Therapeutic drug management of HIV-infected patients with comorbidities

A dissertation by
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Originality Statement

'I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at University of Milan or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at University of Milan and at Luigi Sacco University Hospital, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.'

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Date……...

I certify that I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy.

Clementi Emilio Giuseppe
(Principal Advisor)

I certify that I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy.

Cattaneo Dario

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Preface

Today, antiretroviral (ARV) therapy is potent, convenient and usually well tolerated, capable of reducing human immunodeficiency virus (HIV) blood concentration to undetectable values within a few weeks from treatment initiation and of inducing a robust and sustained CD4 T-cell gain. Despite these unquestioned successes, the problem is far from being solved: even in countries with full access to ARV treatment, life expectancy of people under ARV therapy remains lower with respect to that of uninfected people. Furthermore, large populations of HIV infected individuals who are not diagnosed remain untreated or enter treatment at a very late stage of diseases. Undiagnosed and untreated population represents an infected reservoir that increases HIV transmission.

Patient with Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS) disease face many problems when commencing ARV therapy also called as highly active ARV therapy (HAART). In addition to understanding their HIV disease, they are prescribed with combination ARV therapy and have a higher risk of developing ADRs. Consequently, patients feel that HIV treatment is a burden and turn non-adherent to HAART. Understanding about their HIV disease and the importance of HAART in their daily life can help patients to be adherent to their HAART by changing their intentional non-adherence behavior, negative beliefs and social stigma, which ultimately will improve HAART adherence and effectiveness of HIV treatment. Hence, it is important to understand the knowledge, attitude, belief, and practice of HIV patients towards their HIV disease, ARV medications, and common adverse effects of ARV along with HAART adherence behavior. One important tool for better patient compliance towards HAART is optimizing therapy for minimal side effects by therapeutic drug monitoring.

In the present PhD research work entitled "*Traditional and novel therapeutic approaches for the personalized therapy in HIV patients co-infected with opportunistic infections and other co-morbidities" we* studied the role of therapeutic drug monitoring in HAART therapy for personalized patient care.

The experimental section of the thesis is broadly categories as follows

1. HPLC UV assay method development for ARV drugs quantification
2. LC-MS/MS assay method development for ARV drugs quantification
3. Pharmacokinetics of ARV drugs dosing at conventional doses
4. Association between antiretroviral pharmacokinetics and drug-related metabolic disorders
5. Pharmacokinetic interaction between raltegravir and anti HCV drugs in an HIV-HCV liver transplant recipient

1. Introduction

The concept of managing pharmacotherapy based on plasma drug concentrations has been used for decades in a variety of clinical settings. The interest in therapeutic drug monitoring (TDM)[1] of ARV drugs has grown significantly since HAART [2] became a standard of care in the clinical practice. Beside adherence to the regimen and diet, drug interactions, genetically determined differences in drug distribution and/ or elimination significantly affect systemic drug concentration. High inter-individual variability in plasma concentrations of most ARV drugs and the lower virologic failure rates and adverse events when optimal concentrations are achieved, have already been demonstrated [3]. Thus, TDM can aid optimization of ARV therapy.

In the routine clinical practice TDM of ARV drugs is the method of choice to study drug-drug interactions as multiple agents are concomitantly used in HAART regimens. Moreover, evidence is also available showing that inadequate plasma drug concentrations can favour the development of resistance mutations and endanger present and future treatment options. A number of clinical trials has demonstrated that drug serum concentrations are an important factor for the development of response to therapy for HIV [4]. The main goal of TDM of ARV drugs is to optimise treatment responses and tolerability, and to minimise drug-associated toxicity and resistance. A number of studies in adults suggests that modified doses and regimen choices based on TDM achieve targeted ARV drug concentrations which are associated with improved clinical response and/or tolerability [5–11]. The application of TDM requires, however, the availability of feasible and reliable techniques.

1.1. Analytical techniques in TDM

As new drugs are constantly approved and introduced in HAART regimen the need of TDM is also growing. Chromatography coupled with various spectrophotometer detectors such as ultra violet (UV), photodiode array (PDA) and mass spectrometry (MS) are the instruments of choice of the most clinical pharmacology laboratories[12]. Alternatively immunoassays[13] which are commercially available for lot of drugs are also becoming available for TDM of ARV's.

a. Immunoassays in TDM

TDM of clinical agents and drugs of abuse testing are now performed by immunoassay methods on automated systems. Most immunoassay methods use specimens without any pretreatment and are run on fully automated, continuous, random access systems. The assays require very small amounts of sample (mostly <100µL), reagents are stored in the analyser, and most analysers have stored calibration curves on the system. In immunoassays, the analyte is detected by its complexation with a specific binding molecule, which in most cases is an analyte-specific antibody (or a pair of specific antibodies)[14]. This reaction is further util-

ized in various formats and labels, giving a whole series of immunoassay technologies, systems, and options. With respect to assay design, there are two formats of immunoassays: competition and immunometric (commonly referred as "sandwich")[15]. Competition immunoassays work best for analytes with small molecular weight, requiring a single analyte-specific antibody. In contrast, sandwich immunoassays [16] are mostly used for analytes with larger molecular weight, such as proteins or peptides, and use two different specific antibodies.

Since most TDM immunoassays involve analytes of small molecular size, these assays employ the competition format. In this format, the analyte molecules in the specimen compete with analyte (or its analogues), labelled with a suitable tag provided in the reagent, for a limited number of binding sites provided by, for example, an analyte specific antibody (also provided in the reagent). Thus, in these types of assays, the higher the analyte concentration in the sample, the less of label can bind to the antibody to form the conjugate. If the bound label provides the signal, which in turn is used to calculate the analyte concentration in the sample, the analyte concentration in the specimen is inversely proportional to the signal produced. If the free label provides the signal, then signal produced is proportional to the analyte concentration. The signal is mostly optical—absorbance, fluorescence, or chemiluminescence (Figure 1 and Figure 2).

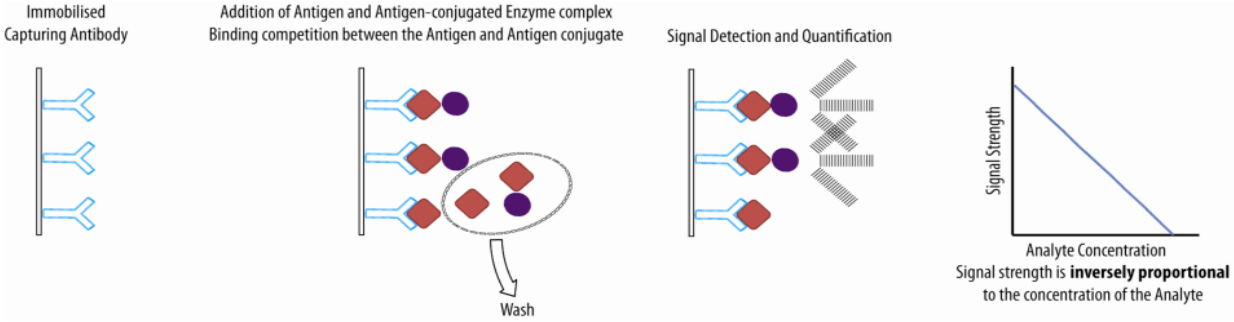

Figure 1. Competition immunoassays

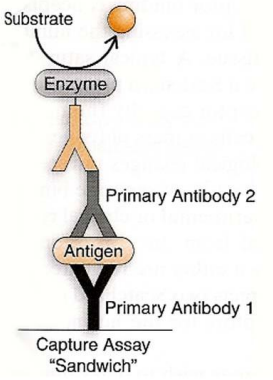

Figure 2. Sandwich immunoassay

There are variations in this basic format. The assays can be homogeneous [17] or heterogeneous [18]. In the former, the bound label has different properties than the free label. For example, in fluorescent polarisation immunoassay (FPIA) [19] , the free label has different Brownian motion than when the relatively small molecular weight (a few hundreds to thousand Daltons) label is complexed with a large antibody (140,000 D). This results in different fluorescence polarisation properties of the label, which is utilized to quantify the bound label [19].

In heterogeneous immunoassays [20], on the contrary, the bound label is physically separated from the unbound labels, and its signal is measured. The separation is often done magnetically, where the reagent analyte (or its analogue) is provided as coupled to paramagnetic particles (PMP), and the antibody is labelled. Conversely, the antibody may be also provided as conjugated to the PMP, and the reagent analyte may carry the label. After separation and wash, the bound label is reacted with other reagents to generate the signal. This is the mechanism occurring in many chemiluminescence immunoassays [21] (CLIA), where the label may be a small molecule which generates chemiluminescent signals. The label also may be an enzyme [enzyme-linked immunosorbent assay (ELISA)], which generates chemiluminescent, fluorometric, or colorimetric signal. In older immunoassay formats, the labels used to be radioactive [radio-immunoassay (RIA)]. But because of safety and waste disposal issues, RIA is rarely used today.

Even though the immunoassay methods are now widely used, there are few limitations of this technique. Antibody specificity is the major concern of an immunoassay. Many endogenous metabolites of the analyte (drug) may have very similar structural recognition motif as the analyte itself. There may be other molecules unrelated to the analyte but producing a recognition motif comparable to that of the analyte. These molecules are generally called cross-reactants. When present in the sample, these molecules may produce false results (both positive and negative interference) in the relevant immunoassay. Other components in a specimen, such as bilirubin, haemoglobin, or lipids, may interfere in the immunoassay by interfering with the assay signal, thus producing incorrect results. A third type of immunoassay interference involves endogenous human antibodies in the specimen, which may interfere with components of the assay reagent such as the assay antibodies, or the antigen-labels. Such interference includes the interference from heterophilic antibodies or various human anti-animal antibodies [22]. Presently, such methodologies provide poor performance for the estimation of ARV drugs concentrations.

b. Gas–liquid chromatography (GC)

Gas–liquid chromatography is a separation technique first described in 1952 by James and Martin. In most GC column, the stationary phase is a liquid and the mobile phase is an inert gas. Typically, the stationary phase has a low vapor pressure so that at column tempera-

ture it can be considered nonvolatile. Introduction of the capillary column dramatically improved resolution of peaks in GC analysis. Resolution equivalent to several hundred thousand theoretical plates is achievable using a fused silica capillary column. Depending on the stationary phase composition, a GC column may have low polarity, intermediate polarity, or high polarity.

