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Simultaneous Determination of Ritonavir and Atazanavir in Human Plasma by LC-MS/MS and Its Pharmacokinetic Application

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ABSTRACT

A simple and rapid liquid chromatography-tandem mass spectrometric (LC-MS/MS) assay method has been developed and validated for simultaneous quantification of two protease inhibitors ritonavir and atazanavir in human plasma. Saquinavir was used as an internal standard. The analytes were extracted from human plasma samples by solid-phase extraction technique using a Orpheus C_{18} extraction cartridges. The reconstituted samples were chromatographed on a C_{18} column by using a 85:15 (v/v) mixture of methanol and 5mM ammonium acetate as the mobile phase at a flow rate of 0.9 mL/min. The calibration curves obtained were linear ($r \geq 0.99$) over the concentration range of 8.0-1600.0 ng/mL for ritonavir and 50.5-5995.2 ng/mL for atazanavir. The results of the intra- and inter-day precision and accuracy studies were well within the acceptable limits. A run time of 2.0 min for each sample made it possible to analyze more than 300 plasma samples per day. The proposed method was found to be applicable to clinical studies.

Keywords: Ritonavir; atazanavir; human plasma; solid-phase extraction; LC-MS/MS; pharmacokinetics

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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 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 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 is a potent in vitro 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 the 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 [13]. Therefore, combination therapy, which may include the 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.

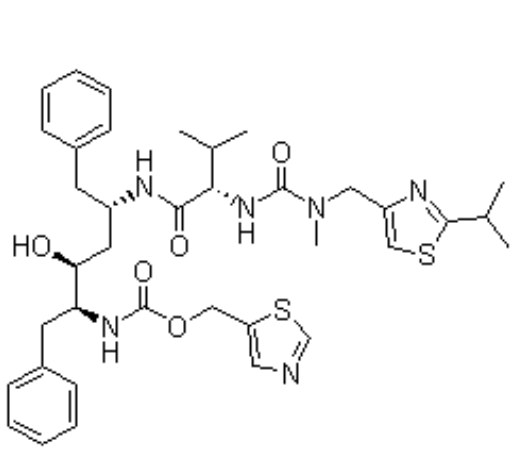
In recent years several methods have been developed to quantify antiretroviral drugs in plasma. But only a few LC-MS/MS methods [14-18] allow the simultaneous monitoring of ritonavir and atazanavir in human plasma. The major disadvantages of all these methods include complicated and expensive extraction procedures, long chromatographic run time, typical mobile phase, and polarity switching. Moreover these methods are not specific for the determination of ritonavir and atazanavir; hence it may create conflicts in final results due to improper characterization of selectivity. Today there is a more urge for the antiretroviral drugs in world and demands a more selective and high throughput method for the determination of these drugs for therapeutic drug monitoring and pharmacokinetics. Thus the aim of the authors was to develop a more specific,

selective, sensitive method, which employs solid-phase extraction technique for sample preparation and liquid chromatography with electropspray ionisation-tandem mass spectrometry for simultaneous determination of ritonavir and atazanavir in human plasma. The application of this assay method to a clinical pharmacokinetic study in healthy male volunteers following oral administration of ritonavir and atazanavir is described.

