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Research Article

Pharmaceutical analysis for Novel drug formulation

Quantification of Atazanavir and Ritonavir in Human Plasma Samples by Rp-Hplc Include Method of Detection in the Title, Eg: Using Pda Detection

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Abstract: A fixed oral dose combination of Atazanavir and Ritonavir is currently used for the treatment of patients with HIV infections. A selective and novel bio-analytical technique was designed to evaluate Atazanavir and Ritonavir by mass spectroscopic investigation in plasma matrices. The method was chromatographed with Agilent TC-C18, 4.6 x 75 mm, 3.5 µm, 80 Å column, 5mM ammonium acetate: acetonitrile (20:80 v/v) mobile phase was used for Chromatographic separation. UV detector was used to detect the Atazanavir and Ritonavir at 235 nm. For extraction of the analyte and internal standard, Liquid-liquid extraction was employed .This method is validated over a linear concentration range of 50.0 – 10000.0 ng/ml for Atazanavir and Ritonavir with a correlation coefficient (r) of = 0.9997 and both drugs were stable in plasma samples.

Keywords: HPLC; Atazanavir, Ritonavir, Human plasma; Bio analytical

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1. INTRODUCTION

Antiretroviral drugs are medications for the treatment of infection by retroviruses, primarily human immunodeficiency virus (HIV). The aim of antiretroviral treatment is to maximally and durably suppress plasma HIV viral load. [1, 2.] To obtain optimal antiviral efficacy and to prevent viral drug resistance, these drugs are administered to patients in combination regimens, which are referred to as highly active antiretroviral therapy (HAART) and is the most effective approach for the treatment of HIV infection [3, 4]. Current HAART treatment guidelines consist of one or two protease inhibitors (PIs) or one non-nucleoside reverse transcriptase inhibitor (NNRTI), together with two nucleoside reverse transcriptase inhibitors (NRTIs) [5] . Ritonavir and atazanavir (Figure-1) are human immunodeficiency virus type-1 (HIV-1) protease inhibitors, which were designed to have a more beneficial pharmacodynamic and/or pharmacokinetic profile compared to the currently licensed PIs [6, 7.] Ritonavir (Figure-2) is a potent *invitro and in-vivo* inhibitor of the HIV virus. It blocks the HIV protease, thereby reducing the viral load in the infected individual. [8, 9.]Atazanavir is an azapeptide PI class of antiretrovirals (ARVs), which has played a significant role in lowering the morbidity and mortality of HIV/AIDS. Its unique HIV resistance profile and favourable pharmacokinetics allows once-daily dosing. Atazanavir is metabolized by CYP3A4 in the liver and is 86% bound to human serum proteins [6, 10-11.]Monotherapy with ritonavir has been shown to be 90% effective[8]. However, monotherapy with a single protease inhibitor may result in both viral resistance [12] and possible cross-resistance to the other protease inhibitors. Therefore, combination therapy, which may include protease inhibitors, is the standard of care. Recently, the Food and Drug Administration (FDA) has approved fixed dose combination of Ritonavir and Atazanavir sulfate tablets (100 mg/300 mg) for use in combination with other antiretrovirals for the treatment of HIV-1 infection. [13] In recent years, several methods have been developed to quantify antiretroviral drugs in plasma. But only a few UV[2-3], HPLC[4-43] , HPTLC[44-45] methods allow the simultaneous monitoring of ritonavir and atazanavir in human plasma. The major disadvantages of all these methods include complicated with expensive extraction procedures and long chromatographic run time. Literature survey reveals that, there is no method reported for quantification of Atazanavir and Ritonavir by using HPLC in Human plasma. The main goal of the present study is to develop and validate the novel simple, sensitive, selective, rapid, rugged and reproducible analytical method for the quantitative determination of Atazanavir and Ritonavirin human plasma by HPLC with a small amount of sample volume.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Atazanavir (AV) and Ritonavir (RV) (Fig - 1 and 2) (Symed labs, Hyderabad, India and Toronto research chemicals, Canada). Ethyl acetate, HPLC grade methanol and acetonitrile were purchased from J.T. Baker, USA. Sodium dihydrogen phosphate (NaH_2PO_4, reagent grade), Ammonium acetate (reagent grade) was purchased from Merck Limited, Worli, Mumbai. Human plasma was obtained from Doctors labs, Hyderabad, India. Ultra pure water from MilliQ-system (Millipore) was used throughout the study.