Microprocessor control of oven temperature and automatic sample injection techniques also enhanced both performance and ease of automation of GC technique in clinical laboratories. The sensitivity and specificity of GC analysis depends on the choice of detector. Mass spectrometry can be used in combination with a GC, and MS is capable of producing a mass spectrum of any compound coming out of the column of gas chromatograph. Nitrogen phosphorus detector is specific for nitrogen- and phosphorus-containing compounds and is very sensitive. Electron capture detector can detect any halogen-containing compounds. Flame ionization and thermal conductivity detectors are also used in GC. The major limitation of GC is that this technique can only be applied to volatile substances with relatively low molecular weights. Polar compounds cannot be analyzed by this technique. However, a relatively polar compound can be chemically converted to a non-polar compound (derivatisation) for analysis by GC.

c. High-Performance Liquid Chromatography (HPLC)

Application of GC as a separation technique is limited to volatile molecules. However, HPLC[23] can be used for separation of both polar and non-polar molecules. Usually, derivatisation is not necessary for HPLC analysis. HPLC is based on the principle of liquid–liquid chromatography where both mobile phase and stationary phase are liquid. LC using column was first described in 1941. In normal LC, the stationary phase is polar and the mobile phase (eluting solvent) is non-polar. In reverse phase chromatography, the stationary phase is non-polar and the mobile phase is polar. Several detectors can be used for monitoring elution of peaks from HPLC column including UV, PDA, MS, fluorescence detectors, conductivity detectors, and refractive index detectors. UV detection is commonly used in clinical laboratories although other detection techniques such as fluorescence technique and electrochemical detection technique are also common.

d. Liquid Chromatography- Mass Spectrometry (LC-MS)

MS when combines with LC offers specificity and selectivity higher than the other methods. A Mass Spectrophotometer [24] is capable of analysing charged particle based on their mass. A typical mass spectrometer consists of an inlet system, which supplies the pure compound (separated from complex biological matrix by GC or HPLC) to the mass spectrometer, an ion source, a mass analyser, and a detector. The ion source is responsible for fragmentation pattern of the compound of interest in characteristic pattern depending on the

functional groups and other structural features of the molecule. The detector plots a chromatogram listing all ions generated and separated by their mass to charge ratios as well as abundance. Mass spectrometer is often used as a detector for compounds eluting from a GC or column of an HPLC. GC/MS is widely used in clinical toxicology [25] laboratories for detection and quantification of drugs of abuse in biological matrixes, such as urine or blood, because of its specificity, sensitivity, and the availability of larger number of mass spectra in standard drug libraries. Coupling of HPLC with MS enables the analysis of thermally labile compounds, polar compounds, or compounds with high molecular weights that cannot be analysed by GC or a combined GC/MS. Electron ionisation (EI) at 70 eV produces a reproducible mass spectrum, which is a common ion source used in GC/MS analysis of therapeutic drugs. The electrospray interface is very common in HPLC/MS analysers used in clinical laboratories. The electrospray interface produces single or multiple charged ions directly from a solvent system by creating a fine spray of highly charged droplets in the presence of a strong electric field with assistance from heat or from pneumatics. The atmospheric pressure chemical ionisation interface produces sample ions by charge transfer from reagent ions. The reagent ions are produced from solvent vapour of the mobile phase. After producing charged particles from the analyte eluting from the column, a mass spectrum is produced by detecting these charged particles (positive or negative ion) in the detector of the mass spectrum. The major types of mass spectrometric analyzers are quadrupole analyser [26], ion trap analysers [27], and time-of-flight analyser [28].

e. Ultra Performance Liquid Chromatography (UPLC)

UPLC applies the same principle as HPLC, the difference is the use of sub 2-µm particle columns in a system holistically designed to maximise the advantages of these columns, creating a powerful, robust and reliable solution. UPLC is based on the principal of use of stationary phase consisting of particles less than 2 µm while HPLC columns are typically filled with particles of 3 to 5 µm. The underlying principles of this evolution are governed by the van Deemter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency)[29]. It was found that HETP decreases to a minimum value and then increases with increasing flow rate. However, with the 1.7 µm particles used in UPLC, HETP is lowered compared to the larger particles and does not increase at higher flow rates. This allow faster separations to be carried out on shorter columns and/or with higher flow rates, leading to column increased resolution between specific peak pairs and increased peak capacity, defined as the number of peaks that can be separated with specified resolution in a given time interval. Efficiency is three times greater with 1.7 µm particles compared to 5 µm particles and two times greater compared to 3.5 µm particles. Resolution is 70% higher than with 5 µm particles and 40% higher than with 3.5 µm particles. High speed is obtained because column length with 1.7 µm particles can be reduced by a factor of 3 compared to 5 µm particles for the same efficiency, and flow rate can be three times higher. This means separations can be nine times faster with equal

resolution. Sensitivity increases because less band spreading occurs during migration through a column with smaller particles (peak width is less and peak height greater)[30,31].

The Van Deemter curve, governed by an equation with three components shows that the usable flow range for a good efficiency with a small diameter particles is much greater than for larger diameters.

$$H=A+B/v+Cv$$

where A, B and C are constants

v is the linear velocity, the carrier gas flow rate

The **A term** is independent of velocity and represents "eddy" mixing, its value correlating positively with packed column particles'dimension and uniformity.

The **B term** represents axial diffusion or the natural diffusion tendency of molecules. This effect is diminished at high flow rates and so this term is divided by v.

The C term is due to kinetic resistance to equilibrium in the separation process. The kinetic resistance is the time lag involved in moving from the gas phase to the packing stationary phase and back again. The greater the flow of gas, the more a molecule on the packing tends to lag behind molecules in the mobile phase. Thus this term is proportional to v and shows that it is possible to increase throughput, and thus the speed of analysis without affecting the chromatographic performance.

1.2. Antiretroviral drugs

In treating patients infected by HIV, different classes of ARV drugs have been developed to target various life cycles of the retrovirus. Drug classes include nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors and two new classes, entry inhibitors and integrase strand transfer inhibitors. **Table 1** shows a summary of the currently available drugs and their respective classes.

Table 1. Classification of Antiretroviral drugs

Sr no	Generic Name
Nucleoside, Nucleotide Reverse Transcriptase Inhibitors (N(t)RTI's)	abacavir (abacavir sulfate, ABC), didanosine (ddI), emtricitabine (FTC), lamivudine (3TC), stavudine (d4T), tenofovir disoproxil fumarate (tenofovir DF, TDF), zidovudine (azidothymidine, AZT, ZDV)
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI's)	delavirdine (delavirdine mesylate, DLV), efavirenz (EFV), nevirapine (extended-release nevirapine, NVP), rilpivirine (rilpivirine hydrochloride, RPV), etravirine (ETV)
Protease Inhibitors (PI's)	atazanavir (atazanavir sulfate, ATV), darunavir (darunavir ethanolate, DRV), fosamprenavir (fosamprenavir calcium, FOS-APV, FPV), indinavir (indinavir sulfate, IDV), nelfinavir (nelfinavir mesylate, NFV), ritonavir (RTV), saquinavir (saquinavir mesylate, SQV), tipranavir (TPV), lopinavir (LPV), amprenavir (APV)
Fusion Inhibitors	enfuvirtide (T-20)
Entry Inhibitors	maraviroc (MVC)
Integrase Inhibitors (INSTI's)	dolutegravir (DTG) , elvitegravir (EVG), raltegravir (raltegravir potassium, RAL)

These classes of drugs are used in a combination regimen referred to as the HAART. This therapy is extremely effective, and is associated with reduced mortality and has allowed HIV infection to become a manageable chronic disease. The HAART regimen involves life-long treatment and requires constant dose optimization to combat development of viral resistance [32]. Treatment is particularly challenging as each drug has large individual pharmacokinetic variability, important adverse effects and considerable potential for drug–drug interactions, and the complex dosing regimen predisposes to non-compliance [33–35]. Because of a possible correlation between drug concentrations and efficacy/toxicity, most ARV agents may be potential candidates for TDM [36]. Monitoring has been proposed to aid HAART to check compliance and individualise dose regimens, as well as to investigate viral resistance when integrated with viral DNA genotyping [33,35,37].

The practice of treating HIV patients with different types of combination drug therapies has lead to the development of simultaneous HPLC-UV and LC-MS/MS methods. These methods have been developed to cover a wide range of possible therapies as each patient may be individualized to receive any combination of ARV drugs.

The majority of HPLC-UV methods involved protein precipitation and solid phase extraction, HPLC-UV assays are economical and feasible in most of clinical setting but also considered as time consuming and less sensitive if compared to mass detection. LC-MS/MS methods have employed protein-precipitation as sample preparation. Although the simplicity of this approach enables high-throughput sample preparation, there is the potential for more interference from endogenous compounds and matrix effects [38]. Other types of sample preparation have therefore been used [39] . Jung and co-workers [40] used protein precipitation followed by liquid–liquid extraction. Semi-automated liquid–liquid extraction in the 96-well plate format has also been reported [41]. Notably, Martin et al. [39] published a simultaneous method for the determination of 11 ARV in human plasma, using two-dimensional chromatography with a total chromatographic run time of 6 min. Briefly, after protein precipitation of the plasma sample, the supernatant was injected onto an on-line solid phase extraction system in which the analytes are firstly retained on an extraction column (Poros), while some of the potential interferences are washed to waste. The analytes are then eluted onto an analytical column (phenyl-hexyl) for chromatographic separation. The advantages of this approach are minimal sample handling, relatively clean extracts and moderately high-throughput.

Electrospray ionization has been the ion source of choice for most published methods. The majority of drugs can be detected by positive ionization. Negative ionization may be required to obtain the necessary sensitivity for some drugs for example Sylvie Quaranta[42], Gehrig AK[43]Jung BH [40] etc.

1.3. Free Drug fraction determination of ARV drugs for TDM

Many of the PI's and NNRTI's are highly bound to alpha-1-acid glycoprotein [44] and variability in free fraction has been reported[45] .Thus the measurement of free (or unbound) drug is more appropriate for therapeutic monitoring for these classes of drugs [34]. Free ARV drugs can be isolated from plasma using commercially available ultrafiltration devices [45]. The subsequent ultrafiltrate then can be prepare for LC-MS/MS analysis by dilution [45] or liquid–liquid extraction [46,47] . These methods were successful in the free drug analysis of ARV drugs using mass spectrometric conditions similar to those applied to the analysis of plasma samples.

a. Intracellular determination of ARV drugs for TDM

As the site of action for many ARV drugs is within the infected cell, and intracellular accumulation of these drugs may display inter-patient variability, it is argued that the measurement of antiviral concentration in peripheral blood mononuclear cells (PBMC's) may more adequately reflect drug efficacy than plasma [32,48–51].

