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Development and Validation of a New Stability Indicating Rp-Hplc Method for Simultaneous Estimation of Ritonavir and Lopinavir

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ABSTRACT:
A new stability indicating RP HPLC method has been developed and validated for simultaneous estimation of Ritonavir and Lopinavirin bulk and dosage forms. The method involves separation on Xterra C18column (250mm x 4.6mm x5µm particle size). The optimized mobile phase consists of 0.1% OPA (pH 2.8) and Methanol (45:55v/v) with a flow rate of 1ml/min and UV detection at 229mn. Retention time was 2.78min (Ritonavir), 3.71min (Lopinavir). Linearity range was 20-100 ug/ml (Ritonavir), 60-300 ug/ml (Lopinavir). Accuracy was in the range of 96.92-100.35% for both drugs. Precision was 0.8% and 0.5% for Ritonavirand Lopinavir. LOD and LOQ are 0.42ug/ml and 1.38 ug/ml for Ritonavir, 0.27ug/ml and 0.89ug/ml for Lopinavir. The method developed is more sensitive, accurate and precise than the methods reported earlier. Retention time and run time were also less and hence the method is economical. When applied for tablet assay, drug content was within 98.55-101.4 % of labelled content. Forced degradation studies indicated thesuitability of the method for stability studies.

KEY WORDS:
Ritonavir and Lopinavir, RP-HPLC Method, Simultaneous estimation, Validation, Forced degradation studies.

I. INTRODUCTION:

Ritonavir (trade name Norvir) is an antiretroviral drug used to treat HIV infection and AIDS. Ritonavir is a protease inhibitor class and it inhibits the same host enzyme that metabolizes other protease inhibitors. This inhibition of the proteases results in increased plasma concentrations of these drugs, thus allowing the clinician to lower their dose and frequency and improving their clinical efficacy. So the simultaneous determination with the other HIV protease inhibitors like lopinavir been shown to be effective against drug-resistant HIV. These drugs are metabolized by cytochrome P-450 (CYP) 3A in the liver. When Lopinavir is administered with Ritonavir as kaletra, ritonavir inhibits the CYP 3A- mediated metabolism of Lopinavir, thereby providing increased plasma levels of Lopinavir [1-3]. Lopinavir is known as N-[4-[[(2,6 dimethylphenoxy) acetyl] amino]-3-hydroxy-5-phenyl-1-(phenylmethyl) pentyl] tetrahydro-alpha-(1methylethyl)-2-oxo 1(2H) pyrrolidine acetamide. Ritonavir is known as 10-hydroxy-2-methyl-5-(1-methylethyl)-1- [2-(1-methylethyl)-4thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12 tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester. Both are freely soluble in methanol and ethanol, isopropanol and practically insoluble in water. A survey of literature reveals that there are two methods reported for the simultaneous determination of lopinavir and ritonavir in pharmaceutical preparations using HPLC [1,4-6]. There are several analytical methods have been reported for the assay of Lopinavir and Ritonavir individually or combination with other drugs in biological samples as well as formulations [7-9]. Simultaneous determinations of Lopinavir and Ritonavir dosage form were also reported by using HPLC, LC-MS, HPTLC and UV Spectroscopy. So our aim is to develop a new rapid and sensitive RP-HPLC of simultaneous determination and to perform the validation as per ICHguidelines [10-17].

II. MATERIALS AND METHODS:

Materials and reagents:

HPLC water (Lichrosolv^{R}Merck Lifesciences Pvt.Ltd., Mumbai, India) Ortho phosphoric acid (Thermo Fischer Scientific Pvt Ltd., Mumbai, India)were used in the study. The working standards of Ritonavir and Lopinavirwere generous gift obtained from Pharma Train Pvt Ltd.,Hyderabad, India. Duraplustablet containing

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Ritonavir 10mg and Lopinavir 30mg was kindly supplied by Sun pharmaceutical IndustriesLtd.

