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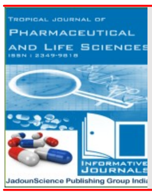

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INFORMATIVE JOURNALS

RELATED SUBSTANCES METHOD DEVELOPMENT AND VALIDATION OF ATAZANAVIR AND RITONAVIR IN TABLET DOSAGE FORM BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple, rapid, selective and stability indicating reversed High performance liquid chromatography method was developed and validated for the simultaneous quantification of process related and degradation impurities present in Atazanavir and Ritonavir tablets. The two proposed drug components and their respective impurities were separated using ACE C18 (250 mm x 4.6 mm), 5µm and mobile phase comprised of Buffer (pH 3.3) and Ogranic solvent in gradient proportion.The flow rate was 0.9ml/min.The detector wavelength of 240nm was used for quantifying the impurities.The selected chromatographic conditions were found to effectively quantitate Atazanavir and Ritonavir and its related impurities. Linearity and range were eatablished from LOQ level to 150% level. Accuracy of the method was established from LOQ level to 150% level.The developed stability indicating method is capable for determination of impurities of Atazanavir and Ritonavir in combined dosage form as well as individual forms also.

Keywords: Atazanavir, Ritonavir, Reversed phase-HPLC.

INTRODUCTION

Atazanavir sulfate and Ritonavir are indicated for the treatment of acquired immune deficiency syndrome (AIDS) and belongs to a class of medicines known as protease inhibitor (PI). These two drugs are used to prevent the reproduction of human immunodeficiency virus (HIV) and categorized as anti-retro viral (ARV) drugs. Combination tablet dosage form containing both Atazanavir and Ritonavir is more effective than individual drug alone since Ritonavir will boost the levels of Atazanavir and intensify its effect. In HIV infected patients, virus multiplies within the cells of the body. Viruses are released from the cells and

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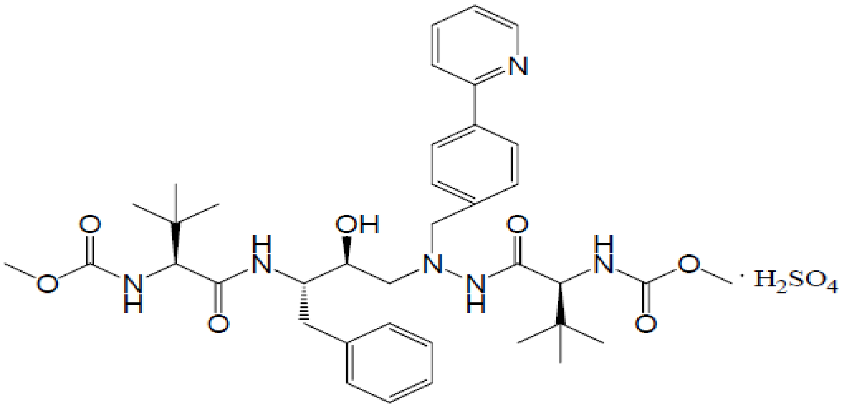
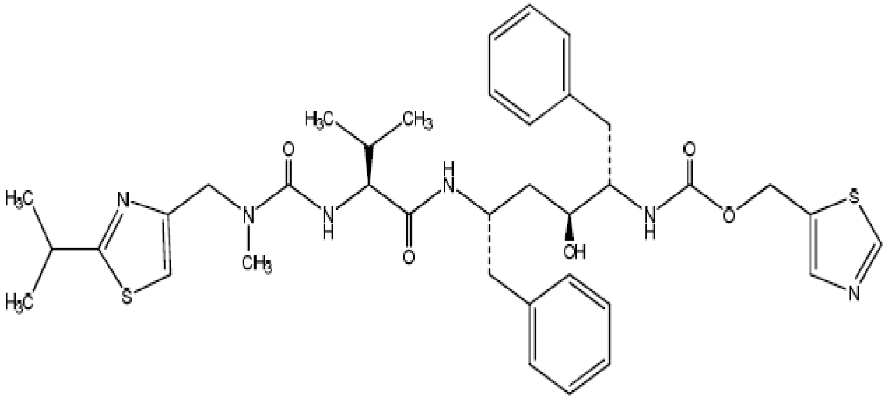
spread throughout the body and infect other cells. During the production of the viruses, new proteins are made. Protease is the enzyme that forms the new structural proteins and enzymes. Atazanavir and Ritonavir are used to block the activity of protease and results in the formation of defective viruses that are unable to infect the body's cells. Due to this, the number of viruses in the body decreases and improves the patient's immunity.

The IUPAC name of Atazanavir sulfate is described as (1,3-thiazol-5-ylmethylN-[(2S,3S,5S)-3-hydroxy-5-[[(2S)-3-methyl-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbamoyl] amino] butanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate). Its molecular formula is $C_{38}H_{52}N_6O_7.H_2SO_4$, which corresponds to a molecular weight of 802.9 (sulfuric acid salt). The molecular weight for free base is 704.9. Atazanavir sulphate is a white to pale-yellow crystalline powder. It is slightly soluble in water and the pH of saturated solution in water is ~1.9 at 24 ± 3°C.

The IUPAC name of Ritonavir is described as 1,3-thiazol-5-ylmethylN-[(2S,3S,5S)-3-hydroxy-5-[[(2S)-3-methyl-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbamoyl]amino] butanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate. Its molecular formula is $C_{37}H_{48}N_6O_5S_2$, and molecular weight is 720.95. Ritonavir is a white-to-light-tan powder. Ritonavir has a bitter metallic taste. It is freely soluble in methanol and ethanol, soluble in isopropanol and practically insoluble in water.