For example HIV PI's exert their action inside cells that are susceptible to HIV infection. The intracellular pharmacokinetics of these drugs, its relation to the plasma pharmacokinetics, and its relation to drug efficacy and toxicity is therefore an active area of research[52–54]. However, determination of the intracellular levels of ARV drugs is an analytical challenge, because of the small amount of target cells, e.g. PBMC's, which can be obtained from a patient's blood sample. Approximately one million PBMC's can be obtained from 1–2 mL blood. This amount of PBMC's has a total intracellular volume of ~0.4 µL, while 500 – 1000 µL plasma can be obtained from that same blood sample. As a consequence, much is known about the plasma pharmacokinetics of HIV PI's in HIV infected children, but no information is available on the intracellular levels of HIV PI's in this group of patients

For sample preparation normally the plasma layer containing the PBMC's is treated with PBS and lysis buffer to disturb the cell membrane. After centrifugation the supernant can be use for further analysis on LC-MS/MS using similar conditions applied to the analysis of plasma samples. LC-MS/MS systems are more common for intracellular ARV drugs analysis as LC-MS/MS has been shown to provide the required sensitivity to detect low cellular ARV concentrations[46,48,53,55,56] .

b. Active metabolite determination of ARV's for TDM

The NRTI's are prodrugs that require cellular enzymes for conversion to the active phosphorylated form [32]. These intracellular phosphorylated metabolites compete with endogenous deoxynucleotide triphosphates for incorporation into the viral DNA. Thus the efficacy of these drugs is related to the cellular concentration of their metabolites. The major difficulties in measuring these metabolites in PBMC's are the low intracellular concentrations and their polar nature. LC-MS/MS methods for the triphosphate metabolites have been recently reported[57,58]. These methods use standard approaches for peripheral blood mononuclear cell isolation. Chromatography is performed using ion-pairing reagents in order to retain the analytes, obtain acceptable peak shape and prevent irreversible adsorption [57,58] . Coulier et al. [57] used negative electrospray ionisation mass spectrometric conditions and measured these analytes in the fmol concentration range. Despite the LC-MS/MS technology to measure intracellular drug concentrations, there are several limitations and factors relevant to TDM purposes: (1) Isolation of peripheral blood mononuclear cell is long and impractical for routine analysis; (2) The potential for discrepancy in the cell number when counted under a microscope; (3) The possibility of drug–drug interactions at the intracellular level; (4) The possibility of analyte binding to intracellular protein and cell membrane [32] .

c. Alternative matrix for therapeutic drug monitoring of ARV's

Dried blood spot samples have been advocated as an alternative matrces for TDM. Dried blood spots allow non-hospital sampling in a resource limited environment and the small sampling volume is patient friendly especially for children and neonates. In addition, it has reduced risk of HIV transmission and samples can be easily transported or stored[59] . The analytes have been found to be stable in dried blood spot for at least seven days at 30 °C [60], thus allowing transport from regional centers. There are several publication which describe simultaneous detection of ARV drug in dried blood spots [55,59–64]. Dried blood spots were typically treated with an organic solvent to extract the analytes and direct injection on to the LC-MS/MS performed. Mass spectrometric conditions are similar to those used for plasma analysis. The measure concentrations in dried blood spots correlated well with that of plasma [55,59]. However, some ARV drugs had 50–80% higher concentrations in dried blood spots, while other ARV drugs had lower concentration compared to plasma. Hence, more investigation into the clinical utility of dried blood spots as an alternative matrix is required.

The sensitivity and selectivity of LC-MS/MS has enabled the successful measurement of ARV's in other matrices such as cerebrospinal fluid, seminal fluid, hair and breast milk has been reported in the literature[47,65–76]. The measurement of these drugs in cerebrospinal fluid has provided a way to better understand the neurological complications of these therapies[47,67], while the investigation of drug pharmacokinetics in seminal fluid and breast milk has facilitated understanding of potential ways to prevent HIV transmission [68,74–76]. The measurement of ARVs in human hair allows the monitoring of long term drug exposure. Huang et al. [71] have reported a LC-MS/MS method capable of measuring 3 ARV drugs in human hair. However, the extraction of analytes from hair is rather time consuming and elaborate and requires further improvement to be practical for monitoring purposes.

1.4. Rationale for TDM of ARV's

a. Protease Inhibitors (PI's)

To date, TDM of PI's has been a useful tool to optimize HIV-1 treatment, prevent drug toxicity, and assess patient compliance[35,77]. PI's are metabolized mainly by CYP450, in particular the CYP3A4 isoenzyme group. With the co-administration of a CYP3A4 inhibitor, RTV or cobicistat plasma exposure of PI's can significantly increase [78,79]. Inducers of CYP3A4 may in turn lower PI concentrations, though this effect can be partially reversed by the action of the boosters [80]. Due to the potent mechanism-based inhibitory effects of RTV on CYP3A4 (50% inhibitory concentration [IC50]: 0.05–1.4 μg/mL), RTV is believed to function as an inhibitor for CYP3A4 substrates with chronic administration [81,82]. TPV,

DRV, SQV, and LPV all require RTV boosting for clinical use; therefore, the interaction profile of these agents is determined in part by the effect of RTV on individual isoenzymes. Many other PI's (ATV, IDV, FOS-APV) are commonly boosted with RTV to optimize their pharmacokinetic profile, simplify their dosing frequency, and improve their side effect profile. Interactions among PI's can be complex due to varied effects on specific CYP enzymes and may result in sub-therapeutic concentrations or additive toxicity.

RTV co-administration (100 mg twice daily) increased the absolute bioavailability of DRV in healthy volunteers from 37% to 82% and produced a 14-fold increase in systemic exposure[83] .Administration of DRV/RTV with food increases bioavailability by 30% irrespective of whether a low or high-fat meal is administered[84]. A 27% increase in NVP AUC and 18–47% increase in C_{min} were observed when NVP (200 mg twice daily) was administered with two separate formulations of DRV (300 mg by oral solution, 400 mg tablet) boosted with RTV twice daily in HIV-infected patients. A large reduction in DRV AUC was observed when given with LPV/RTV (400/100 mg or 533/133 mg twice daily) in HIV-infected patients [85].

Co-administration of IDV (800 mg twice daily) and DRV/RTV (400 mg/100 mg twice daily) in healthy volunteers results in an increase in IDV AUC and *C*min (23% and 125%, respectively) and DRV AUC and *C*min (24% and 44%, respectively) compared with IDV/RTV (800 mg/100 mg twice daily) alone [86]. RAL and MVC have no significant effect on the pharmacokinetics of DRV/RTV.

b. Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI's)

NNRTI's are inducers of hepatic P-cytochrome 450 (CYP450), isoenzyme 2B6, 2C9 and 2C19 (EFV [87]), and 3A4 (EFV, NVP, RPV,[88] and ETV [89]), and their absorption and distribution is affected by the drug transporter P-glycoprotein (P-gp)[90]. NNRTI's, except RPV, interact with PI's[91], MVC[92], analgesics[93], antiarrhythmics[93], rifamycins[94], anticoagulants[95], anticonvulsivants[96], antipsychotics[97], antidepressants[98], antifungals[99], simeprevir[100], calcium channel blockers[101], contraceptives[102], cytotoxics[103] and many other agents.

Important food interactions exist with some NNRTI's. The bioavailability of EFV is increased significantly (AUC by 28%, maximum concentration [C_{max}] by 79%) with a high-fat meal (1000 kcal, 500–600 kcal from fat) resulting in a higher risk for central nervous system side effects (e.g., vivid dreams, somnolence)[104].

ETV exhibits improved bioavailability with food versus fasting, where systemic exposure is reduced by 51%, and should be administered with food [105]. The bioavailability of RPV is decreased by 43% to 50% when taken on an empty stomach or with a high-protein

nutritional drink (8 grams of fat, 300 kcal) compared with a standard meal (21 grams of fat, 533 kcal) but is unaffected by a high fat meal (56 grams of fat, 928 kcal) [106].

EFV and ETV exhibit a mixed interaction profile, whereas NVP primarily functions as an inducer and delavirdine as an inhibitor. EFV is a weak inhibitor of CYP1A2 and CYP2D6 *in vitro*, a strong inhibitor of CYP2C9 and CYP2C19, and an inducer of CYP3A4 and CYP2B6 [87,105,107]. ETV induces CYP3A4, CYP2B6, and uridine diphosphate glucuronosyltransferase (UGT) and inhibits CYP2C9 and CYP2C19 [108]. Drug interaction profile of ETV with other ARV drugs has been studied fairly extensively. Serum concentrations for some PI's are reduced by ETV as a result of CYP3A4 induction. Co-administration of ETV and ATV with or without RTV results in reduction in ATV C_{min} (38–47%) and increased ETV systemic exposure (30–50%). No significant interaction is noted between LPV/RTV and ETV[109]. So at the end ETV should not be administered with unboosted PI's, full-dose RTV, or TPV/RTV but can be safely used with DRV/RTV, LPV/RTV, and SQV/RTV. NVP induces CYP3A4, CYP2B6, CYP2C9, and possibly UGT [110,111].

RPV exhibits weak induction of CYP3A4 and CYP2C19 at higher doses (150–300 mg) *in vivo*, weak induction of CYP1A2 and CYP2B6 *in vitro*, and is prone to having its metabolism altered by strong CYP3A4 inhibitors [112]. RPV (150 mg daily) increases the C_{max}, C_{min}, and AUC of TDF (300 mg daily) by 21–24% but is not thought to be clinically significant [113]. DRV/RTV (800 mg/100 mg daily) increased RPV AUC and C_{min} by 230–280% in healthy subjects; no change in DRV or RTV pharmacokinetics was noted[114]. RPV pharmacokinetics are similarly increased by LPV/RTV (400 mg/100 mg twice daily) where RPV AUC and C_{min} are modestly elevated by 52% and 74%, respectively[115]. Further studies are underway to ascertain the significance of interactions between RPV and other ARV's.

c. Nucleoside/ Nucleotide Reverse Transcriptase Inhibitors (N(t)RTI's)

N(t)RTIs are not substrates for, nor do they inhibit or induce, the cytochrome P450 enzyme system. Therefore, many of the interaction concerns which are common with other classes of ARV agents are not a concern with N(t)RTIs. Some mechanisms of potential interactions with N(t)RTIs include competition for renal elimination pathways between concomitant medications and those N(t)RTIs that are exclusively eliminated via the kidneys, and changes to or alcohol dehydrogenase pathways which can influence the elimination of ZDV and ABC, respectively. TDF has significant interactions to consider with concomitant ARV therapy. Specifically, the dose of ddI must be reduced while ATV must be boosted with RTV if co-administered with TDF **[**116**]**.

d. Entry inhibitors

MVC, the first in this class also called as CCR5 receptor antagonist is one of the most sensitive metabolites of CYP3A4 with no significant involvement of the other CYP450

isoenzymes, and has a weak, poorly significant inhibition on CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 and 3A5[92]. MVC has a linear pharmacokinetics and therefore, C_{trough} can be considered a reliable indicator of adequate drug dose[117,118]. PI's and NNRTI's have a major effect on the plasma concentrations of this drug and require halving or doubling the standard dose[119].