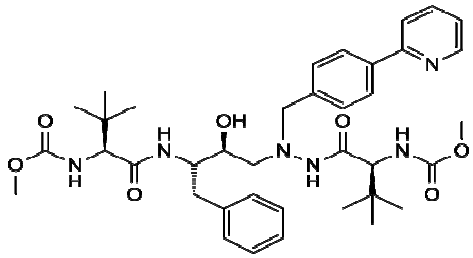

Fig 1. Chemical structure of Atazanavir (AV)

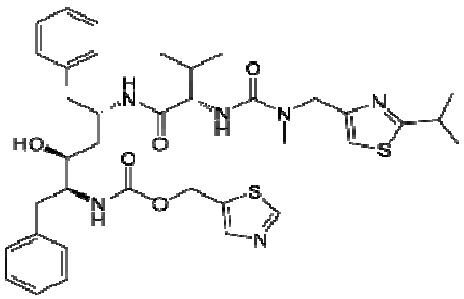

Fig 2. Chemical structure of Ritonavir (RV)

2.2 Instrumentation

The 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany) with UV detection. Data processing was performed on EZCHROM Elite software package.

2.3 Chromatographic conditions

Agilent TC-C18, 4.6 x 75 mm, 3.5 µm, 80 Å column, 5 mM ammonium acetate: acetonitrile (20:80 v/v) mobile phase with a flow-rate of 0.5 mL/min. The column was placed at a temperature of 40 °C. 20 µL of sample was injected into HPLC System. The analytes were eluted at 2.8 minutes (AV) and 3.8 minutes (RV) with total run-time of 8 minutes for each injection.

2.4 Calibration standards and quality control samples

Standard Stock solutions (1 mg/mL) were prepared in methanol. The stock standards were used to prepare calibration standards of 50.0, 100.0, 500.0, 1000.0, 2000.0, 4000.0, 6000.0, 8000.0, 10000.0 ng/mL and QC concentrations at 50.0, 150.0, 3000.0 and 7000.0 ng/mL (LLOQ, LQC, MQC and HQC) in plasma. samples.

2.5 Sample preparation

Liquid-liquid extraction was carried out to extract the analytes from plasma and for this purpose 100µL of respective concentration of plasma sample was taken into polypropylene tubes. This was followed by addition of 100 µL of 5mM NaH_2PO4 solution and 3.0 mL of ethyl acetate and vortexed for approximately 10 minutes. Then, the samples were centrifuged at 4000 rpm for 10 minutes at 20°C. Further, the supernatant was transferred into a labeled polypropylene tubes and evaporated with nitrogen gas at 40°C. Then the samples were reconstituted with mobile phase and vortexed for 2 minutes. Finally, the Sample was transferred into autosampler vials to inject into the HPLC.

2.6 Selectivity and specificity

Six different plasma lots were used for selectivity to identify the potential interferences in plasma samples. The peak area of AV and RV in blank samples should not be more than 20 % of the mean peak area of LOQ of AV and RV.

2.7 Precision and accuracy

Replicate analysis of quality control samples (n = 6) at LQC (low quality control), MQC (medium quality control) and HQC (high quality control) levels for determining the Precision and accuracy. The % CV should be less than 15 %, and accuracy should be within 15 % except LLOQ where it should be within 20 %.

