



## Simultaneous Determination of Ritonavir and Atazanavir in Combined Tablet Dosage Form by HPTLC

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### ABSTRACT

A new, simple, sensitive, precise and accurate High-performance thin-layer chromatographic method for simultaneous determination of Ritonavir and Atazanavir in their combined tablet dosage form has been developed, validated and used for determination of the compounds in commercial pharmaceutical products. Chromatographic separation was achieved on aluminium plates precoated with silica gel 60 F<sub>254</sub> as the stationary phase and chloroform: ethyl acetate: acetone (5:2:3, v/v/v) as a mobile phase. Densitometric measurements of their spots were achieved at 244 nm over the concentration ranges of 800-2800 ng spot<sup>-1</sup> and 2400-8400 ng spot<sup>-1</sup>, with mean recoveries of 98.57 ± 0.35 and 99.16 ± 0.20 for ritonavir and atazanavir respectively. Limit of detection for ritonavir and atazanavir were found to be 300 ng spot<sup>-1</sup> and 200 ng spot<sup>-1</sup> respectively.

**Keywords:** High-performance thin-layer chromatography, Ritonavir, Atazanavir.

### 1. INTRODUCTION

Ritonavir (RTV) chemically (5S,8S,10S,11S)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid 5-thiazolyl methyl ester. Atazanavir (ATV) chemically (3S,8S,9S,12S)-3,12-Bis(1,1-dimethylethyl) -8-hydroxy-4,11-dioxo-9-(phenyl methyl)-6-[[4-(2-pyridinyl)phenyl]methyl]-2,5,6,10,13-penta aza tetradecanedioic acid dimethyl ester, sulphate (1:1).

Ritonavir and Atazanavir inhibit HIV protease, enzyme required to form functional proteins in HIV infected patients. Ritonavir is available in combination of Atazanavir, which inhibits the CYP3A4-mediated metabolism of Lopinavir, increasing the Atazanavir concentrations [1-2].

Literature review reveals that methods have been reported for analysis of RTV and ATV in pharmaceutical formulations and in human plasma individually or in combination with other antiviral drugs using liquid chromatography (LC) [3-14], thin layer chromatography

(TLC) [15] and UV spectroscopy [16-19] and LC/MS [20-22]. As per literature review, there is no single HPTLC method has been reported for simultaneous determination of RTV and ATV in combination tablets. Hence, aim of the present study to develop and validate accurate method and determine both drugs concurrently by simple, rapid, and selective HPTLC method that could be used for quality control and routine analysis. The proposed method was validated in accordance with International Conference on Harmonization (ICH) guideline [23].

### 2. EXPERIMENTAL

#### Materials and Reagents

RTV and ATV powders were procured from Hetero Drugs Ltd. (Hyderabad, India). VIRATAZ-R Tablets was manufactured by Hetero Drugs Ltd. Each tablets claimed to contain 100 mg RTV and 300 mg ATV. Methanol was purchase from Loba Chemie (Mumbai, India). Toluene and Chloroform were purchased from Rankem (RFCL Ltd.,

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New Delhi, India). Ethyl acetate and Acetone were purchased from Finar (Ahmedabad, India). All experiments were performed with analytical grade chemicals.

#### **Instrumentation and Chromatographic Conditions**

Chromatography was performed on 10 cm × 10 cm aluminum plates precoated with 250- $\mu$ m layers of silica gel 60 F<sub>254</sub> (E. Merck, Darmstadt, Germany). The plates were prewashed with methanol and activated at 50 °C for 5 min prior to chromatography. Samples were spotted in the form of bands (3 mm wide) by means of a Camag Linomat IV (Switzerland) sample applicator equipped with a 100- $\mu$ L syringe (Hamilton, Bonaduz, Switzerland) and space between the two bands was 5 mm. The slit dimension was kept at 3 mm × 0.1 mm and scanning speed was set at 100 mm/sec. The monochromator bandwidth was set at 20 nm and baseline correction was used. The mobile phase consisted of chloroform: ethyl acetate: acetone (5:2:3, v/v/v). Linear ascending development was carried out in a 10 cm × 10 cm twin through chamber (Camag, Muttenz, Switzerland) saturated with mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25 ± 2 °C). The development distance was 80 mm. After chromatography plates were dried in a current of air. Densitometric scanning was performed using a Camag TLC Scanner III in the absorbance-reflection mode at 244 nm and operated by CATS software (V 4.01). The source of radiation used was a deuterium lamp emitting a continuous UV spectrum between 200 nm to 400 nm.

