METHOD DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ATAZANAVIR AND RITONAVIR IN BULK AND ITS PHARMACEUTICAL FORMULATIONS

Introduction

The management of HIV/AIDS normally includes the use of multiple antiretroviral drugs in an attempt to control HIV infection. There are several classes of antiretroviral agents that act on different stages of the HIV life-cycle. The use of multiple drugs that act on different viral targets is known as highly active antiretroviral therapy (HAART). HAART decreases the patient's total burden of HIV, maintains function of the immune system, and prevents opportunistic infections that often lead to death. HIV treatment has been proven so successful that in many parts of the world HIV has become a chronic condition in which progression to AIDS has become increasingly rare. Rather than dealing with acute and potentially life-threatening complications, clinicians are now confronted with managing a chronic disease that in the absence of a cure will persist for many decades.

Because of the complexity of selecting and following a regimen, the potential for side-effects, and the importance of taking medications regularly to prevent viral resistance, such organizations emphasize the importance of involving patients in therapy choices and recommend analyzing the risks and the potential benefits. The first effective therapy against HIV was the nucleoside reverse transcriptase inhibitor (NRTI) zidovudine (AZT). It was approved by the US FDA in 1987. Subsequently several more NRTIs were developed but even in combination were unable to suppress the virus for long periods of time and patients still inevitably died. To distinguish from this early anti-retroviral therapy (ART), the term highly active anti-retroviral
therapy (HAART) was introduced. Here are several classes of drugs, which are usually used in combination, to treat HIV infection. Use of these drugs in combination can be termed anti-retroviral therapy (ART), combination anti-retroviral therapy (cART) or highly active anti-retroviral therapy (HAART). Anti-retroviral (ARV) drugs are broadly classified by the phase of the retrovirus life-cycle that the drug inhibits. Typical combinations include 2 NRTIs as a "backbone" along with 1 NNRTI, PI or INSTI as a "base".

Entry inhibitors, also known as fusion inhibitors, are a class of antiretroviral drugs, used in combination therapy for the treatment of HIV infection. This class of drugs interferes with the binding, fusion and entry of an HIV virion to a human cell. By blocking this step in HIV's replication cycle, such agents slow the progression from HIV infection to AIDS. In rare cases, individuals may have a mutation in the CCR5 delta gene which results in a nonfunctional CCR5 co-receptor and in turn, a means of resistance or slow progression of the disease. However as mentioned previously, this can be overcome if an HIV variant that targets CXCR4 becomes dominant.

Nucleoside reverse transcriptase inhibitors (NRTI) and nucleotide reverse transcriptase inhibitors (NtRTI) are nucleoside and nucleotide analogues which inhibit reverse transcription. HIV is an RNA virus and hence unable to become integrated into the DNA in the nucleus of the human cell; it must be "reverse" transcribed into DNA. Since the conversion of RNA to DNA is not done in the mammalian cell it is performed by a viral protein which makes it a selective target for inhibition. NRTIs are chain terminators such that once incorporated, work by preventing other nucleosides from also being incorporated into the DNA chain because of the absence of a 3’ OH group. Both act as competitive substrate inhibitors.
Non-Nucleoside reverse transcriptase inhibitors (NNRTI) inhibit reverse transcriptase by binding to an allosteric site of the enzyme; NNRTIs act as non-competitive inhibitors of reverse transcriptase. NNRTIs affect the handling of substrate (nucleotides) by reverse transcriptase by binding near the active site. NNRTIs can be further classified into 1st generation and 2nd generation NNRTIs.

1st generation NNRTIs include Nevirapine and Efavirenz. 2nd generation NNRTIs are etravirine and rilpivirine.10 HIV-2 is naturally resistant to NNRTIs11. Integrase inhibitors (also known as integrase nuclear strand transfer inhibitors or INSTIs)12 inhibit the viral enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell. Protease inhibitors block the viral protease enzyme necessary to produce mature virions upon budding from the host membrane. Particularly, these drugs prevent the cleavage of gag and gag/pol precursor proteins13.

The life cycle of HIV can be as short as about 1.5 days from viral entry into a cell, through replication, assembly, and release of additional viruses, to infection of other cells.14 HIV lacks proofreading enzymes to correct errors made when it converts its RNA into DNA via reverse transcription. Its short life-cycle and high error rate cause the virus to mutate very rapidly, resulting in a high genetic variability of HIV. Most of the mutations either are inferior to the parent virus (often lacking the ability to reproduce at all) or convey no advantage, but some of them have a natural selection superiority to their parent and can enable them to slip past defenses such as the human immune system and antiretroviral drugs. The more active copies of the virus, the greater the possibility that one resistant to antiretroviral drugs will be made15. When antiretroviral drugs are used improperly, multi-drug resistant strains
can become the dominant genotypes very rapidly. In the era before multiple drug classes were available (pre-1997), the reverse transcriptase inhibitors zidovudine, didanosine, zalcitabine, stavudine, and lamivudine were used serially or in combination leading to the development of multi-drug resistant mutations.\(^{16}\)

Antiretroviral combination therapy defends against resistance by suppressing HIV replication as much as possible, thus reducing the potential pool of spontaneous resistance mutations.\(^{15}\) Most current HAART regimens consist of three drugs: 2 NRTIs ("backbone") + a PI/NNRTI/INSTI ("base") \(^{17}\). Initial regimens use "first-line" drugs with a high efficacy and low side-effect profile. Suppressing the viral load to undetectable levels (<50 copies per ml) is the primary goal of ART\(^{18-20}\). CD4 cell counts are another key measure of immune status and ART effectiveness. When viral suppression on ART is achieved but without a corresponding increase in CD4 counts\(^{21-23}\) it can be termed immunologic non response or immunologic failure. While this is predictive of worse outcomes, there is no consensus on how to adjust therapy to immunologic failure and whether switching therapy is beneficial. DHHS guidelines do not recommend switching an otherwise suppressive regimen.

From the above context, the usage of these drugs has been widely used for the therapy. Inspite of many drugs classified the author had a keen interest in selecting a most recommended drugs which are used as a combination as the new era of conflicts have to be suppressed. Of all, the most widely used are Atazanavir & Ritonavir. Both these drugs come under protease inhibitors\(^{24-27}\), as the main cause of the virus, first attacks the body for weakening the immune system. To overcome the trail, these first line drugs, can be analyzed systematically for the better usage of human health.
Pharmacology & Mechanism Actions of Atazanavir (ATV) and Ritonavir (RTV)

**Atazanavir (ATV):** ATV is an Azapeptide HIV-1 protease inhibitor (PI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1). HIV-1 protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV-1. Atazanavir binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles. Protease inhibitors are almost always used in combination with at least two other anti-HIV drugs. ATV is pharmacologically related but structurally different from other protease inhibitors and other currently available antiretrovirals. ATV selectively inhibits the virus-specific processing of viral Gag and Gag-Pol polyproteins in HIV-1 infected cells by binding to the active site of HIV-1 protease, thus preventing the formation of mature virions. ATV is not active against HIV-2.

**Ritonavir (RTV):** RTV is a protease inhibitor with activity against Human Immunodeficiency Virus Type 1 (HIV-1). Protease inhibitors block the part of HIV called protease. HIV-1 protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV-1. Ritonavir binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles. Protease inhibitors are almost always used in combination with at least two other anti-HIV drugs. Ritonavir inhibits the HIV viral proteinase enzyme which prevents cleavage of the gag-pol polyprotein, resulting in noninfectious, immature viral particles.
Effects, contraindications and adverse effects of ATV & RTV

Few symptoms caused by ATV & RTV are as follows: For ATV: Total bilirubin increased (35-49%), Fever (19%, Rash (3-21%), Cholesterol is increased (6-25%), Nausea (4-14%), CPK increased (6-11%), Cough (21%), Diarrhea (3-11%), Neutrophils decrease (6-10%), Jaundice (5-9%), Headache (1-7%), Peripheral neuropathy (1-4%), Insomnia (1-3%), Fever (2%), Vomiting (3-7%), Dizziness (1-2%) Myalgia (4%), Abdominal pain (2-4%), Depression (1-2%), Edema, Pancreatitis, hyperglycemia, Nephrolithiasis, angioedema, maculopapular rash, pruritus, Cholelithiasis. For RTV: Anemia, Decreased WBCs, Diarrhea, Increased GGT, Increased triglycerides, Insomnia, Incr uric acid, Increased glucose, Increased LFTs, Increased CP, Increased potassium, Increased calcium, Local throat irritation, Leukocytosis, Malaise, Myalgia, Neutropenia, Neutrophilia, Prolonged PT, Paresthesia, Pharyngitis, Rash, Somnolence, Thinking abnormally & Vasodilation.

