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ORIGINAL RESEARCH ARTICLE

Development and validation of stability indicating methods for the simultaneous estimation of antiviral drugs Darunavir and Ritonavir by RP-HPLC in Bulk and Dosage Forms

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ABSTRACT

We have successfully developed and validated a reliable method using reversed phase high performance liquid chromatography (RP-HPLC) that accurately determines stability, amounts of darunavir and ritonavir, in both their pure and dosage form. To achieve this, we utilized a Phenomenex C18 column with dimensions of 250 × 4.6 mm and a particle size of 5 µm. By pumping methanol as mobile phase through the column, at a flow rate of 1mL/min we were able to achieve results. The detection was performed at the wavelengths of 265 nm and 238 nm, for darunavir and ritonavir respectively using PDA detector. The retention times of darunavir and ritonavir were found to be 2.696 and 3.031 min respectively. Linearity was established in concentration range of 10 to 100 µg/mL with r2 ≥ 0.99. This method shows good precision results, the percentage RSD was found to be 0.965 and 1.429. The % recovery was obtained as 99.70 % and 98.83 % for darunavir and ritonavir respectively. The LOD and LOQ values were found to be 4.36 µg/mL and 13.20 µg/mL for darunavir and 4.97 µg/mL and 15.05 µg/mL for ritonavir, respectively.

Keywords: Darunavir; ritonavir; RP-HPLC; method development; validation

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1. Introduction

Darunavir[1], used in combination, with HIV protease inhibitor drugs and ritonavir is a treatment for managing HIV-1 infection. This compound specifically inhibits the virus ability to process Gag and Gag-pol polyproteins in cells thus preventing the formation of mature virions[2]. Darunavir[3] chemical structure is (3R, 3aS, 6aR) hexa-hydro-furo [2,3-b] furan 3yl-N [(2S, 3R) 3-hydroxy 4-[N (2 methyl-propyl)4 amino-benzene-sulfonamido] 1-phenyl-butan-2yl] carbamate (**Figure 1**). On other hand ritonavir[4] has a structure of (5S, 8S, 10S, 11S) 10 hydroxy 2-methyl 5-(1 methylethyl) 1-[2-(1 methyl-ethyl) 4-thiazolyl] 3, 6 dioxo 8, 11-bis (phenylmethyl) 2, 4, 7, 12 tetra-azatri-decan 13-oic acid 5-thiazolyl-methyl ester (**Figure 1**). The combination therapy involving these two drugs has proven effective, in treating AIDS. These drugs belong to the protease inhibitor class of drugs used for combating human immunodeficiency virus infections and managing AIDS.

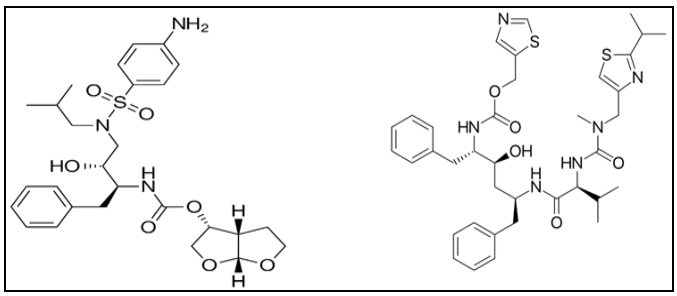

Figure 1. Chemical structure of darunavir (left panel) and ritonavir (i.e., right panel).

From the literature survey, many methods have been developed for the simultaneous or single estimation of darunavir and ritonavir using various instruments such as UV Spectrophotometer, HPLC[5–18], HPTLC[19]. Many works of the literature revealed that numerous RP-HPLC procedures for estimating the darunavir and ritonavir but were noticed to be complicated and highly time consuming and having high retention time.

The current approach outlines a method using RP HPLC for estimation of darunavir and ritonavir, in both bulk and dosage forms. This method ensures less retention time and high sensitivity making it suitable, for analysis.

2. Experimental

2.1. Chemicals and reagents

Darunavir and ritonavir working standard (i.e., API) obtained from V.L. Products, Mumbai, India. The HPLC grade water, acetonitrile, methanol and analytical grade Hydrochloric acid (HCl), sodium hydroxide (NaOH) and hydrogen peroxide (H_2O_2) were purchased from Lab fine chem industries, Mumbai, India.

2.2. Instruments and chromatographic conditions

Chromatography was performed using an HPLC (Shimadzu, Japan) LC-20AD instrument fitted with LC solutions software and equipped with a PDA detector and rheodyne injector. A pH meter (Lab India) and weighing balance (Shimadzu AX200, Japan) were also used. Methanol was employed as the mobile phase, and the PDA detector was utilized in order to carry out the quantification. The column that was utilized was a Phenomenex C18 column that was 250 x 4.6 mm and had a 5µ particle size. A volume of 20 µL of the sample solution was injected. The flow rate was adjusted to 1 mL/min, and the detection of darunavir and ritonavir was performed at wavelengths of 265 nm and 238 nm, respectively, while the temperature was kept at room temperature and the pressure was kept at 80 Pa. The chromatographic data were acquired by the use of the LC solutions.

