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Research Article

STABILITY INDICATING HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC METHOD FOR DETERMINATION OF ATAZANAVIR AND RITONAVIR IN COMBINED TABLET DOSAGE FORMS

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ABSTRACT
A new simple, accurate, precise and selective stability- indicating high performance thin layer chromatographic (HPTLC) method has been developed and validated for simultaneous estimation of Atazanavir and Ritonavir in combined tablet dosage form. The mobile phase selected was Toluene: Ethyl acetate: Methanol (6: 4.5: 0.6, v/v/v) with UV detection at 240 nm. The retention factor for Atazanavir and Ritonavir were found to be 0.25 ± 0.004 and 0.41 ± 0.004. The method was validated with respect to linearity, accuracy, precision and robustness. The drugs were subjected to stress condition of hydrolysis (acid, base), oxidation, photolysis and thermal degradation. Results found to be linear in the concentration range of 1000-8000 ng band^{-1} for Atazanavir and 500-4000 ng band^{-1} for Ritonavir respectively. The method has been successfully applied for the analysis of drugs in pharmaceutical formulation. The % assay (Mean ± S.D.) was found to be 100.40 ± 0.964 for Ritonavir and 99.59 ± 1.103 for Atazanavir.
Keywords: Ritonavir, Atazanavir, Forced degradation, Tablet dosage form

INTRODUCTION

Atazanavir (ATV), chemically, (3S,8S,9S,12S)-3,12-bis(1,1-dimethylethyl)-8-hydroxy-4,11-dioxo-9-(phenylmethyl)-6-[[4-(2-pyridinyl)phenyl] methyl]-2,5,6,10,13-penta aza tetra deca-nedioic acid dimethyl ester which is inhibitor of HIV- 1 protease[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 including Atazanavir, thereby providing increased plasma level of Atazanavir[2]. Literature survey reveals High Performance Liquid Chromatographic (HPLC)[3-9], LC-MS[10] and Ultra Performance Liquid Chromatography (UPLC)[11] methods for determination of ATV as single and in combination with other drugs in human plasma. Also Spectrophotometric method for degradation studies of ATV in dosage form has been also reported[12]. Analytical methods reported for RTV includes HPLC[13-15], LC-MS[16], Densiometry[17-19] and spectrophotometry[20] either as single or in combination with other drugs. No reports were found for stability-indicating HPTLC method for simultaneous determination of ATV 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 Harmonization Guidelines[21,22].

MATERIALS AND METHODS

Reagents and chemicals

Analytically pure samples of RTV and ATV were kindly supplied by Emcure Pharma Pvt. Ltd. (Pune, India) and Cipla Pvt. Ltd. (Kurkumbh, India) respectively. The pharmaceutical dosage form used in this study was Sinthivan tablets (Cipla Pvt. Ltd., Patalganga, India) labeled to contain 300 mg of ATV and 100 mg of RTV were procured from the local market. 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

The samples were spotted in the form of bands of width of 6 mm with space between bands of 10 mm, with a 100 µL sample syringe (Hamilton, Bonaduz, Switzerland) on precoated silica gel aluminum plate 60 F_{254} (10 × 10) with 250 µm thickness (E. MERCK, Darmstadt, Germany) using a CAMAG Linomat 5 sample applicator (Switzerland). The slit dimensions 5 mm × 0.45 mm and scanning speed of 20 mm/sec was employed. The linear ascending development was carried out in 10 cm × 10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland) using Toluene: Ethyl acetate: Methanol (6: 4.5: 0.6 v/v/v) as mobile phase. The optimized chamber saturation time for mobile phase was 15 minutes. The length of chromatogram run was 9 cm and development time was approximately 15 minutes. 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 240 nm for all developments operated by WINCATS software version 1.4.2. 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 RTV and ATV were prepared by dissolving 10 mg of drug in 10 mL of methanol to get concentration of 1 mg mL^{-1} from which 5 mL was further diluted to 10 mL to get stock solution of 500 ng µL^{-1} of RTV and ATV respectively.