The bioavailability of MVC is reduced by 33% with a high-fat meal and by 20% with a low-fat meal [120]. MVC interaction with NNRTI;s and PI's is likely due to shared metabolism through CYP3A4 and altered *p*-glycoprotein function[121]. EFV (600 mg daily) reduces the MVC AUC in healthy volunteers and HIV-infected subjects by 51% and 53%, respectively[122,123]. MVC 200 mg twice daily with EFV produced a MVC AUC comparable to MVC 100 mg twice daily[122]. MVC systemic exposure is reduced by 53% with ETV [83]. No significant change in MVC AUC from historical controls was noted when single-dose MVC (300 mg) was combined with NVP (200 mg twice daily) in HIV-infected subjects [123]. Co-administration of MVC (300 mg twice daily) with the PI LPV/RTV (400 mg/100 mg twice daily) in healthy volunteers increased MVC AUC and C_{max} by approximately 4-fold and 2-fold, respectively [122]. Administration of MVC (100 mg twice daily) with SQV/RTV (1000 mg/100 mg twice daily) in healthy subjects increased MVC AUC and *C*max by 9.8-fold and 4.8-fold, respectively [122]. Inclusion of EFV with either LPV/RTV or SQV/RTV yielded sustained increases in MVC AUC (2.5-fold and 5-fold, respectively)[122]. MVC (300 mg twice daily) with either ATV (400 mg daily) or ATV/RTV (300 mg/100 mg daily) in healthy subjects resulted in increased MVC exposure (3.6-fold and 4.9-fold, respectively) [122]. In contrast, administration of MVC (150 mg twice daily) with TPV/RTV (500 mg/200 mg twice daily) produced no clinically significant change in MVC serum concentrations (GMRs of 1.02 and 0.86 for MVC AUC or *C*max, respectively) [122]. When MVC (150 mg twice daily) was combined with the integrase inhibitor EVG (150 mg daily) and RTV (100 mg daily) in healthy volunteers, the GMR for MVC AUC was 2.86 [124]. A 37% and 28% reduction in RAL AUC and C_{min}, respectively, was observed when RAL (400 mg twice daily) and MVC (300 mg twice daily) were combined in healthy volunteers, but the clinical significance of this is presently unknown due to the wide variability in RAL pharmacokinetics [125].

e. Integrase strand transfer inhibitors (INSTI's)

RAL, the first drug to be approved in this class, is primarily glucuronidated by uridine glucuronosyl transferase (UGT) 1A1, and has limited drug interactions [126]. Interaction between RAL and ATV with or without RTV has been evaluated because of the well-established inhibitory effects of ATV on UGT1A1[127]. RAL however has a high inter- and intra-patient pharmacokinetic variability and needs at least a mini-AUC as described above for correct assessment [128], as the C_{trough} levels may be poorly indicative [129]. ETV decreased mean RAL C_{min} by 34% in healthy volunteers[130]. Concurrent use of RAL and ETV

was associated with incomplete viral suppression in a small case series (n = 4) of HIV-infected patients, but no clinically significant interaction was noted when ETV and RAL were combined with DRV/RTV [131]. An increase in AUC and C_{min} (19% and 750%, respectively) is observed when RAL is administered with a high-fat meal; however, RAL may be administered with or without food [132].

DTG, new drug in this class is also metabolized via UGT1A1 with a minor contribution by CYP3A, and is a substrate for P-glycoprotein, with very few dug-drug interactions [133], the only relevant one being with metformine, whose exposure results nearly doubled by co-administration [134]. There are at present few data on DTG TDM, but it's pharmacokinetics appears to be characterized by low variability [135]. Administration of ATV (400 mg daily) or ATV/RTV (300 mg daily) with DTG (30 mg daily) results in modest elevation of DTG pharmacokinetics in healthy volunteers (AUC increased by 62–91%, C_{min} increased by 90–121%) but is considered safe [136]. DTG AUC and C_{min} are reduced (57% and 75%, respectively) when DTG (50 mg daily) is given with EFV (600 mg daily) but concentrations remain 4–5-fold above the protein-adjusted IC50 for wild-type virus [137]. ETV (200 mg twice daily) significantly reduces the AUC (71%) and C_{min} (88%) of DTG (50 mg daily) in healthy volunteers and should not be co-administered unless LPV/RTV or DRV/RTV are also included[138]. No interaction is evident between DTG and TDF disoproxil fumarate [139]. The drug-drug interaction profile of DTG with MVC and INSTI's remains to be characterized.

f. Fusion inhibitors (FI's)

Enfuvirtide is a synthetic peptide that binds to HIV-1 glycoprotein 41, blocking the fusion of viral and cellular membranes [140]. It exhibits a small volume of distribution (5.48 L), low systemic clearance (1.4 L/h), high plasma protein binding (92%), and high bioavailability (84.3%). Less than 17% of it is deaminated to a minimally active metabolite, and both are primarily eliminated via catabolism to amino acid residues. Following subcutaneous administration, T-20 is almost completely absorbed, with a slow and protracted subcutaneous absorption, resulting in relatively flat steady-state plasma concentration-time profiles. T-20 did not influence concentrations of drugs metabolised by CYP3A4, CYP2D6 or N-acetyltransferase, and had only minimal effects on those metabolised by CYP1A2, CYP2E1 or CYP2C19 [141].

All these drugs therefore have the potential for significant reciprocal drug–drug interactions not to mention those with other co-administered medications, adding to the challenges of constructing long-term effective combination ARV regimens. To that endeavour careful monitoring of drug exposure is an important component of optimum therapeutic outcome. One of the important methods of therapy optimization and personalized patient care system is the TDM and the most important tool of TDM is quantitative assays.

Hence we decided to start work with HPLC-UV and LC-MS/MS assay methods development and validation for ARV drugs followed by TDM and drug-drug interaction studies.

1.5. Plan of Work

a. Development and validation of an HPLC-UV method for the simultaneous quantification of nine antiretroviral agents in the plasma of HIV-infected patients

b. Development and validation of electrospray ionization LC-tandem mass assay for the simultaneous measurement of ten antiretroviral agents in human plasma samples

c. Study of the pharmacokinetics of antiretrovirals given at conventional doses in HIV patients

d. Study of the potential correlations between metabolic and kidney disorders and atazanavir concentrations in HIV infected patients

e. Pharmacokinetic interaction study between RAL and the 3D regimen of ombitasvir, dasabuvir and paritaprevir/RTV in an HIV-HCV liver transplant recipient

2. Experimental and Results

2.1. An HPLC-UV method for the simultaneous quantification of nine antiretroviral agents in the plasma of HIV-infected patients

Many methods for the simultaneous quantification of various ARV drugs using HPLC with UV detection have been published [142–149]. Several chromatographic methods coupled with tandem mass spectrometry (LC-MS/MS) have been validated to quantify newly approved ARV agents like DTG and RPV in plasma samples [150–156]. Methods with mass detectors however, require expensive instrumentations and trained, expert personnel; these conditions are difficult to satisfy in clinical laboratories of many hospitals. Chromatographic analysis coupled with UV detectors (HPLC-UV) represents a cheaper option, more easy to adapt to the hospital setting. No HPLC-UV methods for the simultaneous quantification of ATV, DTG, DRV, EFV, ETV, RPV, RGV, LPV, and TPV in human plasma have been published to date.

The aim of our study was to develop and validate an HPLC coupled with UV detector analytical method for the simultaneous quantification of the new drugs DTG and RPV with the most frequently used seven other ARV drugs in human plasma samples.

2.1.1. Material and Methods

a. Chemicals and reagents

ATV, DTG, EFV, ETV, LPV, RTG was purchased from Spectra 2000 (Rome, Italy), DRV and RPV were kindly provided by Janssen Cilag (Beerse, Belgium), TPV was donated by Boehringer and quinoxaline (QX), used as internal standard (IS), was purchased from Sigma (Milan, Italy). Acetonitrile and methanol (Sigma) were of HPLC grade. Deionised water used in all aqueous solutions was obtained from a Milli-Q water purification system (Millipore, Milan, Italy).

Stock solutions with a concentration of 1 mg/mL were prepared for ATV, DRV EFV, ETV, LPV, RTG, and TPV in methanol. Stock solutions with a concentration of 1 mg/mL were prepared for DTG in methanol/dimethylsulfoxide (98:2) and for RPV in methanol/dimethylsulfoxide (90:10). A working solution of internal standard (IS) was made with QX (7.5 mg/mL) in methanol and stored at 4^0C until use.

Two lots of 6 point of STD and QC's were made, lot ''A'' with DTG and EFV without RGV and LPV, and lot ''B'' with RGV and LPV without DTG and EFV. Calibration ranges, from STD 6 to STD 1, and QC concentrations for all drugs are listed in **Table 2.**

Table 2. UV Wavelengths used to Quantify IS and Each Drug , retention time of each drug, relative concentrations (Lots A and B) of STD 6 to STD 1 of calibration curves and QCs (QC High, QC Medium, and QC Low)

Analyte	**Retention time**	**Wavelengths (nm)**	**Calibration points**						**QC**		
			STD 1	**STD 2**	**STD 3**	**STD 4**	**STD5**	**STD 6**	**Low**	**Medium**	**High**
ATV	15.3	260	60	180	600	1800	6000	12000	90	900	2000
DTG†	5.4	260	20	60	200	800	4000	8000	50	300	2000
DRV	9.6	260	150	300	900	3000	6000	15000	600	1800	8000
EFV†	17.1	260	150	300	900	3000	6000	15000	600	1800	8000
ETV	17.4	305	50	100	500	1000	2000	4000	200	1600	3000
LPV*	17.4	260	150	300	900	3000	6000	15000	600	1800	8000
RGV*	5.6	260	40	120	400	1200	4800	9600	80	600	6000
RPV	13.8	305	20	60	200	400	600	2000	50	300	1000
TPV	18.7	260	500	1000	5000	10000	20000	40000	2000	16000	30000
IS	8.6	260	-	-	-	-	-	-	-	-	-

†DTG and EFV was only present in lot A due to its coelution with RGV and LPV respectively.
**LPV and RGV was only present in lot B due to its coelution with EFV and DTG respectively.*

c. HPLC-UV apparatus and conditions

The chromatographic system consisted of an Alliance e2695 Separation Module equipped with an online degasser and an automatic injector maintained at 10°C and 2998 photodiode array detector, set at 260 for ATV, DRV, DTG, EFV, LPV, RTG and TPV and at 305 nm for ETV and RPV. Data were collected and processed using Empower software for HPLC system (Waters, Milan, Italy).

Separations were performed on an XBridge C18 (4.6 mm X 150 mm, particle size 3.5 µm; Waters) column equipped with a Sentry (4.6 mm X 10 mm ; Waters) guard column. Both columns were maintained at 35°C. Gradient elution was carried out using the mobile phase consisted of acetonitrile (solvent A) and 50 mM acetate buffer at pH 4.5 (solvent B) and was delivered at a flow rate of 1mL/min (**Table 3**). The total run time was 25 minutes.

