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Research Article

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF ATAZANAVIR SULPHATE AND RITONAVIR IN BULK AND FORMULATIONS

DNYANESHWAR SUKHADEV PAWAR*, MANJUSHA DOLE, SANJAY SAWANT, JYOTI M SALUNKE

Smt.Kashibai Navale College of Pharmacy, Kondhwa (Bk), Pune 48, Maharashtra, India. Email: dspmauli13@gmail.com

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ABSTRACT

Objective: To develop a simple, sensitive, rapid and reproducible reversed- phase HPLC method for estimation of Atazanavir Sulphate and Ritonavir simultaneously in bulk and in tablet formulation.

Methods: The assay involved an isocratic elution of these two component on Hi-Q Sil C-18 Column (250 mm × 4.6 mm, 5µm) using a mobile phase composition of Acetonitirile: Methanol: Phosphate Buffer (40:40:20) adjusted to pH 3.1 with orthophosphoric acid. The flow rate was 1.0 mL/min and the analytes monitored at 238nm using photodiode array (PDA) detector. The performance of the method was validated according to the present ICH guidelines for specificity, linearity, accuracy, precision and robustness.

Results: Retention time of Atazanavir sulphate and Ritonavir were found to be 3.7 min and 7.5 min, respectively. Calibration curves were linear with coefficient correlation between 0.99 to 1.0 over a concentration range of 30 to 180 µg/ml of Atazanavir Sulphate and 10 to 60 µg/ml for Ritonavir respectively. Typically the regression equation for the calibration curve was found to be y=12938x-27927 (R^2=0.999) for ATV and y=13050x-2083(R^2=0.999) for RTV.

Conclusion: The described method can be successfully employed for routine quality control analysis of Atazanavir sulphate and Ritonavir in formulation.

Keywords: Atazanavir Sulphate, Ritonavir, RP-HPLC, Validation.

INTRODUCTION

Atazanavir Sulphate (ATV), chemically is (3S, 8S, 9S, 12S)-3,12-Bis(1,1-dimethyl ethyl)-8-hydroxy-4,11-dioxo-9-(phenylmethyl)-6-[[4-(2-pyridinyl)phenyl]methyl]-2,5,6,10,13-pentaazatetrade anedioic acid dimethyl ester; 1-[4-(pyridine-2-yl)phenyl]-5S,2,5-bis[[N-(methoxy carbonyl)-L-tertleucinyl]amino]-4S hydroxyl-6-phenyl-2-azahexane(Figure.1)[1]. It is an oral antiretroviral Protease inhibitors used in the treatment of HIV/AIDS [2]. Ritonavir (RTV) chemically is (5S,8S,10S,11S)-10-hydroxy-2-methyl-5-(1-methyl ethyl)-1-[2-(1-methyl ethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis (phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid 5-thiazolyl methyl ester(Figure.2)[3]. RTV is an antiretroviral drug specifically belongs to protease inhibitors class. Both drugs are official in Indian Pharmacopoeia [3].

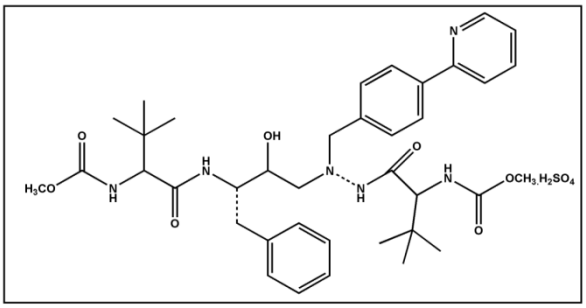

Fig. 1: Atazanavir sulphate (ATV)

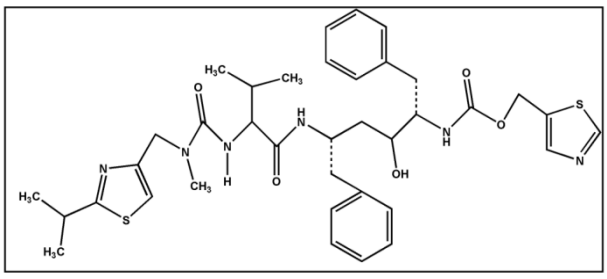

Fig. 2: Ritonavir (RTV)