Selection of Detection Wavelength

After chromatographic development bands were scanned over the range of 200-400 nm and the spectra were overlain. It was observed that both drugs showed considerable absorbance at 240 nm. So, 240 nm was selected as the wavelength for detection.

Analysis of Tablet Formulation
Twenty tablets were weighed accurately and finely powdered. A quantity of powder equivalent to 5 mg of RTV (15 mg ATV) was weighed and dissolved in 10 mL of methanol. The solution was filtered using Whatman paper No. 41 and two µL volume of this solution was applied on TLC plate to obtain final concentration of 1000 ng band^{-1} for RTV and 3000 ng band^{-1} for ATV. After chromatographic development peak areas of the bands were measured at 240 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 homogenous sample.

Stress degradation studies of bulk drug
Forced degradation studies were carried out to provide evidence on how stability of drug varies under the influence of variety of environmental conditions like hydrolysis, oxidation, temperature, etc. and to establish specific storage conditions, shelf-life and retest period.

Acid treatment
1 mL working standard solution of ATV (500 ng µL^{-1}) was mixed with 1 mL of 0.1 N HCl (methanolic) and 8 mL of methanol. The mixture was refluxed for half an hour. 4 µL of the resulting solution was applied to HPTLC. RTV was treated in similar manner to ATV.

Alkali treatment
1 mL working standard solution of ATV (500 ng µL^{-1}) was mixed with 1 mL of 0.1 N NaOH and 8 mL of methanol. The mixture was refluxed for two hour. 4 µL of the resulting solution was applied to HPTLC. RTV was treated in similar manner to ATV.

Oxidative degradation
1 mL working standard solution of ATV (500 ng µL^{-1}) was mixed with 1 mL of 30 % solution of H_2O_2 and 8 mL of methanol. The mixture was refluxed for three hour. 4 µL of the resulting solution was applied to HPTLC. RTV was treated in similar manner to ATV.

Neutral Hydrolysis
1 ml working standard solution of ATV was mixed with 9 ml water. The solution was refluxed for one h in dark place. 4 µL of the resulting solution was applied to HPTLC. RTV was treated in similar manner to ATV.

Photo-degradation
Photolytic studies were carried out by exposure of drug to UV light up to 200 watt hours square meter^{-1} and subsequently to fluorescence light illumination not less than 1.2 million lux hours. Sample was weighed, dissolved in methanol to get concentration of 500 µg mL^{-1}. 4 µL of the resulting solutions was applied to HPTLC.

Degradation under dry heat
Dry heat study was performed by keeping ATV in oven at 60ºC for 7 days. A sample was withdrawn at appropriate times, weighed and dissolved in methanol to get solution of 500 ng µL^{-1}. 4 µL of the resulting solutions was applied to HPTLC. RTV was treated in similar manner to ATV.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions
The primary target in developing this stability indicating HPLTC method is to achieve the resolution between ATV, 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: 4.5: 0.6 v/v/v) as mobile phase. The optimum wavelength for detection and quantitation used was 240 nm. The retention factors for RTV and ATV were found to be 0.25 ± 0.004 and 0.41 ± 0.004 respectively. Representative densitogram of mixed standard solution of RTV and ATV is shown in Figure 1.

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
14 % of ATV and 33 % of RTV degraded in acid condition when refluxed for half an hour. However no additional degradation peaks were seen in the densitogram for ATV. The representative densitogram after acid treatment is shown in Figure 2.

Alkali treatment
36 % of degradation of RTV was observed in alkaline condition while 13 % degradation of ATV was observed with no additional degradation peak. The representative densitogram after alkali treatment is shown in Figure 3.

Oxidative degradation
22 % degradation was observed for ATV when treated with 30 % H_2O_2 while RTV exhibited 8 % degradation. The representative densitogram after oxidative degradation is shown in Figure 4.

