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DEVELOPMENT AND VALIDATION OF A NEW RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF ANTIRETROVIRAL DRUGS: COBICISTAT AND ELVITEGRAVIR

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Keywords:

Cobicistat, Elvitegravir, Anti-HIV agent, CYP3A Inhibitors, Validation, ICH guidelines

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ABSTRACT: A simple, precise, rapid and accurate reverse phase HPLC method was developed for the simultaneous estimation of cobicistat and elvitegravir in the pharmaceutical dosage form. A column of ODS (250mm 4.6mm; i.d and 5µ particle size) was used along with the mobile phase comprising of 0.02M dipotassium hydrogen orthophosphate buffer (pH adjusted to 3.3) and methanol in the ratio of 80:20 (v/v). The flow rate was maintained at 1.0 ml/min and the effluents monitored at 254 nm. The retention time for cobicistat was found to be 2.58 ± 0.3 min and elvitegravir was 3.71 ± 0.3 min. The detection concentration was linear over 125-750 µg/ml for cobicistat and 12.5-75 µg/ml for elvitegravir. Regression equations of cobicistat and elvitegravir were found to be y = 25883x + 19711 and y = 27696x + 6046 respectively with regression co-efficient 0.999. The % RSD for Intra and Inter day precision was < 2%. The accuracy of method was validated by recovery studies and found to be significant within acceptable range 98-102%. The developed method was successfully validated in accordance with ICH guidelines. The present study demonstrates the applicability of chromatographic method to develop a new, sensitive, single RP-HPLC method for the simultaneous quantitative determination of cobicistat and elvitegravir in fixed pharmaceutical dosage form. Hence, this method can be conveniently adopted for routine analysis in quality control laboratories.

INTRODUCTION: Cobicistat and Elvitegravir combined dosage form is used for the treatment of HIV infection in adult patients. 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 buta-noyl] amino]-1, 6-diphenylhexan-2-yl] carbamate which acts as an HIV integrase inhibitor [1, 2].

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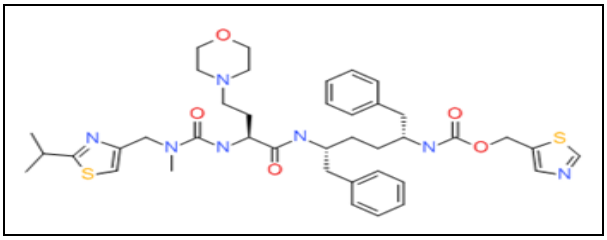

FIG. 1: CHEMICAL STRUCTURE OF COBICISTAT

It has a molecular formula of $C_{40}H_{53}N_7O_5S_2$ and a molecular weight of 776.0 g/mol **Fig. 1**. It is a mechanism-based inhibitor of cytochrome P450 3A (CYP3A) isoforms. Cobicistat does not have any anti-HIV activity on its own. It is a new pharmacokinetic enhancer, metabolized by CYP3A and especially used to increase elvitegravir levels when administered.

Elvitegravir is chemically 6-(3-chloro-2-fluorobenzyl) -1- [(2S) -1-hydroxy-3-methyl butan -2- yl] -7-methoxy -4- oxo-1, 4 dihydro quinoline -3- carboxylic acid. It has a molecular formula of $C_{23}H_{23}ClFNO_5$ and a molecular weight of 447.883 g/mol **Fig. 2**. Elvitegravir inhibits the strand transfer activity of HIV-1 integrase (integrase strand transfer inhibitor; INSTI), 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 [3-5].

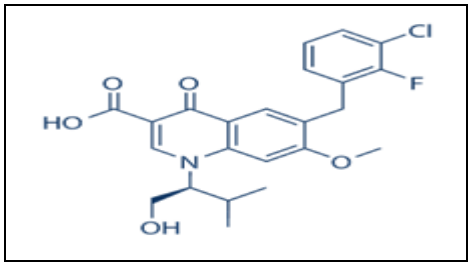

FIG. 2: CHEMICAL STRUCTURE OF ELVITEGRAVIR

Various HPLC and HPTLC methods were reported in the literature for the estimation of cobicistat and elvitegravir individually [6-8], simultaneously [9-10] and other antiretroviral drugs [10-20]. The present method is novel and was successfully validated in accordance with ICH guidelines [21]. The results of the study showed that the proposed RP-HPLC method is useful for the routine simultaneous determination of cobicistat and elvitegravir in the pharmaceutical dosage form.

