflux at 80°C), minor degradation was observed. The drug was exposed to acid hydrolysis under reflux conditions. Entecavir was not sensitive towards the treatment of acid hydrolysis. When the drug was exposed to 0.1 N HCl (24h reflux at 80°C), no degradation was observed. The drug was exposed to 3% hydrogen peroxide at room temperature for 24 h. Entecavir showed sensitivity towards the treatment of hydrogen peroxide. The drug gradually underwent degradation with time in 3% hydrogen peroxide and major degradation was observed (~87.7%).The drug was exposed to water at 60°C for 24 h. No major degradation products were observed after 24 h. The drug was stable towards water hydrolysis. The drug was stable to the effect of photolysis. When the drug powder was exposed to light for an overall illumination of 1.2 million lux hours and an integrated near ultraviolet energy of 200-Watt hours/square meter (W/m.hr) (in a photo stability chamber), no degradation was observed. The drug was stable to the effect of temperature. When the drug powder was exposed to dry heat at 60°C for 10 days, no degradation was observed. Similar stress conditions were applied to excipients (lactose monohydrate, microcrystalline cellulose, crospovidone, povidone, and magnesium stearate, titanium dioxide, hypromellose, polyethylene glycol 400, polysorbate 80 (0.5-mg tablet only), and iron oxide red) and drug products injected. There was no interference of stressed excipient samples with Entecavir and its diastereomeric impurities. Peak purity results for stressed Entecavir samples, derived from PDA detector, (the purity angle within the purity threshold limit) confirm that Entecavir, imp-1, imp-2 and imp-3peaks were homogeneous and pure. No degradation product peaks were observed after 30 min in the extended run time of 100 min for all the Entecavir stressed samples. Assay studies were carried out for stress samples against qualified reference standard. The mass balance (% assay + % of impurities + % of degradation products) of stressed samples was close to 99.5% confirm the stability indicating power of the developed method. The chromatograms of acid degradation, base degradation, oxidation, photo degradation and thermal degradation shown in Fig.6 – 10.