2.8 Matrix effect

To quantify the analyte response suppression/enhancement due to matrix interferences matrix effect will performed. Experiments were performed at MQC levels in triplicate with six different plasma lots. The acceptable precision (% CV) of ≤ 15 % was maintained.

2.9 Recovery

The extraction efficiencies of AV and RVwere determined by analysis of six replicates at each quality control concentration level for AV and RV. The % recovery was calculated with response of un-extracted and extracted samples.

2.10 Limit of quantification/Sensitivity (LOQ)

The limit of quantitation (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOQ was found by analyzing a set of mobile phase and plasma standards with a known concentration of AV and RV.

2.11 Stability (Freeze-thaw, Auto sampler, Room temperature, Long term)

Stock solution stability was performed by comparing the area response of analyte and internal standard in the stability sample, with the area response of sample prepared from fresh stock solution. Stability studies of plasma samples were performed at the LQC and HQC concentration level using six replicates at each level. Analyte was considered stable as the percentage Change was less than 15 % as per USFDA guidelines [46]. The stability of spiked human plasma samples stored at room temperature (bench top stability) was evaluated for 61.0 h. The stability of spiked human plasma samples stored at -30.0 °C in auto sampler (auto sampler stability) was evaluated for 70.0 h. The auto sampler sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h) with the samples that were re-injected after storing in the auto sampler at 20.0 °C for 70.0 h. The re-injection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were re-injected after storing in the auto sampler at 20.0 °C for 70.0 h. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at –30.0 °C and thawed three times with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze-thaw stability evaluation. For long term stability evaluation, the concentrations obtained after 91 days were compared with initial concentrations.

3. RESULTS AND DISCUSSION

3.1 Method development and validation

Chromatographic conditions, in particular, composition of the mobile phase and selection of suitable column was optimized through several trials to achieve the best resolution and to increase the signal of analyte and internal standard. Different extraction methods like solid phase extraction, Liquid-liquid extraction, precipitation methods were optimized for extraction of AV and RV from the plasma sample. A good separation and elution were achieved using 5 mM ammonium acetate: acetonitrile (20:80 v/v) as the mobile phase, at a flow-rate of 0.5 mL/minutes and injection volume of 20 µL. Liquid-liquid extractions was chosen to optimize the drug and internal standard. The retention time was optimized as 2.8 minutes (AV), 3.8 minutes (RV) (Fig- 4 and 5).

3.2 Linearity

Calibration curve was plotted as peak area versus concentration. Calibration was found to be linear over the concentration range of 50.0 – 10000.0 ng/mL. The correlation coefficient (r^2) was greater than 0.9997 for all curves (Table-1).

Table1: Calibration curve details of Atazanavir and Ritonavir

Spiked plasma concentration (ng/mL)	Concentration measured(mean) (ng/mL), (*n* = 5) ±SD	Precision (CV %) (*n* = 5) ±SD	Concentration measured(mean) (ng/mL), (*n* = 5) ±SD	Precision (CV %) (*n* = 5)
	Atazanavir		**Ritonavir**	
50.0	51.0 ± 1.3	2.5	51.2 ± 1.0	2.0
100.0	96.6 ± 4.7	4.9	95.8 ± 3.4	3.5
500.0	498.4 ± 24.7	5.0	495.1 ± 26.3	5.3
1000.0	1000.0 ± 17.1	1.7	1010.5 ± 28.3	2.8
2000.0	2013.0 ± 74.6	3.7	2019.1 ± 70.0	3.5
4000.0	4008.4 ± 206.6	5.2	4067.1 ± 224.9	5.5
6000.0	5956.5 ± 190.7	3.2	5628.5 ± 735.7	13.1
8000.0	7952.2 ± 165.6	2.1	8162.8 ± 191.2	2.3
10000.0	10317.1 ± 487.6	4.7	10440.0±521.5	5.0