Structure of Atazanavir sulphate



Structure of Ritonavir

The aim of the present work is to develop a simple, rapid and simultaneous method to efficiently separate the impurities present in both Atazanavir and Ritonavir in their fixed dose combination product. Since the product is a combination of two molecules, impurities having structural similarity will present in the drug product. The criticality of this method involves in separating both active ingredients along with their specified and unspecified impurities with satisfactory resolution and at shorter runtime. The sensitivity of developed method should be sufficient enough to quantify the lower levels of impurities to ensure safety and efficacy of drug product. The method development was carried out to achieve these specified goals.

The developed method was subjected to forced degradation studies to check the stability indicating nature. Forced degradation study can be able to produce a degradation profile similar to that would be observed in a formal stability study under International Conference on Harmonization (ICH) conditions. Forced degradation studies are able to establish the intrinsic stability of active ingredient in a pharmaceutical dosage formulation and helpful in establishing degradation pathways of finished dosages. These studies can resolve the issues occur during stability exposure by distinguishing degradation products that are related to drug product from those which are produced from excipients present in the formulation.

MATERIALS AND METHODS

Reagents and Chemicals:

Atazanavir Sulphate and Ritonavir drug sample, Potassium dihydrogen phosphate, Orthophosphoric acid, Acetonitrile, Methanol, Water, Tetrahydrofuran, n-Butanol of HPLC grade.

Instruments and Chromatographic Conditions:

HPLC system with UV/PDA detector was used for method development and validation.The separation was achieved on ACE C18 (250 mm x 4.6 mm) 5µ.The column was maintained at 55°C and the eluent was monitored at 240nm using UV detector. A gradient method was used at flow rate of 0.9 ml/min.The injection volume was 20µL.

Preparation of Buffer (pH 3.30)

Accurately weigh and transfer about 4.50 g of potassium dihydrogen phosphate into 1000 mL water and mix well. Adjust the pH to 3.30 ± 0.05 with 10 % v/v Ortho phosphoric acid. Filter through 0.45µm or finer porosity membrane filter.

Preparation of Organic phase

Mix Acetonitrile, tetrahydrofuran and n-butanol in the ratio of 18:8:5 v/v/v.

Preparation of Diluent

Use methanol as diluent.

Preparation of Standard solution

Accurately weigh and transfer about 136 mg of Atazanavir sulfate working standard and 40 mg of Ritonavir working standard into a 200 mL volumetric flask. Add about 100 mL of diluent and sonicate to dissolve the content completely. Make up the volume with diluent and mix well. Further pipette out 5 mL of this solution to 100 mL volumetric flask. Make up the volume with diluent and mix well. Filter the solution through 0.45 µm PVDF filter by discarding first 2 mL of filtrate.

Preparation of Sample solution

Determine the average weight not less than 20 tablets.

Crush not less than 10 tablets in to fine power with mortar and pestle. Accurately weigh and transfer tablet powder equivalent to 300 mg of Atazanavir (about 342 mg of Atazanavir sulfate) to 100 mL volumetric flask. Add about 70 mL of diluent and sonicate in cold water with intermittent shaking for about 30 minutes. Allow the solution to attain room temperature and make up to the mark with diluent and mix well. Filter the solution through 0.45 µm PVDF filter by discarding first 2 mL of filtrate.

Chromatographic conditions

Column	:	ACE C18 (250 mm x 4.6 mm) 5µ
Column oven temperature	:	55°C
Sample temperature	:	10°C
Flow Rate	:	0.9 mL / minute
Detection wavelength	:	240 nm
Injection volume	:	20 µL
Run Time	:	120 minutes
Elution mode	:	Gradient

Gradient Programme

Time (minutes)	Buffer (pH3.3) (% v/v)	Organic solvent (% v/v)
0	92	8
5	92	8
25	80	20
65	50	50
90	25	75
100	25	75
101	92	8
120	92	8

System suitability criteria

The system is suitable for analysis**, if and only if**

1. The USP tailing factor of Atazanavir and Ritonavir peak from the first injection of standard is not more than 1.5.
2. The USP column efficiency (theoretical plates) of Atazanavir peak from the first injection of standard is not less than 100000.
3. % RSD of area counts of Atazanavir and Ritonavir peaks from six replicate injections of standard solution is not more than 5.0

Validation of RP-HPLC method

1. System Precision

The % RSD for the peak area response of six replicate injection for standard solution found to be 0.1% for Atazanavir and 0.2% for Ritonavir which is within the acceptance criteria of 5.0%.

Results obtained for six replicate injections of standard solution

Injection	**Area Counts(µV*sec)**	
	Atazanavir	**Ritonavir**
1	683051	174916
2	682323	175153
3	683469	174464
4	683525	174971
5	682869	174603
6	683952	175498
Mean	683198	174934
% RSD	0.1	0.2

2. Specificity

A.Placebo Interference

a) Blank solution was analysed .No interference from blank at the retention time of known impurity peaks and Atazanavir and Ritonavir peak was observed.

b) Common Placebo solutions was chromatographed. No interference from common placebo at the retention time of known impurity peaks and Atazanavir and Ritonavir peak was observed.

c) Known related substances of Atazanavir and Ritonavir peak were chromatographed individually.

d) Sample solution was chromatographed. Peak purity of Atazanavir and Ritonavir peak in sample indicates that the peak is pure and has has co-eluting peaks indicating specificity of the method.

B.Forced degradation

Forced degradation study was carried out on Atazanavir and Ritonavir tablets, common placebo, placebo with Atazanavir and placebo with Ritonavir under following conditions.

