A novel stability-indicating reversed-phase high-performance liquid chromatographic (HPLC) method has been developed for the quantitative determination of darunavir ethanolate, an HIV-1 protease inhibitor. The chromatographic separation was achieved using an X-Bridge C18 (150 × 4.6 mm × 3.5 μm) HPLC column in isocratic mode employing 0.01 M ammonium formate (pH 3.0) buffer and acetonitrile in the ratio of 55:45 (v/v) with a flow rate of 1.0 mL/min. The detector wavelength was monitored at 265 nm and the column temperature was maintained at 30°C. Darunavir ethanolate was exposed to thermal, photolytic, acidic, base and oxidative stress conditions. Considerable degradation of the drug substance was found to occur under acid, base and oxidative stress conditions. The peak homogeneity data of darunavir ethanolate obtained by photodiode array detection demonstrated the specificity of the method in the presence of degradants. The degradation products were well resolved from primary peak of darunavir, indicating that the method is specific and stability-indicating. The HPLC method was validated as per International Conference on Harmonization guidelines with respect to specificity, precision, linearity, accuracy and robustness. Regression analysis showed a correlation coefficient value greater than 0.999. The accuracy of the method was established based on the recovery obtained for darunavir ethanolate.

Introduction

More than 60 million people have been infected with the human immunodeficiency virus (HIV) infection, known as cause of the acquired immunodeficiency syndrome (AIDS). HIV/AIDS is now the leading cause of death in sub-Saharan Africa, and is the fourth biggest killer worldwide.

HIV is able to evade immunological pressure, to adapt to a variety of cell types and growth conditions and to develop resistance against currently available drug therapies. The latter include nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase (NNRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), HIV-protease inhibitors (PIs), fusion inhibitors and the more recent CCR5 and integrase inhibitors.

Darunavir (DRV) is a new potent HIV-1 PI, chemically related to amprenavir (1), and efficient in vitro against viral strains less sensitive to other PIs (2, 3). DRV belongs to the class of hydroxethyl amino sulfonamide and is a second-generation PI developed by the pharmaceutical company Tibotec. DRV was specifically designed to overcome problems with the older agents in this class, such as indinavir. Currently, darunavir ethanolate is available in the market under the brand name of Prezista, formerly known as TMC114. It is administered twice a day (600 mg) in association with 100 mg of ritonavir as a booster.

Prezista tablets are available in a variety of DRV concentrations (75, 150, 400 and 600 mg). During storage, partial conversion from ethanolate to hydrate may occur; however, this does not affect product quality or performance. Each tablet also contains the inactive ingredients colloidal silicon dioxide, magnesium stearate and microcrystalline cellulose. The tablet film coating, Opadry White (75 and 150 mg doses), contains polyethylene glycol 3350, polyvinyl alcohol partially hydrolyzed, talc and titanium dioxide. The tablet film coating, Opadry Orange (400 and 600 mg doses), contains FD&C Yellow No. 6, polyethylene glycol 3350, polyvinyl alcohol partially hydrolyzed, talc and titanium dioxide.

Prezista 100 mg/mL oral suspension is available as a white to off-white opaque suspension for oral administration. Each milliliter of the oral suspension contains darunavir ethanolate equivalent to 100 mg DRV. In addition, the suspension contains the inactive ingredients hydroxypropyl cellulose, microcrystalline cellulose, sodium carboxymethyl cellulose, methylparaben sodium, citric acid monohydrate, sucralose, masking flavor, strawberry cream flavor, hydrochloric acid (for pH adjustment) and purified water.

DRV is a key component of many salvage therapies in multi-experienced patients. Prezista is an Office of AIDS Research Advisory Council (OARAC) recommended treatment option for treatment-native and treatment-experienced adults and adolescents. Several ongoing phase III trials are showing that the the Prezista/ritonavir combination is highly efficient and superior to the lopinavir/ritonavir combination for first-line therapy. This compound was licensed in June, 2006, in the United States and in February, 2007, in European Union. The phase III clinical study also confirmed that DRV treatment achieved a higher virologic response in pretreated patients than usual boosted PIs (4). As previously evidenced for other PIs (5), the phenotypic inhibitory quotient of DRV is strongly related to the virologic outcome (6). The ethanol solvate of DRV, referred to as darunavir ethanolate, has the chemical name [(1S,2R)-3-[(4-aminophenyl)sulfonyl][2-methylpropyl]amino]-2-hydroxy-1(phenylmethyl)propyl]carbamidic acid (3R,3aS,6a-R)-hexahydrofuro[2,3-b]furan-3-yl ester monoethanolate. Raveendra et al. (7) reported a new RP-HPLC method for the determination of DRV in tablet dosage form. Several research papers have been reported in the literature for the determination of DRV (8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18). All of these methods were reported for determination of DRV alone.
and with other anti-retrovirals in human plasma, based on high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC–MS) and HPLC–MS methods. However, extensive surveys revealed that no stability-indicating HPLC or ultra-performance liquid chromatography (UPLC) methods have been reported for the quantitative determination of darunavir ethanolate in bulk active pharmaceutical ingredient. Furthermore, no official or draft monograph on darunavir ethanolate has been published in any of the pharmacopoeias for compendial applications. Therefore, it was felt necessary to develop an accurate, rapid, specific and stability-indicating method for the determination of assay of darunavir ethanolate. This method is also used for the quantitative analysis of tablet dosage forms. In the developed method, no interferences are observed from blank or excipients at DRV peak retention. The primary advantage of this method is that it is simple and accurate with a shorter run time.

