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Der Pharma Chemica, 2011, 3(2): 127-134
(http://derpharmachemica.com/archive.html)

ISSN 0975-413X
CODEN (USA): PCHHAX

Development and Validation of RP-HPLC and HPTLC methods for Estimation of Ritonavir in bulk and in pharmaceutical formulation

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ABSTRACT

This research paper describes validated reverse phase high performance chromatography (HPLC) and high performance thin layer liquid chromatography (HPTLC) methods for the estimation of Ritonavir in bulk and in pharmaceutical formulation. The RP-HPLC separation was achieved on Eclipse XBD (C_{18}) RP column (150mmX 4.6mm id, 15µparticle size) using acetonitrile: water (60:40%v/v) as the mobile phase at a flow rate of 1.4ml/min at an ambient temperature. Quantification was achieved with ultraviolet (SPD-10AV) detection at 209 nm over the concentration range 5-30µg/ml with recovery range 99.73 to 101.39% for RTV by HPLC method. The HPTLC separation was achieved on the aluminum backed layer of silica gel 60F_{254} using (Toluene: ethyl acetate: methanol: glacial acetic acid) (7.0:2.0:0.5:0.5%v/v/v/v) as mobile phase. Quantification was achieved with HPTLC detection at 263nm over the concentration range of 200 to 1000 ng/ spot with recovery in the range of 98.00-101.11% for RIV. Both the methods are simple, precise, and sensitive and extended for routine analysis in bulk as well as pharmaceutical formulation.

Key words: Ritonavir, RP-HPLC, HPTLC, HIV Protease inhibitor, Validation.

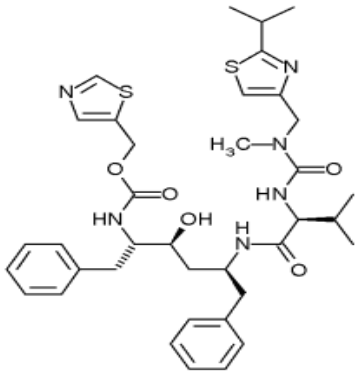
INTRODUCTION

Human immuno deficiency virus is the etiologic agent of acquired immunodeficiency syndrome (AIDS). HIV protease is an enzyme that is essential for viral growth. The HIV genome contains various sites designated as genes, such as gag and gag-pol genes which are translated as polyproteins and form immature viral particles. The latter are precursor protein molecules that

are cleaved by a viral pol-encoded aspartic proteinases to form the desired structural proteins of the mature viral particles.

Inhibition of the HIV protease leads to the production of noninfectious viruses. Cells incubated in the presence of HIV protease inhibitors produce viral particles that are immature. Thus inhibition of HIV protease becomes on of the most important approaches for the therapeutic intervention of HIV infection. Ritonavir [1] is chemically (5S,8S,10S,11S)-10-hydroxy-2methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4- thiazonyl]-3,6- dioxo-8,11-bis(phenyl methyl)-2,4,7,12-tetraazatridecan-13-oic acid 5-thiazolyl methyl ester. It is a peptidomimetic inhibitor of both the HIV-1 and HIV-2 protease.

Figure1. Structure of Ritonavir



Literature survey revealed a selective, sensitive and rapid LC method for the measurements of ritonavir in human plasma and in mouse serum and brain [2-3]. Simultaneous HPLC determination of and other antiretroviral in human plasma [4-8]. UV determination of ritonavir in soft gelatin capsule [9]. Simultaneous LC/MS/MS determination of ritnovir and other antiretrovirals in human plasma [10-13].Determination of NRTIs and phosphorylated metabolites by LC/MS/MS[14]. HPLC and HPTLC method has not been so far reported for the quantification of RIV in bulk and in tablet dosage form. The present research work describes two simple, precise and accurate HPLC and HPTLC chromatographic method for the estimation of bulk and in tablet dosage form.

MATERIALS AND METHODS

Instruments

An Agilent HPLC (1100 series equipped with UV visible detector (SPD-10A), manual injector 20 µl loop eclipse XBD (C_{18}) (150mmX 4.6mm id, 15µparticle size) column and chem. station software were used . For HPTLC a linomat V autosampler scanner III, flat bottom and twin trough developing chamber and receiving cabinet with dual wavelength UV lamps used. HPTLC plates used thicknesses 0.2mm, 10X10cm aluminium backing were used during the study.

Reagents and materials

RIV bulk powder was kindly gifted by hetero labs limited, Hyderabad (India), 99.95% purity. HPLC grade acetonitrile was purchased from S.D fine chemicals (Ahmedabad, India) The water

for HPLC was prepared by triple glass distillation and filtration through a nylon 0.45 µm -0.47 µm membrane filter (Gelman laboratory, Mumbai). Tablets of RIV were purchased from the local pharmacy.