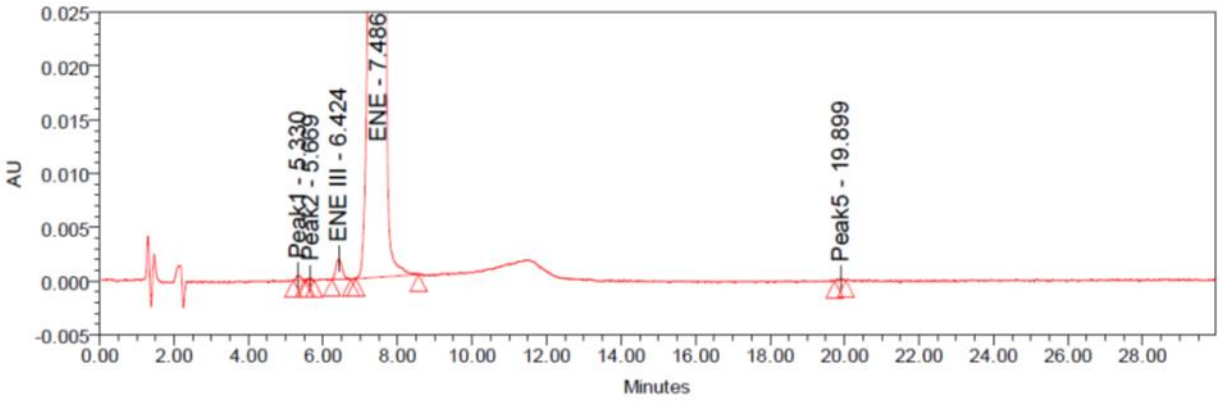

Fig 6: Acid degradation chromatogram

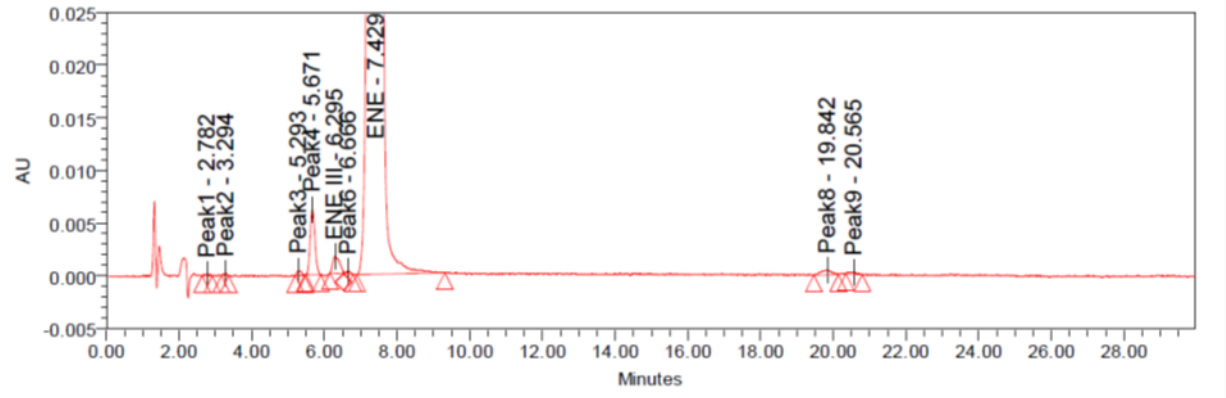

Fig 7: Base degradation chromatogram

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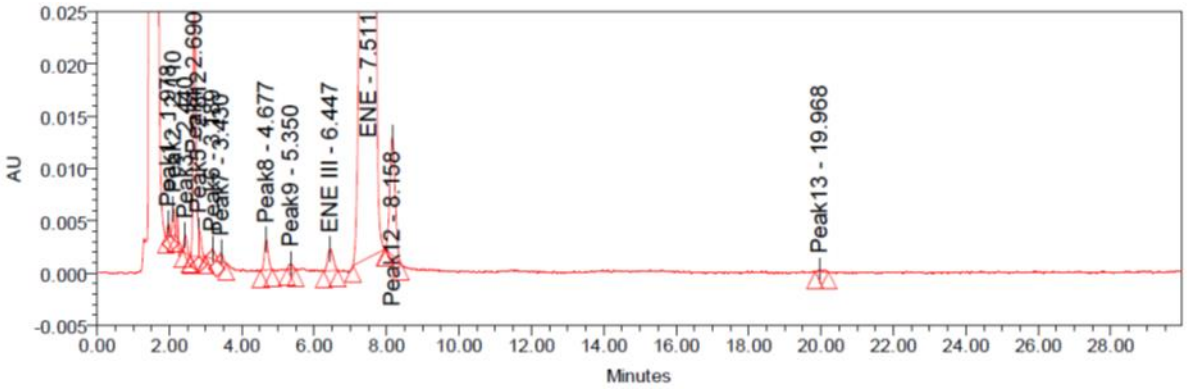