Materials and Methods

**Materials**

Darunavir ethanolate reference standard and test samples were received from the Analytical Research and Development Department of Hetero Research Foundation (Hyderabad, India). The process-related compounds, i.e., Impurity-A and Impurity-B, were also received from the synthetic division of Hetero Research Foundation. These two impurities are formed during the reaction between diamino compound and furanyl derivative under the synthesis of darunavir ethanolate. HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany). Analytical reagent grade ammonium formate and formic acid were purchased from Qualizens Fine Chemicals (Mumbai, India). High-purity water was prepared by using a Millipore Milli-Q Plus purification system.

**Equipment**

The HPLC system used for initial chromatographic development was a Waters Alliance HPLC (Milford, MA) 2695 separation module equipped with quaternary gradient pumps, inbuilt auto injector, 270852 thermostatic compartment and 2487 UV detector. Empower chromatography manager software was used for data acquisition and system suitability calculations. Photo diode array detector was used for determining peak purity.

**Chromatographic conditions**

The chromatographic separation was achieved on an X-Bridge C18 column, 150 × 4.6 mm, 3.5 μm. The mobile phase composition was pH 3.0 ± 0.05 buffer (0.063 g of ammonium formate in 1,000 mL of water and adjusted to pH 3.0 ± 0.05 with formic acid solution) and acetonitrile in the ratio of 55:45 (v/v). The mobile phase was filtered and degassed through 0.22 μm filter paper. The flow rate of the mobile phase was kept at 1.0 mL/min. The column temperature was maintained at 30°C and the detector wavelength was monitored at 265 nm. The injection volume was 20 μL. Buffer and acetonitrile in the ratio of 50:50 (v/v) was used as diluent. All calculations concerning the quantitative analysis were performed with external standardization by measurement of peak areas.

**Preparation of standard solutions**

Two milligrams of the standard sample were placed in a 10 mL volumetric flask, dissolved and diluted to the mark with diluent. A working solution of (0.2 mg/mL) test solution was prepared by dissolving appropriate amounts of the test solution in the diluent. A stock solution of impurity mixture (A and B) at 0.02 mg/mL was also prepared in diluent for specificity.

**Preparation tablets**

The coated layer was removed from an adequate number of Prezista tablets (400 and 600 mg DRV) with a knife and thoroughly ground to a fine powder with mortar and pestle. The powder was transferred into a 100 mL volumetric flask to obtain a 1.0 mg/mL concentration of DRV and sonicated for 20–25 min. The resulting stock solution was used for further dilutions. The resulting solution was filtered through a 0.22 μm pore size Nylon 66 membrane filter.

**Method validation**

Specificity

Specificity is the ability of the method to measure the response of the analyte in the presence of its potential impurities (19). Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule, and to validate the stability-indicating power of the analytical procedures. The specificity of the developed HPLC method for darunavir ethanolate was determined in the presence of its impurities and degradation products. Forced degradation studies were also performed on darunavir ethanolate to provide an indication of the stability-indicating property and specificity of the proposed method. The stress conditions employed for degradation study included: light, 1.2 million lux h [conducted as per International Conference on Harmonization (ICH) Q1B]; thermal (105°C, seven days); humidity (25°C, 90% relative humidity, seven days); acid hydrolysis (0.1N HCl, 1 h of heating at 80°C); base hydrolysis (0.1N NaOH, 30 min of heating at 80°C); water hydrolysis (4 h of heating at 80°C); and oxidation (6% H2O2, 2 h of heating at 80°C).