Neutral Hydrolysis
ATV was found to be stable in neutral condition when refluxed for one hour while 16 % of degradation was observed for RTV with degradation peaks at R_f 0.16, 0.30. The representative densitogram after neutral degradation is shown in Figure 5.

Photo Degradation Studies
ATV and RTV were found to be stable in ultraviolet light (200 Watt hours/Sequre meter) as well as fluroscence light (1.2 million lux hours).

Dry Degradation Studies
The solid state studies showed that the drug substances ATV and RTV were stable to the effect of temperature when the powdered drug substance was exposed to dry heat at 60º C for 7 days. Peak purity results greater than 990 indicate that ATV and RTV peaks are homogeneous in all stress conditions tested. The unaffected assay of ATV 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[22].

Preparation of Calibration Curve

The standard stock solutions of RTV and ATV (500 ng µL each) were applied by over spotting on TLC plate in range of 1, 2, 3, 4, 5, 6, 7, 8 µL and 2, 4, 6, 8, 10, 12, 14, 16 µL respectively. Straight-line calibration graphs were obtained for RTV and ATV in the concentration range 500-4000 ng band^{-1} for RTV and 1000-8000 ng band^{-1} for ATV with high correlation coefficient > 0.993

Precision

Set of three different concentrations in three replicates of mixed standard solutions of RTV and ATV were prepared. All the solutions were analyzed on the same day in order to record any intra day variations in the results. Intra-day variation, as RSD (%), was found to be in the range of 0.22 to 0.63 for RTV and 0.41 to 1.05 for ATV. For Inter day variation study, three different concentrations of the mixed standard solutions in linearity range were analyzed on three consecutive days. Inter day variation, as RSD (%) was found to be in the range of 0.18 to 0.62 for RTV and 0.364 to 0.70 for ATV.

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 RTV and ATV were found 69.20 ng band^{-1} and 143.60 ng band^{-1}, respectively. The LOQ of RTV and ATV were 209.72 ng band^{-1} and 434.56 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 50, 100 and 150 %. Basic concentration of sample chosen was 1000 ng band^{-1} of RTV and 3000 ng band^{-1} of ATV from tablet solution. The drug concentrations were calculated from respective linearity equation. 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.

Robustness Studies

Robustness of the method was determined by carrying out the analysis under conditions during which mobile phase composition, mobile phase saturation, development distance, time from application to development and from development to scanning were altered and the effect on the area of drug were noted. The results are given in Table 3.

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

Stress conditions/ duration	ATV	RTV
	(%) Degradation	(%) Degradation
Acidic / 0.1 N HCl/ Reflux half an hour	14	33
Alkaline /0.1 N NaOH/ Reflux for 2 hours	13	36
Oxidative /30 % H_2O_2 / Reflux for 3 hours	22	08
Neutral/H_2O/ Reflux for 1 hour	00	16
Photolysis UV/200 watt hours/square meter	0	0
Fluorescence / 1.2 million lux hours	0	0
Dry heat/ 60ºC/ 7 days	0	0

Table 2: Recovery Studies of ATV and RTV

Drug	Amount taken (ng band^{-1})	Amount added (ng band^{-1})	Total amount found (ng band^{-1})	% Recovery	% RSD[a]
RTV	1000	500	1518.00	101.20	0.94
	1000	1000	2004.64	100.23	1.07
	1000	1500	2506.62	100.26	1.03
ATV	3000	1500	4479.77	99.55	0.26
	3000	3000	6020.78	100.34	0.45
	3000	4500	7535.32	100.47	1.16

*Average of three determinations

Table 3: Robustness Data in Terms of Peak Area (% RSD)

S. No.	Parameter	(% RSD)*	
		RTV	ATV
1	Mobile phase composition (± 2 %)	0.08	0.02
2	Mobile phase saturation (± 10 %)	0.03	0.02
3	Time from application to development (0, 10, 20, and 30 min)	0.05	0.02
4	Development to scanning (0, 30, 60, and 90 min)	0.06	0.01
5	Development distance (± 10 %)	0.07	0.01

