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High performance thin layer chromatographic determination of atazanavir and ritonavir in combined tablet dosage form

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ABSTRACT

A new simple High Performance Thin Layer Chromatographic (HPTLC) method for determination of Atazanavir and Ritonavir in combined tablet dosage form has been developed and validated. 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 as per the International Conference on Harmonisation (ICH) guidelines. Results found to be linear in the concentration range of 1000-8000 ng/band for Atazanavir and 500-4000 ng/band 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 ± 0.96 for Ritonavir and 99.59 ± 1.10 for Atazanavir. The method can be used for routine analysis of these drugs in combined tablet dosage forms in quality-control laboratories.

Key words: Ritonavir, Atazanavir, Densitometry, Tablet dosage form

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INTRODUCTION

Atazanavir, 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, 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) [3-10] LC-MS [11] and Ultra Perfirmance Liquid Chromatography (UPLC) [12] methods for determination of Atazanavir as single and in combination with other drugs in human plasma. Also Spectrophotometric [13] method for degradation studies of atazanavir in dosage form have been also reported. Analytical methods reported for Ritonavir includes HPLC [14-16], LC-MS [17], Densiometry [18, 19] and spectrophotometry [20] either as single or in combination with other drugs.

No reports were found for the simultaneous estimation of the Atazanavir and Ritonavir in combined dosage form by HPTLC method. This paper describes a simple, accurate, sensitive and validated HPTLC method for simultaneous quantification of these compounds as the bulk drug and in tablet dosage forms. The proposed method is optimized and validated as per the International Conference on Harmonization (ICH) guidelines [21].

MATERIALS AND METHODS

Reagents and chemicals

Analytically pure samples of Ritonavir and Atazanavir 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) used for the method development. The pharmaceutical dosage form used in this study was Sinthivan tablets (Cipla Pvt. Ltd., Patalganga, India) labeled to contain 300 mg of Atazanavir and 100 mg of Ritonavir were procured from the local market.

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 aluminium plate 60 F_{254} (10 cm ×10 cm) 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 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 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 Ritonavir and Atazanavir was prepared by dissolving 10 mg of each drug in 10 mL of methanol to get concentration of 1 mg/mL from which 5 mL was further diluted to 10 mL to get stock solution of 500 ng/µL for both the drugs.

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 as shown in Figure 1.

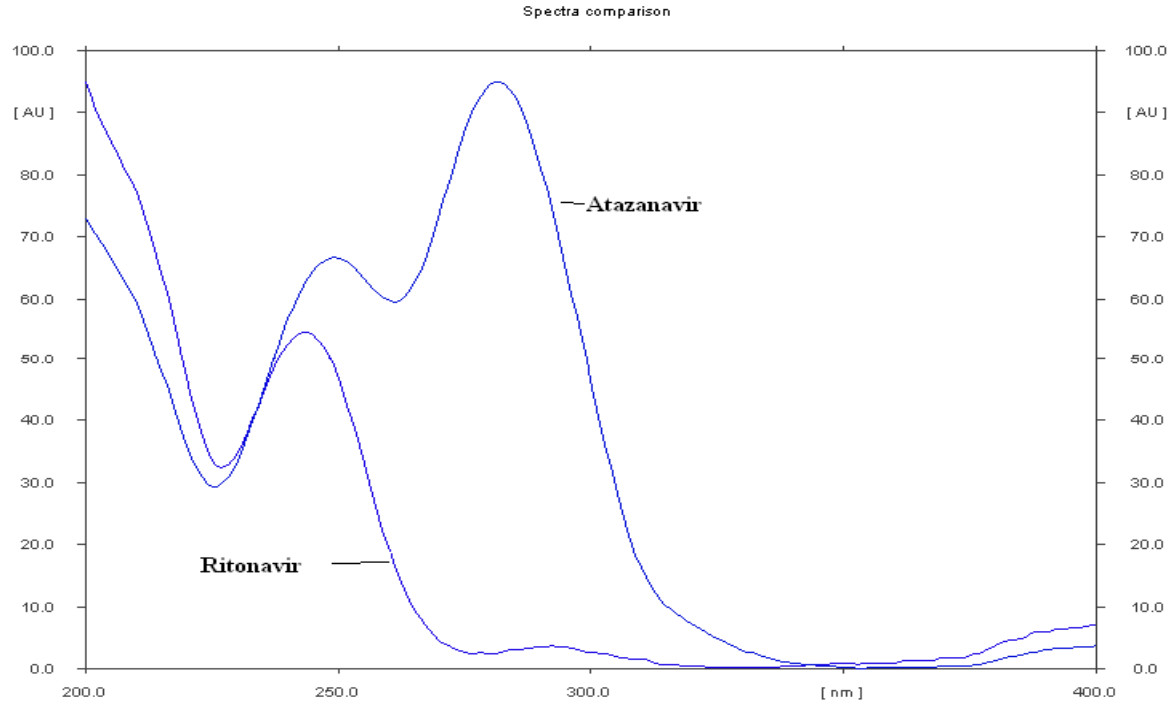

Figure 1: Overlain spectra of Ritonavir and Atazanavir

Analysis of Tablet Formulation

Twenty tablets were weighed accurately and finely powdered. A quantity of powder equivalent to 5 mg of Ritonavir 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 for Ritonavir and 3000 ng/band for Atazanavir . 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.

