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Research Article

# STABILITY-INDICATING VALIDATED REVERSED PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR SIMULTANEOUS DETERMINATION OF COBICISTAT AND ATAZANAVIR SULFATE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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**ABSTRACT**

**Objective:** A simple, rapid, precise, accurate, and economical stability-indicating reversed phase-high performance liquid assay method was developed and validated for simultaneous estimation of cobicistat (COB) and atazanavir (ATV) sulfate in bulk drugs and their combined commercial tablets.

**Methods:** The method has shown adequate separation of COB and ATV from their degradation products. Separation was achieved on a Luna CN (250 mm × 4.6 mm, 5 µm column at a detection wavelength of 239 nm) using a mobile phase consists of o-phthaldialdeyde (Ph2.5) IX buffer, acetonitrile, and methanol in the ratio of 40:40:20 in an isocratic elution mode at a flow rate of 1 ml/min.

**Results:** The retention times for COB and ATV sulfate were found to be 3.606 and 6.113 min, respectively. COB and ATV sulfate, their combination drug product was subjected to acid, base, neutral hydrolysis, thermal, and photolytic stress conditions. Thus, stressed samples were analyzed by the proposed analytical method. Validation of the proposed analytical method was carried out as per ICH guidelines Q2R1. Quantitation was achieved with UV detection at 239 nm based on peak area with linear calibration curves at concentration ranges 50-600 µg/ml for COB and 100-1200 µg/ml for ATV sulfate (R2 = 0.999 for both drugs). The limits of detection were 0.25 µg/ml and 0.5 µg/ml for COB and ATV sulfate, respectively.

**Conclusion:** The method was found to be specific and stability indicating as no interfering peaks of degradants and excipients were observed. The proposed method is hence suitable for application in quality-control laboratories for quantitative analysis of both the drugs individually and in combination dosage forms since it is simple and rapid with good accuracy and precision.

**Keywords:** Stability-indicating assay, Reversed phase-high performance liquid, Cobicistat, Atazanavir sulfate, Forced degradation studies.

## INTRODUCTION

Cobicistat (COB) Thiazol-5-ylmethyl *N*-[1-benzyl-4-[[2-[[(2-isopropylthiazol-4-yl) methyl-methyl-carbamoyl]amino]-4-morpholino-butanoyl] amino]-5-phenylpentyl] carbamate [1] is a cytochrome P450 3A inhibitor having molecular formula C40H53N7O5S, molecular weight 775 g.mol$^{-1}$. It is used for the treatment of human immunodeficiency virus (HIV) infection. COB is of interest for its ability to inhibit liver enzymes that metabolize other medications used to treat HIV. COB is a novel pharmacokinetic boosting agent without activity on HIV.

Atazanavir (ATV) sulfate is a chemically (3S,8S,9S,12S)-3,12-Bis(1,1-dimethylethyl)-8-hydroxy-4,11-dioxo-9-(phenylmethyl)-6-{[4-(2-pyridinyl)phenyl]methyl}-2,5,6,10,13 penta aza tetra decanedioic acid dimethyl ester, sulfate(1:1) [2]. ATV is an antiretroviral agent for the treatment of HIV infection, and consequently, it is clinically useful in the treatment of AIDS. ATV sulfate, azapeptide inhibitor of HIV-1 protease, is indicated in combination with other antiretroviral agents for the treatment of HIV-1 infection. ATV sulfate is a white to pale yellow powder. It is slightly soluble in water.

Brand name: EVOTAZ (150 mg of COB, 300 mg of ATV sulfate) is commercially available combined formulation (Gilead Sciences, Inc.).

### Objective of study

Literature survey revealed that many spectroscopic and chromatographic methods were reported for the estimation of these drugs (COB and ATV sulfate) individually in bulk and pharmaceutical dosage forms, for the determination include COB UV spectrophotometry [3] and high performance liquid (HPLC) [4] method available, and for the determinations of ATV sulfate include HPLC [5,6], simultaneous spectrophotometric determination, and UV spectrophotometric method [7]. However, these analytical methods lack stability-indicating nature. Furthermore, there was no reported analytical method for the estimation of both the drugs in pharmaceutical dosage forms in the presence of their degradation products. In the present investigation, an attempt was made to develop a simple, rapid, precise, and accurate stability-indicating reversed phase-HPLC (RP-HPLC) assay method for simultaneous estimation of COB and ATV sulfate. The major advantage of the proposed method was that COB and ATV sulfate can be determined on a single chromatographic system with the same detection wavelength. This proposed method can be successfully employed for quality-control during manufacture and for assessment of the stability of both drugs in bulk samples and their combined tablet dosage forms. The developed method was validated as per ICH Q2 [8,9] guidelines.

## METHODS

### Drug substance

COB and ATV sulfate (working standard 99.1 and 99.7) were procured from Hetero pharma laboratories, Hyderabad, India. Pharmaceutical tablet formulation of EVOTAZ (150 mg COB, 300 mg ATV) was purchased from a local pharmacy. Methanol (HPLC grade; MERCK), orthophosphoric acid (PH 2.5) (HPLC grade, MERCK), hydrochloric acid (AR), sodium hydroxide (AR), hydrogen peroxide (AR), and HPLC grade water were used for the entrained study.

### Instrumentation

All HPLC experiments were carried out on a Waters Alliance 2695 separation module, with Waters 2996 photodiode array detector in an isocratic mode using Autosampler. Data collections and processing were

done using EMPOWER PDA 2 software. The analytical column used for the separation was: Luna CN, 250 mm × 4.6 mm, 5 µm.I.D., 5 µm particle size, Other equipment's used were ultra-sonicator (model 3210, Branson Ultrasonic Corporation, Connecticut, USA), Analytical balance (contech balance), PH meter (ELICO LI 120).

**Preparation of solutions**

*Preparation of 0.1% o-phthaldialdeyde (OPA) buffer solution*

0.1% OPA was prepared by taking 1 ml of OPA in 1000 ml HPLC grade water.

*Mobile phase*

Mix buffer, acetonitrile, and methanol in the ratio of 40:40:20.

The mobile phase was prepared by mixing OPA mixed buffer (pH-2.5, 0.1%), acetonitrile, methanol and. It was filter to 0.45 µ membrane filter to remove the impurities. Otherwise, they may interfere in the final chromatogram, and it was sonicated for 15 minutes to remove the undissolvable gases and air bubbles.