*Average of three determinations

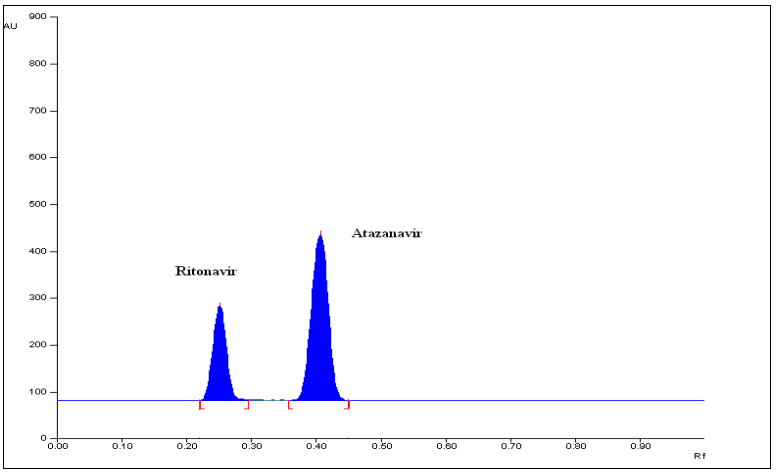


Figure 1: Representative densitogram of mixed standard solution of RTV (2500 ng/band, R_f = 0.25 ± 0.004) and ATV (5000 ng/band, R_f = 0.41 ± 0.004)

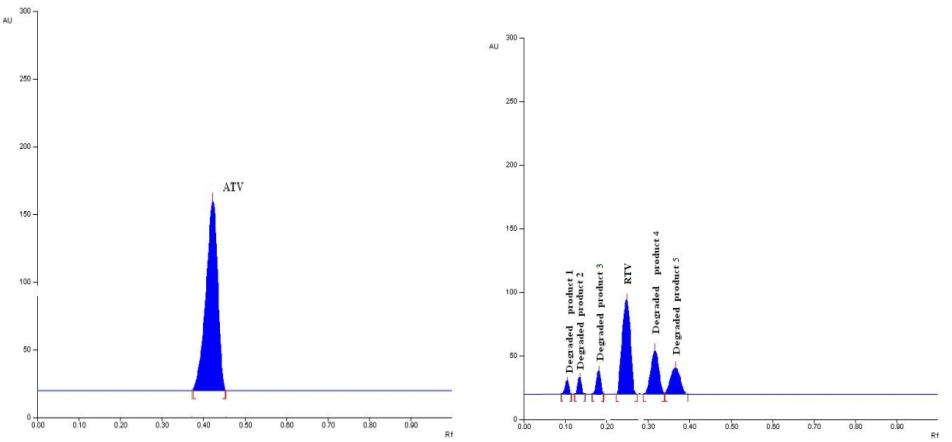


Figure 2: Representative densitogram after acid treatment (a) ATV and (b) RTV with degradation product at R_f 0.10, 0.14, 0.18, 0.32 and 0.36

