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Synthesis, Characterization and Development of Validated Analytical Methods for Process Related Impurity in Ritonavir Hydrochloride Bulk and Formulation

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Abstract

The synthesis, characterization and quantitation of process related impurity in bulk as well as tablet formulation was performed for Ritonavir i.e. chemically 1,3-thiazol-5-ylmethyl *N*-[(2*S*,3*S*,5*S*)-3-hydroxy-5-[[(2*S*)-3-methyl-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbamoyl]amino] butanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate. This synthesis includes Sulfanilic acid, Iso butyl amine and methanol as catalyst. After recrystallization of impurity, preliminary evaluation was done on lab scale including determination of Melting point, solubility. The melting point of synthesized impurity was found to be 230°C. The conformation of synthesized Ritonavir impurity was done using sophisticated instrument such as, UV, FT-IR, NMR and RP-HPLC method was developed to identify and quantify the Ritonavir impurity in bulk as well as formulation according to ICH Q2B guidelines. The developed RP-HPLC method was found to be linear, precise, accurate, robust and rugged. Based on RP-HPLC data of the Ritonavir sample after spiking with the impurity we conclude that impurity is present in Ritonavir. Finally 4-amino-N-(2-methyl propyl) benzene sulphonamide impurities were quantified from bulk Ritonavir Hydrochloride and its marketed tablet formulation. The amount of said impurity present in tablet was found to be 0.32 % while it was found to be 0.84%. in the bulk.

Key words: Ritonavir Hydrochloride, Validation, Impurity, RP-HPLC, FT-IR.

1.INTRODUCTION

Ritonavir is chemically, 1,3-thiazol-5-ylmethyl *N*-[(2*S*,3*S*,5*S*)-3-hydroxy-5-[[(2*S*)-3-methyl-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbamoyl]amino]butanoyl]amino]-1,6 diphenyl hexan-2-yl] carbamate (Figure No. 1). Ritonavir is an antiretroviral protease inhibitor that is widely used in combination with other protease inhibitors in the therapy and prevention of human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS) [1]. Ritonavir is indicated in combination with other antiretroviral agents for the treatment of HIV-1 infected patients (adults and children of 2 years of age and older) [2, 3, 4, 5].

Through the manufacturing process of an active pharmaceutical substance or product, some intermediates are formed [6]. These intermediates may affect the safety and efficacy issues of the pharmaceutical products [7, 8]. An impurity is defined by International Conference on Harmonization guidelines as Any component of the medicinal product which is not the chemical entity defined as the active substance or an excipient in the product though impurity profiling is considered to be the analytical activities with the help of aim which shows detection, identification and elucidation of the structure which quantitatively determines the impurities in bulk drug and pharmaceutical formulations. Impurities named differently by a number of groups of scientists who deal with them [9-14].

Efficient removal of process-related impurities or process residuals is important to pharmaceutical and biopharmaceutical development. Process impurities are related to the manufacturing process and constitute the chemicals, reagents and catalysts used during production process or intermediates and by-products formed during production process [15, 16]. 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 high performance liquid layer chromatography, UPLC-MS/MS, LC-MS/MS [17-25].

Isolation followed by characterization of impurities is required for acquiring and evaluating data that establishes biological safety which reveals the need and scope of impurity profiling of drugs in pharmaceutical research [26, 27]. Study was undertaken with the aim of development, optimization and validation of an efficient isocratic reversed-phase high-performance liquid chromatographic method to study the process related impurity of Ritonavir Hydrochloride bulk and formulation.

2.EXPERIMENTAL

2.1Materials and methods

Chemicals and reagents

Ritonavir Hydrochloride was obtained as gift sample from Cipla Pvt. Ltd., Mumbai. Methanol, Toluene, Hexane, Ethyl acetate, pyridine, Ammonia, Benzene, Ethyl aceto-acetate, Acetone and the HPLC grade solvents Acetonitrile, Methanol and Water all the chemicals are of AR grade were purchased from SM chemicals Pune.