Current therapeutic and clinical uses of ATV & RTV

They are antiretroviral drug of the protease inhibitor (PI) class. Both the drugs are used to treat infection of human immunodeficiency virus (HIV). ATV is distinguished from other PIs in that it can be given once-daily (rather than requiring multiple doses per day) and has lesser effects on the patient’s lipid profile (the amounts of cholesterol and other fatty substances in the blood). Like other protease inhibitors, it is used only in combination with other HIV medications. RTV is an HIV protease inhibitor that works by interfering with the reproductive cycle of HIV.

Dosage Regimen: Atazanavir: 300 mg + Ritonavir: 100 mg
<table>
<thead>
<tr>
<th>Official Name</th>
<th>Chemical Name(s)</th>
<th>Structure</th>
<th>Feature(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atazanavir</td>
<td>methyl N-[(1S)-1-{N'-[(2S,3S)-2-hydroxy-3-{(2S)-2-[(methoxycarbonyl)amino]-3,3-dimethylbutanamido]-4-phenylbutyl]-N'-{[4-(pyridin-2-yl)phenyl]methyl}hydrazine carbonyl}-2,2-dimethylpropyl]carbamate</td>
<td><img src="image.png" alt="Structure" /></td>
<td>N-acyl-alpha Amino Acids and Derivatives, acyl group at terminal nitrogen atom.</td>
</tr>
</tbody>
</table>
Table 4.2: Structural features of Ritonavir (RTV)

<table>
<thead>
<tr>
<th>Official Name</th>
<th>Chemical Name(s)</th>
<th>Structure</th>
<th>Features</th>
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**Drug/Characteristic Profile of Atazanavir (ATV)**

**Name** : Atazanavir

**Description** : Atazanavir is an antiretroviral drug of the protease inhibitor (PI) class. Like other anti retrovirals, it is used to treat infection of human immunodeficiency virus (HIV). Atazanavir is distinguished
from other PIs in that it can be given once-daily (rather than requiring multiple doses per day) and has lesser effects on the patient’s lipid profile (the amounts of cholesterol and other fatty substances in the blood). Like other protease inhibitors, it is used only in combination with other HIV medications.

**Brand names**: Latazanavir, Reyataz, Zrivada

**Categories**: Anti Retroviral Agent

**Molecular Weight**: 802.934 gms/mol

**Chemical Formula**: $C_{38}H_{52}N_{6}O_{7}$

**IUPAC Name**: methylN-[(1S)-1-{N'[(2S,3S)-2-hydroxy-3-[(2S)-2-methoxycarbonyl)amino]-3,3-dimethylbutanamido]-4-phenylbutyl]-N'{-[4-(pyridin-2-yl)phenyl]methyl}hydrazine carboxyl]-2,2-dimethylpropyl] carbamate

**CAS Number**: 198904-31-3

**Physicochemical descriptive properties:**

**State**: Light yellow colour powder

**Solubility**: Soluble in water, and 100% ethanol. Insoluble in ether, and acetone

**Melting Range**: 190-198°C
**pKa** : 4.5

**Odour & taste** : Odourless and bitter

**Storage** : Store between 15°C-25°C and protect from moisture

**Pharmacological Actions & Clinical Pharmacology**

**Indication:** Used in combination with other antiretroviral agents for the treatment of HIV-1 infection, as well as post exposure prophylaxis of HIV infection in individuals who have had occupational or non occupational exposure to potentially infectious body fluids of a person known to be infected with HIV when that exposure represents a substantial risk for HIV transmission.

**Mechanism of Action:** Atazanavir selectively inhibits the virus-specific processing of viral Gag and Gag-Pol polyproteins in HIV-1 infected cells by binding to the active site of HIV-1 protease, thus preventing the formation of mature virions. Atazanavir is not active against HIV-2.

**Pharmacokinetics**

**Absorption** : Atazanavir is rapidly absorbed with a $T_{\text{max}}$ of approximately 2.5 hours. Administration of Atazanavir with food enhances bioavailability and reduces pharmacokinetic variability. Oral bioavailability is 60-68%.
**Toxicity**

: Most common adverse reactions (≥2%) are nausea, jaundice/scleral icterus, rash, headache, abdominal pain, vomiting, insomnia, peripheral neurologic symptoms, dizziness, myalgia, diarrhea, depression, and fever.

**Metabolism**

: Atazanavir is extensively metabolized in humans, primarily by the liver. The major biotransformation pathways of ATV in humans consisted of monooxygenation and dioxygenation. Other minor biotransformation pathways for ATV or its metabolites consisted of glucuronidation, N-dealkylation, hydrolysis, and oxygenation with dehydrogenation. In vitro studies using human liver microsomes suggested that ATV is metabolized by CYP3A.

**Elimination**

: Following a single 400 mg dose of 14C-ATV, 79% and 13% of the total radioactivity was recovered in the feces and urine, respectively. Unchanged drug accounted for approximately 20% and 7% of the administered dose in the faeces and urine, respectively.

**Half-life**

: Elimination half-life in adults (healthy and HIV infected) is approximately 7 hours (following a 400 mg daily dose with a light meal). Elimination half-life in hepatically impaired is 12.1 hours (following a single 400 mg dose).

**Year of Introduction:** 2003 A.D
Drug/characteristic Profile for Ritonavir (RTV)

Name : Ritonavir

Description : An HIV protease inhibitor that works by interfering with the reproductive cycle of HIV.

Brand names : Busvir, Empetus, Norvir

Categories : Anti Retroviral agent

Molecular Weight : 720.944gms/mol

Chemical Formula : C$_{37}$H$_{48}$N$_6$O$_5$S$_2$

IUPAC Name : 1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-[[methyl([2-(propan-2-yl)-1,3-thiazol-4-yl]methyl)]carbamoyl]amino]butanamido]-1,6-diphenylhexan-2-yl]carbamate

CAS Number : 155213-67-5

Physicochemical Properties:

State : white or almost white, crystalline powder

Solubility : practically insoluble in water, freely soluble in acetone, soluble in alcohol.

Melting Range : 149-153°C
**pKa**

: 3.9

**Storage**

: Store between 15°C-25°C and protect from moisture

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**Pharmacological Actions & Clinical Pharmacology**

**Indication**

: Indicated in combination with other antiretroviral agents for the treatment of HIV-infection. Ritonavir is a protease inhibitor with activity against Human Immunodeficiency Virus Type 1 (HIV-1). Protease inhibitors block the part of HIV called protease. HIV-1 protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV-1. Ritonavir binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles. Protease inhibitors are almost always used in combination with at least two other anti-HIV drugs.

**Mechanism of Action:**

Ritonavir inhibits the HIV viral proteinase enzyme which prevents cleavage of the gag-pol polyprotein, resulting in noninfectious, immature viral particles.

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**Pharmacokinetics**

**Absorption**

: The absolute bioavailability of Ritonavir has not been determined.
Toxicity: Human experience of acute overdose with RTV is limited. One patient in clinical trials took RTV 1500 mg/day for two days. A post-marketing case of renal failure with eosinophilia has been reported with RTV overdose. The approximate lethal dose was found to be greater than 20 times the related human dose in rats and 10 times the related human dose in mice.

Metabolism: Five metabolites have been identified. The isopropylthiazole oxidation metabolite (M-2) is the major metabolite and has antiviral activity similar to that of RTV, however, plasma concentrations are low. The cytochrome P<sub>450</sub> enzymes CYP3A and CYP2D6 are primarily involved in the metabolism of RTV.

Elimination: In a study of five subjects receiving a 600 mg dose of <sup>14</sup>C-ritonavir oral solution, 11.3 ± 2.8% of the dose was excreted into the urine, with 3.5 ± 1.8% of the dose excreted as unchanged parent drug. In that study, 86.4 ± 2.9% of the dose was excreted in the faeces with 33.8 ± 10.8% of the dose excreted as unchanged parent drug. Upon multiple dosing, RTV accumulation is less than predicted from a single dose possibly due to a time and dose-related increase in clearance.