Chromatographic conditions

(a) RP-HPLC method

Eclipse XBD C_{18} column (150mmX 4.6mm id, 15µparticle size) was used at an ambient temperature. The mobile phase acetonitrile: water (60: 40 % v/v) was pumped at a flow of 1.4ml/min. The mobile phase was filtered through nylon 0.45 µm -0.47 µm membrane filter and degassed before use. The elution was monitored at 209nm and injection volume was 20 µl.

(b). HPTLC method

Solutions of RTV was applied to silica gel $60F_{254}$ HPTLC plates (10X10cm) by means of a Linomat V automatic spotter equipped with a 100 µl syringe and operated with settings of band length, 6mm, distance between bonds, 8mm, distance from the plate edge, 10mm and distance from the bottom of the plate 10mm. the plate was developed in a twin trough chamber previously saturated for 30min with the mobile phase toluene: ethyl acetate: methanol: glacial acetic acid (7.0:2.0:0.5:0.5%v/v/v/v) to 8cm. The spots on the air dried plate were scanned with a scannerIII at 263nm using deuterium source.

Preparation of RIV standard stock solutions

(a).RP-HPLC method

Accurately weighed RIV (25mg) was transferred too a 25ml volumetric flask and dissolved and diluted to a mark with acetonitrile to obtain a standard solution of RIV (1000 µg/ml). This solution (2.5ml) was further diluted to 50ml with mobile phase to obtain a working standard solution with RIV (50 µg/ml) for the RP-HPLC method.

(b). HPTLC method

Accurately weighed RIV (10mg) was transferred too a 100ml volumetric flask and dissolved and diluted to a mark with methanol to obtain a standard solution of RIV (100 µg/ml). This solution was used as a working standard solution.

Preparation of working sample solution

Twenty tablets of EMPETUS (containing 100mg of RTV) were weighed and powered, the tablet power equivalent to 25mgof RTV was transferred to 25ml standard flask and 15ml acetonitrile was added. The solution was sonicated for 15min, and the final volume was made with same to obtain solution of RIV (1000µg/ml). The mixture was then filtered through a nylon 0.20mm-0.47mm membrane filter. The above solution was suitably diluted with mobile phase to obtain final dilution of RIV (50µg/ml) for HPLC method and dilute with acetonitrile to obtain final dilution of RIV (100µg/ml) for HPTLC method.

Method validation

Both the methods are validated for its linearity range, accuracy, precision, sensitivity and specificity. Method validation is carried out as per ICH guidelines [15-16].

Linearity

(a).Calibration curve (linearity) of the RP-HPLC method

Calibration curve was constructed by plotting peak area Vs concentration of RTV solutions, and the regression equation was calculated. The calibration curve was plotted over the concentration range 5-30µg/ml. accurately measured standard working solution of RTV (1,2,3,4,5 and 6ml) were transferred to a series of 10ml volumetric flasks and diluted up to the mark with mobile phase. Aliquots (20 µl) of each solution were injected under the operating chromatographic condition described above.

(b).Calibration curve (linearity) of the HPTLC method

Calibration curve was plotted over a concentration range of 200 to 1000 ng/spot for RIV. Accurately prepared standard solution of RIV (2, 4, 6, 8, 10 and 12) was applied to the plate. The calibration curve was constructed by plotting peak area versus concentration with the help of win cats software.

Accuracy

The accuracy of the methods was determined by calculating recoveries of RTV by the standard addition methods. Known amounts of standard solution RIV (20-120%) for the RP-HPLC method and HPTLC method were added to prequantified sample solution of tablet powder. The amounts of RTV were estimated by applying these values to the regression equation of the calibration curve.

Method precision

The precision of the instruments was checked by repeatedly injecting (n=6) solutions of RTV (15µg/ml) for the RP-HPLC method and by repeated scanning of the same spot (n=6) of RTV (600ng/spot) with out changing the position of plate for the HPTLC method Repeatability was reported in terms of percentage relative standard deviation(%RSD).

Intermediate Precision (Reproducibility)

The interday and intraday precision of the proposed methods were determined by the corresponding responses three times on the same day and on three different days over a period of one week for three different concentration of RTV (5,20 and 30µg/ml) for RP-HPLC method and RTV (200, 800,1200ng/spot)for HPTLC method. The results in terms of percentage relative standard deviation (%RSD).

Limit of detection and limit of quantification

The limit of detection (LOD) limit of quantification (LOQ) of the drug carry was calculated using the following equation as per international conference harmonization (ICH) guidelines.