2.2Instrumentation

2.2.1. UV- Visible Spectrophotometer

Labindia UV 3200 double beam spectrophotometer with 1 cm path length was used for spectral measurements.

2.2.2. High Performance Liquid Chromatography (HPLC)

The High Performance Liquid Chromatography (HPLC) of model 2080, Thermo system with PDA detector having Quaternary gradient system was used for analysis. CHEMSIL ODS-C18 (250 mm X 4.6 mm), 5µ column is used.

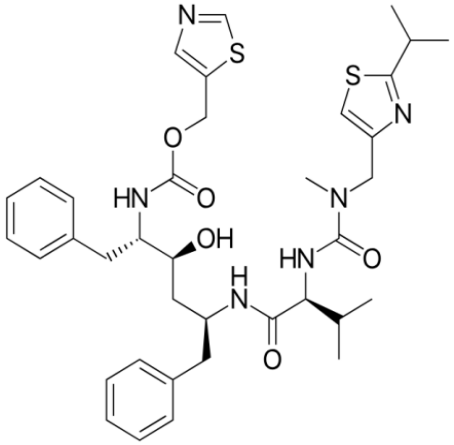


Figure No.1: Structure of Ritonavir hydrochloride

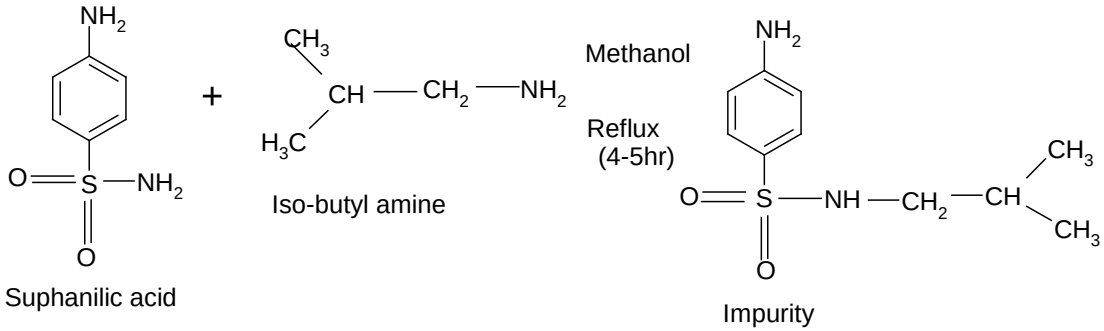


Figure no.2 Scheme for synthesis of 4-amino-N-(2-methyl propyl) benzene sulphonamides

2.2.3. Fourier Transforms Infrared Spectroscopy:

The Fourier transform Infrared Spectrophotometer Model No 8400s Shimadzu Inc. was used for the determination of the functional group of the synthesized impurity.

2.3.Experimental

2.3.1. Method for synthesis of impurity of Ritonavir Hydrochloride (4-amino-N-(2-methyl propyl) benzene sulphonamide)

The synthesis of 0.01 mole batch of 4-amino-N-(2-methyl propyl) benzene sulphonamide was carried out using key ingredients such as Sulfanilic acid, Iso butyl amine and Methanol. In this procedure take 4 gm of sulphanilic acid to this add 10 ml methanol and shake it vigoursly latter it was followed by addition of 4 ml Iso butyl amine .The reaction mixture was stirred and heated to reflux for 4-5 hours, and then cooled in ice bath. The precipitate was filtered to produce the compound. The product was washed with cold water then later purified by recrystallization and column chromatography by using methanol. Scheme for synthesis of 4-amino-N-(2-methyl propyl) benzene sulphonamides as shown in figure No.2.

2.3.2. Mobile phase preparation

The Methanol: Acetonitrile: Water was selected as mobile phase in ratio of 60: 20:20 and was filtered on membrane filter (0.45µ) to remove dissolve gases solvents were sonicated for 15-30 min.

2.3.3. Standard stock Preparation

Standard stock solution was prepared by dissolving accurately weighed 100 mg of Ritonavir Hydrochloride and Impurity in Methanol and the volume was made up to 100 ml with Methanol in 100 ml volumetric flask (Stock solution-I, 1000 mcg / ml).

