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Original Article

DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING HPTLC METHOD FOR DETERMINATION OF DARUNAVIR ETHANOLATE AND RITONAVIR

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ABSTRACT

Objective: To develop and validate a new simple, accurate, precise and selective stability-indicating high performance thin layer chromatographic (HPTLC) method for simultaneous estimation of Darunavir ethanolate and Ritonavir in combined tablet dosage form as per ICH guidelines.

Methods: Chromatographic separation of drugs was performed on precoated silica gel aluminium plate 60 F_{254} (10 ×10) with 250 µm thicknesses (E. MERCK, Darmstadt, Germany) using a CAMAG Linomat 5 sample applicator (Switzerland). The mobile phase selected was Toluene: Ethyl acetate: Methanol (6: 2.5: 1.5, v/v/v) with UV detection at 250 nm.

Results: The retention factor for Darunavir ethanolate and Ritonavir was found to be 0.29±0.005 and 0.50±0.07. Results were found to be linear in the concentration range of 200-1000 ng band^{-1}for both the drugs. Intra-day variation, as RSD (%), was found to be in the range of 0.45 to 1.54 for Darunavir ethanolate and 0.22 to 0.64 for Ritonavir. Interday variation, as RSD (%) was found to be in the range of 0.64 to 1.48 for Darunavir ethanolate and 0.13 to 1.24 for Ritonavir. The lower values of % RSD obtained have proved that the method is precise and robust.

Conclusion: Stability-indicating HPTLC method was developed and validated for simultaneous estimation Darunavir ethanolate and Ritonavir and can be used for assessing the stability of Darunavir ethanolate and Ritonavir in bulk drug and pharmaceutical dosage form.

Keywords: Darunavir, Ritonavir, HPTLC, Forced degradation.

INTRODUCTION

Darunavir ethanolate (DVR), chemically, [(1R, 5S, 6R)-2, 8-dioxabicyclo [3.3.0] oct-6-yl] N-[(2S, 3R)-4-[(4-aminophenyl) sulfonyl-(2-methylpropyl) amino]-3-hydroxy-1-phenyl-butan-2-yl] carbamate is a protease inhibitor drug used to treat HIV infection [1]. Ritonavir (RTV), 2, 4, 7, 12-Tetraazatridecan-13-oic acid, 10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3, 6-dioxo-8, 11-bis (phenylmethyl)-5-thiazolylmethyl ester [5S-(5R*, 8R*, 10R*, 11R*)] is a potent cytochrcome P-450 (CYP)3A inhibitor and usually used as pharmacokinetic booster for other protease inhibitor [2].

Literature survey reveals Spectrophotometric [3, 4], thin layer chromatography [5], High Performance Liquid Chromatographic (HPLC) [6-13] methods for determination of DVR in bulk and pharmaceutical preparations. Analytical methods reported for RTV includes HPLC [14-17], LC-MS [18], Densiometry [19-21] and spectrophotometry [22] either as single or in combination with other drugs. Also reverse phase high performance liquid chromatographic method for simultaneous determination of DVR and RTV in binary mixture is also reported [23].

No reports were found for stability-indicating HPTLC method for simultaneous determination of DVR and RTV in tablet dosage form. This paper describes simple, precise, accurate and sensitive HPTLC method development and validation as well as stability study (hydrolysis, oxidation, photo-degradation and thermal degradation) as per International Conference on Harmonisation Guidelines [24, 25]. The developed method would be suitable for simultaneous analysis of DVR and RTV in combination without any interference from the excipients and it can be successfully applied to estimate the amount of DVR and RTV in the formulations by easily available low cost materials.

The method can be used for routine analysis of marketed products of DVR and RTV in combined tablet formulation as well as for single drug analysis and also can be used for routine analysis of pharmaceutical formulations in quality-control laboratories.