Instrumentation:

Chromatography was performed on a WATERS 2695 HPLC column (waters corporation, Mildord, USA) with an autosampler and equipped with a 2996 series of PDA detector with a spectral bandpass of 1.2nm. Components were detected using UV and that processing was achieved by Empower 2 software. A hot air oven was used for thermal degradation of the samples and a UV crossinker, with series of 23400 model UV chamber, equipped with a UV fluorescence lamp with the wavelength range between 200 & 300nm was selected for photolytic degradation. Ultrasonic bath (Toshcon by Toshniwal), digital Ph meter(Systronics model 802) were used in the study.

Chromatography conditions:

The chromatographic conditions was performed on XTerra C_{18} column (250 X 4.6mm,5µm particle size) at an ambient column temperature. The samples were eluted using 0.1%Orthophosphoric acid (pH adjusted to2.8):Methanol(45:55v/v) as the mobile phase at a flow rate of 1 ml/min and samples were degassed by ultra sonication for 20 min and filtered through 0.45µm Nylon(N66)47mm membrane filter. The measurements were carried out with an injection volume of 10µL, flow rate was set to 1.0 mL/min, and UV detection was carried out at 229nm. All determinations were done at ambient column temperature (30°C). The chromatograms of the prepared standard stock solutions of Ritonavir and Lopinavirwere recorded under optimized chromatographic conditions (**Fig. 1**).

Diluent

Buffer and Methanol in 50:50 v/v ratio.

Preparation of Standard Solutions:

Stock standard solution:

Standard stock solution of Ritonavirand Lopinavirwere prepared by dissolving 20 mg of Ritonavirand 60mg of Lopinavirin 10ml of diluent (Buffer: Methanol, 50:50v/v) in a 10ml clean dry volumetric flask and the standard solutions was filtered through 0.45µm nylon membrane filter and degassed by sonicator to get the concentration of 2000µg/ml of Ritonavir and 6000µg/ml of Lopinavir. The above standard stock solution suitably diluted with diluents to obtain working concentration drugs.

Working Standard Solution:

Working standard solution of Ritonavir and Lopinavirwere prepared by taking 0.3ml of stock solutions of Ritonavir and Lopinavirin to clean dry 10ml volumetric flask and make up volume with diluent to get a concentration of 60µg/ml of Ritonavir and 180µg/ml of Lopinavir.

Preparation of Sample Solutions of Ritonavir and Lopinavir:

Twenty tablets were accurately weighed and powdered and tablet powder equivalent to 20 mg of Ritonavirand 60 mg of Lopinavir was taken into 10 ml clean dry volumetric flask, diluent was added and sonicated to dissolve completely and volume was made up to volume with the diluent. The above sample solution suitably diluted to get a concentration of 60µg/ml of Ritonavir and 180µg/ml of Lopinavir.

III. RESULTS AND DISCUSSION:

Optimization of chromatographic conditions:

Ritonavir and Lopinavirweresoluble in polar solvent, so the developed method of estimation was carried out on reverse phase high performance liquid chromatography. To develop a rugged and suitable Preliminary trials were taken with different composition of buffer and organic phase of mobile phases with pH range of 2.5–5. After evaluating all these factors, a XTerra C_{18} column was found to be giving satisfactory results. The selection of methanol and buffer were based on chemical structure of both the drugs. Best results were obtained with 0.1% O-phosphoric acid pH adjusted to 2.8 with sodium hydroxide solution that improved the peak shapes of Ritonavir and Lopinavir. Mobile phase composition consisting of a mixture of buffer-pH 2.8 (0.1% Ortho phosphoric acid): Methanol (45:55v/v).Flow rates between 0.5 to1.2ml/min were tried. Flow rate of 1ml/min was observed to be enough to get both the drugs eluted within less than 10min. Under above described experimental conditions, all the peaks were well defined and free from tailing. The concern of small deliberate changes in the mobile phase composition, flow rates, and column temperature on results were evaluated as a part of testing for methods robustness.

