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Research Article

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Development and validation of RP-HPLC method for the estimation of ritonavir oral suspension

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ABSTRACT

Ritonavir was originally developed as an inhibitor of HIV protease. It is one of the most complex inhibitors. It is now rarely used for its own antiviral activity, but remains widely used as a booster of other protease inhibitors. cytochrome P450-3A4 (CYP3A4). The drug's molecular structure inhibits CYP3A4, so a low dose can be used to enhance other protease inhibitors. The chromatographic conditions comprise of a column: Zorbax Bonus RP 18, 4.6x100mm, 3.5µ. A mixture of 0.01M Potassium dihydrogen phosphate buffer (pH 6.8), Acetonitrile in the ratio of 50:50 was used as Mobile phase. Quantitation was achieved by UV detection at 239 nm. A linear response (r^2 - 0.999) was observed in the range of 200 to 800ppm (from about 40% to 160%of target concentration) for Ritonavir. The method was validated for Accuracy, Precision, Linearity, Specificity, robustness LOD,LOQ and stability are within the ICH specifications. The proposed method has been used to estimate the Ritonavir. It is also desirable to have less run time; Henceforth the analysis of assay sample will become fast and reliable. The method was validated to meet requirements of global regulatory filling.

Key words: Ritonavir Oral Suspension, UV detection, Linearity.

INTRODUCTION

One of the deadliest and unmanageable chronic health catastrophes is HIV/AIDS. It requires lifelong treatment with potent life saving essential drugs that include nucleoside reverse transcriptase inhibitors, non nucleoside reverse transcriptase inhibitors and protease inhibitors. Amongst these Ritonavir drug is a protease inhibitor used as a second line regimen to treat patients with HIV [1]. Ritonavir (Figure 1) is chemically designated as 10-Hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatri -decan -13-oic acid, 5-thiazolylmethyl ester, [5S-(5R*,8R*,10R*,11R*)] It is official in Indian Pharmacopoeia [2] and United States Pharmacopoeia [3] Literature survey revealed several analytical methods for the determination of Ritoavir and lopinavir in tablets, capsules, and syrups which employ techniques such as high-performance liquid chromatography (HPLC) [3-5], Ultra performance liquid chromatography (UPLC) [6], and high performance thin layer chromatography (HPTLC) [7]. In biological fluids, the active principles as well as their metabolites have been quantitatively determined by HPLC with UV detection, LC/MS/MS [8,9] Spectroscopic method[10-14], Micellarelectrokinetic chromatography method [15] and Tandem mass spectrometry [16] Apart from the above no other methods for quantitative determination of Ritonavir oral suspension. The developed method was simple,

precise, specific and accurate. The statistical analysis proved that method is reproducible and selective for the analysis of Ritonavir in bulk drug and its oral suspension.

EXPERIMENTAL SECTION

Instruments and reagents

An analytically pure sample of Ritonavir was procured as gift sample from Matrix laboratories (Hyderabad, India). Analytical HPLC grade Acetonitrile, Water -Millipore water from Merck, Photassium dihydrogen phosphate AR Grade from Chem India was used as solvent for dilution. HPLC Agilent 1200 infinity series with PDA Array Detector with OPEN LAB software was used. Ritonavir oral suspension 400mg/5mL was procured from local pharmacy.

Selection of chromatographic method

Proper selection of the method depends upon the nature of the sample, molecular weight and the solubility. Reverse phase chromatography technique was selected for initial separations from the knowledge of properties of the compounds.

CHROMATOGRAPHIC CONDITIONS

The HPLC system used was HPLC Agilent 1200 infinity series with PDA Array Detector. A ZORBAX BONUS RP 18 column 3.5µ 4.6x100mm was used at ambient temperature. Separation was achieved using a mobile phase consisting of the mixture 0.01M Phosphate buffer (pH 6.8), Acetonitrile in the ratios of 50:50 v/v. pH of solutions were measured using a Crison Model. The detection wavelength was set at 239 nm. The flow rate was 1.0 ml/min. Data analysis was carried out using open lab software. The standard optimized chromatogram was show in fig 2.

Buffer preparation: (0.01M Potassium dihydrogen phosphate buffer)

Dissolved 1.3g of Potassium dihydrogen phosphate in 1000ml of milliQ water and adjusted pH to 6.8 ± 0.05 With NaOH solution.