2.3.4. Sample preparation of Ritonavir Hydrochloride

Twenty tablets were weighed and finely powdered. The powder equivalent to 100 mg of Ritonavir Hydrochloride was accurately weighed and transferred to volumetric flask of 100 ml capacity containing 50 ml of the Methanol and sonicated for 30 min. This solution was carefully filtered through Whatman filter paper and the final volume was made with Methanol to get the solution of 1000 mcg/ml.

Chromatographic condition

Column : CHEMSIL ODS-C18 (250 mm X 4.6 mm), 5µ column
Flow Rate : 1 ml/min
Injection Volume : 20 µL
ColumnTemperature : Ambient
Wavelength : 250 nm
Mobile phase : Methanol: Acetonitrile: Water (60:20:20)
Run time : 10 min

3. RESULT AND DISCUSSION

Physicochemical properties: Results for physicochemical property are enlisted in **Table No.1-3**

TABLE NO.1: PRELIMINARY TEST FOR BULK AND IMPURITY

Sr. No.	Substance	Characteristics	Observation
1.	Impurity	State	Solid
		Colour	Cream colour
		Nature	Crystalline
2.	Bulk	State	Solid
		Colour	White
		Nature	Amorphous powder

TABLE NO. 2: MELTING POINT OF SUBSTANCES

Sr. No.	Substance	Melting point
1.	Impurity	230ºC
2.	Bulk Ritonavir drug	132ºC

TABLE NO. 3: SOLUBILITY OF IMPURITY

Sr. No.	Solvents	Solubility	Part of solute soluble per part of solvent
	Methanol	Very soluble	10 mg in 1 ml
	Ethanol	Slightly soluble	1 mg in 10 ml
	Water	Very soluble	10 mg in 1 ml
	Acetone	Insoluble	< 100 ml
	Acetonitrile	Very slightly soluble	1 mg in 100 ml

3.1. Preliminary tests

3.1.1. Melting point

The melting point of Ritonavir hydrochloride and impurity were determined.

3.1.2. Solubility

The solubility data was used for UV method development and selection of mobile phase for RP-HPLC method.

3.2. Spectroscopic study

3.2.1. UV Spectrophotometric determination

The λmax for Ritonavir impurity was found to be 250nm. (Figure No.3) Calibration curve data was constructed in the range of 2-14 µg/ml. Beer's law was obeyed over this concentration range. The correlation coefficient (R2) was found to be 0.994. The regression equation Y= 0.059x + 0.035 was found to be linear.

3.2.2. IR Data

IR spectra obtained for Impurity is shown in **Figure No. 4**

IR (KBr) cm^{-1}: 3565 (N-H stretching), 3445 (N-H stretching), 3162 (Aromatic stretch), 3038 (CH_3 stretch), 2920 (CH_2 stretch), 2850 (CH stretch), 1650 (NH bend), 1453 (CH bend), 1377 (CH_3 bend), 1318 (S=O sulphonamide), 1028 (S=O sulfoxides), 695 (Aromatic out of plane bend).

3.3. RP-HPLC Method Development

Liquid Chromatographic (LC) method was developed for the separation of possible related substances of Ritonavir, specifically for impurity in Ritonavir. The RP-HPLC method was developed by using Thermo 2080 system. The PDA detector and C-18 column with dimension on 25 x 0.46 cm was used for the method development. CHROMQUEST chromatographic software was used for data acquisition. With flow rate 1.0ml/ min at wavelength 250 nm. The methanol: acetonitrile: water in proportion of (60:20:20) as a mobile phase was selected for development and validation of method for synthesized impurities and various parameters according to ICH guidelines (Q2B). HPLC chromatograms are shown in **Figure No.5-9**