Fig 8: Oxidation chromatogram

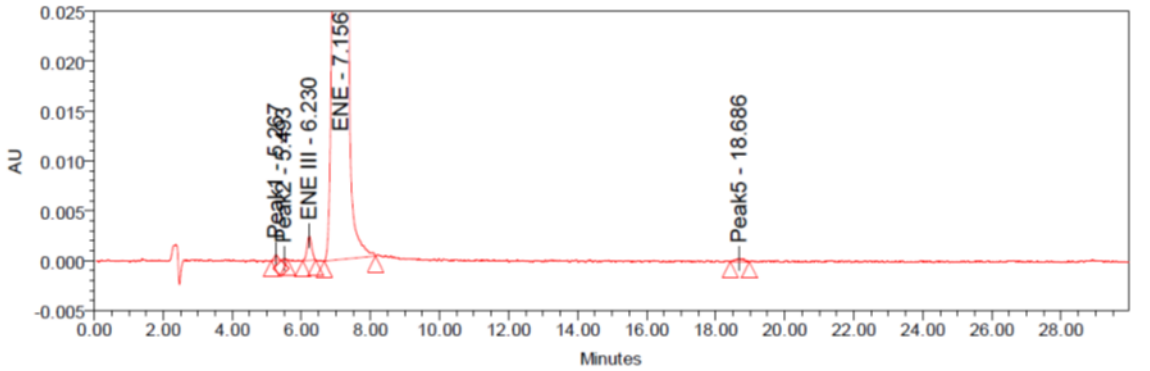

Fig 9: Photo degradation chromatogram

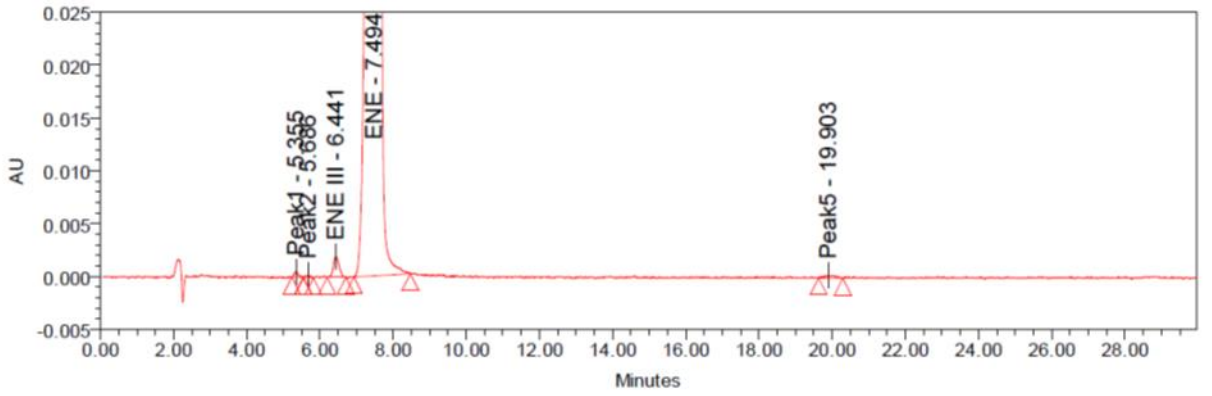

Fig 10: Thermal degradation chromatogram

Linearity and Range

Linearity of the method was evaluated by determining nine concentration levels from LOQ to 200% of 500 µg ml^{-1} analyte concentration. The correlation coefficient obtained for Entecavir was 0.999. The best-fit linear equation obtained for Entecavir was y = 458908x + 1036.13. The correlation coefficient obtained for Imp-1, Imp-2 and Imp-3 were greater than 0.999. The best-fit linear equation obtained for imp-1 was y = 272349x-156.41, for imp-2 was y = 427959x-313.01 and for imp-3 was y = 342134x-101.08.The results of linearity shown in Table 3 – 6. The linearity graphs of Imp-1, Imp-2 and Imp-3 and Entecavir shown in Fig. 11 – 14.

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TABLE 3: LINEARITY OF ENTECAVIR

Sample No.	% Level	Concentration (% w.r.t. analyte concentration)	Mean Peak Area Response
1	QL	0.021	10629
2	50	0.049	24420
3	80	0.078	35847
4	100	0.097	45280
5	120	0.117	54681
6	150	0.146	68485
Slope		458908	
Y-intercept		1036.13	
Correlation Co-efficient square (r^2)		0.9991	
Residual sum of squares		2051636	

TABLE 4: LINEARITY OF Imp-1 (ENE-III)

Sample No.	% Level	Concentration (% w.r.t. analyte concentration)	Mean Peak Area Response
1	QL	0.009	2320
2	50	0.072	19519
3	80	0.115	31010
4	100	0.144	39046
5	120	0.173	47004
6	150	0.216	58705
Slope		272349	
Y-intercept		-156.41	
Correlation co-efficient square (r^2)		1.0000	
Residual sum of squares		32011	

TABLE 5: LINEARITY OF Imp-2 (ENE-IV)

Sample No.	% Level	Concentration (% w.r.t. analyte concentration)	Mean Peak Area Response
1	QL	0.008	3415
2	50	0.068	28504
3	80	0.108	45594
4	100	0.135	57488
5	120	0.162	69185
6	150	0.203	86660
Slope		427959	
Y-intercept		-313.01	
Correlation co-efficient square (r^2)		0.9999	
Residual sum of squares		309709	

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TABLE 6: LINEARITY OF Imp-3 (ENE-II)

