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ORIGINAL ARTICLE

Method Development and Validation for the Simultaneous Quantification of Elvetigravir and Cobicistat in Bulk and Dosage Form

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ABSTRACT
*A new method was established for simultaneous estimation of Elvetigravir and Cobicistat by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Elvetigravir and Cobicistat by using an Xterra C18 5µm (4.6*150 mm) column with a flow rate was 1 ml/min, mobile phase ratio of phosphate buffer (0.05M) pH 7: MEOH (30:70%v/v) (pH was adjusted with orthophosphoric acid), and the detection wavelength was 270 nm. A WATERS HPLC Auto Sampler, Separation module 2695, PDA Detector 996, Empower-software version-2.The retention times were 2.399 and 3.907 min, respectively. The % purities of Elvetigravir and Cobicistat were found to be 100.7% and 101.4%, respectively. The system suitability parameters for Elvetigravir and Cobicistat, such as the theoretical plates and tailing factor, were found to be 1.3, 5117.5, and 1.4, 3877.3, and 8.0, respectively. The analytical method was validated according to the ICH guidelines (ICH, Q2 (R1)). The linearity study for Elvetigravir and Cobicistat was found in the concentration range of 10µg-50µg and 20µg-100µg and the correlation coefficient (r2) was found to be 0.997 and 0.997, the% mean recovery was found to be 100% and 100.5%, respectively, and the%RSD for repeatability was0.2 and 0.4 % RSD for intermediate precision was 0.5 and 0.1%, respectively. The precision study was precise, robust, and reproducible. LOD value was 2.95 and 3.04, and LOQ value of 9.87 and 10, respectively. Hence, the suggested RP-HPLC method can be used for routine analysis of Elvetigravir and Cobicistat in API and Pharmaceutical dosage forms.*
KEY WORDS: *Elvitegravir , Cobicistat, RP-HPLC, Method validation.*

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INTRODUCTION

High-performance liquid chromatography (HPLC) is the fastest growing analytical technique for analysis of drugs. Its simplicity, high specificity, and wide range of sensitivity make it ideal for the analysis of many drugs in both dosage forms and biological fluids. High-performance liquid chromatography (HPLC) is a term used to describe liquid chromatography, in which the liquid mobile phase is forced through the column at high speed, and the analysis time is reduced by 1-2 orders of magnitude relative to classical column chromatography. The use of much smaller particles of the adsorbent or support increases the column efficiency substantially, and the importance of chromatography is increasing rapidly in pharmaceutical analysis for the exact differentiation, selective identification, and quantitative determination of structurally closely related compounds. Another important field of application of chromatographic methods is purity testing of the final products and intermediates. The reasons for the popularity of this method are its sensitivity, ready adaptability to accurate quantitative determinations, suitability for separating non-volatile or thermally fragile species, and widespread applicability to substances that are of prime interest to the industry. [1-6]

Cobicistat is chemically as 1,3-thiazol-5-ylmethyl N- [(2R,5R)-5-[[(2S)-2-[[methyl [(2propa2-yl-1, 3-thiazol- 4-yl) methyl] carbamoyl] amino]-4 morpholin-4-yl butanoyl] amino]-1, 6- iphenylhexan-2-yl] Carbamate**,** is a mechanism-based inhibitor of cytochrome P450 3A (CYP3A) isoforms. Inhibition of CYP3A-mediated metabolism by cobicistat increases the systemic exposure of CYP3A substrates Atazanavir and Darunavir, thereby enabling increased anti-viral activity at a lower dosage. Cobicistat does not have any anti-HIV activity on its own.[7] Elvitegravir is chemically as**:** 6-(3-Chloro-2-fluorobenzyl)-1-[(2S)-1-hydroxy 3methylbutan-2yl] 7- methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid, an HIV-1 integrase strand transfer inhibitor (INSTI).Integrase is an HIV-1 encoded enzyme that is required for viral replication inhibition of integrase prevents the integration of HIV-1 DNA into host genomic DNA, blocking the Formation of the HIV-1 provirus and propagation of the viral infection. Elvitegravir does not inhibit human topoisomerases. [8] In the present work, attempts were made to develop an analytical method for the simultaneous estimation of Cobicistat and Elvitegravir pharmaceutical formulations by RP the RP-HPLC method.

