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SIMULTANEOUS ESTIMATION OF ATAZANAVIR AND RITONAVIR IN TABLET DOSAGE FORM BY HPTLC METHOD

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ABSTRACT

A simple, sensitive and rapid high performance thin layer chromatographic method has been developed and validated for the simultaneous estimation of atazanavir and ritonavir in pharmaceutical formulations. The chromatographic development was carried out on HPTLC plates pre-coated with silica gel 60G F_{254} using a mixture of toluene: ethyl acetate: 0.1% formic acid in the ratio of 6.0:4.0:1.0 v/v as mobile phase. The calibration curve was found to be linear over the concentration range of 150-900 ng/spot for ATV and 50-300 ng/spot for RTV with a regression coefficient for both analytes were greater than 0.999. The %RSD values for intra-day and inter-day variation were not more than 2.0. The method has demonstrated high sensitivity and specificity. The method is new, simple and economic for routine estimation of atazanavir and ritonavir in bulk and pharmaceutical formulation to help the industries as well as researchers for their sensitive determination of atazanavir and ritonavir rapidly at low cost in routine analysis.

Keywords: HPTLC, Method Development, Atazanavir, Ritonavir, Method Validation

INTRODUCTION

Nucleoside reverse transcriptase inhibitors (NRTIs) were the first class of drugs that were introduced as antiretroviral agents for the treatment of infection with human immune deficiency virus (HIV). Additional drug classes were developed. They are protease inhibitors (PIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), fusion inhibitors[1]. Atazanavir sulfate (figure 1), chemically (3S,8S,9S,12S)-3,12-Bis(1,1-dimethylethyl)-8hydroxy-4,11-dioxo-9-(phenylmethyl)-6-[[4-(2-pyridinyl) phenyl] methyl]-2,5,6,10,13 penta azatetra decanedioic acid dimethyl ester, sulfate (1:1)[2-5]. Ritonavir (figure 2), chemically 10 – Hydroxy – 2 – methyl – 5 - (I – methyl ethyl) –I - [2 -(l - methy lethyl) – 4 - thiazolyl] - 3, 6 – dioxo - 8, I1 – bis (phenyl methyl) - 2,4,7,12- tetraazatridecan – 13 – oic acid, 5 -thiazolylmethyl ester, It is reversibly binds to the active site of the HIV protease, preventing polypeptide processing and subsequent virus maturation. Virus particles are produced in the presence of ritonavir but are non-infectious[6-8].

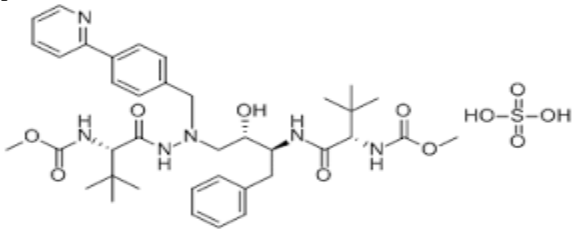

Figure 1: Chemical Structure of Atazanavir Sulfate

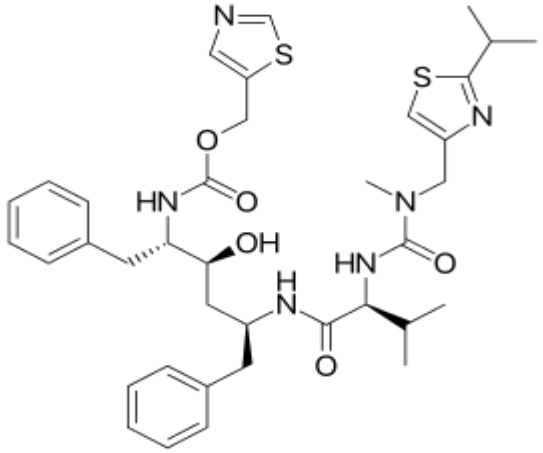

Figure 2: Chemical Structure of Ritonavir

The literature survey reveals that several analytical methods have been reported for the quantification of these drugs individually or in combination with other drugs in pharmaceutical dosage forms or in human plasma by UPLC-MS/MS[9,10], LC-MS/MS[11-14], high performance liquid layer chromatography[15-17]. Today, HPTLC is rapidly becoming a routine analytical technique due to its advantages of low operating costs high sample throughput, and need for minimum sample preparation. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus reducing the analysis time and cost per analysis. Accordingly, the aim of the present study involves development and validation of HPTLC method for the simultaneous estimation of atazanavir and ritonavir in combined tablet dosage form, which is fast, sensitive with better resolution and peak symmetry. Finally, the developed method was validated to assess the validity of research data means determining whether the method used during the study can be trusted to provide a genuine, account of the intervention being evaluated.