Sample No.	% Level	Concentration (% w.r.t. analyte concentration)	Mean Peak Area Response
1	QL	0.009	3066
2	50	0.075	25476
3	80	0.120	40724
4	100	0.150	51329
5	120	0.180	61691
6	150	0.226	77129
Slope		427959	
Y-intercept		-313.01	
Correlation co-efficient square (r^2)		0.9999	
Residual sum of squares		309709	

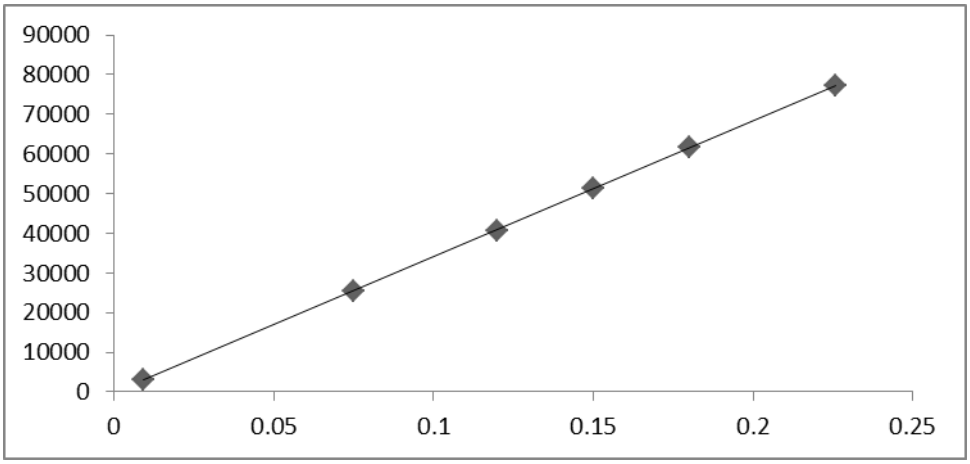

Fig 11: Linearity graph of Imp-1 (ENE-III)

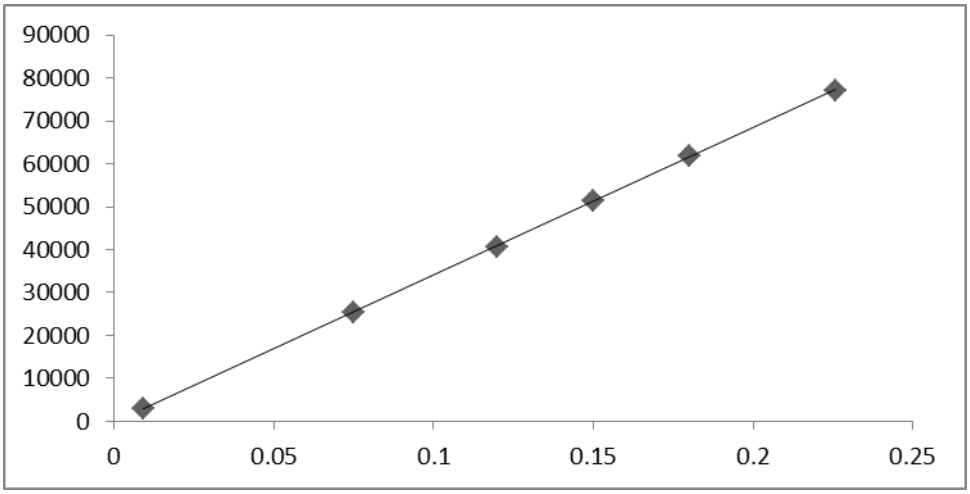

Fig 12: Linearity graph of Imp-2 (ENE-IV)

