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ESTIMATION OF ENTECAVIR IN PURE AND PHARMACEUTICAL FORMULATION BY USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract:

A rapid and precise Reverse Phase High Performance Liquid Chromatographic method has been developed for the validated of Entecavir, in its pure form as well as in tablet dosage form. Chromatography was carried out on a Phenomenex Gemini C18 (4.6 x 250mm, 5µm) column using a mixture of Acetonitrile and Water (15:85% v/v) as the mobile phase at a flow rate of 0.9ml/min, the detection was carried out at 215nm. The retention time of the Entecavir was 3.1 ±0.02min. The method produce linear responses in the concentration range of 30-150µg/ml of Entecavir. The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

Keywords: *Entecavir, RP-HPLC, validation.*

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INTRODUCTION:

HPLC is also called as high pressure liquid chromatography since high pressure is used to increase the flow rate and efficient separation by forcing the mobile phase through at much higher rate. The pressure is applied using a pumping system. The development of HPLC from classical column chromatography can be attributed to the development of smaller particle sizes. Smaller particle size is important since they offer more surface area over the conventional large particle sizes [1]. The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages.

1. Improved resolution of separated substances
2. column packing with very small (3,5 and 10 µm) particles
3. Faster separation times (minutes)
4. Sensitivity
5. Reproducibility
6. continuous flow detectors capable of handling small flow rates
7. Easy sample recovery, handling and maintenance [2].

Types of HPLC Techniques

Based on Modes of Chromatography

These distinctions are based on relative polarities of stationary and mobile phases

Reverse phase chromatography: In this the stationary phase is non-polar and mobile phase is polar. In this technique the polar compounds are eluted first and non polar compounds are retained in the column and eluted slowly. Therefore it is widely used technique.

Normal phase chromatography: In this the stationary phase is polar and mobile phase is non-polar. In this technique least polar compounds travel faster and are eluted first where as the polar compounds are retained in the column for longer time and eluted [3,4].

Based on Principle of Separation

Liquid/solid chromatography (Adsorption): LSC, also called adsorption chromatography, the principle involved in this technique is adsorption of the components onto stationary phase when the sample solution is dissolved in mobile phase and passed through a column of stationary phase. The basis for separation is the selective adsorption of polar compounds; analytes that are more polar will be attracted more strongly to the active silica gel sites. The solvent strength of the mobile phase determines the rate at which adsorbed analytes are desorbed and elute. It is widely used for separation of isomers and classes of compounds differing in polarity and number of functional groups. It works best with compounds that have relatively low or intermediate polarity [3].

Liquid/Liquid chromatography (Partition Chromatography): LLC, also called partition chromatography, involves a solid support, usually silica gel or kieselguhr, mechanically coated with a film of an organic liquid. A typical system for NP LLC column is coated with ß, ß'-oxy dipropionitrile and a non-polar solvent like hexane as the mobile phase. Analytes are separated by partitioning between the two phases as in solvent extraction. Components more soluble in the stationary liquid move more slowly and elute later [5,6].

Ion exchange: In this the components are separated by exchange of ions between an ion exchange resin stationary phase and a mobile electrolyte phase. A cation exchange resin is used for the separation of cations and anion exchange resin is used to separate a mixture of anions [7-10]

Size exclusion: In this type, the components of sample are separated according to their molecular sizes by using different gels (polyvinyl acetate gel, agarose gel). ex: separation of proteins, polysaccharides, enzymes and synthetic polymers [11-15].

Chiral chromatography: In this type of chromatography optical isomers are separated by using chiral stationary phase.

Affinity chromatography: In this type, the components are separated by an equilibrium between a macromolecular and a small molecule for which it has a high biological specificity and hence affinity [16].

Based on elution technique

Isocratic separation: In this technique, the same mobile phase combination is used throughout the process of separation. The same polarity or elution strength is maintained throughout the process.

Gradient separation: In this technique, a mobile phase combination of lower polarity or elution strength is followed by gradually increasing polarity or elution strength [3].

