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ORIGINAL RESEARCH

Determination of Darunavir and Cobicistat Simultaneously Using Stability Indicating RP-HPLC Method

M. Venkata Siva Sri NALINI, P. Rama Krishna VENI, B. HARİBABU

ABSTRACT

A simple, sensitive and precise stability indicating reverse phase high performance liquid chromatographic method has been developed and validated for the estimation of darunavir and cobicistat simultaneously in combined dosage form. The stationary phase used was Phenomenex C18 (150 x 4.6 mm, 5 μm particle size). The mobile phase used was a mixture of 0.1 M NaH_2PO_4 and methanol (70:30 *v/v*). Quantification was done with photodiode array detection at 260 nm over the concentration range of 80-240 μg/mL and 15-45 μg/mL for darunavir and cobicistat, respectively. The method had accuracy in the range of 100.11-100.31% for darunavir and 99.87-99.89% for cobicistat. Darunavir and cobicistat were also subjected to acid, base, oxidation, heat, photo and UV degradation. The degradation products obtained were well resolved from the darunavir and cobicistat with different retention times. Since the method can effectively separate the darunavir and cobicistat from its degradation products, it can be used as stability-indicating method.

Keywords: RP-HPLC, darunavir, cobicistat, stability indicating method.

M. Venkata Siva Sri Nalini, B. Haribabu
Department of Chemistry, Acharya Nagarjuna University, Nagarjuna nagar, Guntur-522510, Andhra Pradesh, India.

P. Rama Krishna Veni
Department of Applied Sciences and Humanities, Sasi Institute of Technology and Engineering, Tadepalligudem-534101, Andhra Pradesh, India.

Corresponding author
B. Haribabu
Tel.: +91-8500338866
E-mail: drharibabuanu2015@gmail.com

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1. INTRODUCTION

Darunavir (Figure 1), chemically known as [(3a*S*,4*R*,6a*R*)-2,3,3a,4,5,6a-hexahydrofuro [2,3-b] furan-4-yl] *N*-[(2*S*,3*R*)-4-[(4-aminophenyl)sulfonyl-(2-methylpropyl)amino]-3-hydroxy-1-phenylbutan-2-yl] carbamate, is a antiretroviral drug belonging to the class known as protease inhibitors (1). Darunavir was approved by US and Europe to treat patients infected with human immunodeficiency virus in combination with other antiretroviral drugs (2, 3). Darunavir inhibits the human immunodeficiency virus aspartyl protease enzyme. This enzyme cleaves the human immunodeficiency virus polyprotein into its functional fragments (4). Several methods are published for darunavir estimation in bulk, formulations and biological matrices using infrared spectroscopy (5), UV spectrophotometry (6, 7), visible spectrophotometry (8-10), LC-MS (11, 12), UPLC-MS (13), HPLC (14-16) and HPTLC (17-19).

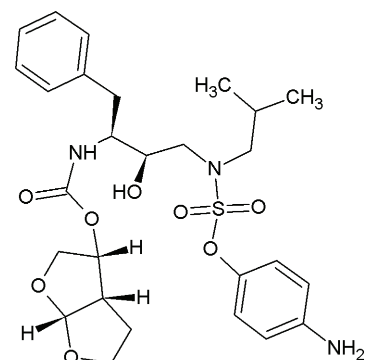

Figure 1. Chemical structure of darunavir

Cobicistat (Figure 2), chemically described as 1,3-thiazol-5-ylmethyl *N*-[(2*R*,5*R*)-5-[[(2*S*)-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbamoyl]amino]-4-morpholin-4-ylbutanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate, is a pharmacokinetic enhancer and Cytochrome P450 3A Inhibitor (20). By inhibiting cytochrome P450 3A enzyme and intestinal transport proteins, cobicistat increases the bioavailability and absorption of several human immunodeficiency virus medications such as atazanavir, darunavir and tenofovir alafenamide (21). Few methods have been described for the determination of cobicistat in bulk and in its tablet dosage form include many techniques as UV spectrophotometry (22), visible spectrophotometry (23) and HPLC (24-27).