Method Validation

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

Preparation of Calibration Curve

The standard stock solutions of Ritonavir and Atazanavir (500 ng/µLeach) were applied by overspotting on TLC plate in range of 1, 2, 3, 4, 5, 6, 7 and 8 µL and 2, 4, 6, 8, 10, 12 , 14 and 16 µL respectively with the help of CAMAG 100 µL sample syringe, using Linomat 5 sample applicator. The plate was developed and scanned under above established chromatographic conditions. Each standard in six replicates was analyzed and peak areas were recorded. Calibration curves of Ritonavir and Atazanavir were plotted separately of peak area vs respective concentration of Ritonavir and Atazanavir.

Precision

Set of three different concentrations in three replicates of mixed standard solutions of Ritonavir and Atazanavir were prepared. All the solutions were analyzed on the same day in order to record any intra day variations in the results. For Inter day variation study, three different concentrations of the mixed standard solutions in linearity range were analyzed on three consecutive days.

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

LOD and LOQ for both the drugs were calculated by using the values of slopes and intercepts of the calibration curves.

Robustness Studies

In the robustness study, the influence of small, deliberate variations of the analytical parameters on peak area of the drugs was examined. Factors varied were mobile phase composition (± 2 %), mobile phase saturation (± 10 %) , development distance (± 10 %). One factor at a time was changed to estimate the effect. Robustness of the method was checked at a concentration level of 1000 ng/ band for Ritonavir and 2000 ng/ band for Atazanavir. The results are given in Table 1.

Table 1: Robustness Data in Terms of Peak Area (% RSD)

Sr . No.	Parameter	(% RSD)*	
		Ritonavir	Atazanavir
1	Mobile phase composition	0.087	0.021
2	Mobile phase saturation	0.039	0.021
3	Development distance	0.076	0.016

**Average of three determinations.*

Table 2: Recovery Studies of Atazanavir and Ritonavir

Drug	Amount taken (ng/band)	Amount added (ng/band)	Total amount found (ng/band)	% Recovery[a]	% R.S.D.[a]
Ritonavir	1000	500	1518.00	101.20	0.94
	1000	1000	2004.64	100.23	1.07
	1000	1500	2506.62	100.26	1.03
Atazanavir	3000	1500	4479.77	99.55	0.26
	3000	3000	6020.78	100.34	0.45
	3000	4500	7535.32	100.47	1.16

[a] *Average of three determinations; RSD is the relative standard deviation*

Recovery Studies

To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample solution at three different levels 50, 100 and 150 %. Chromatogram was developed and the peak

areas were noted. At each level of the amount, three determinations were carried out. The results of recovery studies were expressed as percent recovery and are shown in Table 2.

RESULTS AND DISCUSSION

Different mobile phases containing various ratios of Formic acid, Ethyl acetate, Methanol, Chloroform, Toluene and Glacial acetic acid were examined (data not shown). Finally the mobile phase containing Toluene: Ethyl acetate: Methanol (6: 4.5: 0.6, v/v/v) was selected as optimal for obtaining well defined and resolved peaks. The optimum wavelength for detection and quantitation used was 240 nm. The retention factors for Ritonavir and Atazanavir were found to be 0.25 ± 0.004 and 0.41 ± 0.004 respectively. Representative densitogram of mixed standard solution of Ritonavir and Atazanavir is shown in Figure 2.