*Preparation of 0.1N HCl*

0.1 N HCl was prepared by taking 0.08 ml of conc. HCl in 100 ml of HPLC grade water.

*Preparation of 0.1N NaOH*

0.1N NaOH was prepared by taking 0.4 g of NaOH in 100 ml of HPLC grade water.

*Preparation of hydrogen peroxide*

Hydrogen peroxide was prepared by taking 3 ml of hydrogen peroxide in 100 ml of HPLC grade water.

**Standard solution**

*Preparation of standard solution*

Solution A

COB: Weigh accurately about 500 mg of COB working standard into a 100 mL volumetric flask. Add 70 mL of diluent, sonicate to dissolve, and dilute to volume with diluent.

Solution B

ATV sulfate**:** Weigh accurately about 1 g ATV sulfate working standard into a 100 mL volumetric flask. Add 70 mL of diluent, sonicate to dissolve, and dilute to volume with diluent.

Further dilute each 5 mL of solution A and B to 50 mL with the diluent.

**Preparation of sample solution**

Weigh 20 tablets and crush to powdered then take the equivalent of 150 mg of COB a (300 g of ATV sulfate) sample into a 100 mL volumetric flask. Add 70 mL of diluent, sonicate for 5 minutes, dissolve and dilute to volume diluent. Filter through 0.45 µ nylon syringe filter. Further dilution was made in by taking 5 ml above solution in 50 ml volumetric flask. The final volume was made up to the mark with diluent to get required test concentration 150 µg/ml of COB and 300 µg/ml of ATV sulfate.

**Procedure**

Inject 10 µL of standard preparation 5 times and sample preparation in the chromatograph. Record the chromatograms and measure the peak responses for COB and ATV sulfate. The system suitability parameters should be met. From the peak responses, calculate the content of COB and ATV sulfate in the sample.

**Chromatographic condition**

Luna CN, 250 mm × 4.6 mm, 5 µm column at a detection wavelength of 239 nm, using mobile phase consists of orthophosphoric acid

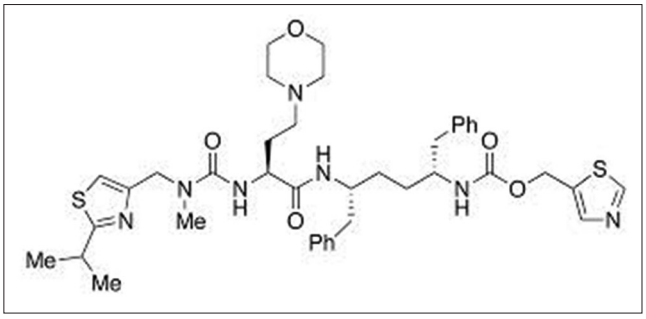

**Fig. 1: Cobicistat**

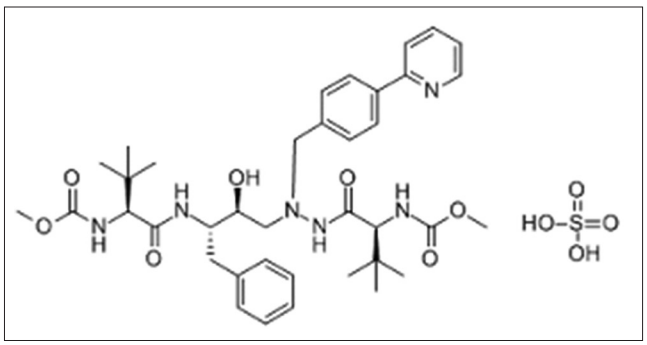

**Fig. 2: Atazanavir**

**Table 1: Method development conditions**

| Trial | Type of column | Mobile phase composition | Injection volume | Flow | Defect |
|---|---|---|---|---|---|
| 1. | Luna C18 250×4.6 mm, 5 µ | Buffer+ACN 60+40 | 10 µl | 1 ml/min | Here only one peak is eluted |
| 2 | Luna CN 250×4.6 mm, 5 µ | Buffer+ACN 70+30 | 10 µl | 1 ml/min | Here peak retention times are very less |
| 3 | Luna CN 250×4.6 mm, 5 µ | Buffer+ACN 70+30 | 10 µl | 1 ml/min | Here peak retention times are very less |
| 4 | Luna CN 250×4.6 mm, 5 µ | Buffer+ACN 60+40 | 10 µl | 1 ml/min | Here second peak retention time is very less |
| 5 | Luna CN 250×4.6 mm, 5 µ | Buffer+ACN 50+50 | 10 µl | 1 ml/min | Here ghost peak is obtained |
| 6 | Luna CN 250×4.6 mm, 5 µ | Buffer+ACN 40+60 | 10 µl | 1 ml/min | Here second peak splitted into two peaks |
| 7 | Luna CN 250×4.6 mm, 5 µ | Water+ACN 70+30 | 10 µl | 1 ml/min | Here second peak splitted into two peaks |
| 8 | Luna CN 250×4.6mm, 5µ | 0.1% OPA+ACN 50+50 | 10 µl | 1 ml/min | Broad peak is obtained |
| 9 | Luna CN 250×4.6 mm, 5 µ | 0.1% OPA+MEOH 30+70 | 10 µl | 1 ml/min | Broad peak is obtained |
| 10 | Luna CN 250×4.6 mm, 5 µ | 0.1% OPA+ACN 50+50 | 10 µl | 1 ml/min | Only one peak is obtained |
| 11 | Luna CN 250×4.6 mm, 5 µ | 0.1% OPA+CAN+MEOH 40+ 40+20 | 10µl | 1 ml/min | Only one peak is obtained |
| 12 | Luna CN 250×4.6 mm, 5 µ | 0.1% OPA: ACN: MEOH (40:40:20) | 10 µl | 1 ml/min | This method is suitable for validation |

(0.1%) pH 2.5, acetonitrile, and methanol in the ratio of 40:40:20. in an isocratic elution mode. The contents of the mobile phase were degassed with a heliumspurge for 15 minutes and pumped from the respective solvent reservoirs to the column at a flow rate of 1 ml/minutes. The column temperature was maintained at 30°C and run time 10 minutes. The injection volume of samples was 20 µl. The retention times for COB and ATV sulfate were found to be 3.606 and 6.113 minutes (optimized chromatographic conditions as shown in the Table 2).