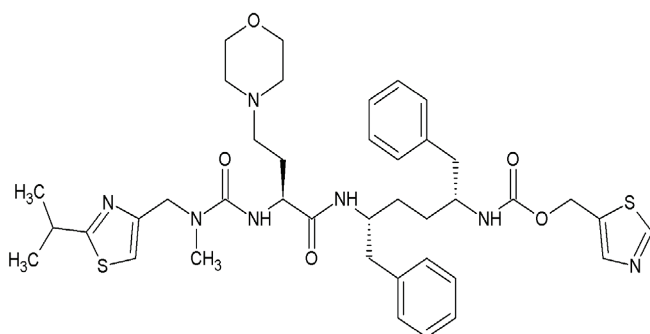

Figure 2. Chemical structure of cobicistat

The US Food and Drug Administration approved the combination of darunavir and cobicistat for the treatment of human immunodeficiency virus type 1 infection (28-30). Darunavir acts as human immunodeficiency virus type 1 protease inhibitor and cobicistat increases the systemic exposure of darunavir. To the best of our knowledge only one stability indicating RP-HPLC method is found in the literature for the quantification of darunavir and cobicistat simultaneously (31). In the reported method, darunavir and cobicistat were separated on an ODS C18 column, using a 45:55 (*v/v*) mixture of phosphate buffer (pH 7.0) and acetonitrile as mobile phase, delivered at 1.0 mL/min. Detection was performed at 253 nm.

The aim of this study is to develop and validate a sensitive and economical stability indicating RP-HPLC assay procedure, suitable for the simultaneous analysis of darunavir and cobicistat in their binary combination tablets. The summary of reported (31) and proposed stability indicating HPLC methods are shown in Table 1. The low values of percentage RSD, LOD and LOQ indicates that the developed method is more precise and sensitive than the reported HPLC method (31). The use of methanol in the preparation of mobile phase, rather than acetonitrile, makes the method economical than the reported method (31).

Table 1. Summary of reported and proposed methods

Drug	Retention time (min)	Linearity (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)	RSD (%)	Recovery (%)	Reference
Darunavir	2.320	160-480	19.07	57.78	0.282	99.30 -100.53	31
Cobicistat	3.529	30-90	10.0	24.7	1.000	100.04-100.94	
Darunavir	3.734	80-240	0.220	0.733	0.245	100.11-100.31	Proposed method
Cobicistat	5.279	15-45	0.097	0.323	0.307	99.87-99.89	

2. MATERIALS AND METHODS

2.1. Instrumentation

Chromatographic analyses of darunavir and cobicistat were carried out with Waters HPLC system, consisted of a binary HPLC pump model 2695, PDA detector model 2998, vacuum degasser and Waters Empower2 software.

2.2. Chemicals and solvents

Reference standards darunavir and cobicistat were obtained from Lara Drugs Private Limited (Telangana, India). Methanol of HPLC grade was obtained from Merck Private

Limited, (Mumbai, India). Other chemicals such as NaH_2PO_4, HCl, NaOH and H_2O_2 employed were of analytical grade (Sd Fine Chemicals Ltd., Mumbai, India). Mili-Q water was used for the preparation of the mobile phase. Commercial tablets, Prezcobix® (Janssen Ortho, LLC, Gurabo; labeled to contain 800 mg and 150 mg of darunavir and cobicistat, respectively) were acquired from local pharmacies.

2.3. Chromatographic conditions

The separation of darunavir and cobicistat was performed using a mobile phase containing 0.1 M NaH_2PO_4 and methanol (70:30 *v/v*), pumped at a flow rate of 1.0 mL/min through a Phenomenex C18 (150 x 4.6 mm, 5 µm particle size), thermostatized at 30 °C. The analytes were detected at 260 nm and the injection volume was 10 µL.

