Synopsis of PhD Thesis Entitled

"Application of Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) for the Quantitation of HIV Protease Inhibitor Drugs in Biological Fluids"

Submitted
To

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IN

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BY

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UNDER THE SUPERVISION OF

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Application of Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) for the Quantitation of HIV Protease Inhibitor Drugs in Biological Fluids

Majority of physiological processes rely on regulation of proteolytic enzyme activity and there can be dramatic consequences when equilibrium between an enzyme and its substrates is disturbed. In this perspective, the discovery of small-molecule, like protease inhibitors, that can modulate catalytic activities has an enormous therapeutic effect [1]. Protease inhibitors are a class of compounds used in the treatment of human immunodeficiency virus (HIV) infection and their development is regarded as major success of structure-based drug design [2]. They are highly effective against HIV and have, since the 1990s, been a key component of anti-retroviral therapies for HIV/AIDS [3].

This class of drugs inhibits the HIV-1 protease, which acts to process viral proteins essential for the completion of the viral life cycle and subsequent infection of other cells [4]. Since these drugs are typically part of multi-drug regimens, there is a need for specific methods to assess pharmacokinetic parameters in combination therapy as well as in monotherapy to identify complex drug interactions and the emerging role of therapeutic drug monitoring (TDM) [5].

For the quantitative determination of drugs in biological matrix such as whole blood, plasma, serum, tissue and urine, bioanalysis of drug substance and drug product has been considered as a sub discipline of analytical chemistry. It has witnessed a significant flow, both in the development of more selective and effective drugs and in understanding their therapeutic and toxic effects. Bioanalytical methods include analytical methods that are used in monitoring of drugs in biological materials. Such methods have different purposes:

(i) To evaluate the pharmacokinetics and the metabolism in drug discovery of new candidates,

(ii) To compare the pharmacokinetic profiles of newly developed generic drug formulations,

(iii) To perform routine drug monitoring in order to establish appropriate dosage schemes, to reveal inter-individual metabolic variability and to minimize adverse effects, and

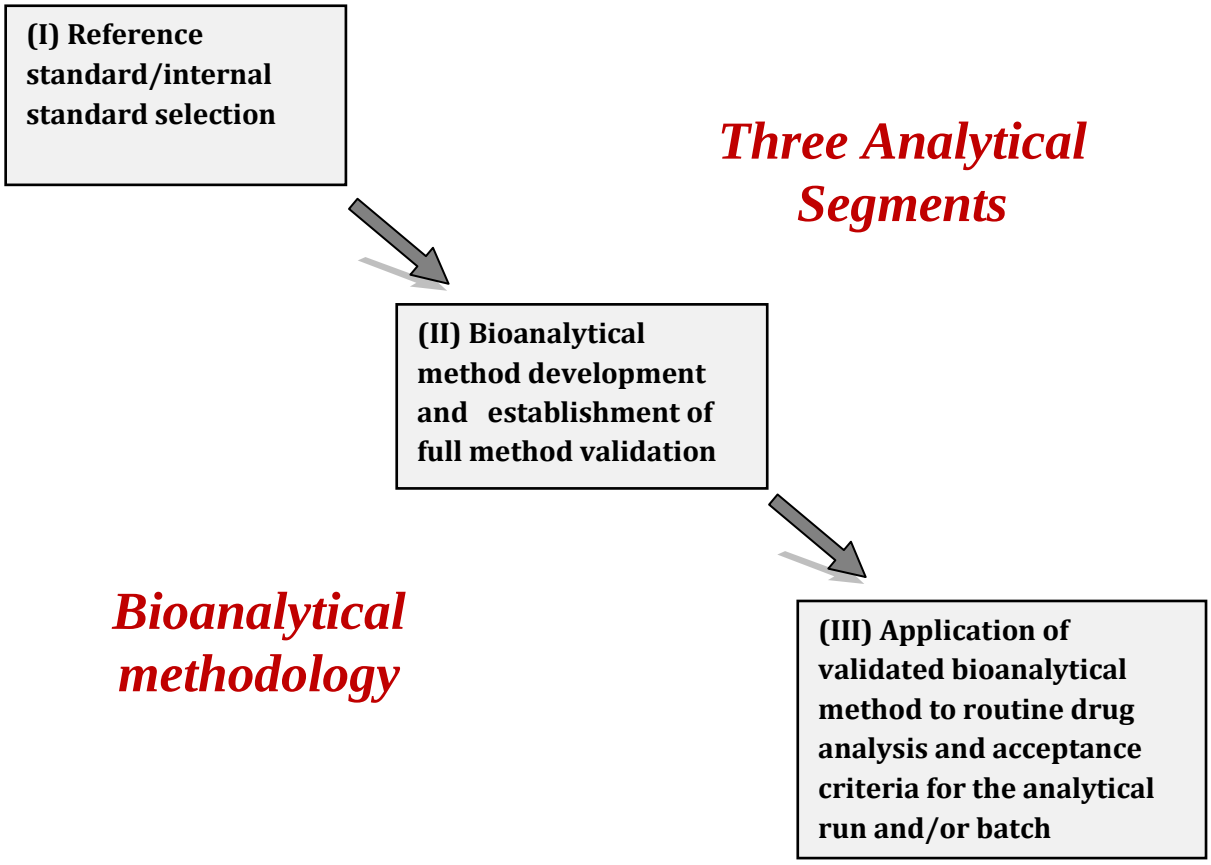
(iv) To determine drugs and their metabolites in a specific biological fluid.

The proposed role for TDM of protease inhibitors is that it may allow practitioners to better determine and maintain appropriate plasma concentrations, identify interactions with other medications, and assess adherence to medication [6]. At the same time it also allows the

optimization of pharmacotherapy and provides a basis for the evaluation and interpretation of patient compliance, bioavailability and bioequivalence. With a greater number of drugs being co-administered, more data is needed to elucidate this information. Thus, the development of efficient analytical techniques capable of determining information about multiple species is desired.

The use of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS has emerged as the developmental method of choice in supporting clinical and pre-clinical pharmacokinetic studies. Additionally, this hyphenated analytical tool is becoming more and more important in clinical-chemical analysis. This is based on the ability of this technique to provide superior specificity, extraordinary selectivity, speed and sensitivity, as compared to commonly used high pressure liquid chromatography with ultra-violet absorbance detection (HPLC-UV) methods [7]. In the pharmaceutical industry LC-MS/MS is already an established method for quality control and quantification of drugs in different matrices.

The process by which a specific bioanalytical method is developed, validated and used in routine sample analysis can be divided into three elements, which are shown in the form of a flow chart. The main analytical segments that comprise the bioanalytical methodology are method development, method validation and application in routine sample analysis.



✓ **THE CONTENTS OF THE THESIS ARE DIVIDED INTO SEVEN CHAPTERS**

CHAPTER – 1

Introduction

BACKDROP

This chapter provides a comprehensive description of bioanalytical method development and validation for single drug and their binary or ternary mixtures in biological human matrices and their significance in the bioequivalence studies. Special prominence has been directed towards the use of liquid chromatography as the separation technique with mass spectrometer as the detector in development and validation of methods and their relevant application in various fields. Validation parameters are explained as per the current guidelines laid down by United States Food and Drug Administration (USFDA). The foremost objectives of the work endeavored are presented with an aim to develop rational, sensitive and selective methods which are competent enough for rapid determination of drugs using low biological sample volume.

The importance of sample preparation techniques such as solid phase extraction (SPE), liquid-liquid extraction (LLE) and protein precipitation (PP) have been discussed. Efficient and quantitative extraction of drugs from biological matrices assists in achieving desired sensitivity and selectivity during the method development.