Validation of Method Developed:

The proposed method was validated according to the ICH guidelines[12]for the following parameters

System suitability:

The Retention time of Ritonavir and Lopinavirusing optimum conditions was 2.78min and 3.72min respectively. For two of them, the peak symmetries were <1.5 and the theoretical plates numbers were >2000 and %RSD of areas of six standard injections of Ritonavir and Lopinavir

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was less than 2. These values are within the acceptable range of United States pharmacopoeia definition and the chromatographic conditions. The results obtained are shown in **Table 1.**

Table 1:System suitability results of Ritonavirand Lopinavir

Parameter	Ritonavir	Lopinavir
Peak area	188339(0.46%)*	1092032(0.14%)*
Theoretical plates	2675.81±0.302	3626.04±0.258
Retention time	2.789±0.031	3.726±0.057
Tailing factor	1.10±0.07	1.27±0.05

*RSD (%)

Specificity:

The specificity of the method was evaluated by assessing interference from excipients in the pharmaceutical dosage form prepared as a placebo solution. Optimized chromatogram ofRitonavir and Lopinaviris shown in **Fig. 1** clearly shows the ability of the method to assess the analyte in the presence of other excipients.

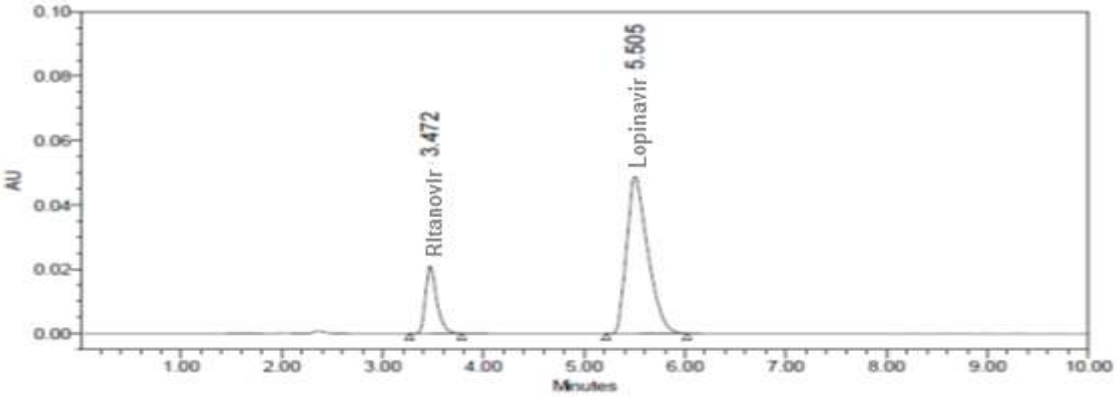

Fig. 1: Optimized Chromatogram of Ritonavir and Lopinavir

Linearity and Range:

Linearity was assessed for the two oral anti diabetic drugs at concentration ranges 20-100µg/ml for Ritonavirand 60-300µg/ml for Lopinavir. A linear relationship was established at these ranges between Area under the peak (AUP) and concentration. Good linearity was proved by high values of coefficient of determinations(**Fig.2 and Fig.3**). The results were tabulated in **Table 2**

TABLE 2: Linearity data of Ritonavir and Lopinavir

Level	Concentration of Ritonavir (µg/ml)	Peak area	Concentration of Lopinavir(µg/ml)	Peak area
1	20	61652	60	352575
2	40	129447	120	713850
3	60	189375	180	1082534
4	80	244442	240	1408995
5	100	309206	300	1792376

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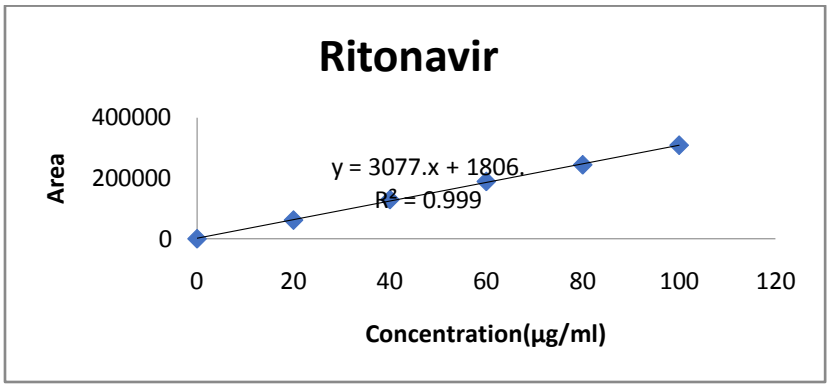