2.4. Preparation of standard solutions

The stock and working standard solutions were prepared with mobile phase. The standard stock solution of darunavir (8 mg/mL) and cobicistat (1.5 mg/mL) was prepared by transferring accurately weighed amounts (800 mg of darunavir and 150 mg of cobicistat) into 100 mL volumetric flask. The drugs were dissolved by shaking gently with 30 mL of mobile phase and made upto the mark with the same solvent. The working standard solution (darunavir - 160 µg/mL and cobicistat - 30 µg/mL) was prepared by transferring 2 mL of stock standard solution into 100 mL volumetric flask and the volume was made upto the mark with the mobile phase. All the solutions were filtered through 0.45 µm membrane filters before use.

2.5. Calibration curves

Standard calibration curves were prepared with five calibrators over a concentration range of 80-240 µg/mL for darunavir and 15-45 µg/mL for cobicistat. 10 µL of solutions were injected in triplicate and chromatographed under the optimized conditions as described above. The peak areas measured were plotted against the concentration of the corresponding drug and the regression equation was derived.

2.6. Preparation of tablet sample solution

Ten tablets were weighed and their average weight was determined. The tablets were crushed to a homogenous powder and an amount equivalent to 800 mg of darunavir and 150 mg of cobicistat was accurately weighed and transferred into a 100 mL volumetric flask to which 30 mL of mobile phase was added. After sonication for 15 min, the mixture in the flask was diluted to the mark with mobile phase and mixed. An aliquot of 2 mL was transferred to a 100 mL flask and filled to the mark with mobile phase. For analysis, the samples were filtered through 0.45 µm membrane filter. The contents of the analytes were obtained from the corresponding regression equation/corresponding calibration curve.

2.7. Forced degradation

Forced degradation studies were performed on tablet sample using different stress conditions such as acidic, basic, oxidative, thermal and photolytic stresses and then the samples are filtered through 0.45 µm membrane filter and subjected to HPLC analysis.

Acidic degradation

Acidic degradation was carried out using 0.1 N HCl. For this, tablet powder equivalent to 800 mg of darunavir and 150 mg of cobicistat were taken in 100 mL volumetric flask. 10 mL of 0.1 N HCl was added and sonicated for 30 min. After completion of the stress, the solution was neutralized using 0.1N NaOH and filled up to the mark with mobile phase.

Basic degradation

Base degradation study was carried out using 0.1 N NaOH. For this, tablet powder equivalent to 800 mg of darunavir and 150 mg of cobicistat were taken in 100 mL volumetric flask. 10 mL of 0.1 N NaOH was added and sonicated for 30 min. After completion of the stress, the solution was neutralized by using 0.1 N HCl and completed upto the mark with mobile phase.

Oxidative degradation

Oxidative degradation was carried out using 30 % H_2O_2. To perform this, tablet powder equivalent to 800 mg of darunavir and 150 mg of cobicistat were taken in 100 mL volumetric flask. 10 mL of 30 % H_2O_2 was added to it. The contents of the flask were sonicated for 30 min. After completion of the stress, the volume of the flask was made up to the mark with mobile phase.

Thermal degradation

Thermal degradation was performed in hot air oven at 105 °C. For this study, tablet powder equivalent to 800 mg of darunavir and 150 mg of cobicistat was taken in glass petri dish and placed in oven at 105 °C for 30 min. After specified time, the sample was cooled, transferred into a 100 mL volumetric flask and dissolved in 30 mL of mobile phase and the volume was made upto mark with mobile phase.

Photolytic degradation

For photolytic degradation study, 800 mg of darunavir and 150 mg of cobicistat tablet powder was taken in glass petri dish

and placed in the direct sunlight for 24 h. After completion of the stress, the drug sample was cooled, transferred into a 100 mL volumetric flask and dissolved in 30 mL of mobile phase and upto mark with mobile phase.

UV degradation

For UV degradation study, tablet powder equivalent to 800 mg and 150 mg of darunavir and cobicistat, was taken in glass petri dish and exposed to UV radiation of a wavelength of 256 nm for 12 h in UV chamber. After completion of the stress, the sample was cooled, transferred into a 100 mL volumetric flask and dissolved in 30 mL of mobile phase and upto mark with mobile phase.

