A Novel Ultra Performance Liquid Chromatography-PDA Method Development and Validation for Darunavir in Bulk and Its Application to Marketed Dosage Form

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Aims and Objective: The aim of this study was to develop and validate a novel ultra-performance liquid chromatographic method for estimation of darunavir in a bulk and tablet dosage form. Materials and Methods: The chromatographic separation was achieved using DIKMA Endoversil (2.1 mm x 50 mm, 1.7 µm) column. A mixture of 40% buffer (0.1% octa sulfonic acid) and 60% acetonitrile was used as a mobile phase with the isocratic elution mode and eluent was monitored at 281 nm using UV detector. The method was continued and validated in accordance with International Conference on Harmonization Guidelines. Validation study revealed the specificity and reliability of the method. Results: In this method, darunavir was eluted with retention time of 0.516 min. Calibration curve plots were found linear over the concentration ranges 10–50 µg/mL for darunavir. Limit of detection was 0.02 µg/mL and limit of quantification was found 0.07 µg/mL. The present method was also found stable in force degradation study. Conclusion: The empirical evidences of all the study results revealed the suitability of the estimation of darunavir in bulk and tablet dosage form without any interference from the excipients.

Keywords: Darunavir, ICH guidelines, method development, method validation, UPLC

INTRODUCTION

Darunavir (C27H37N3O7S) is a recommended treatment option for adults and adolescents, regardless of whether they have received HIV treatment in the past as recommended by the Office of AIDS Research Advisory Council.[1] In a study of patients who had never received HIV treatment, darunavir (chemical structure as shown in Figure 1) was as effective as lopinavir/ritonavir at 96 weeks with a once-daily dosing.[2] It was approved by the FDA on October 21, 2008 for people not previously treated for HIV.[3] As with other antiretrovirals, darunavir does not cure HIV/AIDS.[4] Darunavir is a nonpeptidic inhibitor of protease (PR) that lodges itself in the active site of PR through a number of hydrogen bonds.[5] Darunavir is an HIV protease inhibitor[6,7] which prevents HIV replication by binding to the enzyme’s active site, thereby preventing the dimerization and the catalytic activity of the HIV-1 protease.[8-10] The extensive review of literature reveals that very few methods are available for the estimation of darunavir,[11-15] and even it was not included in any pharmacopoeia. Among the reported methods, one reported that high-performance liquid chromatography (HPLC) method by Patel et al. used 40:60 v/v ratio of water:acetonitrile and reported 5.02 min retention time with 0.38 µg/mL as a quantitation limit. They also reported 2–20 µg/mL as

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a linearity range. Another reported RP-HPLC method by Satyanarayana et al. where they reported 5.859 min as a retention time and used mixture of buffers in 40 volume and acetonitrile in 60 volume. The reported peak shape by them was not satisfactory. The reported stability indicating HPLC method by Correa et al. showed 6–21 µg/mL as a linearity range, which was considered a narrow range. They reported 8 min as a elution time for darunavir, which was considered too long and even the peak shape was not satisfactory. Their reported quantitation limit was also quite high, that is, 2.89 µg/mL. The darunavir estimation by Chaudhary et al. reported 11.8 min elution time for darunavir, which was really too long time required for the estimation. Their reported linearity range was also narrow, that is, 1–30 µg/mL. There are also few reported UPLC methods[16,17] available for the simultaneous estimation of darunavir with ritonavir and atazanavir in tablet dosage form and in human plasma. Only one reported UPLC method[18] for darunavir related to our study used for the estimation of it along with four impurities. They reported the use of gradient elution and a long retention time of 9.5 min for the darunavir. After considering the above drawbacks from the existing method, efforts were taken to develop stability indicating estimation method for darunavir using ultra-performance liquid chromatography (UPLC). The authors successfully resolved the drawbacks in this method by significantly reducing the retention time and increasing the linearity range with a high-resolution optimized chromatogram of darunavir. The present method was also considered sensitive as the detection and quantitation limit were very less in comparison to other reported methods. Therefore, the present research work describes simple, stability indicating, accurate, specific, robust, rugged and rapid UPLC method and its subsequent validation study as per International Conference on Harmonization (ICH) Guidelines Q2 (R1),[19,16] for the estimation of Darunavir in bulk drug and in its dosage forms.