Fig.2. Linearity graph of Ritonavir

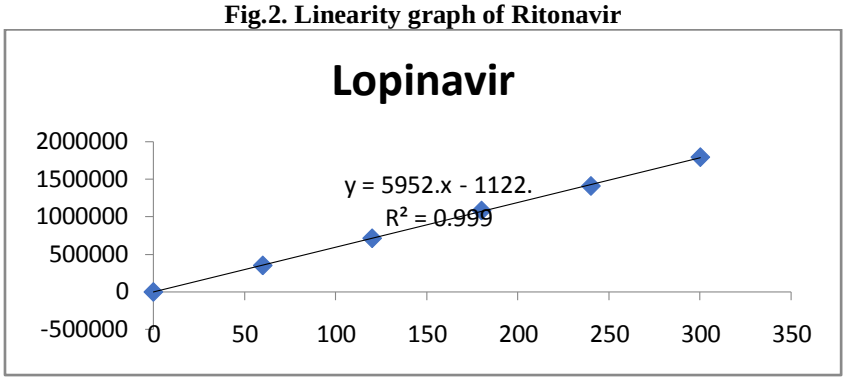

Fig.3. Linearity graph of Lopinavir

Limit of Detection (LOD)/Limit of Quantitation (LOQ):

The LOD was determined on the basis of signal to noise ratios and was determined using analytical response of three times the background noise. LOQ was determined as the lowest amount of analyte that was reproducibly quantified above the baseline noise following triplicate injections. Both LOQ and LOD were calculated on the peak area using the following equations:

LOQ= 10 x N/ B

LOD= 3 x N/ B

The limit of detection and limit of quantification were evaluated by serial dilutions of Ritonavir and Lopinavir stock solution in order to obtain signal to noise ratio of 3:1 for LOD and 10:1 for LOQ. The LOD value for Ritonavir and Lopinavir was found to be 0.42µg/mL and 0.27µg/mL, respectively, and the LOQ value 1.386µg/mL and 0.89µg/mL, respectively.

Precision:

System Precision:

System Precision was carried to ensure analytical system is working properly. One dilution of both the drugs in six replicates was injected into HPLC system & was analyzed and the results were found within the acceptance limits (RSD<2).

Method Precision(Repeatability):

Precision is expressed as the closeness of agreement between a series of measurements obtaining from multiple sampling of the same homogeneous sample. Six replicate injections of a known concentration of sample preparation of Ritonavir (60 µg/mL) and Lopinavir(180 µg/mL) have been analyzed by injecting them into a HPLC column on the same day and on consecutive days. The results of precision are given in **Table 4** .

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Table 4: Method Precision data for Ritonavirand Lopinavir

Ritonavir				**Lopinavir**		
S. No	Concentration (µg/ml)	Peak Area	% Assay	Concentration (µg/ml)	Peak Area	% Assay
1	60	186186	98.8	180	1055440	100.22
2	60	184635	101.6	180	1060313	98.04
3	60	188760	98.4	180	1050116	100.29
4	60	188378	101.2	180	1061756	101.20
5	60	189416	100.9	180	1076488	101.24
6	60	191859	98.6	180	1095045	99.05
Average		188205.7	99.9	Average	1066526.3	100
SD		2528.9	1.5	SD	16529.5	1.3
%RSD		1.3	1.5	%RSD	1.5	1.3

Accuracy:

The percentage recovery was calculated by preparing standard drug concentrations of Ritonavir and Lopinavirwith concentration levels of 50%, 100% and 150%. Good recovery of the spiked drugs was obtained at each added concentration, and the mean percentage recovery of Ritonavir and Lopinavirwas achieved between 97.44-100.35 ± 0.753% and 96.92-100.23±0.327.The results are given in **Tables 5,6**.