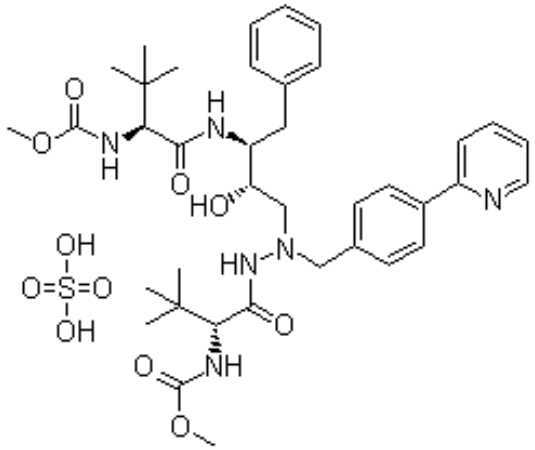
MATERIALS AND METHODS

Materials and reagents

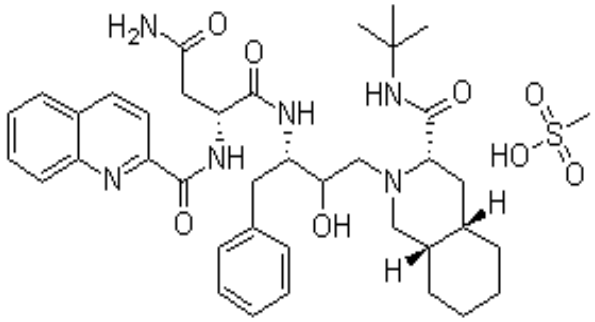
The reference samples of ritonavir (99.30%), atazanavir sulfate (99.9%) and saquinavir mesylate (99.80%) were obtained from Hetero Drugs. Ltd, Hyderabad, India. Chemical structures are presented in **Fig**ure 1. Water used for the LC-MS/MS analysis was prepared by using Milli Q water purification system procured from Millipore (Bangalore, India). HPLC grade methanol was purchased from J.T. Baker (Phillipsburg, USA). Analytical grade formic acid and ammonium acetate was purchased from Merck Ltd (Mumbai, India). The control human plasma sample was procured from Decan's Pathological Labs (Hyderabad, India).



Ritonavir



Atazanavir sulfate



Saquinavir mesylate (IS)

Figure 1. Chemical structures of ritonavir, atazanavir and saquinavir (IS).

Instrumentation and chromatographic conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a Hypurity Advance C_{18} column (50 mm × 4.6 mm, 5 µm; Thermo Scientific Corporations), a binary LC-20AD prominence pump, an auto sampler (SIL-HTc) and a solvent degasser (DGU-$20A_3$) were used for the study. Aliquots of the processed samples (10 µL) were injected into the column, which was kept at ambient temperature. An isocratic mobile phase consisting of a 85:15 (v/v) mixture of methanol and 5mM ammonium acetate was used to separate the analytes and delivered at a flow rate of 0.9 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantification was achieved with MS-MS detection in positive ion mode for both the analytes and the internal standard using an MDS Sciex API-3000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray ™ interface at 500 ºC. The ion spray voltage was set at 5500 V. The source parameters viz. the nebulizer gas, curtain gas and collision gas were set at 8, 9 and 8 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP), focusing potential (FP) and collision cell exit potential (CXP) were 12, 25, 10, 400, 5 V for ritonavir, 45, 70, 10, 300, 7 V for atazanavir and 34, 57, 10, 190, 22 V for saquinavir. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 721.3 precursor ion to the *m/z* 296.1 for ritonavir, *m/z* 705.6 precursor ion to the *m/z* 168.1 for atazanavir and *m/z* 671.5 precursor ion to the *m/z* 570.2 product ion for the IS. Quadrupoles Q1 and Q3 were set on unit resolution. The analysis data obtained were processed by Analyst software™ (version 1.4.2). As earlier publications have discussed the details of fragmentation patterns of ritonavir [19], atazanavir [20] and IS [21], we are not presenting the data pertaining to this.

Preparation of plasma standards and quality controls

Stock solutions of ritonavir, atazanavir and the saquinavir were dissolved in methanol at a concentration of 1 mg/mL. From these stock solutions, appropriate dilutions were made to produce working standard solutions using a 50:50 (v/v) mixture of methanol and water as a diluent. Calibration curve (CC) standard solutions of ritonavir and atazanavir in blank plasma were prepared by spiking with an appropriate volume of the working solutions, giving final concentrations of 8, 16, 40, 80, 320, 640, 960, 1280 and 1600 ng/mL for ritonavir, and 50, 101, 306, 612, 1223, 2446, 3597, 4796, and 5995 ng/mL for atazanavir. The CC samples were analyzed along with the quality control (QC) samples for each batch of plasma samples. The QC samples were prepared at five different concentration levels of 8.0 (LLOQ), 23.8 (LQC), 104.7 (MQC-1), 805.5 (MQC-2) and 1388.9 (HQC) ng/mL for ritonavir and 50.6 (LLOQ), 151.1

(LQC), 899.5 (MQC-1), 2998.5 (MQC-2) and 4997.4 (HQC) ng/mL for atazanavir in blank plasma. All the prepared plasma samples were stored at –70 ± 10 ºC.