TABLE NO. 4: OBSERVATIONS FOR PRECISION BY UV

Sr. No.	Concentration (ppm)	Absorbance at 250nm	Intraday After 4 hrs	Interday After 24 hrs
1.	6	0.382	0.384	0.383
2.	6	0.383	0.383	0.385
3.	6	0.381	0.385	0.381
4.	6	0.386	0.386	0.386
5.	6	0.385	0.383	0.382
6.	6	0.383	0.384	0.383
	Mean	**0.383**	**0.3843**	**0.383**
	SD	**0.00184**	**0.00179**	**0.0018**
	% RSD	**0.5075**	**0.4834**	**0.4953**
	Max. limit for %RSD	Below 2.0%	Below 2.0%	Below 2.0%

TABLE NO.5: OBSERVATIONS FOR PRECISION BY RP-HPLC

Sr.No.	Concentration(ppm)	Area (mv) at 250nm	Intraday After 6 hrs	Interday After 24 hrs
1.	1	226.066	233.656	227.775
2.	1	233.252	233.228	233.680
3.	1	228.832	230.832	229.560
4.	1	230.132	232.324	231.862
	Mean	**229.57**	**232.51**	**230.71**
	SD	**2.97**	**2.24**	**2.18**
	% RSD	**1.29**	**1.23**	**1.19**
	Max. limit for %RSD	Below 2.0%	Below 2.0%	Below 2.0%

TABLE NO. 6: RESULT OF RECOVERY STUDY BY UV

Sr. No.	Drug / Formulation	Percentage recovery			Mean	S. D.	%RSD
		20%	50%	100 %			
1.	Bulk	97.91	98.72	96.97	97.86	0.85	0.86
2.	Tablet	97.65	97.88	98.58	98.03	0.86	0.87

TABLE NO.7: RESULT OF RECOVERY STUDY BY RP-HPLC

Sr. No.	Drug / Formulation	Percentage recovery			Mean	S. D.	%RSD
		20%	50%	100 %			
1.	Bulk	92.62	91.82	93.58	92.67	1.22	1.32
2.	Tablet	93.75	91.69	92.87	92.77	1.42	1.54

TABLE NO. 8: RESULT OF ROBUSTNESS STUDY BY CHANGE IN SOLVENT I AND II BY UV

Sr.No.	Concentration (ppm)	Absorbance at 250nm	
		I	II
1.	6	0.384	0.385
2.	6	0.382	0.383
3.	6	0.385	0384
4.	6	0.384	0.385
	Mean	0.383	0.384
	S.D.	0.0024	0.0021
	%RSD	0.60	0.53
	Max limit for % RSD	below 2.0%	below 2.0%

TABLE NO. 9: RESULT OF ROBUSTNESS STUDY BY CHANGE IN SOLVENTS I AND II BY RP-HPLC

Sr.No.	Concentration (ppm)	Area(mv) at 250nm
1.	1	229.556
2.	1	230.690
3.	1	232.703
4.	1	234.162
5.	1	231.730
	Mean	231.76
	S.D.	1.77
	%RSD	0.76
	Max limit for % RSD	below 2.0%

TABLE NO. 10: RESULT OF RUGGEDNESS STUDY BY CHANGE IN ANALYST I AND II BY UV METHOD

Sr. No.	Conc. (ppm)	Absorbance at 250 nm	
		I	II
1.	6	0.385	0.384
2.	6	0.386	0.385
3.	6	0.384	0.383
	Mean	0.385	0.384
	S.D	0.0014	0.00172
	%RSD	0.4182	0.4511
	Max limit for % RSD	below 2.0%	below 2.0%

TABLE NO. 11: RESULT OF RUGGEDNESS STUDY BY CHANGE IN ANALYST I AND II BY RP-HPLC METHOD.