MATERIALS AND METHODS

Materials: Pure atazanavir sulfate (ATV) and ritonavir (RTV) used as working standards, were gifts from Hetero Drugs Pvt. Ltd., Hyderabad, India. All chemicals and reagents employed were of analytical grade, and purchased from Rankem, India. A commercial Synthivan tablets containing 300 mg of ATV and 100 mg of RTV were obtained from local pharmacies and used within their shelf life period.

Instrumentation and chromatographic conditions: Instruments used in the study were Camag HPTLC: A conventional CAMAG 20 × 10 cm twin-trough chamber and ultra sonicator were used for the development of chromatogram. Automatic Linomat 5 sampler was used as sample applicator with Hamilton syringe. Experiment performed on aluminium foil pre-coated with silica gel 60G F_{254} plates (10 × 10 cm, layer thickness 0.2 mm) (E. Merck, Darmstadt, Germany). Before use, the plates were prewashed with methanol and water mixture then dried in the current of dry air and activated at 120 °C for 5 min. Samples were sprayed at a speed of 150 nL/second to the plates with band length of 6 mm bands and distance of 7.8 mm between each track. The plates were developed by the ascending technique, to a total distance of 8 cm, at 25 ± 5°C, relative humidity 50-60%, in a CAMAG twin-trough glass chamber with a stainless steel lid, using a mobile phase of toluene: ethyl acetate: 0.1% formic acid in the ratio of 6.0:4.0:1.0 v/v and the chamber saturation time of 30 minutes. After development of plates, it was dried in an oven. Densitometric scanning was performed at 252 nm with a CAMAG TLC Scanner III in reflectance–absorbance mode controlled by winCATS software (version 1.4.8.2012; CAMAG) resident in the system.

Preparation of standard solutions: A mixed standard stock solution of ATV (1000 µg/mL) and RTV (1000 µg/mL) was prepared by accurately weighing 100 mg of each ATV and RTV, and dissolved in 100 mL volumetric flasks containing 30 mL methanol and the flasks were sonicated to dissolve the contents and made up to the mark with methanol. Aliquots of these solutions were transferred into 100 mL volumetric flask containing 30 mL methanol, sonicated for 2 min and the remaining volume was made up to mark with methanol to get final concentration of 150 µg/mL for ATV and 50 µg/mL for RTV.

Method Validation

The method was validated in accordance with ICH guidelines[18].

i. **Specificity and sensitivity:** The specificity of the developed method was established analyzing the sample solutions containing ATV and RTV standards, and marketed tablets in relation to interferences from formulation ingredients. The spot for ATV and RTV in the sample was confirmed by comparing Rf values of the spot with that of the standard.
ii. The sensitivity of measurement was estimated in terms of the limit of quantification (LOQ) and the limit of detection (LOD). The LOQ and LOD were

calculated by the use of equations LOD = 3 × N/B and LOQ = 10 × N/B where N is the standard deviation of the peak area of the drug (n = 3), taken as a measure of noise and B is the slope of the corresponding calibration plot.

iii. ***Linearity:*** Calibration curves were constructed by plotting peak areas versus concentrations of ATV and RTV, and the regression equations were calculated. From the mixed standard stock solution containing 150 µg/mL of ATV and 50 µg/mL of RTV, aliquots of standard solution were spotted on TLC plate to obtain final concentration of 150-900 ng/spot and 50-300 ng/spot for ATV and RTV, respectively. Each concentration was applied three times to the TLC plate.

iv. ***Accuracy:*** The accuracy was carried out by adding known amounts of each standard drug corresponding to three concentration levels - 50, 100 and 150 % - of the labeled claim to the analytes. At each level, three determinations were performed and the results were recorded. The accuracy was expressed as percent analyte recovered by the proposed method.

v. ***Precision:*** The precision of the method was checked by repeatability of injection, repeatability (intra-assay), intermediate precision (inter-assay) and reproducibility. Injection repeatability was studied by calculating the percentage relative standard deviation (%RSD) for ten determinations of peak areas of ATV (450 ng/spot) and RTV (150 ng/spot), performed on the same day. For both intra- and inter-assay variation, sample solutions of ATV (450, 600 and 750 ng/spot) and RTV (150, 200 and 250 ng/spot) were injected in triplicate.

vi. ***Robustness:*** The robustness of the proposed method was determined by carrying out the analysis, during which mobile phase composition and duration of saturation time (varied by 5 min) were altered.

vii. **Stability studies:** To test the stability of the drugs on the TLC plates, the freshly prepared solutions of the analyte were applied to the plates and developed plates were scanned at different intervals of 2, 6, 24, 48 and 72 h.