2.3. Preparation of standard solutions

Accurately weighed 10mg of darunavir and ritonavir, transferred into 10ml of volumetric flask and 3/4th of diluent (HPLC grade methanol) was added to this flask and sonicated for 10 min. Flask was made up to the mark with diluent to get concentration of 1000 µg/mL and labelled as standard stock solution. Appropriate concentrations are prepared using this standard solution by the dilution.

2.4. Analytical method validation

In accordance with the standards established by ICH, each of the analytical validation parameters for the suggested approach was formulated.

2.4.1. Linearity

It is the ability of the method to obtain linear response with the analyte concentration in the specified range. The linearity was carried out for darunavir and ritonavir concentrations ranging from 10 µg/mL to 100 µg/mL. The coefficient of determination was calculated for both the analytes.

2.4.2. Accuracy

Accuracy refers to the degree of agreement between the measured value and true value. Accuracy was evaluated by performing recovery studies.

2.4.3. Precision

A measurement of the degree to which different outcomes of the same quantity agree with one another is referred to as precision. In order to assess the intra-day and inter-day changes, three duplicate concentration solutions with concentrations of 30, 40, and 50 µg/mL were tested on the same day and on three different days. A calculation was made to determine the % RSD of peak area and retention time.

2.4.4. Limit of Detection and Limit of Quantitation

For the calculations that determined the limits of detection (LOD) and quantification (LOQ), the signal-to-noise ratio was set at 3:1 for detection and 10:1 for quantification. For each of these medications, the LOD and LOQ values were determined to be 3 µg/mL, 10 µg/mL respectively.

$$LOD = 3.3\sigma/s \quad (1)$$

$$LOQ = 10/s \quad (2)$$

where, 'σ' is the standard deviation of y-intercepts of the regression line and 's' is the slope of the calibration line[20].

2.5. Stability

The ICH guidelines exhibit significant degradation conditions such as light, dry heat, acidic, basic, photolytic, etc. The forced degradation studies are illustrated by ICH[21] Q1A and Q1B.

2.5.1. Acid degradation

Acid degradation study was carried out using 0.1M HCl. Darunavir and ritonavir drug samples were taken in 10 mL volumetric flask. To this 10 mL of 0.1M HCl was added and sonicated for 10 minutes before being refluxed at 60 °C for 6 h. It was cooled and neutralized with 0.1M NaOH then diluted to 10 µg/mL. The sample was injected into HPLC and analyzed[22].

2.5.2. Base degradation

Analysis of base degradation darunavir and ritonavir drug samples were taken in 10 mL volumetric flasks. To this 10 mL of 0.1M NaOH was added then sonicated for 10 minutes and refluxed at 60 °C for 6 h. The solution was cooled and neutralized with 0.1M HCl then dilute to 10 µg/mL[23].

2.5.3. Oxidative degradation

This study was carried out using 30% w/v H_2O_2. For this, drug sample of darunavir and ritonavir was taken in 10 mL of flask. To this 10 mL of 30% w/v H_2O_2 was added and sonicated for 10 min. and reflux at 60 °C for 6 h. It was cooled and dilute the sample to 10 µg/mL[22].

2.5.4. Thermal degradation

Darunavir and ritonavir drug samples were placed in a glass petri dish in a hot air oven at 100 °C for one day. After specified time, the sample was cooled, transferred to a 10 mL volumetric flask, dissolved in the solvent, and dilute to10 µg/mL[22].

2.5.5. Photolytic degradation

Darunavir and ritonavir drug samples were placed in a glass petri dish and exposed to UV light for 24 hours for the photolytic degradation study. After the stress, the drug sample was transferred to a 10 mL flask, dissolved in the mobile phase and dilute the sample to 10 µg/mL[22].

3. Results and discussion

3.1. Method development

In order to obtain effective separation and resolution between two analytes, the chromatographic conditions that need to be optimised include the mobile phase, the flow rate, and the kind of column. On Kromasil 100-C18 (100 × 4.6 mm, 5 µm) and Phenomenex C18 (250 × 4.6 mm, 5 µm) columns, HPLC-grade methanol and acetonitrile were mixed in a variety of proportions and tested for their performance. The flow rate, another factor that plays a role in determining appropriate peak shapes, was also investigated. Acceptable separation was obtained in a relatively short amount of time, 4.5 minutes, using a Phenomenex C18 column at a flow rate of 1 mL/min for the mobile phase. The optimized chromatogram is shown in **Figure 2**.