Sample processing

A 200 µL aliquot of human plasma sample was mixed with 20 µL of the internal standard working solution (25 µg/mL of saquinavir). To this, 500 µL of 2% formic acid was added after vortex mixing for 10 s. The sample mixture was loaded onto a Orpheus C_{18} cartridge (100 mg/1 mL) that was pre-conditioned with 1.0 mL of methanol followed by 1.0 mL water and 1.0 mL of 2% formic acid. The extraction cartridge was washed with 1.0 mL of 100mM ammonium acetate followed by 1.0 mL of water. Ritonavir, atazanavir and IS were eluted with 1.0 mL of mobile phase. Aliquot of 10 µL of the extract was injected into the LC-MS/MS system.

Method validation

The validation of the above method was carried out as per US FDA guidelines [22]. The parameters determined were selectivity, matrix effect, linearity, precision, accuracy, recovery, stability and dilution integrity. Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma obtained from six different sources including one lipemic and one hemolyzed plasma. Sensitivity was determined by analyzing six replicates of plasma samples spiked with the lowest level of the calibration curve concentrations. Matrix effect was checked with six different lots of K2-EDTA plasma. Three replicate samples each of LQC and HQC were prepared from different lots of plasma (36 QC samples in total). For checking the linearity standard calibration curves containing at least 9 points (non-zero standards) were plotted (8.0-1600.0 ng/mL for ritonavir and 50.5-5995.2 ng/mL for atazanavir). In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences. Intra-day precision and accuracy were determined by analyzing six replicates at five different QC levels on two different days. Inter-day precision and accuracy were determined by analyzing six replicates at five different QC levels of five different runs. Recoveries of ritonavir, atazanavir and saquinavir were determined by comparing the peak area of extracted analyte standard with the peak area of non-extracted standard. Recoveries of ritonavir and atazanavir was determined at a concentration of 23.8, 151.1 (LQC), 805.5, 2998.5 (MQC-2) and 1388.9, 4997.4 (HQC) ng/mL, respectively, whereas for IS was determined at concentration of 25 µg/mL. Dilution integrity was performed to extend the upper concentration limit with acceptable precision and accuracy. Six replicates each at a concentration of about 1.6 times of the uppermost calibration standard were diluted two- and four-fold with blank plasma. The diluted samples were processed and analyzed.

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2-8 °C) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability (14 h), processed samples stability (Autosampler stability for 60 h, wet extract stability for 55 h and reinjection stability for 36 h), freeze-thaw stability (4 cycles), long-term stability (52 days) were performed at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15% SD) and precision (≤15% RSD).

Pharmacokinetic study design

A pharmacokinetic study was performed in healthy male subjects (n = 6). The ethics committee approved the protocol and the volunteers provided with informed written consent. Blood samples were collected following oral administration of ritonavir (100 mg) and atazanavir (300 mg) at pre-dose and 0.33, 0.67, 1, 1.33, 1.67, 2, 2.33, 2.67, 3, 3.33, 3.67, 4, 4.33, 4.67, 5, 5.5, 6, 6.5, 7, 8, 10, 12, 16, 24, 36, 48 and 72 h, in K2-EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at –70 °C till their use. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. Along with the clinical samples, the QC samples at low, middle 1, middle 2 and high concentration levels were also assayed in triplicate. Plasma concentration-time profile of ritonavir and atazanavir was analyzed by non-compartmental method using WinNonlin Version 5.1.

RESULTS AND DISCUSSION

Method development

Mass parameters were tuned in both positive and negative ionization modes for the analytes. Good response was found in positive ionization mode. Data in the MRM mode were considered, which showed better selectivity. Chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve good resolution and increased intensity of the signals of the analytes, as well as short run time. The use of ammonium acetate in the mobile phase improved the detection of the analytes. It was found that a mixture of methanol and 5mM ammonium acetate (85:15, v/v) could achieve this purpose and was finally adopted as the mobile phase. Hypurity advance C_{18} (50 mm × 4.6 mm, 5 µm) column gave good peak shapes and response even at lowest concentration level for both the analytes and IS. The mobile

phase was operated at a flow rate of 0.9 mL/min. The retention time of ritonavir, atazanavir and the IS were low enough (0.8, 1.0, 0.85 min) allowing a small run time of 2.0 min. A simple solid-phase extraction (SPE) technique was employed for the sample preparation in this work and provides high recoveries of the drugs. At the initial stages of this work, several compounds were tried for finding out a suitable IS in this analysis and finally saquinavir was found to be the best for the purpose.