**Method development**

To saturate the column, the mobile phase was pumped for about 30 minutes thereby to get the baseline corrected. The separate standard calibration lines were constructed for each drug. A series of aliquots were prepared from the above stock solutions using HPLC grade water to get the concentrations 50-600 µg/ml COB and 100-1200 µg/ml ATV sulfate. Each concentration was injected 6 times into the chromatographic system. Each time peak area and retention time were recorded separately for both the drugs. Calibration curves were constructed by taking average peak area on Y-axis and concentration on X-axis separately for both the drugs as shown in the Figs. 3 and 4. From the calibration curves, regression equations were calculated, this regression equation was used to calculate drug content in the formulation.

**Estimation of COB and ATV in laboratory synthetic mixture**

For the estimation of COB and ATV sulfate, laboratory synthetic mixture was prepared with COB and ATV sulfate APIs and excipients with the strength of 150 mg of COB, (300 mg of ATV sulfate) and excipients in glass motor and mix well weigh accurately about powder equivalent to 150 mg of COB was transferred to100 ml volumetric flask add 70 mL of diluent, sonicate to dissolve, and dilute to volume with diluent. Filter through 0.45 µ Nylon syringe filter. Further dilute 5-50 mL with the diluent.

This solution was estimated by the above-developed method. The assay procedure was repeated 6 times (n=6) the drug content was estimated using above calculated regression equation, the results of laboratory mixture are shown in Table 4

**Estimation of COB and ATV sulfate in tablet dosage form**

Weigh 20 tablets and crush to powdered then take the equivalent of 150 mg of COB and 300 g of ATV sulfate sample into a 100 mL volumetric flask. Add 70 mL of diluent, sonicate for 5 minutes, dissolve and dilute to volume diluent. Filter through 0.45 µ nylon syringe filter. Further dilution was made in by taking 5 ml of solution in 50 ml volumetric flask. The final volume was made up to the mark with diluent to get required test concentration 150 µg/ml of COB and 300 µg/ml of ATV sulfate. This solution was estimated by the above-developed method. The assay procedure was repeated 6 times (n=6), and the drug content was estimated using above calculated regression equation; the results of laboratory mixture are shown in Table 4.

**Method validations**

The analytical method was validated for various parameters as per ICH guidelines.

**Accuracy**

Accuracy was evaluated in triplicate, at three different concentration levels equivalent to 50, 100, and 150% of the target concentration of active ingredient, by adding a known amount of each of the placebo to a pre-analyzed concentration of both drugs and calculating the % of recovery, and the results obtained were shown in Table 5.

**Linearity**

The linearity of the method was determined in the concentration range of 50-600 µg/ml for COB and 100-1200 µg/ml for ATV. Each solution was injected in triplicate. The average peak area versus concentration data of both drugs was treated by least squares linear regression analysis and the results obtained as shown in Table 6.

**Table 2: Optimized chromatographic conditions**

| | |
|---|---|
| Column | Luna CN, 250 mm×4.6 mm, 5 µm |
| Flow rate | 1 ml/min |
| Wavelength | 239 nm |
| Column temperature | 30°C |
| Injection volume | 10 µl |
| Run time | 8 minutes |
| Diluents | 0.1% OPA: ACN: MEOH |
| Elution | Isocratic |
| Mobile phase | 0.1% OPA: ACN: MEOH (40:40:20) |

**Table 3: Results of laboratory synthetic mixture**

| Compound name | Label claim (mg) | Test concentration (µg/ml) | Amount found (µg/ml) | %Assay | %RSD |
|---|---|---|---|---|---|
| Cobicistat | 150 | 150 | 149.5 | 99.5 | 1.594 |
| Atazanavir sulfate | 300 | 300 | 300 | 100 | 0.044 |

RSD: Relative standard deviation

**Table 4: Results of tablet dosage form**

| Compound name | Brand name | Label claim (mg) | Test concentration (µg/ml) | Mean amount estimated (µg/ml) (n=6) | %Assay | %RSD |
|---|---|---|---|---|---|---|
| Cobicistat | EVOTAZ | 150 | 150 | 150.3 | 100.2 | 1.645 |
| Atazanavir sulfate | | 300 | 300 | 300.15 | 100.05 | 0.161 |

RSD: Relative standard deviation

**Table 5: Accuracy**

| Drug | Level of recovery | Pre analyzed conc. (µg/ml) | Amount found (µg/ml) (n=6) | %Recovery | %RSD |
|---|---|---|---|---|---|
| Cobicistat | 50 | 75 | 76.4 | 100.56 | 0.440 |
| | 100 | 150 | 150.8 | 100.16 | 0.170 |
| | 150 | 225 | 249.9 | 99.98 | 0.120 |
| Atazanavir sulfate | 50 | 150 | 149.6 | 99.92 | 0.080 |
| | 100 | 300 | 300.6 | 100.06 | 0.390 |
| | 150 | 375 | 375.9 | 100.06 | 0.480 |

RSD: Relative standard deviation

**Specificity and selectivity**

Specificity is the degree to which the procedure applies to a single analyzer and is checked in each analysis by examining blank matrix samples for any interfering peaks. The specificity of the method was evaluated with regard to interference due to the presence of any other placebos. Two different samples were injected and studied with respective placebos. The HPLC chromatograms recorded for the drug matrix (mixture of the drug and placebos) showed almost no interfering peaks within retention time ranges. Figs. 3, 5, and 6 show the respective chromatograms for COB and ATV with blank and placebo. The figures show that the selected drugs were cleanly separated. Thus, the HPLC method proposed in this study was selective.

**Precision**

Precision is the degree of repeatability of an analytical method under normal operation conditions.