Half-life: 3-5 hours

Year of Introduction: 2001 A.D
OBJECTIVE OF INVESTIGATION

The objective of current investigation is to provide combined estimation of the selected drugs in dosage forms as well as in its pure forms by using a simple, reliable, precise, accurate, robust and reproducible analytical method with stability indication. The stability indication nature of the analytical method provides confidence to use the developed method in a regulatory environment of pharmaceutical industry without any further modification. This makes that the developed analytical method will be use full for the estimation of these drugs in either combined dosage forms or in its pure form.

LITERATURE SURVEY

Both, Atazanavir (ATV) and Ritonavir (RTV) are official in USP & BP respectively. The official methods of analysis for ATV & RTV are Colorimetry and Liquid Chromatography. The USP prescribes Colorimetry & HPLC method for the analysis of ATV & RTV. A survey of literature reveals that, other reported methods for ATV include Spectrophotometry, HPLC and Tandem Mass Spectroscopy in varied Matrices like formulations and biological fluids. The details of the HPLC methods are given below.

The analytical methods reported for RTV are based upon Spectrophotometry, HPLC and other related analytical techniques like Tandem Mass Spectroscopy. The details of HPLC methods are presented below. As highlighted earlier, the use of the above two drugs in combination has become, very wide spread. It is however, surprising to note that, not even a single method is available till now, for the simultaneous estimation of these two drugs in combination products for stability and degradation studies. The present project seeks to bridge this gap by developing such methods. The details of these successful efforts, by the author are incorporated in this chapter.
Past Studies on Atazanavir and Ritonavir

1. A validated RP HPLC method for the estimation of Atazanavir sulphate in capsule dosage form on Egilent TC C18 (2) 250 x 4.6 mm, 5 μ column using mobile phase composition of water: acetonitrile (20:80 v/v) pH adjusted to 3. Flow rate was maintained at 1 ml/min at an ambient temperature. Quantification was achieved with ultraviolet (DAD) detection at 255 nm. The retention time obtained for Atazanavir sulphate was at 3.7 min. The detector response was linear in the concentration range of 10 – 80 μg/ml. This method has been validated and shown to be specific, sensitive, precise, linear, accurate, rugged, robust and fast. Hence, this method can be applied for routine quality control of Atazanavir sulphate in capsule dosage forms as well as in bulk drug. (Sathish Kumar Konidala, K. Sujana and A. Prameela Rani. 2012) (28)

2. A validated stability indicating RP-HPLC method for the estimation of Atazanavir sulphate in capsule dosage form on Agilent TC C18 (2) 250 × 4.6 mm, 5 μ column using mobile phase composition of 0.02 M ammonium dihydrogen phosphate buffer:acetonitrile:methanol (30:25:45 v/v) and pH adjusted at 2.5 with ortho-phosphoric acid. Flow rate was maintained at 1 ml/min at an ambient temperature. Quantification was achieved with ultraviolet detection at 288 nm. (Charushila H. Bhirud, Shivanand N. Hiremath, 2013) (29)

3. A validated RP HPLC method for the estimation of Atazanavir in capsule dosage form on YMC ODS 150 × 4.6 mm, 5 μ column using mobile phase composition of ammonium dihydrogen phosphate buffer (pH 2.5) with acetonitrile (55:45 v/v). Flow rate was maintained at 1.5 mL/min with 288 nm UV detection. The retention time obtained for Atazanavir was at 4.7 min. The detector response was linear in the concentration range of 30 - 600 μg/mL. This method has been validated and shown to be specific, sensitive, precise, linear, accurate, rugged, robust and fast. Hence, this method can be applied for routine quality control of Atazanavir in capsule
dosage forms as well as in bulk drug. (K. Srinivasu, J. Venkateswara Rao, N. Appala Raju, and K. Mukkanti, 2009) (30)

4. Reversed phase high performance liquid chromatographic method was developed and validated for estimation of Atazanavir Sulphate in tablet dosage form. A Zodiac C$_{18}$, 250x4.6 mm i.d, 5 $\mu$m particle size, with mobile phase consisting of a buffer of 1.85 g ammonium acetate in 1000 ml water and acetonitrile in the ratio of 60:40 v/v was used. The flow rate was 1.0 ml/min and the effluents were monitored at 205 nm. The retention time was 2.840 min. The detector response was linear in the concentration of 18-42 mcg/ml, with the regression coefficient of 0.999. The percentage assay of Atazanavir Sulphate was 98.81%. The method was validated by determining its accuracy, precision and system suitability. The results of the study showed that the proposed RP-HPLC method can be applied for the determination of Atazanavir Sulphate in quality control samples and formulations without interferences of the excipients present. (P. Anupama, A. Viswanath, P. Sreenivasa babu and R. Sasidhar, 2013) (31)

5. The present study describes a simple, accurate, precise and cost effective UV-Spectrophotometric method for the estimation of Atazanavir sulphate, an Anti-HIV drug, in bulk and pharmaceutical dosage form. The drug was first dissolved in 20% glacial acetic acid and final volume was made up with distilled water. The $\lambda_{\text{max}}$ or the absorption maxima of the drug was found to be 299 nm. A linear response was observed in the range of 10-50 $\mu$g/ml with the regression coefficient of 0.999. The method was validated for different parameters as per the ICH (International Conference for Harmonization) guidelines. This method can be used for the determination of Atazanavir sulphate in quality control of formulation without interference of the excipients. (Konidala, Sathish Kumar; Sujana, K., 2012) (32)
Atazanavir sulphate (ATV) is a new HIV-1 protease inhibitor anti retroviral drug. The present study describes a simple, accurate, reproducible and precise UV Spectrophotometric method for the estimation of ATV in 0.025 N HCl. ATV is insoluble in 0.025 N HCl, so methanol was used as a co-solvent. The absorbance maximum ($\lambda_{\text{max}}$) was found to be 300 nm with linearity in the range of 5-60µg/ml and regression equation as $Y = 0.013 - 0.001$. Regression coefficient was calculated as 0.999. The method was validated for different parameters such as sandell’s sensitivity, molar absorptivity, accuracy, precision, ruggedness, robustness, detection limit, quantification limit, etc (as per the ICH guidelines). Molar absorption coefficient and sandell’s sensitivity comes out to be 1.06 X 104 l. mol-1cm-1 and 0.1538µg/ml/0.001 A.U. respectively. LOD and LOQ were found to be 0.2071µg/ml and 0.627µg/ml respectively. The relative standard deviation (RSD) in case of accuracy, precision, ruggedness and robustness was less than 2.0% proving that method was highly accurate, precise and robust. This method can be used for the determination of ATV in pharmaceutical formulations without interference of the excipients.(Mittal A, Kalia A, Malhotra D.2011) (33)

An accurate, sensitive, precise and robust reverse phase high performance liquid chromatographic method for the simultaneous estimation of lopinavir and ritonavir in combined dosage form has been developed and validated. Chromatographic separation was conducted on Phenomenex Gemini C18 (250 mm×4.6 mm, 5µ) column at room temperature using Potassium hydrogen phosphate buffer (pH adjusted to 6.0 ± 0.1 with diluted potassium hydroxide solution), acetonitrile and methanol in the ratio of 50:35:15v/v and at a flow rate of 1.0 ml / min, while UV detection was performed at 254 nm. The retention time for lopinavir and ritonavir was found to be 6.0±0.2 and 3.7±0.1 min, respectively. The method was found to be linear in the range of 400-600µg/ml for lopinavir and 100-150 µg/ml for Ritonavir. The developed method was
validated in terms of accuracy, precision, LOD, LOQ, robustness and solution stability. The proposed method can be successfully used for the estimation of lopinavir and Ritonavir in bulk and combined dosage forms. (A. Suneetha, S.kathirvel and g.ramachandrika; 2011) (34)