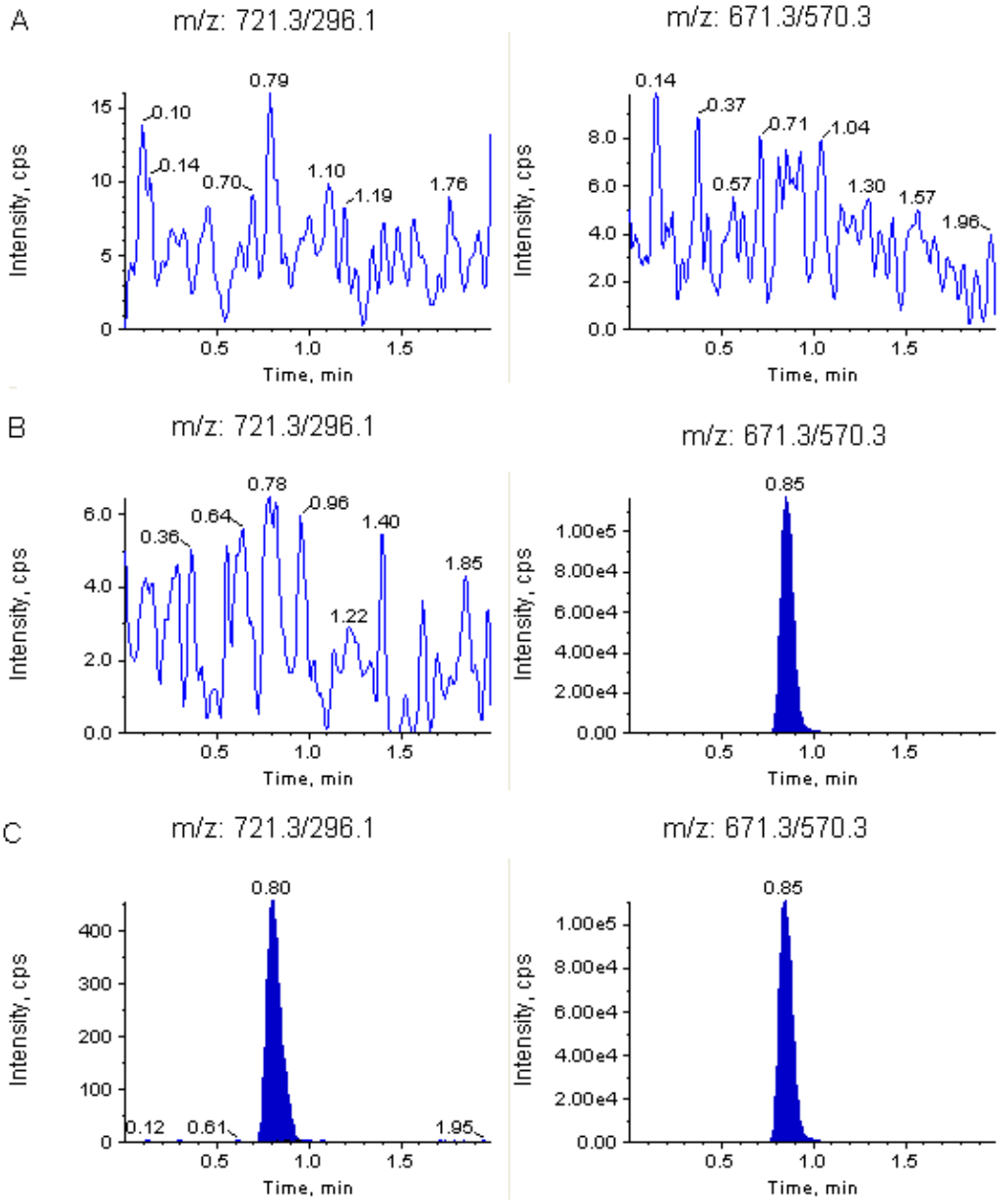


Figure 2. Typical MRM chromatograms of ritonavir (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS (C).

Selectivity and chromatography

The degree of interference by endogenous plasma constituents with the analytes and the IS was assessed by inspection of chromatograms derived from processed blank plasma sample. As shown in **Figures 2 & 3,** no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free plasma at the retention time of the analytes.