Sr.No.	Conc. (ppm)	Area (mv) at 250 nm	
		I	II
1.	1	229.56	229.620
2.	1	230.412	230.52
3.	1	228.94	228.632
4.	1	232.55	231.490
	Mean	230.36	230.06
	S.D	1.57	1.22
	%RSD	0.68	0.530
	Max limit for % RSD	below 2.0%	below 2.0%

TABLE NO. 12: SYSTEM SUITABILITY PARAMETERS OF IMPURITY

Property	Values	Required limits
Retention time (t_R)	1.847	RSD ≤ 1%
Theoretical plates (N)	3513	N ≥ 2000
Tailing factor (T)	0.98	T ≤ 2
Resolutions (R)	2.040	R ≥ 2

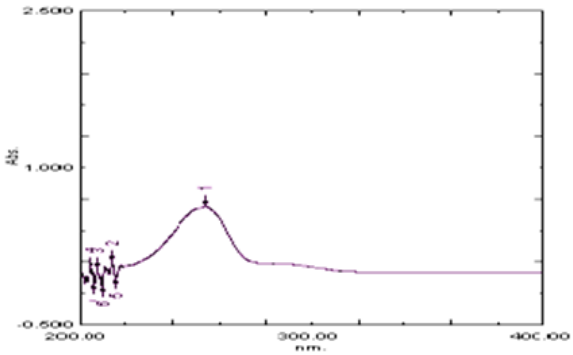


Figure No.3: UV spectrum of impurity

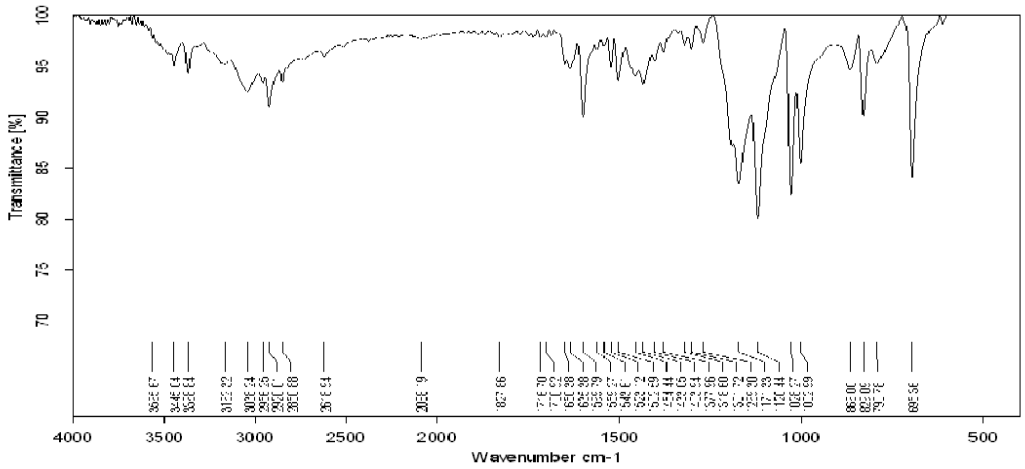
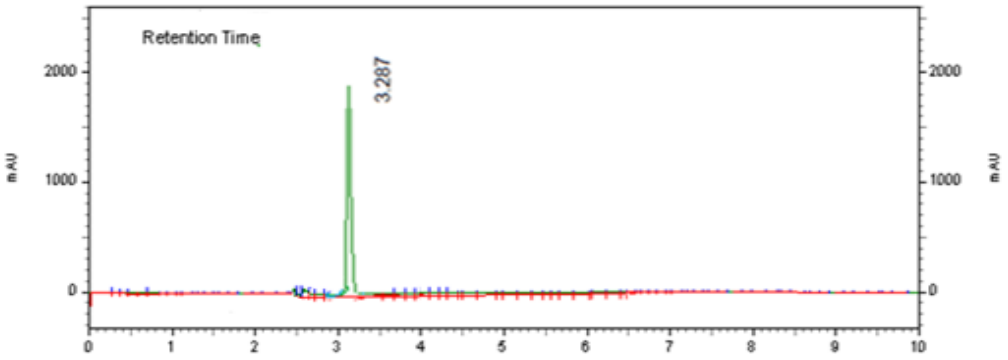
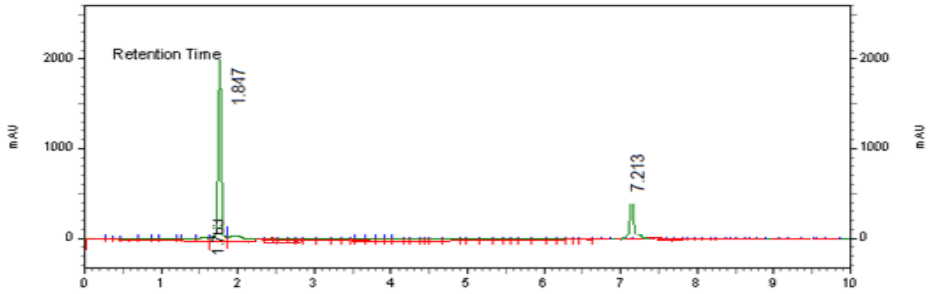


