Method development and validation for Cabotegravir and Rilpivirine by using HPLC and its degradants are characterized by LCMS and FTIR

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Abstract

**Background:** Using a Symmetry C18 (4.6 × 150 mm, 3.5) column, a high-performance liquid chromatographic method for quantification of Rilpivirine and Cabotegravir in active pharmaceutical ingredients was developed and validated. The mobile phase is made up of buffer, acetonitrile, and 0.1 percent formic acid in a 20:80v/v ratio. The flow rate was kept constant at 1.0 ml/min, and detection was accomplished through absorption at 231 nm with a photodiode array detector.

**Results:** The calibration curve was linear, with a regression coefficient (R2) value of 0.999 and concentrations ranging from 30 to 450 g/ml of Rilpivirine and 20–300 g/ml of Cabotegravir. The method’s LOD and LOQ were 0.375 g/ml, 1.238 g/ml, and 0.25 g/ml, 0.825 g/ml for Rilpivirine and Cabotegravir, respectively.

**Conclusions:** In the forced degradation studies, the degradants were characterized by using LCMS and FTIR. The current application was found to be simple, economical, and suitable, and validated according to ICH guidelines.

**Keywords:** Rilpivirine, Cabotegravir, LCMS, FTIR development, Characterization

**Background**

Cabotegravir is a medicine utilized for the treatment of acquired immunodeficiency syndrome [1, 2]. It is available in tablet form and as an intramuscular injection [3, 4], as well as an injectable blend with Rilpivirine under the brand name Cabenuva. The injection forms are used once every month or every two months. Cabotegravir in combination with Rilpivirine has been shown to treat human immunodeficiency virus type 1 in adults (Human immune virus-1). And, if the virus has not developed resistance to the inhibitors, the combination injection is planned for the treatment of adults who do not have detectable human immune virus levels in their blood despite their current antiretroviral treatment [5, 6] and integrase strand transfer inhibitors [7]. Before beginning injection therapy, the tablets are used to see if a person responds to the medication. The two drugs are the first antiretroviral drugs to be available in an injectable formulation with a long half-life. This implies that rather than a day by day pills, individuals get intramuscular injections month to month.

Rilpivirine, also known as Edurant and Rekambys, is a Tibotec prescription medication which is used to treat human immune virus/acquired immune deficiency syndrome [8]. It is a second-generation non-nucleoside reverse transcriptase inhibitor with a lower side-effect profile, higher strength, and a longer half-life than older non-nucleoside reverse transcriptase inhibitors like efavirenz [9]. The well-known side effects of the injectable formulation include reactions at the injection site (in
up to 84 percent of patients), such as pain and swelling, just cerebral pain [10, 11], and fever [12, 13] or feeling hot. Depressive disorders, insomnia [14, 15], and rashes are less common (less than 10%). Depressive disorders [16], headache, rashes, and insomnia are some of the less common side effects of the tablets. These side effects occurred when Rilpivirine was combined with one or more other anti-human immune virus drugs. Heart rhythm prolongation [17, 18] has been observed at extremely high doses of the drug, but it is not clinically relevant at standard doses. Figure 1 shows structures of Cabotegravir, Rilpivirine, and its degradation products.

**Fig. 1** Structures of Cabotegravir (AP1), Rilpivirine (AP2) and DPs (DP1, DP2, DP3, and DP4)
Methods

Chemicals
Merck Pvt. Ltd. Worli, Mumbai, India, provided water, formic acid, and acetonitrile (High-performance liquid chromatography grade). Spectrum Pharma research solutions Pvt. Ltd., Hyderabad, provided the reference standards and APIs for Rilpivirine and Cabotegravir.

Instrumentation

HPLC
Waters Alliance LC (model 2695) monitored with empowering 2 data handling systems and fitted with asymmetry C18 (150 × 4.6 mm, 3.5µ) and a detector of photodiode array (model 2998) was used for this study.

LC-MS/MS conditions
In the stress degradation study, HPLC was linked to a mass spectrophotometer with the splitter placed before the ESI source, allowing only 35% of eluent to enter. The following were the standard operating source conditions for Cabotegravir and Rilpivirine MS scans on positive ESI mode: the fragmented voltage was set at 80 V, the capillary at 3000 V, the skimmer at 60 V, nitrogen was used as a drying and nebulizing gas (45 psi), and highly filtered nitrogen gas was used as collision gas.