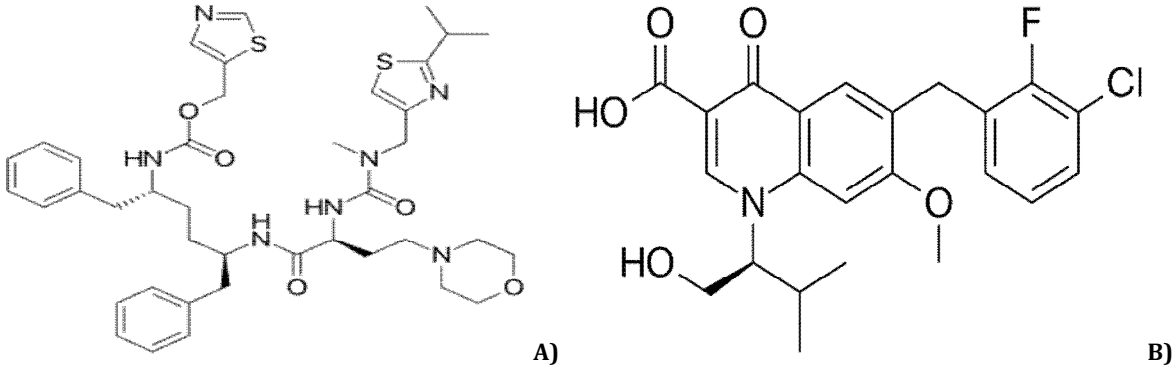

Fig. 1: Chemical Structures of (A) Cobicistat and (B) Elvitegravir.

MATERIAL AND METHODS

Materials and Chemicals

HPLC grade water, methanol, acetonitrile and analytical grade potassium dihydrogen were obtained from Merck, India. Elvetigravir and cobicistat reference compounds acquired from Hetero drugs, Hyderabad, India.

Instruments

The instruments used in this study are listed in Table 1.

Table 1: List of Equipment's

S.No.	Instrument	Model No.	Software	Manufacturer's name
1	HPLC Alliance PDA Detector	Waters 2695 Waters 996	Empower	Waters
2	UV double beam spectrophotometer	UV 3000	UV Win 5	Lab India
3	Digital weighing balance	BSA224SCW	-	Satorius
4	pH meter	AD102U	-	Lab India
5	Ultra sonicator	SE60US	-	-
6	Suction pump	VE115N	-	-

Optimized chromatographic conditions:

Chromatographic conditions:

Column : Xterra C18 5µm (4.6*150mm)
Mobile phase ratio : Phosphate buffer (0.05M) pH 7: MEOH (30:70%v/v)
Detection wavelength : 270nm
Flow rate : 1ml/min
Injection volume : 10µl
Column temperature : Ambient
Auto sampler temperature : Ambient

Preparation of Phosphate buffer (PH: 7):
Weighed 0.50 grams of KH_2PO_4 and potassium dihydrogen phosphate (0.301 g) were placed in a 1000 ml beaker, dissolved, and diluted to 1000 ml with HPLC water, and the pH was adjusted to 7 with orthophosphoric acid.
Preparation of mobile phase:
A mixture of pH 7 Phosphate buffer 300 mL (pH 7, 30%) and 700 mL MEOH (70%) was degassed in an ultrasonic water bath for 5 min. This solution was filtered through 0.45 µ filter under vacuum filtration.
Diluent Preparation:
The mobile phase was used as a diluent.
Preparation of the individual Elvetigravir standard preparation:
10mg of Elvetigravir working standard was accurately weighed and transferred into a A clean dry volumetric flask (10 mL) and DMF (2 ml of DMF were then added. Then, it was sonicated to dissolve it completely and volume-up to the mark with the diluent. (Stock solution). Further 10.0 ml of the above stock solution was pipetted into a 100 ml volumetric flask and diluted to the mark with diluent.
Preparation of individual Cobicistat standards
10mg of Cobicistat working standard was accurately weighed and transferred into a
A clean dry volumetric flask (10 mL) and DMF (2 ml of DMF were then added. Then, it was sonicated to dissolve it completely and volume-up to the mark with the diluent. (Stock solution). Further 10.0 ml from the above stock solution was pipetted into a 100 ml volumetric flask was dilution upto the mark with diluent.
Preparation of Sample Solution :(Tablet)
Accurately 10 tablets are weighed and crushed in mortar and pestle and weight equivalent to 10 mg of Cobicistat and Elvetigravir (marketed formulation) sample into a 10mL clean dry volumetric flask and about 7mL of Diluents is added and sonicated to dissolve it completely and made volume upto the mark with the same solvent. (Stock solution) Further 3 ml of above stock solution was pipetted into a10ml volumetric flask and diluted upto the mark with diluent.
Method validation
Developed method was validated as per the ICH Q2R1 guidelines.