MATERIALS AND METHODS

Chemicals and reagents

Analytically pure samples of DVR and RTV were kindly supplied by Emcure Pharma Pvt. Ltd. (Pune, India) and Cipla Pvt. Ltd. (Kurkumbh, India) respectively. Toluene and Methanol (AR grade) were obtained from Thomas Baker Pvt Ltd (Mumbai, India). Ethyl acetate was obtained from Loba Chemie Pvt Ltd. (Mumbai, India).

Instrumentation and Chromatographic conditions

Chromatographic separation of drugs was performed on precoated silica gel aluminium plate 60 F_{254} (10 ×10) with 250 µm thickness (E. MERCK, Darmstadt, Germany) using a CAMAG Linomat 5 sample applicator (Switzerland). Samples were applied on the plate as a band with 6 mm width using Camag 100 µl sample syringe (Hamilton, Switzerland).

Linear ascending development was carried out in 10 x 10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland) using Toluene: Ethyl acetate: Methanol (6: 2.5: 1.5, v/v/v) as mobile phase. The optimized chamber saturation time for mobile phase was 15 min. The length of chromatogram run was 9 cm and development time was approximately 15 min. TLC plates were dried in a current of air with the help of a hair drier. Densitometric scanning was performed on CAMAG thin layer chromatography scanner at 250 nm for all developments operated by WINCATS software version 1.4.3. The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 200 to 400 nm.

Preparation of standard stock solutions

Standard stock solution of DVR and RTV were prepared by dissolving 10 mg of drug in 10 ml of methanol to get concentration of 1 mg ml^{-1} from which 1 ml was further diluted to 10 ml to get stock solution of 100 ng µl^{-1} of DVR and RTV respectively.

Selection of detection wavelength

After chromatographic development bands were scanned over the range of 200-400 nm and the spectra was overlain. It was observed

that both drugs showed considerable absorbance at 250 nm. So, 250 nm were selected as the wavelength for detection.

Analysis of tablet formulation

The binary mixture of DVR and RTV was prepared in the ratio of 3:1. Accurately weighed DVR (300 mg) and RTV (100 mg) was transferred to a 100 ml volumetric flask containing 30 ml methanol. Common excipients, which are used in the tablet formulation, were added in this mixture and the content was sonicated for 15 min. The flask was allowed to stand at room temperature for 15 min, and the volume was made up to the mark with methanol to obtain the final concentration of 3000 ng band^{-1} for DVR and 1000 ng band^{-1} for RTV. The solution was filtered using Whatman paper No. 41. One ml of the above solution was further diluted with methanol to get final concentration of 300 ng band^{-1} for DVR and 100 ng band^{-1} for RTV. Two µl volumes of this solution were applied on TLC plate to obtain final concentration of 600 ng band^{-1} for DVR and 200 ng band^{-1} for RTV. After chromatographic development peak areas of the bands were measured at 250 nm and the amount of each drug present in sample was estimated from the respective calibration curves. Procedure was repeated six times for the analysis of the homogenous sample.

Stress degradation studies of bulk drug

The drugs were subjected to stress condition of hydrolysis (acid, base), oxidation, photolysis and thermal degradation to provide evidence on how stability of drug varies under the influence of variety of environmental conditions and to establish specific storage conditions, shelf-life and retest period.

Acid treatment

1 ml working standard solution of DVR (100 ng µl^{-1}) was mixed with 1 ml of 0.1 N methanolic HCl and 8 ml of methanol. Solution was kept for 4 h in dark place. The 4 µl of the resulting solution was applied on TLC plate and developed under optimized chromatographic condition. RTV was treated in similar manner to DVR.

Alkali treatment

1 ml working standard solution of DVR (100 ng µl^{-1}) was mixed with 1 ml of 0.1 N methanolic NaOH and 8 ml of methanol. The solution was kept for 4 h in the dark place. The 4 µl of the resulting solution was applied on TLC plate and developed under optimized chromatographic condition. RTV was treated in the similar manner to DVR.

