Development and Validation of Stability-Indicating UPLC-TUV Method for Simultaneous Estimation of Darunavir and Ritonavir in Bulk and Tablet Dosage Form

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ABSTRACT

A straightforward, explicit, accurate and monetary ultra-performance liquid chromatographic with tunable ultraviolet indicator (UPLC-TUV) strategy was produced for the simultaneous estimation of Darunavir and Ritonavir in bulk and tablet dosage form. The Separation was accomplished on a BEH C18 section (4.6 mm X 50 mm, 5 μm) at a frequency of 270 nm, utilizing a mobile phase acetonitrile and water (50:50 V/V) in an isocratic elution mode at a stream pace of 0.3 mL/min. The maintenance time for Darunavir and Ritonavir was discovered to be 0.739 min and 0.401 min, individually. The proposed strategy was validated for precision, linearity range, accuracy, roughness, and constrained degradation concentrates according to ICH rules. The adjustment bends of Darunavir and Ritonavir were linear over the scope of 100-600 μg/mL and 12.5 to 75 μg/mL. The LOD's were discovered to be 1.93 and 0.03 for Darunavir and Ritonavir, separately. The LOQ's were discovered to be 5.84 for Darunavir and 0.08 for Ritonavir. The strategy was discovered to be precise and stability-showing has no meddling pinnacles of debases and excipient were noticed. The created technique was appropriate for quality-control labs for quantitative examination of both in bulk and joined dose structure. The created strategy is new and better in innovation when contrasted than the announced techniques and less maintenance time, high theoretical plate check and structure of the mobile phase with great division, precise and stability-showing has no meddling pinnacles of debases and excipient were noticed. The technique was appropriate for quality-control research centers for quantitative examination of both in bulk and consolidated measurements structure.

INTRODUCTION

Ritonavir (RIT) is synthetically called as 1,3-thiazol-5-yl methyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-[(2-propane-2-yl-1,3-thiazol-4yl) methyl] carbamoyl] amino]-1,6-diphenyl hexan-2-yl] carbamate. This is utilized as the inhibitor of the HIV-protease enzyme. This is one of the best unpredictable inhibitor. It is as currently very less used for its antiviral properties; however, it still is broadly used as a promoter of other inhibitors of protease. All the high explicitly,
RIT is utilized to repress a specific liver compound that ordinarily processes proteases inhibitors, cytochromes P450-3A4 (CYP3A4). The atomic drug structure strains CYP3A4; consequently, a low portion can be used to elevate other proteases inhibitors (Yekkala et al., 2008; Pathak and Rajput, 2009). The compound structure of RIT is appeared in Figure 1.

Figure 1: Chemical Structure of Ritonavir

Darunavir (DAR) is chemically known as (3R, 3aS, 6aR)-hexahydrofuro[2,3-b]furan-3-yl[(2S,3R)-4-(4-amino-N-isobutyl phenyl sulfonamide)-3-hydroxy-1-phenylbutazone-2-yl]carbamate. It is an HIV-protease inhibitor that inhibits replication of HIV by interacting with the compound's dynamic site, along these lines inhibiting the dimerization and the reactant action of the HIV-1 protease. DAR specifically represses the breakage of HIV encoded Gag-Pol polyproteins in infection contaminated cells, which prevents the development of developing irresistible infection particles (Behera et al., 2011). The chemical structure of DAR is shown in Figure 2.

Figure 2: Chemical moiety of Darunavir

Stability testing and stresses testing (constrained degradation contemplates) are basic parts of medication advancement system. These investigations help us to comprehend the system of a medication's decomposition, which further aids in getting data on physical and compound calculates that outcome instability. These elements are then controlled to balance out the medication or medication plan, bringing about expanded shelf-life or improved viability (Ghosh et al., 2007). Stress testing is characterized as the stability testing of medication substances and medication items under conditions surpassing those utilized for quickened testing. As per International Conference on Harmonization (ICH) rule Q1A (R2), the stability testing of medication substances ought to be done under various pressure conditions (hydrolysis, oxidation, photolysis, and warm degradation) to validate the stability-demonstrating matchless quality of logical strategies utilized for the examination of stability tests (International Conference on Harmonization, 2003). The standard conditions for photograph stability testing are portrayed in ICH rule Q1B (International Conference on Harmonization, 1996). These tests permit exact and exact evaluation of medications and their degradation and interaction items.