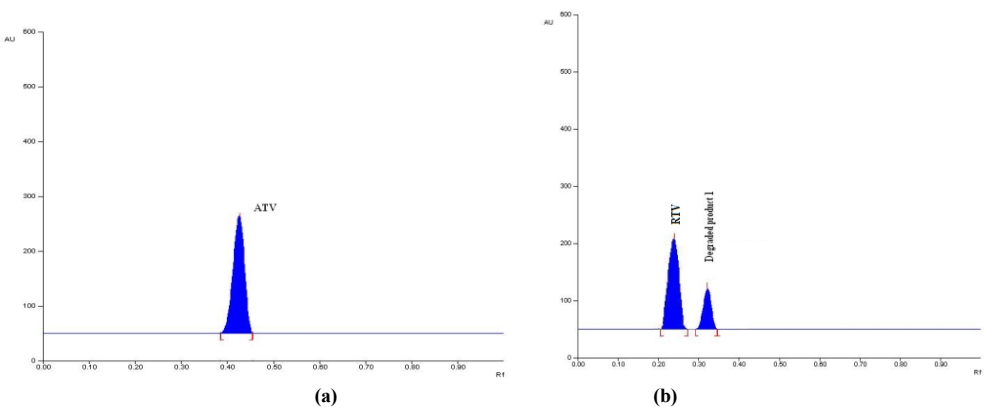
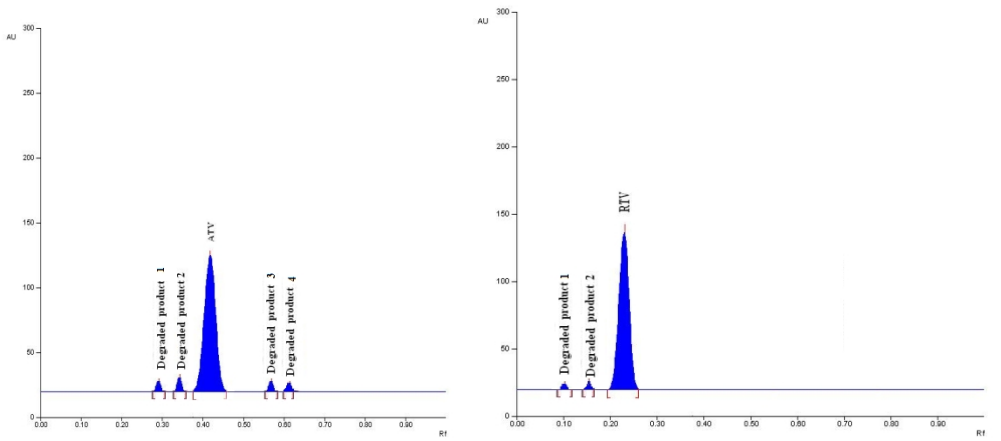
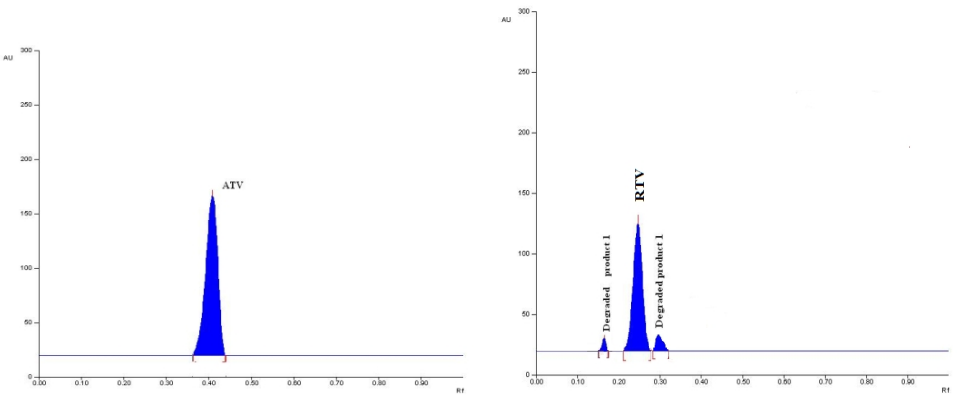


Figure 3: Representative densitogram after alkali treatment (a) ATV and (b) RTV with degradation product at R_f 0.32



(a) **(b)**

Figure 4: Oxidative degradation densitogram of (a) ATV with degradation peaks at R_f 0.28, 0.35, 0.56 and 0.61 and (b) RTV with degradation peaks at R_f 0.10, 0.14



(a) **(b)**

Figure 5: Neutral degradation densitogram of (a) ATV and (b) RTV along with its degradation product (R_f 0.16, 0.30)

CONCLUSION

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

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