The photo degradation was conducted by exposing the darunavir ethanolate sample in solid state to light, providing an overall illumination of not less than 1.2 million lux hours and an integrated near-ultraviolet energy of not less than 200 Wh/m², which lasted approximately 10 days in a photo-illumination chamber. The generated stressed samples of darunavir ethanolate were checked for peak purity by using a Waters photodiode array detector (PDA). The purity factor is within the threshold limit obtained in all stressed samples, which demonstrates the peak homogeneity of the analyte. Assay studies were conducted for stress samples against qualified reference standards and the mass balance [assay (%) + impurities (%) + degradation products (%)] was calculated. The assay was also
calculated for bulk samples by spiking impurities (A and B) at specification levels (i.e., 0.15% with respect to analyte concentration of 0.2 mg/mL). A typical HPLC assay chromatogram of darunavir ethanolate is shown in Figure 1.

**Precision**
The system precision of the assay method was evaluated by conducting six replicate injections of darunavir ethanolate standard solution. The percentage of relative standard deviation (RSD) was calculated for the area of the darunavir ethanolate peak from six replicate injections. The method precision of the assay method was evaluated by conducting six independent assays of the test sample of darunavir ethanolate against qualified reference standards. The RSD was calculated for six assay values obtained. The intermediate precision of the method was also evaluated with different analyst and a different instrument in the same laboratory.

**Linearity**
The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of the analyte in the sample. Linearity test solutions for the assay method were prepared from a stock solution at five concentration levels ranging from 80 to 120% of the assay analyte concentration (80, 90, 100, 110 and 120%). Regression analysis was performed by least-squares using the peak area versus concentration data.

**Accuracy and recovery**
Standard addition and recovery experiments were conducted to determine the accuracy of the method for the quantification of darunavir ethanolate. The accuracy of the assay method was evaluated in triplicate at three concentration levels, i.e., 80, 100 and 120% of the analyte concentration (0.2 mg/mL) in bulk drug samples and the percentage recoveries were estimated.

**Robustness**
To determine the robustness of the developed assay method, experimental conditions were purposely altered and the assay content of the darunavir ethanolate was evaluated. To study the effect of flow rate on the assay, it was changed to 0.80 to 1.2 mL/min. The effect of the percent organic strength on assay was studied by varying it by ± 5.0%. The effect of pH was studied at 2.8 and 3.2 instead of 3.0. The effect of column temperature was studied at 28 and 32°C instead of 30°C. In all of the varied conditions, the components of the mobile phase were held constant, as stated previously. In all of the deliberately varied chromatographic conditions, the selectivity and the performance of the method were unchanged, which proves the robustness of the method.

**Test solution stability and mobile phase stability**
The test solution stability of darunavir ethanolate for the assay method was conducted by keeping both the test solution and reference standard in tightly capped volumetric flasks at room temperature for 12 and 24 h. The same sample solutions were assayed at 6-h intervals up to the study period. The mobile phase stability was also conducted by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions at 6-h intervals up to 24 h. The prepared mobile phase was kept constant during the study period. The percentage recovery of assay of darunavir ethanolate was calculated for the study period during mobile phase and solution stability experiments.

**Results and Discussion**

**Method development and optimization**
The primary criteria for the development of a successful HPLC method for the determination of darunavir ethanolate was that the method should be able to separate impurities and degradants within a shorter run time and should be accurate, reproducible, robust and stability-indicating.

![Auto-Sealed Chromatogram](image)