7A. We developed a simple HPLC method for the simultaneous determination of lopinavir (LPV), ritonavir (RTV) and efavirenz (EFV) to evaluate the efficiency of co-administration of LPV/RTV and EFV in Japanese patients enrolled in a clinical study. The monitoring of LPV plasma concentration is important because co-administration of LPV/RTV with EFV sometimes decreases plasma concentrations of LPV caused by EFV activation of cytochrome P-450 3A. A solution of acetonitrile, methanol and tetramethylammonium perchlorate (TMAP) in dilute aqueous trifluoroacetic acid (TFA) has been used as the mobile phase in a HPLC method to elute LPV and RTV. We found that a solvent ratio of 45 : 5 : 50 (v/v/v) of acetonitrile/methanol/0.02 M TMAP in 0.2% TFA optimized separation of LPV, RTV and EFV. A column temperature of 30 degrees C was necessary for the reproducibility of the analyses. Standard curves were linear in the range 0.060 to 24.06 micro g/ml for LPV, 0.010 to 4.16 micro g/ml for RTV, and 0.047 to 37.44 micro g/ml for EFV. Coefficients of variation (CVs) of LPV, RTV and EFV in intraday and interday assays ranged from 1.5 to 4.0%, 2.5 to 16.8% and 1.0 to 7.7%, respectively. Accuracies ranged from 100 to 110%, 101 to 116% and 99 to 106% for LPV, RTV and EFV, respectively. The extraction recoveries were 77-87, 77-83 and 81-91% for LPV, RTV and EFV, respectively.(Usami Y, Oki T, Nakai M, Sagisaka M, Kaneda T.,2003) (35)

8. Two sensitive and reproducible methods were described for the quantitative determination of the antiretroviral drug Ritonavir in the presence of its degradation products. The first method was based on high performance liquid chromatographic (HPLC) separation of the drug from its stress degradation products with the use of a reversed phase Agilent Eclipse XDB-
C18 column (5 μm, 4.6 × 150 mm) and a mobile phase consisting of acetonitrile : 0.05 M phosphoric acid (55 : 45, v/v) at a flow rate of 1.0 mL/min. The retention time of the drug was found to be 4.82 ± 0.002 min. Quantification was achieved with diode array detection (DAD) at 210 nm based on peak area and a linear calibration curve in the concentration range of 1-500 μg/mL. The proposed method made use of diode array detection as a tool for peak purity and identification. The second method involved a high performance thin layer chromatographic (HPTLC) separation followed by densitometric measurement of the spots at 240 nm. The separation was carried out on Fluka TLC aluminium sheets of silica gel with fluorescent indicator (254) nm and the mobile phase was acetonitrile - water (1 : 2, v/v), adjusted to pH 5.0 using 1 M Orthophosphoric acid solution. The proposed procedure gave compact spots for Ritonavir (retention factor, Rf = 0.41 ± 0.014). The linear regression equation was generated by least-squares treatment of the calibration data in the range of 0.8-12.5 μg/spot. The reliability and analytical performance of the proposed methods, including linearity, range, precision, accuracy, detection and Quantitation limits, were statistically validated. The proposed methods were applied to Norvir capsules and no chromatographic interference was observed. When Ritonavir was subjected to stress conditions; according to ICH guidelines, the proposed methods could effectively separate the drug from its degradation products, and were thus considered as good stability-indicating procedures. (Mohammad h. Abdelhay, azza a. Gazy, rasha a. Shaalan and heba k. Ashour, 2012) (36)

9. A simple, precise, accurate and selective reverse phase high pressure liquid chromatographic method has been developed and validated for the simultaneous estimation of Lopinavir and Ritonavir in bulk and pharmaceutical dosage form. The method was carried out on A HiQ sil C18 column (250 x 4.6mm id,5μm) in Isocratic mode with mobile phase consisting of
methanol, acetonitrile and potassium dihydrogen phosphate (pH was adjusted to 5 by using Triethylamine) in the ratio of 70:10:20 at a flow rate 1.5 mL/min. The detection was carried out at 260 nm for both drugs. The retention times of Lopinavir and Ritonavir were found to be 3.73 and 6.16 min respectively. The developed method was validated in terms of linearity, precision, accuracy, limit of detection/Quantitation and robustness. The proposed method is easy to perform and successfully applied for the estimation of these drugs in bulk and tablet dosage form. (Jyoti M. Salunke, Satyendra Singh, Sheela Malwad, Vinit D. Chavhan, Mauli S. Pawar, Chinmay Patel, Dipak Salunke, Minal R. Ghante and S. D. Sawant, 2013) (37)

10. A simple, sensitive and precise reverse phase high performance liquid chromatographic method has been developed for the simultaneous estimation of Ritonavir and Lopinavir in pharmaceutical dosage forms. The mobile phase consisted of Acetonitrile: buffer (0.05M Potassium Dihydrogen orthophosphate, PH – 3) in the ratio of 50:50 v/v delivered at a flow rate of 2.0 ml / min and wavelength of detection at 215 nm. The retention times of Ritonavir and Lopinavir were 10.253 min and 12.490 min respectively. The developed method was validated according to ICH guidelines. The proposed method can be used for determination of these drugs in combined dosage forms. (D. Varun, Bahul Z Awen, Ch. Babu Rao, K. Mukkanti, P. Nagaraju, 2010) (38)

11. A simple, robust and selective and sensitive spectrophotometric method has been developed for the determination of Ritonavir in pharmaceutical formulations. The method was based on the scanning of methanolic solution of the drug and methanolic solution of formulation. The method showed high sensitivity with linearity range from 10 to 20 μg/mL. The lower limit of detection (LOD) was found to be 1.1 μg/mL and the limit of quantization (LOQ) was determined as the lowest concentration was found to be 3.3 μg/mL. The variables that affected
the reaction were carefully studied and optimized. The proposed method was applied successfully for the determination of Ritonavir in pharmaceutical formulations. The percentage recovery was found to be 99.426 ± 0.59 (n = 9) for pharmaceutical formulation. (K. Seetaramaiah, A. Anton smith, K. Ramyateja, G. Alagumanivasagam and R. Manavalan, 2011) (39)

12. A reversed phase high-performance liquid chromatographic method was developed and validated for the quantitative determination of two antiviral drugs viz. lopinavir and Ritonavir. Chromatography was carried out by gradient technique on a reversed-phase C18 Column, Phenomenex (250 x 4.6 mm, 5 μ) with mobile phase mixture of Buffer: Acetonitrile (45:55 v/v) was used as a mobile phase and the pH was adjusted into 4.5 by using with O-phosphoric acid, at a flow rate of 1.2 ml/min. The UV range was detected at 240 nm for lopinavir and Ritonavir respectively. The different analytical performance parameters such as linearity, precision, accuracy, and specificity, limit of detection (LOD) and limit of quantification (LOQ) were determined according to International Conference on Harmonization ICH Q2B guidelines. The linearity of the calibration curves for each analyte in the desired concentration range is good (r2 >0.9). The recovery of the method was between 102.1% and 100.1% for lopinavir and Ritonavir respectively. Hence the proposed method is highly sensitive, precise and accurate and it successfully applied for the reliable quantification of API content in the commercial formulations of lopinavir and Ritonavir.(Jagadeeswaran M, Gopal N, Pavan kumar K and Siva kumar T, 2012) (40)

13. A simple, precise, specific and accurate reverse phase HPLC method has been developed for the determination of Atazanavir and Ritonavir in pharmaceutical dosage forms. The chromatographic separation was achieved on Symmetry C8 (4.6 x 100mm, 5 μm,
Make: ACE) column using a mixture of Buffer: Acetonitrile (45:55) as the mobile phase at a flow rate 0.9ml/min. The retention time of Atazanavir and Ritonavir was 2.9 min and 4.1min. The analyte was monitored using UV detector at 235 nm. Results of analysis were validated statistically and by recovery studies. The method was validated according to the ICH guidelines with respect to linearity, accuracy, precision and robustness. The proposed method can be successfully used to determine the drug contents of marketed formulation. (Anusha Tiyyagura, Ashwini Gunda, Annapurna Renee Chitturi, Aravind sai2, Abbaraju prasana Laxmi and Avinash Kodoori, 2012) (41)

14. The present investigation describes about a simple, economic, selective, accurate, precise reverse phase high performance liquid chromatographic method for the simultaneous estimation of Atazanavir and Ritonavir in pure and pharmaceutical dosage forms of Atazanavir and Ritonavir were well separated using a XTera C18 (100 x 4.6mm, 3.5μm) and Mobile phase consisting of Buffer(pH-2.5): Acetonitrile (40:60) adjusted to pH- 2.5 at the flow rate 1.2 ml/min and the detection was carried out at 247nm with PDA detector. The Retention time for Atazanavir and Ritonavir were found to be 1.982 & 2.576 respectively. The developed method was validated for recovery, specificity, precision, accuracy, linearity according to ICH guidelines. The method was successfully applied to Metronidazole and Norfloxacin combination pharmaceutical dosage form. (Nuli Vasavi, Afroz Patan, 2013) (42)