Mobile Phase:

Mixed buffer and Acetonitrile in the ratio of 50:50v/v respectively and degassed.

Standard preparation:

Weighed accurately and transferred about 100mg of Ritonavir working standard, into 200mlvolumetric flask. Added about 170ml of diluent and sonicated to dissolve. Made up to the volume with diluent and mixed well. Take above solution 10 ml in 50 ml volumetric flask dilute up to mark with diluent (200ppm). Mix well and filter through 0.45µm filter.

Sample preparation:

Accurately transferred 1.25 mL of Ritonavir oral suspension 400mg/5mL (100mg) transferred in to 200ml volumetric flask. Added 170 mL of diluent and sonicated for 20minutes with intermittent shaking in cold water, made up to the volume with diluent and mixed well. Take above solution 10 ml in 50 ml volumetric flask dilute up to mark with diluent (200ppm). Filtered the solution through 0.45 µm PVDF membrane filter.

Assay procedure:

Separately inject equal volumes (about 10 µL) of diluents as blank, standard preparation and sample preparations in to the chromatograms and measure the peak area responses for the analyte peaks and calculate the %Assay. The sample chromatogram was shown in fig 3 and the assay values are reported in table 1.

METHOD VALIDATION [17-19]

System suitability testing:

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type

of procedure being validated and the parameters like tailing factor, retention time, theoretical plates per unit, resolution factor are determined and the results are tabulated in table 2

Accuracy:
A known quantity of the pure drug was added along with placebo to prepare samples at the level of 50%, 100%, 150%, of the target concentration in triplicate. The contents were determined from the respective chromatograms. The concentration of the drug product in the solution was determined using assay method. The % RSD, mean recoveries was calculated, which shows that method is accurate. The results obtained are tabulated in table 3.

System Precision
Weighed accurately and transferred about 100mg of Ritonavir working standard, into 200ml volumetric flask. Added about 170ml of diluent and sonicated to dissolve. Made up to the volume with diluent and mixed well. solution preparation was injected six times into the chromatograph and Chromatograms were recorded. The results obtained are tabulated in table 4.

Method precision:
The precision of the method (repeatability) was investigated by performing six determinations of the same batch of product of known concentration The resulting data are provided in Tables 5 which shows that the repeatability

Specificity:
Specificity is the ability to assess unequivocally the analyte in the presence of components that might be expected to be present, such as impurities, and matrix components. Diluent, placebo, standard solution, and sample solution were separately injected into the HPLC system. Ritonavir peaks were examined not affected by diluent and placebo.The results observed are tabulated in table 6.and in figure 4 and 5.

Linearity:
Weighed accurately and transferred about 100mg of Ritonavir standard, into 100mlvolumetric flask. Added 70 mL of diluent and sonicated for 20 minutes with intermittent shaking in cold water, made up to the volume with diluent and mixed well. Then from the above solution take 4, 6,8,10,12,14,16 ml of the solution and make up the volume to 20 ml with diluents to get the concentrations of 200,300,400,500,600,700 and 800 µg/ml respectively. The solution was injected into the chromatograph. The area of Ritonavir was recorded for each level and correlation coefficient was calculated. A graph of **Concentration** (ppm) was plotted on X-axis and **Area** on Y-axis. The results are reported in table 7 and figure 6.

LIMIT OF DETECTION
Pipette 1mL of 10µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent. Pipette 1mL of 10µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent. Further pipette 0.8mL of above diluted solution into a 10 ml of volumetric flask and dilute up to the mark with diluents. The results are tabulated in table 8.

LIMIT OF QUANTIFICATION
Pipette 1mL of 10µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.Pipette 1mL of 10µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent. Further pipette 2.5mL of above diluted solution into a 10 ml of volumetric flask and dilute up to the mark with diluent. The results are tabulated in table 9.

Stability of solutions
a) Bench top stability of standard and sample Solutions:
A study to establish the stability of standard and sample solutions on bench top was conducted for 24 hours. The similarity factor for standard solution and % assay for sample solutions were estimated at initial and after 24 hours against freshly prepared standard solution.The difference in % assay of sample solutions between initial and after 24 hours was calculated and the similarity factor of Ritonavir in standard solution was calculated after 24 hours. The results are tabulated in table 10.

b) Bench top stability of mobile phase

A study to establish the stability of mobile phase on bench top was conducted for 5 days. The system suitability was performed as per the test method by using the same mobile phase on different days (initial, after 2 days and 5 days) and areas were recorded. The results obtained are tabulated in table 11.