Figure No. 4: IR spectrum of impurity



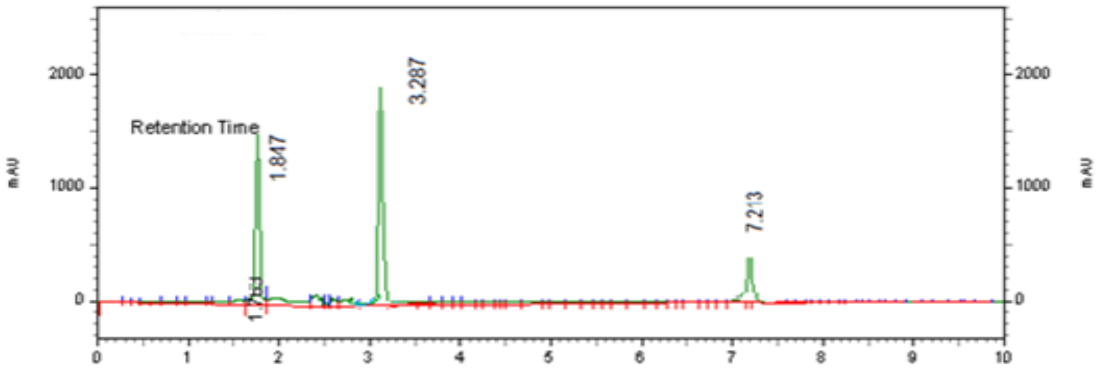
Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
3.287	214.815	84.567	96.9	98.3	0.07

Figure No. 5: HPLC chromatogram of drug Ritonavir Hydrochloride



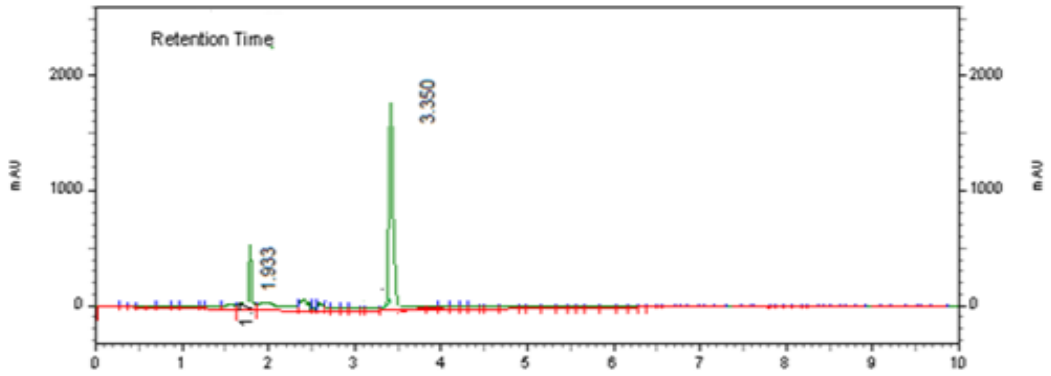
Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1.847	394.595	84.567	96.9	98.3	0.07
7.213	4.815	0.576	1.2	0.7	0.09