15. The assay involved an isocratic elution of these two component on Hi-Q Sil C-18 Column (250 mm x 4.6 mm, 5μm) using a mobile phase composition of Acetonitrile: Methanol: Phosphate Buffer (40:40:20) adjusted to pH 3.1 with orthophosphoric acid. The flow rate was 1.0 mL/min and the analytes monitored at 238nm using photodiode array (PDA) detector. The performance of the method was validated according to the present ICH guidelines for specificity,
linearity, accuracy, precision and robustness. (Dnyaneshwar sukhadev pawar, manjusana dole, sanjay sawant, jyoti m salunke, 2013) (42)

**PROPOSED WORK**

As mentioned earlier, inspite of the wide therapeutic use of the ATV & RTV combinations, no analytical methods for the simultaneous quantification by stability studies, of these two drugs are available till date. The author has made efforts to fulfil this need and succeeded in developing a simple, precise, accurate and sensitive RP-HPLC method for this purpose. The protocols and guidelines prescribed by the ICH have been followed in the development and validation of these procedures. The details are presented in the following sections as mentioned below.

The present project has been focused on the development of simple and precise analytical method for the widely used dosage formulation combination - (Synthivan®, CIPLA Pharmaceutical Pvt Ltd, Mumbai, India, containing two drugs (Atazanavir & Ritonavir), an Antiretroviral therapeutic activity drug, meant for the treatment of infected human immunodeficiency virus (HIV).

This part of the thesis, reports sensitive and precise RP-HPLC method for the determination of drug in bulk samples and also in pharmaceutical formulations. The reported methods are applicable for the estimation of either for ATV or RTV individually or in combination with other drugs from pharmaceutical dosage forms or biological fluids. But till date there were not even a single method reported on degradation studies to prove that the method is stability indicating method. Of late, however, the role of antiretroviral agents in
strengthening the viral defensive factors is gaining importance. All though these drugs have brought about remarkable changes in viral therapy, the efficacy of these drugs is still debatable.

From the above cited content, the author has selected a widely used novel end drugs (Atazanavir & Ritonavir), which has been clinically proven to fight and heal the infected virus for analytical investigations. However there is still some uncertainty & hysterical role of these drugs in prevention of infection and its adverse effect profile. The author is fascinated in achieving the methodology for the above said drugs and also for routine analytical purpose in both industrial and academic levels. The present work describes the development of a validated stability indicating analytical RP-HPLC method, which can quantify these components simultaneously from a combined dosage form.

**EXPERIMENTAL**

**Materials and Instrumentation**

**Drugs**

Atazanavir (ATV) and Ritonavir (RTV) Pure API samples as gift samples were procured from Chandra Labs, Hyderabad.

**Marketed formulations**

**Synthivan® Capsules (Mfd. by CIPLA Pvt., Ltd, Mumbai)**

Each capsule contains

Atazanavir ..............................................300 mg

Ritonavir .................................................100 mg
Chemicals & Reagents Used

► HPLC grade Acetonitrile (Merck specialities Private Limited, Mumbai)

► Ammonium Acetate (Merck specialities Private Limited, Mumbai)

► HPLC grade Double distilled water (Merck specialities Private Limited, Mumbai)

► All dilutions were performed in standard class-A, volumetric glassware.(Borosil)

Instrumentation

Agilent 1120 compact LC chromatographic system, with DAD detector and a fixed injector equipped with 20 µL loop was used for the chromatographic separation. The chromatogram was recorded at and peaks quantified by means of Ezchrome software. Chromatographic separation was carried out on a C<sub>18</sub> column [Zodiacsil, 250 mm x 4.6 mm i.d, 5µ particle size]. Sartorius electronic balance was used for weighing the samples. Ultra-sonic bath sonicator was used for degassing and mixing of the mobile phase.

Chromatographic conditions

Chromatographic separation of Atazanavir and Ritonavir was carried on a RP-C<sub>18</sub> Zodiacsil column. The mobile phase was composed of Acetonitrile and Ammonium acetate buffer (pH 4.0) in the ratio of 40:60 v/v. It was filtered through a 0.45 µ membrane filter and degassed for 15 minutes. The flow rate of the mobile phase was maintained at 1 ml/min and the runtime was set for 30 minutes. Detection was carried out at 205 nm at ambient temperature.
ANALYTICAL METHODOLOGY

Preparation of Primary Standard Stock Solutions

Standard stock solutions were prepared by dissolving 60 mg of Atazanavir and 25 mg Ritonavir working standard in two separate each of 100 ml volumetric flasks using 15ml of mobile phase and made up to the mark with mobile phase to obtain a final concentration of 600 µg/mL and 250 µg/mL of each Atazanavir and Ritonavir. From the above stock solutions, 5 and 4 ml aliquots each were pipetted in to a 100 ml volumetric flask and dissolved in 25ml of the mobile phase and made up to the mark with the solvent to obtain a final concentration of 30 µg/mL and 10 µg/mL for Atazanavir and Ritonavir respectively.

Preparation of Sample solutions

Twenty capsules were weighed and finely powdered. Accurately weighed and transferred equivalent to 300 mg of ATV and 100 mg of RTV into a 100 ml volumetric flask, added 70 ml of diluent, and sonicated for 30 minutes with intermittent shaking at controlled temperature and diluted to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transferred 2.0 ml of the above solution into a 100 ml volumetric flask and diluted to volume with diluent to obtain a final concentration levels of 30 and 10 µg/mL of Atazanavir and Ritonavir respectively.
Table: 4.3: Chromatographic Conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>RP-C_{18} Zodiacsil 250 mm x4.6 mm i.d, 5\mu particle size</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Acetonitrile and Ammonium acetate buffer (pH 4.0) in the ratio of 40:60 v/v</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Run time</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Temperature</td>
<td>Ambient</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>20 \mu L</td>
</tr>
<tr>
<td>Detection &amp; Wavelength</td>
<td>DAD Detector, 205 nm</td>
</tr>
<tr>
<td>Retention times</td>
<td>2.833 for ATV &amp; 5.753 for RTV</td>
</tr>
</tbody>
</table>

**Recommended Procedure**

After systematic and detailed study of the various parameters involved as described under the results and discussion in this chapter, the following procedure was adopted for the determination of ATV and RTV in bulk samples and pharmaceutical formulations.

**Procedure**

Initially the mobile phase was pumped for about 30 minutes to saturate the column thereby to set the baseline corrected. Then 20 \mu L of the standard and sample solutions were injected separately. A quantitative determination of the active ingredients was made by
comparison of the peak area of the sample injection with the corresponding peak area of the standard injection. The amount of ATV and RTV present in the sample were calculated through the standard calibration curve.

RESULTS AND DISCUSSIONS

The appropriate wavelength in the UV-region (205 nm), was selected for the measurement of the active ingredients in the proposed method. The method was validated by linear fit curve and all the other parameters were calculated similar to the Spectrophotometric method and were discussed in the following pages. The typical chromatogram indicating the separation of ATV & RTV with Zodiacsil RP-C18 column (250 mm x 4.6 mm i.d; 5µ particle size) and mobile phase consisting of Acetonitrile and Ammonium acetate buffer (pH 4.0) in the ratio of 40:60 v/v in gradient mode was shown in Figures: 4.1 and 4.2 respectively. Chromatogram of Blank Standard samples tested by the same procedure showed no interfering peaks as shown in below Figure: 4.1.

Figure: 4.1: A typical HPLC Chromatogram showing the no interference of diluent for Atazanavir and Ritonavir
Figure 4.2: A typical HPLC Chromatogram showing the Control Sample profile of Atazanavir and Ritonavir by proposed method

Parameters Fixation

In developing this method, systematic study of the effects of various parameters were undertaken by varying one parameter at a time and controlling all other parameters. The following studies were conducted for this purpose.
Mobile Phase Characteristics

In order to get sharp peaks and baseline separation of the components, the author has carried out a number of experiments by varying different components like composition of organic phase in mobile phase, pH of the aqueous phase, total pH of the selected mobile phase, modifiers and flow rate by changing one at a time and keeping all other parameters constant respectively. The optimum conditions evolved from the above studies were incorporated in the recommended procedure.