Neutral hydrolysis

1 ml working standard solution of DVR (100 ng µl^{-1}) was mixed with 1 ml of water and 8 ml methanol. The solution was kept for 24 h in the dark place. The 4 µl of the resulting solution was applied on TLC plate and developed under optimized chromatographic condition. RTV was treated in the similar manner to DVR.

Oxidative degradation

1 ml working standard solution of DVR (100 ng µl^{-1}) was mixed with 1 ml of 6 % solution of H_2O_2 and 8 ml of methanol. The solution was kept for 4 h in the dark place. The 4 µl of the resulting solution was applied on TLC plate and developed under optimized chromatographic condition. RTV was treated in the similar manner to DVR.

Photo-degradation

Photolytic studies were carried out by exposure of drug to UV light up to 200 watt h/m^2 and subsequently to fluorescence light illumination not less than 1.2 million lux h. Sample was weighed, dissolved in methanol to get concentration of 100 ng µl^{-1}. 4 µl of the resulting solution was applied to HPTLC.

Degradation under dry heat

Dry heat study was performed by keeping DVR in an oven at 60ºC for the period of 2 h. A sample was withdrawn at appropriate times, weighed and dissolved in methanol to get the solution of 100 ng µl^{-1}. 4 µl of the resulting solutions was applied to HPTLC. RTV was treated in the similar manner to DVR.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The primary target in developing this stability indicating HPLTC method is to achieve the resolution between DVR, RTV and its degradation products. The separation was achieved by linear ascending development in 10 cm × 10 cm twin trough glass chamber using Toluene: Ethyl acetate: Methanol (6: 2.5: 1.5, v/v/v) as mobile phase. The optimum wavelength for detection and quantitation used was 250 nm. The retention factors for DVR and RTV were found to be 0.29±0.005 and 0.50±0.07 respectively. Representative densitogram of mixed standard solution of DVR and RTV is shown in fig. 1.

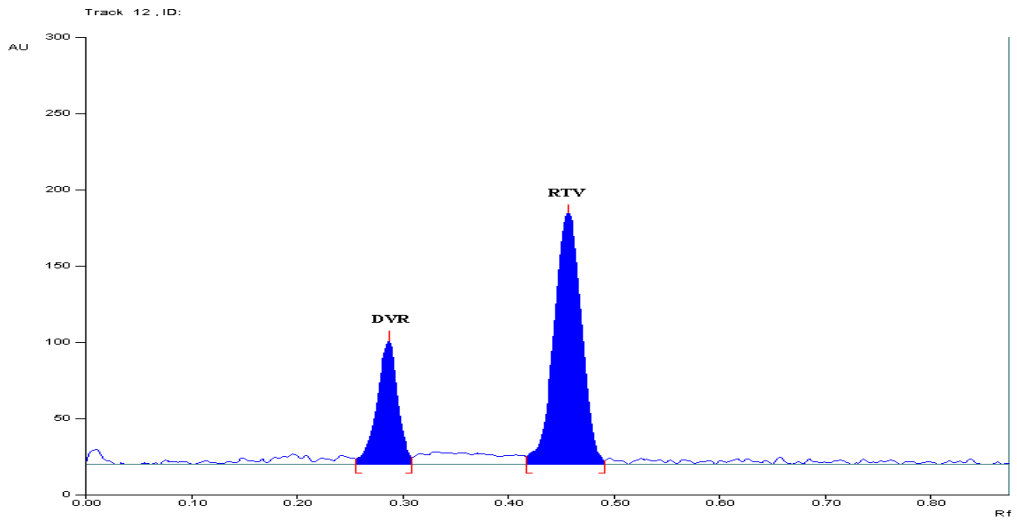


Fig. 1: Representative densitogram of mixed standard solution of DVR (400 ng band^{-1}, R_f = 0.29±0.005) and RTV (400 ng band^{-1}, R_f = 0.50±0.07)