After degradation, the degraded samples were aptly diluted with mobile phase to give a final concentration of 160 μg/mL (darunavir) and 30 μg/mL (cobicistat). The samples were filtered through 0.45 μm membrane filter before injecting into the chromatographic system. Three injections were made and the chromatograms were recorded.

3. RESULTS AND DISCUSSION

3.1. Optimization of the method

RP-HPLC procedure was optimized to develop a stability indicating assay method that can be used for quality control analysis in laboratories.

For method optimization, various conditions such as different columns and mobile phase mixtures were tried. Two different analytical columns, YMC Pack Pro C18 (250 x 4.6 mm, 5 μm) and Phenomenex C18 (150 x 4.6 mm, 5μm) were tried. For the separation of darunavir, cobicistat and their forced degradation products, Phenomenex C18 (150 x 4.6 mm, 5μm) column maintained at a temperature of 30°C was found to be efficient.

Two different mobile phase mixtures like ammonium acetate with methanol and 0.1 M NaH_2PO_4 with methanol were tested. On the basis of preliminary experiments, a mobile phase composed of 0.1 M NaH_2PO_4 with methanol was chosen for analysis that showed good peak shape and resolution. Additional attempts for mobile mixture composition demonstrated that the mobile phase with composition of 70 % 0.1 M NaH_2PO_4 and 30 % methanol (*v/v*) with the flow rate of 1.0 mL/min exhibits proper separation of drug peaks. For the detection and quantification of darunavir and cobicistat, 260 nm was selected as the optimum detection wavelength. At 260 nm best detector response for both the drugs was obtained.

Under the described chromatographic conditions above, all peaks of darunavir and cobicistat were shaped well and free from tailing. The retention times were 3.739 and 5.285 min for darunavir and cobicistat, respectively (Figure 3).

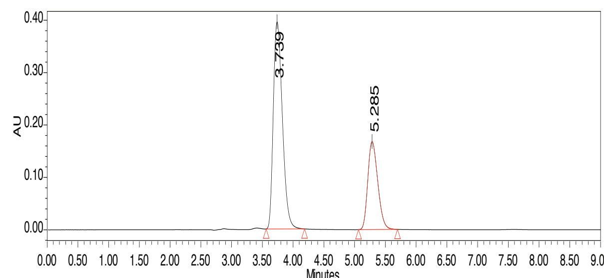

Figure 3. Chromatogram of darunavir and cobicistat under optimized chromatographic conditions

3.2. Method Validation

After development, the method was subjected to validation as per ICH guidelines (32).

System suitability

The system suitability parameters were evaluated by injecting standard solution of 160 μg/mL darunavir and 30 μg/mL cobicistat. The results are presented in Table 2. The system was found to be suitable, as the parameters are within the acceptable limits.

Table 2. Results of system suitability

Parameters	Darunavir	Cobicistat	Recommended limits
Retention time	3.734 (%RSD- 0.124)	5.279 (%RSD – 0.369)	RSD ≤2
Peak area	4083217 (%RSD -0.152)	1885067 (%RSD - 0.215)	RSD ≤2
USP resolution	-	5.40	> 1.5
USP plate count	3098	5010	> 2000
USP tailing factor	1.34	1.24	≤ 2

Linearity

The linearity of the method was evaluated by analyzing a series of solutions containing darunavir and cobicistat in the concentration range of 80-240 μg/mL and 15-45 μg/mL, respectively. The calibration curves were constructed. The regression coefficients of the curves were found to be ≥ 0.9990 for the two drugs, enabling the linear behavior of the method in the established concentration range. Darunavir and cobicistat showed linearity in the range of 80-240 μg/mL and 15-45 μg/mL, respectively. Linear regression equations and correlation coefficient are presented in Table 3.