Literature survey reveals that for the quantitative estimation of Atazanavir sulphate UV Spectrophotometric[4-6] and HPLC[7,8] method have been reported, likewise very few methods have been reported for the quantitative estimation of Ritonavir i.e. UV[9-10] and HPLC [11-14].One Spectrophotometric method has been reported for the simultaneous estimation of Atazanavir sulphate and Ritonavir in tablets [15].An attempt has been made to develop a simple, economical, precise, accurate and reproducible HPLC method for estimation of Atazanavir sulphate and Ritonavir in bulk as well as pharmaceutical formulations according to ICH guidelines.

MATERIAL AND METHODS

Chemicals and Reagents

Pure sample of Atazanavir sulphate was received as a gift sample from Micro Lab, Bangalore and Ritonavir was procured from Hetero Health Care Ltd, Hyderabad. The percentage purity of ATV and RTV was 99.85% w/w and 99.1% w/w respectively. Tablet formulation containing Atazanavir sulphate 300mg and Ritonavir 100mg (Synthivan, Cipla Pharmaceuticals, Patalganga), was used for the analysis. HPLC water was obtained by using Elga DV 25, Purelab option-Q water purification system. HPLC grade Acetonitrile was procured from Merck (Mumbai, India). Analytical grade Orthophosphoric acid was purchased from Research lab (Mumbai, India). Digital balance (Contech CB series) was employed for weighing purpose.

Instrumentation

HPLC system (Jasco MD-2089 Plus) equipped with quaternary pump with an on-line degasser was used. The column compartment Hi-Q Sil C-18 (250 mm × 4.6 mm) and photodiode array (PDA) detector was employed throughout the analysis. Chromatographic data was acquired using chrom-NAV software.

Preparation of Mobile Phase

The mobile phase was prepared by mixing of Acetonitrile, Methanol and Phosphate Buffer (40:40:20 v/v/v) and the pH adjusted to 3.1 by using o-phosphoric acid. The mobile phase was sonicated for 15min and then it was filtered through 0.45µ Whatman filter paper.

Preparation of 0.025 M Phosphate Buffer

Phosphate buffer (0.025 M) was prepared by dissolving accurately weighed quantity of 850 mg of potassium dihydrogen orthophosphate in a 250.0 ml of double distilled water.

Table 1: Optimised Chromatographic condition

Parameter	Optimised Condition
Instrument	Jasco HPLC MD-2089 Plus
Column	Hi-Q Sil C-18 (250 mm × 4.6 mm)
Detector	PDA
Detection wavelength	238nm
Mobile Phase	Acetonitrile:Methanol:0.025M Phosphate Buffer(40:40:20v/v/v) pH 3.1 was adjusted with dilute 10% o-phosphoric acid
Flow rate	1ml/min
Injection Volume	20µl
Temperature	Ambient

Preparation of Standard Solution

About 50 mg of each reference standard of ATV and RTV were accurately weighed & transferred to 50 ml volumetric flasks. Both the drugs were dissolved in 50 ml of mobile phase with shaking and then volume was made up to the mark with mobile phase to get 1000 µg/ml of standard stock solution of each drug. Then it was ultrasonicated for 10 minutes and filtered through 0.20 µ membrane filter. For calibration curve, stock solutions of ATV and RTV were appropriately diluted to obtain working standard solutions in the increasing concentration range.

Analysis of Marketed Formulation

Twenty tablets containing RTV and ATV (100:300mg) were weighed and average weight was determined. Tablet powder equivalent to 100 mg of RTV and 300 mg of ATV transferred to 100 ml volumetric flask, dissolved in mobile phase. Solution was ultrasonicated for 20 min. filtered through Whatman filter paper No. 42. Filtrate was diluted with mobile phase to obtain final concentration within linear range. Chromatogram was recorded at 238 nm. Content of drugs in sample solution was calculated by comparing mean peak area of sample with that of the standard. The typical Chromatogram of ATV and RTV in tablet dosage form were shown in Figure 3.