Mode of degradation	**Condition**	**%Degradation for sample**	
		Atazanavir	**Ritonavir**
Control sample	Unstressed	Not applicable	Not applicable
Acid degradation	1ml of 1N HCL	1.2	1.2
Base degradation	5 ml of 1N sodium hydroxide	3.2	8.7
Peroxide degradation	5 ml of 30% H_2O_2, 60°C at 60 minutes.	0.6	0.9
Thermal degradation	24 hours at 60 °C	1.2	1.4
Humidity degradation	25°C/90% RH for NLT 7 days.	1.7	1.8
Photolytic degradation	1.2 million luxhours/ 200watt hours/square meter	1.3	1.3

No peak were detected at the retention time of Atazanavir and Ritonavir in the chromatograms of diluent and common placebo solution. The purity angle was less than purity threshold and there was no tick mark in the purity flag column for Atazanavir and Ritonavir peak in all final stressed sample solutions. Peak purity plot of stressed sample solution indicate that the Atazanavir and Ritonavir peak is homogeneous and has co-eluting peaks, indicating specificity of the method.

3. Method Precision

The %RSD for six replicate sample preparation were found to be 14.4% for Hydantoin Amino alcohol and 0.5% for Highest unknown impurity and 10.6% for Total impurities which is within the acceptance criteria of 15.0%.

Sample No.	**Hydantoin aminoalcohol (Ritonavir Impurity E) (%w/w)**	**Highest unknown impurity (Ritonavir) (%w/w)**	**Total Impurities (%w/w)**
1	0.282	0.122	0.411
2	0.365	0.123	0.498
3	0.276	0.123	0.409
4	0.381	0.124	0.515
5	0.374	0.123	0.507
6	0.375	0.123	0.508
Mean	0.342	0.123	0.475
% RSD	14.4	0.5	10.6

4. Accuracy

The accuracy of the method was determined by analyzing known amount of N-deacylvaline ritonavir (Ritonavir Impurity A**),** Hydroxy- ritonavir (Ritonavir Impurity D), Hydantoin aminoalcohol **(**Ritonavir Impurity E), Ritonavir hydroperoxide (Ritonavir Impurity F) and Oxazolidinone derivative (Ritonavir Impurity J) spiked into twelve different flasks having control sample and in four different levels, each in triplicate viz., LOQ level, 50%,100%,& 150% of the specification limit. The samples were prepared and analysed by the proposed method and the known amount of impurities recovered after making corrections for the amount already present was calculated.

Results obtained for N-deacylvaline ritonavir (Ritonavir Impurity A)

Sample	Concentration (%w/w)		Recovery (%)	Mean % Recovery	%RSD
	Amount Added	**Amount recovered**			
LOQ (1)	0.057	0.053	93.0	94.2	2.0
LOQ (2)	0.057	0.055	96.4		
LOQ (3)	0.057	0.053	93.1		
50% (1)	0.096	0.090	93.8	94.2	2.6
50% (2)	0.096	0.088	91.9		
50% (3)	0.096	0.093	96.9		
100% (1)	0.191	0.181	94.8	94.2	1.2
100% (2)	0.191	0.177	92.9		
100% (3)	0.191	0.181	94.9		
150% (1)	0.287	0.277	96.5	95.6	0.8
150% (2)	0.287	0.273	95.0		
150% (3)	0.287	0.274	95.4		

Results obtained for Hydroxy- ritonavir (Ritonavir Impurity D)

Sample	Concentration (%w/w)		Recovery (%)	Mean % Recovery	%RSD
	Amount Added	Amount recovered			
LOQ (1)	0.031	0.028	89.5	90.7	1.7
LOQ (2)	0.031	0.029	92.5		
LOQ (3)	0.031	0.028	90.2		
50% (1)	0.153	0.147	96.1	96.2	1.2
50% (2)	0.153	0.145	95.1		
50% (3)	0.153	0.149	97.4		
100% (1)	0.305	0.292	95.7	95.2	0.5
100% (2)	0.305	0.290	95.0		
100% (3)	0.305	0.289	94.8		
150% (1)	0.458	0.441	96.3	96.0	0.6
150% (2)	0.458	0.437	95.4		
150% (3)	0.458	0.442	96.4		

Results obtained for Hydantoin aminoalcohol (Ritonavir Impurity E)

Sample	Concentration (%w/w)		Recovery (%)	Mean % Recovery	%RSD
	Amount Added	Amount recovered			
LOQ (1)	0.104	0.079	75.8	80.0	5.7
LOQ (2)	0.104	0.088	84.8		
LOQ (3)	0.104	0.083	79.4		
50% (1)	1.300	1.269	97.6	98.1	0.6
50% (2)	1.300	1.274	98.0		
50% (3)	1.300	1.283	98.7		
100% (1)	2.601	2.493	95.8	95.9	0.4
100% (2)	2.601	2.508	96.4		
100% (3)	2.601	2.488	95.6		
150% (1)	3.901	3.867	99.1	97.9	1.1
150% (2)	3.901	3.779	96.9		
150% (3)	3.901	3.810	97.7		

Results obtained for Ritonavir hydroperoxide (Ritonavir Impurity F)

Sample	Concentration (%w/w)		Recovery (%)	Mean % Recovery	%RSD
	Amount Added	Amount recovered			
LOQ (1)	0.062	0.049	79.0	80.6	2.0
LOQ (2)	0.062	0.051	82.3		
LOQ (3)	0.062	0.050	80.6		
50% (1)	0.103	0.085	82.5	82.2	0.7
50% (2)	0.103	0.084	81.6		
50% (3)	0.103	0.085	82.5		
100% (1)	0.206	0.183	88.8	89.3	5.2
100% (2)	0.206	0.194	94.2		
100% (3)	0.206	0.175	85.0		
150% (1)	0.309	0.276	89.3	89.1	0.8
150% (2)	0.309	0.273	88.3		
150% (3)	0.309	0.277	89.6		

Results obtained for Oxazolidinone derivative (Ritonavir Impurity J)