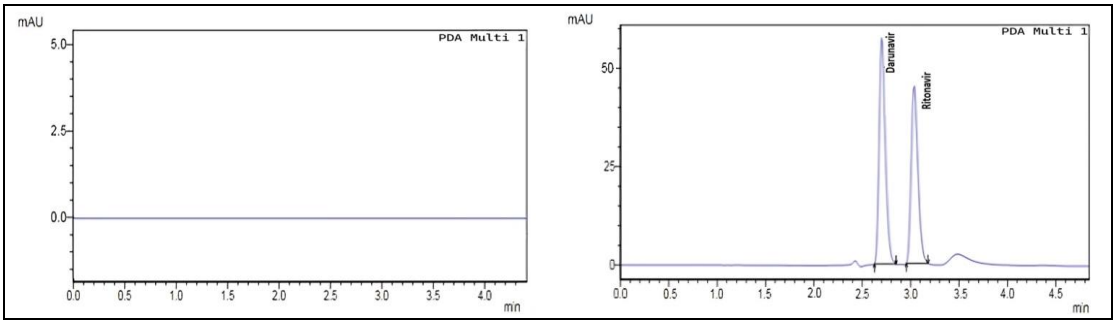

Figure 2. Chromatograms of blank (left panel) and analytes (right panel) at 10 µg/mL concentration.

3.2. Linearity

In order to generate the calibration curve, we first plotted (**Figure 3**) the peak area versus the concentration of the analyte. **Table 1** presents the linearity test findings that were obtained.

Table 1. Analytical validation parameters.

Parameter	Darunavir	Ritonavir
Linearity	10–100 µg/ml	10–100 µg/ml
Slope	10,788	10,744
Coefficient of determination	0.9983	0.9978
Regression equation	y = 10788x + 8898.3	y = 10744x + 15339
Correlation coefficient	0.9983	0.9978
LOD	4.36 µg/ml	4.97 µg/ml
LOQ	13.20 µg/ml	15.05 µg/ml
Retention time (min)	2.692	3.031

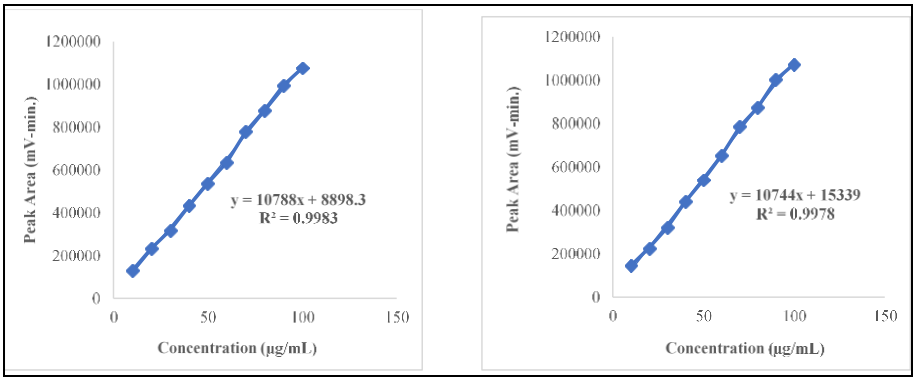

Figure 3. Linearity graph of darunavir (left panel) and ritonavir (right panel).

3.3. Accuracy

Working standards of 30, 40 and 50 µg/mL were injected into the system. Percentage standard recovery at each level was determined. The results of recovery are illustrated in **Table 2**.

Table 2. Accuracy data.

Drug	Concentration (µg/mL)	Peak Area (mV-min)	Amount Recovered (µg/mL)	% Recovery
Darunavir	30	315,366	29.60	98.70
	50	539,467	50.00	100.00
	80	874,133	79.47	99.34
Ritonavir	30	314,975	29.65	98.83
	50	539,981	50.24	100.48
	80	865,832	79.14	98.93

3.4. Precision

The method was found to be precise with the percentage RSD value in the range of 0.86 and 1.42 (**Table 3**).

Table 3. Precision data.

Drug	Concentration (µg/mL)	Intraday %RSD	Interday %RSD
Darunavir	30	0.95	0.96
	50	0.37	1.04
	80	1.12	1.36
Ritonavir	30	1.42	1.42
	50	0.31	0.94
	80	1.04	1.52

3.5. LOD & LOQ

The LOD and LOQ values were found to be 4.36 µg/mL and 13.20 µg/mL for darunavir and 4.97 µg/mL and 15.05 µg/mL for ritonavir, respectively.

3.6. Stability

Among all the degradation conditions, base degradation resulted in maximum degradation with percent degradation of 38.70% for darunavir and 91.1% for ritonavir. The chromatograms are shown in **Figure 4** and

the results are illustrated in **Table 4**.

Table 4. Degradation studies data.