MATERIALS AND METHODS:

Materials: Cobicistat and elvitegravir were obtained as a gift sample from Hetero Drugs Ltd. Hyderabad. Methanol (Merck Specialities Private Limited, India), potassium dihydrogen phosphate and ortho-phosphoric acid (Rankem Ltd., India) used were of analytical grade. Commercially available cobicistat tablets (TYBOST® 150mg) and elvitegravir tablets (VITEKTA ®-150mg) were procured from the local market.

Instruments: Quantitative HPLC was performed on Waters Alliance 2695 separations module is a high performance liquid chromatographic system with a quaternary, low-pressure mixing pump and inline vacuum degassing powered with Empower-2 Software. An ODC column of 250mm 4.6mm: i.d and 5µ particle size was used. PG Instruments T60 with special bandwidth of 2 mm and 10 mm and matched quartz was be used for UV measurements.

Selection of UV Wavelength: The sensitivity of the method that uses UV-Visible detector depends upon the proper selection of wavelengths. An ideal wavelength is that gives maximum absorbance and good response for both the drugs to be detected.

Standard solutions of cobicistat and elvitegravir were scanned in the UV range (200-400nm), and the spectrums obtained were overlaid, and the overlain spectrum was recorded. From the overlain spectrum, 254 nm was selected as the detection wavelength for the present study **Fig. 3**.

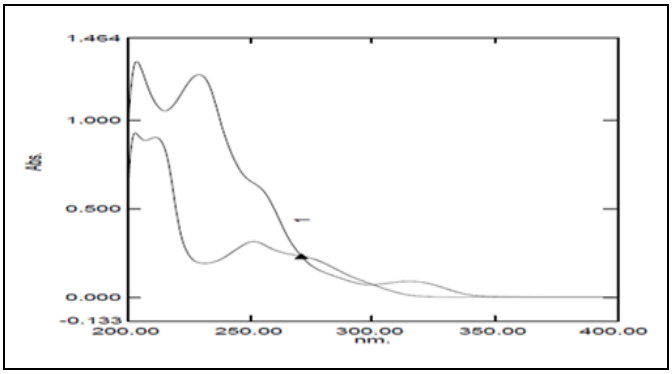

FIG. 3: OVER LINE SPECTRUM OF COBICISTAT AND ELVITEGRAVIR

Preparation of Standard Solution: Accurately weighed and transferred 50 mg of cobicistat and 5 mg of elvitegravir working Standards into 10 ml clean dry volumetric flasks, added 3/4 ml of diluent, sonicated for 5 min and makeup to the final volume with diluents. 1 ml each from the above two stock solutions was taken into a 10 ml volumetric flask, made up to the mark to obtain the final concentration of 500 µg/ml of cobicistat and 50 µg/ml of elvitegravir respectively.

Preparation of Working Standard: Twenty tablets of cobicistat and two tablets of elvitegravir were accurately weighed, the average weight of tablets were found and crushed to a fine powder. From the triturate of tablets, an amount equivalent to 2500 mg of cobicistat and 250 mg of elvitegravir were weighed and transferred into 100ml volumetric flask and make up to the mark with diluent. The solution was sonicated for 25 min and filtered through Whatman filter paper no. 41. From both the solutions pipette out 0.2 ml each, transfer into a 10 ml volumetric flask, made up to the mark

with diluent to obtain final concentration of 500 µg/ml of cobicistat and 50 µg/ml of elvitegravir working standards.