Robustness:

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The standard solution and sample solution were injected into the chromatograph at varied conditions of flow, column temperature and mobile phase ratio.

a)Change in flow rate:

Standard solution preparation was injected 5 times into the chromatograph at different flow rate (± 0.1 mL/min) i.e., at 0.9 mL/min and 1.1 mL/min respectively and chromatograms were recorded. The results are tabulated in table 12.

b) Change in column temperature form 25°C

Standard solution preparation was injected 5 times into the chromatograph at different column temperature (± 5 %) i.e., at 20°C and 30°C respectively and chromatograms were recorded. The results are tabulated in table 13.

c) Change in mobile phase pH:

Standard solution preparation was injected 5 times into the chromatograph at different pH i.e., at 6.6 and 6.9 respectively and chromatograms were recorded. The results are tabulated in table 14.

RESULTS AND DISCUSSION

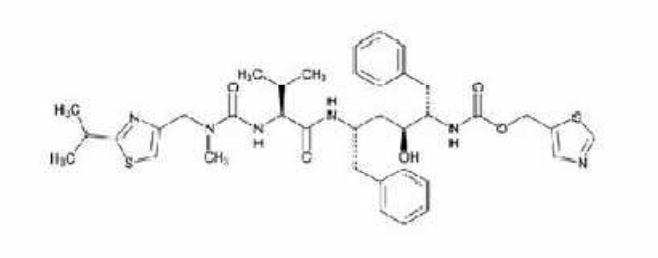

Fig 1: Chemical Structure of Ritanovir

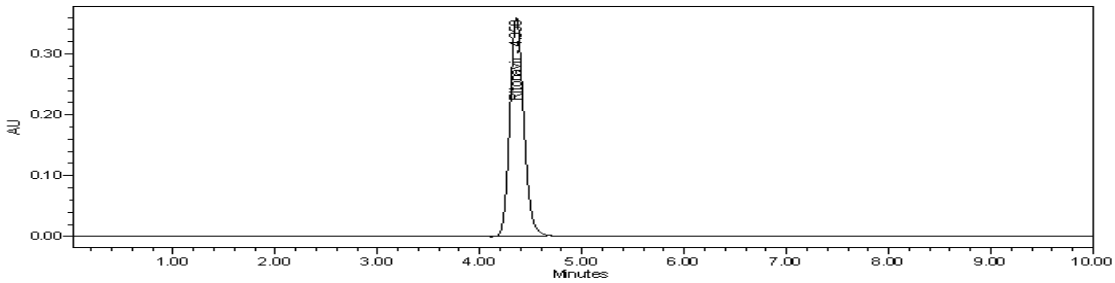

Fig 2: The standard optimized chromatogram

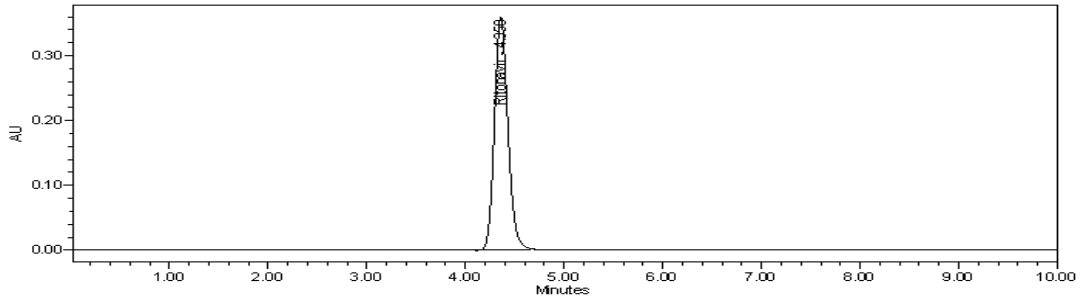

Fig 3: Typical Chromatogram of sample

Table 1: Result of Assay

SL.No	Drug taken in the %level	Amount added in µg	Amount found in µg	%recovered	%Mean recovery	%RSD
1	100	100.8	100.15	100.1	99.9	0.2
2	100	100.2	99.80	99.8		
3	100	100.7	99.96	99.9		