FTIR conditions
FTIR was utilized to get insights concerning the presence of different functional groups like keto, Aldehydes, cyano alcohol, and amides present in the degradation samples. Perkin Elmer range 100 models were utilized with KBr as a dispersion medium to make the specimen pellets. Approximately 1.5–3.5 mg of specimens were mixed with 4.5 mg of KBr for a complete examination.

Buffer preparation
1 mL formic acid is dissolved in 1lt high-performance liquid chromatography-grade water and filtered through 0.45 filter paper.

Mobile phase preparation
After thoroughly mixing buffer and acetonitrile in 20:80 ratios, the mixture was sonicated for 5 min and filtered through a 0.22 m membrane filter. The HPLC analysis was carried out on a reversed phase-HPLC system with isocratic elution mode using a mobile phase of 0.1 percent formic acid and acetonitrile (80:20) on a Symmetry C18 column (150 × 4.6 mm, 3.5) with a flow rate of 1 mL/min and a photodiode array detector at 231 nm. As a diluent, the mobile phase was used.

Standard solution preparation (Rilpivirine 300 g/ml and Cabotegravir 200 g/ml)
Weigh out 300 mg of Rilpivirine and 200 mg of Cabotegravir working standards into a 100 ml volumetric flask, add 70 ml of diluent, sonicated for 30 min to dissolve it, and makeup to the mark with diluent. Transfer 5 ml of this stock solution to a 50 ml volumetric flask and dilute to the desired concentration with diluent.

Chromatographic condition optimization
Different mobile phase combinations were tested to determine theoretical plate count, resolution, tailing, and other system suitability parameters. Finally, the separation was accomplished using a freshly prepared mobile phase composed of buffer: acetonitrile in a 20:80 ratio with a flow rate of 1.0 ml/min. The injection volume of 10 l and the ambient temperature were kept constant throughout the process to obtain the symmetric peak of Rilpivirine and Cabotegravir at 231 nm.

Results

To achieve the best chromatographic conditions, various columns such as C18, C8, and CN-propyl, as well as mobile phases, were tested. The best chromatographic separation occurred on a Symmetry C18 column with acetonitrile and 0.1 percent formic acid in (80:20) mobile phase at a flow rate of 1 ml/min and PDA detection at 231 nm (Fig. 2). Table 1 depicts the optimized chromatographic conditions.

System suitability
Six replicates of a standard solution containing 300 g/ml of Rilpivirine and 200 g/mL of Cabotegravir were used to assess system suitability. The results show that the system's suitability is within the acceptable range. Table 2 and Fig. 3 show the results.

Linearity
The current application's linearity was determined by plotting a graph between concentration and corresponding peak area for Rilpivirine and Cabotegravir over concentration ranges of 30–450 g/ml and 20–300 g/mL, respectively (Fig. 4) For both drugs, the correlation coefficient was found to be 0.999. Table 3 contains a summary of the linearity results.

Limit of Detection (LOD) and Limit of Quantification (LOQ)
The LOD and LOQ for Rilpivirine and Cabotegravir were 0.375 g/mL, 1.238 g/mL, and 0.25 g/mL, 0.825 g/mL, respectively. Table 4 summarizes the findings.
Precision

Repeatability (or) method precision

The percentage of RSD value for six replicate injections of known concentrations of Rilpivirine and Cabotegravir performed on the same day was discovered to be 2%, indicating that the method precision is within the limit. Table 5 summarizes the findings.

Accuracy

Rilpivirine and Cabotegravir were prepared in three concentration levels: 50%, 100%, and 150%. The recovery percentage was found to be within the acceptable range of 98–102 percent. Based on these findings, it was determined that the developed method is precise and accurate. Table 6 summarizes the findings.

Robustness

The current method’s robustness was tested by varying the mobile phase composition and flow rate. The percentage of RSD was discovered to be within an acceptable range. Table 7 contains a summary of the robustness results.

Degradation effects and its characterization

Rilpivirine and Cabotegravir samples were subjected to a variety of stress degradation conditions to observe the drugs partial degradation. The stress studies revealed the conditions under which the drug becomes unstable; these measures can be implemented during formulation to avoid potential instabilities. LC-MS and FTIR are used to characterize these degradation products.