Preparation of Mobile Phase: Prepare a mixture of 80 ml buffer and 20 ml methanol, degas in an ultrasonic water bath for 5 min. Then this solution is filtered through 0.45 µ filter under vacuum filtration. The mobile phase is used as diluent.

Preparation of Buffer (0.01 KH_2PO_4): Accurately weigh 1.36 gm of Potassium di-hydrogen orthophosphate in a 1000ml of volumetric flask, add about 900 ml of Milli-Q water and degas to sonicate. Finally, make up the volume with water and pH adjusted to 3.3 with orthophosphoric acid solution.

Analytical Method Validation: The HPLC method was validated according to the International Conference on Harmonization (ICH) guidelines (2005) [21]. The following characteristics were considered for validation: specificity, linearity, range, accuracy, precision, LOD, LOQ, and robustness.

The specificity was evaluated by comparing the representative chromatograms of samples containing possible interfering substances and samples containing cobicistat and elvitegravir. Linearity was determined from the plot peak area *vs.* concentration for the six concentrations of cobicistat and elvitegravir. The regression equation and regression coefficient were calculated using least square methodology.

Precision is of two types: repeatability or intra-day variability and intermediate precision or inter-day variability. The intraday precession was testing for six different solutions of cobicistat and elvitegravir on the same day. Inter day precision tested by analyzing solutions of both drugs six times on different days. The results were reported as % RSD.

The LOD and LOQ were determined from the specific calibration curve obtained using six standard solutions that were the closest to the LOQ.

Robustness was evaluated by deliberately varying the temperature of the analytical column, the flow rate, and by using similar columns.

RESULTS AND DISCUSSION:

System Suitability: The system suitability studies were evaluated by comparing with standard chromatogram and by obtaining the parameters retention time, column efficiency, and tailing factor **Table 1**. All the system suitability parameters are within range and satisfactory as per ICH guidelines **Fig. 4, 5** [21].

TABLE 1: SYSTEM SUITABILITY DATA

Property	Cobicistat	Elvitegravir
Retention time (t_R)	2.58 ± 0.3 min	3.71 ± 0.3min
Theoretical plates (N)	6477 ± 163.48	7979 ± 163.48
Tailing factor (T)	0.86 ± 0.117	1.34 ± 0.117