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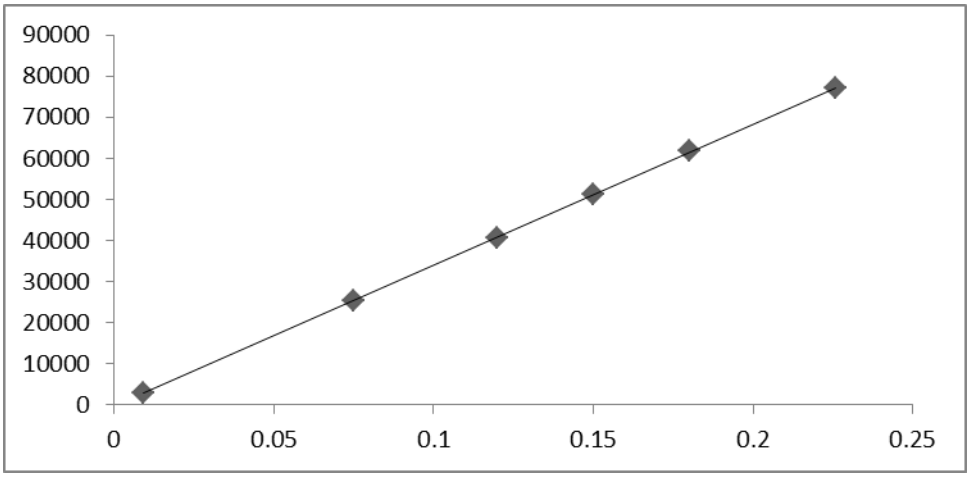

Fig 13: Linearity graph of Imp-3 (ENE-II)

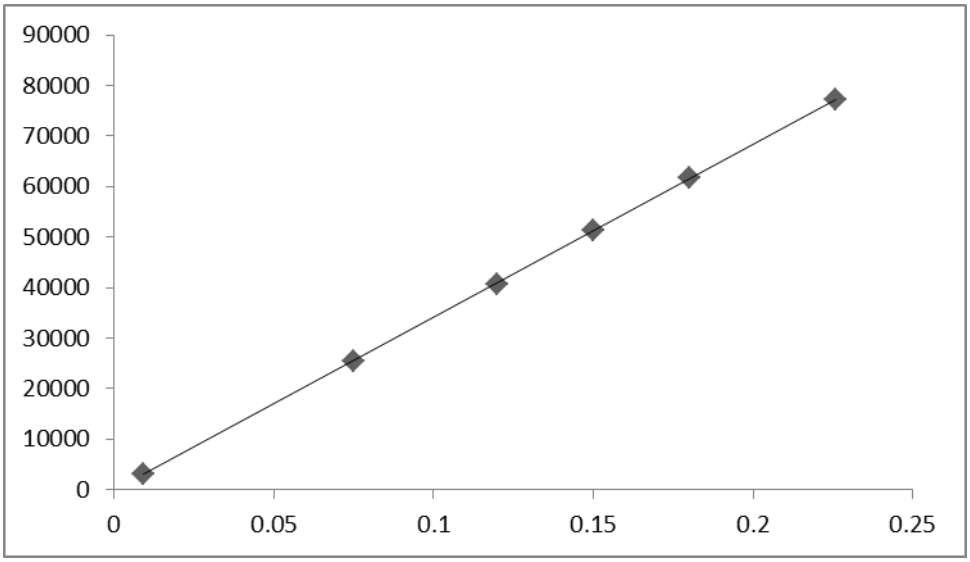

Fig 14: Linearity graph of Entecavir (ENE)

Precision

The precision of the diastereomeric impurities was checked by injecting six individual preparations of (3.75 µg ml^{-1}) Entecavir spiked with 0.15% of each impurity. The % RSD for percentage of imp-1, imp-2, imp-3 and Entecavir was below 0.4%. In the intermediate precision, the % RSD for imp was within 0.8%. The intermediate precision of the method was evaluated by different analyst, column and by using different instrument, % RSD's were within 1.8%, confirming the ruggedness of the method.

Sensitivity

Sensitivity was determined by establishing the LOD and LOQ for imp-1, imp-2, imp-3 and Entecavir estimated at a signal to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration. The limit of detection for imp-1, imp-2, imp-3 and Entecavir were 0.002%, 0.002%, 0.003% and 0.008% for 20 µL injection volume. The limit of quantification for imp-1, imp-2, imp-3 and Entecavir were 0.007%, 0.006%, 0.008% and 0.025% respectively for 20 µL injection volume. The precision study at the LOQ level performed. The % RSD for the areas of each impurity was within 1.1%. Thus, the method was found to be highly sensitive.

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Accuracy

Recovery study was carried out in triplicate at 50, 100 and 150% of the analyte concentration (500 µg ml^{-1}). The percentage of recovery for imp-1, imp-2, imp-3 and Entecavir was 101.3 to 105.1. The percentage recovery of Entecavir in bulk drug samples ranged from 99.2 to 101.5 and in pharmaceutical dosage forms ranged from 98.8 to 101.1%., indicated that the method was suitable for the determination of diastereomeric impurities in drug substances and drug product.