Detection Characteristics

The schematic experimentation has been carried out to test whether ATV & RTV have been linearly eluted from the column successively and systematically. In this method, different amounts of the active ingredients were taken and all the solutions were analyzed by respective procedures separately. Quantitative determinations were made by comparing the peak area from a sample injection to the corresponding peak area from the standard injection in the method. The linear fit was illustrated graphically in Figures: 4.5 - 4.6. Least linear correlation and regression data analysis for the method was carried out for the slope; intercept, standard error of estimate; correlation coefficient & % RSD were illustrated in Tables: 4.4 and 4.5 respectively. The results are represented in Table: 4.6.
Table: 4.4 Linear Correlations and Regression Data Sheet / Summary for Atazanavir

\[ \sum X = 150, \sum X^2 = 4860, \quad \sum Y = 18990.79, \quad \sum Y^2 = 76836875.1499 \]

Therefore, \( \sum XY = 610867.08 \)

<table>
<thead>
<tr>
<th>R</th>
<th>( r^2 )</th>
<th>Slope</th>
<th>Y Intercept</th>
<th>Standard error of estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.999</td>
<td>0.999</td>
<td>114.287</td>
<td>369.543</td>
<td>39.5553</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>T</th>
<th>df</th>
<th>P</th>
<th>One-tailed</th>
<th>Two-tailed</th>
</tr>
</thead>
<tbody>
<tr>
<td>54.821</td>
<td>3</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
</tr>
</tbody>
</table>

0.95 and 0.99 : Confidence Intervals of rho

<table>
<thead>
<tr>
<th>( \rho )</th>
<th>Lower Limit</th>
<th>Upper Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.95</td>
<td>0.986</td>
<td>1</td>
</tr>
<tr>
<td>0.99</td>
<td>0.963</td>
<td>1</td>
</tr>
</tbody>
</table>

Values entered:

<table>
<thead>
<tr>
<th>Pairs</th>
<th>X</th>
<th>Y</th>
<th>Residuals</th>
</tr>
</thead>
<tbody>
<tr>
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<td>2451.56</td>
<td>24.848</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>3102.59</td>
<td>-9.845</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>3785.73</td>
<td>-12.428</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>4438.88</td>
<td>-45.001</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
<td>5212.03</td>
<td>42.426</td>
</tr>
</tbody>
</table>
Table: 4.5 Linear Correlations and Regression Data Sheet / Summary for Ritonavir

\[
\begin{align*}
\sum X &= 48.13, \quad \sum X^2 = 511.8493, \\
\sum Y &= 2808.79, \quad \sum Y^2 = 1677152.994
\end{align*}
\]

Therefore = \(\sum XY = 29231.2523\)

<table>
<thead>
<tr>
<th>(r)</th>
<th>(r^2)</th>
<th>Slope</th>
<th>(Y) Intercept</th>
<th>Standard error of estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.999</td>
<td>0.998</td>
<td>45.187</td>
<td>126.7851</td>
<td>7.2813</td>
</tr>
<tr>
<td>(t)</td>
<td>(df)</td>
<td>(p)</td>
<td>One-tailed</td>
<td>(&lt;.0001)</td>
</tr>
<tr>
<td>43.242</td>
<td>3</td>
<td></td>
<td>Two-tailed</td>
<td>(&lt;.0001)</td>
</tr>
</tbody>
</table>

0.95 and 0.99 : Confidence Intervals of rho

<table>
<thead>
<tr>
<th>(\rho)</th>
<th>Lower Limit</th>
<th>Upper Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.95</td>
<td>0.985</td>
<td>1</td>
</tr>
<tr>
<td>0.99</td>
<td>0.963</td>
<td>1</td>
</tr>
</tbody>
</table>

Values entered:

<table>
<thead>
<tr>
<th>Pairs</th>
<th>(X)</th>
<th>(Y)</th>
<th>Residuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.04</td>
<td>349.568</td>
<td>-4.961</td>
</tr>
<tr>
<td>2</td>
<td>7.56</td>
<td>468.631</td>
<td>0.23</td>
</tr>
<tr>
<td>3</td>
<td>10.08</td>
<td>591.079</td>
<td>8.806</td>
</tr>
<tr>
<td>4</td>
<td>11.34</td>
<td>642.098</td>
<td>2.889</td>
</tr>
<tr>
<td>5</td>
<td>14.11</td>
<td>757.414</td>
<td>-6.964</td>
</tr>
</tbody>
</table>
**Table: 4.6: Least Linear Regression Data Analysis**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Atazanavir</th>
<th>Ritonavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. Range (µg/mL)</td>
<td>18 - 42</td>
<td>5.04 – 14.11</td>
</tr>
<tr>
<td>Slope (m)</td>
<td>114.287</td>
<td>45.187</td>
</tr>
<tr>
<td>Intercept (b)</td>
<td>369.543</td>
<td>126.7851</td>
</tr>
<tr>
<td>Standard error of estimate</td>
<td>39.5553</td>
<td>7.2813</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.999</td>
<td>0.998</td>
</tr>
<tr>
<td>% RSD</td>
<td>1.0</td>
<td>1.10</td>
</tr>
</tbody>
</table>

**Performance Calculations**

To ascertain the system suitability for the method, a number of parameters such as retention time, theoretical plates, and HETP, tailing factor, % Peak area, and LOD, LOQ, resolution and peak asymmetry have been calculated with the observed readings and the results are recorded in Table: 3.7.

**Table: 4.7: Validation Summary / System Suitability**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Atazanavir</th>
<th>Ritonavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical Plates (N)</td>
<td>3103</td>
<td>7471</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.79</td>
<td>1.30</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>2.833</td>
<td>5.753</td>
</tr>
<tr>
<td>Resolution</td>
<td>-------</td>
<td>12.392</td>
</tr>
<tr>
<td>% Peak Area</td>
<td>86.59</td>
<td>13.41</td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>1.14</td>
<td>0.53</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>3.46</td>
<td>1.62</td>
</tr>
</tbody>
</table>
Method validation

The following parameters were used to validate the method for the proposed assay procedure of ATV & RTV in pharmaceutical dosage forms. The developed HPLC method for the simultaneous determination of ATV and RTV was validated as per the ICH guidelines\textsuperscript{43, 44}.

Precision

The precision of an analytical method is the closeness of replicate results obtained from analysis of the same homogeneous sample. Precision was considered at different levels, i.e. method, system, Inter day and Intraday. Precision of the developed method was assessed by measuring the response on the same day (Intraday precision) and next two consecutive days (Inter day precision). The precision of the method was assessed by six replicate injections of 100\% test concentration. Intra and inter-day precision of the method was assessed by determination of standard deviation and \% RSD for the analyte response. The result was given in Table 4.8. Similarly the representative chromatograms for method precision were shown in Figure: 4.3.
Figure: 4.3: Representative Chromatograms for Method Precision (Six Replicates)
Table: 4.8: Method Precision (Inter and Intraday) studies for Atazanavir and Ritonavir by proposed method

<table>
<thead>
<tr>
<th>Method Precision (Inter &amp;Intra Day)</th>
<th>Atazanavir</th>
<th>Ritonavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.9</td>
<td>99.3</td>
<td>99.9</td>
</tr>
<tr>
<td>99.6</td>
<td>100.4</td>
<td>98.4</td>
</tr>
<tr>
<td>100.5</td>
<td>101.2</td>
<td>98.2</td>
</tr>
<tr>
<td>98.9</td>
<td>98.7</td>
<td>99.3</td>
</tr>
<tr>
<td>100.7</td>
<td>98.6</td>
<td>100.2</td>
</tr>
<tr>
<td>101.1</td>
<td>100.0</td>
<td>101.7</td>
</tr>
<tr>
<td><strong>Overall Avg.</strong></td>
<td><strong>100.0</strong></td>
<td><strong>99.9</strong></td>
</tr>
<tr>
<td><strong>Overage Std Dev.</strong></td>
<td><strong>0.95</strong></td>
<td><strong>1.09</strong></td>
</tr>
<tr>
<td><strong>Over all % RSD</strong></td>
<td><strong>1.00</strong></td>
<td><strong>1.10</strong></td>
</tr>
</tbody>
</table>

**Linearity and range**

The standard curve was obtained in the concentration range of 18 - 42 μg/mL for ATV and 5.04 – 14.11 μg/mL for RTV. The linearity of this method was evaluated by linear regression analysis. Slope, intercept and correlation coefficient \([r^2]\) of standard curve were calculated and given in Figure-4.5 (Atazanavir) and Figure-4.6 (Ritonavir) to demonstrate the
linearity of the proposed method. The result of regression analysis was given in the Table 4.6. Similarly the representative chromatograms for linearity were shown in Figure: 4.4. From the data obtained which given in Table: 4.9 (Atazanavir and Ritonavir) the method was found to be linear within the proposed range.