Sample	Concentration (%w/w)		Recovery (%)	Mean % Recovery	%RSD
	Amount Added	Amount recovered			
LOQ (1)	0.094	0.084	89.4	92.6	8.0
LOQ (2)	0.094	0.095	101.1		
LOQ (3)	0.094	0.082	87.2		
50% (1)	0.157	0.140	89.2	89.2	0.7
50% (2)	0.157	0.139	88.5		
50% (3)	0.157	0.151	89.8		
100% (1)	0.313	0.315	100.6	100.3	9.4
100% (2)	0.313	0.343	109.6		
100% (3)	0.313	0.294	90.7		
150% (1)	0.470	0.435	92.6	93.3	1.9
150% (2)	0.470	0.433	92.1		
150% (3)	0.470	0.448	95.3		

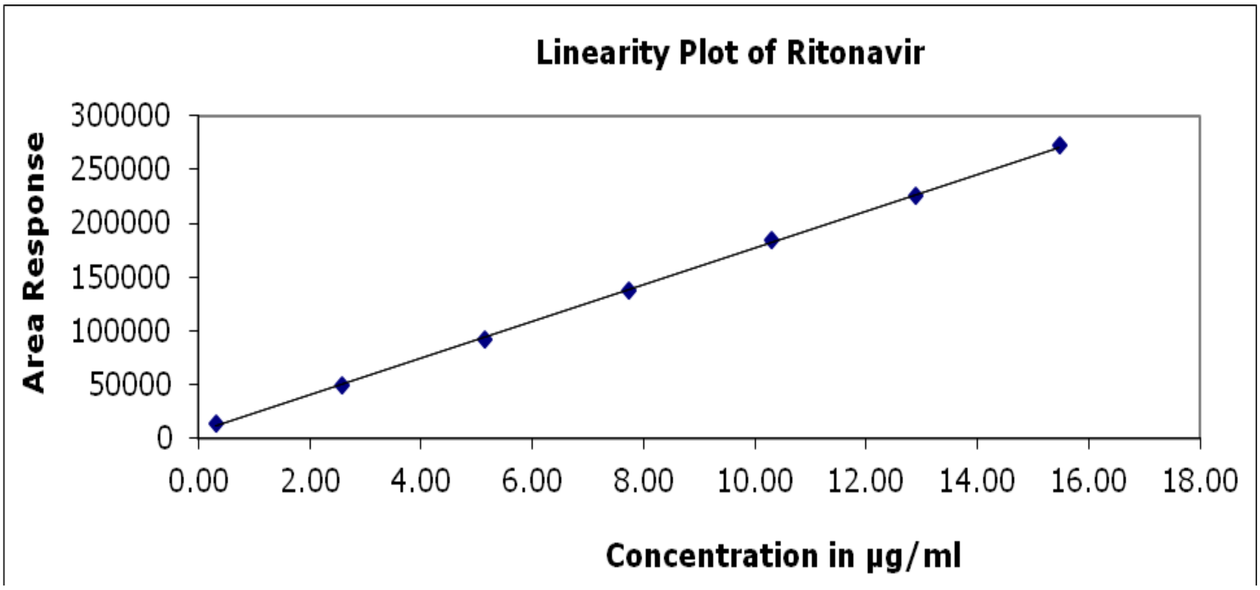
5. Linearity

Linearity solutions prepared from LOQ to 150% of Atazanavir and Ritonavir, N-deacylvaline ritonavir (Ritonavir Impurity A), Hydroxy- ritonavir (Ritonavir-Impurity D), Hydantoin aminoalcohol (Ritonavir Impurity E), Ritonavir hydroperoxide (Ritonavir-Impurity F), Oxazolidinone derivative (Ritonavir Impurity J) of specification limit.

Linearity Results for Ritonavir

S. No.	Linearity level	Ritonavir Concentration (µg/mL)	Area (µV*sec)
1	Level-1 -LOQ	0.3093	14179
2	Level-2 -25%	2.5772	49240
3	Level-3 -50%	5.1545	92508
4	Level-4 -75%	7.7317	137813
5	Level-5 -100%	10.3090	184613
6	Level-6 -125%	12.8862	225548
7	Level-7 -150%	15.4635	272027
Correlation coefficient (r)			0.99982
Slope			17106.2222
Intercept			6401.7683
% Deviation of the Y-intercept			3.5