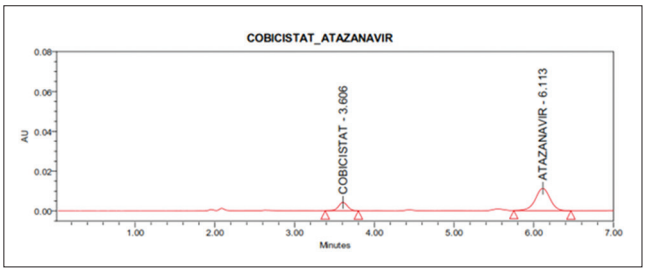


**Fig. 3: Typical chromatogram of COB and ATZ**

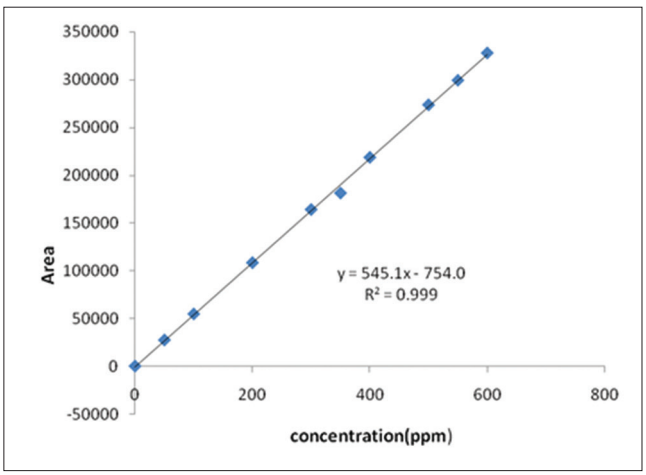


**Fig. 4: Calibration curve of cobicistat**

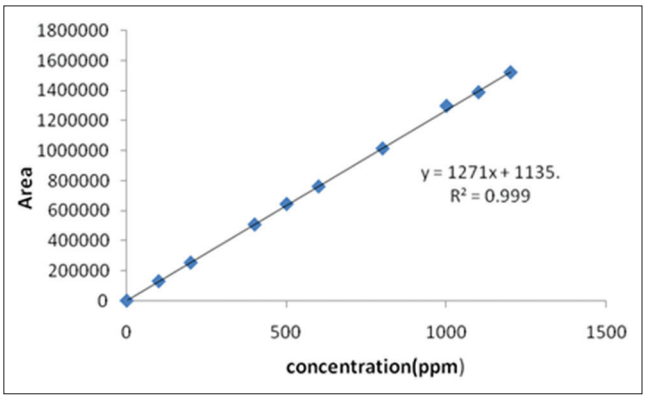


**Fig. 5: Calibration curve of atazanavir sulfate**

Precision is of 3 types:

1. System precision
2. Method precision
3. Intermediate precision
   a. Intraday precision
   b. Interday precision.

Method precision was achieved by repeating the same procedure of preparation solution six times and injecting.

System precision is checked using the standard chemical substance to ensure that the analytical system is working properly. In this peak area, % of drug of six determinations is measured, and % relative standard deviation (RSD) should be calculated.

In method precision, a homogenous sample of the single batch should be analyzed 6 times. This indicates whether a method is giving constant results for a single batch. In this analyze, the sample six times and calculate the % RSD, and the results are shown in the Tables 7 and 8.

**Limit of detection (LOD) and limit of quantification (LOQ)**

LOD: It is the lowest amount of analyte in a sample that can be detected but not necessarily quantities as an exact value under the stated,

**Table 6: Linearity studies of proposed method**

| Parameters | Cobicistat | Atazanavir sulfate |
|---|---|---|
| Linearity range (µg/ml) | 50-600 | 100-1200 |
| Regression equation | y=545.1x - 754.0 | y=1271x+1135 |
| Slope | 545.1 | 1271 |
| Intercept | –754.0 | 1135 |
| Correlation coefficient (r) | 0.999 | 0.999 |
| LOD (µg/ml) | 0.25 | 0.5 |
| LOQ (µg/ml) | 0.75 | 1.5 |

LOD: Limit of detection, LOQ: Limit of quantification

**Table 7: Method precision**

| S. No. | Injection name | Peak area | |
|---|---|---|---|
| | | Cobicistat | Atazanavir sulfate |
| 1 | Method precision-1 | 1496616 | 6356398 |
| 2 | Method precision-2 | 1491875 | 6339154 |
| 3 | Method precision-3 | 1497193 | 6333461 |
| 4 | Method precision-4 | 1497193 | 6344368 |
| 5 | Method precision-5 | 1497885 | 6360424 |
| 6 | Method precision-6 | 1493353 | 6360424 |
| Mean | | 1495686 | 6349038 |
| SD | | 2458.00 | 11623.93 |
| %RSD | | 0.1643 | 0.1831 |

RSD: Relative standard deviation, SD: Standard deviation

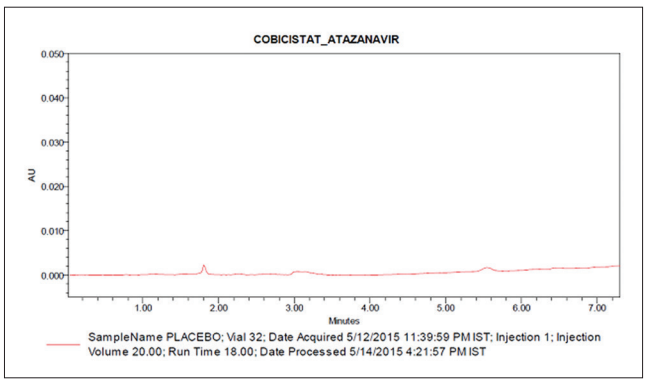


**Fig. 6: Chromatogram of placebo**

**Table 8: System precision**

| S. No. | Injection name | Peak area | | USP tailing | | USP plate count | |
|---|---|---|---|---|---|---|---|
| | | Cobicistat | Atazanavir sulfate | Cobicistat | Atazanavir | Cobicistat | Atazanavir |
| 1 | Sys pres-1 | 1464704 | 6256273 | 1.00 | 0.77 | 4836 | 4905 |
| 2 | Sys pres-2 | 1466284 | 6258054 | 1.00 | 0.76 | 4895 | 4900 |
| 3 | Sys pres-3 | 1460646 | 6254362 | 1.01 | 0.76 | 5060 | 4906 |
| 4 | Sys pres-4 | 1469540 | 6232951 | 1.02 | 0.76 | 5134 | 4957 |
| 5 | Sys pres-5 | 1467433 | 622638 | 1.02 | 0.76 | 5139 | 4900 |
| 6 | Sys pres-6 | 1468093 | 6222696 | 1.02 | 0.76 | 5117 | 4891 |
| Mean | | 1466117 | 6241162 | | | | |
| SD | | 3140.38 | 16967.058 | | | | |
| %RSD | | 0.2142 | 0.2791 | | | | |