Figure: 4.4 Representative Chromatograms for Linearity
Table: 4.9: Linearity studies for Atazanavir and Ritonavir by proposed method

<table>
<thead>
<tr>
<th>% Level</th>
<th>Conc. µg/mL</th>
<th>Area</th>
<th>Conc. µg/mL</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>18.00</td>
<td>2451.56</td>
<td>5.04</td>
<td>349.568</td>
</tr>
<tr>
<td>80</td>
<td>24.00</td>
<td>3102.59</td>
<td>7.56</td>
<td>468.631</td>
</tr>
<tr>
<td>100</td>
<td>30.00</td>
<td>3785.73</td>
<td>10.08</td>
<td>591.079</td>
</tr>
<tr>
<td>120</td>
<td>36.00</td>
<td>4438.88</td>
<td>11.34</td>
<td>642.098</td>
</tr>
<tr>
<td>140</td>
<td>42.00</td>
<td>5212.03</td>
<td>14.11</td>
<td>757.414</td>
</tr>
</tbody>
</table>

| Slope   | 114.3       | 45.179 |
| Intercept | 369.5   | 126.85 |
| % Y-Intercept | 323.3 | 280.8 |
| Residual Sum of Squares | 39.6    | 7.31   |
| CC(r)   | 0.9995      | 0.9992 |
| RSQ(r2) | 0.9990      | 0.9984 |
| LOD     | 1.14        | 0.53   |
| LOQ     | 3.46        | 1.62   |
Figure: 4.5: Calibration curve for Atazanavir

Figure: 4.6: Calibration curve for Ritonavir
System suitability

System suitability for chromatographic separation was checked on each day of validation to evaluate the components of the analytical system in order to show that the performance of the system meet the standards required by the method. System suitability parameters established for the developed method include number of theoretical plates (efficiency), Resolution, Tailing factor. The HPLC system was equilibrated using the initial mobile phase composition, followed by 5 injections of the standard solution of 100% concentration containing 30 µg/mL of ATV and 10 µg/mL of RTV. These 5 consecutive injections were used to evaluate the system suitability on each day of method validation. The result was given in the below table 4.10.

Table 4.10: System suitability parameters for Atazanavir and Ritonavir by proposed method

<table>
<thead>
<tr>
<th>Name of the Compound</th>
<th>Retention Time</th>
<th>Theoretical plate</th>
<th>Tailing factor</th>
<th>USP Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atazanavir</td>
<td>2.833</td>
<td>3103</td>
<td>1.79</td>
<td>----</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>5.753</td>
<td>7471</td>
<td>1.30</td>
<td>12.392</td>
</tr>
</tbody>
</table>

Specificity & Interference studies

The effect of wide range of excipients & additives were studied and determined under optimum conditions. A study to establish the interference of blank was conducted. Diluent was injected into the chromatograph in the defined above chromatographic conditions and the blank
chromatograms were recorded. Chromatogram of Blank solution (Figure: 4.1) showed no peaks at the retention time of ATV and RTV peaks. This indicates that the diluent solution used in sample preparation do not interfere in estimation of ATV and RTV in capsules. Similarly typical representative chromatogram of standard is also shown (Figure: 4.2).

**Analysis of Formulation**

To find out the suitability of the method for the assay of pharmaceutical formulation containing ATV and RTV were analyzed by the proposed method. It was found that the proposed method do not differ significantly in the precision and accuracy from the reference method. The results are recorded in Table: 4.11.

**Accuracy**

To determine the accuracy of the proposed method, different amounts of bulk samples of ATV & RTV within linearity limits were taken and analyzed by the proposed method. The results are presented in Table: 4.11 & 4.12 and Figure 4.15. The accuracy of an analytical method is the closeness of results obtained by that method to the true value for the sample. It is expressed as recovery (%), which is determined by the standard addition method. In the current study recovery at three spike levels 50%, 100% and 150% were carried out. The % recovery at each spike level was calculated and was given in Tables: 4.11 & 4.12. Similarly the representative chromatograms for recovery studies at different levels were shown in Figures: 4.7 – 4.9.
Recovery Studies

Recovery studies were conducted by analyzing the formulations in the first instance for the active ingredients in the concentration of 50%, 100% & 150% of the working standard solution for both the two drugs (ATV & RTV) by the proposed method. Each concentration was injected three times and the peak areas were recorded. The known amount of the pure drug 10%
of the working standard solution contains was added to each three previously analyzed formulations and the total amount of the drug was again determined by the proposed method (each concentration was injected three times) by keeping the active ingredient concentration within the linearity limits. The chromatograms are shown in Figures: 4.7 – 4.9 and the results are recorded in Table: 4.11, 4.12 & 4.14.

Figure: 4.7: Reference chromatogram for Recovery-50 % level

Figure: 4.8: Reference chromatogram for Recovery-100 % level
Figure: 4.9: Reference chromatogram for Recovery-150 % level

Table: 4.11: Recovery studies for Atazanavir by proposed method

<table>
<thead>
<tr>
<th>% Level</th>
<th>Recovery Range</th>
<th>% RSD at each level</th>
<th>Over all %RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>98.9-99.5</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>99.5-99.9</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>150</td>
<td>98.9-101.0</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>

Table: 4.12: Recovery studies for Ritonavir by proposed method

<table>
<thead>
<tr>
<th>% Level</th>
<th>Recovery Range</th>
<th>% RSD at each level</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>98.2-99.9</td>
<td>0.7</td>
</tr>
<tr>
<td>100</td>
<td>98.4-100.1</td>
<td>0.9</td>
</tr>
<tr>
<td>150</td>
<td>99.4-99.8</td>
<td>0.3</td>
</tr>
</tbody>
</table>
LOD and LOQ

The detection limit of the method was investigated by injecting standard solutions Synthivan® into the HPLC column. By using the signal-to-noise (S/N) method, the peak-to-peak noise around the analyte retention time is measured. Subsequently, the concentration of the analyte which would yield a signal equal to certain value of noise to signal ratio was also estimated. A signal-to-noise ratio (S/N) of 3 was generally accepted for estimating LOD and signal-to-noise (S/N) ratio of 10 was used for estimating LOQ.

The LOQ can be determined by signal-to-noise ratio of 10:1, or even approximated by multiplying the LOD by 3. This method is commonly applied to analytical methods that exhibit the baseline noise. The LOD was found out to be 1.14 & 3.46 µg/mL for ATV & RTV respectively. The LOQ was found to be 0.53 & 1.62 µg/mL for ATV & RTV.

Robustness

The robustness of the method was determined by assessing the ability of the developed method to remain unaffected by the small changes in the parameters such as percent organic content, pH of the mobile phase, buffer concentration, temperature, injection volume and flow rate. A deviation of ± 2 nm in the detection wavelength, ± 0.2 ml/min in the flow rate, ± 5% change in the organic phase were tried individually. The result was given in the Table 4.13. Similarly the representative chromatograms for robustness parameters like flow variation and wavelength variation were shown in Figure. 4.10.
Figure: 4.10 - Representative Chromatograms for robustness parameters
Table: 4.13: Robustness studies for Atazanavir and Ritonavir by proposed method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% RSD</th>
<th>Atazanavir</th>
<th>Ritonavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>203 nm</td>
<td>0.46</td>
<td>0.56</td>
</tr>
<tr>
<td>(±2 nm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>207 nm</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>0.8 mL/min</td>
<td>0.65</td>
<td>0.72</td>
</tr>
<tr>
<td>(±02 ml /min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.2mL/min</td>
<td>0.87</td>
<td>0.82</td>
</tr>
</tbody>
</table>

FORCED DEGRADATION STUDIES

Control Sample

Weigh and finely powder not fewer than 20 capsules. Accurately weigh and transfer equivalent to 300 mg of Atazanavir and 100 mg of Ritonavir into a 100 ml volumetric flask, add 70 ml of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 2.0 ml of the above solution into a 100 ml volumetric flask and dilute to volume with diluent. (Figure: 4.2)

Acid Degradation Sample

Weigh and finely powder not fewer than 20 capsules. Accurately weigh and transfer equivalent to 300mg of Atazanavir and 100 mg of Ritonavir into a 100 ml volumetric flask, add 70 ml of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature. Then add 5 ml of 1N HCl, refluxed for 30 min at 60°C, then cooled to room temperature,
neutralize with 1N NaOH and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 2.0 ml of the above solution into a 100 ml volumetric flask and dilute to volume with diluent (Figure: 4.11).