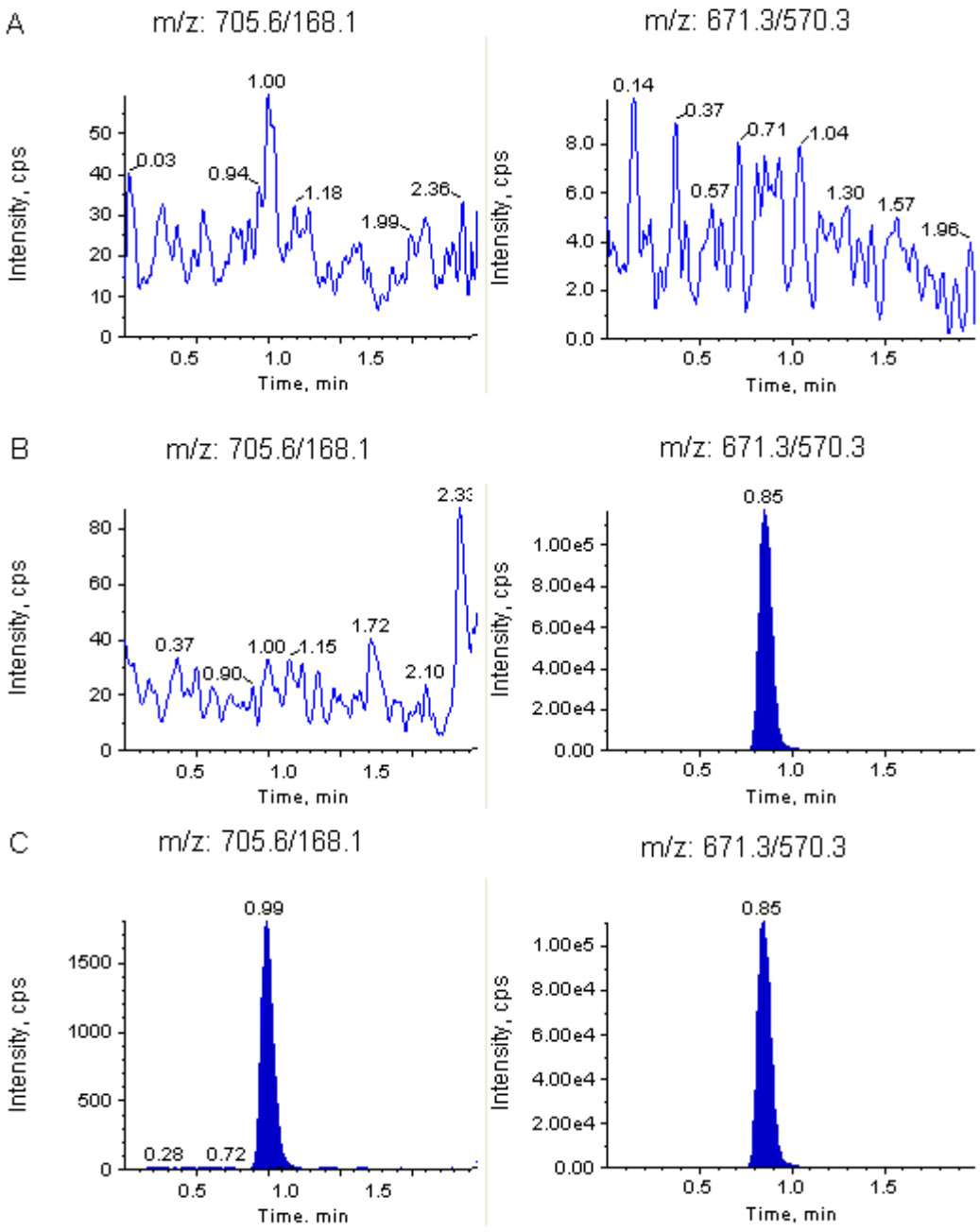


Figure 3. Typical MRM chromatograms of atazanavir (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS (C).

Sensitivity

The lowest limit of reliable quantification for the analytes was set at the concentration of the LLOQ. The precision and accuracy at LLOQ concentration were found to be 4.3% and 87.0% and 2.5% and 90.6% for ritonavir and atazanavir, respectively.