**Materials and Methods**

**Chemicals and reagents**

Pharmaceutical grade working standards darunavir (99.97%) was obtained as a gift sample from Cipla Pharmaceutical Pvt Ltd., Verna Industrial Estate, Goa, India. The tablets of darunavir were obtained from Local Market of Hyderabad. All chemicals and reagents were required for the method development and validation and stability studies were purchased from Finer Chemicals Ltd, Fisher Scientific and Merck.

**Instrumentation conditions**

The analysis was performed using UPLC Acquity Waters, PDA detector. Software: Empower 2 equipped with auto sampler and PDA detector, analytical balance 0.1 mg Sensitivity (Afcoset ER-200A), pH meter (Adwa – AD 1020), Ultra Sonicator. The column used was DIKMA Endoversil (2.1mm x 50mm, 1.7 µm) UPLC Column with a flow rate of 0.3mL/min (isocratic) Detection was carried out at 281 nm

**Preparation of 0.1% octa sulfonic acid**

Accurately 1 g of octa sulfonic acid was taken in a 1000-mL volumetric flask, dissolved, and diluted to 1000 mL with UPLC water, and the volume was adjusted to pH 3.0 with orthophosphoric acid.

**Preparation of mobile phase**

Accurately 400 mL (40%) of the above buffer and 600 mL of acetonitrile HPLC grade (60%) were mixed and degassed in an ultrasonic water bath for 10 min and then filtered through 0.45 µ filter under vacuum filtration.

**Standard solution preparation**

Accurately weigh 25 mg of darunavir working standard and transferred into a 25-mL clean dry volumetric flask, add about 10 mL of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (stock solution). Further pipette out 0.3 mL of the above stock solution and transferred into a 10 mL volumetric flask and dilute up to the mark with diluent to obtain 30 µg/mL of darunavir.

**Assay of marketed dosage form**

The weight of five tablets were measured, triturated in a Mortar-Pestle. Transfer the tablet powder equivalent to 25 mg darunavir (marketed formulation = 132.5 mg of tablet powder) sample into a 25-mL clean dry volumetric flask add about 10 mL of diluent and sonicate it up to 30 min to dissolve completely and make volume up to the mark with the same solvent. Then it was filtered through 0.44 micron injection filter which was considered as stock solution. Further pipette out 0.3 mL of the solution from the above stock solution and transferred into a 10 mL volumetric flask and dilute up to the mark with diluent. Inject 10 µL of the standard and sample solution in triplicate into the chromatographic system and measure the areas for darunavir peaks and calculate the assay percentage.

**Method validation**

**Specificity**

It was carried out using placebo interference test of the sample solution using 500 mg of placebo equivalent to one tablet dissolved in 100 mL of mobile phase and the
placebo solution was treated like a standard solution. The solution was injected into the chromatographic system to evaluate the possible interfering peaks.

**System suitability**

This study was carried out to justify whether the analytical system is working properly. It was carried out by injecting the six replicates of the standard solution of darunavir. The %RSD of the various optimized parameters such as theoretical plates, peak area, retention time, and asymmetric factor were calculated.

**Accuracy**

To justify the accuracy of the developed method, a recovery study was conducted at various levels (80%, 100%, and 120%) of pure darunavir. The various amounts of standard darunavir were added to a fixed concentration to darunavir tablet sample solution to achieve the various levels. This study was carried out three times and the percentage recovery as well as percentage mean recovery were calculated.

**Intra-day and inter-day precision**

The precision of the method was studied by determining the 100% concentration darunavir solution. It was evaluated by analyzing the six sample solutions in triplicate (n = 6) of 10 µg/mL of darunavir solution. The intra- and inter-day precision was determined by analyzing for six times on the same day (intra-day study) and repeated on the second and third day (inter-day study). The chromatograms were recorded. The peak area and retention time of darunavir were determined and relative standard deviation (RSD) was calculated.