Table 3. Results of linearity, LOD, LOQ and precision

Parameter	Darunavir	Cobicistat
Linearity (μg/mL)	80-240	15-45
Regression equation (y = mx + c)*	y = 25519x + 417.8	y = 62840x - 2764
% RSD of slope	0.351	0.145
%RSD of intercept	0.256	0.532
Regression coefficient (R^2)	0.9999	0.9990
LOD (μg/mL)	0.220	0.097
LOQ (μg/mL)	0.733	0.323
RSD (%)**	0.245	0.307

*y – Peak area; m- Slope; x – Concentration of drug (μg/mL); c – Intercept
** RSD for six determinations

Precision

The precision of the method was evaluated by analyzing standard solution of darunavir and cobicistat with a concentration of 160 μg/mL and 30 μg/mL, respectively. Six replicates were analyzed to determine the precision. The percent RSD of peak areas was calculated and was found to be below 1.0 %. This indicates the precision of the method for the simultaneous estimation of darunavir and cobicistat. The results are shown in Table 3.

Limit of detection (LOD) and Limit of quantification (LOQ)

The limits of detection and quantification were evaluated based on residual standard deviation of the response and the slope. The LOD and LOQ values for darunavir and cobicistat are presented in Table 3. The values indicate the adequate sensitivity of the method.

Robustness

The robustness of the method was studied by varying the chromatographic conditions with respect to the flow rate of the mobile phase and column temperature. The study was conducted at three different flow rates (0.9 mL/min - 1.0 mL/min - 1.1 mL/min) and at three different column temperatures (28 °C, 30 °C, 32 °C). The effect of these changes on the different chromatographic parameters was studied. The results are summarized in Table 4. Negligible difference was found in system suitability parameters for darunavir and cobicistat such as USP plate count, resolution and the USP tailing factor, therefore the method found to be robust.

Table 4. Results of method robustness

Parameter	Investigated value	Area	USP Plate Count	USP Tailing	USP resolution
		Darunavir			
Temperature (ºC)	28	4043878	3046	1.34	-
	30	4083217	3098	1.34	
	32	4044492	3086	1.35	-
Flow rate (mL/min)	0.9	4855186	3496	1.36	-
	1.0	4083217	3098	1.34	
	1.1	3472273	3091	1.33	-
		Cobicistat			
Temperature (ºC)	28	1875692	4796	1.25	5.42
	30	1885067	5010	1.24	5.40
	32	1878809	4983	1.25	5.44
Flow rate (mL/min)	0.9	2256364	5113	1.24	5.78
	1.0	1885067	5010	1.24	5.40
	1.1	1614172	4875	1.24	5.07

Accuracy

To determine the accuracy of the method, recovery studies were carried out by application of the standard addition technique. Known amounts of the darunavir and cobicistat at three different concentration levels (50 %, 100 % and 150 %) were added to a pre-analyzed tablet sample; the prepared samples were then analyzed by the proposed method and the percentage recoveries were then calculated. Good percentage recoveries were obtained, confirming the accuracy of the proposed method (Table 5).

Table 5. Results of method accuracy

Spiked Level	Darunavir Added (µg/mL)	Found (µg/mL)	Recovery (%)	Mean (%)	Cobicistat Added (µg/mL)	Found (µg/mL)	Recovery (%)	Mean (%)
50%	79.20	79.44	100.31		15.00	14.96	99.72	
50%	79.20	79.40	100.25	100.31	15.00	15.00	100.03	99.89
50%	79.20	79.48	100.36		15.00	14.99	99.92	
100%	158.40	158.46	100.04		30.00	29.99	99.97	
100%	158.40	158.61	100.13	100.11	30.00	29.96	99.86	99.89
100%	158.40	158.64	100.15		30.00	29.95	99.83	
150%	237.60	238.02	100.18		45.00	44.96	99.91	
150%	237.60	237.98	100.16	100.15	45.00	44.89	99.75	99.87
150%	237.60	237.83	100.10		45.00	44.97	99.94	

Forced degradation studies

When darunavir and cobicistat was subjected to different forced degradation conditions (acid, base, oxidative, thermal, photolytic and UV), significant degradation was observed. The percentage of degradation and percent relative standard deviation values are summarized in Table 6. The degradants produced in all the forced degradations were well separated from darunavir and cobicistat (Figure 4). The method therefore proved to be stability-indicating.