Acid degradation

Initially, no degradant peaks were formed when Rilpivirine and Cabotegravir were studied in 0.1 N HCl. When the acid concentration was increased to 1 N HCl and heated at 60 °C for 30 min, 16.5 percent of Rilpivirine and 15.3 percent of Cabotegravir were degraded, and two degradations were observed. (DP1and DP2) products were formed on acid hydrolysis.
Alkali degradation
Rilpivirine and Cabotegravir were stressed under 0.1 N NaOH, and no degradant products are formed. When the strength of alkali was increased to 1 N NaOH and heated at 60 °C for 30 min, 12.5% of Rilpivirine and 12.1% of Cabotegravir degradation were observed and one degradation (DP3) product was formed on alkali hydrolysis.

Oxidative degradation
Rilpivirine and Cabotegravir were studied in 10% peroxide condition, and no degradant peaks are formed. Then, the strength of peroxide was increased to 30% and refluxed for 3 h, one degradant (DP4) was formed.

Table 3 Results of linearity

| S. no | Rilpivirine | Cabotegravir |
|-------|-------------|--------------|
|       | Concentration (µg/ml) | Area | Concentration (µg/ml) | Area |
| 1     | 30.00       | 270,078      | 20.00       | 143,686 |
| 2     | 75.00       | 637,392      | 50.00       | 358,414 |
| 3     | 150.00      | 1,273,025    | 100.00      | 679,383 |
| 4     | 300.00      | 2,544,502    | 200.00      | 1,311,917 |
| 5     | 375.00      | 3,060,044    | 250.00      | 1,610,677 |
| 6     | 450.00      | 3,643,429    | 300.00      | 1,914,838 |

Table 4 Results of LOD AND LOQ

| Drug   | LOD   | LOQ   |
|--------|-------|-------|
| Rilpivirine | 0.375 | 1.238 |
| Cabotegravir | 0.25  | 0.825 |

heated at 60 °C for 30 min, 12.5% of Rilpivirine and 12.1% of Cabotegravir degradation were observed and one degradation (DP3) product was formed on alkali hydrolysis.
Fig. 5  Forced degradation chromatograms of A acid, B alkali, C control, D peroxide, E photo, F reductive, and G thermal degradation
Fig. 5 continued
Reductive degradation
The first trial of Rilpivirine and Cabotegravir were studied in 10% sodium bisulfate solution, for reduction degradation process, and no degradant was formed. After that, the above solution refluxed for 3 h, and no degradants were formed.

Thermal degradation
For thermal degradation, the sample was exposed at 105 °C for 3 h, and no degradant products are formed. After that, the above solution was refluxed for 3 h, and no degradation peaks were formed.

Photolytic degradation
For the first trial of photolytic degradation, the sample was exposed to UV light for 6Hrs and the exposed sample was analyzed, and no degradants peaks are formed. After that, the exposed sample was refluxed for 3 h, and no degradants peaks were formed.

The details of degradation products and chromatograms are represented in Table 8 and Fig. 5. FTIR spectra show the frequencies (Fig. 6) of all different functional groups like acid, amide, cyano, and keto. The details of FTIR spectra for all degradation products are provided in Table 9.
Table 5 Results of method precision

| S. no | Area of Rilpivirine | Area of Cabotegravir |
|-------|---------------------|---------------------|
| 1     | 2,542,689           | 1,344,264           |
| 2     | 2,553,130           | 1,371,927           |
| 3     | 2,505,117           | 1,355,421           |
| 4     | 2,577,854           | 1,384,891           |
| 5     | 2,591,109           | 1,355,421           |
| 6     | 2,514,689           | 1,391,485           |
| Mean  | 2,547,431           | 1,361,886           |
| SD    | 33,914.023          | 25,865.819          |
| %RSD  | 1.33                | 1.90                |

Table 6 Accuracy results

| Accuracy (%) | Amount of Rilpivirine | % Recovery | Amount of Cabotegravir | % Recovery |
|--------------|-----------------------|------------|------------------------|------------|
| 50           | 150                   | 100.6      | 100                    | 100.9      |
| 100          | 300                   | 99.8       | 200                    | 98.6       |
| 150          | 450                   | 101.0      | 300                    | 100.4      |