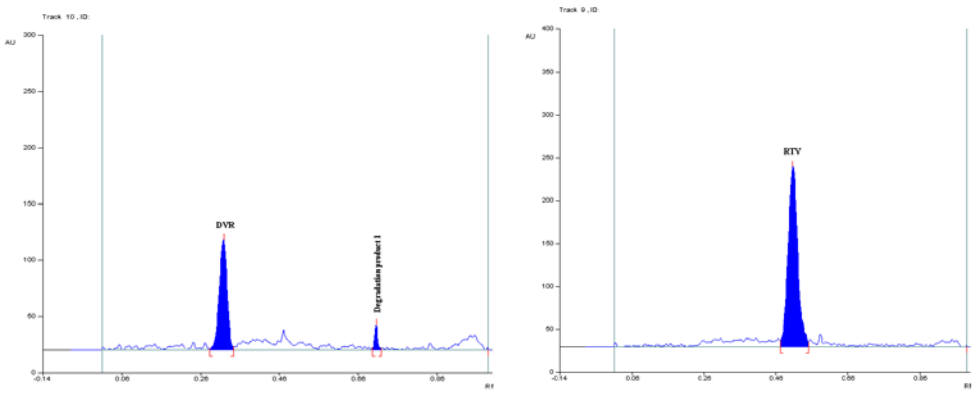
Result of forced degradation studies

Forced degradation study showed the method is highly specific and no degradation products were eluted at retention time of drugs.

Acid treatment

12 % of DVR and 16 % of RTV degraded in acid condition when refluxed for 4 h. However, no additional degradation peaks were

seen in the densitogram for RTV. The representative densitogram after acid treatment is shown in fig. 2.



(a) **(b)**

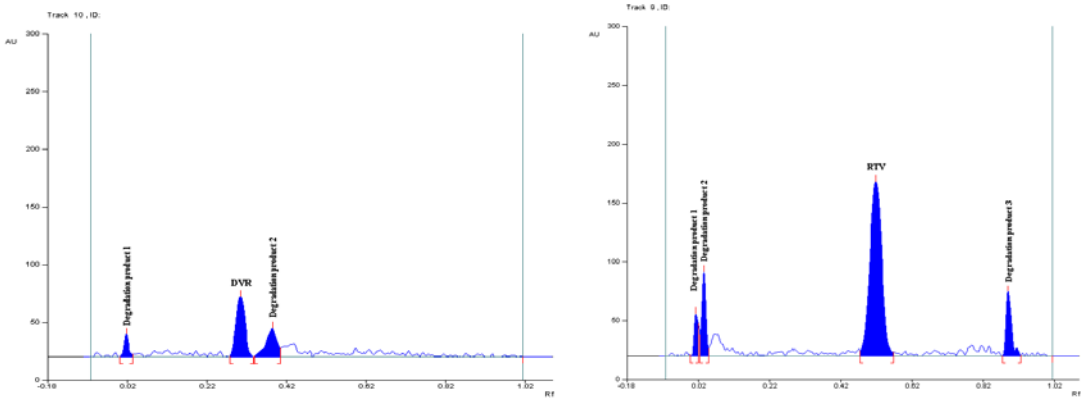
Fig. 2: Representative densitogram after acid treatment (a) DVR with degradation product at Rf 0.72 and (b) RTV

Alkali treatment

22 % of degradation of DVR was observed in alkaline condition while 25 % degradation of RTV was observed with degradation products. The representative densitogram after alkali treatment is shown in fig. 3.