Matrix effect

No significant matrix effect was observed in all the six batches of human plasma for the analytes at low and high quality control concentrations. The precision and accuracy for ritonavir at LQC concentration were found to be 2.5% and 109.1%, and at HQC level they were 1.0% and 103.2% respectively. Similarly, the precision and accuracy for atazanavir at LQC concentration were found to be 6.8% and 99.8%, and at HQC level they were 2.5% and 101.6%, respectively.

Linearity

The nine-point calibration curve was found to be linear over the concentration range of 8.0–1600.0 ng/mL for ritonavir and 50.5–5995.2 ng/mL for atazanavir. After comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration-detector response relationship for both the analytes in human plasma. The mean correlation coefficient of the weighted calibration curves generated during the validation was 0.99.

Precision and accuracy

As shown in **Table 1,** the precision and accuracy of each analyte in the intra-day and inter-day runs were within ± 15% at LQC, MQC-1, MQC-2 and HQC concentrations and within ± 20% at LLOQ QCs.

Extraction efficiency

Six replicates at low, medium and high quality control concentration for ritonavir and atazanavir were prepared for recovery determination. The recoveries of analytes and IS were good and reproducible. The mean overall recoveries (with the precision range) of ritonavir, atazanavir and IS were 89.1±0.8% (0.9-2.5%), 78.5±1.6% (1.4-3.7%) and 90.2% (1.89-3.40%), respectively.

Dilution integrity

The upper concentration limits can be extended to 2702.7 ng/mL for ritonavir and 9745.0 ng/mL for atazanavir by 1/2 and 1/4 dilutions with screened human blank plasma. The mean back calculated concentrations for 1/2 and 1/4 dilution samples were within 85-115% of their nominal value. The coefficients of variation (%CV) for 1/2 and 1/4 dilution samples were less than 5%.

Table1. Precision &Accuracy data for Ritonavir and Atazanavir in human plasma samples.

Analyte	Concentration added (ng/mL)	Intra-day precision and accuracy (*n*=12; 6 from each batch)			Inter-day precision and accuracy (*n*=30; 6 from each batch)		
		Concentration found (mean; ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean; ng/mL)	Precision (%)	Accuracy (%)
Ritonavir	8.04	7.3	4.8	90.6	7.5	5.3	93.6
	23.8	21.3	2.5	89.4	22.2	6.2	93.2
	104.7	100.5	2.8	96.0	102.5	3.8	97.9
	805.5	772.7	2.2	95.9	803.0	5.6	99.7
	1388.9	1351.2	2.0	97.3	1359.3	3.0	97.9
Atazanavir	50.6	47.2	3.4	93.2	47.4	3.1	93.7
	151.1	149.7	2.2	99.1	147.0	4.0	97.3
	899.5	902.6	1.6	100.3	895.5	3.6	99.5
	2998.5	2929.1	2.1	97.7	2876.4	4.5	95.9
	4997.4	4605.5	6.9	92.2	4623.3	5.3	92.5

Table 2. Stability data for ritonavir and atazanavir in human plasma samples (*n*=6).

Analytes	Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Accuracy/ Stability (%)	Precision (%)
Ritonavir	Process[a]	23.8	23.2 ± 1.0	97.8	4.5
		1388.9	1435.0 ± 49.7	103.3	3.5
	Process[b]	23.8	23.7 ± 1.3	99.5	5.5
		1388.9	1465.0 ± 19.2	105.5	1.3
	Bench top[c]	23.8	23.3 ± 0.6	97.8	2.5
		1388.9	1471.4 ± 37.1	105.9	2.5
	FT[d]	23.8	23.2 ± 0.7	97.8	2.8
		1388.9	1458.0 ± 39.6	105.0	2.7
	Reinjection[e]	23.8	21.3 ± 0.6	89.7	2.6
		1388.9	1370.6 ± 22.1	98.7	1.6
	Long-term[f]	23.8	22.8 ± 0.7	96.1	3.3
		1388.9	1459.1 ± 26.8	105.1	1.8
Atazanavir	Process[a]	151.1	168.5 ± 4.0	111.5	2.4
		4997.4	5297.4 ± 58.8	106.0	1.1
	Process[b]	151.1	163.1 ± 1.7	107.9	1.1
		4997.4	5165.1 ± 60.3	103.4	1.2
	Bench top[c]	151.1	156.4 ± 2.7	103.5	1.8
		4997.4	4860.3 ± 85.5	97.3	1.8
	FT[d]	151.1	162.3 ± 2.9	107.4	1.8
		4997.4	5105.3 ± 51.8	102.2	1.0
	Reinjection[e]	151.1	142.6 ± 1.8	94.3	1.3
		4997.4	4398.1 ± 45.4	88.0	1.0
	Long-term[f]	151.1	158.5 ± 4.5	104.9	2.9
		4997.4	4959.9 ± 40.3	99.2	0.8