Table 7 Results of robustness

| Parameter       | % RSD of Rilpivirine | % RSD of Cabotegravir |
|-----------------|----------------------|-----------------------|
| FP (1.2 ml/min) | 0.36                 | 0.52                  |
| FM (0.8 ml/min) | 0.79                 | 0.33                  |
| OP (88:12)      | 0.64                 | 0.84                  |
| OM (72:28)      | 1.39                 | 1.11                  |

Table 8 Stress degradation results

| Results: % degradation results | Rilpivirine | Cabotegravir | Number of DPs formed |
|-------------------------------|-------------|--------------|----------------------|
| Area                          | % degradation | Area | % degradation |                        |
| Control                       | 2,535,823   | 0   | 1,335,136 | 0.1                  | –                     |
| Reduction                     | 2,277,678   | 12.2 | 1,190,347 | 13.9                 | –                     |
| Base                          | 2,244,102   | 12.5 | 1,173,639 | 12.1                 | DP3                   |
| Acid                          | 2,208,598   | 16.5 | 1,161,321 | 15.3                 | DP1 and DP2           |
| Thermal                       | 2,197,980   | 11.4 | 1,150,247 | 10.9                 | –                     |
| Photo                         | 2,176,871   | 10.2 | 1,138,264 | 12.8                 | –                     |
| Peroxide                      | 2,114,687   | 13.5 | 1,335,136 | 13                   | DP4                   |

**Acid degradation product (DP1) characterization**

The DP1, which was observed under acid degradation conditions. The ESI spectrum showed the most intense [M+H]⁺ ion of m/z-313.69. The MS/MS spectrum of DP1 displayed abundant product ions at m/z-241.23 (loss of C₇H₄N₂O₃), m/z-201.23 (loss of C₁₀H₁F₂NO₂), m/z-143.13 (loss of C₁₂H₁₀N₂O₂). IR spectrum of DP1 showed a peak at 3406.8 cm⁻¹ indicates the presence of hydroxyl group, 3318.2 cm⁻¹ correspondings to N–H stretching, 3162.3 cm⁻¹ indicates the presence of C–H alkene stretching, 1725.3 cm⁻¹ belongs to C=O stretching present in amide and, 1288.6 cm⁻¹, 1051.7 cm⁻¹ indicates the presence of the C–N stretching.

**Acid degradation product (DP2) characterization**

The fragmentation mechanism of DP2 and the MS/MS spectrum showed more intense protonated molecular ion of m/z-299.76 which was noticed under acid conditions. The spectrum displayed abundant product ions at m/z-248.71 (loss of C₃H₃N from m/z-299.76), m/z-155.62 (loss of C₅H₅N₃ from m/z 248.71). IR spectrum of DP2 showed at 3115.3 cm⁻¹, 3327.8 cm⁻¹ indicates the presence of N–H stretching in primary amines, 2236.2 cm⁻¹, 1487.8 cm⁻¹ belongs to C≡N stretching and, 1028.7 cm⁻¹ corresponding to C–N stretching. Finally, the DP2 was characterized as (E)-3-(4-(2-amino pyrimidine-4-ylamino)-3-(chloromethyl)-5-methyl phenyl) acrylonitrile.

**Alkali degradation product (DP3) characterization**

The molecular ion of m/z 384.43 with molecular formula C₂₂H₂₆N₆O which was noticed under alkali condition and fragmented into product ions at m/z-315.27 (loss of C₃H₅NO), m/z-211.22 (loss of m/z C₈H₁₀N₃ from m/z 315.27), and m/z-96.05 (loss of C₇H₆N₂ from m/z 211.22).
Fig. 6  FTIR Spectrums of A Cabotegravir, B Rilpivirine, C DP1, D DP2, E DP3, F DP4
Fig. 7  Mass spectrums of A Cabotegravir, B Rilpivirine, C DP1, D DP2, E DP3, F DP4
Fig. 7 continued
Fig. 7 continued
IR spectrum of DP3 showed at 3489.5 cm\(^{-1}\), 3357.1 cm\(^{-1}\) indicates the presence of N–H stretching, 2257.3 cm\(^{-1}\) belongs to C≡N stretching, 1727.6 cm\(^{-1}\) belongs to C=O stretching in amides, 3222.8 cm\(^{-1}\) C–H alkene stretching and 2897.8 indicates C–H aliphatic stretching.