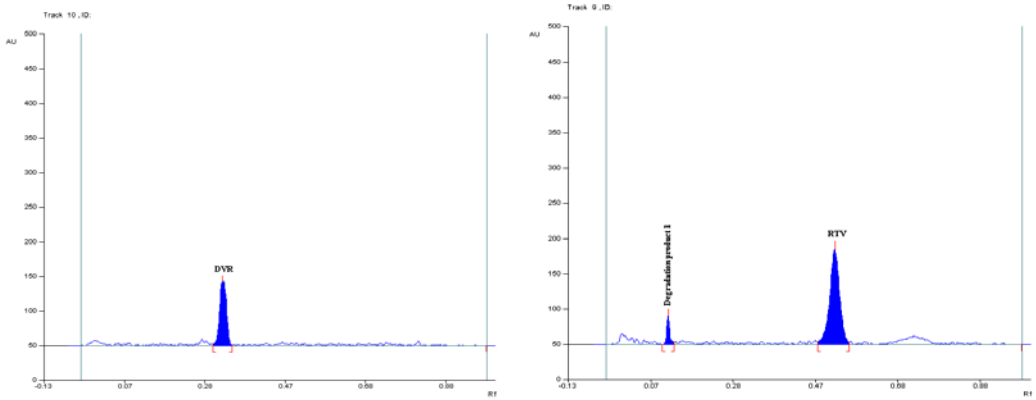
Oxidative degradation

17 % degradation was observed for DVR when treated with 6 % H_2O_2 while RTV exhibited 26 % degradation. The representative densitogram after oxidative degradation is shown in fig. 4.



(a) **(b)**

Fig. 3: Representative densitogram after alkali treatment (a) DVR with degradation product at Rf 0.02, 0.39 and (b) RTV with degradation product at Rf 0.02, 0.05, 0.89



(a) **(b)**

Fig. 4: Oxidative degradation densitogram of (a) DVR and (b) RTV with degradation product at Rf 0.15

Neutral hydrolysis

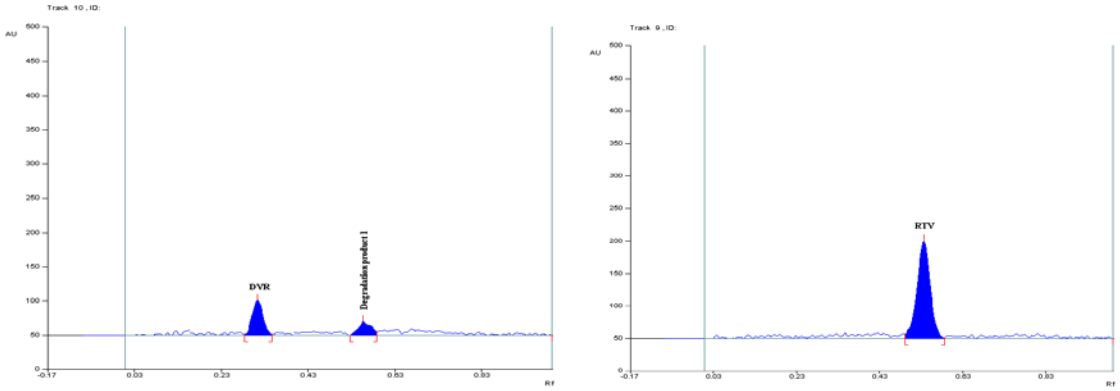
16 % degradation was observed for DVR in neutral condition when refluxed for 24 h with degradation peak at Rf 0.57 while 11 % of degradation was observed for RTV. The representative densitogram after neutral degradation is shown in fig. 5.

Photo degradation studies

DVR exhibited 10 % and 15 % of degradation when exposed to ultraviolet light (200 Watt h/m²) and fluorescence light (1.2 million lux h) and RTV shown 9 % and 12 % of degradation when exposed to fluorescence light (1.2 million lux h). The representative densitogram after photo degradation is shown in fig. 6.