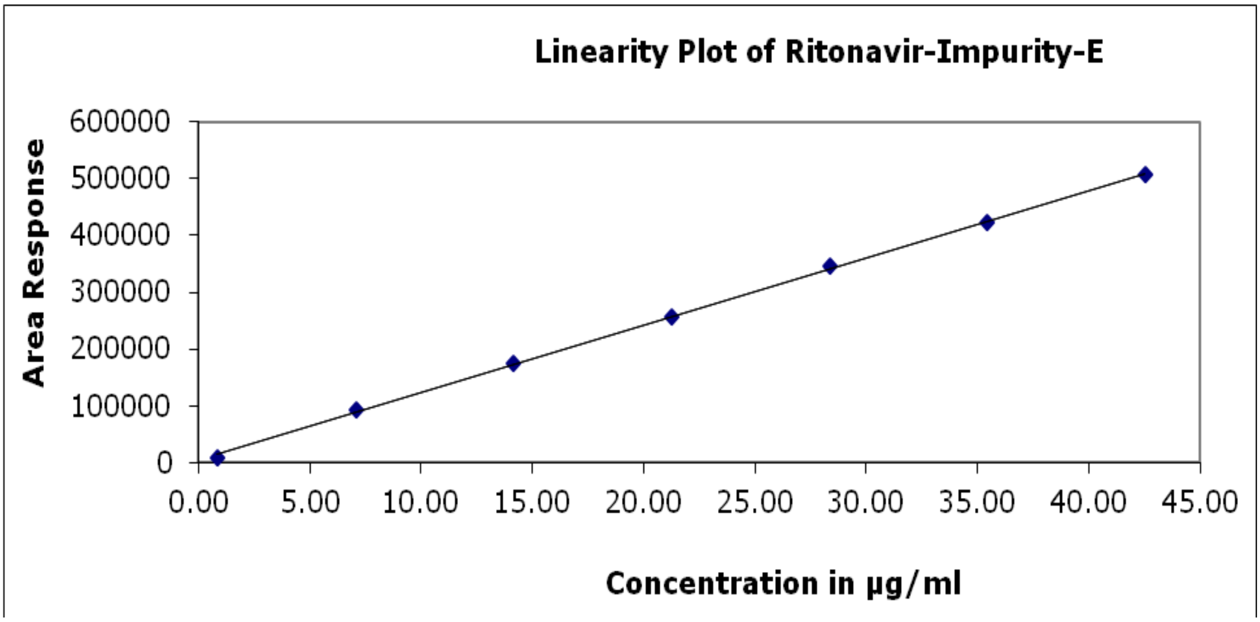
Linearity plot for Ritonavir



Linearity Results for Hydantoin aminoalcohol (Ritonavir Impurity E)

S. No.	**Linearity level**	**Hydantoin aminoalcohol (Ritonavir Impurity E) Concentration (µg/mL)**	**Area (µV*sec)**
1	Level-1 -LOQ	0.8502	9149
2	Level-2 -25%	7.0853	92773
3	Level-3 -50%	14.1707	173963
4	Level-4 -75%	21.2561	258053
5	Level-5 -100%	28.3415	345594
6	Level-6 -125%	35.4269	423353
7	Level-7 -150%	42.5123	507119
Correlation coefficient (r)			0.99980
Slope			11877.1623
Intercept			4667.1144
% Deviation of the Y-intercept			1.4
RRF			0.69

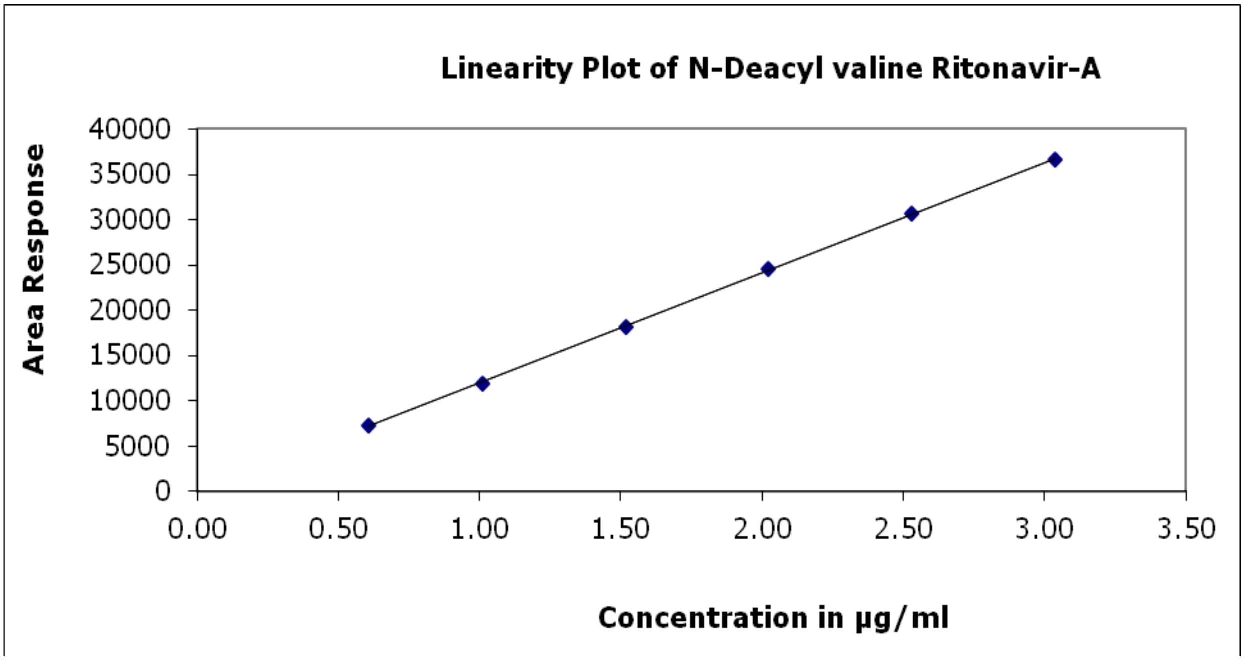
Linearity plot for Ritonavir Impurity-E



Linearity Results for N-deacylvaline ritonavir (Ritonavir Impurity A)

S. No.	Linearity level	N-deacylvaline ritonavir (Ritonavir Impurity A) Concentration (µg/mL)	Area (µV*sec)
1	Level-1 -LOQ	0.6071	7319
2	Level-3 -50%	1.0119	11948
3	Level-4 -75%	1.5178	18110
4	Level-5 -100%	2.0237	24595
5	Level-6 -125%	2.5297	30644
6	Level-7 -150%	3.0356	36573
Correlation coefficient (r)			0.99990
Slope			12143.6327
Intercept			-176.8626
% Deviation of the Y-intercept			-0.7
RRF			0.74