#### **Preparation of Standard Stock Solutions**

A standard stock solution of RTV was prepared by dissolving 10 mg drug in 10 mL methanol to furnish concentration of 1 mg mL<sup>-1</sup>. A standard stock solution of ATV was prepared by dissolving 11.40 mg of Atazanavir sulphate (equivalent to 10 mg Atazanavir) in 10 mL methanol to furnish concentration of 1 mg mL<sup>-1</sup>. From the standard stock solution, the mixed standard solution was prepared using methanol to contain 100  $\mu$ g mL<sup>-1</sup> of RTV and 300  $\mu$ g mL<sup>-1</sup> of ATV. The stock solution was stored at 2-8 °C and protected from light.

#### **Method Validation**

Validation of optimized TLC method was carried out with respect to following parameters.

#### **Linearity and Range**

From the mixed standard solution 100  $\mu$ g mL<sup>-1</sup> of RTV and 300  $\mu$ g mL<sup>-1</sup> of ATV, 8 to 28  $\mu$ L solutions were spotted on the TLC plate to obtain final concentration of 800-2800 ng spot<sup>-1</sup> for RTV and 2400-8400 ng spot<sup>-1</sup> for ATV. Each concentration was applied five times on the TLC plate. The plate was then developed using mobile phase.

Calibration curves were constructed by plotting peak areas versus concentrations of RTV and ATV and regression equations were calculated.

#### **Precision**

It expresses within laboratory variations as on different days analysis or equipment within the laboratory. Intraday precision of the proposed method was evaluated by assaying freshly prepared solutions of RTV and ATV in triplicate at different concentrations (800, 1600 and 2400 ng spot<sup>-1</sup> for RTV and 2400, 4800 and 8400 ng spot<sup>-1</sup> for ATV) on the same day. Interday precision of the proposed method was evaluated by assaying freshly prepared solutions of RTV and ATV in triplicate at different concentrations (800, 1600 and 2400 ng spot<sup>-1</sup> for RTV and 2400, 4800 and 8400 ng spot<sup>-1</sup> for ATV) on three different days. The precision of the instruments were checked by repeatedly spotting ( $n = 7$ ) standard solution of RTV (1600 ng spot<sup>-1</sup>) and ATV (4800 ng spot<sup>-1</sup>) for this method. Repeatability studies ( $n = 7$ ) were performed by analysis of standard solution of RTV (1600 ng spot<sup>-1</sup>) and ATV (4800 ng spot<sup>-1</sup>).

#### **Limit of Detection and Limit of Quantification**

According to ICH recommendation, the approach based on the standard deviation of the response and slope was used for determining the limit of detection (LOD) and limit of quantification (LOQ) limits.

#### **Accuracy**

The interference of excipients in the pharmaceutical formulations was studied in detail by proposed methods. For this reason, standard addition method was applied to the pharmaceutical formulation containing these compounds. This study was performed by spiking known amount of studied drugs to a known concentration of the commercial pharmaceutical product.

#### **Specificity**

The specificity of the method was determined by analyzing standard drug and test samples. The spot for RTV and ATV in the samples was confirmed by comparing the  $R_f$  and spectrum of the spot to that of a standard. The peak purity of RTV and ATV was determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E).

#### **Analysis of Marketed Formulations**

To determine the content of RTV and ATV in a combination tablet (Brand name: VIRATAZ-R, Label claim: 100 mg Ritonavir and 300 mg Lopinavir per tablet), twenty tablets were weighed, their mean weight determined and finely powdered. The weight of the tablet triturate equivalent to 50 mg of RTV was transferred into a 50 mL volumetric flask containing 30 mL methanol, sonicated for 45 min, and diluted to 50 mL with methanol. The resulting solution was filtered through Whatman filter

paper No. 41. Then 1 mL of the filtered solution was diluted to 10 mL with methanol to produce a concentration of 100 and 300 µg mL<sup>-1</sup> for RTV and LPV respectively, and 17 µL of this solution (1700 and 5100 ng spot<sup>-1</sup> for RTV and ATV respectively) was applied to a HPTLC plate which was developed in an optimized mobile phase. The analysis was repeated in triplicate. The possibility of excipient interference with the analysis was examined.