RESULTS AND DISCUSSION
METHOD development
Selection of Detection wavelength
10 mg of Elvetigravir and Cobicistat was dissolved in mobile phase. The solution was scanned from 200-400 nm the spectrum was obtained[19]. The overlay spectrum was used for selection of wavelength for Elvetigravir and Cobicistat. The isobestic point was taken as detection wavelength (Fig. 2).

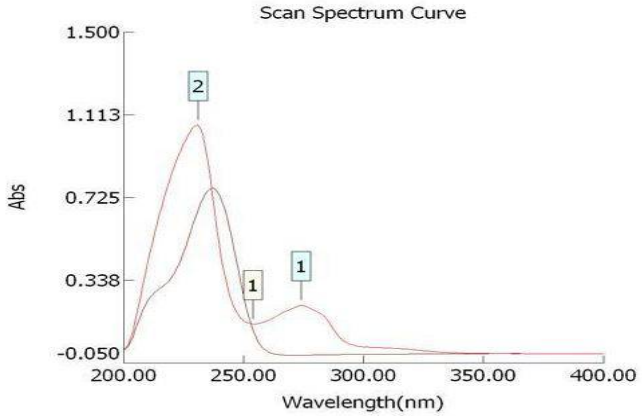

Fig. 2: Overlay spectrum of Cobicistat and Elvetigravir

Selection of column
Column is selected based on solubility, polarity and chemical differences among Analytes [Column: xterra C18 (4.6 x 150mm, 5µm, Make: Waters)]

Selection of mobile phase

Phosphate buffer (0.05M) pH 7: MEOH (30:70%v/v) has been selected as mobile phase. Buffer pH should be between 2 to 8. If the buffer pH is below 2 siloxane linkages are cleaved. If the buffer pH is above 8 dissolution of silica takes place. pH controls the elution properties by controlling the ionization characteristics. It also decreases the retention and improves separation. Good Response, Area, Tailing factor, Resolution will be achieved.

Selection of flow rate:

Flow rate selected was 1ml/min. It was selected based on retention time, column back pressure, peak symmetry and separation of impurities.

System Suitability:

Tailing factor for the peaks due to Cobicistat and Elvetigravir in Standard solution should not be more than 2.0. Theoretical plates for the Cobicistat and Elvetigravir peaks in Standard solution should not be less than 2000.

Optimization of the method

The chromatographic conditions were successfully developed for the separation of Elvetigravir and Cobicistat by using an Xterra C18 5µm (4.6*150 mm) column with a flow rate was 1 ml/min, mobile phase ratio of phosphate buffer (0.05M) pH 7: MEOH (30:70%v/v) (pH was adjusted with orthophosphoric acid, d and the detection w wave length was 2270 nm(Fig. 3).