**Peroxide degradation product (DP4) characterization**

DP4 degradation product for \(m/z\)-282.34 which was noticed under peroxide degradation condition. The product ions formed at \(m/z\)-213.28 (loss of C\(_3\)H\(_2\)NO), \(m/z\)-94.05 (loss of C\(_6\)H\(_5\)N). IR spectrum of DP3 showed at 3488.1 cm\(^{-1}\), 3345.6 cm\(^{-1}\) indicates the presence of N–H stretching, 2368.1 cm\(^{-1}\) belongs to C≡N stretching, 1737.1 cm\(^{-1}\) belongs to C=O stretching in amides, 3228.3 cm\(^{-1}\) C–H alkene stretching, 2868.2 indicates C–H aliphatic stretching, and 1258.3 cm\(^{-1}\), 1087.4 related to C–N stretching. It was characterized as (E)-3-(4-(6-aminopyridine-2-ylamino)-3,5-dimethyl phenyl)acrylamide. Finally, FTIR data clears that the
presence of cyano, primary amide, amine, hydroxyl group, and keto functional groups in DP4.

**Discussion**
We developed a high-performance liquid chromatography method for indicating stability, and forced degradation products are characterized using LC-MS and FTIR under ICH guidelines [19]. Very few articles were reported in the last few decades for determining the Cabotegravir and Rilpivirine by using HPLC [20–22]. In the present study, we intended to explore a specific, sensitive, and new HPLC method towards the analysis of Cabotegravir, Rilpivirine, and characterization of its degradation products by LC-MS and FTIR.

**Conclusion**
Till today there is no HPLC method to estimate the combination of Rilpivirine and Cabotegravir. A single HPLC method was validated and developed under ICH
guidelines for the estimation of these two drugs. All validation parameters are within the limits, including system suitability, method precision, accuracy, LOD, LOQ, and robustness. This method has several advantages, including a shorter run time, a lower cost, accessibility, sensitivity, reliability, and reproducibility. The drugs' degeneration actions were investigated under acid, base, oxidation, reduction, photolytic, and thermal stress conditions. The drugs were discovered to be stable under reduction, thermal, and photolytic conditions, but unstable under acid, alkali, and oxidative conditions. LCMS and FTIR experiments were used to characterize the degradation products.

### Abbreviations

HPLC: High-performance liquid chromatography; LOQ: Limit of quantitation; LOD: Limit of detection; ICH: International Conference on Harmonization; FTIR: Fourier Transform Infrared spectroscopy; MS: Mass spectrometry.

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### Authors’ contributions

Author ‘SMT AND AV’ designed the study, performed the method development and validation, wrote the protocol, and wrote the first draft of the manuscript. Author ‘MR’ and ‘BSNM’ helped in the analyses of the study. Author ‘KAE’ managed the literature searches. All authors read and approved the final manuscript.

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### Availability of data materials

The data for verification are provided with a Supplementary file and the rest. Of the data, if required, will be available upon request.

### Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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### Table 9 FTIR data of Cabotegravir, Rilpivirine, and its degradation products

| S. no | API 1 | API 2 | DP1 | DP2 | DP3 | DP4 | Region (cm\(^{-1}\)) | Assignment |
|-------|-------|-------|-----|-----|-----|-----|-----------------|------------|
| 1     | 3400  | 3502  | 3406.8 | 3515.3 | 3489.5 | 3488.1 | 3500–3300 | N–H stretching |
|       | 3325.4 | 3318.2 | 3327.8 | 3357.1 | 3345.6 |       | 2840–3000 | C–H Aliphatic stretching |
| 2     | 3157.6 | 3161.9 | 3162.3 | – | 3222.8 | 3228.3 | 3200–3000 | C–H Alkene stretching |
| 3     | – | – | – | – | 2897.8 | 2868.2 |       |       |
| 4     | – | 2235.7, 2148.3 | – | 2236.2, 2152.5 | 2257.3 | 2368.1 | 2354 | C≡N Stretching |
| 5     | 1722.8 | – | 1725.3 | – | 1727.6 | 1737.1 | 1680–1630 | C=O Stretching (Amides) |
| 6     | – | – | – | 1487.8 | – | – | 1600–1450 |       |
| 7     | 1287.8 | 1025.5 | 1288.6 | 1028.7 | 1287.9 | 1258.3 | 1350–1000 | C–N Stretching |
|       | 1049.3 | 1051.7 | 1057.1 | 1087.4 |       |       |       |       |
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