**3. RESULTS AND DISCUSSION**

**Method Development**

Different mobile phases containing chloroform, toluene, ethyl acetate, methanol and acetone in different proportions were examined. Chloroform: ethyl acetate: acetone (5:2:3, v/v/v) was finally selected because it resulted in acceptable resolution of the bands with R<sub>f</sub> values of 0.28 ± 0.02 for RTV and 0.58 ± 0.02 for ATV. The densitogram obtained from a mixed standard solution of RTV and ATV is shown in Figure 1. A wavelength of 244 nm was selected for the quantification of the drugs.

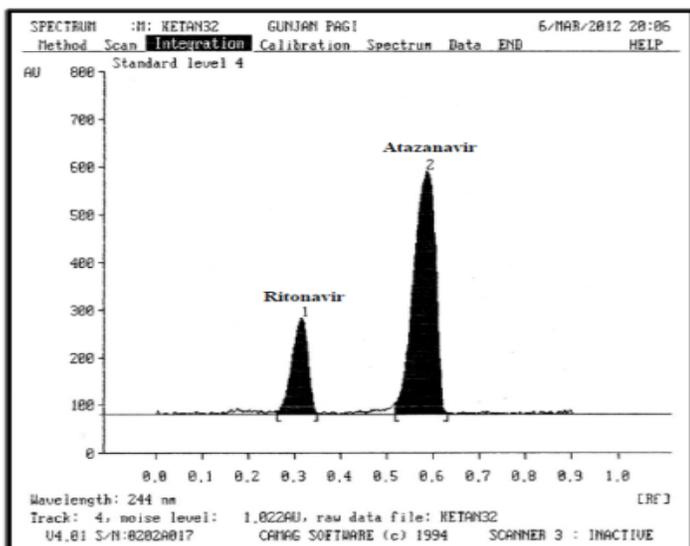


Figure 1: Chromatogram of standard Ritonavir (Peak 1, R<sub>f</sub> = 0.28) and Atazanavir (Peak 2, R<sub>f</sub> = 0.58)

**Validation**

**Linearity**

The calibration curves were found to be linear over the ranges 800-2800 ng spot<sup>-1</sup> and 2400-8400 ng spot<sup>-1</sup> for RTV and ATV respectively. Characteristic parameters for the regression equation and correlation coefficients are given in Table 1. The linearity of the calibration curves was validated by the high value of correlation coefficients of the regression.

**Precision**

The results of the repeatability, intraday and interday precision are shown in Table 1. The low values of relative standard deviation (RSD) of the repeatability, intraday and interday determinations show that the proposed method is precise.

**Limit of Detection and Limit of Quantification**

The limit of detection and limit of quantification of the drugs are given in Table 1. These data shows that this method is sensitive for the determination of RTV and ATV.

**Accuracy**

The recovery experiments were carried out by the standard addition method. The mean percent recoveries obtained were 98.57 ± 0.35 and 99.16 ± 0.20 for RTV and ATV respectively (Table 1).

**Specificity**

The peak purity of RTV and ATV were assessed by comparing their respective spectra at the peak start, apex and peak end positions of the spot i.e., r(S, M) = 0.9996 and r(M, E) = 0.9993 for RTV and r(S, M) = 0.9999 and r(M, E) = 0.9999 for ATV . A good correlation was also obtained between the standard and sample spectra of RTV (0.9996) and ATV (0.9990) respectively (Figure 2 and 3).