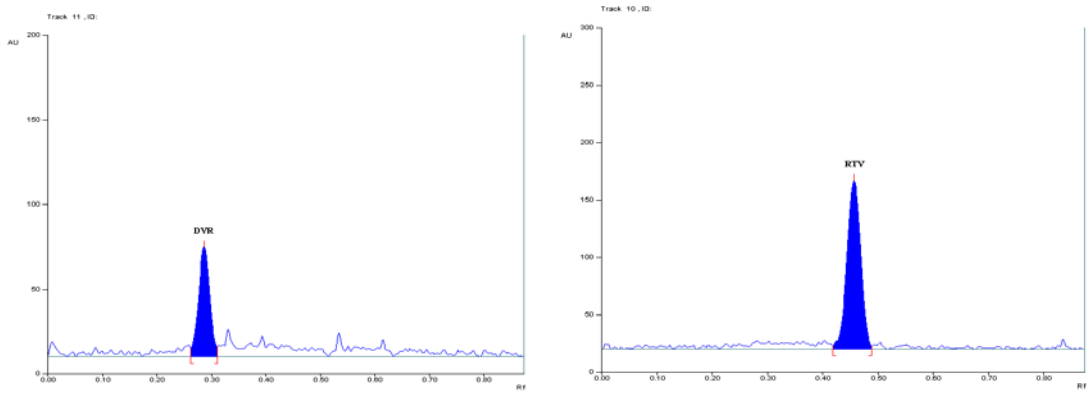
Dry degradation studies

21 % and 19 % of degradation was observed for DVR and RTV when the powdered drug substances were exposed to dry heat at 60º C for 24 h. The representative densitogram obtained from sample subjected to dry heat is shown in fig. 7.



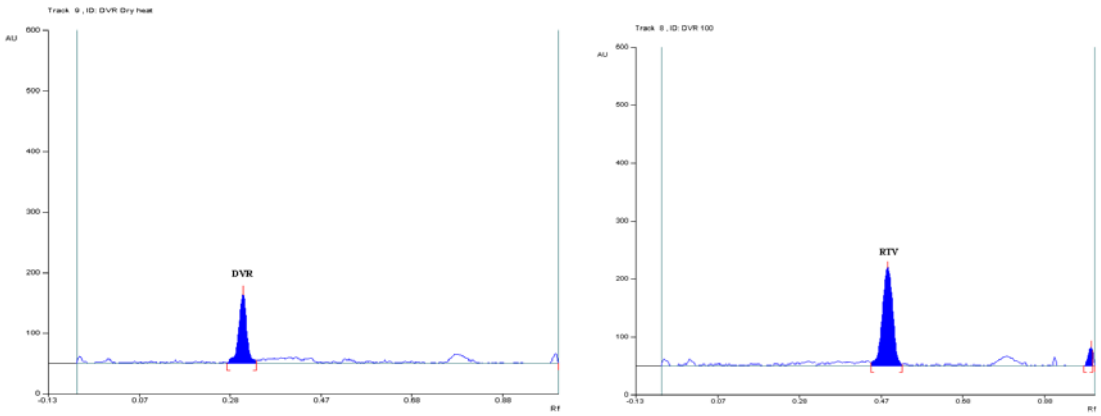
(a) **(b)**

Fig. 5: Neutral degradation densitogram of (a) DVR along with its degradation product (Rf 0.57) and (b) RTV



(a) **(b)**

Fig. 6: Photo degradation densitogram of (a) DVR and (b) RTV



(a) **(b)**

Fig. 7: Densitogram obtained from sample subjected to dry heat (a) DVR and (b) RTV

The results of the stress testing of the drugs carried out revealed that the degradation products were formed in hydrolytic (acid and base), oxidative conditions. The developed method is able to separate the peaks of active pharmaceutical ingredients from their degradation products formed under various stress conditions and excipients used in tablet dosage forms and hence, it can be used for monitoring quality during stability studies of pharmaceutical preparations.

Peak purity results greater than 990 indicate that DVR and RTV peaks are homogeneous in all stress conditions tested. The unaffected assay of DVR and RTV in the tablet confirms the stability indicating power of the method. The forced degradation studies data are summarized in table 1.

Method validation

The method was validated for linearity, accuracy, intra-day and inter-day precision and robustness, in accordance with ICH guidelines [24, 25].