[a] after 60 h in autosampler at 10°C; [b] after 55 h in refrigerator at 2-8°C; [c] after 14 h at room temperature; [d] after four freeze and thaw cycles; [e] after 36 h of Reinjection; [f] at -70°C for 52 days

Table 3. Pharmacokinetic parameters of ritonavir and atazanavir (*n*=6, Mean±SD).

Parameter	Ritonavir	Atazanavir
C_{max} (ng/mL)	1216.5±123.9	3747.6±241.0
t_{max} (h)	3.5±0.9	2.0±0.6
AUC_{0-t} (ng h/mL)	8051.4±1544.6	43329.6±4683.0
AUC_{0-inf} (ng h/mL)	8093.2±1563.3	44584.9±5157.0
$t_{1/2}$ (h)	5.9±1.4	12.1±4.1

Stability studies

In the different stability experiments carried out viz. bench top stability (14 h), autosampler stability (60 h), repeated freeze-thaw cycles (4 cycles), reinjection stability (36 h), wet extract stability (55 h at 2-8 °C) and long term stability at –70 °C for 52 days the mean % nominal values of the analytes were found to be within ±15% of the predicted concentrations for the analytes at their LQC and HQC levels **(Table 2).** Thus, the results were found to be within the acceptable limits during the entire validation.

Pharmacokinetic study results

In order to verify the sensitivity and selectivity of this method in a real-time situation, the present method was used to test for ritonavir and atazanavir in human plasma samples collected from healthy male volunteers (*n* = 6). The mean plasma concentrations vs time profiles of ritonavir and atazanavir are shown in **Figure 4** and the corresponding pharmacokinetic parameters are listed in the **Table 3.** The results confirm that the assay is suitable for clinical pharmacokinetic studies of ritonavir and atazanavir.

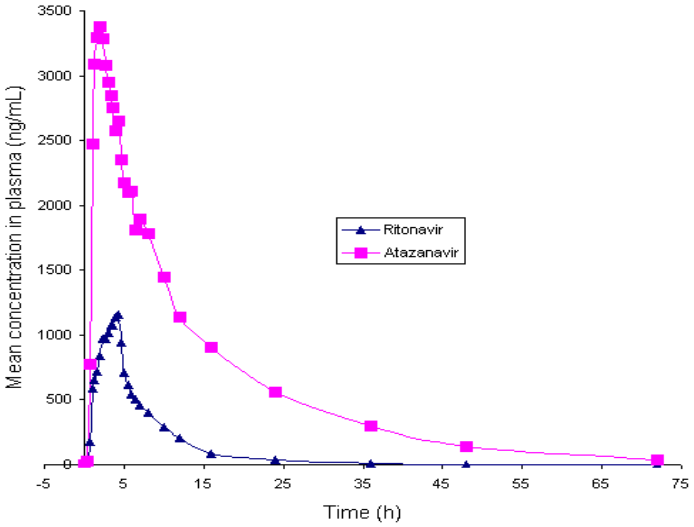


Figure 4. Mean plasma concentration-time profile of ritonavir and atazanavir in human plasma following oral dosing of ritonavir (100 mg) and atazanavir (300 mg) tablet to healthy volunteers (*n*=6).

CONCLUSIONS

The LC-MS/MS assay method described in this paper is rapid, simple, specific and sensitive for quantification of ritonavir and atazanavir in human plasma and is fully validated as per the FDA guidelines. Simple, rapid, reproducible validated methods are essential for the determination of ritonavir and atazanavir concentrations in human plasma for bioequivalence studies. The method was found to be suitable for pharmacokinetic studies in humans. The solid-phase extraction method gave consistent and reproducible recoveries for the analytes from plasma. A sample turnover rate of less than 2.0 min makes it an attractive procedure in high-throughput bioanalysis of ritonavir and atazanavir. From the results of all the validation parameters, we can conclude that the developed method can be useful for BA/BE studies and routine therapeutic drug monitoring (TDM) with desired precision and accuracy.

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