RSD: Relative standard deviation, SD: Standard deviation

**Table 9: Robustness studies for cobicistat and atazanavir**

| Method parameters | Conditions | Retention time ($R_t$) | | Area | | %Recovery | |
|---|---|---|---|---|---|---|---|
| | | Cobicistat | Atazanavir | Cobicistat | Atazanavir | Cobicistat | Atazanavir |
| Flow + | +20 % | 3.111 | 4.713 | 1216049 | 5185839 | 100.5 | 100.1 |
| Flow – | –20 % | 4.685 | 6.926 | 41361 | 193458 | 100.7 | 100.5 |
| Organic + | +2 % | 2.981 | 4.909 | 36319 | 172902 | 100.7 | 100.8 |
| Organic – | –2 % | 4.910 | 6.407 | 35600 | 152424 | 100.5 | 100.6 |
| Wavelength + | +5 nm | 3.744 | 5.658 | 1346078 | 6035134 | 100.4 | 100.4 |
| Wavelength – | –5 nm | 3.744 | 5.658 | 1333524 | 5786418 | 100.2 | 100.4 |

experimental conclusions. The detection limit is usually expressed as the concentration of the analyte. The standard deviation and response of the slope and the results obtained.

LOD = 3.3*standard deviation (ϭ)/s

LOQ: The quantitation limit of an analytical procedure is the lowest amount of an analyte of a sample which can be quantitatively determined with suitable precision and accuracy.

LOQ = 10* standard deviation (ϭ)/s

The results of LOD and LOQ are shown in Table 6.

**Robustness**

To evaluate the robustness of the method, the chromatographic conditions were deliberately altered, and degree of reproducibility was evaluated. During robustness testing, each condition was varied separately, all other conditions being held constant at the optimized values. Robustness of the proposed method was assessed with respect to small alterations in the flow rate (1.0±0.2 ml/min) and temperature (30°C±2°C) and the results obtained as shown the Table 9

**System suitability parameters**

For assessing system suitability, six replicates of working standards samples of COB and ATV were injected and studied the parameters such as plate number (N), tailing factor (K), resolution, relative retention time, and peak asymmetry of samples. The results were tabulated in Table 10.

**Degradation sample preparation**

For the forced degradation studies of 150 mg COB and 300 mg ATV sulfate, drug samples were weighed accurately and transfer to 100 ml volumetric flask containing 70 ml of diluent, sonicate for 5 minutes, the volume was made up to the mark with diluent and filter the solution using 0.45 µ nylon syringe filter.

**Acid hydrolysis**

From the test stock solution, 5 ml was taken in 50 ml volumetric flask, add 2.5 ml of 5N HCl, and heated at 70°C for 1 hr on a water bath. The flask was removed from the water bath and allowed to cool at room temperature. Add 2.5 ml of 5N NaOH to neutralize the solution cooled at room temperature and diluted to volume with diluent and mixed.

**Base hydrolysis**

From the test stock solution, 5 ml was taken in 50 ml volumetric flask, add 2.5 ml of 5N NaOH, and heated at 70°C for 1 hr on a water bath. The flask was removed from the water bath and allowed to cool at room temperature. Add 2.5 ml of 5 N HCl to neutralize the solution cooled at room temperature and diluted to volume with diluent and mixed.

**Peroxide degradation (30% $H_2O_2$)**

*Procedure*

From the test stock solution, 5 ml was taken in 50 ml volumetric flask, add 1 ml of 30% $H_2O_2$, and heated at 70°C for1 hr on a water bath. The flask was removed from the water bath and allowed to cool at room temperature and diluted to volume with diluent and mixed.

**Reduction degradation (10% sodium bisulfate)**

*Procedure*

From the test stock solution, 5 ml was taken in 50 ml volumetric flask, add 1 ml of 10% sodium bisulfate, and heated at 70°C for 1 hr on a water bath. The flask was removed from the water bath and allowed to cool at room temperature and diluted to volume with diluent and mixed.

**Hydrolysis degradation**

*Procedure*

From the test stock solution, 5 ml was taken in 50 ml volumetric flask, add 10 ml of water and sonicated to disperse, dissolve and heated at 70°C for 3 hrs on a water bath. The flask was removed from the water bath and allowed to cool at room temperature and diluted to volume with diluent and mixed.

**Thermal degradation (105°C/6 hrs)**

*Procedure*

For the thermal degradation 150 mg COB and 300 mg ATV, drug samples were weighed accurately and transfer to petri dish heat the sample in an oven for about 6 hrs at 105°C. Moreover, transfer the sample into

a 100 ml volumetric flask dissolve and dilute to volume with diluent. Filter the solution using 0.45 µ nylon filter. Transfer 5 ml of above stock solution to 50 ml volumetric flask and make up the volume with diluent to get the concentration of 150 µg COB and 300 µg ATV.

**Humidity degradation (25°C/92% RH for 72 hrs)**

*Procedure*

The sample was exposed at 25°C/92% RH for at least 72 hrs, and the exposed sample was analyzed as per Appendix A.

**Photolytic degradation (1.2 million lux hrs)**

*Procedure*

For the photolytic degradation of 150 mg COB and 300 mg ATV, drug samples were weighed accurately and transfer to the petri dish. The sample was exposed to UV light in a photolytic chamber at 1.2 million lux hrs for 24 hrs, after 24 hrs and the sample was transferred into a 100 ml volumetric flask dissolve and dilute to volume with diluent. Filter the solution using 0.45 µ nylon filter. Transfer 5 ml of above stock solution to 50ml volumetric flask and make up the volume with diluent to get the concentration of 150 µg COB and 300 µg ATV.

Using the peak purity test, the purity of the drugs peaks were checked at every stage of above-mentioned studies. And the results are shown in Table 11 and Figs. 16-21.