A literature survey revealed that there are several methods for the determination of DAR (Babu et al., 2013; Eswarudu et al., 2018) alone and DAR combined with other drugs also reported (Nalini et al., 2016; Fayet et al., 2009). The different analytical methods for the estimation of RIT (Behera et al., 2011; Koppala et al., 2015) alone and combined with other drugs have been reported (Mardia et al., 2012; Saritha et al., 2013). There are very few analytical methods for the simultaneous determination of DAR and RIT estimation are reported (Damle and Deosthali, 2016; Prathap et al., 2018). Not a solitary report has been found in regards to any explanatory UPLC-TUV technique for the estimation of DAR and RIT drug mix simultaneously in bulk drug and pharmaceutical plan alongside validated stability-demonstrating information (Rao et al., 2016; Gupta et al., 2011).

Henceforth, we endeavoured to create stability-showing explicit, delicate, accurate and precise UPLC-TUV strategy for estimation of these medications simultaneously in isocratic elution method (Prathap et al., 2018). The extent of the current work is to grow the streamlining of the chromatographic conditions to discover the level of degradation items accurately and precisely at any runtime utilizing UPLC-TUV which in another manner is more explicit and furthermore spares time and dissolvable. Furthermore, stress testing of the medication substance can help recognize
the feasible degradation items, which can thusly assist with setting up the degradation pathways and the natural stability of the atom and validate the stability-demonstrating intensity of the investigative systems utilized.

MATERIALS AND METHODS

Instrumentation
The partition was continued Waters Acquity UPLC-TUV 2996 with Empower 2 programming that comprised of a paired dissolvable manager outfitted with a programmed sampler. An Acquity UPLC BEH C18, section (4.6 mm X 50 mm X 5 μm) was utilized for detachment of dynamic fixings. Analytes were screened with a TUV locator at a frequency of 270 nm. Ultra-sonicator was utilized to eliminate imbibed gases and gas rises in the mobile phase.

Chemicals and Reagents
Active pharmaceutical ingredients of DAR and RIT are acquiring as blessing tests from Glenmark Laboratories, Mumbai, India. HPLC grade acetonitrile, HPLC grade water or same and the whole synthetics (AR Grade) used in the study was procured from Merck (Mumbai, India). Pharmaceutical tablet dosage form, i.e., Durant-R-450 (400 mg of DAR and 50 mg of RIT) was purchased from a local pharmacy.

Chromatographic Conditions
UPLC was performed on Acquity UPLC BEH C18 section (4.6 mm X 50 mm, 5 μm) segment and the mobile phase comprised of Acetonitrile (ACN) and water (50:50 V/V) which siphoned at a stream rate equivalents to 0.3 mL/min at 30°C. The mobile phase was sifted through a 0.2 μ film channel and afterwards sonicated for 5 min. The volume of infusion was 0.30 μL and runtime was 3 min. The eluents were recognized at a frequency of 270 nm.

Standard Solutions of stock preparation
Accurately measured 100 mg of DAR and 12.5mg of RIT and moved to singular 50 mL volumetric flasks independently. The $\frac{3}{4}$ th of mixes were mixed in both flasks and were vortexed for 10 min. The flasks were comprised of mixes and marked as standard arrangement 1 and 2 (2000 μg/mL of DAR and 250 μg/mL of RIT). 1 mL from each stock arrangement was pulled out and poured into a 10ml flask and made up with diluents (400 μg/mL for DAR and 50 μg/mL for RIT).

Sample Solutions for stock preparation
Ten tablets were weighed and moved into a 100 mL volumetric flask, 50 mL of diluents was mixed in both flasks and were vortexed for 10 min, further, the volumes were made up with diluents and separated by HPLC channels (4000 μg/mL for DAR and 500 μg/mL for RIT). The 0.5 mL of sifted test stock arrangement was moved to a 10ml flask and added up with diluents (400 μg/mL for DAR and 50 μg/mL for RIT).

Validation of method
The proposed technique was confirmed according to ICH rules regarding Specificity, Linearity, Precision, exactness and constrained degradation considers.