Table 6. Results of forced degradation

Type of degradation	Darunavir Peak area	Assay (%)	RSD (%)	Degradation (%)	Cobicistat Peak area	Assay (%)	RSD (%)	Degradation (%)
Undegraded	4083217	100.00	0.238	0.0	1885067	100.00	0.592	0.0
Acid	3913344	94.98	0.329	5.02	1732987	91.66	0.361	8.34
Base	3908665	94.86	0.365	5.14	1770676	93.65	0.518	6.35
Oxidative	3958440	96.07	0.592	3.93	1751511	92.64	0.248	7.36
Thermal	3931076	95.41	0.425	4.59	1785100	94.41	0.351	5.59
Photolytic	3968848	96.32	0.361	3.68	1791612	94.76	0.568	5.24
UV	3900256	95.51	0.258	4.49	1785624	94.72	0.425	5.28

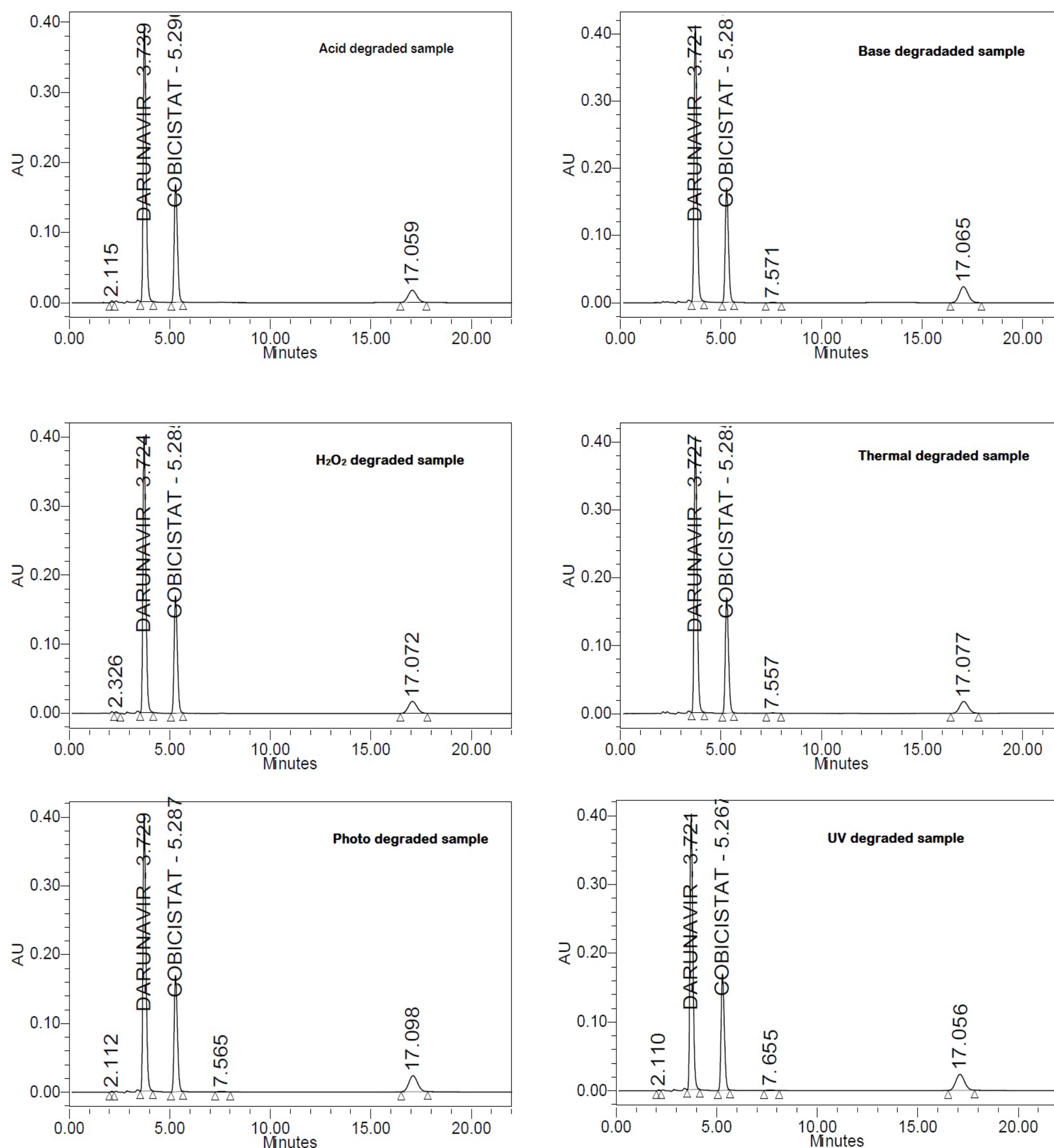

Figure 4. Chromatograms of darunavir and cobicistat after forced degradation

Specificity

The specificity of the method was evaluated by verifying the purity of darunavir and cobicistat peaks in the mixture of forced degraded samples by photodiode array analysis. The peak purity angle values for darunavir and cobicistat peaks were found to be less than the purity threshold value. This indicates the absence of any co-eluting peak in darunavir and cobicistat peaks. The purity angle values and purity threshold values are summarized in Table 7.

Table 7. Specificity of the method

Type of degradation	Darunavir		Cobicistat	
	Purity Angle	Purity Threshold	Purity Angle	Purity Threshold
Acid	0.137	0.266	0.119	0.249
Base	0.144	0.270	0.121	0.249
Oxidative	0.149	0.268	0.128	0.249
Thermal	0.140	0.270	0.116	0.250
Photolytic	0.141	0.269	0.120	0.250
UV	0.139	0.268	0.121	0.249

Selectivity

The chromatograms of mobile phase blank, placebo blank, test sample (160 µg/mL darunavir and 30 µg/mL cobicistat) and standard (160 µg/mL darunavir and 30 µg/mL cobicistat) were compared to give reason for the selectivity of method. The method was selective since excipients in the formulation and components of the mobile phase did not interfere in the simultaneous analysis of darunavir and cobicistat (Figure 5).