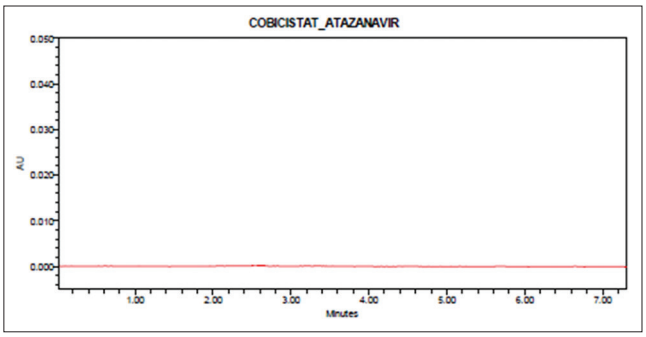


**Fig. 7: Chromatogram of blank**

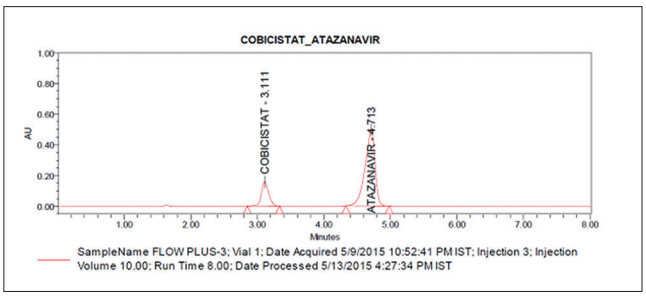


**Fig. 8: Chromatogram of flow plus**

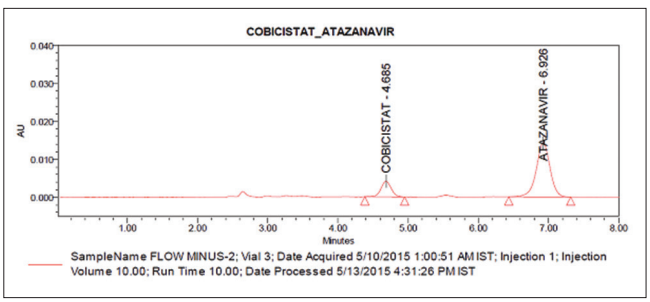


**Fig. 9: Chromatogram of flow minus**

## RESULTS AND DISCUSSIONS

The conditions tested for method development (Table 2) indicates that all the system suitability parameters according to the ICH guidelines were achieved by Luna CN, 250 mm × 4.6 mm, 5 µm.

Column using mobile phase mixed buffer, acetonitrile, and methanol by an isocratic program with a flow rate of 1 ml/minutes throughout the isocratic program with a detection wavelength of 239 nm for all the compounds with an injection volume of 10 µl.

To validate the RP-HPLC method, a series of tests were made using the most promising conditions. A calibration curve was made and concentration examined within the detection range of 50-600 µg/ml 100-1200 µg/ml correlation coefficient was found to be 0.999, 0.999 for all the compounds, respectively. The precision (expressed as the RSD was determined for COB and ATV for repeated analysis and the values are presented in Table 8. The assay values obtained by proposed method and recovery experiment values obtained were performed by adding different amounts placebo to pre-analyzed concentration summarized in Table 6.

The stability of sample was checked by forced degradation in different conditions and % of degradation was calculated. The peak purity of the analyte was passed in all conditions (purity angle should be less than the threshold value). The results as shown in Table 11 indicate that any other impurity is not merging with the main peak (Figs. 16-21). The analyte sample solution was stable up to 24 hrs at room temperature.