Table 2: Result of Assay study

Label claim (mg)		Amt of Drug Found (mg)		% Label Claim*±SD		% RSD	
RTV	ATV	RTV	ATV	RTV	ATV	RTV	ATV
100	300	99.85	298.99	99.34±0.385	98.82±0.164	0.387	0.165

*average of six determinations

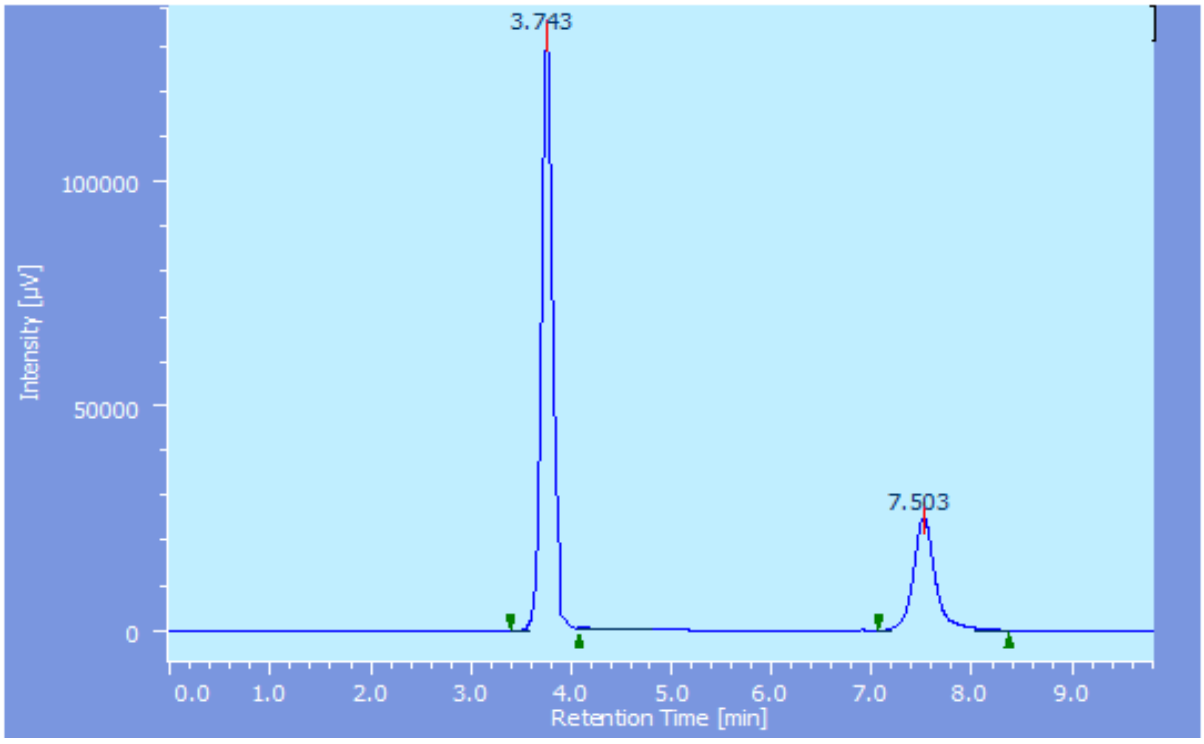

Fig. 3: Typical Chromatogram of ATV (t_R = 3.743) and RTV (t_R =7.503) in Tablet dosage form

Method Validation

The chromatographic conditions were validated by evaluating linearity, accuracy method and system precision, system suitability, solution stability, limit of detection (LOD), limit of quantification (LOQ), robustness studies in accordance with ICH guidelines Q2 (R1). [16-22]

Linearity

The linearity of the method is its ability to elicit test results that are directly proportional to the concentration of the analyte in samples. ATV was found to be linear in the concentration range of 30-180 µg/ml and RTV showed linearity in the concentration range of 10-60 µg/ml at the 238nm. The correlation coefficient was found to be

0.999 at each wavelength for both drugs as shown in Figure.4 and Figure. 5.