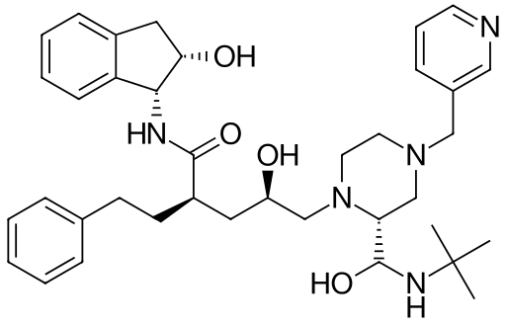
AIM AND OBJECTIVES

- To develop and validate high throughput, sensitive and rugged bioanalytical methods for routine sample analyses based on LC-MS/MS detection.
- To realize efficient and selective extraction methodologies for quantitative extraction of selected drugs from human plasma by employing suitable extraction technique viz. SPE, PP or LLE.
- The developed methods should have the following merits:
  High sensitivity (lower limit for quantitation), High throughput (shorter analytical run time), Quantitative and precise recovery of drugs and their active metabolites, Rugged enough for routine sample analysis (human subjects)
- To apply these validated methods on their formulations for the study of bioequivalency or pharmacokinetics in healthy volunteers/subjects.

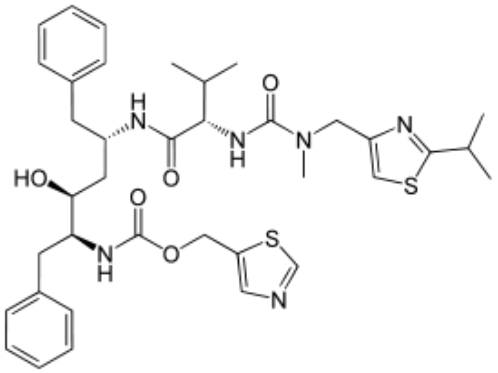
The following important protease inhibitors as single analyte or in binary and ternary combinations have been studied for their bioanalytical method development, method

validation and their application to bioequivalence / bioavailability studies in human plasma in the present work.

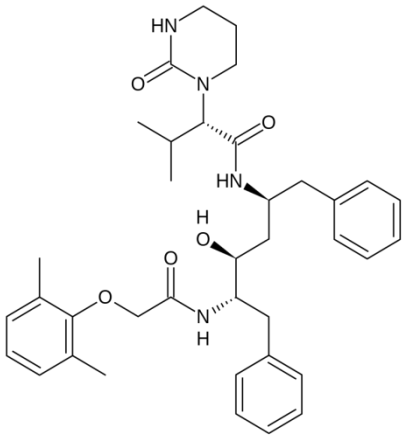
1. ***Indinavir***
2. ***Lopinavir and Ritonavir***
3. ***Ritonavir, Lopinavir and Indinavir***
4. ***Atazanavir***
5. ***Atazanavir, Darunavir and Ritonavir***



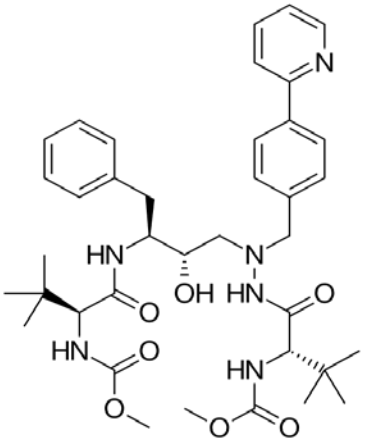
Indinavir



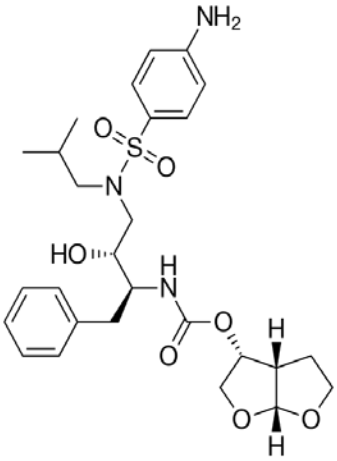
Ritonavir



Lopinavir



Atazanavir



Darunavir

Figure 1: Structure of selected HIV protease inhibitors in the present work

LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Chromatography, a physical method of separation in which the components/solutes to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction. It is an analytical tool widely

employed for the separation, identification of chemical/pharmaceutical components in complex mixtures. The components must interact with the stationary phase to be retained and separated by it. The mobile phase may be a gas, liquid or a supercritical fluid which moves over or through the stationary phase, carrying the components along with it. Mass spectrometer is generally used for quantification of compounds in different biological matrices/complex mixtures. Analysis of drugs LC-MS/MS is extensively used in pharmaceutical industry.

Mass spectrometry has progressed to become a powerful analytical tool for both quantitative and qualitative applications. Over the past decade, mass spectrometry has undergone tremendous technological improvements allowing for its application to proteins, peptides, carbohydrates, DNA, drugs, and many other biologically relevant molecules. Due to ionization sources such as electrospray ionization and matrix-assisted laser desorption/ ionization (MALDI), mass spectrometry has become an irreplaceable tool in the biological sciences. The mass spectrometry principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments and to measure their mass-to-charge ratio.

High performance liquid chromatography (HPLC) coupled with mass spectrometry is an extremely powerful and indispensable methodology practiced in virtually every stage of pharmaceutical discovery and development process, including biological target discovery, biological assay for high throughput screening, characterization of physiochemical properties of drug candidates, drug metabolism and pharmacokinetics. Compounds are separated on column in HPLC and then enter into mass spectrometer where they are first ionized in the source (parent ions). ESI, APCI and APPI are the ionization techniques mostly used for analysis of pharmaceutical compounds in biological fluids.

SAMPLE PREPARATION

With LC-ESI-MS/MS, several issues must be considered. One in particular is unstable instrument response due to ion-suppression which arises when co-eluting ions within a matrix reduce the ion intensity of the analytes, thus affecting quantitative reproducibility. One of the protease inhibitors indinavir has been shown to be subject to such matrix effects. For reliable quantitation, the belief that very little, if any, sample preparation is needed is typically untrue. Therefore, it is critical that any method developed by LC-MS/MS for quantitation of clinical samples be thoroughly characterized, especially for accuracy in various matrices. This is increasingly important as a greater number of analytes are included in one analysis. Thus, it is required to extract the drug from biological matrix before injecting it into LC-MS/MS. Also,

the sample clean-up plays an important role as it results in proper quantification of drugs. Due to sample clean-up, ion suppression or enhancement caused by endogenous impurities from biological fluids can be minimized. Protein precipitation, liquid-liquid extraction and solid phase extraction are the extraction methods generally used to extract the drugs from biological matrices.

BIOANALYTICAL METHODOLOGY

A bioanalytical method is a set of all the procedures involved in the collection, processing, storing, and analysis of a biological matrix for an analyte [8]. Analytical methods employed for quantitative determination of drugs in biological fluids are the key determinants in generating reproducible and reliable data for sample analysis**.** Method development involves evaluation and optimization of the various stages of sample preparation, chromatographic separation, detection and quantitation.

Method validation involves all the procedures required to demonstrate that a particular method for quantitative determination of the concentration of an analyte (or a series of analytes) in a particular biological matrix is reliable for the intended application [9-12]. Validation is also a proof of the repeatability, specificity and suitability of the method. Bioanalytical methods must be validated if the results are used to support the registration of a new drug or a new formulation of an existing one. Validation is required to demonstrate the performance of the method and reliability of analytical results [13-14]. The fundamental parameters to ensure the acceptability of the performance of a bioanalytical method validation are (1) system suitability, (2) system performance, (3) carryover effect, (4) selectivity towards endogenous and exogenous matrix components, (5) sensitivity, (6) accuracy and precision, (7) recovery, (8) stability and (9) dilution integrity [15-17].

At patent expiration of a brand drug, generic versions that demonstrate bioequivalence to the innovator’s product may be marketed via the Abbreviated New Drug Application (ANDA) process. In order to demonstrate the bioequivalence of two proprietary preparations of the same drug molecule, studies must be conducted to show an equivalent rate and extent of bioavailability of the two products.