3.3 Selectivity

No significant endogenous peaks observed at respective retention time of AV and RV. The results indicate that the method exhibited good specificity and selectivity. (Fig.4 and 5)

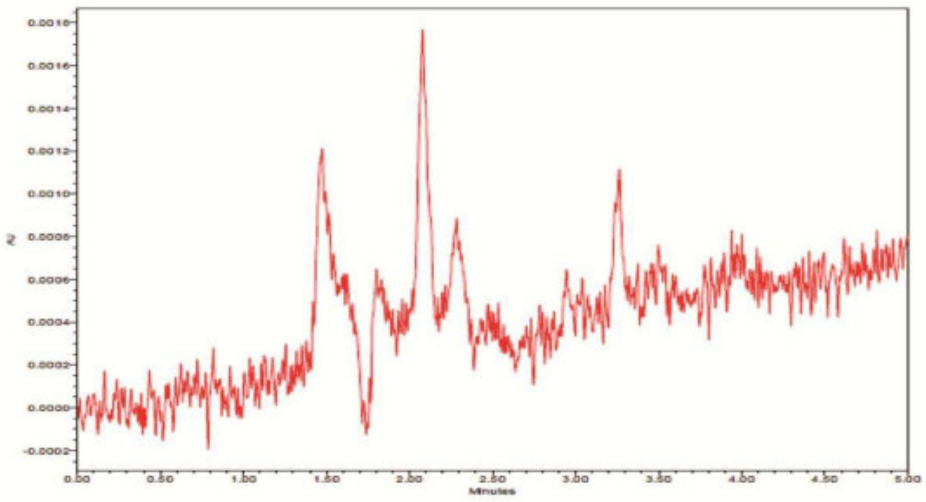

Fig 3.Typical chromatogram of mobile phase

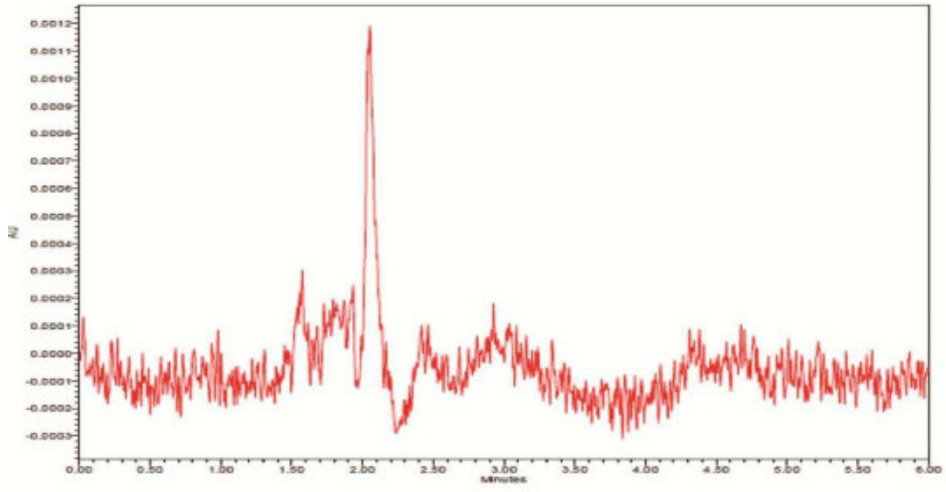

Fig 4. Typical chromatogram of blank plasma

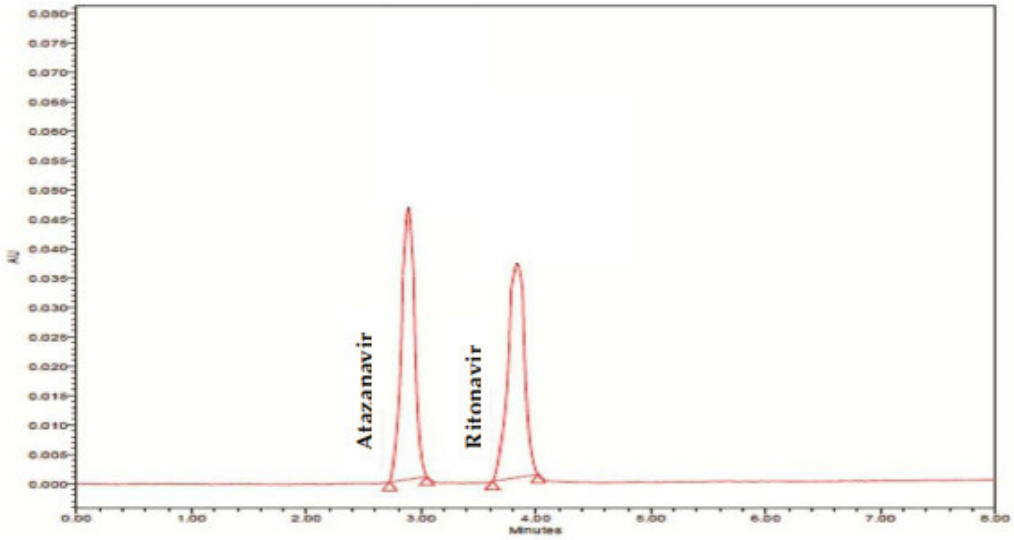

Fig 5. Typical chromatogram of LLOQ (Lower limit of quantification)

3.4 Precision and Accuracy

Precision and accuracy for this method was controlled by calculating the within-run and between-run variations at three concentrations (150.0, 3000.0 and 7000.0 ng.mL^{-1}) of QC samples in six replicates. As shown in Table 2, the within-run precision and accuracy were between 1.2 to 4.5 & 91.7 to 105.5 % for AV and 1.4 to 4.3 & 84.8 to 106.4 % for RV. Similarly, the between-run precision and accuracy were between 1.6 to 7.4 & 102.2 to 110.6% for AV and 1.1 to 5.1 & 99.4 to 107.1% for RV. These results indicated the adequate reliability and reproducibility of the developed method within the analytical range (Table-2).

Table2: Precision and accuracy

Spiked plasma concentration (ng/mL)	Within-run (n=6)			Between-run (n=30)		