Precision

The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate statistically valid estimate of % Relative Standard Deviation (%RSD). Precision was done to express within laboratory variation, on different days. Six replicates of 20µg/ml RTV and 60 µg/ml ATV concentration of the working sample solution were analyzed. %RSD was found to be less than 2% indicating high precision of the method. [18, 19]

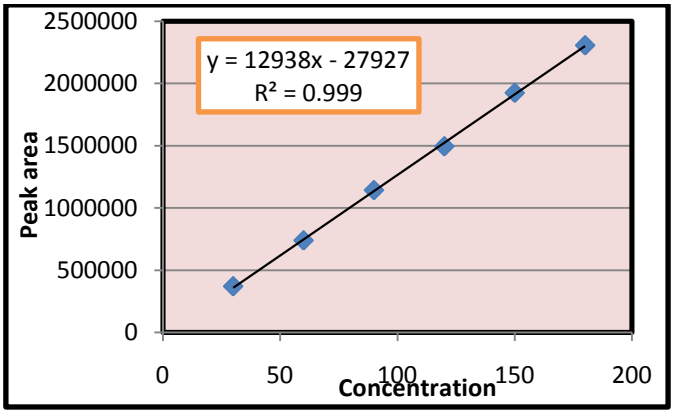

Fig.4: Calibration plot of ATV at 238 nm.

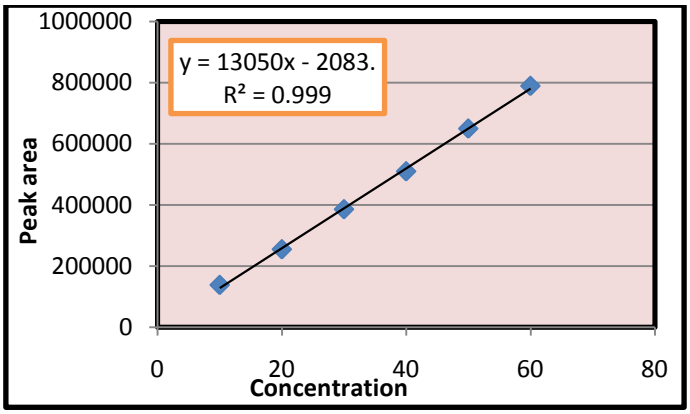

Fig.5: Calibration plot of RTV at 238 nm

Table 3: Precision study of ATV and RTV

Precision	Label claim (mg)	% Drug found*	SD	% RSD	SE
Interday	RTV 100	99.48	0.6167	0.6199	0.2531
Intraday	ATV 300	99.36	0.3367	0.3389	0.1383
	RTV 100	99.39	0.595	0.5986	0.2429
	ATV 300	99.47	0.3517	0.3535	0.1436

*average of six determinations

Accuracy [16, 18]

Accuracy of the method was calculated by performing recovery studies. It is carried out by preparing the samples at concentration levels of 80%, 100% and 120% of test concentration. The samples were prepared in triplicate for each level. The results of studies along with its evaluation are given in table 4.

Specificity

Results of tablet solution showed that there is no interference of the excipients when compared with the working standard solution as shown Figure 5. The peak purity index was found to be greater than 0.9999 indicating no impurities are present in tablet dosage form. Thus, the method was said to be specific.