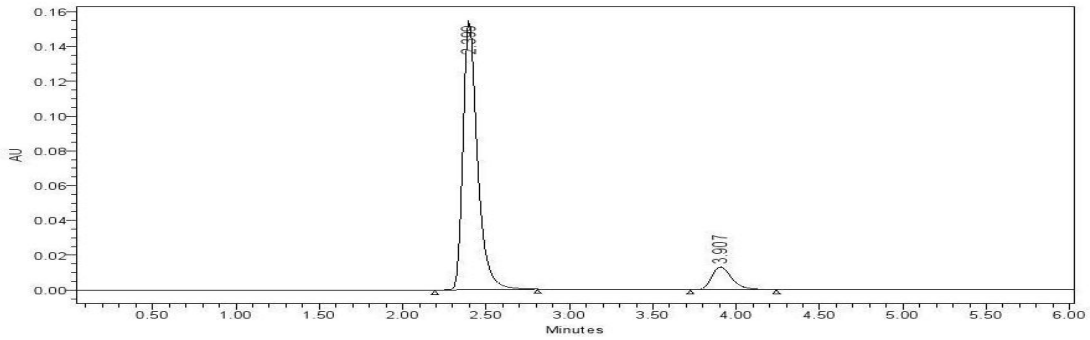

Fig. 3. Chromatogram of Optimized method

Assay calculation:

$$Assay\,\% = \frac{sample\ area}{Standard\ area} \times \frac{dilution\ sample}{dilution\ of\ standard} \times \frac{P}{100} \times \frac{Avg.wt}{Lc} \times 100$$

Where, P is Percentage purity of working standard and Lc means label claim of drug in mg/ml. The system suitability parameters for Cobicistat and Elvetigravir such as theoretical plates and tailing factor were found to be 5117.5, 1.3 and 3877.3, 1.4. Resolution was8.1. The % purity of Cobicistat and Elvetigravir in pharmaceutical dosage form was found to be 100.7% and 101.4% respectively.

METHOD VALIDATION

Accuracy:

Preparation of standard solution (Elvetigravir and Cobicistat):

Accurately weighed 10 mg of Cobicistat and 10mg of Elvetigravir working standard were transferred into a 10mL and 100ml of clean dry volumetric flasks [9-12].

About 7mL and 70ml of Diluents are added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further 3ml and 0.3ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted upto the mark with diluents.

Preparation of Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration):

Accurately 5mg of Cobicistat and 5mg of Elvetigravir working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock Solution). Further 3ml and 0.3ml of the above Cobicistat and Elvetigravir stock solution were pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

***For preparation of 100% solution (With respect to target Assay concentration)*:**

Accurately 10mg of Cobicistat and 10mg of Elvetigravir working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was

added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock Solution). Further 3ml and 0.3ml of the above Cobicistat and Elvetigravir stock solution were pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

For preparation of 150% solution (With respect to target Assay concentration):

Accurately 15mg of Cobicistat and 15mg of Elvetigravir working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock Solution). Further 3ml and 0.3ml of the above Cobicistat and Elvetigravir stock solution were pipetted into a 10ml volumetric flask and diluted up to the mark with diluent.

Procedure:

The standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions were injected. The Amount found and Amount added for Cobicistat & Elvetigravir and the individual recovery and mean recovery values were calculated. Correlation coefficient should be not less than 0.999 (Table 2 and 3).

Table 2: Accuracy results of Elvetigravir

% Concentration (at specification Level)	Area	Amount added (mg)	Amount found (mg)	% Recovery	Mean Recovery
50%	2332744	5	5.10	101.8%	100.5%
100%	3132697	10	9.99	99.9%	
150%	3918997	15	14.9	99.1%	

Table 3: Accuracy results of Cobicistat

% Concentration (at specification level)	Area	Amount Added(mg)	Amount Found(mg)	% Recovery	Mean Recovery
50%	353867	5	5.0	101.3%	100.0%
100%	4735088	10	9.94	99.4%	
150%	5911798	15	14.8	99.2%	

Precision

A) Repeatability:

Preparation of standard stock solution:

Accurately 10 mg of Cobicistat and 10mg of Elvetigravir working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flasks and about 7mL and 70ml of Diluent was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further it was pipette (3ml and 0.3ml) into a 10ml volumetric flask and diluted up to the mark with diluents.

Procedure:

The standard solution was injected for five times and the areas for all five injections in HPLC were measured. The %RSD for the area of five replicate injections was found to be within the specified limits.

Discussion: The Method precision study was performed for the %RSD of Cobicistat and elvitegravir was found to be 0.39 and 0.30 (NMT 2).