System suitability tests
System suitability tests were utilized to check that the goal and reproducibility were sufficient for the performed investigation. The system suitability tests incorporated the number of hypothetical plates, goal, top following, limit factor and selectivity factor.

Specificity and Sensitivity
The specificity is the capacity of an explanatory technique to analyse asymmetrically the analyte of interest within the segment sight that is required to be available, in the example framework.

Linearity (Calibration curve)
To develop adjustment plots, the stock standard arrangements were weakened with diluents to get ready working standard arrangements in the concentration scope of 100-600 μg/mL for DAR and 12.5 to75 μg/mL for RIT. Every arrangement (n=6) was infused in three-fold and chromatographed under the previously mentioned conditions. Linear connections were acquired.

At that point, normal medication standard pinnacle regions were plotted against the comparing concentrations for each medication. A regression equation was determined Figures 3 and 11.

Limits of Detection and Quantification (LOD and LOQ)
The limits of detection are the place where an expected worth is greater than the weakness related to it. It is minimal measure of an analyte in a model that can be distinguished anyway not generally assessed.

The restriction of assessment is the most negligible injected entirety that produces quantitative estimations in the target framework with agreeable precision in chromatography. As far as possible is particularly used for the assurance of contaminations and debasement things.

Precision
The precision of a logical system is the degree of comprehension among singular test results when...
the strategy is applied reliably to different looking at a homogenous model. The precision of the logical procedure is ordinarily imparted as the standard deviation or relative standard deviation (coefficient of assortment) of a movement of estimations. For methodology precision, six rehashes of test plans were implanted into a chromatographic framework and decided the rate test and rate RSD.

**Accuracy**
The accuracy of an insightful method is the closeness of test results got by that procedure to the veritable worth. The accuracy of the strategy was passed on by choosing the recovery learns at three distinctive concentration levels (50%, 100%, and 150%) in three-overlay.

**Forced degradation of DAR and RIT**
Constrained degradation considers were led to determine the stability of the technique. The lowering examines were completed by satisfying different stress conditions for the item like corrosive stress, base stress, oxidation stress, dry warmth stress, UV stress, and unbiased stress.

**Acidic degradation**
To 1 mL of 2N hydrochloric acid was added to 1 mL of the stock solution of DAR and RIT. At that point, the solution was refluxed for 30 mins at 60 °C.

The resultant solution was weakened to get 400 µg/mL for DAR and 50 µg/mL for RIT solution. The 0.3 µL solutions were infused into the framework and the chromatograms were recorded to assess the steadiness of the model (Figure 4).

**Alkali Degradation**
One mL of 2N sodium hydroxide was added to 1 mL of the stock solution of DAR and RIT. At that point, the solution was refluxed for 30 mins at 60 °C.

The resultant solution was weakened to get 400 µg/mL for DAR 50 µg/mL for RIT, individually. The 0.3 µL solutions were infused into the framework and the chromatograms were recorded to assess the steadiness of the model (Figure 5).

**Oxidation Stress**
To 1 mL of 20% hydrogen peroxide (H2O2) was added independently to 1 mL of stock solution of DAR and RIT. The solutions were saved for 30 min at 60 °C. For UPLC study, here resultant solution was weakened to acquire 400 µg/mL for DAR and 50 µg/mL for RIT, individually. The 0.3 µL solutions were into the framework and the chromatograms were recorded to assess the steadiness of the model (Figure 6).

**Dry-Heat Degradation**
The standard medication solution was put in a stove at 105 °C for six h to consider dry warmth degradation. For the UPLC study, the resultant solution was weakened to 400 µg/mL for DAR and 50 µg/mL for RIT solution. The 0.3 µL solutions were infused into the system. At that point, the chromatograms were recorded to survey the stability of the example (Figure 7).

**Photo Stability studies**
The photochemical stability of the medication was additionally concentrated by uncovering the (400 µg/mL for DAR and 50 µg/mL for RIT solutions to the UV light by saving the measuring glass in UV-Chamber for 3 days or 200-Watt hours/m2 in
photograph stability chamber. For the UPLC study, the resultant solutions (0.3 μL) were infused into the system. At that point, the chromatogram was recorded to survey the stability of the example (Figure 8).