Figure No. 6: HPLC chromatogram of synthesized impurity



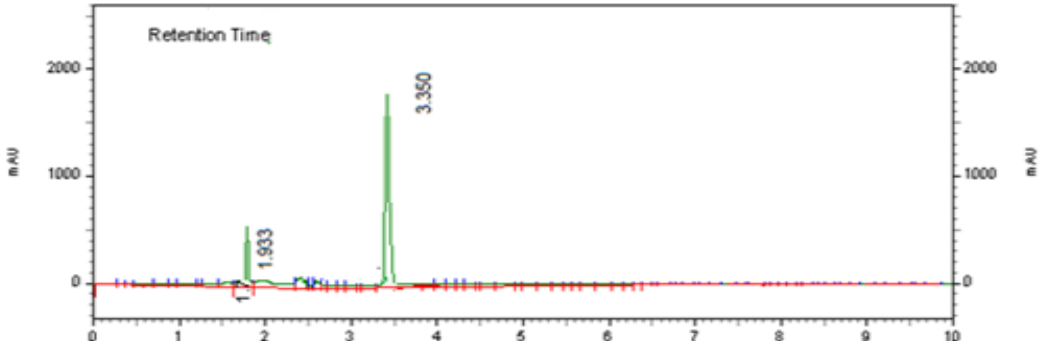
Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1.847	94.595	31.88	64.8	63.4	0.09
3.287	214.815	84.567	96.9	98.3	0.07
7.213	4.815	0.576	1.2	0.7	0.09

Figure No. 7: HPLC chromatogram of Ritonavir Hydrochloride and impurity mixture



Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1.933	3.845	0.730	7.1	7.6	0.08
3.350	94.595	8.734	90.8	90.6	0.09

Figure No.8: HPLC chromatogram of Ritonavir Tablet



Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1.933	3.845	0.730	7.1	7.6	0.08
3.350	94.595	8.734	90.8	90.6	0.09

Figure No. 9: HPLC chromatogram of Ritonavir tablet and impurity mixture

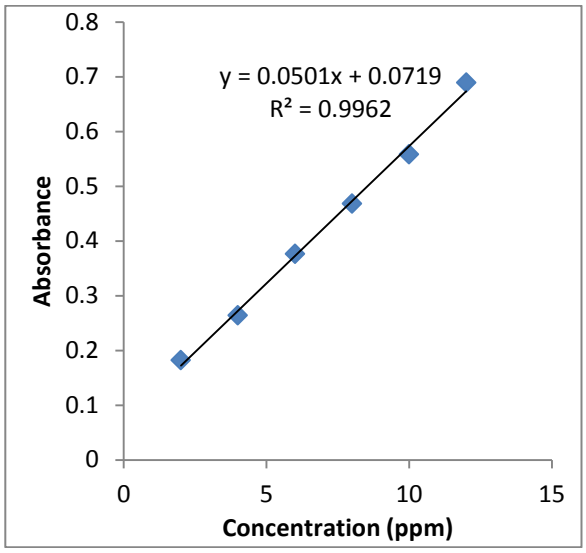


Figure No.10: Graph of linearity of impurity by UV

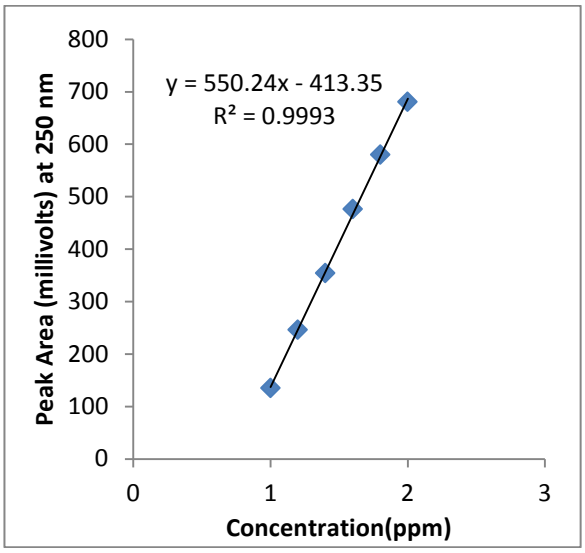


Figure No.10: Linearity graph for impurity by RP-HPLC

3.4. Method Validation

Validation of optimized method was performed to demonstrate system suitability, linearity, precision, accuracy study, ruggedness and robustness as per ICH Q2B guidelines. System-suitability parameters are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (t_R), number of theoretical plates (N) and tailing factor (T) were evaluated for six replicate injections of the drug at a concentration of 100 mcg/ml.