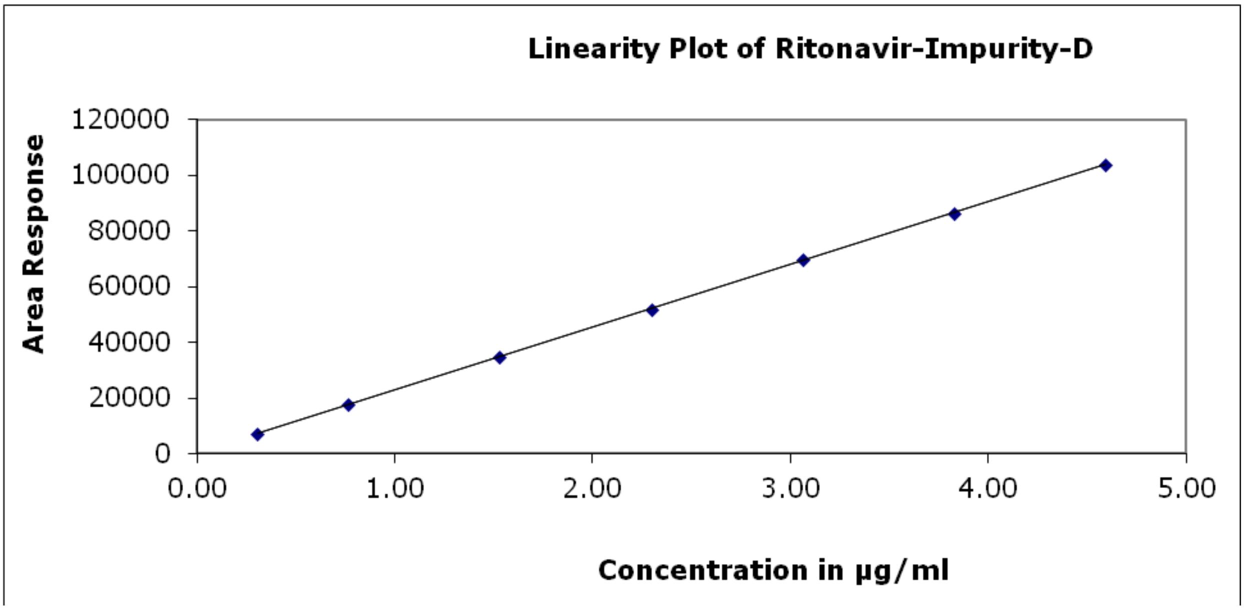
Linearity plot for N-Deacyl valine Ritonavir (Ritonavir Impurity A)



Linearity Results for Hydroxy- ritonavir (Ritonavir-Impurity D)

S. No.	Linearity level	Hydroxy- ritonavir (Ritonavir-Impurity D) Concentration (µg/mL)	Area (µV*sec)
1	Level-1 -LOQ	0.3061	7140
2	Level-2 -25%	0.7653	17755
3	Level-3 -50%	1.5306	34564
4	Level-4 -75%	2.2959	51609
5	Level-5 -100%	3.0612	69849
6	Level-6 -125%	3.8265	86187
7	Level-7 -150%	4.5918	103961
Correlation coefficient (r)			0.99995
Slope			22555.2780
Intercept			238.3129
% Deviation of the Y-intercept			0.3
RRF			1.38

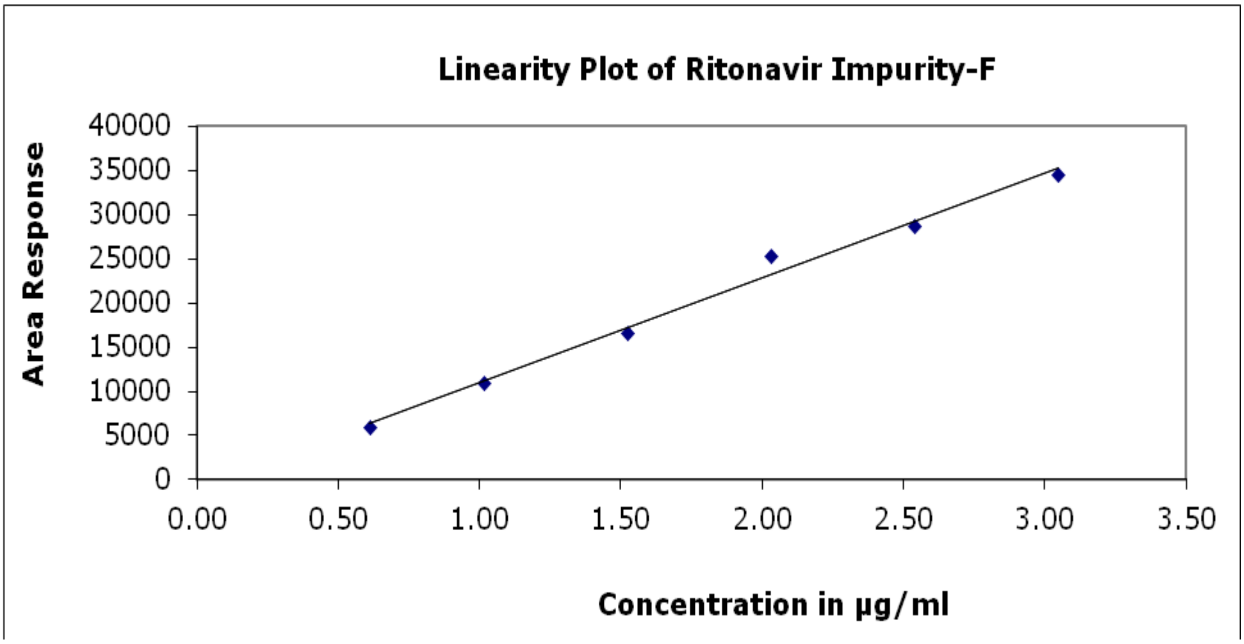
Linearity plot for Ritonavir Impurity-D



Linearity Results for Ritonavir hydroperoxide (Ritonavir-Impurity F)