B) Intermediate Precision (Ruggedness):

To evaluate the intermediate precision (also known as ruggedness) of the method, precision was performed on different days by using different make column of same dimensions.

Preparation of standard stock solution:

Accurately 10 mg of Cobicistat and 10mg of Elvetigravir working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flasks and about 7mL and 70ml of Diluent was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further this Stock was pipette (3ml and 0.3ml) into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure

The standard solution was injected for five times and the area for all five injections measured in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Discussion: The intermediate precision was performed for %RSD of Cobicistat and Elvetigravir was found to be 0.11 and 0.16 respectively (NMT 2).

Specificity

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The specificity was performed by injecting blank (Fig. 4 to 6).

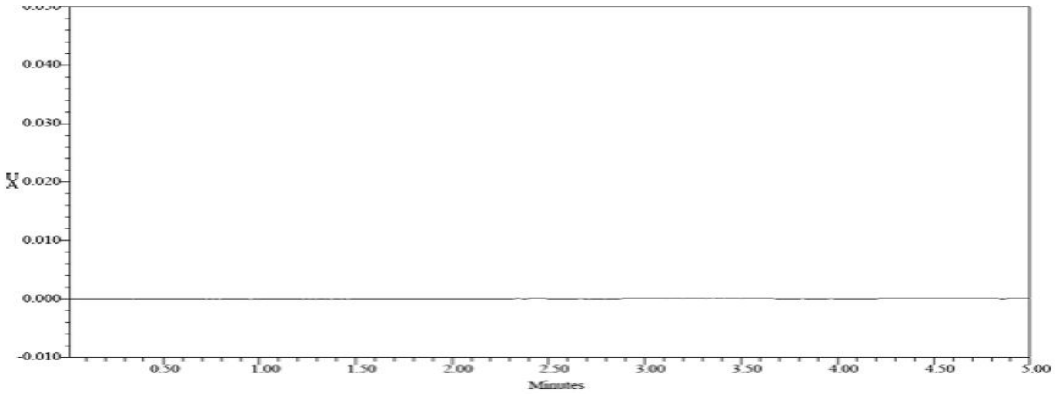

Fig.4. Chromatogram of blank Injection

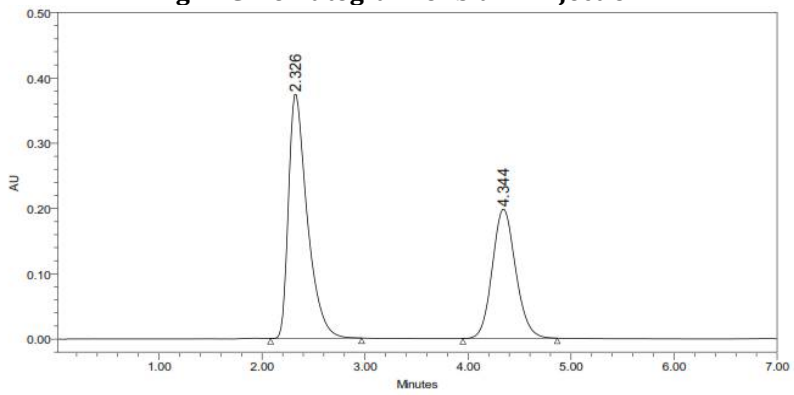

Fig. 5. Chromatogram of standard injection

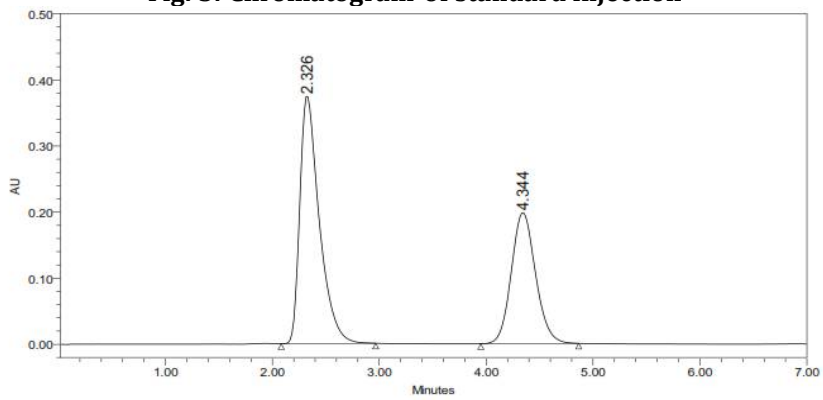

Fig. 6: Chromatogram of Sample Injection

The specificity test was performed for Cobicistat and Elvetigravir. It was found that there was no interference of impurities in retention time of analytical peak.