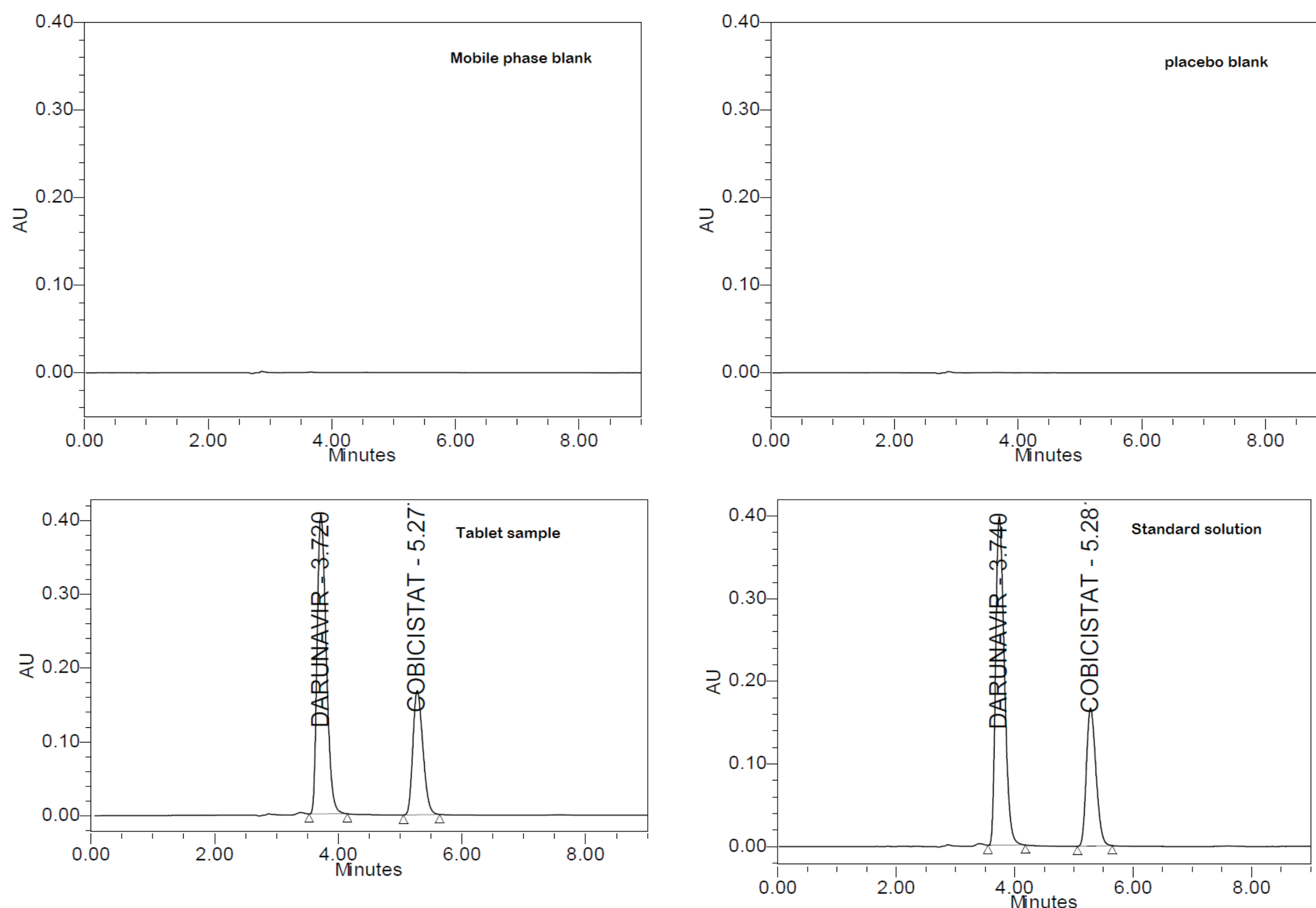

Figure 5. Chromatograms of method selectivity

4. CONCLUSION

The developed stability indicating RP-HPLC method has been successfully applied for simultaneous determination of darunavir and cobicistat in their combined tablet sample. The method was found to be rapid, simple and accurate. When the developed method was completely validated, the results showed satisfactory data for all the method validation parameters. From the values percentage RSD, LOD and LOQ, it was found that the developed method is more precise and sensitive than the reported HPLC method (31). So the proposed method can be easily and conveniently adopted for routine quality control analysis of darunavir and cobicistat.

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Darunavir ve Kobistat'ın Ters Faz-Yüksek Basınçlı Sıvı Kromatografisi Yöntemiyle Eş-zamanlı Miktar Tayini

ÖZ

Kombine dozaj formundan darunavir ve kobisistat'ın eş zamanlı miktar tayini ve stabilitesinin gösterilebilmesi için basit, duyarlı, güvenilir bir tes faz-yüksek