S. No.	Linearity level	Ritonavir hydroperoxide (Ritonavir-Impurity F) Concentration (µg/mL)	Area (µV*sec)
1	Level-1 -LOQ	0.6096	5962
2	Level-3 -50%	1.0160	10997
3	Level-4 -75%	1.5240	16683
4	Level-5 -100%	2.0321	25425
5	Level-6 -125%	2.5401	28735
6	Level-7 -150%	3.0481	34561
Correlation coefficient (r)			0.99498
Slope			11856.7580
Intercept			-888.8497
% Deviation of the Y-intercept			-3.8
RRF			0.73

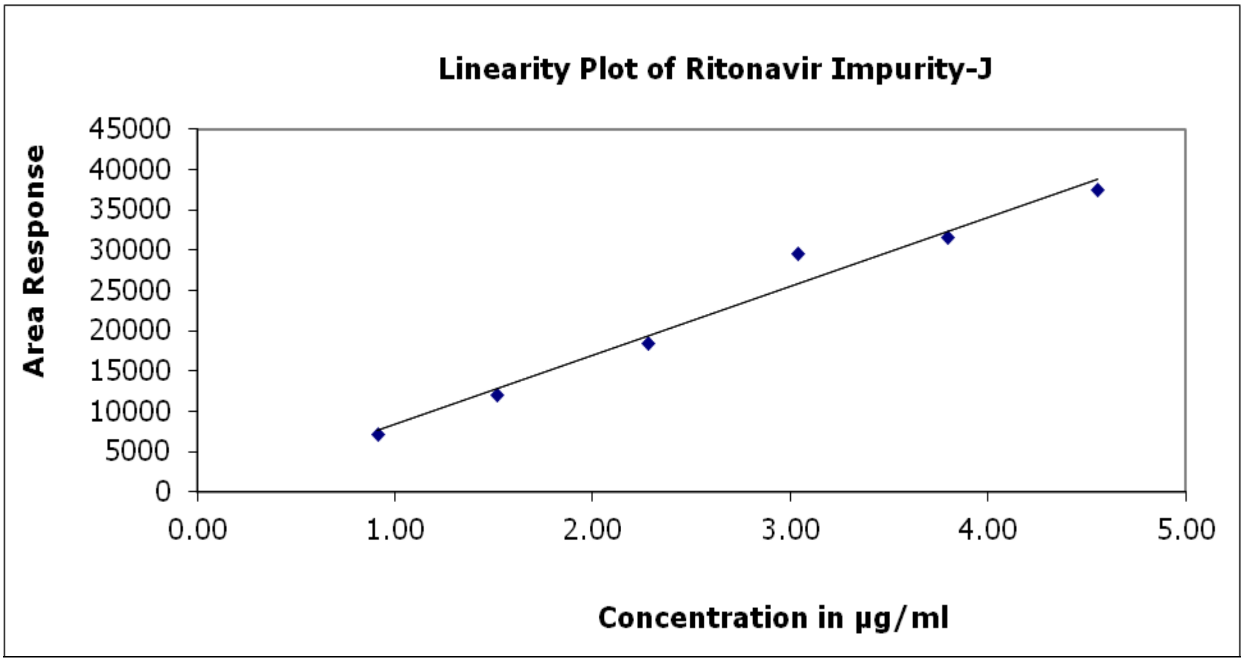
Linearity plot for Ritonavir Impurity-F



Linearity Results for Oxazolidinone derivative (Ritonavir Impurity J)

S. No.	Linearity level	Oxazolidinone derivative (Ritonavir Impurity J) Concentration (µg/mL)	Area (µV*sec)
1	Level-1 -LOQ	0.9106	7227
2	Level-3 -50%	1.5176	12083
3	Level-4 -75%	2.2765	18521
4	Level-5 -100%	3.0353	29671
5	Level-6 -125%	3.7942	31656
6	Level-7 -150%	4.5530	37603
Correlation coefficient (r)			0.98737
Slope			8589.1533
Intercept			-235.7379
% Deviation of the Y-intercept			-0.9
RRF			0.53