LOD:

LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines (Fig. 7).

Formula:

$$LOD = 3.3\ X\ \frac{\sigma}{S}$$

Where; σ - Standard deviation (SD) S – Slope

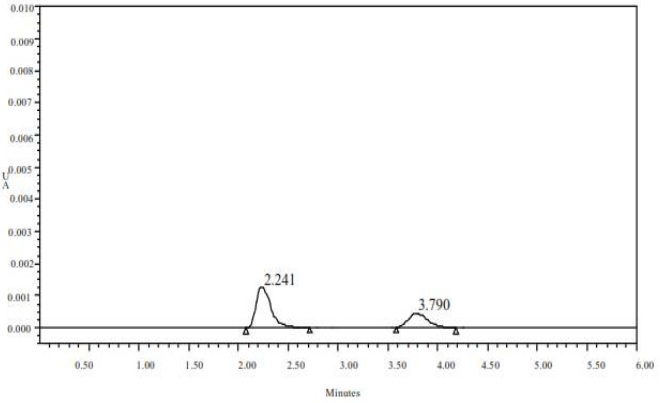

Fig. 7: Chromatogram of LOD

Elvetigravir.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 41µV Signal
Obtained from LOD solution : 125 µV
S/N = 125/41= 3.04

Cobicistat

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 41 µV Signal Obtained from LOD solution : 121 µV
S/N = 121/41 = 2.95

Acceptance Criteria:

S/N Ratio value shall be 3 for LOD solution.
The LOD for Cobicistat and Elvetigravir were 2.95and 3.04 respectively.

LOQ:

LOQ's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula [9-13]. Again, the standard deviation of the response can be determined based on the standard deviation of y- intercepts of regression lines (Fig. 8).

Formula:

LOQ = 10 σ /Slope

Where

σ - Standard deviation
S – Slope

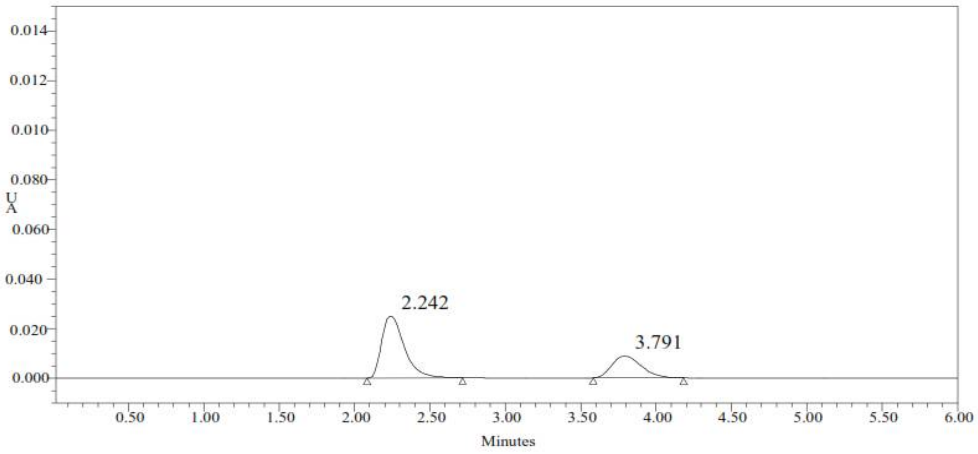

Fig. 8: Chromatogram of LOQ

Elvetigravir

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 41 µV Signal Obtained from LOQ solution: 412µV
S/N = 412/41 = 10.0

Cobicistat

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 41 µV Signal Obtained from LOQ solution : 405µV
S/N = 405/41 = 9.87

Acceptance criteria:

S/N Ratio value shall be 10 for LOQ solution.