RESULTS AND DISCUSSIONS

The HPTLC method, as described, was validated and successfully employed for the simultaneous quantification of ATV and RTV in tablets. There is need to consider the successive steps for the development of HPTLC method. In particular, the problems relating to the standardization of sample preparations and selection of mobile phase needs to be emphasized. The mobile phase [i.e., toluene: ethyl acetate: formic acid (85%) in the ratio of 6.0:4.0:1.0 v/v] was found to give a sharp and well-defined peak at Rf of 0.39±0.01 and 0.73±0.01 for RTV and ATV, respectively (Figure 3). Better resolution was obtained when the chamber was saturated for 30 minutes with the mobile phase at a room temperature. Thus, this system and aforementioned conditions were selected for the analysis. A calibration curve was constructed by plotting peak area against concentration (ng/spot). The results of regression analysis are shown in Table 1. They confirm the linearity of the standard curves over the concentration range of 150-900 ng/spot for ATV and 50-300 ng/spot for RTV. The regression coefficients of ATV (r^2 = 0.9992) and RTV (r^2 = 0.9994) signify that a decent linear relationship exhibited between peak area versus concentration over a wide range.

The peak purity of EFV and 3TC was assessed by comparing the spectra at three different levels, that is, peak start (*S*), peak apex (*M*), and peak end (*E*) positions of the spot and the results obtained as r(S, M) = 0.9992 and r(M, E) = 0.9996 for RTV; r(S, M) = 0.9992 and r(M, E) = 0.9996 for ATV. Good correlation was obtained between standard and sample spectra of ATV and RTV. Limit of detection for ATV and RTV was 9.62 ng/spot and 4.35 ng/spot, respectively, whereas limit of quantification was 29.15 ng/spot and 13.19 ng/spot, respectively.

The developed method showed high and consistent recoveries at all studied levels. The results obtained from recovery studies are presented in Table 2. The mean % recovery ranged from 98 to 102. Additionally, the obtained recoveries were found to be normally distributed with low %RSD ($\leqslant$2) at all concentration levels. The recovery study results signifying that the developed method was accurate.

Injection repeatability values (%RSD) of ATV and RTV were found to be 1.015 and 1.097, respectively. The intra- and inter-assay precision results were expressed as %RSD values and were shown in Table 3. The low %RSD values proved that the method was precise. There was no significant difference between %RSD values, which indicates that the optimized method was reproducible. The results obtained in the new conditions were in accordance with the original results as shown in Table 4, though the R_f varied very slightly and the %RSD values for peak area was less

than 2.0 indicating the highly robust nature of the developed method. There was no significant deviation in peak area (RSD < 1.5%) observed on analysis up to 72 h. No decomposition of the drug was observed during chromatogram development. These observations suggest that the drug is stable under the typical processing and storage conditions of the analytical procedure. The results of the assay yielded 99.60% for ATV and 99.84 % for RTV, of label claim of the tablets. The assay results show that the method was selective for the simultaneous determination of ATV and RTV without interference from the excipients used in the tablet dosage form and the results were shown in the Table 5.

CONCLUSION

A convenient, rapid, accurate and precise HPTLC method was developed for the simultaneous determination of atazanavir and ritonavir in tablets. The assay provides a linear response across a wide range of concentrations. This method can be said to be more economical as compared to other methods reported in literature. The method suitable for the determination of these drugs in tablets, and hence can be used for routine quality control of atazanavir and ritonavir in this dosage form.