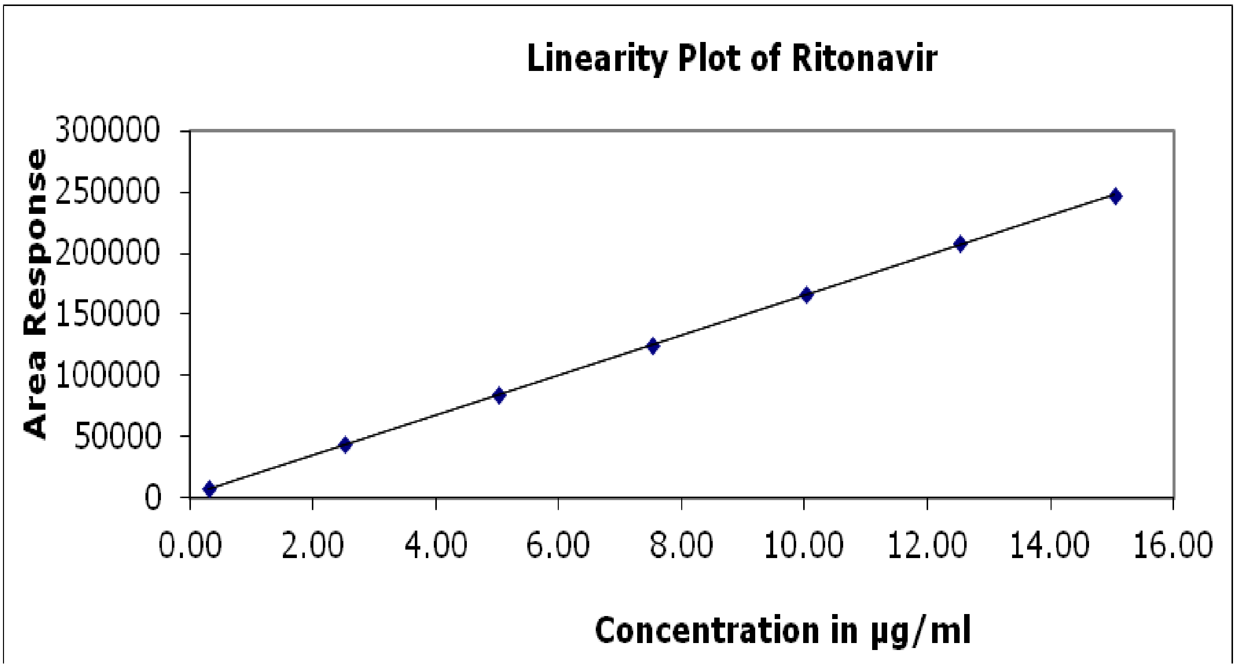
Linearity plot for Ritonavir Impurity-J



Linearity Results for Ritonavir

S. No.	Linearity level	Ritonavir Concentration (µg/mL)	Area (µV*sec)
1	Level-1 -LOQ	0.3022	7609
2	Level-2 -25%	2.5068	44671
3	Level-3 -50%	5.0136	84079
4	Level-4 -75%	7.5204	125074
5	Level-5 -100%	10.0272	166186
6	Level-6 -125%	12.5340	208205
7	Level-7 -150%	15.0408	247990
Correlation coefficient (r)			0.99997
Slope			16313.9913
Intercept			2867.1041
% Deviation of the Y-intercept			1.7

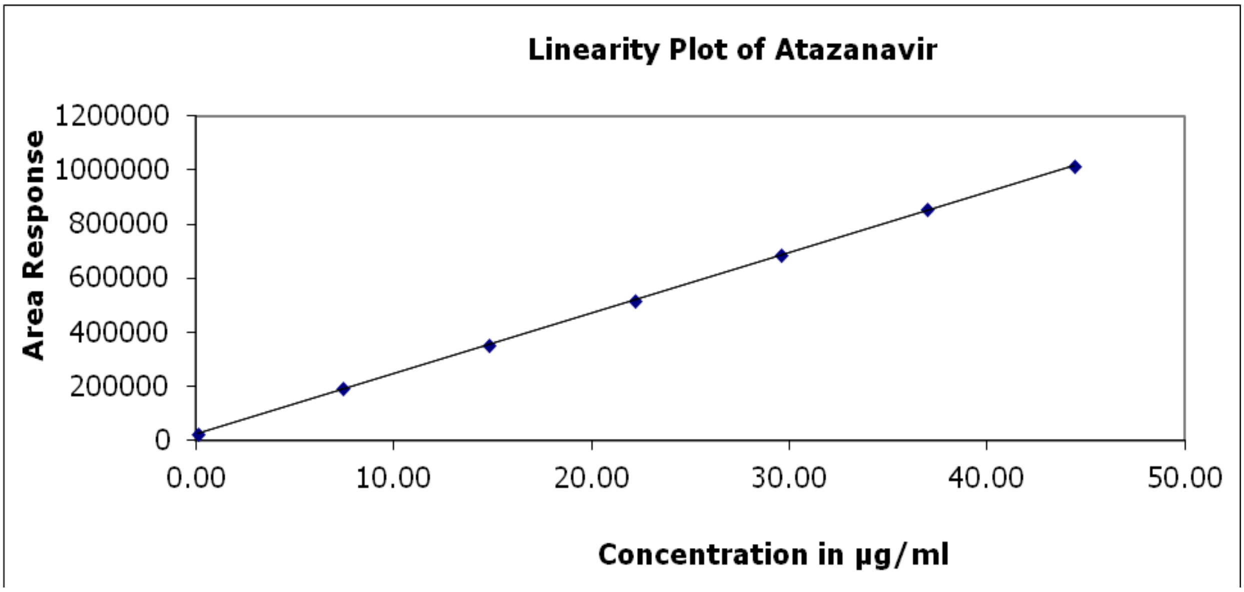
Linearity plot for Ritonavir



Linearity Results for Atazanavir

S. No.	Linearity level	Atazanavir Concentration (µg/mL)	Area (µV*sec)
1	Level-1 -LOQ	0.1041	24577
2	Level-2 -25%	7.3969	192991
3	Level-3 -50%	14.7939	354378
4	Level-4 -75%	22.1909	518812
5	Level-5 -100%	29.5879	685593
6	Level-6 -125%	36.9849	853044
7	Level-7 -150%	44.3819	1015310
		Correlation coefficient (r)	0.99998
		Slope	22356.9451
		Intercept	24218.6104
		% Deviation of the Y-intercept	3.5

Linearity plot for Atazanavir



6.Robustness

Following parameters were changed one by one and their effect was observed on system suitability for standard preparation.

1.Flow rate of Mobile Phase was changed (±0.2 ml/min) 0.7ml/min and 1.1ml/min.
2. Column oven Temperature was changed (± 5°C) 50°C and 60°C.
3. Detector wavelength was changed (± 2nm) 238nm and 242nm.
4. Mobile phase buffer pH was changed (± 0.2 pH) pH 3.1 and pH 3.5.
5. Mobile phase organic composition was changed (± 10% Acetonitrile).

The system suitability parameter was found to be within the acceptance criteria in all above conditions.

CONCLUSION

From the above discussion it can be concluded that the proposed method is specific, precise, accurate, linear and robust.Results are in good agreement which indicates there is no interference of excipients.Therefore the proposed method can be used for routine analysis of Atazanavir and Ritonavir drug product.

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