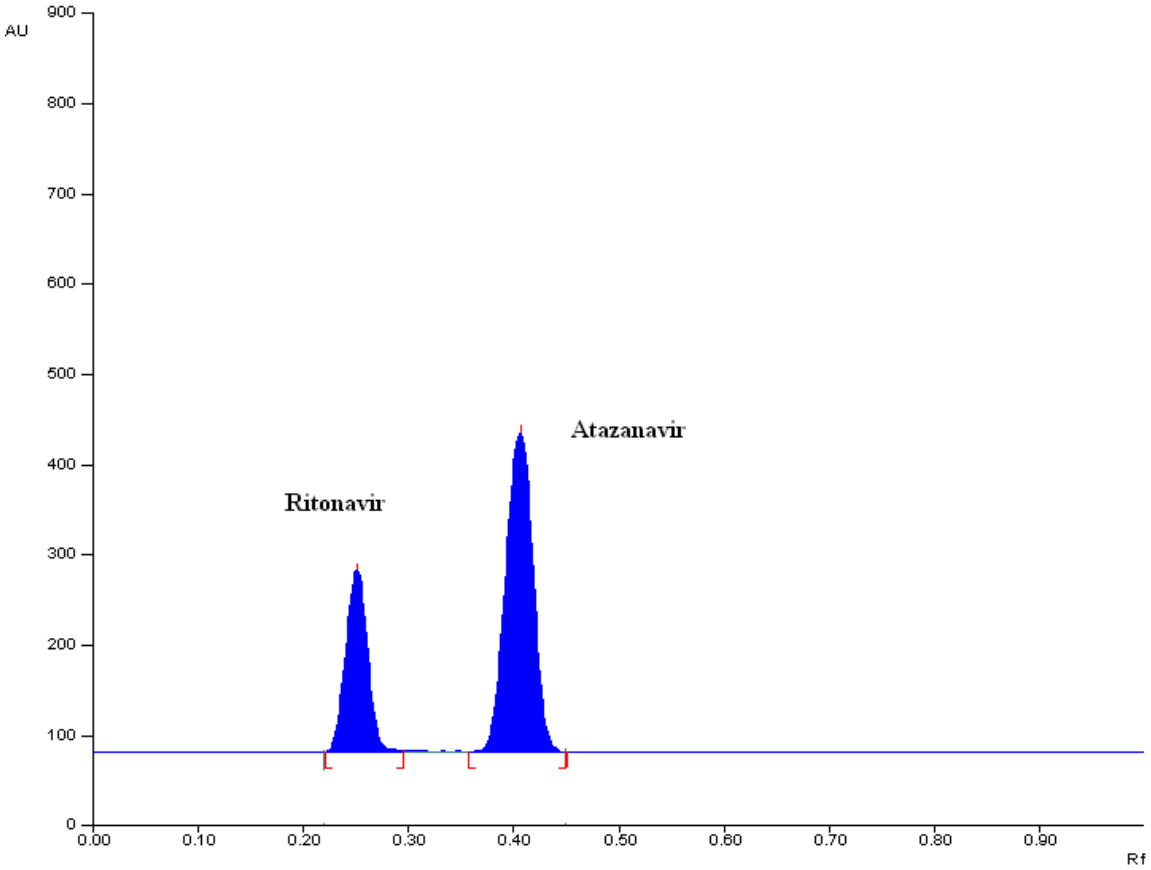

Figure 2: Representative densitogram of mixed standard solution of Ritonavir (2500 ng/band, R_f = 0.25 ± 0.004) and Atazanavir (5000 ng/band, R_f = 0.41 ± 0.004)

Straight-line calibration graphs were obtained for Ritonavir and Atazanavir in the concentration range 500-4000 ng/band for Ritonavir and 1000-8000 ng/band for Atazanavir with high correlation coefficient > 0.993. The proposed method was also evaluated by the assay of commercially available tablets containing Ritonavir and Atazanavir. The % assay (Mean ± S.D.) was found to be 100.40 ± 0.96 for Ritonavir and 99.59 ± 1.10 for Atazanavir. Robustness of the method checked after deliberate alterations of the analytical parameters showed that areas of peaks of interest remained unaffected by small changes of the operational parameters (% RSD < 2).

For Ritonavir, the recovery study results ranged from 100.23 to 101.20 % with % RSD values ranging from 0.94 to 1.07. For Atazanavir, the recovery results ranged from 99.55 to 100.47 % with % RSD values ranging from 0.26 to 1.16. The method was found to be accurate and precise, as indicated by recovery studies as recoveries were close to 100 % and % RSD not more than 2. Intra-day variation, as RSD (%), was found to be in the range of 0.22–0.63 for Ritonavir and 0.41–1.05 for Atazanavir. Interday variation, as RSD (%) was found to be in the range of 0.18–0.62 for Ritonavir and 0.36–0.70 for Atazanavir. The summary of validation parameters of proposed method are given in Table 3.

Table 3: Summary of validation parameters of proposed method

Parameters	Ritonavir	Atazanavir
Linearity range (ng/band)	500 - 4000	2000 -8000
Correlation co-efficient	0.994	0.996
LOD[a] (ng/band)	69.20	143.40
LOQ[b] (ng/band)	209.72	434.56
Accuracy (% Recovery)	100.23-101.20	99.55-100.47
Precision (% R.S.D.)[c]		
Intraday (n[d] = 3)	0.22-0.63	0.41-1.05
Inter day (n = 3)	0.18-0.62	0.36-0.70

[a]*LOD = Limit of detection* [c]*R.S.D. = Relative standard deviation*
[b]*LOQ =Limit of quantitation* [d]*n = Number of determination*

CONCLUSION

The validated HPTLC method employed here proved to be simple, fast, accurate, precise and robust, thus can be used for routine analysis of atazanavir and ritonavir in combined tablet dosage form.

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