3.4.1. Linearity

The calibration curve for impurity of Ritonavir Hydrochloride was constructed over the absolute concentration range of 2-14 µg/ml. Concentration versus peak area was plotted and regression coefficient was found to be 0.9962 by UV method and 0.9993 by RP-HPLC method. Linearity graph for impurity by UV and RP-HPLC are shown in **Figure No. 10 and 11** respectively.

3.4.2. Precision

Precision was carried out for Inter and Intraday analysis for tablet dosage form. Precision was evaluated by carrying out six independent sample preparations of a single lot of formulation. Precision of the developed method was demonstrated by intra-day and inter-day variation studies. Results obtained for precision study by UV and RP-HPLC are shown in **Table No.4 and 5** respectively. The intra and inter-day precision was carry out and difference in % RSD was found not much vary so it can concluded that method should precise.

3.4.3. Accuracy

Recovery study for the developed method was carried out at three different levels i.e. 20 %, 50% and 100% by adding standard drug solution to the sample solution. Results obtained for recovery study by UV and RP-HPLC are shown in **Table No. 6 and 7** respectively. The % recovery was calculated and found to be within the range.

3.4.4. Robustness

To evaluate the robustness of the developed method, small deliberate variations in optimized method parameters were done. The effect of change in solvent was studied. Results obtained by UV and RP-HPLC determination are shown in **Table No. 8 and 9** respectively. The method was unaffected by changes in solvent. % RSD was found to be 0.60 and 0.53 for solvent I and II by UV method. % RSD was found to be 0.76 and 0.73 for solvent I and II by RP-HPLC method

3.4.5. Ruggedness

Change in analyst I and II:

Result for ruggedness study by UV and RP-HPLC are shown in **Table No. 10 and 11** respectively. % RSD was found to be 0.4182 and 0.4511 for analyst I and II by UV method. % RSD was found to be 0.68 and 0.530 for analyst I and II by RP-HPLC method.

3.4.6. Limit of Quantification and Limit of Detection

Limit of detection (LOD) and Limit of quantification (LOQ) for Ritonavir were found to be 0.04953 µg/ml and 0.1501 µg/ml respectively by UV method and LOD 0.0261 and LOQ 0.079 µg/ml respectively by RP-HPLC method.

3.4.7. System Suitability Parameters

To verify that analytical system is working properly and can give accurate and precise results the system suitability parameters were set. Different system suitability parameters are shown in **Table No. 12** and system suitability parameters were found to be in the range.

3.5. Quantitation of impurity

The objective of this study was to develop a rapid and sensitive RP-HPLC method for the process related impurity in Ritonavir Hydrochloride bulk and formulation. By using most commonly employed RP C 18 column with UV detection and isocratic mode of operation. Finally 4-amino-N-(2-methyl propyl) benzene sulphonamide impurity was quantified from bulk Ritonavir Hydrochloride and its marketed tablet formulation. It was revealed that the amount of impurity present in tablet was found to be 0.32 % and in the bulk was found to be 0.84%.

4. CONCLUSION

Nowadays, it is mandatory requirements in various Pharmacopoeias to know the impurities present in API's. Isolation along with characterization for any process related impurity is necessary for acquiring and evaluating data that establishes biological safety which reveals the need and scope of impurity profiling of drugs in pharmaceutical research. A competent isocratic reversed-phase high-performance liquid chromatographic method was developed, optimized and validated to study the process related impurity of Ritonavir Hydrochloride bulk and formulation. The developed method was found to be specific, sensitive, linear, precise and accurate. The amount of impurity present in tablet was found to be 0.32 % and in the bulk was found to be 0.84%.

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