Relative bioavailability or bioequivalence between drugs, administered by the same extra vascular route, may be evaluated by comparing pharmacokinetic parameters related to bioavailability, i.e., to the quantity absorbed and to the rate of the absorption process. Bioequivalent drugs are pharmaceutical equivalents (same pharmaceutical formulation and

quantity of the same active ingredient) that, when given in the same molar dose, in the same condition, does not present significant statistical differences regarding bioavailability [18].

CHAPTER - 2

Experimental

This chapter provides details of materials, working standards, chemicals and solvents used during method development, validation and study sample analysis. It gives a brief idea on experiments need to be performed in method validation and their acceptance criteria. Method summary that includes chromatographic conditions with extraction procedure and bioequivalence study conditions are also illustrated in a tabular form.

Table 1: Optimized liquid chromatographic conditions for the selected drugs

Name of drug	Mobile phase (Buffer solution/additive:organic solvent), pH	Flow rate (mL/min)	Column make, type and dimensions	Total LC Run time (min)
Indinavir	2 mM ammonium formate, pH 4.1 adjusted with formic acid & acetonitrile (20: 80, *v/v*)	0.400	Zorbax XDB-C8, 50 × 2.1 mm, 5µm	3.0
Lopinavir & Ritonavir	10 mM ammonium formate, pH 4.0 adjusted with formic acid & methanol (10:90, *v/v*)	0.300	Waters Acquity UPLC BEH C18, 50 × 2.1mm, 1.7 µm	1.2
Ritonavir, Lopinavir & Indinavir	0.1% formic acid and methanol (40:60, *v/v*)	0.200	Waters Acquity UPLC BEH C18, 50 × 2.1mm, 1.7 µm	2.7
Atazanavir	5 mM ammonium formate in water: methanol (10:90, *v/v*)	0.700	Hypersil Gold C18, 50 × 4.6mm, 5µm	2.5
Atazanavir, Darunavir & Ritonavir	10 mM ammonium formate, pH 4.0 adjusted with formic acid and acetonitrile	0.300	Waters Acquity UPLC C18, 50 × 2.1mm, 1.7 µm	2.0

Table 2: Summary of MS transitions of the selected drugs

Drug Name	MS Detection in positive polarity with unit resolution in Q1 and Q3		
	Precursor Ion (*m/z*)	**Product Ion (*m/z*)**	**Dwell time (msec)**
Indinavir	614.4	421.2	200
Lopinavir	629.3	447.4	200
Ritonavir	721.3	296.3	200
Atazanavir	705.3	167.9	200
Darunavir	548.1	392.0	200

CHAPTER - 3

Determination of Indinavir, an HIV-1 Protease Inhibitor in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry

An LC-MS/MS method is discussed for the determination of indinavir in human plasma and the validation data is presented. The analyte and internal standard are isolated from plasma by a simple acetonitrile precipitation of plasma proteins followed by centrifugation. LC-tandem mass spectrometry in positive ion, multiple reaction monitoring mode used pairs of ions at *m*/*z* of 614.4/421.2 for indinavir and *m*/*z* of 628.4/421.2 for internal standard, respectively. The calibration curve had a linear range from 10.0 to 10000 ng/ml when linear least square regression weighing 1/*x* was applied to the concentration versus peak area plot. The advantages of this method are the fast sample preparation, wide dynamic assay range and quick analysis taking only 2.5 min for each sample run. The robust nature of this assay has been further verified during routine use over several months involving multiple analysts. The method was successfully applied to a bioequivalence study of test and reference formulation of indinavir in selected subject samples.

Indinavir is a potent inhibitor of human immunodeficiency virus (HIV) proteases [19, 20] now prescribed in combination therapy potentially with another protease inhibitor and one or more nucleoside analogs or non-nucleoside reverse transcriptase inhibitors or with two reverse transcriptase inhibitors. Determination of the indinavir concentrations in body fluids

including serum, plasma and cerebrospinal fluid is of importance in conducting clinical studies of this drug with regard to efficacy, toxicity and dose ranging. Modern pharmacokinetic studies require parts per billion characterization and quantification, as well as, the ability to provide analytical results with rapid turnaround from large batches of samples [21].

The proposed validated method for the estimation of indinavir in human plasma is highly sensitive and rapid compared to published reports. The method offers significant advantages over those previously reported, in terms of lower sample requirements (100 µL), simplicity of extraction procedure and overall analysis time. The linear dynamic range established was adequate to measure the plasma concentration of indinavir in a clinical study involving Indian subjects. Extracted ion chromatograms from a patient sample with indinavir and added internal standard (methyl derivative of indinavir) are illustrated in **Figure 2**.

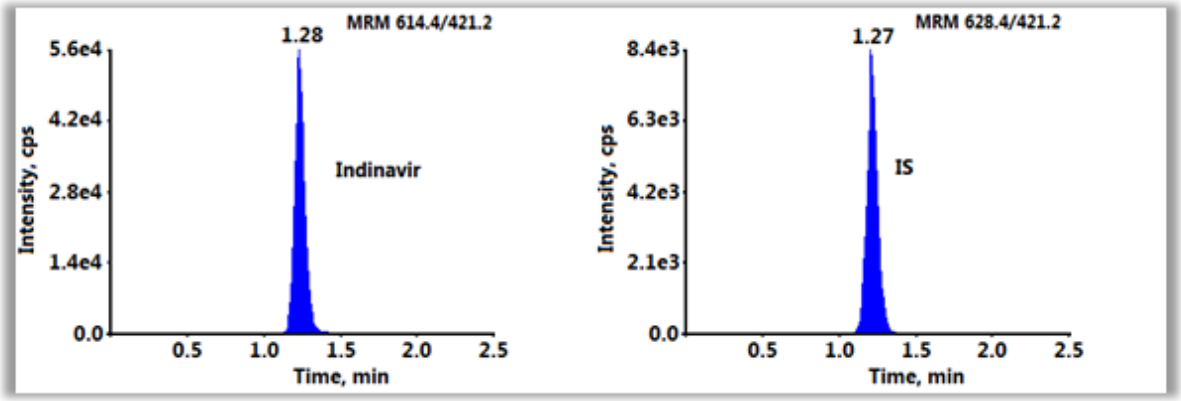


Figure 2: Extracted ion chromatograms of plasma sample with indinavir and internal standard

CHAPTER – 4

Simultaneous Determination of Lopinavir and Ritonavir in Human Plasma by UPLC-ESI-MS/MS and its Application to a Bioequivalence Study

Lopinavir (LPV) is a novel peptidomimetic HIV protease inhibitor developed from and structurally related to ritonavir (RTV). Both these drugs are currently marketed under the brand name Kaletra in a fixed-dose combination (133mg LPV and 33mg RTV) [22, 23]. The low-dose of ritonavir significantly improves the pharmacokinetic properties and hence the activity of LPV against HIV-1 protease. RTV inhibits the cytochrome P450 CYP3A isoenzymes that inactivate LPV, thereby increasing its circulating levels. Co-formulated

LPV/RTV-based regimens provide adequate and durable suppression of viral load and sustained improvements in CD4+ cell counts [24]. Due to their extensive binding to plasma proteins, essentially α1-acid glycoprotein and albumins (98-99%), they have limited distribution in the body. LPV and RTV are extensively metabolized by the liver and are eliminated in the urine and faces. Their half-life range varies from 3-6h [25]. The simultaneous determination of these drugs in biological matrices, along with their pharmacokinetic study can assist in checking their effectiveness, treatment compliance, to prevent adverse events, and to formulate optimum dosages. Due to extensive use of both these protease inhibitors in HAART, it has become essential to develop competent bioanalytical assays for their routine measurement in subject samples.