basınçlı sıvı kromatografisi yöntemi geliştirilmiş ve doğrulanmıştır. Stasyoner faz olarak Phenomenex C18 kolon (150 x 4.6 mm, 5 µm partikül çapı), mobil faz olarak 0.1 M NaH_2PO_4 : metanol (70:30 *h/h*) karışımı kullanılmıştır. Miktar tayini PDA detektör yardımıyla 260 nm'de yapılmıştır, konsantrasyon aralığı darunavir ve kobisistat için sırasıyla 80-240 µg/mL ve 15-45 µg/mL olarak tespit edilmiştir. Yöntemin doğruluğu darunavir ve kobisistat için sırasıyla % 100.11-100.31 ve % 99.87-99.89 olarak tespit edilmiştir. Darunavir ve kobisistat; asit, baz, oksidasyon, ısı, ışık ve UV degredasyonuna maruz bırakılmıştır. Darunavir ve kobisistat'tan farklı alıkonma zamanlarına sahip olan degredasyon ürünleri başarıyla ayrılmıştır. Geliştirilen yöntem, darunavir ve kobisistat'ı degradasyon ürünlerinden başarıyla ayırabildiği için stabiliteyi gösteren bir yöntem olarak da kullanılabilir.

Anahtar kelimeler: Ters faz-yüksek basınçlı sıvı kromatografisi yöntemi, darunavir, kobisistat, stabilite gösterir yöntem.

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