Table 3. Chromatographic Condition (Gradient)—Mobile Phase: Solvent A (CH3COONa 50 mM With Glacial acetic acid, Final pH = 4.5) and Solvent B (HPLC Grade Acetonitrile)

Time	**Solvent A CH_3CN**	**Solvent B 50 mM acetate buffer at pH 4.5**	**Flow (mL/min)**
0.0	40	60	1
8.90	40	60	1
15.00	70	30	1
18.00	70	30	1
19.00	40	60	1

c. Sample preparation

Patients receiving standard dosing of different ARV drugs underwent blood sampling after obtaining their informed consent for the measurement of plasma drug concentrations. Blood samples were collected in EDTA tubes and plasma was obtained after centrifugation at 3000 rpm for 10 minutes at $+4^0$C (Sigma 3- 16pk). Plasma samples were then underwent heat inactivation procedure for HIV (35 minutes at 58°C). To avoid thawing cycles, each patient plasma sample was aliquoted into micro tube of 2 ml and stored at -20^0C until analysis. On the day of analysis an aliquot of 500 µL volume of plasma samples was pipetted into labelled disposable polypropylene eppendorf tubes and added with 50 µL of internal standard solution. The tubes were vortex-mixed for 30s, and then added with 500 µL of 50 mM acetate buffer pH 4.5, for protein precipitation. The tubes were vortex-mixed for further 60s and centrifuged at 10,000x*g* for 10 min at 4°C.

SPE C18 cartridges were placed on a Vac Elut 20 Manifold (Agilent Technologies) and activated with 1 mL of methanol followed by 1 mL of HPLC solvent B before sample loading. Loading was carried out under gravity. Then the cartridges were washed with 500

µL of HPLC solvent B, followed by 250 µL of HPLC grade water and then elution was carried out using 1 mL of methanol and acetonitrile solution (90:10, vol/vol). Eluted solution were collected into a polypropylene tube and taken to dryness at 50°C in a model Speedvac centrifugal evaporator (Bioinstruments, Italy). The residue was re-suspended in 150 µL H_2O:CH_3CN (60:40) centrifuged, filtered and then transferred to polypropylene vials. 30 µL were then injected in HPLC.

d. Specificity and Selectivity

Interference from endogenous compounds was investigated by analysis of different blank plasma samples. Potential interference by ARV drugs concomitantly administered to the patients was also evaluated by spiking blank plasma with them. To test potential concomitant medication or xenobiotic interference, plasma from different patients (n=30) given different combinations of anti-HIV drugs or antibiotics were analysed.

e. Accuracy, Precision, Calibration, and Limit of Quantification

Intraday and interday accuracy and precision were determined by assaying 6 spiked plasma samples at 3 different concentrations (QC's) for all drugs. Accuracy was calculated as the percent deviation from the nominal concentration. Interday and intraday precision were expressed as the SD at each QC concentration. Each calibration curve was obtained using 6 calibration points, and the ranges are listed in **Table 2**. Calibration curves were constructed by linear least-squares regression ($1/x^2$ weighting) of peak height ratios (analyte/IS) versus nominal concentrations. The method was considered linear if the coefficient of regression (r^2) calculated as mean of 10 curves was equal or better than 0.99 [157]. The calibration curves for estimating all the drugs concentrations in unknown samples consisted of six concentration of plasma samples. These samples were prepared in every analysis together with a blank plasma samples.

The within-day and between-day coefficient of variation (CV) and the accuracy of the method were assessed by calculating daily and overall CV's and bias values for QC (five replicates at each concentration per analytical run) that were assayed in five separate analytical runs. The assay was considered acceptable if CV at each concentration was <15% for both within-and between-day variability and the deviation of the mean from the true value was between ±15% [157]. The lowest identifiable discrete that yielded a signal to noise ratio of 3:1 and reproducible concentration that showed an imprecision of 20% and accuracy of 73%-125% for each analyte was accepted as lower limit of quantification (LOQ) and was set as the first calibration curve point (**Table 4**).

Table 4. Intraday and Interday Precision study of HPLC-UV method

Within day	**ATV**	**DTG**	**DRV**	**ETV**	**EFV**	**RGV**	**RPV**	**LPV**	**TPV**
LOQ Theoretical concentration (ng/mL)	60	20	150	50	150	40	20	150	500
Mean ±SD (ng/mL)	60.3 ± 7.1	19.9 ± 1.6	155.2±11.6	51.7 ± 5.7	153.2±13.4	41.9 ± 5.0	19.8 ± 0.9	146 ± 15.53	529.0±50.14
CV,%	11.73	8.1	7.50	11.0	8.7	12.0	4.6	10.64	9.48,
Accuracy,%	100.5	99.6	103.5	103.4	102.1	104.8	99.2	97.3	105.8
Low QC Theoretical concentration (ng/mL)	90	50	600	200	600	80	50	600	2000
Mean ±SD (ng/mL)	90.9±6.69	46.7 ± 2.9	625.0±76.83	194.8±25.1	638.0±50.1	81.9±7.9	43.5 0.8	620.2±65.2	2030.4±224.7
CV,%	7.36	6.1	12.3	13.0	7.8	9.7	1.8	10.5	10.0
Accuracy,%	101.0	93.5	104.2	97.0	106.3	102.4	87.1	193.4	101.5
Medium QC Theoretical concentration (ng/mL)	900	300	1800	1600	1800	600	300	1800	16000
Mean ±SD (ng/mL)	874.5± 64.7	337.0 ± 21.4	1886.6±68.1	1647.5±119.8	1834.7±186.2	620.2 ±51.2	264.1 ± 6.1	1828.3±127.3	13962.2±849.4
CV,%	7.4	6.4	3.6,	7.3	10.2	8.3	2.3	7.0	6.1
Accuracy,%	97.2	112.4	104.8	103.0	101.9	103.4	88.0	101.6	87.3
High QC Theoretical concentration (ng/mL)	2000	2000	8000	4000	8000	6000	1000	8000	30000,
Mean ±SD(ng/mL)	2169.7± 280.5	2215.5 ± 201.3	8625.6±530.6	4283.2±305.6	8177.4±787.0	6097.9±662.5	935.2 ± 32.4	8600.3±585.1	30761.4±1981.4
CV,%	12.93	9.1	6.1	7.1	9.72	10.9	3.5	6.8	6.44
Accuracy,%	108.5	110.7	107.8	107.1	102.2	101.6	93.5	107.5	102.5
Between day	**ATV**	**DTG**	**DRV**	**ETV**	**EFV**	**RGV**	**RPV**	**LPV**	**TPV**
LOQ Theoretical concentration (ng/mL)	60	20	150	50	150	40	20	150	500
Mean ±SD (ng/mL)	63.3±6.8	19.9 ± 0.3	149.1±15.1	50.1±5.7	148.3±12.0	42.1±4.75	19.7 ± 0.3	150.6 ±11.6	516.5±59.0
CV,%	10.7	1.5	10.1	11.31	8.11	11.25	1.4	7.7	11.4
Accuracy,%	105.5	99.8	99.4	100.2	98.9	105.3	98.5	100.4	3.3
Low QC Theoretical concentration (ng/mL)	90	50	600	200	600	80	50	600	2000
Mean ±SD (ng/mL)	99.7± 6.6	53.7 ± 6.8	611±15.0	216.4±31.0	603.4±53.29	85.9±8.23	44.9 ± 4.6	570±73.4	2040.2±148.9
CV,%	6.7	12.7	2.46	14.34	8.84	9.6	12.3	12.9	7.3
Accuracy,%	110.8	107.4	101.8	108.2	99.43	107.4	89.7	95.0	102.0
Medium QC Theoretical concentration(ng/mL)	900	300	1800	1600	1800	600	300	1800	16000

Mean ±SD (ng/mL)	964.4 ±57.4	340.4 ± 16.1	1949.2±52.6	1538.2±177.5	1850.5±124.7	567 ±18.1	272.9 ± 30.3	1840.5 ±109.9	14989.2±2023
CV,%	5.95	4.7	2.7	11.5	6.7	3.2	11.1	6.0	13.5
Accuracy,%	107.1	113.5	108.3	96.1	102.8	94.5	91.0	102.3	93.7
High QC Theoretical concentration (ng/mL)	2000	2000	8000	4000	8000	6000	1000	8000	30000
Mean ±SD (ng/mL)	1814.8±78.9	2182.4 ± 252.5	8373.6±828.5	4162±297.6	8031.6±345.7	6120.8±253.6	881.5± 88.1	8039±1049.1	30102.2±1469.4
CV,%	4.35	11.6	9.9	7.15	4.3	4.1	10.0	13.1	4.88
Accuracy,%	90.74	109.1	104.7	104.0	99.6	102.0	88.2	99.51	100.3

f. Recovery

Recovery from plasma, using the extraction procedures, was assessed by comparing the peak height ratio obtained from multiple analyses of spiked plasma samples (QC's) with the peak height ratio from direct injections of the same amount of all analytes and IS. The assay was accepted if recovery exceeded 60%.

g. Stability

Drug stability in plasma samples were studied as per the FDA guidelines [157]. Stability studies evaluated the stability of all the analytes during sample collection and handling, after long-term (intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Stability study also included the evaluation of analyte stability in stock solution. The stability study of drugs and IS in plasma extracts included the analytical variability study which was done by keeping the samples for overnight at room temperature.

h. Analysis of Samples from Patients

The developed method has been routinely use for the TDM of ATV, DRV, DTG, ETV, EFV, LPV, RGV, RPV and TPV carried out in our routine clinical practice for the optimization of HAART therapy of HIV infected patients .

2.1.2. Results

Retention times of all the analytes are shown in **Table 2**. Retention time of DTG and EFV are found to be similar with RGV and LPV respectively. For this reason, 2 different lots of STD and QC's were made. DTG and EFV along with other ARV's were present in LOT A. RGV and LPV along with other ARV's were present in LOT B. Representative chromatograms of Std 1 and blank plasma of LOT A, LOT B at 260 nm and ETV and RPV at 305 nm is shown in **Figure 3**. Whereas the comparative chromatograms of Std 6 and Std 1 of LOT A and LOT B at 260 nm and ETV and RPV at 305 nm is shown in **Figure 4**. Mean regression coefficient of determination (r^2) of all calibration curves was better than 0.99. A linear through zero regression was chosen due to good linear response for all the drugs up to STD 6.