Study of Neutral Degradation

The stability testing in nonpartisan conditions was concentrated by refluxing the medication in water for 6hrs at a temperature of 60ºC. For the UPLC study, the resultant solution was weakened to 400 μg/mL for DAR and 50 μg/mL for RIT, individually. The 0.3 μL of solutions were infused into the framework and the chromatograms were recorded to assess the steadiness of the model (Figure 9).

RESULTS
Method Progress and Optimization

The primary objective of the UPLC-TUV method is to separate DAR, RIT and the degradation product generated from the analyte during stress studies. The best peak shape and maximum separation were achieved with the mobile phase composition of acetonitrile and water (50:50% V/V) peak symmetry and reproducibility was obtained on UPLC BEH C18 column (4.6 mm X 50 mm, 5 μm). The optimum wavelength for detecting the analyze was found to be 270 nm, a flow rate of 0.3 mL/min yielded the optimum differentiation and symmetry of the peak. (Table 1)

The system suitability test was performed by infusing six duplicates of standard solution into the chromatograph and the chromatograms were recorded. The overall standard deviation of the zone for individual tops, for six, reproduce infusions of standard solution, ought to be under 2.0%. The following component for the DAR and RIT pinnacles ought to be under 2.0. The hypothetical plates for the DAR and RIT pinnacles ought not to be under 2000. The outcomes acquired for hypothetical plates, USP following element, were all well inside satisfactory limits (Table 2).

Specificity is checked in every examination by analyzing clear and fake treatment tests for any meddling pinnacles. The specificity of the technique was assessed as to obstruction because of the indication of some other excipients. The figures indicate that the chose drugs were unmistakably isolated. There were no meddling tops at the maintenance season of RIT and DAR.

Method Validation

Linearity (Calibration Curve), LOD and LOQ

The alignment plot was linear over the concentration range examined (100-600 μg/mL) for DAR, (12.5-50 μg/mL) for RIT, individually. From regression examination, normal relationship coefficient
Table 1: Optimized Chromatographic Conditions

<table>
<thead>
<tr>
<th>Optimized Chromatographic Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
</tr>
<tr>
<td>Mobile-phase</td>
</tr>
<tr>
<td>Flow rate</td>
</tr>
<tr>
<td>Column temperature</td>
</tr>
<tr>
<td>Injection volume</td>
</tr>
<tr>
<td>Detection wavelength</td>
</tr>
<tr>
<td>Run time</td>
</tr>
</tbody>
</table>

Table 2: System suitability parameter

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ritonavir</th>
<th>Darunavir</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time</td>
<td>0.400</td>
<td>0.736</td>
<td>For information</td>
</tr>
<tr>
<td>Plate count</td>
<td>2330.2</td>
<td>5153.4</td>
<td>NLT 2000</td>
</tr>
<tr>
<td>Tailing</td>
<td>1.4</td>
<td>1.4</td>
<td>NMT 2.0</td>
</tr>
<tr>
<td>Resolution</td>
<td>-</td>
<td>8.8</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3: Optical Characteristic of ritonavir and darunavir

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ritonavir</th>
<th>Darunavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µg/mL)</td>
<td>12.5-75</td>
<td>100-600</td>
</tr>
<tr>
<td>Regression equation</td>
<td>Y = 2488X + 743</td>
<td>Y = 2065X + 4365</td>
</tr>
<tr>
<td>Slope</td>
<td>2488</td>
<td>2065</td>
</tr>
<tr>
<td>Intercept</td>
<td>743</td>
<td>4365</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9999</td>
<td>0.999</td>
</tr>
<tr>
<td>LOD-(µg/mL)</td>
<td>0.030</td>
<td>1.93</td>
</tr>
<tr>
<td>LOQ-(µg/mL)</td>
<td>0.080</td>
<td>5.84</td>
</tr>
</tbody>
</table>

Table 4: Method precision studies of ritonavir and darunavir

<table>
<thead>
<tr>
<th>S.No</th>
<th>Ritonavir</th>
<th>Darunavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>126099</td>
<td>859465</td>
</tr>
<tr>
<td>2</td>
<td>126805</td>
<td>850520</td>
</tr>
<tr>
<td>3</td>
<td>126445</td>
<td>851042</td>
</tr>
<tr>
<td>4</td>
<td>126517</td>
<td>855424</td>
</tr>
<tr>
<td>5</td>
<td>126911</td>
<td>850149</td>
</tr>
<tr>
<td>6</td>
<td>126832</td>
<td>854764</td>
</tr>
<tr>
<td>Average</td>
<td>126602</td>
<td>853561</td>
</tr>
<tr>
<td>SD</td>
<td>308.0</td>
<td>3660.9</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.2</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The regression results demonstrate that the proposed strategy was linear in the concentration range contemplated and can be utilized for the detection and evaluation of RIT and DAR in an exceptionally wide concentration range. (Table 3)