The UPLC-MS/MS methodology presented for simultaneous estimation of LPV and RTV in human plasma is highly selective and rugged for routine measurement of these drugs in combination therapy. The wide linear dynamic range of 2.9–1452 ng/mL for RTV and 29.6–14379 ng/mL for LPV ensures measurement of the drugs in all currently available formulations of Kaletra with different strengths. The method involved an efficient and specific sample preparation by solid phase extraction followed by isocratic chromatographic separation in 1.2min. The small plasma requirement for processing is beneficial, especially for patients infected with HIV. The method was developed following USFDA and ICH guidelines for regulated bioanalysis, which included key elements for bioanalytical method validation of small molecules and bioavailability/bioequivalence study. The method was successfully applied to a bioequivalence study of [200 (lopinavir) + 50 (ritonavir)] mg tablet formulation in 36 healthy human subjects under fasting conditions. **Figure 3** shows the mean plasma concentration-time profile following oral administration of test and reference tablet formulation to 36 healthy Indian male subjects under fasting condition.

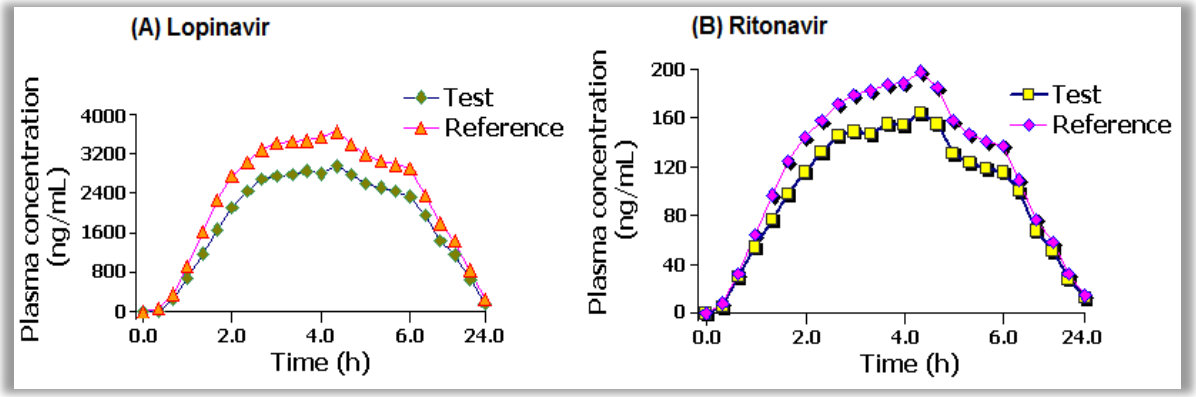


Figure 3: Mean plasma concentration-time profile of (A) lopinavir and (B) ritonavir

CHAPTER-5

Simultaneous Quantitation of HIV-protease Inhibitors Ritonavir, Lopinavir and Indinavir in Human Plasma by UPLC-ESI-MS/MS

The best way to avoid drug resistance is to stop or reduce HIV production in the body. The less HIV made in the body, the less chance of a virus created that's resistant to anti-HIV drugs. To keep HIV levels as low as possible, it's recommended that protease inhibitors be taken in combination with at least two other anti-HIV drugs. This is called Highly Active Anti-Retroviral Therapy or HAART [26, 27]. Studies have shown that when certain protease inhibitors are combined, the anti-HIV effect is increased, which can help prevent or overcome resistance. This is called Protease Boosting. Some doctors will order a drug resistance test to determine a patient's resistance profile, in order to decide the best drug combination to use.

Thus present method describes a selective, sensitive and high-throughput ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS-MS) method has been developed and validated for the quantification of HIV-protease inhibitors ritonavir (RTV), lopinavir (LPV) and indinavir (IDV) in human plasma.

Sample clean-up involved protein precipitation of both drugs and fluconazole used as internal standard from 100 mL human plasma. All the analytes were chromatographically separated on a Waters Acquity UPLC BEH C18 (2.1 × 50 mm, 1.7 mm particle size) analytical column using 0.1% formic acid and methanol (40:60, *v/v*) as the mobile phase. The parent→product ion transitions for ritonavir (*m/z* 721.40 → 296.10), lopinavir (*m/z* 629.40 → 447.40), indinavir (*m/z* 614.4 → 421.0) and IS (*m/z* 307.10 → 220.10) were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring and positive ion mode. The method was validated over the concentration range of 30–15,000 ng/mL for LPV & IDV and 3–1500 ng/mL for RTV.

The inherent selectivity of MS-MS detection was also expected to be beneficial in developing a selective and sensitive method. The present study was conducted using electrospray ionization as the ionization source because it gave high intensity for ritonavir, lopinavir, indinavir and IS, and a good linearity in regression curves. The intensity found was much higher in the positive mode for ritonavir, lopinavir and indinavir because they have similar sites for protonation. Also, the use of formic acid, pH 3.00, in the mobile phase further augmented the response of protonated precursor $[M + H]^+$ ions in the Q1 MS full scan spectra. **Figure 4** shows the most abundant and consistent product ions produced in Q3 MS spectra for RTV, LPV, IDV and IS respectively.

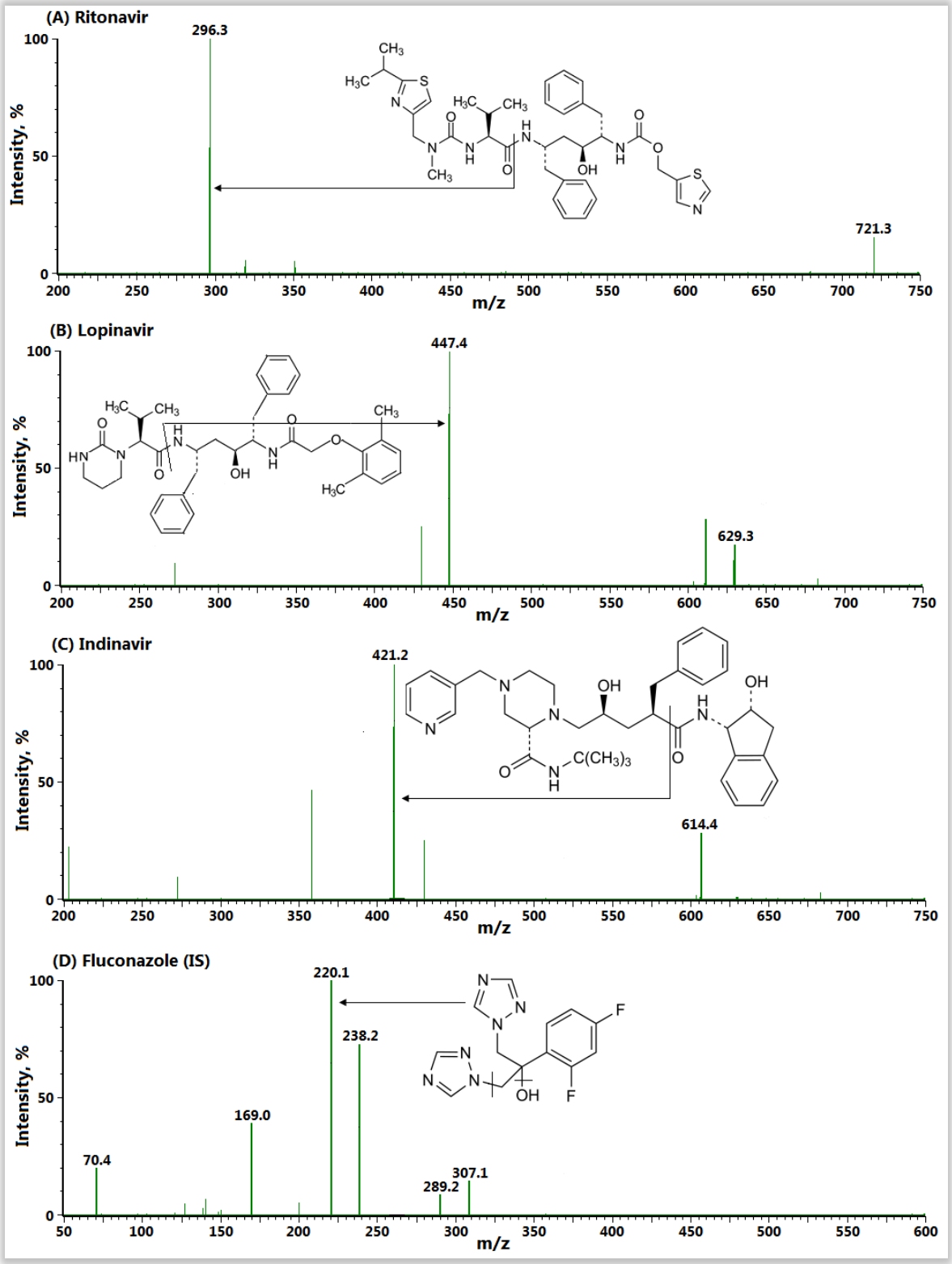


Figure 4: Product ion mass spectra in positive ionization mode: ritonavir (m/z 721.3→296.3, scan range 200–750 amu) (A); lopinavir (m/z 629.3→447.4, scan range 200–750 amu) (B); indinavir (m/z 614.4→421.2, scan range 200–750 amu) (C) and fluconazole (IS, m/z 307.1→220.1, scan range 50–600 amu) (D)