Based on the scale of operation

Analytical HPLC: Where only analysis of samples are done. Recovery of samples for reusing is normally not done, since the sample used is very low. Ex: µg quantities.

Preparative HPLC: Where the individual fractions of pure compounds can be collected using fraction collector. The collected samples are reused. Ex: separation of few grams of mixtures by HPLC [17].

Based on type of analysis

Qualitative analysis: Which is used to identify the compound,detect the presence of impurities to find out the number of components. This is done by using retention time values.

Quantitative analysis: This is done to determine the quantity of individual or several components of mixture. This is done by comparing the peak area of the standard and sample [18-24].

The basic liquid chromatograph consists of six basic units. The mobile phase supply system, the pump and programmer, the sample valve, the column, the detector and finally a means of presenting and processing the results.

Mobile phase (solvent) reservoirs and solvent degassing The mobile phase supply system consists of number of reservoirs (200 mL to 1,000 mL in capacity). They are usually constructed of glass or stainless steel materials which are chemically resistant to mobile phase**.**

Mobile phase

Mobile phases in HPLC are usually mixtures of two or more individual solvents. The usual approach is to choose what appears to be the most appropriate column, and then to design a mobile phase that will optimize the retention and selectivity of the system. The two most critical parameters for nonionic mobile phases are strength and selectivity [8,24].

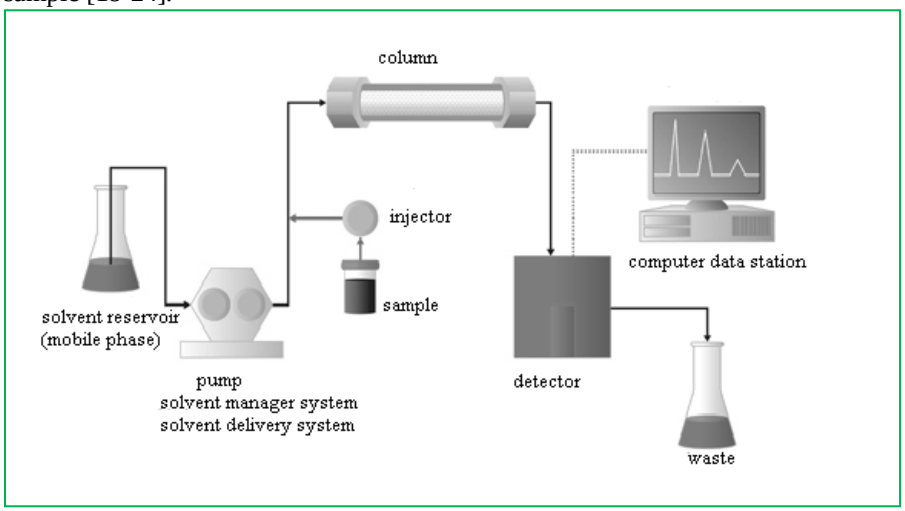

Fig.1: Components of HPLC instrument block diagram. [22]

Mobile phase preparation

Mobile phases must be prepared from high purity solvents, including water that must be highly purified. Mobile phases must be filtered through ≤1 µm pore size filters and be degassed before use.

Entecavir 2 - amino-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylidenecyclopentyl]-6,9-dihydro-3H-purin-6-one. For the treatment of chronic hepatitis B virus infection in adults with evidence of active viral replication and either evidence of persistent elevations in serum aminotransferases (ALT or AST) or histologically active disease.

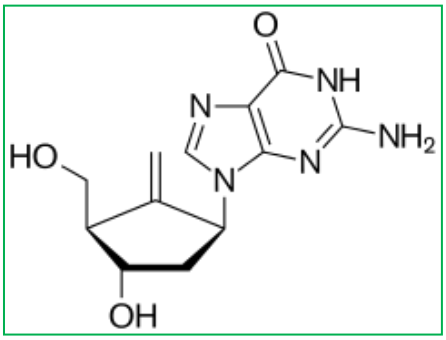

Fig. 2: chemical structure of Entecavir

MATERIALS AND METHODS:

Accurately weigh and transfer 10 mg of Entecavir working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Instrumentation and Chromatographic conditions