Precision

During method precision, six samples were prepared as per the analytical method. The %RSD for samples was calculated. (Table 4)
### Table 5: Accuracy studies of ritonavir and darunavir

<table>
<thead>
<tr>
<th>Drug name</th>
<th>% recovery level</th>
<th>Pre-analysed conc (ppm)</th>
<th>Added amount (ppm)</th>
<th>Recovered Amount (ppm)</th>
<th>% of Recovery</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ritonavir</td>
<td>50</td>
<td>50</td>
<td>25</td>
<td>25.217</td>
<td>100.87</td>
<td>0.53</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>50</td>
<td>50</td>
<td>50.391</td>
<td>100.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>50</td>
<td>75</td>
<td>74.873</td>
<td>99.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Darunavir</td>
<td>50</td>
<td>400</td>
<td>200</td>
<td>199.500</td>
<td>99.75</td>
<td>0.56</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>400</td>
<td>400</td>
<td>397.642</td>
<td>99.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>400</td>
<td>600</td>
<td>595.718</td>
<td>99.21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 6: Result of forced degradation studies for ritonavir and darunavir

<table>
<thead>
<tr>
<th>Stress Conditions</th>
<th>Ritonavir (%) Degradation</th>
<th>Darunavir (%) Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid</td>
<td>6.61</td>
<td>6.05</td>
</tr>
<tr>
<td>Base</td>
<td>4.57</td>
<td>4.73</td>
</tr>
<tr>
<td>Peroxide</td>
<td>4.57</td>
<td>4.50</td>
</tr>
<tr>
<td>Thermal</td>
<td>1.23</td>
<td>1.03</td>
</tr>
<tr>
<td>Photolytic</td>
<td>1.18</td>
<td>1.46</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>0.43</td>
<td>1.46</td>
</tr>
</tbody>
</table>

### Accuracy

Accuracy as recuperation was assessed by the recently investigated test solution at three distinctive concentration levels (50%, 100%, and 150%). The recovery of as of late inspected test arrangement drug concentration added was found to be 99.45% for RIT and 100.49% for DAR and with the estimation of RSD under 1% exhibiting that the proposed strategy was precise for the concurrent assessment of the two prescriptions from their mix drug things in the presence of their degrading items. (Table 5)

### Forced Degradation Studies

Degradation contemplates were performed under various conditions and there was no stamped degradation besides in acid stress. The degradation contemplates inferred that there is no obstruction of degradants with the analytes top. An acid degradation study was performed. 6.61% of RIT and 6.05% of DAR were degraded. Alkali degradation study was performed 4.57% of RIT and 4.73% of DAR were degraded. (Figure 10)
DISCUSSION

The created technique is new and better in innovation when contrasted than the announced strategies and less maintenance time, high hypothetical plate check and organization of the mobile phase with great division, precise and stability-demonstrating has no meddling pinnacles of corrupts and excipient were noticed. The strategy was appropriate for quality-control labs for quantitative examination of both in bulk and consolidated measurement structure (Deshpande and Butle, 2015)

CONCLUSIONS

An epic UPLC-TUV technique was created for the simultaneous estimation of the RIT and DAR in a pharmaceutical dosage form. The maintenance season of RIT and DAR was discovered to be 0.400 min and 1.736 min. The %RSD of the RIT and DAR were and discovered to be 0.2 and 0.4 separately. % Recovery was gotten as 100.78% and 99.41% for RIT and DAR individually. LOD, LOQ values got from regression equations of RIT and DAR were 0.03, 0.08 and 1.93, 5.84 separately. Regression equation of RIT is y = 2488x + 743 and y = 2065x + 4365 of DAR. Maintenance times were diminished and that run time was diminished, so the technique created was basic and conservative that can be received in standard quality control tests in Industries.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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