Table 4: Result of Recovery study

Level of % Recovery*	Amt. taken (µg/ml)		Amt. of std. added (µg/ml)		% Mean Recovery ± S.D		% RSD	
	RTV	ATV	RTV	ATV	RTV	ATV	RTV	ATV
80	20	60	16	48	99.63±0.797	99.31±0.03	0.799	0.0302
100	20	60	20	60	100.78±0.243	100.82 ±0.563	0.241	0.559
120	20	60	24	72	99.57±0.470	100.43±0.143	0.472	0.142

*average of three determinations

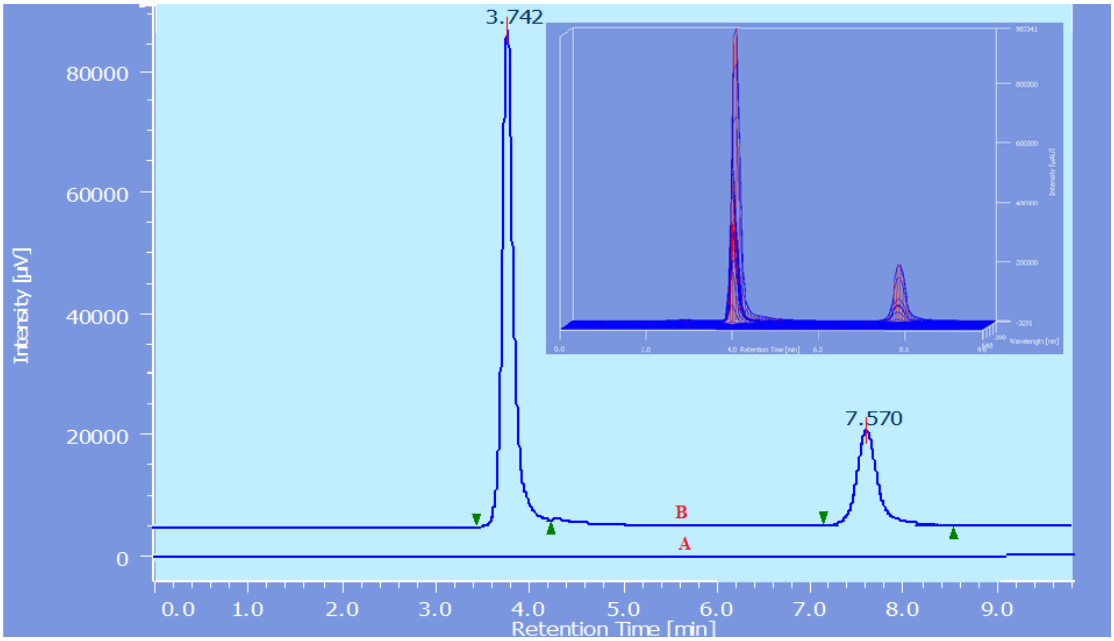

Fig. 6: Overlay of placebo (A) and formulation (B) chromatograms along with in lay of 3 D plots

Limit of Detection

Limit of detection was calculated using following equation as per ICH guidelines. [19, 20]

$$LOD = 3.3 \times \sigma/s$$

Where,

σ = Standard deviation of the response and

s = Slope of the corresponding calibration curve.

Results are shown in the table no.5

Limit of Quantification

Limit of quantification was calculated using following equation as per ICH guidelines. [21, 22]

$$LOQ = 10 \times \sigma/s$$

Where,

σ = Standard deviation of the response and

s = Slope of the corresponding calibration curve

Results are shown in the table no.5

Table 5: LOD and LOQ result of Ritonavir and Atazanavir sulphate

Validation Parameters	Ritonavir(RTV)	Atazanavir sulphate(ATV)
LOD (µg/ml)	6.21	11.14
LOQ (µg/ml)	9.12	33.77

Robustness

The robustness of the method was determined by subjecting the method to slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP- HPLC method developed is robust. The results are summarized in table no.6.

System suitability parameters

The column efficiency, resolution and peak symmetry were calculated for the standard solutions. (Table.7). The values obtained demonstrated the suitability of the system for the analysis of this drug combination and the system suitability parameters fall within ± 2% of standard deviation range during performance of the method. Here tailing factor for peaks of ATV and RTV was less than 2% and resolution was satisfactory. The peaks obtained for ATV and RTV were sharp and have clear base line separation.