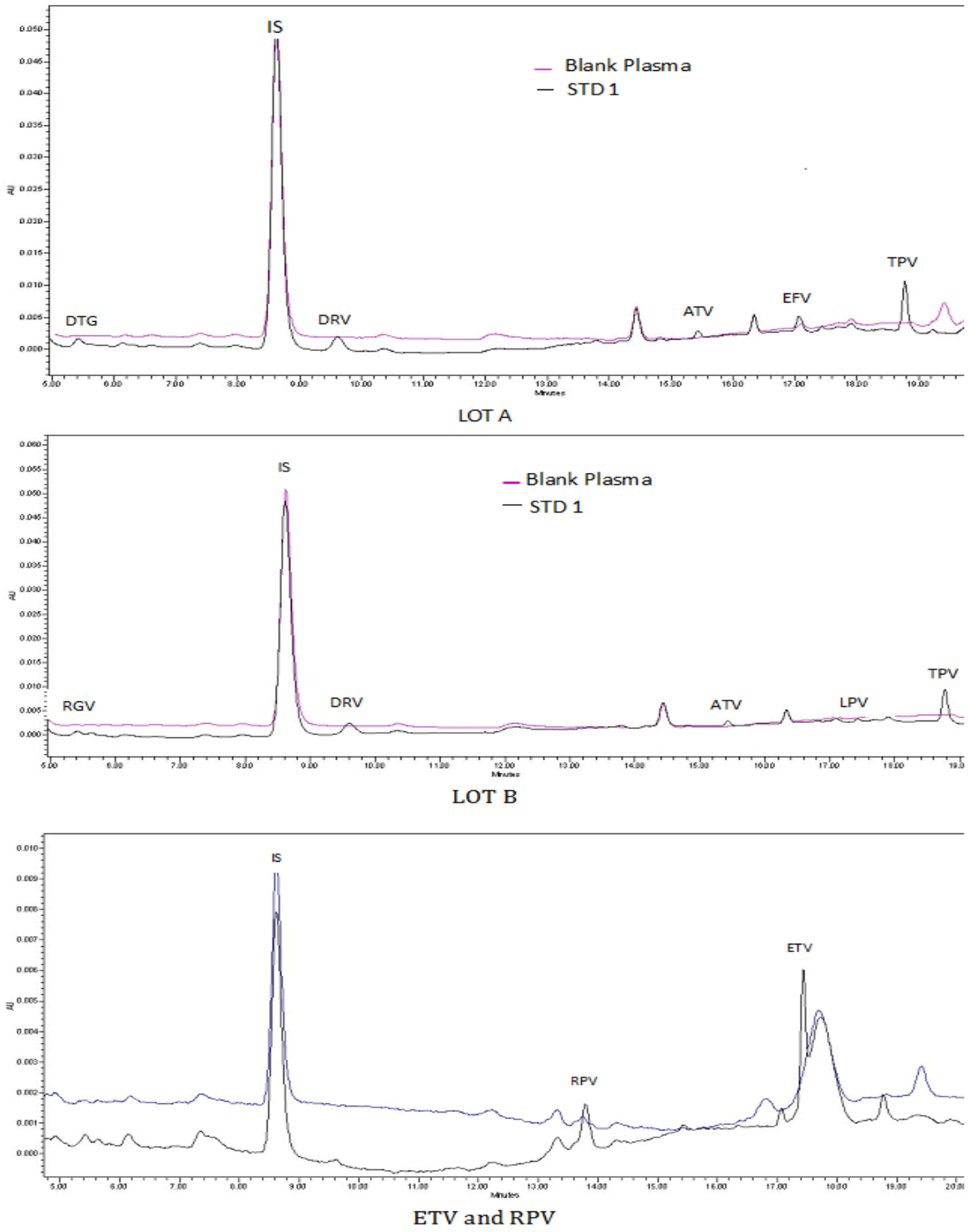

Figure 3. Representative chromatograms of Std 1 and blank plasma of Lot A and LOT B at 260 nm and RPV and ETV at 305 nm

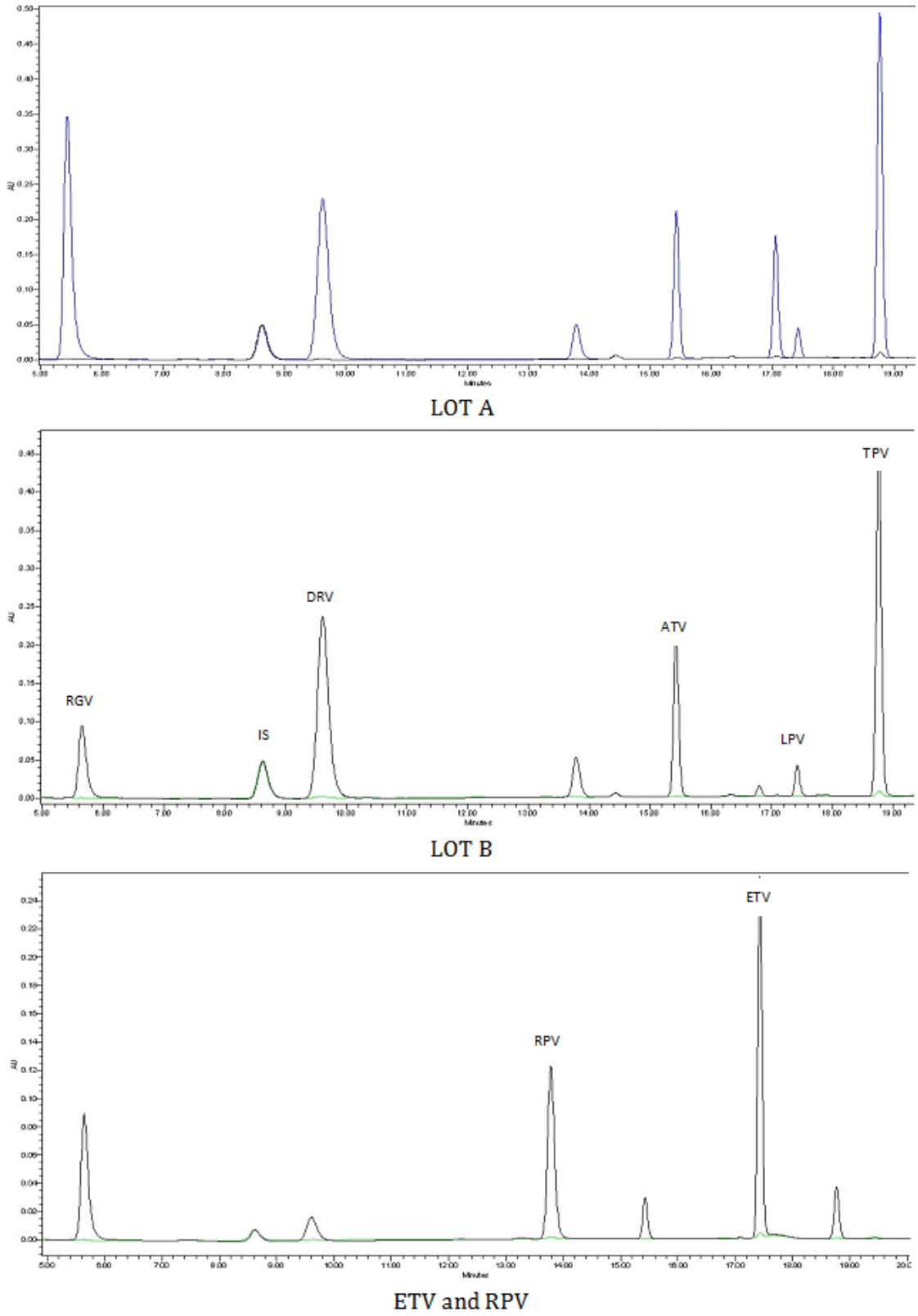

Figure 4. Comparative chromatograms of Std 6 and Std 1 of LOT A and LOT B at 260 nm and RPV and ETV at 305 nm

a. Specificity and Selectivity

The assay did not show any significant interference with ARV's or other concomitant drugs administered to the patients at therapeutic doses, excluding the above mentioned overlapping of DTG with RGV and EFV with LPV. Five drug-free plasma samples did not show any interfering peaks in the retention time windows, considering the specific wavelength for each drug .

b. Accuracy, Precision, and Limit of quantification

Results of the validation of the methods are listed in **Table 4** for all analytes. All observed data [intraday and interday precision as percent relative standard deviation (RSD%) and % coefficient of variation (% CV)] were below 15.0%, in line with FDA guidelines [157]. A linear response was shown for all drugs up to STD 6. The LLOQ and limit of detection for each analyte are listed in **Table 4**.

c. Recovery

Multiple aliquots at each of the 3 QC concentrations were assayed; the mean recovery of all drugs ranged from 80% and 125%.

d. Stability

The stability study of drugs and IS in plasma extracts, kept for 24 hours in the autosampler rack at room temperature, showed a variation of less than 5% for IS and all drugs at each concentration. Taking into account the analytical variability, the processed samples were stable throughout the HPLC-UV analysis, which was always completed within 24 hours.

e. Analysis of Samples from Patients

Our method is regularly applied to the analysis of ARV drugs from the plasma of HIV infected blood samples. The developed method was applied for the determination of DRV plasma concentration in 371 samples of which 7.8% were found to have levels less the LOQ. Measured trough concentrations ranged from 205 to 31488 ng/mL in 342 DRV samples, with an inter-individual coefficient of variation (CV%) of 70.6 % (**Figure 5**, panel A). ATV plasma concentration in 1022 samples were analysed of which 13.5 % were found to have concentration less the LOQ. Measured trough concentrations ranged from 60 to 6144 ng/mL in 885 ATV samples, with an inter-individual CV% of 96.8% (**Figure 5**, panel B). ETV concentration found less then LOQ was 8.1 % in 74 patient samples. Measured EFV trough concentrations ranged from 53 to 3158 ng/mL in 68 ETV samples, with an inter-individual CV% of 85.6% (**Figure 5**, panel C). LPV concentration found less then LOQ was 16.7 % in

209 patient samples. Measured trough concentrations ranged from 174 to 26125 ng/mL in 174 LPV samples, with an inter-individual CV% of 66.6% (**Figure 5**, panel D). EFV concentration found less then LOQ was 12.6 % in 207 patient samples. Measured trough concentrations ranged from 454 to 10078 ng/mL in 181 EFV samples, with an inter-individual CV% of 65.5% (**Figure 5**, panel E). RGV concentration found less then LOQ was 17.1 % in 408 patient samples. Measured trough concentrations ranged from 42 to 9814 ng/mL in 338 RGV samples, with an inter-individual CV% of 158.63 (**Figure 5**, panel F). TPV concentration found less then LOQ was 58.3 % in 12 patient samples. Measured EFV trough concentrations ranged from 14450 to 28065 ng/mL in 5 TPV samples, with an inter-individual CV% of 29.3% (**Figure 5**, panel G). Measured RPV trough concentrations ranged from 22 to 173 ng/mL, with an inter- individual CV% of 48.0% (**Figure 5**, panel H) in 48 samples and no samples found below LOQ level. Measured DTG trough concentrations ranged from 649 to 2878 ng/mL with an inter-individual variability of 60.8% in 3 samples. The same distribution was observed also for the other sampling times: DTG concentrations at 1, 2, 3 and 4 hours after the morning drug intake ranged from 1835 to 3420 ng/mL (CV% 30.4%;), from 2112 to 5666 ng/mL (CV% 45.1%); from 2836 to 6462 ng/mL (CV% 40.2%) and from 2167 to 5195 ng/mL (CV% 40.1%), respectively (**Figure 5**, panel I).