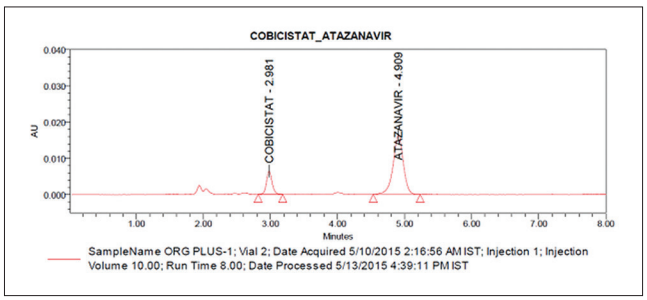


**Fig. 10: Chromatogram of mobile phase plus**

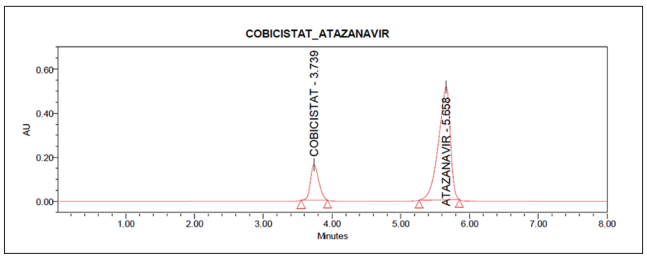


**Fig. 11: Chromatogram of mobile phase of minus**

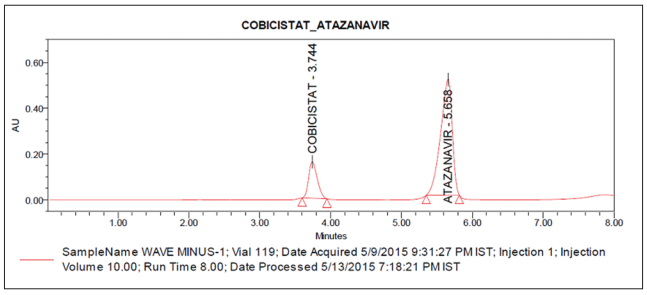


**Fig. 12: Chromatogram of wavelength of plus**

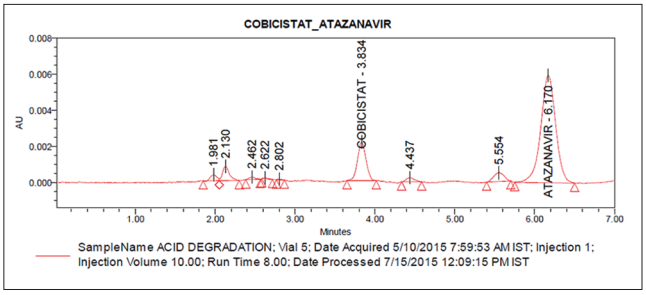


**Fig. 13: Chromatogram of wavelength of minus**

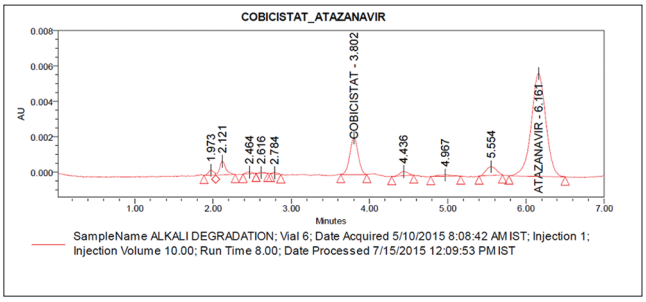


**Fig. 14: Chromatogram of acid degradation**

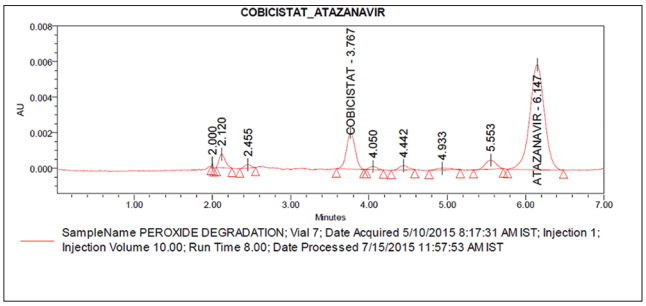


**Fig. 15: Chromatogram of alkali degradation**

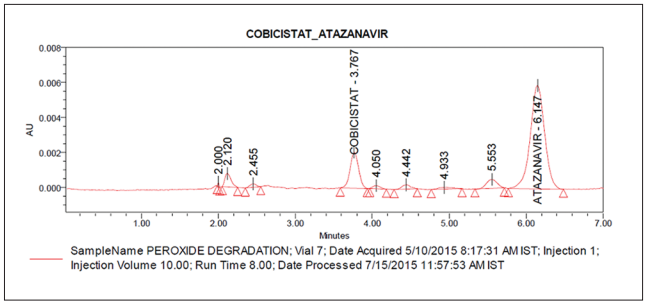


**Fig. 16: Chromatogram of peroxide degradation**

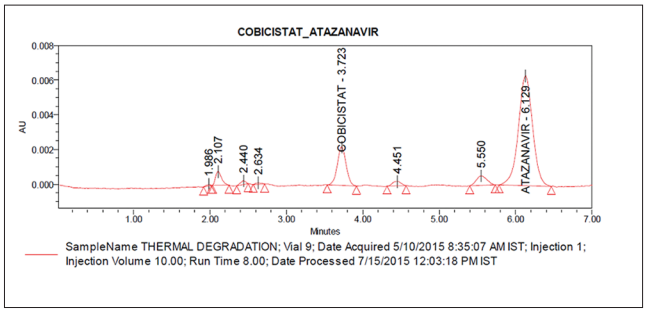


**Fig. 17: Chromatogram of thermal degradation**

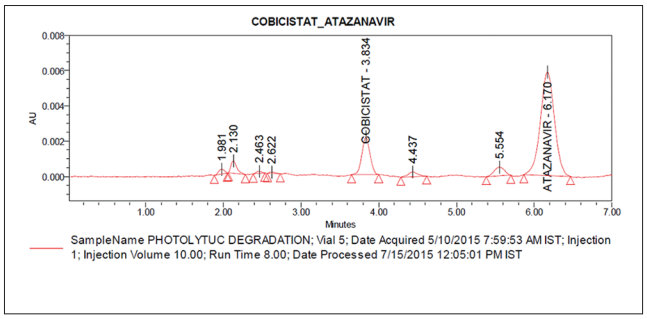


**Fig. 18: Chromatogram of photolytic degradation**

The reliability of the method was determined by made small deliberate variations in method parameters, and the % RSD values (Table 10, Figs. 10-15) obtained, an indication of its reliability on normal usage. A method was developed for the determination of COB and ATV in tablets which is rapid, stable, and specific. The results indicate that the described method can be used for quantitative analysis of the compounds.

**Linearity, LOD, and LOQ**

The calibration plot was linear over the concentration range investigated (50-600 µg/ml; n=3) (100-1200 µg/ml; n=3) for COB and ATV, respectively (Figs. 4 and 5). Average correlation coefficient r=0.999 for all the drugs with % RSD values ≤2.0 across the concentration ranges studied was obtained from the regression analysis. The LOD that produced the requisite precision and accuracy was found to be 0.25 µg/ml for COB, 0.5 µg/ml for ATV. The resultant % RSD values were ≤1.00% (Table 5). The LOQ for COB and ATV were found to be 0.75 and 1.5 µg/ml, respectively. The regression results indicate that method was linear in the concentration range studied and can be used for detection and quantification of COB and ATV in a very wide concentration range.

**Accuracy and precision**

Accuracy as recovery was evaluated by spiking previously analyzed test solution with additional placebo at three different concentration levels (Table 6). Recovery of previously analyzed test solution drug concentration added was found to be 100.23% for COB and 100.0133% for ATV the value of RSD <1% indicating that the proposed method is accurate for the simultaneous estimation of all drugs from their combination drug products in the presence of their degradation products. The low RSD values indicate the repeatability and reproducibility of the method (Table 6).

**Specificity and selectivity**

Specificity is checked in each analysis by examining blank and placebo samples for any interfering peaks. The specificity of the method was evaluated with regard to interference due to the presence of any other excipients. The Figs. 8 and 9 show that selected drugs were clearly separated.

**Robustness**

To evaluate the robustness of the method, the chromatographic conditions were deliberately altered, and degree of reproducibility was evaluated. During robustness testing, each condition was varied separately, all other conditions being held constant at the optimized values. Robustness of the proposed method was assessed with respect to small alterations in the flow rate (1.0±0.2 ml/min), organic composition, and wavelength (239±2) and the results obtained as shown in Table 9 and Figs. 7-12.

**System suitability**

For assessing system suitability, six replicates of working standards samples of COB and ATV were injected and studied the parameters such as plate number (N), tailing factor (K), resolution, relative retention

time, and peak asymmetry of samples. The results were tabulated in Table 10.