**Detection and quantitation limit**

The limit of detection was defined as the concentration for which a signal-to-noise ratio of 3 was obtained and for quantitation limit, a signal-to-noise ratio of 10 was considered. Standard solution of darunavir was prepared by sequential dilution and injected into the chromatographic system in decreasing order of concentration in the range of 0.01–10 µg/mL of darunavir.

**Linearity**

To carry out the linearity, working standard solutions of darunavir were prepared as described earlier; aliquot from these solutions was diluted with the mobile phase in five different concentrations to 1–50 µg/mL of darunavir. Calibration curve was plotted for darunavir by considering concentration versus peak area, obtained data was subjected to regression analysis.

**Robustness**

Robustness of the developed method was studied by deliberately changing the chromatographic conditions. Six sample solutions were prepared and analyzed in triplicate under the established condition by varying the analytical conditions such as flow rate, mobile phase ratio, and detection wavelength at three different levels. One factor was changed at one time to estimate the effect. All the optimized parameter was found within the limit. For the calculation of %RSD, the tailing factor was considered.

**Force degradation study of darunavir**

Force degradation study was carried out by following different ICH prescribed stress conditions such as acidic, basic, oxidative, thermal, and photolytic stress conditions. All the types of degradation studies have been performed in triplicate and the mean peak area has been considered for the calculation.

**Acid degradation**

The acid degradation study was performed in environmental test chamber (Acamus Technologies, India) at 60°C and 75% relative humidity using 1 M HCL. 0.3 mL of stock solution (1 mg/mL) was taken in 10 mL of volumetric flask. 0.3 mL of 1 M HCL was added to the flask, then kept in an environmental test chamber for 16 h. Then the stress period solution was neutralized using 1 M NaOH and made up the volume up to mark with mobile phase.

**Base degradation**

Base degradation study was performed under the condition of 60°C and 58% relative humidity using the same environmental chamber. 0.3 mL of stock solution was taken in 10 mL volumetric flask mixed with 1 M of 0.3 mL of 1 M NaOH for 16 h. After the suitable stress period, the solution was neutralized with 1 M HCL and made up the volume up to mark with mobile phase.

**Oxidative degradation**

It was performed in versatile environmental chamber at 40°C, 75% relative humidity using 6% H₂O₂. For this purpose, 0.3 mL of stock solution was taken in 10-mL volumetric flask and 0.3 mL of 6% H₂O₂ was added into flask and kept at 60°C for 16 h. Finally, the volume was made up to mark with the mobile phase.

**Photolytic degradation**

This study was carried out in sunlight (60,000–70,000 lux) during day time and in UV light at 254 nm for the
period of 48h. 0.3 mL of stock solution was taken in 10 mL volumetric flask and made up the volume up to the mark with mobile phase was used for the study.

RESULT AND DISCUSSION

Method optimization
Initially, the various mobile phases were tried with different ratios of buffer and organic solvents, for example, methanol and acetonitrile. Finally, the mobile phase was optimized with 0.1% octasulfonic acid buffer (pH 3.0) and acetonitrile in a proportion of 40: 60 v/v. Like mobile phase, various columns such as C18 column, BEH column, phenomenex, and endoversil column were tested for optimized separation. Finally, endoversil (2.1 cm x 50 mm, 1.7 μm) was found to be ideal as it showed good peak shape and parameters in the acceptable range at the flow rate of 0.3 mL/min at ambient temperature. The photodiode array (PDA) detection was considered 281 nm. Under these optimized conditions, darunavir was separated at 0.516 min, which was considered a too fast separation. The optimized chromatogram is shown in Figure 2.