Robustness

The robustness study were performed using the Minitab 17 software using the 6 as number factors viz., flow, column oven temperature, mobile phase organic ratio, particle size of the packing material, length of the column and wavelength. By change in experimental conditions in the Table 2, the resolution between Entecavir, imp-1, imp-2 and imp-3 was evaluated. The flow rate of the mobile phase was 1.0 ml min^{-1}. To study the effect of flow rate on the resolution, 0.1 units changed it from0.9 to 1.1ml min^{-1}. The effect of column temperature on resolution was studied at 35°Cand 45°C instead of 40 °C. The effect of mobile phase organic ratio, 10% v/v changed it from 45 ml to 55 ml of acetonitrile. The effect of particle size of the packing material, 3.5 µ changed it from 3.0 µ to 5.0 µ. The effect of length of the column, 150 mm changed it from 100 mm to 250 mm. The effect of wavelength of detection, 254 nm changed it from 252 nm to 256 nm. In all the deliberate varied chromatographic conditions carried out (flow rate, column oven temperature, mobile phase organic ratio, particle size of the packing material, length of the column and wavelength of detection), the resolution between closely eluting impurities, namely imp-1, imp-2 peaks and Entecavir, imp-3 was greater than 1.5, illustrating the robustness of the method.

TABLE 2: DESIGN OF EXPERIMENTS FOR ROBUSTNESS STUDY

Experiment	Flow (mL/min)	Column oven temperature (°C)	Acetonitrile (% v/v)	Particle size (µm)	Length of the column (mm)	Wavelength (nm)
1	0.9	45	45	5	250	252
2	1.1	45	55	3	250	256
3	1.1	45	55	5	250	256
4	0.9	35	55	5	250	256
5	1.1	35	55	3	100	256
6	1.1	35	55	5	250	252
7	1.1	45	45	5	250	252
8	0.9	45	55	3	250	252
9	0.9	45	55	5	100	252
10	1.1	35	55	5	100	252
11	0.9	45	55	5	100	256
12	0.9	35	45	5	100	252
13	0.9	35	45	3	100	252
14	0.9	45	45	3	100	256
15	0.9	35	55	3	100	256
16	1.1	45	55	3	100	252
17	1.1	35	45	3	250	252
18	1.1	45	45	3	100	252
19	0.9	45	45	3	250	256
20	1.1	35	45	5	100	256
21	1.1	35	45	3	250	256
22	1.1	45	45	5	100	256
23	0.9	35	45	5	250	256
24	0.9	35	55	3	250	252

Using the Minitab 17 software with the 6 as number factors - DOE

Solution Stability

The solution stability of Entecavir and its diastereomeric impurities was carried out by leaving spiked sample solution in a tightly capped volumetric flask at room temperature for 48 hours. The mobile phase stability was also carried out by freshly prepared sample solutions against freshly prepared reference standard solutions at 48 hours. The %RSD of Entecavir during solution stability and mobile phase stability experiments was within 1.1%. No significant change was observed in the content of imp-1, imp-2 and imp-3 during solution stability and mobile phase stability experiments. The data confirms that standard and sample solutions were stable upto 48 hours.

Numerous methods are available [1-12] for the determination of Entecavir by several techniques like, HPLC, UV, LCMS, etc. All the methods have determined only the LOD, LOQ, % recovery and linearity of Entecavir and not the diastereomeric impurities of Entecavir. The data mentioned in the Table 1 for comparison of different techniques. But the present method describes about the diastereomeric impurities of Entecavir and Entecavir in both bulk drug substances and drug products. All the methods describe the Entecavir as the analyte.

CONCLUSION

A new, sensitive and stability-indicating RP-LC method was successfully developed for quantitative determination of diastereomeric impurities of Entecavir in both bulk drug and pharmaceutical dosage forms. The method was found to be accurate and precise with good and consistent recoveries. The validated method may be used for the routine analysis of determination of diastereomeric impurities of Entecavir from bulk drug, pharmaceutical preparation and other quality control samples of product development.

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