**Degradation studies**
Results are tabulated in Table 11.

**Acid hydrolysis (Fig. 14)**
Upon performance of acid degradation studies, 25.4% of COB and 20.6% of ATV were degraded.

**Base hydrolysis (Fig. 15)**
Upon performance of base degradation studies, 24.9% of COB and 20.6% of ATV were degraded.

**Peroxide hydrolysis (Fig. 16)**
Upon performance of peroxide degradation studies, 23.8% of COB and 21.9% of ATZ were degraded.

**Thermal degradation (Fig. 17)**
Upon performance of thermal degradation studies, 21% of COB and 21% of ATV were degraded.

**Photolytic degradation (Fig. 18)**
Upon performance of photolytic degradation studies, 28.2% of COB and 24.3% of ATV were degraded.

**Reduction degradation (Fig. 19)**
Upon performance of reduction degradation studies, 20% of COB and 23.7% of ATV were degraded.

**Humidity degradation (Fig. 20)**
Upon performance of humidity degradation studies, 26.0% of COB and 25.0 % of ATV were degraded.

**Hydrolysis degradation (Fig. 21)**
Upon performance of hydrolysis degradation studies, 28.3% of COB and 26.5% of ATV were degraded.

**Table 10: System suitability parameters of cobicistat and atazanavir**

| Parameters | Values obtained (n=6) | | Acceptance criteria |
|---|---|---|---|
| | Cobicistat | Atazanavir | |
| Plate count | 5115 | 5408 | NLT 2000 |
| Tailing factor | 1.02 | 0.97 | NMT 2 |
| $R_t$ (min) | 3.628 | 6.128 | For information |
| Resolution | 0 | 9.19 | NLT 1.5 |

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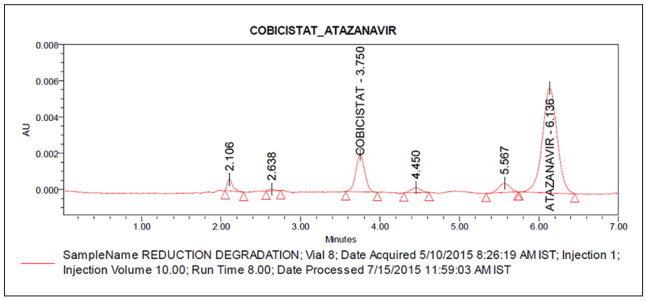


**Fig. 19: Chromatogram of reduction degradation**

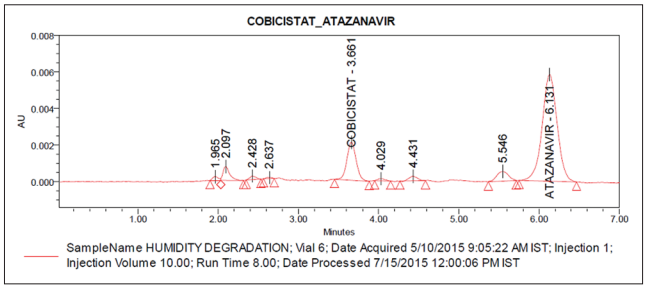


**Fig. 20: Chromatogram of humidity degradation**

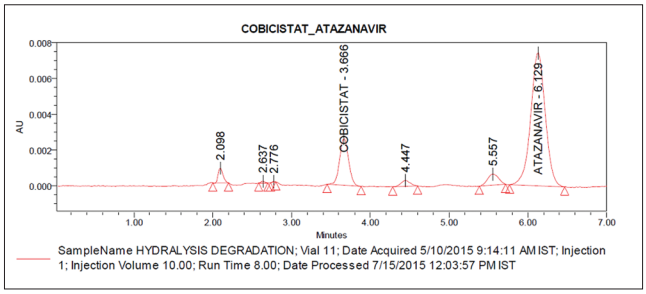


**Fig. 21: Chromatogram of hydrolysis degradation**

**Table 11: Stability studies for cobicistat and atazanavir sulfate**

| Experiment | Drugs | %Assay | %Degradation | %Mass balance | Purity angle | Purity threshold |
|---|---|---|---|---|---|---|
| Acid (5N HCL) | Cobicistat | 74.6 | 25.4 | 99.87 | 39.402 | 90 |
| | Atazanavir sulfate | 79.4 | 20.6 | 99.50 | 1.374 | 3.154 |
| Alkali (5N NaOH) | Cobicistat | 75.1 | 24.9 | 99.90 | 37.678 | 90 |
| | Atazanavir sulfate | 77.3 | 22.8 | 100.06 | 1.402 | 3.32 |
| Peroxide | Cobicistat | 76.2 | 23.8 | 99.83 | 36.888 | 90 |
| | Atazanavir sulfate | 77.9 | 21.9 | 99.75 | 1.42 | 3.5 |
| Reduction | Cobicistat | 80 | 20 | 99.90 | 41.151 | 90 |
| | Atazanavir sulfate | 76.3 | 23.7 | 99.95 | 1.258 | 2.973 |
| Thermal | Cobicistat | 79 | 21 | 99.99 | 40.642 | 90 |
| | Atazanavir sulfate | 79 | 21 | 99.95 | 1.244 | 3.147 |
| Photolytic | Cobicistat | 71.2 | 28.2 | 99.22 | 39.025 | 90 |
| | Atazanavir sulfate | 75.7 | 24.3 | 99.95 | 1.206 | 2.957 |
| Humidity | Cobicistat | 74 | 26 | 99.90 | 40.584 | 90 |
| | Atazanavir sulfate | 75 | 25 | 99.95 | 1.404 | 3.32 |
| Hydrolysis | Cobicistat | 71.7 | 28.3 | 99.88 | 40.972 | 90 |
| | Atazanavir sulfate | 73.5 | 26.5 | 99.95 | 1.102 | 2.782 |

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## CONCLUSION

A simple, rapid, accurate, and precise stability-indicating HPLC analytical method has been developed and validated for the routine quantitative analysis of COB and ATV sulfate in API and combined dosage forms. The results of stress testing undertaken according to the ICH guidelines reveal that the method is specific and stability-indicating. The proposed method has the ability to separate these drugs from their degradation products in tablet dosage forms and hence can be applied to the analysis of routine quality-control samples and samples obtained from stability studies.

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