	Concentration measured (ng/mL) (mean± S.D.)	Precision (CV %)	Accuracy %	Concentration measured (ng/mL) (mean±S.D.)	Precision (CV %)	Accuracy %
Atazanavir						
50.0	51.4±2.3	4.5	102.7	55.5±4.1	7.4	110.6
150.0	152.9±1.4	2.2	105.5	151.9±1.7	1.6	102.2
3000.0	3103.8±102.0	3.3	103.2	3133.0±108.2	3.5	104.3
7000.0	7197.1±89.9	1.2	91.7	7178.7±275.5	1.8	103.9
Ritonavir						
50.0	42.4±1.0	2.4	84.8	49.9±6.9	3.8	99.4
150.0	152.6±2.3	1.4	106.4	151.5±1.6	1.2	101.6
3000.0	3072.4±132.6	4.3	102.4	3216.1±162.6	5.1	107.1
7000.0	7160.1±105.8	1.4	98.0	7174.3±123.9	1.1	102.1

3.5 Matrix effect

The analyte suppression/enhancement in the signal at MQC level was found to be % CV 1.27 for AV and % CV 1.20 for RV respectively. These results indicate that there is no effect on ion suppression and ion enhancement.

3.6 Recovery

The extraction recoveries of AV determined at three different concentrations (150.0, 3000.0 and 7000.0 ng.mL^{-1}) were found to be 99.6 ± 3.53, 88.2 ± 2.7 and 97.60 ± 4.7 %. Similarly, extraction recoveries of RV determined at three different concentrations (150.0, 3000.0 and 7000.0 ng.mL^{-1}) were found to be 95.5 ± 9.7, 91.6 ± 10.21 and 92.3 ± 4.7 %. The overall average recoveries of AV, RV was found to be 94.98 ± 6.1, 93.5 ± 1.8 %. Recoveries of the analytes were consistent, precise and reproducible.