**Base Degradation Sample**

Weigh and finely powder not fewer than 20 capsules. Accurately weigh and transfer equivalent to 300 mg of Atazanavir and 100 mg of Ritonavir into a 100 ml volumetric flask, add 70 ml of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature. Then add 5 ml of 1N NaOH, refluxed for 30 min at 60°C, then cooled to room temperature, neutralize with 1N HCl and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 2.0 ml of the above solution into a 100 ml volumetric flask and dilute to volume with diluent. (Figure: 4.12).

**Peroxide Degradation Sample**

Weigh and finely powder not fewer than 20 capsules. Accurately weigh and transfer equivalent to 300 mg of Atazanavir and 100 mg of Ritonavir into a 100 ml volumetric flask, add 70 ml of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature. Then add 5 ml of Hydrogen Peroxide, refluxed for 30 min at 60°C, then cooled to room temperature, and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 2.0 ml of the above solution into a 100 ml volumetric flask and dilute to volume with diluent. (Figure: 4.13)

**Thermal Degradation Sample:**

Powder collected from 20 capsules is exposed to heat at 105°C for about 5 days. Accurately weigh and transfer equivalent to 300 mg of Atazanavir and 100 mg of Ritonavir into
a 100 ml volumetric flask, add 70 ml of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 2.0 ml of the above solution into a 100 ml volumetric flask and dilute to volume with diluent. (Figure: 4.14). Similarly Humidity, UV-Light exposure, Sunlight exposure and Water hydrolysis stress samples are prepared and checked for their purity by proposed method.

Figure: 4.11: A typical HPLC Chromatogram showing the Control Sample profile of ATV and RTV in Acidic hydrolysis by proposed method
Figure: 4.12: A typical HPLC Chromatogram showing the Control Sample profile of ATV and RTV in Base hydrolysis by proposed method.

Figure: 4.13: A typical HPLC Chromatogram showing the Control Sample profile of ATV & RTV in Peroxide hydrolysis by proposed method.
SUMMARY: RESULTS AND DISCUSSION

Column chemistry, solvent selectivity, solvent strength (volume fraction of organic solvent(s) in the mobile phase), detection wavelength and flow rate were varied to determine the chromatographic conditions for giving the best separation. Several mobile phase compositions were tried to resolve the peaks of Atazanavir and Ritonavir. The optimum results were attained with acetonitrile and ammonium acetate buffer (pH 4.0) in the ratio of 40:60 (v/v) because it could resolve the peaks of Atazanavir with retention time at 2.833 min and Ritonavir retention time at 5.753 min. The two peaks were symmetric and sufficiently resolved. System suitability was carried out by injecting 5 replicate injections of 100% concentration of Atazanavir and Ritonavir. The resolution was found to be greater than 2 and the other parameters are presented in Table 4.10.
Specificity of the chromatographic method was tested by injecting mobile phase as blank and sample concentration prepared from marketed formulation. The response was compared with that obtained from the standard drug. The chromatogram confirms the presence of Atazanavir and Ritonavir at 2.833 min and 5.753 min respectively without any interference. Thus the developed method was specific for analyzing the commercial formulations for Atazanavir and Ritonavir. An optimized chromatogram with the retention times of Atazanavir and Ritonavir was shown in the Figure: 4.2.

The peak areas corresponding to the concentration range of Atazanavir 18.0 - 42.0 µg/mL and Ritonavir 5.0 - 14.0 µg/mL prepared in triplicate was plotted against the respective concentrations. The calibration curves were linear in the range studied for Atazanavir and Ritonavir, respectively, with mean correlation coefficients (n=3) of 0.999 and higher, the representative calibration curve is shown in Figure 4.5 & 4.6. The regression analysis was given in Table 4.6.

Accuracy of the proposed method was assessed by standard addition method at 50%, 100% and 150% levels of recovery to the pre analyzed sample in triplicate. The recovery of the added standard to the sample was calculated and it was found to be 98.9 - 101.0 % w/w for Atazanavir and 98.2 - 100.1 % w/w for Ritonavir respectively and the % RSD was less than 2 for both the drugs which indicates good accuracy of the method. The result of recovery was given in Table 4.11 & 4.12.

LOD and LOQ were calculated from the average slope and standard deviation of y intercepts of the calibration curve. Limit of Detection for Atazanavir and Ritonavir were 1.14 µg/mL and 0.53 µg/mL respectively where as Limit of Quantitation of Atazanavir and Ritonavir
were 3.46 µg/mL and 1.62 µg/mL respectively indicating high sensitivity of the method. LOD and LOQ value was given in Table 4.9 & 4.11. The method is precise with a % RSD of less than 2 for both Atazanavir and Ritonavir respectively. The results of intraday and inter day precision was given in Table 4.8. Robustness was carried out by change in the flow rate (±0.2 ml/min), mobile phase variation (±5%) and variation in wavelength (± 2 nm). Solution of 100% concentration is prepared and injected in triplicate for each varied operational condition and % R.S.D was found to be less than 2. The result was given in Table 4.6. The proposed method was applied for the assay of commercial formulation containing Atazanavir and Ritonavir. Each sample was analyzed in triplicate. The mean recovery values were 100.14 and 100.45 % for Atazanavir and Ritonavir. The result of estimation was given in Tables: 4.11 & 4.12.

Table 4.14: Assay studies for Atazanavir and Ritonavir by proposed method on Marketed sample

<table>
<thead>
<tr>
<th>Drug</th>
<th>Amount claimed in mg/capsule</th>
<th>Amount obtained (mg) by proposed method</th>
<th>% recovery by the proposed method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atazanavir</td>
<td>150</td>
<td>148.12</td>
<td>98.73</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>400</td>
<td>389.28</td>
<td>97.25</td>
</tr>
</tbody>
</table>
CONCLUSION

There are no reports on the simultaneous HPLC determination of ATV & RTV in combination pharmaceutical formulations by stability and degradation studies from the literature, prior to the commencement of these investigations. The author has developed a sensitive, accurate and precise RP-HPLC procedure for the simultaneous estimation of ATV & RTV in bulk drug and also in pharmaceutical formulations.

The proposed RP-HPLC method for simultaneous assay ATV and RTV in combined dosage forms was validated, and found to be applicable for routine quantitative analysis. In the HPLC method, the standard and sample preparations required less time and no tedious extraction were involved thereof. The low values of standard deviations are indicative of the high precision of the method developed. The results of linearity, precision, accuracy and specificity, were proved to be within the limits.

The absence of additional peaks in the chromatogram indicated non-interference of the common excipients used in the capsules. It is thus, demonstrated that the developed RP-HPLC method is simple, linear, accurate, sensitive and reproducible. Thus, the developed method can easily be used for the routine quality control of bulk and pharmaceutical formulations of ATV & RTV with a short analysis time.

It can be seen from the results presented that the proposed procedure has good precision and accuracy. The above proposed method obviates the need for any preliminary treatment and is simple, sensitive and reliable. Thus, the present procedures constitute the first ever reported RP-HPLC method with good precision, accuracy and sensitivity for the simultaneous estimation of ATV & RTV in pure stage and also in combination products.
REFERENCES


36. Mohammad H. Abdelhay, Azza A. Gazy, Rasha A. Shaalan and Heba K. Ashour, two sensitive and reproducible methods were described for the quantitative determination of the antiretroviral drug ritonavir in the presence of its degradation products, Journal Of Pharmaceutical And Biomedical Analysis.;66(11), 648-671, 2012