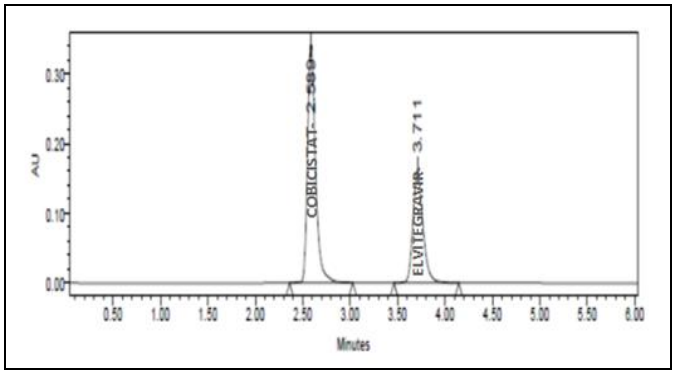

FIG. 4: OPTIMIZED CHROMATOGRAM OF COBICISTAT AND ELVITEGRAVIR STANDARDS

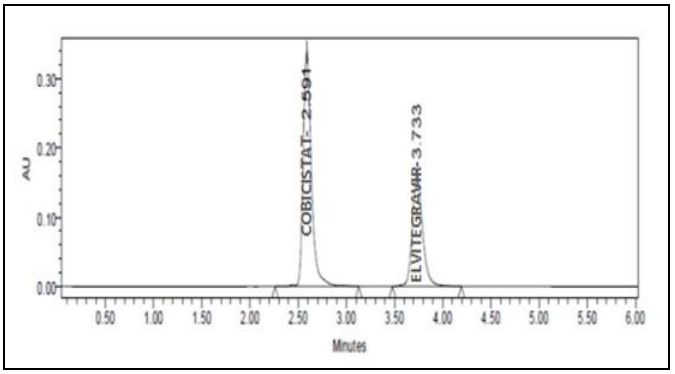

FIG. 5: OPTIMIZED CHROMATOGRAM OF COBICISTAT AND ELVITEGRAVIR SAMPLE

Linearity: The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of the analyte in the sample. A value of correlation coefficient (r^2) > 0.998 is considered as the evidence of an acceptance of the data to the regression line. Serial dilutions of cobicistat (125-750 µg/ml) and elvitegravir (12.5-75 µg/ml) were injected into the column and detected at a wavelength set at 254 nm **Table 2**. The calibration curve was obtained by plotting the concentration *vs.* peak area **Fig. 6, 7**.

Regression equation of cobicistat and elvitegravir are found to be y = 25883x + 19711 and y = 27696x + 6046 respectively. The regression co-efficient was 0.999.

TABLE 2: CALIBRATION DATA OF COBICISTAT AND ELVITEGRAVIR

S. no.	Concentration of Cobicistat (µg/ml)	Response	Concentration of Elvitegravir (µg/ml)	Response
1	0	0	0	0
2	125	3481464	12.5	352868
3	250	6645306	25	689638
4	375	10066263	37.5	1052360
5	500	13408035	50	1405358
6	625	16372442	62.5	1753133
7	750	19348128	75	2059078

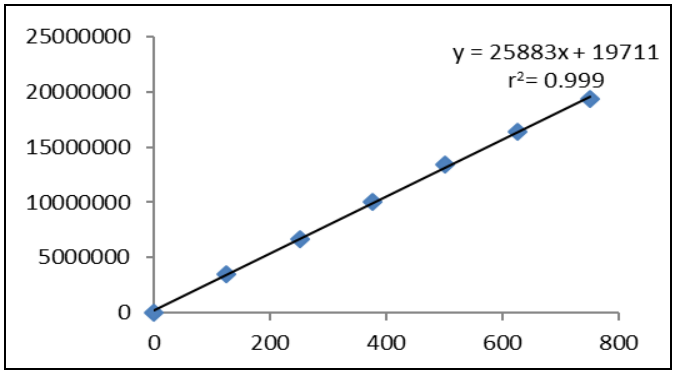

FIG. 6: CALIBRATION CURVE OF COBICISTAT
(X-Axis = Concentration, Y-Axis = Peak area)

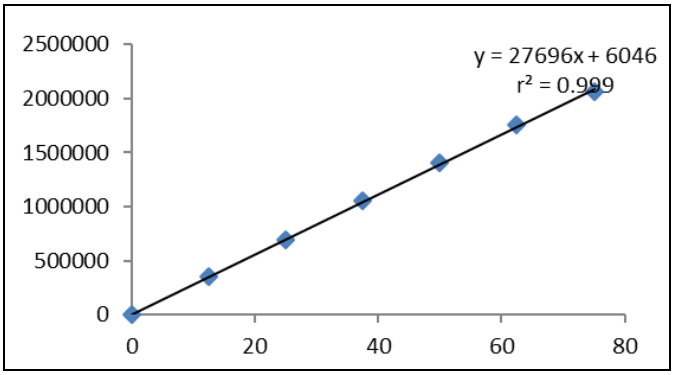

FIG. 7: CALIBRATION CURVE OF ELVITEGRAVIR
(X-Axis = Concentration, Y-Axis = Peak area)

Assay Studies: Six homogeneous samples of both samples and standards were injected. The percentage assay of the drugs in the formulation was estimated. The average % assay was calculated and found to be 99.87% and 100.16% for cobicistat and elvitegravir respectively **Fig. 8**. The assay data were tabulated in **Table 3**.

Assay % = AT / AS × WS / DS × DT / WT × P / 100 × Avg. Wt / Labelled Claim (LC) × 100

Where,
AT = average area counts of sample preparation.
AS = average area counts of standard preparation.
WS = Weight of working standard taken in mg.
DS = Weight of sample taken in mg.
P = Percentage purity of working standard
LC = Label claim of a drug in mg/ml.