Table 5: Recovery data of Ritonavir

Sample name	**Amount added(µg/ml)**	**Amount found (µg/ml)**	**%Recovery**	**Statistical Analysis**
S1:50%	30	29.6	98.66	Mean=97.44
S2:50%	30	29.2	97.33	S.D=1.16
S3:50%	30	28.9	96.33	%RSD=1.1
S4:100%	60	60.04	100.06	Mean=99.76
S5:100%	60	59.42	99.03	S.D=0.63
S6:100%	60	60.12	100.2	%RSD=0.6
S7:150%	90	90.54	100.6	Mean=100.35
S8:150%	90	90.28	100.311	S.D=0.22
S9:150%	90	90.14	100.15	%RSD=0.2

Table 6: Recovery data of Lopinavir

Sample name	**Amount added(µg/ml)**	**Amount found (µg/ml)**	**%Recovery**	**Statistical Analysis**
S1:50%	90	90.43	100.47	Mean=96.92
S2:50%	90	90.26	100.28	S.D=1.97
S3:50%	90	89.12	90.02	%RSD=1.9
S4:100%	180	180.34	100.18	Mean=100.23
S5:100%	180	180.17	100.09	S.D=0.17
S6:100%	180	180.77	100.42	%RSD=1.7
S7:150%	270	270.11	100.04	Mean=100.12
S8:150%	270	270.30	100.11	S.D=0.09
S9:150%	270	270.63	100.23	%RSD=0.1

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Robustness:

Robustness of the proposed analytical method is a measure of its capacity to remain unaffected, and it reflects the reliability of the analysis with respect to deliberate changes in the parameters such as flow rate (1.0 ± 0.2 mL), column temperature (30 ± 5°C), mobile phase ratio of the mobile phase. The result shown that during all variance conditions, assay value of the test preparation solution was not affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory; hence the analytical method would be concluded as robust.

IV. CONCLUSION:

RP-HPLCmethodforthesimultaneousestimationofRitonavir and LopinavirintheircombinedosageformwasestablishedandvalidatedaspertheICHguidelines.Linearitywasachievedfor Ritonavir and Lopinavirintherangeof 20-100µg/mlforRitonavirand60-300µg/mlforLopinavirwithcorrelationcoefficients(r^2=0.999).ThepercentagerecoveriesofRitonavirandLopinavirwereachievedintherangeof96.92-100.35%.whichwaswithintheacceptancecriteria. ThepercentageRSDwasNMT2%whichprovedtheprecisionofthedevelopedmethod.Thedevelopedmethodissimple,sensitive,rapid,linear,precise,rugged,accurate,specific,androbust.

V. ACKNOWLEDGMENTS:

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REFERENCES:

[1]. Hatzimouratidis K, Amar E, Eardley I, Giuliano F, Hatzichristou D, Montorsi F, Vardi Y, Wespes E. Guidelines on male sexual dysfunction: erectile dysfunction and premature ejaculation. European urology.57(5);2010:804-14.

[2]. Huang SA, Lie JD. Phosphodiesterase-5 (PDE5) inhibitors in the management of erectile dysfunction. Pharmacy and therapeutics. 38(7);2013:407.

[3]. Zhang XH, Morelli A, Luconi M, Vignozzi L, Filippi S, Marini M, Vannelli GB, Mancina R, Forti G, Maggi M. Testosterone regulates PDE5 expression and in vivo responsiveness to Ritonavir in rat corpus cavernosum. European urology. 47(3);2005:409-16.

[4]. Kendirci M, Salem E, Hellstrom WJ. Lopinavir, a novel selective serotonin transport inhibitor for the treatment of premature ejaculation. Therapeutics and clinical risk management. 3(2); 2007:277.