Type of Degradation	Darunavir			Ritonavir		
	Rt	%Drug	%Degraded	Rt	%Drug	%Degraded
Acid	2.69	17.38	82.62	2.94	6.54	93.46
Base	2.68	8.26	91.74	2.85	18.44	81.56
Oxidative	2.63	84.47	15.53	2.88	9.38	90.62
Thermal	2.71	69.72	30.28	3.04	99.98	0.02
Photolytic	2.68	68.06	31.94	3.06	67.64	32.36

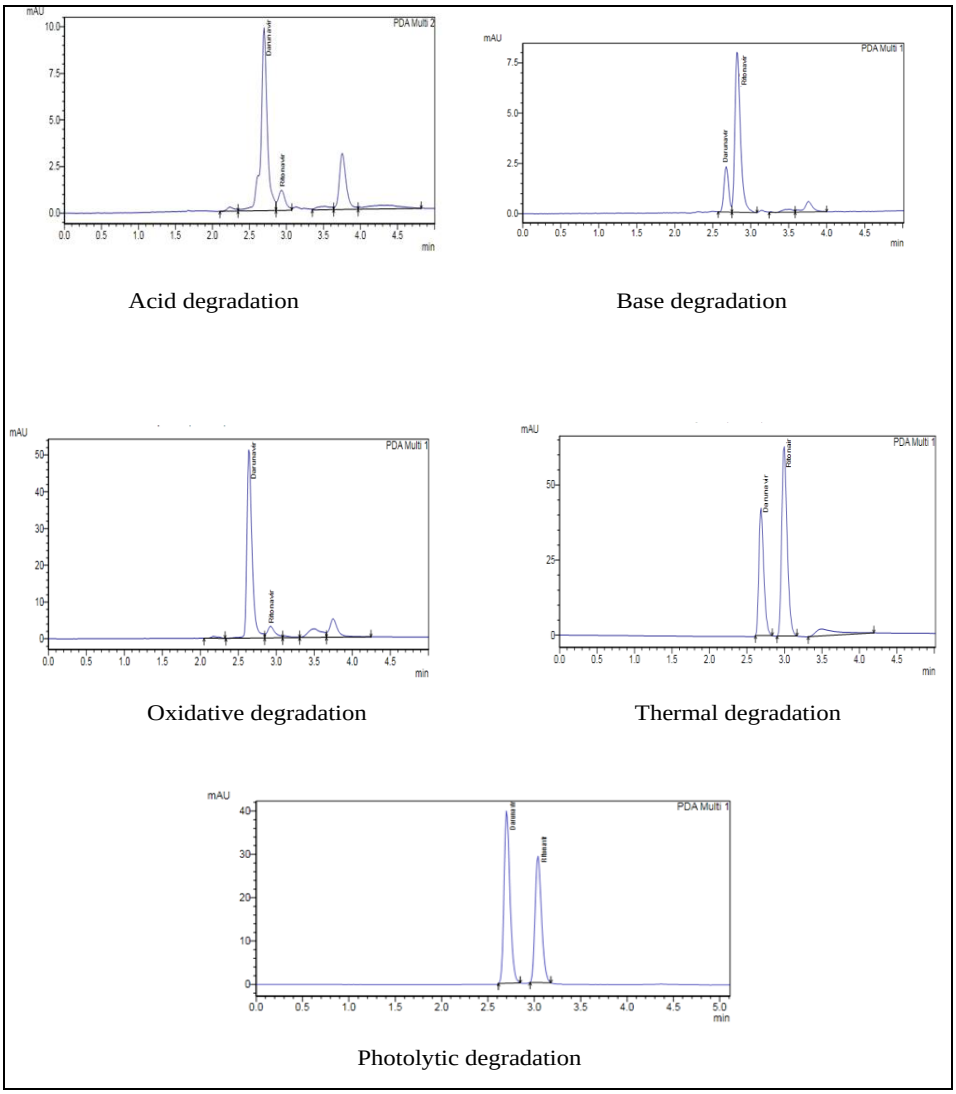

Figure 4. Various degradation chromatograms.

4. Conclusion

The developed and validated stability indicating technique for the simultaneous estimate of darunavir and ritonavir in bulk and dosage form was straightforward, speedy, accurate, and exact. The newly designed technique underwent comprehensive validation, and the findings demonstrated that it satisfies all of the method validation requirements. Based on the figures obtained for % RSD, LOD, and LOQ, it was determined that the newly developed approach is more exact and sensitive than the HPLC methods that were

previously published.

Author contributions

Conceptualization, RK; methodology, SD; software, RK; validation, RK and SD; formal analysis, RKJ; investigation, RK; resources, SD; data curation, SD; writing—original draft preparation, RK; writing—review and editing, SD; visualization, RKJ; supervision, SD; project administration, SD; funding acquisition, RKJ. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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