Table 6: Robustness Study

Factors	Level	ATV (RT min)	RTV (RT min)
A : Flow Rate (ml/min)			
0.9	-0.1	3.813	7.940
1.0	0	3.742	7.570
1.1	+0.1	3.437	7.155
Mean ± SD		3.664± 0.161	7.555± 0.060
B : Mobile phase v/v (Acetonitile: Methanol: Buffer)			
38: 42: 20	-2	3.798	7.601
40: 40: 20	0	3.742	7.570
42: 38:20	+2	3.758	7.598
Mean ± SD		3.766± 0.0213	7.589± 0.0133
C : pH of mobile phase			
3.0	-0.1	3.790	7.692
3.1	0	3.742	7.570
3.2	+0.1	3.873	8.090
Mean ± SD		3.802± 0.0477	7.784± 0.204

Table 7: System suitability parameters

S. No	Parameter	ATV	RTV	Formula	Limits
1.	Retention time	3.742	7.570	---------	1<K<20
2.	Resolution (R_s)	6.195		$R_s = 2(t_{R1}-t_{R2}) / (W_1+W_2)$	R_s>2
3.	Tailing Factor (T_f)	1.296	1.246	$T_f = W_{0.05\%}/2F$	T_f< 2
4.	No. of Theoretical Plates (N)	5325	6461	---------	N>2000
5.	RSD of Replicate injection	0.3875	0.1659	%RSD=S.D×100/ Mean Recovery	Not more than 2%

RESULT AND DISCUSSION

Optimization of the mobile phase was performed based on resolution, tailing factor and peak area obtained for both ATV and RTV. The optimized mobile phase containing Acetonitrile: methanol: Phosphate buffer (pH 3.1) (40:40:20 v/v/v) and the pH adjusted to 3.1 with o-phosphoric acid was found to be satisfactory and gives two symmetric and resolved peaks for ATV and RTV and the retention times of ATV and RTV were found to be 3.742 min and 7.570 min respectively. Typically the regression equation for the calibration curve was found to be y=12938x-27927 (R^2=0.999) for ATV and y=13050x-2083(R^2=0.999) for RTV. The correlation coefficient was indicative of high significance. The high percentage recovery indicates that the proposed method is highly accurate. The % RSD was found to be less than 2% in the developed method, indicating high degree of precision. The changes in flow rate, pH of mobile phase and composition of mobile phase did not affect the percentage assay of the drug, confirming the robustness of the method. No interfering peaks were found in the chromatogram indicating that the excipients used in tablet formulations did not interfere with the estimation of drug. Proposed HPLC method indicates that method is specific can be used for routine quality control analysis.

CONCLUSION

The proposed methods for simultaneous estimation of ATV and RTV in combined dosage form were found to be simple, specific, precise, accurate, economical and rapid. The complete separation of the ATV and RTV was accomplished in less than 10 min and the method can be successfully applicable for routine analysis of these drugs in combined dosage forms.