Parameters	HPTLC method	
	RTV	ATV
Linearity Range (ng spot <sup>-1</sup> )	800-2800	2400-8400
Slope	2.942	2.262
Intercept	1517.20	9071.70
Correlation coefficient (r)	0.9991	0.9972
Repeatability of measurement of peak area (% RSD, n = 7)	0.71	0.59
Repeatability of sample application (% RSD, n = 7)	1.88	1.89
Intraday precision (% RSD, n = 3)	0.71-1.91	0.80-1.93
Interday precision (% RSD, n = 3)	1.08-1.97	0.89-1.98
Accuracy (% Recovery ± SD)	98.57 ± 0.35	99.16 ± 0.20
LOD (ng spot <sup>-1</sup> )	300	200
LOQ (ng spot <sup>-1</sup> )	366.90	1447.32

Table 1: Summary of validation parameter for the proposed method

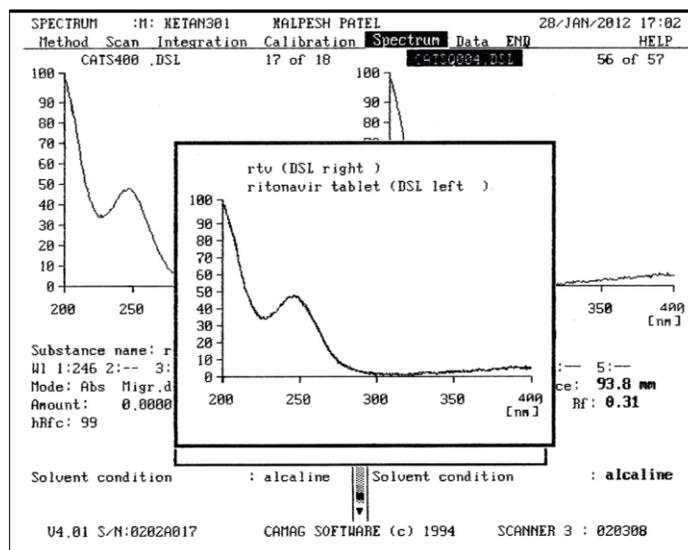
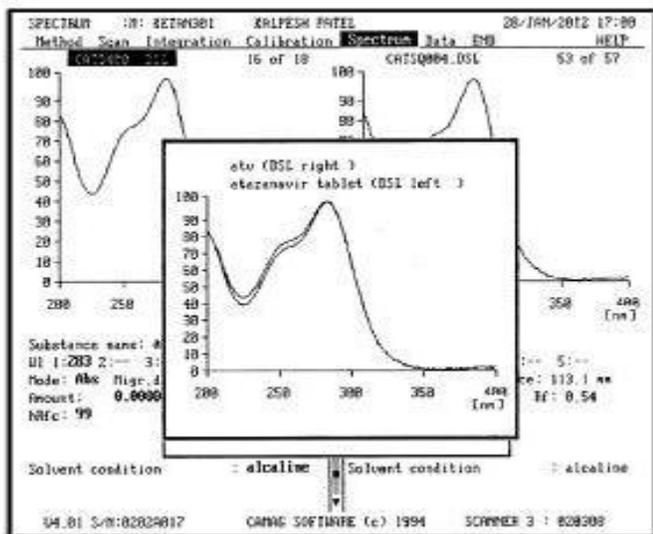


Figure 2: Correlation of Ritonavir spectrum in Standard and Formulation



**Figure 3: Correlation of Atazanavir spectrum in Standard and Formulation**

**Analysis of Marketed Formulations**

The proposed method was successfully applied to determine RTV and ATV in pharmaceutical formulations (Table 2). The results obtained for RTV and ATV were comparable with the corresponding label claims thereby suggesting that there is no interference from any of the excipients which are normally present in tablets.

Samples	RTV		ATV	
	Label claim	% Recovery Mean $\pm$ S.D. <sup>a</sup>	Label claim	% Recovery Mean $\pm$ S.D. <sup>a</sup>
VIRATAZ-R	100 mg	97.08 $\pm$ 0.43	300 mg	101.10 $\pm$ 1.98

<sup>a</sup> Average of three experiments

**Table 2: Determination of RTV and ATV in pharmaceutical formulation by HPTLC method**

**4. CONCLUSION**

A new simple, sensitive, accurate, reproducible and precise HPTLC method for determination of RTV and ATV in combination tablets has been developed and validated. Statistical analysis proves that the method is suitable for analysis of RTV and ATV in pharmaceutical formulation without any interference from excipients. The proposed HPTLC method is less expensive, simple, rapid, and more flexible than HPLC.

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**Conflict of Interest: None Declared**