Method validation
For the subsequent validation study and force degradation study, the optimized chromatographic conditions were used and validated as per ICH guidelines. System suitability study was conducted to verify the proper working of the equipment used for analytical measurements. Several parameters such as tailing factor and theoretical plates were be taken into consideration. The %RSD of peak area, theoretical plates, tailing factors, and retention time were 0.4%, 0.3%, 0.3%, and 0.5%, respectively, which confirmed the suitability of the instrument to carry out the validation study.[25] The accuracy of test method was performed at three different concentration levels: 50%, 100%, and 150%. The prepared solutions for these levels were injected in a triplicate manner and average % recovery was calculated, which were found 100.38% for 50% level, 99.35% for 100% level, and 99.34% for 150% level of the labeled amount with an overall average of 99.67%. The details of the results are summarized in Table 1, showing the accuracy of the developed method. Fixed-dose tablet dosage from “Daruvir,” from Cipla Pharmaceutical Ltd, containing 300 mg of darunavir was selected for the application of the developed method. The obtained amount of the drug was found to be significantly close to the label claimed amount. The content of the tablet was found to be 298.17 mg, with a percentage purity of 99.39%. The result is presented in Table 1 and the chromatogram of assay is shown in Figure 3. Precision study was conducted under the division of repeatability and

| Table 1: Summary of validation parameters |
|------------------------------------------|
| Parameters                               | Darunavir |
| Linearity range (μg/mL)                   | 5–50      |
| Co-relation coefficient                  | 0.999     |
| LOD μg/mL                                | 0.02      |
| LOQ μg/mL                                | 0.07      |
| Repeatability (%RSD)                     | 0.43      |
| Intermediate precision (%RSD)            | 0.56      |
| Mean % recovery                          | 99.67     |
| Assay (darunavir tablets, each tablet contains 300 mg) (% assay) | 99.39 |

Figure 1: Chemical structure of darunavir

Figure 2: Optimized chromatogram of darunavir
intermediate precision. In repeatability, the amount found was calculated and %RSD was found to be 0.43. In intermediate precision, the % amount found was calculated and %RSD was found to be 0.56, as shown in Table 1, which justified the precision of the developed method. The result of linearity values of the developed method was expressed by plotting a graph between the concentration of the test solution on the X-axis and response of the corresponding solutions on the Y-axis and correlation coefficient was found to be 0.999, as shown in Table 1, which is within the limit and linear correlation was found between the concentration and the peak area in the specific range. The limit of detection was found to be 0.02 µg/mL and limit of quantitation was 0.07 µg/mL, thus signifying the sensitivity of the developed method. For the robustness study, the % RSD of tailing factor was considered. The developed method was found robust with the change in flow rates from ±0.03 mL/min, the mobile phase ratio of ±2, and also with change of column temperature ±2°C. The results of force degradation study under various stressed conditions were obtained and found no significant degradation. Slight decomposition was seen on exposure of darunavir drug solution to acidic (3.11%), alkaline (3.93%), oxidation (3.15%), thermal (4.87%), and photolytic (3.66%). The details of the result indicated that the drug found significantly resistant towards the above degradations as shown in Table 2. Chromatograms of degradations studies are shown in Figure 4. Based on peak purity results, obtained from the analysis of forced degradation samples using the described method, it can be concluded that the absence of coeluting peak along with the main peak of indicated that the developed method is specific\textsuperscript{26} for the estimation of darunavir in the presence of degradation products.

**Conclusion**

Based on the empirical evidences of this developed method, authors can strongly claim about the novelty of the present developed method over the available methods. This is the first stability indicating UPLC method which is “rapid” as it significantly reduced the total analysis time within 0.516 min, which is the lowest analysis time required. The present method is “stability indicating” as this has shown less degradation pattern in stressed conditions and good separation of famciclovir among the other degraded peaks. The results of all validation parameters were within the acceptance criteria of ICH Q2B guidelines. Hence, the present developed method can be used as a novel, reliable, validated useful method, which can be applied

| Sample name | Mean area | % Degraded | Purity angle | Purity threshold | Peak purity |
|-------------|-----------|------------|-------------|-----------------|-------------|
| Acid        | 499,327   | 3.11       | 0.589       | 1.619           | Passes      |
| Base        | 495,047   | 3.94       | 0.865       | 3.529           | Passes      |
| Peroxide    | 499,327   | 3.15       | 0.426       | 0.964           | Passes      |
| Thermal     | 490,258   | 4.87       | 0.379       | 1.146           | Passes      |
| Photo       | 496,473   | 3.66       | 0.786       | 2.744           | Passes      |
for routine analytical and quality control assay of darunavir in the tablet dosage form.

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Conflicts of interest
There are no conflicts of interest.

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