TABLE 3: ASSAY DATA COBICISTAT AND ELVITEGRAVIR

S. no.	Cobicistat % Assay	Elvitegravir % Assay
1	99.24	100.13
2	100.61	99.55
3	99.61	100.92
4	100.53	99.50
5	100.16	100.54
6	99.07	100.31
Avg.	99.87	100.16
Std. Dev	0.66	0.56
% RSD	0.66	0.56

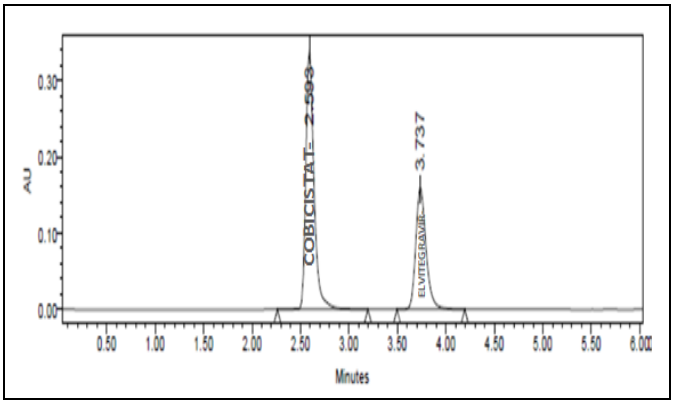

FIG. 8: STANDARD ASSAY CHROMATOGRAM OF COBICISTAT AND ELVITEGRAVIR

TABLE 4: INTER DAY PRECISION STUDIES OF COBICISTAT AND ELVITEGRAVIR

S. no.	Cobicistat (500 µg/ml)	Elvitegravir (50 µg/ml)
1	13541278	1387585
2	13515981	1412087
3	13030796	1353382
4	12991186	1350963
5	13056331	1357619
6	13087151	1362676
Mean	13203787	1370719
Std. Dev.	253708	24178.3
%RSD	1.92	1.76

Precision: The precision of the method was determined by repeatability (intraday precision) and intermediate precision (interday precision) of

both standard and sample solutions. Precision was determined in six replicates of the analyte on the same day (intra-day precision) and daily for 6 times over a period of one week (inter-day precision). The results were expressed as % RSD of the measurements. The interday and intraday precession studies of cobicistat and elvitegravir were performed at concentrations of and found within the acceptable limit.

Interday precision was performed with 24 h time lag and the % RSD obtained for cobicistat and elvitegravir were 1.92% and 1.76% **Table 4**.

Intraday Precision Studies (Repeatability): Interday precision was performed and % RSD for cobicistat and elvitegravir were found to be 0.66% and 0.56% respectively **Table 5**.

TABLE 5: REPEATABILITY RESULTS FOR COBICISTAT AND ELVITEGRAVIR

S. no.	Cobicistat (500 µg/ml)	Elvitegravir (50 µg/ml)
1	13347448	1386574
2	13531146	1378592
3	13397408	1397495
4	13520404	1377842
5	13470132	1392342
6	13324749	1389042
Mean	13431881	1386981
Std. Dev.	88258	7714.8
%RSD	0.66	0.56

Recovery: Three different concentrations (50%, 100%, 150%) of cobicistat and elvitegravir were injected in a triplicate manner and amount recovered, and percentage recovery were tabulated in **Table 6**.

TABLE 6: ACCURACY RESULTS FOR COBICISTAT AND ELVITEGRAVIR

Sample	Amount added (µg/ml)	Amount Recovered (µg/ml)	Recovery (%)	% RSD
Cobicistat	250	250.70	100.28	0.11
	500	499.78	99.96	0.24
	750	749.4	99.92	0.43
Elvitegravir	25	25.08	100.33	0.55
	50	49.89	99.79	0.42
	75	74.97	99.97	0.86

Limit of Detection (LOD) and Limit of Quantification (LOQ): 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 the y-intercepts of regression lines.

$$* \text{LOD} = 3.3 \times /S$$

$$**\text{LOQ} = 10 \times \kappa S$$

Where;
p= the standard deviation of the response *
S = Slope of the calibration curve

LOD for Cobicistat and Elvitegravir were found to be 2.51 µg/ml and 0.72 µg/ml respectively **Table 7**.