Table 2: System suitability testing

Peak Table
Peak Name: Ritonavir

	SampleName	Vial	Injection	Peak Name	RT	Area
1	STANADRD	101	1	Ritonavir	4.358	3388949
2	STANADRD	101	2	Ritonavir	4.332	3387682
3	STANADRD	101	3	Ritonavir	4.339	3424050
4	STANADRD	101	4	Ritonavir	4.336	3394325
5	STANADRD	101	5	Ritonavir	4.347	3402068
6	STANADRD	101	6	Ritonavir	4.346	3398582
Mean					4.343	3399276
% RSD					0.2	0.4

Table 3 Results of Recovery

Sample No.	% Level (about)	"ml" Added	"ml" recovered	% Recovery	Mean % Recovery	% RSD
1	50%	50.12	50.20	100.2	100.1	0.2
2		50.06	50.15	100.2		
3		50.13	50.02	99.8		
1	100%	100.08	100.15	100.1	99.9	0.2
2		100.02	99.80	99.8		
3		100.07	99.96	99.9		
1	150%	150.03	149.94	99.9	99.7	0.2
2		149.98	149.20	99.5		
3		150.08	149.52	99.6		

Table 4: Results of System Precision

Injection Number	Peak area
1	3388949
2	3387682
3	3424050
4	3394325
5	3402068
6	3398582
Mean	3399276
% RSD	0.4

Table 5 Results for Method Precision

Sample Number	% Assay
1	98.2
2	98.1
3	98.5
4	99.4
5	97.7
6	97.9
Mean	98.3
% RSD	0.5