Figures 5 demonstrate the selectivity experiments with the chromatograms of peak response of LPV, RTV and IDV at LLOQ and IS. The combination of protein precipitation and UPLC–MS-MS detection gave very good selectivity for the analytes and IS. No endogenous peaks were observed at the retention times of LPV (1.71 min), RTV (1.54 min), IDV (0.63 min) and IS (0.68 min) for any of the batches. The analysis time was short, which makes it an attractive method for routine analysis. None of the concomitant medications and antiretrovirals showed interfering signals at the retention times of LPV, RTV, IDV or IS. This demonstrates that the method is highly selective and free from interference due to matrix components and other prescribed medications.

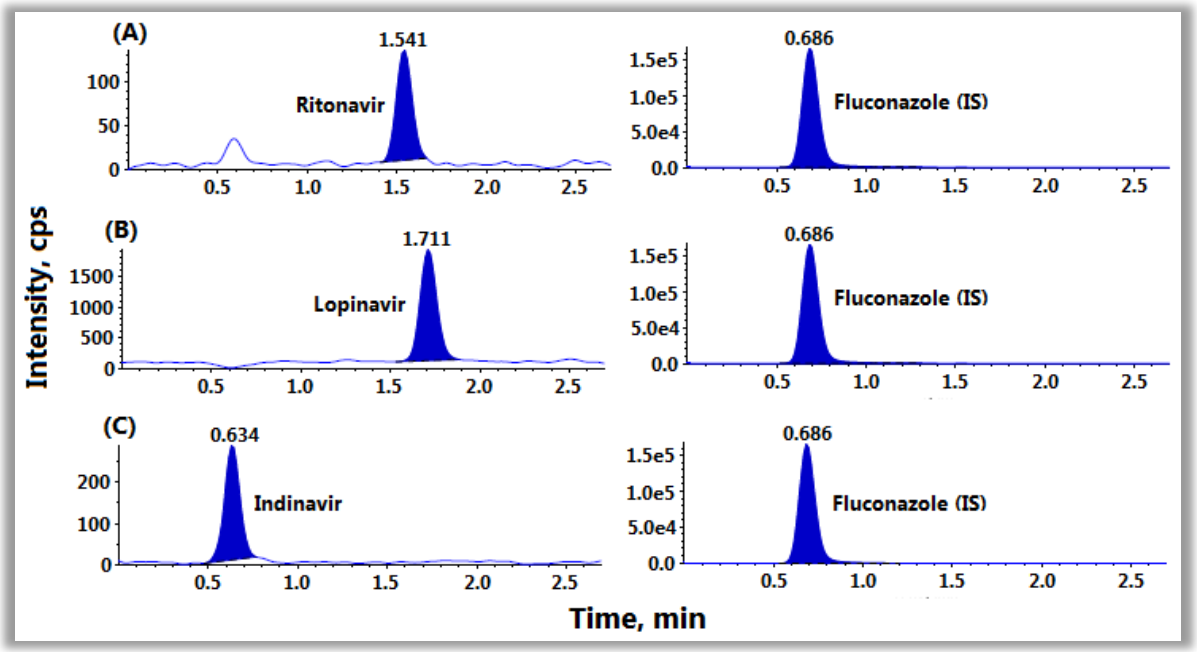


Figure 5: MRM ion-chromatograms of (A) ritonavir (m/z 721.40→ 296.10), (B) lopinavir (m/z 629.40→ 447.40), (C) indinavir (m/z 614.4→ 421.0) at LLOQ and IS (m/z 307.10→ 220.10)

Stock solutions for short term and long term stability of the analytes and IS were stable at room temperature for minimum period of 7 h and between 2-8°C for 7 days respectively. RTV, LPV and IDV in control human plasma (bench top) at room temperature were stable at least for 6 h at 25±3°C and for minimum of three freeze and thaw cycles. Spiked plasma samples stored at –20°C and –70°C for long term stability experiment were stable for minimum of 60 days. Autosampler stability of the spiked quality control samples maintained at 5^0C was also determined up to 23 h. Different stability experiments in plasma and the values for the precision and percent change are shown in **Tables 3**.

Table 3. Stability of lopinavir, ritonavir and indinavir in human plasma under different conditions (n=3)

Storage conditions	% Mean accuracy		
	Lopinavir	**Ritonavir**	**Indinavir**
Bench top stability, 6 h, Ambient			
LQC	101.2	113.0	92.4
HQC	97.7	100.1	91.3
Freeze & thaw stability in plasma, 3 cycles			
LQC	105.3	110.6	94.1
HQC	102.7	100.7	92.5
Wet extract stability, 23 h			
LQC	107.8	110.5	86.6
HQC	102.6	94.8	106.2
Long term stability in plasma; 60 days, -78 °C			
LQC	109.8	106.3	105.6
HQC	101.9	104.5	101.2

CHAPTER – 6

Selective and Reliable Determination of Atazanavir in Human Plasma by LC-ESI-MS/MS

A selective, rugged and high throughput SPE-LC-MS/MS method has been developed for reliable determination of atazanavir in human plasma. The chromatographic separation was achieved on a Hypersil Gold C18 (50 mm × 4.6 mm, 5 µm) analytical column using 5mM ammonium formate in water: methanol (10:90, *v/v*) as the mobile phase under isocratic conditions. The method was validated over a wide dynamic concentration range of 10-6000 ng/mL. The mean relative recovery and absolute matrix effect across quality controls were 84.9 and 93.2 % respectively. The precision value for relative matrix effect between eight different lots of plasma, expressed as % CV of the slopes of the calibration lines was 2.41. The stability of atazanavir under different storage conditions varied from -8.4 to 5.4%. The method was successfully applied to a bioequivalence study of 300 mg atazanavir capsule formulation in 24 healthy Indian males under fasting condition.

Atazanavir (ATV) is an azapeptide human immunodeficiency virus (HIV) type 1 protease inhibitor, which has played a significant role in lowering the morbidity and mortality of HIV. Its unique HIV resistance profile and favourable pharmacokinetics allows once-daily dosing. ATV is metabolized hepatically by CYP3A4 and is a strong inhibitor of this enzyme [28-30]. It is 86% bound to human serum proteins and protein binding is independent of concentration. The oral bioavailability of ATV is significantly enhanced in the presence of food. It is rapidly absorbed with a T_{max} of ~ 2.5h. It can be used alone as first line protease

inhibitor or in combination therapy. ATV is available commercially under the brand name Reyataz® (Bristol-Myers Squibb) capsules in dose strength of 100, 150, 200 and 300 mg atazanavir sulphate [31].

Based on the outcome a reliable and rugged method has been proposed for the analysis of ATV in human plasma with desired sensitivity. Ion suppression/enhancement was studied by post column infusion of analyte and post extraction spiking technique **(Figure 6)**. The present method is highly selective for ATV in presence of endogenous plasma matrix components and nine other antiretroviral drugs (amprenavir, darunavir, ritonavir, lopinavir, tipranavir , saquanavir, nelfinavir, nevirapine and etravirine).