[5]. Attia AM, Abo-elmaaty SM. Evaluation of Lopinavir hydrochloride in treatment of primary premature ejaculation. Menoufia Medical Journal. 29(2); 2016:393.

[6]. Unnisa A, Babu Y, Suggu SK, Chaitanya S. RP-HPLC-PDA method development and validation for the analysis of Ritonavir in bulk, pharmaceutical dosage forms and in-vitro dissolution samples. Journal of Applied Pharmaceutical Science. 4; 2014:072-6.

[7]. Raul SK, Kumar BV, Pattnaik AK. A RP-HPLC Method Development and Validation for the Estimation of Ritonavir in Bulk and Pharmaceutical Dosage Forms. Research Journal of Pharmacy and Technology.5(12);2012:1573-6.

[8]. Rezaei M, Ramazani A. A novel validated method for the determination of Lopinavir HCl by RP-HPLC in bulk and tablet dosage forms. Current Pharmaceutical Analysis.14(6);2018:622-6.

[9]. Patil RB, Deshmukh TA, Patil VR. Stability indicating HPLC method for Lopinavir HCl in bulk and in formulation. Int. J. Pharm. Pharm. Sci.6;2014:687-90.

[10]. Crack GP. Development and Validation of Stability Indicating RP-HPLC Method for Estimation of Ritonavir and Lopinavir HCL in Solid Dosage Form.

[11]. Rajeshwari M, Chenthilnathan A, Rama K. Validated RP-HPLC method for simultaneous estimation of Ritonavir and Lopinavir hydrochloride in combined pharmaceutical dosage forms. Int. J. Pharm. Biol. Sci.4;2014:72-82.

[12]. Validation of Analytical Proceudures , ICH Harmonised Tripatite Guidelines ,Q_2 B 1997.

[13]. Ray JE, Marriott D, Bloch MT, McLachlan AJ. Therapeutic drug monitoring of atazanavir: surveillance of pharmacotherapy in the clinic. Br J Clin Pharmacol. 2005;60(3):291-9

[14]. Sulebhavikar AV, Pawar UD, Mangoankar KV, Prabhunavelkar ND. HPTLC Method for Simultaneous Determination of Lopinavir and Ritonavir in Capsule Dosage Form. E J Chem. 2008;5(4):706-12.

[15]. Ponnilavarasan, Rajasekaran A, Dharuman JG, Kalaiyarasi D, Senthilkumar M. RP-HPLC method for simultaneous estimation of antiretrovira l drugs lopinavir and ritonavir in tablet dosage form. Dig J NanomaterBiostruct. 2010;5(3):771-78.
[16]. John R, Edna P, Dianne C. Simultaneous determination of indinavir, ritonavir and lopinavir (ABT 378) in human plasma by high performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 2002;775:225- 30.
[17]. Phechkrajang CM, Thin EE, Sratthaphut L, Nacapricha D, Wilairat. Quantitative Determination of Lopinavir and Ritonavir in Syrup Preparation by Liquid Chromatography. Mahiuniv J Pharm Sci. 2009;36(1- 4):1-12.
[18]. Dias CL, Bergold AM, Froehlich PE. UV Derivative Spectrophotometric determination of Ritonavir Capsules and Comparison with LC Method. Anal Lett. 2009;42(12): 1900-10.
[19]. Suneetha A, Kathirvel S, Ramachandrika G. A validated RP-HPLC method for simultaneous estimation of lopinavir and ritonavir in combined dosage form. Int J Pharm Pharm Sci. 2011;3(1):49-51.
[20]. Nagulwar VP, Bhusari KP. Simultaneous Estimation of Ritonavir and Lopinavir by Absorption ratio (Q-analysis) UV Spectrophotometric Method in Combined Tablet Dosage Form. Der Pharmacia Lettre. 2010;2(1):196-200.
[21]. Validation of Analytical Procedures: Text and Methodology (Q2-R1). Geneva: ICH Guidelines, Switzerland; 2005.