$LOD = 3.3 \times \alpha / S$

$LOQ = 10 \times \alpha / S$

Analysis of RTV in Tablet powder

Tablets of RTV (EMPETUS containing 100mg of RTV) were purchased from local pharmacy. The responses of the solutions of that tablet dosage forms were measured at 209nm and 263nm

for quantification by using HPLC and HPTLC instruments described as above. The amount of RTV present in sample solutions was determined from regression equation of both the methods.

RESULTS AND DISCUSSION

(a).RP-HPLC method

To optimize the RP-HPLC parameters, several mobile phases of different compositions were tried. A satisfactory separation and good peak symmetry for RTV were obtained with a mobile consisting of acetonitrile: water (60: 40v/v). Quantification was achieved with UV detection at 209nm based on peak area. Complete resolution of the peaks with clear baseline was obtained. (Fig-2). System suitability parameters was calculated and compared with the standard limit as per USP [17]. The results were shown in (Table-3)

(b). HPTLC method

Several mobile phases were tried to accomplish good distance of RIV. Using mobile phase Toluene: ethyl acetate: methanol: glacial acetic acid (7.0:2.0:0.5:0.5%v/v/v/v), better distance was attained at Rf value of 0.73 for RTV. A wavelength of 263nm was used for quantification of the drug. Resolution of the peaks with clear baseline separation was formed (Fig-3). System suitability parameters were shown in (Table-3)

Validation of the proposed method

Linearity

Linear correlation was obtained between peak area used absorbance Vs concentration of RIV in the range of 5-30µg/ml and 200-1200ng/ml respectively for HPLC and HPTLC method. The linearity of the calibration curve was validated by the high value of correlation co-efficient of regression (Tab-1).

Accuracy

The accuracy experiments were carried out by the standard addition method. The recoveries obtained by 99.73 to 101.39% for HPLC and 98.00 to 101.11% by HPTLC method for RTV. The high values indicate that both methods are accurate.

Method Precision

The %RSD[18] values for RTV were found to be 0.54 % using RP-HPLC and 0.72% for HPTLC method. The low values %RSD indicates the proposed methods were precise(Table-2).

Intermediate Precision

The low% RSD values of intraday and interday (0.669 and 0.526%) and (0.697and 0.789%) for RTV respectively by RP-HPLC and HPTLC methods, reveal that the proposed methods are precise (Table-2).

LOD and LOQ

LOD for RTV was found to be 0.2078 and 0.0164 µg/ml respectively for RP-HPLC and HPTLC method. LOQ for RTV was found to be 0.7512 and 0.0497 µg/ml respectively for RP-HPLC and HPTLC method. These data show that both the methods are sensitive for the determination of RTV.(Table-2).