3.7 Limits of Quantification/Sensitivity (LOQ)

The LOQ signal-to-noise (S/N) values was found for six injections of AV and RV at concentrations of 31.95 ng/mL and 40.23 ng/mL respectively. ..

3.8 Stability (Freeze-thaw, Auto sampler, Room temperature, Long term)

Stock solution stability was performed to check the stability of AV & RV in stock solutions prepared in methanol and stored at 2.0 -8.0 °C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 25 days. The % change for AV & RV were -0.02 %and 0.01 % respectively indicated that stock solutions were stable at least for 25 days. The room temperature and auto sampler stability for AV and RV was investigated at LQC and HQC levels. The results revealed that AV and RV were stable in plasma for at least 60.0 h at room temperature, and

70.0 h in an auto sampler. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with AV and RV at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that AV and RV were stable in a plasma samples up to 91 days at a storage temperature of -30.0 °C. The results obtained from all these stability studies are tabulated in Table-3. Precision (% CV) is less than 15% for Room temperature, long-term, Freeze and thaw, auto sampler stability.

Table.3: Stability of Atazanavir and Ritonavir in spiked human plasma samples

Stability experiments	Storage condition	Spiked plasma concentration (ng/mL)	Concentration measured (*n*=6) Mean ± SD	CV (%) (*n*=6)	Accuracy (%)
Atazanavir					
Bench top (Room temperature)	RT 61 hr	150.0	148.3 ± 8.1	5.5	98.9
		7000.0	6728.3±206.3	3.1	81.5
Processed (extracted sample)	Autosampler 70 hr	150.0	162.3 ± 2.4	1.5	108.2
		7000.0	7536.7±294.5	3.9	90.4
Freeze & Thaw stability	-30 ^{0}C Cycle-3	150.0	156.5 ± 4.0	2.5	104.3
		7000.0	7381.7±173.4	2.3	90.4
Long term stability	- 30 ^{0}C, 91 days	50.0	160.3±13.2	8.2	106.9
		7000.0	7450.0±229.1	3.1	90.5
Ritonavir					
Bench top (Room temperature)	RT 61 hr	150.0	156.3 ± 8.7	5.6	104.2
		7000.0	7411.7±213.7	2.9	92.6
Processed (extracted sample)	Autosampler 70 hr	150.0	161.7 ± 4.9	3.0	107.8
		7000.0	7675.0±473.5	6.2	95.9
Freeze & Thaw stability	-30 ^{0}C Cycle-3	150.0	159.7 ± 7.6	4.7	106.4
		7000.0	7540.0±323.0	4.3	94.3
Long-term stability	- 30 ^{0}C,91 days	50.0	159.0 ± 6.3	4.0	106.0
		7000.0	7608.3±297.2	3.9	95.1

4. CONCLUSION

In summary, for the first time, we have developed and validated a sensitive and rapid HPLC method for the measurement of Atazanavir and Ritonavir by HPLC. The method showed precise recovery for both Atazanavir and Ritonavir. The proposed method showed good performance with respect to all the validation parameters tested and optimized working conditions. The method was successfully employed for estimation of safinamide in spiked human plasma samples. The proposed method can be used for routine quality control, stability studies and also suitable for therapeutic drug monitoring (Bioavailability and bioequivalence studies) of pharmaceutical dosage forms containing Atazanavir and Ritonavir.

5. AUTHORS CONTRIBUTION STATEMENT

Concept and Supervision, Analysis and/or Interpretation and Critical Reviews were done by N. Rama Rao; Literature Search, Collection of Materials, datas, data processing and writing was done by P.Abhinandana.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

Conflict of interest declared none.

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