Table6 -Results of Placebo Interference

Preparation	Peak found at RT of analyte (Yes/No)
1	No
2	No

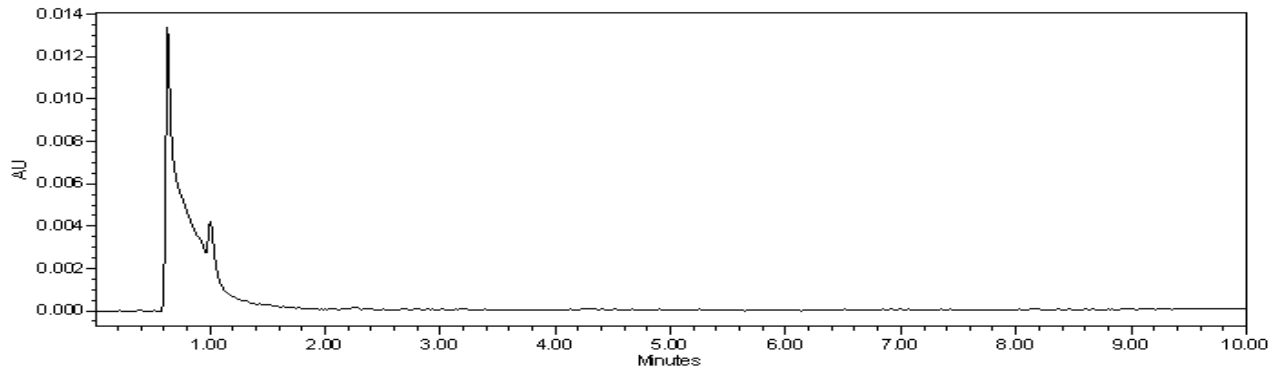

Fig 4: Typical Chromatogram of Placebo

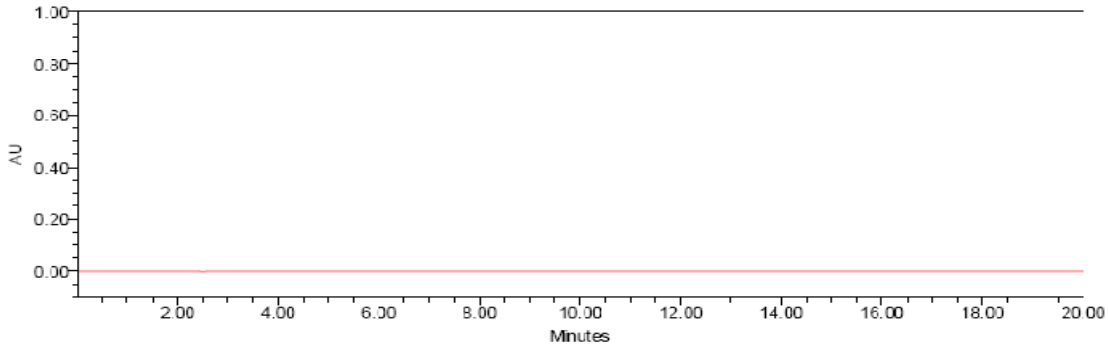

Fig 5: Typical Chromatogram of Blank

Table 7: Linearity Data

Solution	Dil.From stock	Percentage	Concentration(µg/ml)	Area
1	4ml in 20ml	40	200	1355584
2	6ml in 20ml	60	300	2053915
3	8ml in 20ml	80	400	2711167
4	10ml in 20ml	100	500	3388949
5	12ml in20ml	120	600	4063500
6	14ml in 20ml	140	700	4759774
7	16ml in 20ml	160	800	5422334

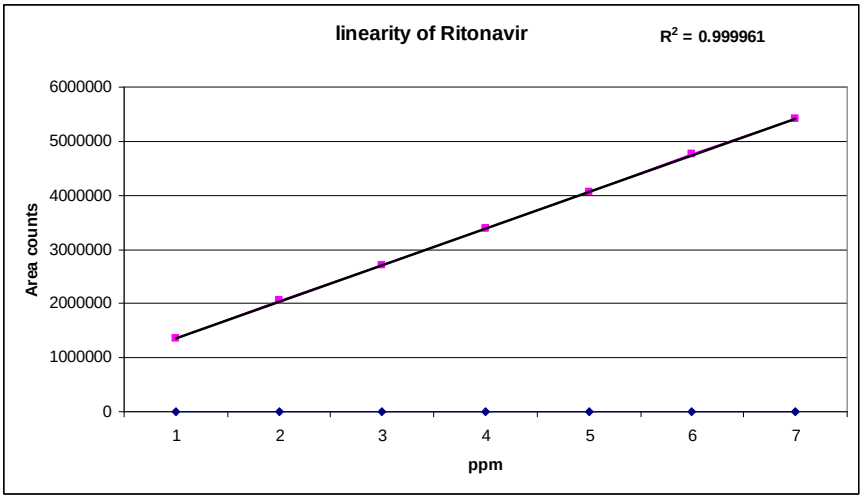

Figure 6: Linearity Plot of Ritonavir

Table 8: Limit of Detection

Component	Working conc. (µg/ml)	LOD Conc. (µl/ml)	Signal To Noise Ratio
Ritonavir	0.08	0.024	2.8

Table 9: Limit of Quantification:

Component	Working conc. (µg/ml)	LOQ Conc. (µl/ml)	Signal To Noise Ratio
Ritonavir	0.25	0.07	10.32

Table 10: Results of Bench top Stability for Standard and Sample solutions

Time in Hours	Similarity factor of Standard	%Assay		Difference from initial	
		Sample-1	Sample-2	Sample-1	Sample-2
Initial	NA	98.7	99.3	NA	NA
24	1.01	99.3	99.7	0.6	0.4

Table 11: Results of Mobile Phase Stability on Bench top

System suitability Parameters	Observed value		
	At Initial	After 2 day	After 5 days
The %RSD for Ritonavir peak areas from five injections of standard solution	0.7	0.7	0.9
The tailing factor for Ritonavir peak from the chromatogram of Standard solution	1.1	1.1	1.2

Table 12: Results of Robustness-Change in Flow rate from 1 mL/min

System suitability Parameters	Observed value with Flow Rate		
	0.9 mL/min	1.0 mL/min	1.1 mL/min
The %RSD for Ritonavir peak areas from five injections of standard solution	0.5	0.4	0.7
The tailing factor for Ritonavir peak from the chromatogram of Standard solution	1.1	1.1	1.2

Table 13: Results of Robustness-Change in Column Temperature from 30°C

System suitability Parameters	Observed value with Column oven Temperature		
	20°C	25°C	30°C
The %RSD for Ritonavir peak areas from five injections of standard solution	0.6	0.4	0.5
The tailing factor for Ritonavir peak from the chromatogram of Standard solution	1.2	1.1	1.1