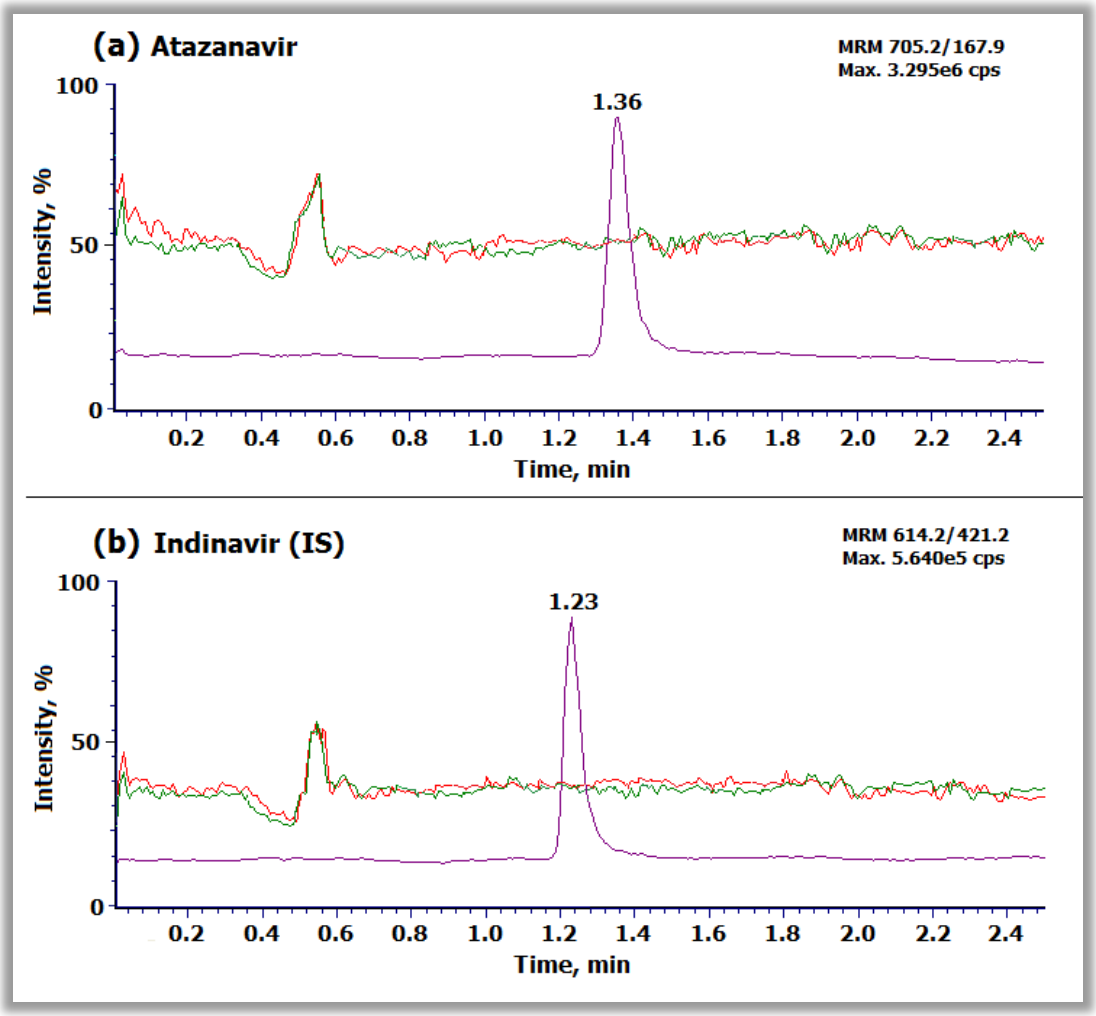


Figure 6: Post column infusion LC-MS/MS chromatograms of two blank plasma extracts from SPE with overlaid chromatograms of atazanavir and indinavir (IS)

The validated method has been successfully used to quantify ATV concentration in human plasma samples after administration of a single 300mg dose of test and reference formulation of atazanavir sulphate. **Figure 7** shows the plasma concentration *vs.* time profile

of atazanavir in human subjects under fasting condition. Incurred sample reanalysis (ISR) study has now become an essential part of the bioanalytical process to assess the quality of bioanalytical assays. It reaffirms the reproducibility and reliability of a validated bioanalytical method. This was done by random selection of subject samples (10% of total samples analyzed). Out of 105 incurred samples studied, 62 samples showed % change for assay reproducibility within ±5 %, while the remaining 43 samples were within ±15 % which falls within acceptance limit [32] as shown in **Figure 8**. This authenticates the reproducibility of the proposed method.

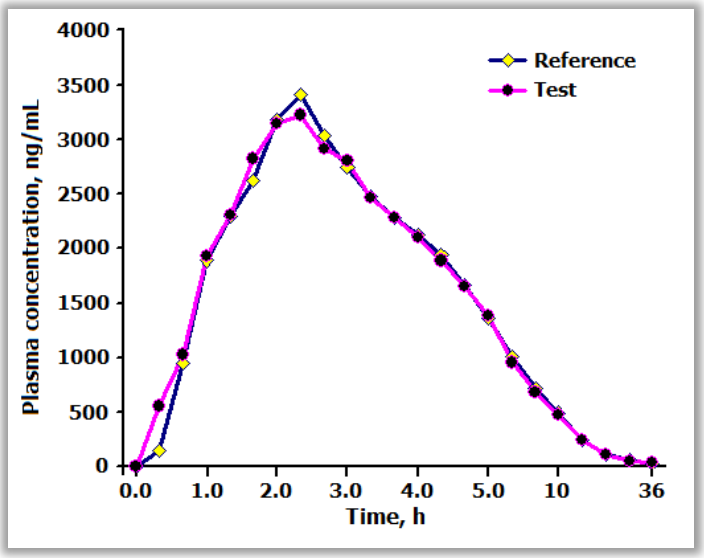


Figure 7: Mean plasma concentration-time profile of atazanavir

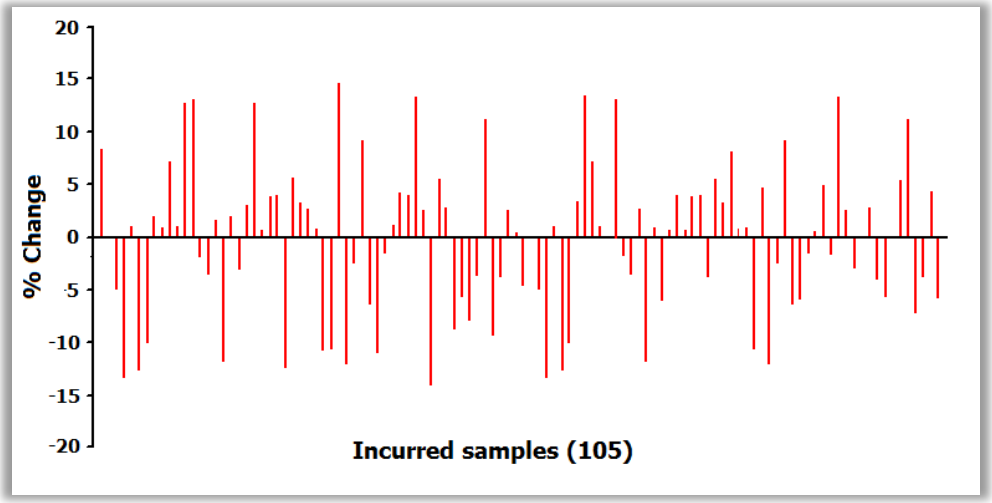


Figure 8: Graphical representation of results for 105 incurred samples of atazanavir

CHAPTER-7

Development and Validation of a Method for the Simultaneous Quantitation of HIV Protease Inhibitors Atazanavir, Darunavir and Ritonavir in Human Plasma by UPLC-MS/MS

The work describes a selective, sensitive and robust method for simultaneous determination of three protease inhibitors atazanavir, darunavir and ritonavir in human plasma by ultra performance liquid chromatography-tandem mass spectrometry. The sample pretreatment consisted of solid phase extraction of analytes and their deuterated analogs as internal standards from 50 µL human plasma. Chromatographic separation of analytes was performed on Waters Acquity UPLC C18 (50 × 2.1 mm, 1.7 µm) column under gradient conditions using 10 mM ammonium formate, pH 4.0-acetonitrile as the mobile phase. The method was established over a concentration range of 5.0-6000 ng/mL for atazanavir, 5.0-5000 ng/mL for darunavir and 1.0-500 ng/mL for ritonavir.