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REFERENCES

1. The Merck Index, An Encyclopedia Of Chemical, Drug's and Biologicals, Maryadele J.O. Neil.Eds,14th edition, Merck Research Lab, Division of Merck and Co. Inc., Whitehouse Station, NJ: 2006; 142.
2. B.M.Kane, In: D.J.Triggle (Ed.), HIV/AIDS Treatment Drugs, Chelsea house, New York, 2008; 61.
3. Indian Pharmacopoeia, published by the controller of publication, New Delhi, 2010; Vol : I-III: 149, 167, 421, 844-45, 2066-68.
4. Anindita Behera, Swapan Kumar Moitra and Sudam Chandra Si; Spectrophotometric method for determination of atazanavir sulfate in capsule dosage form, *Quim. Nova* 2011; 34(8): 1349-1353.
5. Sathish Kumar Konidala and Sujana. K; Development and validation of UV spectroscopic method for determination of atazanavir sulphate in bulk and formulation, *International Journal of Pharmacy and Pharmaceutical Sciences* 2012; 4(3): 614-617.
6. S.G. Khanage, V.K .Deshmukh, P.B Mohite, V.M Dhamak, S.Appala Raju; Development of Derivative Spectrophotometric Estimation of Atazanavir sulfate in Bulk Drug and Pharmaceutical Dosage Forms, *Int.J.Pharm.& Health Sci*. 2010; 1(3): 149-154.
7. Dario Callaneo, Franco Maggiolo, Diego Ripamonti and Norberto Perico; Determination Of Atazanavir in Human Plasma by HPLC with UV Detection; *Journal of Chromatographic Science* 2008; 16: 485-489.
8. Behera, D. G. Sankar, S. K. Moitra, and S. C. Si; Development, Validation and Statistical Correlation of RP–LC Methods for Determination of Atazanavir Sulfate in Capsule Dosage Form; *E-Journal of Chemistry* 2012; 9(4): 1778-1787.
9. Seetaramaiah, A. Anton smith, K. Ramyateja, G. Alagumanivasagam and R. Manavalan; Spectrophotometric determination of Ritonavir in Bulk and pharmaceutical formulation;*Sci. Revs. Chem. Commun.*2012; 2(1): 1-6.
10. Behera, *et al*; Assay and stability study of Ritonavir by Spectrophotometric method, *Chronicles of Young Scientists* 2011; 2(3): 161-167.
11. Anindita Behera, Swapan Kumar Moitra, Sudam Chandra Si, Dannana Gowri Sankar; A simple validated isocratic RP – LC method for estimation of Ritonavir in bulk and tablet dosage form, *Der Pharmacia Letter* 2011; 3(1): 145-151.
12. Ravi N. Tiwari and Chandrakant G. Bonde; LC, LC-MS/TOF and MS^n studies for the separation, identification and Characterization of degradation products of Ritonavir, *Anal. Methods* 2011; 3: 1674–1681.
13. Suneetha, S. Kathirvel and G.Ramachandrika; A validated Rp-Hplc method for simultaneous estimation of lopinavir and Ritonavir in combined dosage form, *International Journal of Pharmacy and Pharmaceutical Sciences* 2011; 3(1): 49-51.
14. Ponnilavarasana, A. Rajasekarana, J. G. Dharumana, D. Kalaiyarasia, M. Senthilkumara; Rp–Hplc method for simultaneous estimation of antiretroviral drugs lopinavir and ritonavir in tablet dosage form, *Digest Journal of Nanomaterials and Biostructures,* July-September 2010; 5(3): 771-778.
15. R. K. Nanda, A. A. Kulkarni, P.B. Yadav, Simultaneous Spectrophotometric estimation of Atazanavir sulphate and Ritonavir in tablets, *Der Pharma Chemica* 2011; 3(3): 84-88.
16. RajendraprasadY, RajasekharK.K.,ShankarananthV.,Yaminikrishna H.V.,Saikumar S, Venkataraghav Reddy P, *J. of Pharmacy Research* 2009; 2: 1593-94.
17. ICH, Q2A validation of analytical procedure: Methodology International Conference on Harmonization, Geneva, October 1994.
18. ICH, Q2B Validation of analytical procedure: Methodology International Conference on Harmonization, Geneva, March 1996.
19. Gandhi SV , Khan SI., Jadhav RT. , Jadhav SS., Jadhav GA*, J. of AOAC International*.,2009, 92,1064-67.
20. Reviewer Guidance: Validation of Chromatographic Methods, Center for Drug Evaluation and Research (CDER), PDA, Incorporation Publication Service, 1994.
21. ICH Harmonised Tripartite Guidelines, 2005. Validation of Analytical Procedures: Text and Methodology Q2 (R1).
22. Wankhede SB, Nanda RK, Gadewar VS, Thombare VG, Chitlange SS, Indian Drugs, 2008; 45, 726-30.