The analysis was performed by using Phenomenex Gemini C18 column, 4.6×250mm internal diameter with 5 micron particle size column and UV detector set at 215nm nm, in conjunction with a mobile phase of Acetonitrile:Water in the ratio of 15:85 v/v (pH 5 adjusted with OPA) at a flow rate of 0.9 ml/min. The retention time of Entecavir was found to be 3.144minute. The 10µl of sample solution was injected into the system

Preparation of standard solution:

Accurately weigh and transfer 10 mg of Entecavir working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.9ml of the above Entecavir stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol: Water, Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Acetonitrile:Water in proportion 15:85 v/v respectively.

Optimization of Column:

The method was performed with column like Phenomenex Gemini C18 (4.6×250mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 0.9ml/min flow

(Optimized chromatogram):

Column : Phenomenex Gemini C18 (4.6×250mm) 5µ
Column temperature: Ambient
Wavelength : 215nm
Mobile phase ratio: Acetonitrile:Water(15:85 v/v)
Flow rate : 0.9ml/min
Injection volume : 10µl
Run time : 6minutes

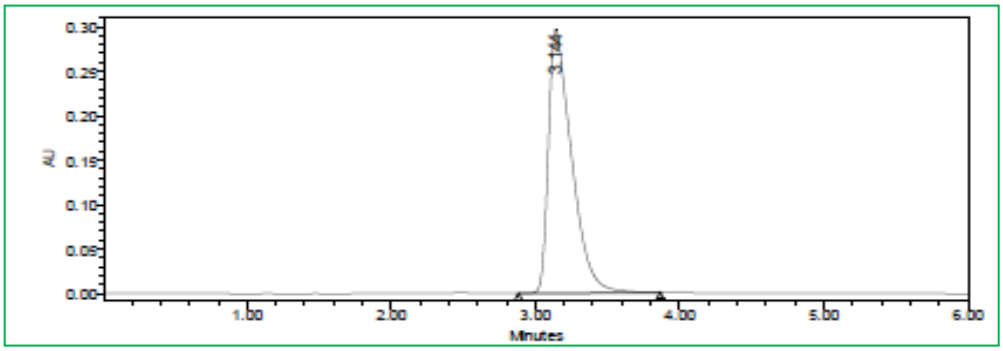

Fig. 3: Typical chromatogram of mixture of Standard solution.

VALIDATION

PREPARATION OF MOBILE PHASE:

Preparation of mobile phase:

Accurately measured 850ml (85%) of HPLC Water and 150ml (15%) of HPLC Acetonitrile in to a 1000ml of volumetric flask and degassed in a digital ultrasonicator for 10 minutes.

Diluent Preparation:

The Mobile phase was used as the diluent.