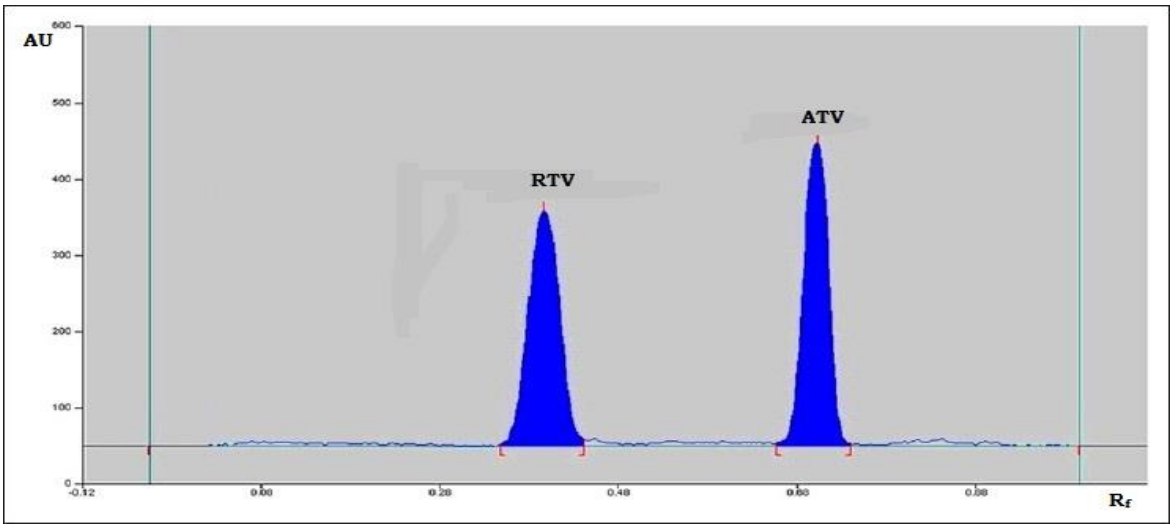

Figure 3: HPTLC chromatogram of standard ATV and RTV

Table 1: Linearity data of the proposed method

Analyte	Conc. (ng/spot)	Peak Area (Mean ± SD)*	RSD (%)	Linear regression equation
ATV	150	2143±12	0.566	y= 11.903x – 308.87 R^2= 0.9992
	300	3963±33	0.839	
	450	5532±23	0.417	
	600	7386±24	0.325	
	750	9191±136	1.484	
	900	11134±165	1.481	
RTV	50	992±13	1.263	y= 26.989x – 450.93 R^2= 0.9994
	100	2166±28	1.293	
	150	3544±49	1.37	
	200	4956±56	1.131	
	250	6299±55	0.876	
	300	7676±31	0.403	

No. of Replicates (N=3); SD: Standard Deviation; RSD: Relative Standard Deviation

Table 2: Results of recovery studies by standard addition method

Analyte	Amount of standard drug spiked		Amount of sample taken (mg)	% Recovery (Mean ± SD) {three replicates}	RSD (%)	SEM
	% Spiked	Quantity (mg)				
ATV	50	150	300	99.31±0.988	0.994	0.5702
	100	300	300	100.44±1099	1.094	0.6364
	150	450	300	99.26±0.971	0.978	0.5605
RTV	50	50	100	100.27±0.681	0.680	0.3934
	100	100	100	99.02±0.539	0.545	0.3114
	150	150	100	100.21±1.145	1.143	0.6612

**%RSD Values*

Table 3: Precision data of the proposed method

Analyte	Analyte Conc. (ng/spot)	Intra-assay precision*	Inter-assay precision*	Reproducibility*	
				Analyst one	Analyst two
ATV	450	1.113	1.314	1.106	0.464
	600	1.036	0.839	1.463	1.206
	750	0.295	0.926	0.462	1.111
RTV	150	0.837	0.948	0.686	0.689
	200	1.169	1.482	0.569	1.037
	250	0.648	1.154	1.589	0.659

**%RSD Values*

Table 4: Results for robustness of the proposed method

Parameter	Original	Used	Analyte	R_f Values	
				Mean± SD	RSD (%)
Development Distance	8 cm	7.5	ATV	0.75±0.01	1.333
		8.0		0.73±0.01	0.787
		8.5		0.76±0.01	0.756
		7.5	RTV	0.38±0.01	1.533
		8.0		0.39±0.01	1.493
		8.5		0.36±0.01	1.619
Wavelength	252 nm	250	ATV	0.71±0.01	0.809
		252		0.74±0.01	0.777
		254		0.78±0.01	0.743
		250	RTV	0.35±0.01	1.665
		252		0.39±0.01	1.493
		254		0.41±0.01	1.397

Table 5: Assay results for atazanavir and ritonavir in tablets

Product	Analyte	Label claim per tablet (mg)	% analyte estimated (Mean ±SD)*	RSD (%)	SEM
Synthivan	ATV	300	99.60±1.628	1.634	0.940
	RTV	100	99.84±1.037	1.039	0.599

** n = 6; SEM = standard error of mean*

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