The LOQ was performed for Cobicistat and Elvetigravir was found to be 9.87and 10 respectively.

Linearity

Preparation of stock solution:

Ten tablets were weighed and crushed in a mortar and pestle, and weight equivalent to 10 mg of Cobicistat and Elvetigravir (marketed formulation) sample were transferred into a 10mL clean dry volumetric flask and about 7mL of Diluent was added and sonicated to dissolve it completely and made up to the mark with the same solvent [10-14]. (Stock solution)

Preparation of Level I (20ppm ppm Cobicistat&10pm Elvetigravir)

The stock solution (1 ml of stock solution was placed in a 10 ml of volumetric flask and diluted to the mark with a diluent.

Preparation of Level II (40ppm ppm Cobicistat&20ppm ppm Elvetigravir)

2ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level III (60ppm ppm Cobicistat&30ppm ppm Elvetigravir)

A stock solution (3 ml of stock solution was placed in a 10 ml of volumetric flask and diluted to the mark with a diluent.

Preparation of Level – IV (80ppm of Cobicistat&40ppm of Elvetigravir):

4ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level – V (100ppm of Cobicistat&50ppm of Elvetigravir)

5ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluent.

Procedure:

Each level was injected into the chromatographic system and the peak area was measured. A graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) was plotted and the correlation coefficient was calculated (Fig. 9 and 10).

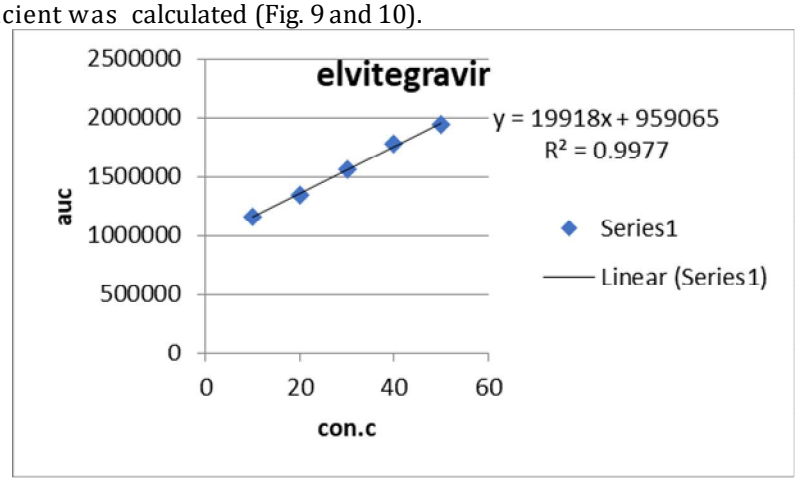

Fig. 9: Calibration curve of Elvetigravir

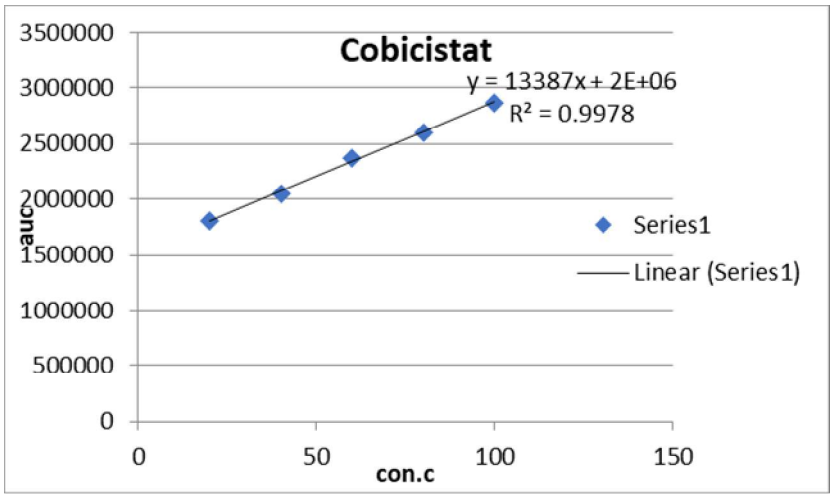

Fig. 10: Calibration curve of Cobicistat

The linearity study was performed for a concentration range of 10µg - 50µg and 20µg-100µg of Cobicistat and Elvetigravir, and the correlation coefficients were 0.999 and 0.999, respectively. (NLT 0.999).