Linearity

The linearity of was obtained in the concentration ranges from 30-150 µg/ml

Table 1: Linearity data of Entecavir

Concentration Level (%)	Concentration µg/ml
60	30
80	60
100	90
120	120
140	150

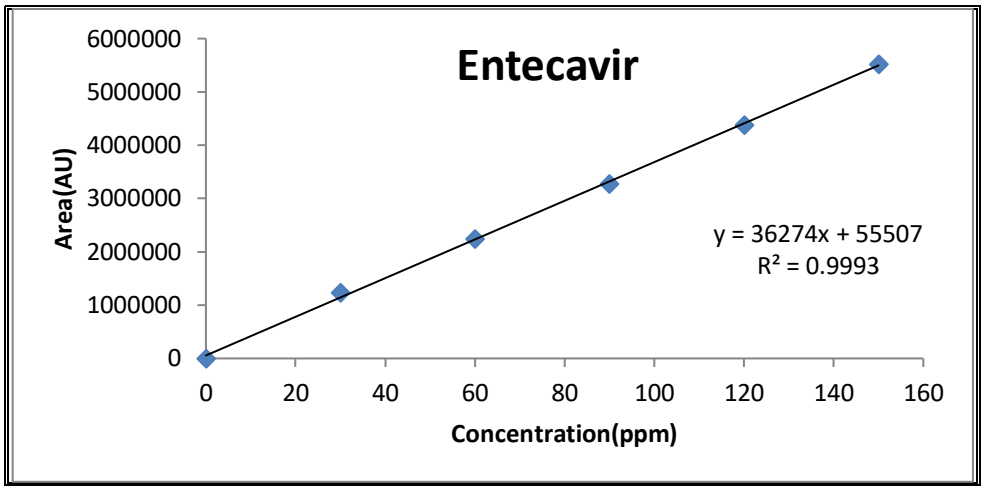

Fig.4: Calibration graph of Entecavir

LINEARITY PLOT

Linearity of detector response of assay method was found by injecting seven standard solutions with concentration ranging from 60-140 µg/mL for Entecavir. The graph was plotted for concentration versus peak area. The results were shown in Table-1 and fig 4.

Precision

Repeatability

The precision of test method was determined by preparing six test preparations using the product blend and by mixing the active ingredient with excipients as per manufacturing formula. And the relative standard deviation of assay results was calculated. The results were shown in Table 2

Table 2: Results of repeatability for Entecavir

S. No	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Entecavir	3.146	332245	298209.0	9961	1.26
2	Entecavir	3.144	335716	300291.0	8161	1.1
3	Entecavir	3.144	336456	300996.9	8297	1.22
4	Entecavir	3.143	331847	302799.8	6816	1.11
5	Entecavir	3.146	337437	297067.0	5038	1.1
Mean			334740.2			
Std.dev			2537.987			
%RSD			0.758196			

Accuracy

Entecavir tablets content were taken at various concentrations ranging from 50 % to 150 % (50 %, 75 %, 100 %, 125 %, and 150 %) to accurately quantify and to validate the accuracy. The assay was performed in triplicate. The results were shown in Table-3

Table 3: The accuracy results for Entecavir

%Concentration (at specification Level)	Peak area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	3992721	45	44.8	99.8	99.8
100%	6254827	90	89.8	99.5	
150%	9381167	135	134.9	99.8	

LIMIT OF DETECTION (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.The LOD value for Entecavir 4.7 µg/ml.

Quantitation limit (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined. The LOQ values for Entecavir 14.3 µg/ml

ROBUSTNESS

The robustness was performed for the flow rate 0.9 ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Entecavir. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The standard sample of Entecavir were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor and plate count. Table 4

Table 4: Results for Robustness of Entecavir

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 0.9mL/min	381656	3.158	5857	1.12
Less Flow rate of 0.8mL/min	381641	3.526	7673	1.25
More Flow rate of 1.0mL/min	381645	2.035	8947	1.5
Less organic phase (about 5 % decrease in organic phase)	385663	3.528	9947	1.1
More organic phase (about 5 % Increase in organic phase)	389467	2.004	8576	1.6

SUMMARY AND CONCLUSION:

The analytical method was developed by studying different parameters.First of all, maximum absorbance was found to be at 215nm and the peak purity was excellent. Injection volume was selected to be 10µl which gave a good peak area. The column used for study was Phenomenex Gemini C_{18} because it was giving good peak.35ºC temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time. Mobile phase is Water: Acetonitrile (85:15% v/v) was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study. In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Entecavir in bulk drug and pharmaceutical dosage forms. This method was simple, since diluted samples are directly used without any preliminary chemical derivatisation or purification steps. Entecavir was freely soluble in acetonitrile ethanol, methanol and sparingly soluble in water. Water: Acetonitrile (85:15% v/v) was chosen as the mobile phase. The solvent system used in this method was economical.

Table 5:Summary data for Entecavir

Parameters	Entecavir
Retention Time (min.)	3.144
Linearity (µg/ml)	3-150
Correlation Coefficient (r^2)	0.999
Slope	36274
Y - intercept	55507
LOD (µg/ml)	4
LOQ (µg/ml)	14.3
Repeatability (% RSD) n=6	0.758196
Intraday Precision (% RSD) n=6	0.543924
Interday Precision (% RSD) n=6	0.818445
Accuracy (%)	99.8

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