TABLE 7: LOD RESULTS FOR COBICISTAT AND ELVITEGRAVIR

S. no.	Drug name	Conc. (µg/ml)	RT (min)	Area (µV* sec)
1	Cobicistat	2.51	2.589	56211
2	Elvitegravir	0.72	3.732	5016

LOQ: Limit of quantification of cobicistat and elvitegravir was calculated by method and LOQ for cobicistat and elvitegravir were found to be 7.62 µg/ml and 2.18 µg/ml respectively **Table 8**.

TABLE 8: LOQ RESULTS FOR COBICISTAT AND ELVITEGRAVIR

S. no.	Drug name	Conc. (µg/ml)	RT (min)	Area (µV* sec)
1	Cobicistat	7.62	2.590	56211
2	Elvitegravir	2.18	3.733	5016

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

For the determination of a method's robustness, deliberate change in the Flow rate was made to evaluate the impact on the method. Small deliberate changes in a method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized changes in the result and are within range as per ICH Guidelines **Table 9, 10**.

TABLE 9: ROBUSTNESS RESULTS FOR COBICISTAT AND ELVITEGRAVIR

S. no.	Robustness condition	Cobicistat %RSD	Elvitegravir %RSD
1	Flow minus	0.3	0.0
2	Flow Plus	0.5	0.6
3	Mobile phase minus	0.0	0.1
4	Mobile phase Plus	0.3	0.2
5	Temperature minus	0.2	0.1
6	Temperature Plus	0.5	0.1

TABLE 10: DETECTION CHARACTERISTICS OF COBICISTAT AND ELVITEGRAVIR

Parameters	Cobicistat	Elvitegravir
Calibration range (µg/ml)	125-750 µg/ml	12.5-75 µg/ml
Optimized wavelength	254nm	254nm
Retention time	2.58±0.3min	3.71±0.3 min
Regression equation (Y)	y = 25883x + 19711	y = 27696x + 6046
Correlation coefficient (r^2)	0.999	0.999
Precision (% RSD)	1.92%	1.76%
% Assay	99.87%	100.16%
Limit of Detection (µg/ml)	2.51 µg/ml	0.72 µg/ml
Limit of Quantitation (µg/ml)	7.62 µg/ml	2.18 µg/ml

CONCLUSION: From the typical chromatogram of drugs, it is shown that the retention time for cobicistat is 2.58 min and elvitegravir is 3.71min. The mobile phase comprises of 0.02M di-potassium hydrogen orthophosphate buffer (pH adjusted to 3.3) and methanol in the ratio of 80:20 (v/v). The flow rate was 1.0 ml/min and the effluents were monitored at 254 nm. Over 1.0 ml/min gradient mode of separation, which was found to be most suitable to obtain a peak well defined and free from tailing.

In the present developed HPLC method, the standard and sample preparation required less time and no tedious extraction was involved. A good linear relationship (r^2=0.999) was observed between the concentration range of 125-750 µg/ml for cobicistat and 12.5-75 µg/ml for elvitegravir. Low values of standard deviation are indicative of the high precision of the method.

The percentage assay of Cobicistat is 99.87%, and Elvitegravir is 100.16%. The absence of additional peaks in the chromatogram indicates non-interference of the common excipients used in the formulation. The limit of detection (LOD) and limit of quantification (LOQ) for cobicistat were found to be 2.51 µg/ml and 7.62 µg/ml; for elvitegravir were 0.72 µg/ml and 2.18 µg/ml respectively.

This demonstrates that the developed HPLC method is simple, linear, accurate, sensitive and reproducible. Thus, the developed method can be easily used for the routine quality control of the pharmaceutical dosage form of the drugs within a short analysis time.

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CONFLICT OF INTEREST: Nil

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