Range:

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 1µg-5µg and 100µg- 500µg of Elvetigravir and Cobicistat respectively.

Robustness:

As part o f the robustness, deliberate change in the flow ate, mobile phase composition was made to evaluate the impact on the method [15-18].

a) The flow rate was varied at 0.8ml/min to 1.2 ml/min. Standard solution 3ppm of Elvetigravir and 300ppm of Cobicistat was prepared and analyzed using the varied flow rates along with method flow rate.

b) The organic composition in the mobile phase was varied from 65% to75 % standard solution 3 µg/ml of Elvetigravir and 300 µg/ml of Cobicistat were prepared and analyzed using the varied mobile phase composition along with the actual mobile phase composition in the method.

Table 4: System suitability results for change in mobile phase.

S.No	Change in Organic Composition in the Mobile Phase	System suitability results (Elvetigravir)		System suitability results (Cobicistat)	
		USP Plate count	USP Tailing	USP Plate count	USP Tailing
1	10% Less	1748.5	1.22	883.3	1.56
2	Actual	1548.2	1.2	1234.0	1.1
3	10% More	1948.0	1.2	969.2	1.6

Table 5: System suitability results for change in flow rate.

S.No	Flow Rate(ml/min)	System suitability results (Elvetigravir)		System suitability results (Cobicistat)	
		USP Plate count	USP Tailing	USP Plate count	USP Tailing
1	0.8	1748.5	1.22	883.3	1.56
2	1.0	1548.2	1.2	1234.0	1.1
3	1.2	1948.0	1.2	969.2	1.6

System suitability

Elvetigravir (5 mg) and Cobicistat working standard (500 mg) were accurately weighed and transferred into a 100 ml clean dry volumetric flask, and approximately 20 ml of diluent was added and sonicated to dissolve it completely and make the volume up to the mark with the same solvent (stock solution). Further 10 ml of Elvetigravir and Cobicistat was pipetted out from the above stock

solution into a 100ml volumetric flask and was diluted up to the mark with diluent. All the parameters were within the limit for the system suitability [18,19]. Tailing factor for the peaks due to Cobicistat and Elvetigravir in Standard solution were less than 2.0. The theoretical plates for the Cobicistat and Elvetigravir peaks in the standard solution should be greater than 2000.

CONCLUSION

A new method was established for simultaneous estimation of Elvetigravir and Cobicistat by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Elvetigravir and Cobicistat by using Xterra C18 5µm (4.6*150mm) column, flow rate was 1ml/min, mobile phase ratio was Phosphate buffer (0.05M) pH 7: MEOH (30:70%v/v) (pH was adjusted with orthophosphoric acid), detection wave length was 270nm. The retention times were 2.399 and 3.907 min, respectively. The % purities of Elvetigravir and Cobicistat were found to be 100.7% and 101.4%, respectively. The system suitability parameters for Elvetigravir and Cobicistat, such as the theoretical plates and tailing factor, were found to be 1.3, 5117.5, and 1.4, 3877.3, and 8.0, respectively. The linearity study for Elvetigravir and Cobicistat was found in the concentration range of 10µg-50µg and 20µg-100µg and the correlation coefficient (r2) was found to be 0.997 and 0.997, the% mean recovery was found to be 100% and 100.5%, respectively, and the % RSD for repeatability was0.2 and 0.4 % RSD for intermediate precision was 0.5 and 0.1%, respectively. The precision study was precise, robust, and reproducible. LOD value was 2.95 and 3.04, and LOQ value of 9.87 and 10, respectively. Hence, the suggested RP-HPLC method can be used for routine analysis of Elvetigravir and Cobicistat in API and Pharmaceutical dosage forms.

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