Fig 2.A Typical HPLC Chromatogram of Ritonavir at 209nm

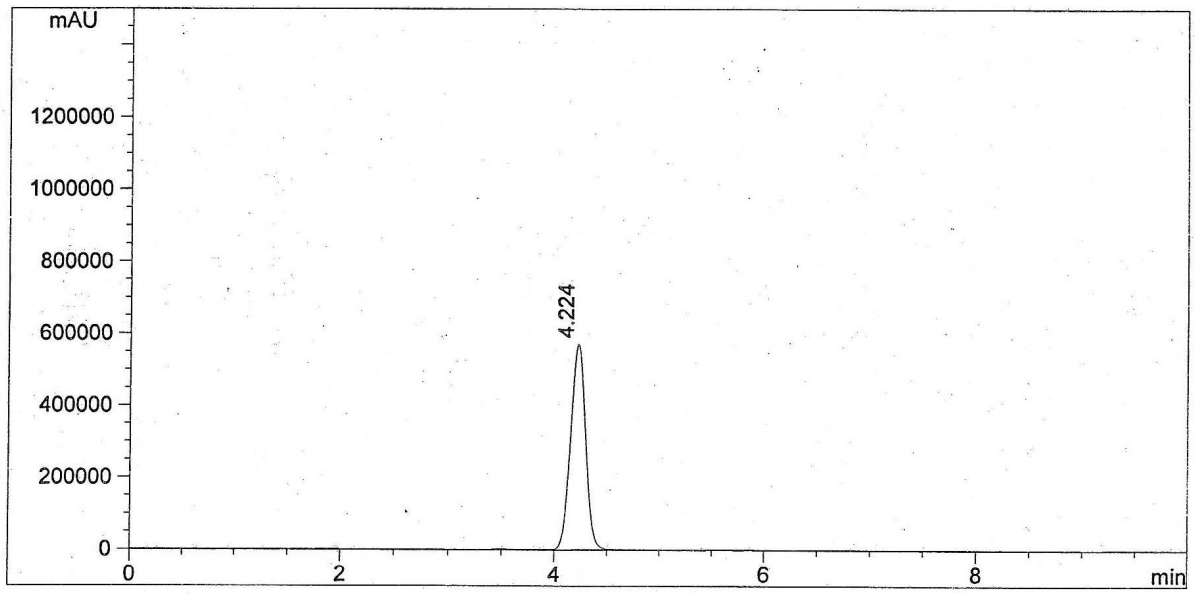


Fig 3.A Typical HPTLC Chromatogram of Ritonavir at 263nm

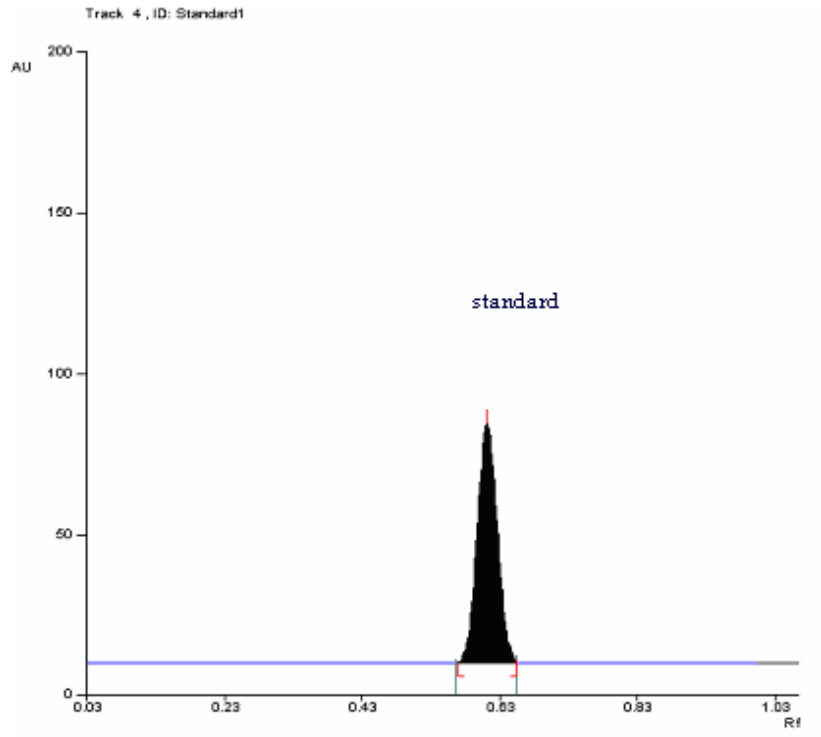


TABLE.1. Regression analysis of the calibration curve for RTV for the proposed RP-HPLC and HPTLC

Parameters	Ritonavir	
	RP-HPLC	HPTLC
Concentration range	5-30µg/ml	200-1200ng/spot
Slope	28685.30	10096.68
Intercept	53182.33	2544.321
Correlation coefficient	0.9990	0.9994

TABLE.2. Summary of validation parameters for RTV by proposed RP-HPLC and HPTLC

Parameters	Ritonavir	
	RP-HPLC	HPTLC
LOD[1]	0.2078	0.0164
LOQ[2]	0.7512	0.0499
Accuracy	99.73-101.26%	98.00 - 101.11%
Repeatability (%RSD[3])	0.340	0.470
Precision(%RSD[3])		
Interday (n=3)	0.669	0.697
Intraday (n=3)	0.526	0.789

[1]LOD=Limit of detection
[2]LOQ= Limit of quantification
[3]%RSD= percent relative standard deviation

TABLE.3. System suitability parameters for RTV for the proposed RP-HPLC

Parameters	Ritonavir	Limits as per USP
Tailing factor	1.020	Less than 2
Asymmetric factor	0.995	Less than 2
Theoretical plates	4610	More than 2000
Capacity factor	1.49	1 to 10
HETP	0.05759	-
Theoretical plate per unit length	321.12	-

TABLE.4. System suitability parameters for RTV for the proposed HPTLC

Parameters	RTV±%RSD
Rf value	0.73±0.01
Area average	44121±0.64

TABLE.5 Analysis of RTV formulation by the proposed RP-HPLC and HPTLC

Brand Name	Ritonavir	
	RP-HPLC	HPTLC
Empetus	100.23±0.3400	101.02±0.1428

Analysis of RTV in Tablet powder

The proposed method validated methods were successfully applied to determine RTV in their tablet dosage forms. The results obtained for RTV were comparable with the corresponding labeled amounts (Table-5)

CONCLUSION

The results of analysis of tablet dosage form by the proposed methods are highly reproducible and reliable and are in good agreement with the label claim of the drug. The addi5tives usually present in pharmaceutical formulations of the assayed samples did not interfere with determination of RTV by the proposed method. So both the methods can be used for the routine analysis of the RTV in their tablet dosage form.

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