Detection and quantitation of analytes and ISs was carried out using multiple reaction monitoring (MRM) for protonated precursor→ product ion transitions on Quattro Premier XE^{TM} mass spectrometer from Waters – Micro Mass Technologies (MA, USA), in the positive electro spray ionization mode as ATV, DRV and RTV have several secondary amino groups which can be readily protonated. Q1 mass spectra of ATV, DRV, RTV, ATV-d6, DRV-d9 and RTV-d6 contained protonated precursor $[M+H]^+$ ions at *m/z* 705.2, 548.1, 721.3, 711.2, 557.1 and 727.4 respectively. The most abundant and consistent product ions in Q3 mass spectra for ATV, DRV and RTV were observed at *m/z* 167.9, 392.0 and 296.3 by applying collision energy of 44, 17 and 20 eV respectively. These product ion fragments can be attributed to the substructure 4-(pyridin-2-yl)phenylmethyl group in ATV **(Figure 9a)**, elimination of *p*-aminophenyl sulfonyl group from the precursor ion of DRV **(Figure 9b)** and breaking of amide linkage in RTV **(Figure 9c)** respectively. All mass parameters were suitably optimized to obtain a stable and adequate response for the analytes. A dwell time of 200 ms was sufficient and no interference was observed between the MRMs of the analytes and their deuterated ISs.

Accuracy, precision, matrix effect, recovery and stability of the analytes were evaluated as per US FDA guidelines. The efficiency of sample preparation, short analysis time and high selectivity permits simultaneous estimation of these inhibitors. The validated method can be useful to determine plasma concentration of these protease inhibitors for therapeutic drug monitoring and in high throughput clinical studies.

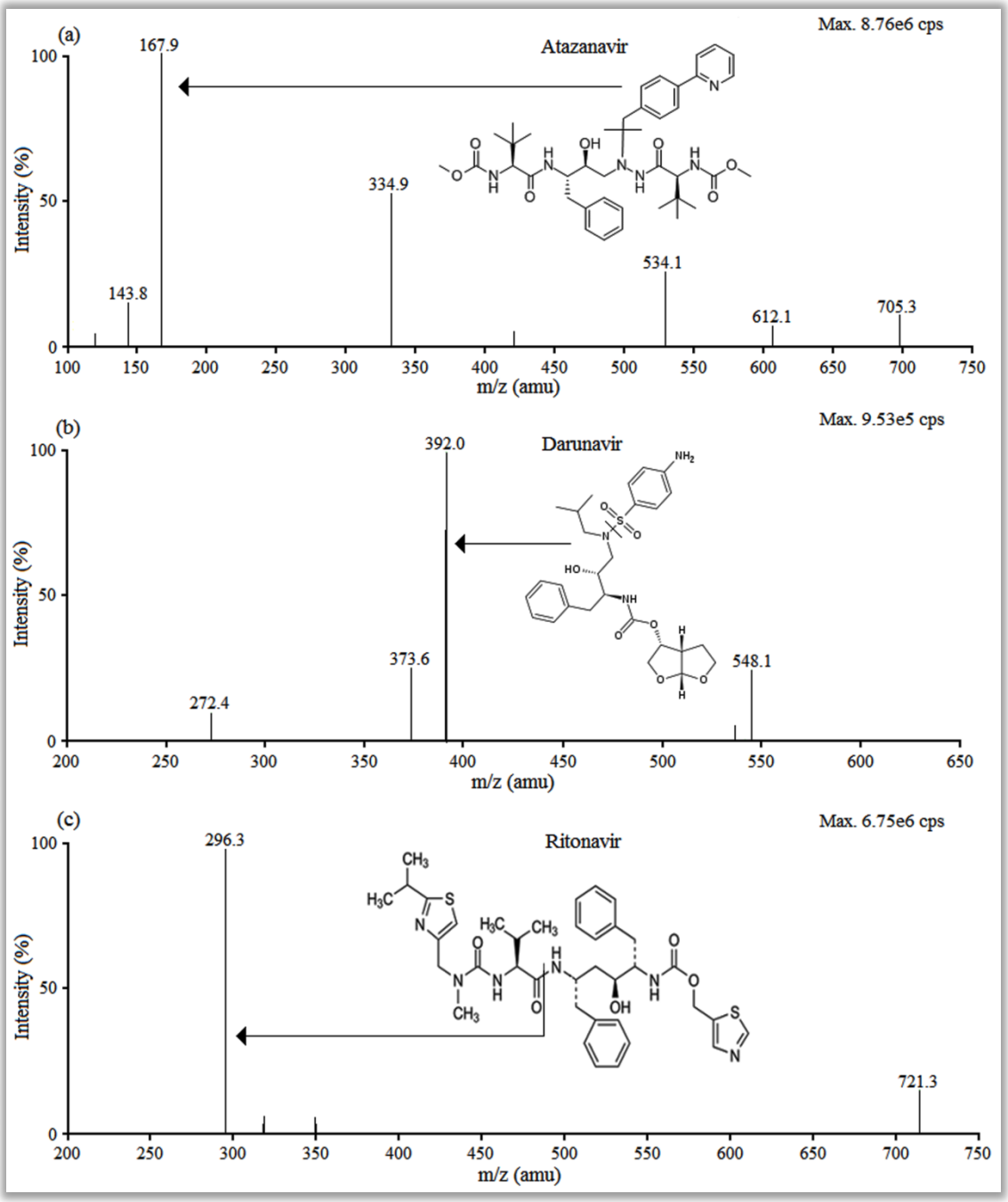


Figure 9: Product ion mass spectra of (a) atazanavir (m/z 705.3 → 167.9, scan range 100-750 amu) and (b) darunavir (m/z 548.1 → 392.0, scan range 200-650 amu) and (c) ritonavir (m/z 721.3 → 296.3, scan range 200-750 amu) in the positive ionization mode

All the analytes were eluted within 2.0 min with retention time of 0.69, 1.02 and 1.54 for ATV, DRV and RTV respectively. The resolution factor (R_s) between ATV & DRV and DRV & RTV was 2.06 and 3.25 respectively. MRM chromatograms for a real subject sample in **Figure 10** confirm the selectivity of the method to distinguish and quantify the analyte from endogenous components in the plasma matrix.

