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- 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-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylidencyclopentyl]-6,9-dihydro-3H-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 Entecav® 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 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 Agilent HC-C18 (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 nm 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 (Contec®).

**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 µg/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 µg/mL Entecavir).

**Preparation of mobile phase**

Prepare a filtered and degassed mixture of Buffer and Acetonitrile in the ratio 95:5 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 μm 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 λmax 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 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, 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 ± (% 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 |          |       |
|                  |               | 401220 |          |       |
| 80               | 8.28          | 400814 | 401015   | 0.1   |
|                  |               | 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 |          |       |
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 ±2% which indicated good accuracy of the developed method.

Table 3: 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             | 109038 | 0.00212                 | 98.0          |      |       |
|           | 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          |      |       |
| 50        | 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          |      |       |
| 80        | 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          |      |       |
| 100       | 0.01293             | 655303 | 0.01272                 | 98.4          | 98.4 | 0.1   |
|           | 0.01293             | 655152 | 0.01271                 | 98.3          |      |       |
|           | 0.01293             | 655715 | 0.01272                 | 98.4          |      |       |
| 120       | 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 er.

| 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 an 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 compared 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 more light on these studies.

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REFERENCES
1. Beale, J. M., Block, J. H. Wilson and Gisvold’s Textbook of Organic Medicinal and Pharmaceutical Chemistry. Philadelphia: Lippincott Williams and Wilkins, 2011.
2. Brunton, L., Parker, K., Blumenthal, D., Buxton, I. Goodman & Gilman’s Manual of Pharmacology and Therapeutics. New York: The McGraw-Hill, 2008.
3. Satyanarayana, L., Naidu, S. V., Narasimha Rao, M., Rishi Priya, L., & Suresh, K. The Estimation of Entecavir in Tablet Dosage Form by RP-HPLC. Research Journal of Pharmacy and Technology, 2011; 4(11): 1699-1701.
4. Appala, R. N., Rao, J. V., Prakash, K. V., Mukkanti, K., & Srinivasu, K. Estimation of Entecavir in Tablet Dosage Form by RP-HPLC. Asian Journal of Chemistry, 2009; 21(3): 2317.
5. Balasekhar Reddy, C., Awen, B. Z., Chandu, B. R., & Rihanaparveen, S. LC–ESI-MS/MS method for the quantification of entecavir in human plasma and its application to bioequivalence study. Journal of Chromatography B, 2011; 879(11): 769-776.