Table 14: Results of Robustness-Change in Mobile Phase pH from 6.7

System suitability Parameters	Observed value with buffer		
	pH 6.6	pH 6.8	pH 6.9
The %RSD for Ritonavir peak areas from five injections of standard solution	0.5	0.5	0.6
The tailing factor form Ritonavir peak from the chromatogram of Standard solution	1.1	1.1	1.1

CONCLUSION

Ritonavir was originally developed as an inhibitor of HIV protease. On literature survey it was found that HPLC, Colorimetric, Spectroflourimetry & UV methods were reported for the estimation of Ritonavir in its formulations. A simple and sensitive Spectrophotometric method has been described for the assay of Ritonavir either in pure form or in pharmaceutical Liquid dosage form, Absorption maxima of Ritonavir were found to be at 254nm. RP –HPLC method was reported for estimation of Ritonavir. In view of the need for a suitable method for routine analysis of Ritonavir for its assay with less solvent consumption, high resolution, more sensitive, and reduced run time an attempts were being made to develop simple, precise and accurate method by using Reverse Phase High Performance Liquid Chromatography. RP-HPLC method for estimation of Ritonavir is developed and validated for various parameters as per ICH guidelines. The system suitability parameters proved that the proposed method is suitable for estimation of Ritonavir. Good agreement was seen in the estimation results of pharmaceutical formulation for its assay by developed method. Hence it can be concluded that the proposed method was a good approach for obtaining reliable results and found to be suitable for the routine analysis of Ritonavir.

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REFERENCES

[1] Sean CS Martindale: The complete drug reference, 34th edition, Pharmaceutical Press, New Delhi, **2008**, 201-203
[2] Indian Pharmacopoeia, vol. III, **2007**, 1058.
[3] United States Pharmacopoeia 30, National Formulary 25, **2007**, 3143.
[4] Usami Y, Oki T, Nakai M, Sagisaka M, Kaneda T. *Chem. Pharm Bull.*, **2003**, 5(1), 715-718.
[5] Donato EM, Dias CL, Rossi RC, Valente RS, Froelich PE et al. *Journal of Chromatography.*, **2006**, 63(1), 437-443.
[6] Phechkrajang CM, Thin EE, Sratthaphut L, Nacapricha D, Wilairat P. *Mahi Univ J Pharm Sci*., **2009**, 36(1) 1-12.
[7] Marina VA, Julia P, Jorge PR, Eduardo S, Rafael L. *J Braz Chem Soc*., **2011**, 22(2), 134-141.
[8] Sulebhavikar AV, Pawar UD, Mangoankar KV, Prabhu NDN. *E-J Chem*., **2008** 5(3) 706-712.
[9] Temghare GA, Shetye SS, Joshi SS. *E-J Chem*., **2009** 6(2) 223-230.
[10] Myasein F, Kim E, Zhang J, Wu H, El-Shourbagy TA. *Anal Chem Acta* ., **2009**, 65(1),112-116.
[11] Carolina Lupi Dias, Ana Maria Bergold, Pedro Eduardo Froehlich. *Anal Letters*., **2009**; 42(12):1900-1910
[12] Vaishali P Nagulwar, Kishore PB. *Der Pharmacia Lettre* **2010**, 2(1), 196-200.
[13] K. Seetaramaiah, a. Anton smith, k. Ramyateja, G. Alagumanivasagam Manavalan. *Sci. Revs. Chem. Commun.* **2012**, 2(1), 1-6
[14] K.Chiranjeevi, K.P.Channabasavaraj, P.Srinivas Reddy, P.T.Nagaraju. *International Journal of ChemTech Research,* **2011**.3(1), 58-62,
[15] Carvalho AZ, El-Attug MN, Zayed SE, Hove EV, Duppen JV et al. *J Pharm Biomed Anal* **2010**, 53(2), 1210-1216.
[16] Estrela RC, Ribeiro FS, Seixas BV, Suarez-Kurtz G (**2008**) *Rapid Commun Mass Spectrom* 22(1), 657-664.
[17] International Conference on Harmonization (ICH), Validation of Analytical Procedures: Text on Validation of Analytical Procedures Q2A, **1994**.
[18] International Conference on Harmonization (ICH), Validation of Analytical Procedure: Methodology Q2B, **1996**.
[19] International Conference on Harmonization (ICH), Validation of Analytical Procedures: Text and Methodology Q2 (R1), **2005**.