Preparation of calibration curve

The standard stock solutions of DVR and RTV (100 ng μl^{-1} each) were applied by overspotting on TLC plate in the range of 2, 4, 6, 8, 10, 12 and 14 µl. Straight-line calibration graphs were obtained in the concentration range 200-1400 ng $band^{-1}$ for both the drugs with the high correlation coefficient>0.998.

Table 1: Data of forced degradation studies of DVR and RTV

Stress conditions/duration	DVR	RTV
	(%) Degradation	(%) Degradation
Acidic/0.1 N HCl/Reflux for 4 h	12	16
Alkaline/0.1 N NaOH/Reflux for 4 h	22	25
Oxidative/6 % H_2O_2/Reflux for 24 h	17	26
Neutral/H_2O/Reflux for 24 h	16	11
Photolysis		
UV/200 watt h/m^2	10	09
Fluorescence/1.2 million lux h	15	12
Dry heat/60ºC/24 h	21	19

Precision

Set of three different concentrations in three replicates of mixed standard solutions of DVR and RTV were prepared. All the solutions were analyzed on the same day in order to record any intraday variations in the results. Intra-day variation, as RSD (%), was found to be in the range of 0.45 to 1.54 for DVR and 0.22 to 0.64 for RTV. For Inter day variation study, three different concentrations of the mixed standard solutions in linearity range were analyzed on three consecutive days. Interday variation, as RSD (%) was found to be in the range of 0.64 to 1.48 for DVR and 0.13 to 1.24 for RTV. The lower values of % RSD obtained have proved that the developed method is precise.

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

LOD and LOQ were calculated as 3.3 σ/S and 10 σ/S, respectively; where σ is the standard deviation of the response (y-intercept) and S is the slope of the calibration plot. The LOD of DVR and RTV was found 65 ng $band^{-1}$ and 30 ng $band^{-1}$, respectively. The LOQ of RTV and ATV was 192 ng $band^{-1}$ and 91 ng $band^{-1}$, respectively.

Recovery studies

To check accuracy of the method, recovery studies were carried out by adding standard drug to sample at three different levels 80, 100 and 120 %. Basic concentration of sample chosen was 300 ng $band^{-1}$ of DVR and 100 ng $band^{-1}$ of RTV. The drug concentrations were calculated from respective linearity equation. The results of recovery studies indicated that the method is accurate for estimation of drugs in tablet dosage form. The results obtained are shown in table 2.

Specificity

The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than 991, indicating the no interference of any other peak of degradation product, impurity or matrix.

Table 2: Recovery studies of DVR and RTV

Drug	Amount taken (ng $band^{-1}$)	Amount added (ng $band^{-1}$)	Total amount found (ng $band^{-1}$)	% Recovery	% RSD[a]
	600	480	1078.41	99.81	0.65
DVR	600	600	1186.38	98.80	0.62
	600	720	1312.75	99.43	0.95
	200	160	4479.77	101.10	0.82
RTV	200	200	6020.78	99.33	0.52
	200	240	7535.32	99.61	1.48

*Average of three determinations

Table 3: Robustness data in terms of peak area (% RSD)

S. No.	Parameter	(% RSD)*	
		DVR	RTV
1	Mobile phase saturation (±10 %)	0.70	0.50
2	Wavelength	1.00	0.78
3	Time from application to development (0, 10, 20, and 30 min)	0.54	0.52
4	Development to scanning (0, 30, 60, and 90 min)	1.00	0.61

*Average of three determinations

Robustness studies

Robustness of the method was determined by carrying out the analysis under conditions during which mobile phase saturation time, wavelength, time from application to development and from development to scanning were altered and the effect on the area of the drug was noted. When deliberate variations were made to the method conditions, there were no marked changes in chromatographic behaviour, indicating the method is robust. The results are given in table 3.

CONCLUSION

The developed method is stability indicating and can be used for assessing the stability of DVR and RTV in bulk drug and pharmaceutical dosage form. The developed method is accurate, precise, specific and robust.

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CONFLICT OF INTERESTS

Declared None

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