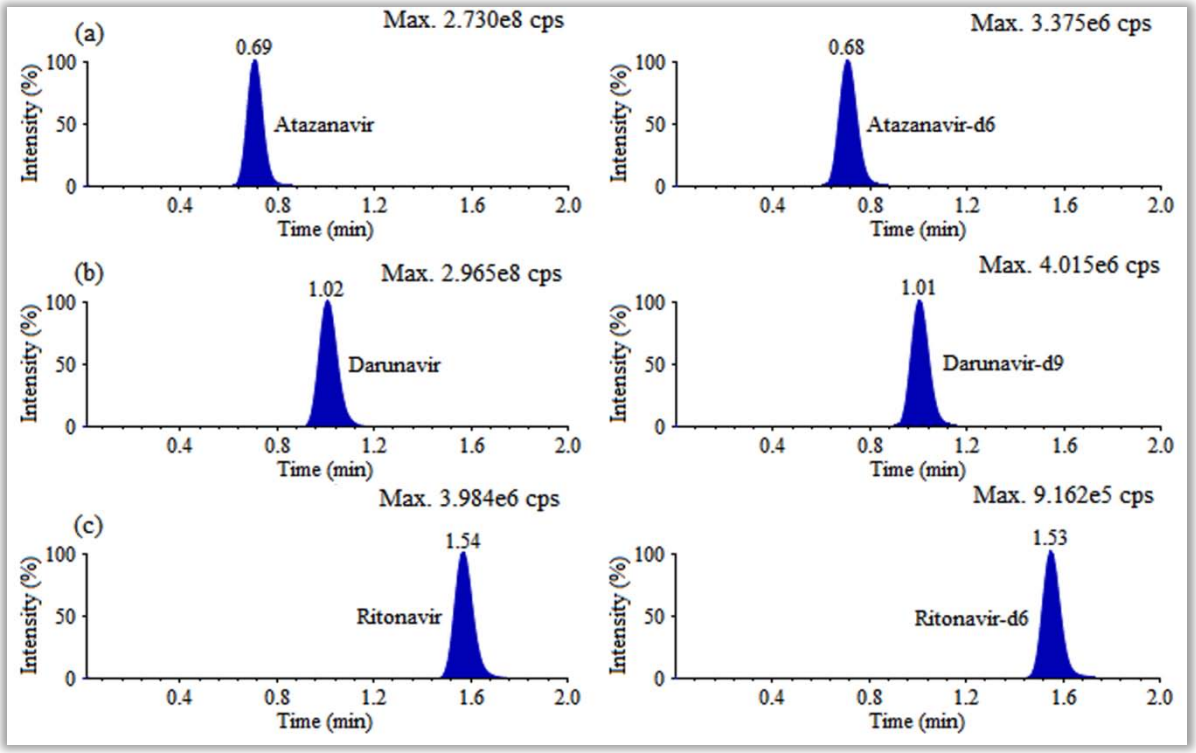


Figure 10: MRM ion chromatograms of atazanavir (a) atazanavir (b) darunavir and (c) ritonavir in real subject sample

The extraction recovery for the analytes are presented in **Table 4**. The mean extraction recovery ranged from 97.35-101.06 for ATV, 97.73-102.30 % for DRV and 98.37-102.12 % for RTV across QC levels.

Table 4. Extraction recovery of ATV, DRV & RTV

QC level	Extraction Recovery (B/A), %					
	ATV	ATV-d6	DRV	DRV-d9	RTV	RTV-d6
LQC	97.35	99.18	98.94	100.07	100.72	98.35
MQC-2	98.33	97.47	102.30	103.05	98.65	99.71
MQC-1	101.06	101.35	97.73	98.81	98.37	97.50
HQC	100.16	101.93	98.48	99.47	102.12	102.47

LQC: low quality control; MQC: medium quality control; HQC: high quality control
A: Mean area response of six replicate samples prepared by spiking in extracted blank plasma
B: Mean area response of six replicate samples prepared by extracting spiked blank plasma

In spite of several existing assay methods for the simultaneous determination of PIs, very few studies have reported the use of UPLC-MS/MS for therapeutic drug monitoring. In this present work, we have developed and fully validated a reliable, precise and sensitive UPLC-MS/MS method for the simultaneous quantification of atazanavir, darunavir and

ritonavir in human plasma. The assay is superior to reported methods with respect to sensitivity, analysis time and matrix effect. The method is rapid and requires small plasma volume for sample processing. Use of deuterated internal standards further reinforces the accuracy and precision of the proposed method and can be suitable for pharmacokinetic/bioequivalence studies.

✓ *REFERENCES:-*

1. M. Cuccioloni, M. Mozzicafreddo, L. Bonfili, V. Cecarini, A.M. Eleuteri, M. Angeletti, *Chem. Biol. Drug Des.* 74 (2009) 1.
2. X. Chen, *et al.*, *Bioorg. Med. Chem. Lett.* 13 (2003) 3657.
3. M. Adachi, *et al.*, *Proc. Natl. Acad. Sci. U.S A.* 106 (2009) 4641.
4. J.A. Levy, HIV and the Pathogenesis of AIDS, 2nd ed, American Society for Microbiology, Washington, DC, 1998, p. 343.
5. V.A. Simon, M.D. Thiam, L.C. Lipford, *J. Chromatogr. B* 913 (2001) 447.
6. V.A. Frerichs, R. DiFrancesco, G.D. Morse, *J. Chromatogr. B* 787 (2003) 393.
7. B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, *Anal. Chem.* 70 (1998) 309.
8. L.R. Snyder, J.J. Kirkland, J.L. Glajch, *Practical HPLC Method Development*, Second Edition., John Wiley & Sons, Inc., 1997.
9. G. Hendriks, D.R.A. Uges, J.P. Franke, *J. Pharm. Biomed. Anal*. 47 (2008) 126.
10. C.J. Briscoe, M.R. Stiles, D.S. Hage, *J. Pharm. Biomed. Anal.* 44 (2007) 484.
11. C.A. James, M. Breda, E. Frigerio, *J. Pharm. Biomed. Anal.* 35 (2004) 887.
12. M. Yadav, V. Upadhyay, P. Singhal, S. Goswami, P.S. Shrivastav, *J. Chromatogr. B* 877 (2009) 680.
13. M. Yadav, T. Mishra, P. Singhal, S. Goswami, P.S. Shrivastav, *J. Chromatogr. Sci.* 47 (2009) 140.
14. B.N. Patel, N. Sharma, M. Sanyal, P.S. Shrivastav, *J. Chromatogr. Sci.* 48 (2010) 704.
15. N.K. Patel, G. Subbaiah, H. Shah, M. Kundlik, P.S. Shrivastav, *J. Chromatogr. B* 877 (2009) 221.
16. B.N. Patel, N. Sharma, M. Sanyal, A. Prasad, P.S. Shrivastav, *Biomed. Chromatogr.* 22 (2008) 1213.
17. B.N. Patel, N. Sharma, M. Sanyal, P.S. Shrivastav, *J. Sep. Sci.* 31 (2008) 301.
18. S.K. Niazi, *Handbook of Bioequivalence Testing*, Informa Healthcare USA, Inc., New York (2007).

19. B.D. Dorsey, R.B. Levin, S.L. McDaniel, J.P. Vacca, J.P. Guare, P. L. Darke, J. A. Zugay, E.A. Emini, W.A. Schleif, J.C. Quintero, *J. Med. Chem.* 37 (1994) 3443.
20. D. Adkin, K. Eng, K. Rossen, R. Puric, K. Wells, R. Volante, P. Reider, *Tetrahedron Lett.* 35 (1994) 673.
21. S.G. Deeks, M. Smith, M. Holodny, J. Kahn, *J. Am. Med. Assoc.* 277 (1997) 145.
22. N. von Hentig, *Drugs* 43 (2007) 221.
23. T.M. Martin, G.D. Morse, J. Kurpewski, R. Difrancesco, A.M. Caliendo, T.P. Flanigan, K.T. Tashima, *AIDS* 20 (2006) 1085.
24. D.J. Porche, *J. Assoc. Nurses AIDS Care* 12 (2001) 101.
25. R.S. Cvetkovic, K.L. Goa, *Drugs* 63 (2003) 769.
26. P. Clevenbergh, S. Mouly, P. Sellier, E. Badsi, J. Cervoni, V. Vincent, *et al., Current HIV Res.* 2 (2004) 309.
27. M. Boffito, E. Acosta, D. Burger, C.V. Fletcher, C. Flexner, R. Garaffo, *et al., Antivir. Ther.* 10 (2005) 375.
28. A. Breckenridge, *Medicine* 37 (2009) 374.
29. E. De Clercq, *Adv. Antiviral Drug Design* 4 (2004) 1.
30. J. E. Ray, D. Marriott, M. T. Bloch, A. J. McLachlan, *Br. J. Clin. Pharmacol.* 60 (2005) 291.
31. Bristol-Myers Squibb Company, Reyataz®, package insert, 2006. http://packageinserts.bms.com/pi/pi.reyataz.pdf. Last assessed on 10/08/2015
32. M. Yadav, P.S. Shrivastav, *Bioanalysis* 3 (2011) 1007.

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