**Figure 1.** Typical HPLC chromatogram for the assay of darunavir ethanolate reference standard.

<table>
<thead>
<tr>
<th>HPLC Method Development Trials</th>
<th>Buffer</th>
<th>Solvent</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td>HPLC column</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Inertsil C8, 250 x 4.6 mm, 5 μm</td>
<td>KH₂PO₄, pH 6.5</td>
<td>Methanol</td>
</tr>
<tr>
<td>2</td>
<td>Kromasil C18, 250 x 4.6 mm, 5 μm</td>
<td>KH₂PO₄, pH 6.5</td>
<td>Methanol</td>
</tr>
<tr>
<td>3</td>
<td>Zorbax SB-CN, 250 x 4.6 mm, 5 μm</td>
<td>KH₂PO₄, pH 6.5</td>
<td>Methanol</td>
</tr>
<tr>
<td>4</td>
<td>Inertsil C8, 250 x 4.6 mm, 5 μm</td>
<td>KH₂PO₄, pH 4.5</td>
<td>Methanol</td>
</tr>
<tr>
<td>5</td>
<td>Inertsil C8, 150 x 4.6 mm, 5 μm</td>
<td>KH₂PO₄, pH 4.5</td>
<td>Methanol</td>
</tr>
<tr>
<td>6</td>
<td>Zorbax SB18, 150 x 4.6 mm, 5 μm</td>
<td>KH₂PO₄, pH 4.5</td>
<td>Methanol</td>
</tr>
<tr>
<td>7</td>
<td>Zorbax SB18, 150 x 4.6 mm, 5 μm</td>
<td>KH₂PO₄, pH 3.0</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>8</td>
<td>Zorbax SB18, 150 x 4.6 mm, 5 μm</td>
<td>KH₂PO₄, pH 6.0</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>9</td>
<td>X-Bridge C18, 150 x 4.6 mm, 3.5 μm</td>
<td>KH₂PO₄, pH 3.0</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>10</td>
<td>X-Bridge C18, 150 x 4.6 mm, 3.5 μm</td>
<td>Ammonium formate, pH 3.0 (for mass specificity)</td>
<td>Acetonitrile</td>
</tr>
</tbody>
</table>
The primary objective of the chromatographic method was to achieve the separation of Imp-A and Imp-B from darunavir ethanolate by using different stationary phases like C18, C8 and cyan, different mobile phases containing buffers like phosphate, acetate and formate with different pH (2–7) and using organic modifiers like acetonitrile and methanol in the mobile phase (Table I). The chromatographic separation was achieved on an X-Bridge C18 150 × 4.6 mm, 3.5 μm column. The monitored system suitability parameters were USP tailing factor and system precision. The USP tailing factor for the DRV peak was not more than 2.0 and the RSD for the peak area of five replicate injections of standard solution of DRV was not more than 1.0. The developed method is specific for darunavir ethanolate and its two impurities, i.e., Impurity-A [(1S,2R)-1-benzyl-2-hydroxy-3-isobutyl-[4-(3-methylureido)benzenesulfonyl]-amino]-propyl-carbamic acid (3R,3aS,6aR)-hexahydro furo [2,3-b]furan-3-ylic ether and Impurity-B [Carboxylic acid (1R,2S)-1-[(4-aminobenzenesulfonyl)-isobutylamino]-methyl]-2-[(3R,3aS,6aR)-hexahydropyrolo[2,3-b]furan-3-yl]oxy carboxylic amino-[3-phenylpropylether] (3R,3aS,6aR)-hexahydrofurano[2,3-b]furan-3-ylic ether. The chemical structures of darunavir ethanolate, Impurity-A and Impurity-B are shown in Figure 2.

Method validation results

Precision
The RSD for system precision and method precision studies for the assay of darunavir ethanolate was found to be within 1.0. The percent assay of six individual test preparations of darunavir ethanolate ranged from 99.4 to 100.0% with RSD of 0.24%. The percent assay of six individual test preparations of darunavir ethanolate in the intermediate precision study ranged from 99.6 to 100.3% with RSD of 0.29%, thus confirming the precision of the method.

Linearity
The plot of peak area of DRV versus concentration ranging from 0.16 to 0.24 mg/mL was linear, and the data were subjected to statistical analysis using a linear regression analysis with least-squares. The linear regression equation and correlation coefficient were $y = 18013925x + 43395.80$, 0.999, respectively. The RSD values for the peak areas of DRV at each level are within 1.0. These results show that an excellent correlation existed between the peak area and concentration of the analyte.

Accuracy and recovery
The accuracy of the assay method was evaluated in triplicate at three concentration levels, 0.16, 0.20, and 0.24 mg/mL, with respect to specification level, which is 0.2 mg/mL. The same procedure was adopted for tablet powder. The percentage recovery of darunavir ethanolate found in bulk drug samples and tablet dosage forms was found in the ranges of 99.77–100.17% and 92.63–100.4%, respectively.

Robustness
No significant change in the assay value was observed for any of the deliberately varied chromatographic conditions. The mean percent assay of darunavir ethanolate was found between 99.8 and 100.2% from robustness studies involving deliberate changes in flow rate, pH of the mobile phase, mobile phase composition and column temperature. The system suitability parameters like tailing factor (1.14 to 1.20) and RSD values are well within the limits, which confirm the robustness of the developed method.

Test solution stability and mobile phase stability
The RSD of the assay of darunavir ethanolate during solution stability and mobile phase stability experiments was within 1.0%. No significant changes were observed in the content of the assay of darunavir ethanolate during solution stability and mobile phase stability experiments. The solution stability and mobile phase stability experiment data confirm that the sample solutions and the mobile phase are stable up to 24 h.

Results of forced degradation studies
The darunavir ethanolate sample was found to be stable upon exposure to light, heat, water and humidity. Degradation of the drug substance was observed under acid, base and oxidative stress conditions (Figure 3). Peak purity test results derived
Conclusions

The newly developed RP-HPLC isocratic method for the determination of darunavir ethanolate assay in bulk active pharmaceutical ingredients and tablet dosage forms was found to be specific, precise, accurate and robust. The stability-indicating nature of the proposed method was established by performing forced degradation studies, which provided the degradation behavior of darunavir ethanolate under various conditions. The method validation data showed satisfactory results for all tested method parameters. Hence, the developed HPLC method can be used for routine analysis of production samples and for stability of bulk samples of darunavir ethanolate.

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