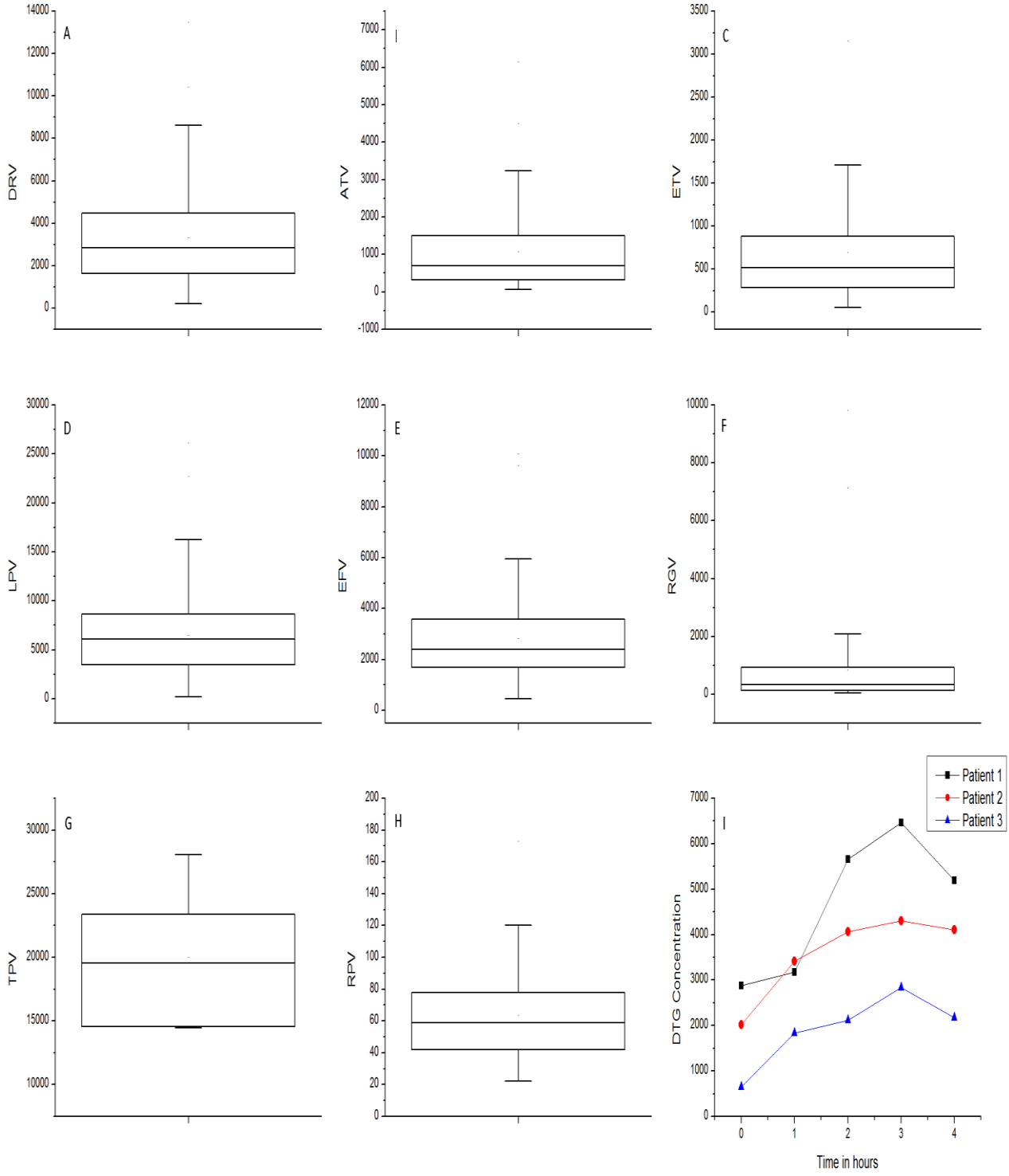

Figure 5. Box-plot of DRV (n=342, panel A), ATV (n=885, panel B) , ETV (n=68, panel C) , LPV (n=174, panel D), EFV (n=181, panel E), RGV (n=3338, panel F) , TPV (n=5, panel G), RPV (n=48, panel H) plasma trough concentrations and time course of DTG plasma concentration (n=15 of 3 patients. Panel I)

2.2. Development and validation of electrospray ionization LC tandem mass assay for the simultaneous measurement of ten antiretroviral agents in human plasma samples

This part of the study describes the development and validation of a straightforward analytical method for the simultaneous analysis in plasma of the ten ARV agents APV, ATV, EFV, RAL, MVC, NVP, NFV, IDV, SQV and RTV by liquid chromatography coupled with tandem triple quadrupole mass spectrometry detection. This method is characterized by a very low limit of quantification, greatly below the clinically relevant range of concentrations encountered in patients. This method is currently applied in our TDM service for patients' follow-up and for clinical research projects.

2.2.1. Experimental

a. Chemicals and reagents

APV, ATV, EFV, EFV-d4, TDV, MRV-d6, NFV, NVP, RTG, RTV and SQV were purchased from Sigma2000 (Rome, Italy), and MVC was obtained from Pfizer. Quinoxaline (QX), used as internal standard (IS), was purchased from Sigma (Milan, Italy). Ammonium acetate , formic acid, acetonitrile , methanol and all other chemicals (Sigma) were of HPLC grade. Deionised water used in all aqueous solutions was obtained from a Milli-Q water purification system (Millipore, Milan, Italy). Blank plasma used for the assessment of matrix effect and for the preparation of calibration and control samples was provided by the Lugi sacco hospital, Milan, Italy. Stock solutions with a concentration of 1 mg/mL were prepared for all drugs and internal standard (IS) in methanol. A working solution of internal standard (IS) was made with QX (200 ng/mL) in methanol and stored at 4^0C until use.

b. Chromatographic and Mass-Spectrometric Conditions

A Waters Alliance 2695 HPLC separation module (Waters, Milford, Massachusetts, U.S.A.) with cooled autosampler and column oven was used to perform the assays. Chromatographic separation was achieved on a Waters XBridge C18. 2.1 mm X 100 mm, particle size 3.5 µm HPLC column maintained at 50^0C. The mobile phases consisted of water + ammonium acetate 2 mM + formic acid 0.1% (mobile phase A) and methanol + formic acid 0.1% (mobile phase B). The gradient elution was performed at a flow rate of 0.3 mL/min (**Table 5**). The HPLC was connected to a Quattro Premier XE LC-MS/MS system (Waters, Milford, Massachusetts, U.S.A.). Samples were injected into the LC-MS/MS system to determine ARV drug concentrations. Experiments were performed using electrospray ionization (ESI) in the positive ion mode except for EFV, performed in the negative ion mode. The protonated molecules were used as the precursor ion for the MS/MS experiment and the most suitable product ion was selected. Drug retention times, parent and daughter ions, cone volt-

age, and collision energy were optimized for each compound and are listed in **Table 6**. Data were processed using MassLynx software (version 4.1, Waters).

Table 5. Chromatographic Condition (Gradient): Buffer A (Ammonium Acetate 2 mM/Formic Acid 0.1%) and Buffer B (Methanol/Formic Acid 0.1%)

Time	A%	B%	C%	D%	Flow	Curve
0.00	90.0	10.0	0.0	0.0	0.300	1
0.10	90.0	10.0	0.0	0.0	0.300	1
4.00	10.0	90.0	0.0	0.0	0.300	6
9.00	0.0	90.0	0.0	0.0	0.300	6
10.00	0.0	100.0	0.0	0.0	0.300	6
10.10	0.0	100.0	0.0	0.0	0.500	1
11.00	0.0	100.0	0.0	0.0	0.500	1
11.10	90.0	10.0	0.0	0.0	0.300	11

Table 6. Drug Retention Times, Precursor and Product Ions, Cone Voltage, and Collision Energy for all drugs and Internal Standards (MVCD6 and EFVD4) by LC-MS/MS in Plasma Samples

Analyte	M/Z	Cone voltage	Collision energy	Retention time
QX	313.2>246.3	55	40	7.05
RGV	445.05>108.95	45	28	6.6 (met a 5.5)
ATV	705.35>335.3	50	30	7.6
MVC	514.25>280	35	35	5.2
MVCD6	520.3>280	35	35	5.2
EFV	314.0>244	30	17	6.7
EFVD4	318.0>248	30	17	6.7
APV	506.3>156.0	30	25	6.7
NVP	267.1>226.1	40	28	5.8
SQV	671.35>570.4	45	35	7.1
IDV	614.35>421.35	45	30	6.6
NFV	568.3–330.2	40	30	6.9
RTV	721.3>269.25	25	15	7.7

c. Preparation of Stock Solutions, Calibration Standards, and Quality Control Samples

Stock solutions were prepared in methanol to obtain a final concentration of 1 mg/mL, for all analyte and internal standard. Aliquots of stock solutions were then stored at -20^{0}C for no longer than 12 months. Stock solutions of all analyte except EFV were further diluted in methanol to obtain three working solutions with concentrations of 20, 200 and 2,000 ng/mL for MCV, 100, 1000 and 15,000 ng/mL for ATV, 200, 2000 and 20,000 ng/mL for NVP and APV, 100 , 1000, 10,000 ng/mL for RGV, IDV, SQV, 150, 1500 and 15,000 ng/mL for

RTV, 500, 5000 and 50,000 ng/mL for NFV. Stock solutions of EFV were further diluted in methanol to obtain four working solutions with concentration of 100, 1000, 10000, and 100,000 ng/mL. Calibration standards were prepared by dilution of various volumes of the working solution in blank human plasma as shown in **Table 7**. Quality control (QC) samples were obtained with different stock solutions and were prepared in blank plasma at concentrations of 60, 600, and 4,000 ng/mL for ATV; 80, 800, and 8,000 ng/mL for APV and EFV; 200, 1000, and 8,000 ng/mL for IDV; 10, 90, and 700 ng/mL for MVC; 100, 1000, and 5,000 ng/mL for NFV; 80, 800, and 7,000 ng/mL for NVP; 30, 200, and 2,000 ng/mL for RGV; 100, 500, and 2,000 ng/mL for RTV; 200, 1,000, and 4,000 ng/mL for SQV. Aliquots of the QC samples were kept at -20^0C for a maximum of 12 months.

Table 7. Standard points of calibration curve of LC-MS/MS method

STD	ATV(µL)	MVC APV NVP(µL)	EFV(µL)	RGV(µL)	RTV(µL)	IDV SQV NFV(µL)
STD 1	20	25	50	10	10	10
STD 2	50	50	10	50	20	20
STD 3	10	10	20	10	50	50
STD 4	50	20	50	30	10	10
STD 5	10	50	10	50	20	20
STD 6	20	10	20	10	50	50
STD 7	40	20	50	30	10	10
STD 8	60	50	10	50	20	20

d. Sample Processing

The extraction method was based on a simple protein precipitation procedure: 200 µL of methanol containing the QX (200 ng/ml) were added to 100 µL of plasma sample and standard point of calibration curve. 10 µL of deuterated internal standard of MVC and EFV were then added to the resultant mix. After vortex mixing for 30 seconds, the samples then treated with 600 µL of CH_3CN to precipitate out the plasma proteins and vortex again for 30 sec. Tubes then again centrifuged at 10,000 g for 8 minutes. A 100 mL volume of supernatant was then diluted with 200 µL of CH_3OH : CH_3COONH_4 (60 : 40) in another eppendroff tubes . Mix was then filtered at 10,000 g for 4 minutes and 20 µL of each sample were injected onto the analytical column except EFV (40 µL of EFV injected on analytical column)

e. Linearity

Calibration standards were prepared and analyzed in 6 replicates in 6 independent runs for each compound. Calibration curves were fitted using the quadratic regression and applying a 1/X weighting. To obtain acceptable linearity, deviations of the mean calculated concentrations over 6 runs had to be within ± 15% of nominal concentrations for the nonzero

calibration standards [157]. The limits of quantification (LOQ) were determined in 6 replicates in the same run for each compound. A precision of ± 20% and an accuracy of 80% to 120% were allowed for the LOQ[157].

f. Precision and Accuracy

Intra-assay values were determined by analyzing 6 replicates of each spiked QC sample in a single assay. Inter-assay values were determined by analyzing 1 QC sample per day at each concentration over 6 different days. Intra-assay and inter-assay precisions were expressed as the percentage coefficient of variation (%CV) at each QC concentration and could not exceed ±15%[157]. Accuracy was calculated as the percent deviation from the nominal concentrations and was acceptable if within ±15%[157].

g. Selectivity and Specificity

Interferences from endogenous plasma compounds were investigated by analyzing 6 different blank plasma samples. To investigate the potential interferences of co-medications, the ARV drugs concomitantly administered to the patients were assayed by spiking different blank plasma containing DRV, ETV, LPV, DTG, RPV and TPV, respectively.

h. Matrix effect

Matrix effect was assessed as per the European medicines agency guideline on bioanalytical method validation, 2012. Absolute matrix effect was assessed for all analytes by comparing the chromatographic peak areas of spiked blank plasma extracts (i.e., after protein precipitation with methanol and acetonitrile) from 6 different sources with STD2 and STD8 to peak areas obtained from the same concentration of analytes in the same composition of the extract (i.e., 200 µl of methanol + 600 µl of acetonitrile + 200 µl of CH_3OH : CH_3COONH_4) without plasma.

Furthermore, matrix effects over an entire chromatographic run were evaluated using a post-column infusion of the analytes to ensure that no interfering peaks of the blank plasma (***n*** = 7) extract were found at the retention time corresponding to each analytes. The blank plasma was extracted and injected into the LC-MS/MS system with concurrent postcolumn infusion of analytes.

i. Stability

Drug stability in plasma samples were studied as per the FDA guidelines [157]. Stability studies evaluated the stability of all the analytes during sample collection and handling, after long-term (intended storage temperature) and short-term (bench top, room temperature)

storage, and after going through freeze and thaw cycles and the analytical process. Stability study also included the evaluation of analyte stability in stock solution. The stability study of drugs and IS's in plasma extracts included the analytical variability study which was done by keeping the samples for overnight at room temperature.

j. Analysis of Samples from Patients

The developed method has been routinely use for the TDM of APV, ATV, EFV, IDV, MVC, NFV, NVP, RGV, RTV and SQV carried out in our routine clinical practice for the optimization of HAART therapy of HIV infected patients .

2.2.2. Results

ARV drugs were detected and quantified overall total run time of 8 minutes. All analytes were optimized in terms of cone voltage, collision energy and injection volume prior to their quantification by LC-MS/MS. For each selected transition of APV (m/z 506.3–156.0), ATV (m/z 705.35–335.3), EFV (m/z 314.0–244), EFVD4 (m/z 318.0–248), IDV (m/z 614.35–421.35), MVC (m/z 514.25–280), MVCD6 (m/z 520.3–280), NVP (m/z 267.1–226.1), NFV (m/z 568.3–330.2), RGV (m/z 445.05–108.95), SQV (m/z 671.35–570.4), RTV (m/z 721.3- 269.25) and QX (m/z 313.2–246.3) the analyte retention times and collision energies are shown in **Table 6.** Representative chromatograms of all analytes STD 1 and blank plasma sample of calibration curve are shown in **Figure 6** whereas representative MRM chromatograms of internal standards: QX , EFV-D4 , and MVC-D6 are shown in **Figure 7**.

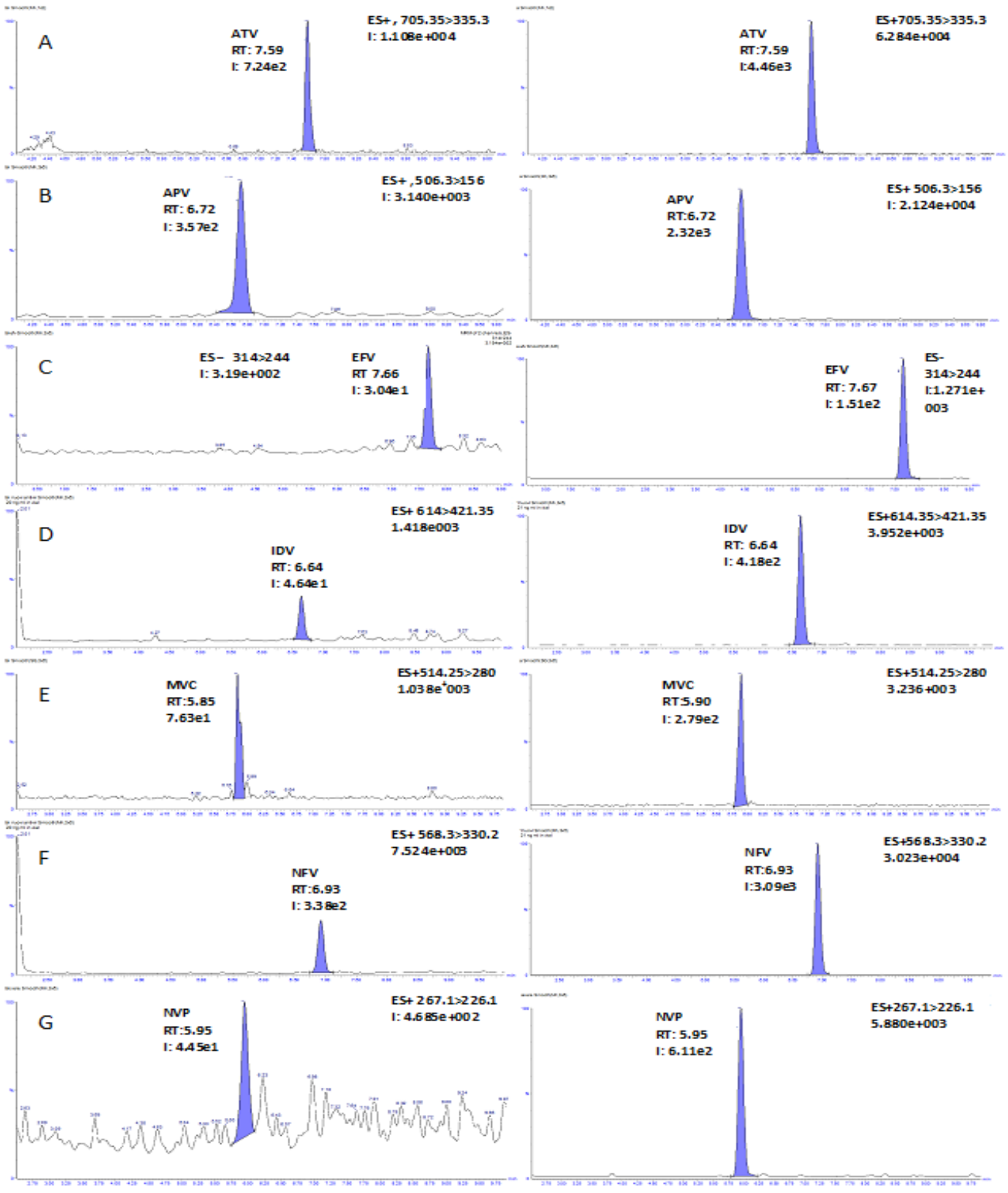

Figure 6. Representative MRM chromatograms of an extracted blank plasma sample and an extracted STD 1 of ATV (A), APV (B), EFV (C) IDV(D), MVC(E), NFV(F), NVP (G),RGV (H), RTV (I), SQV (J)

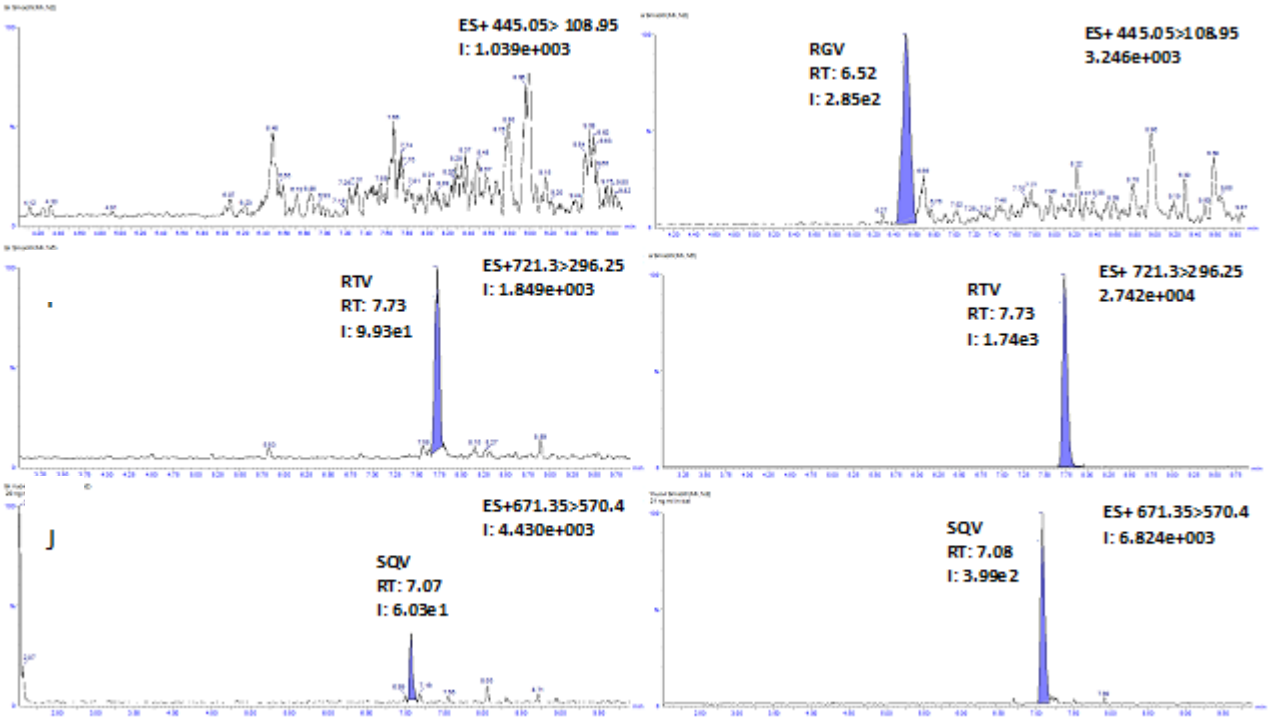

Figure 6. (Continued).

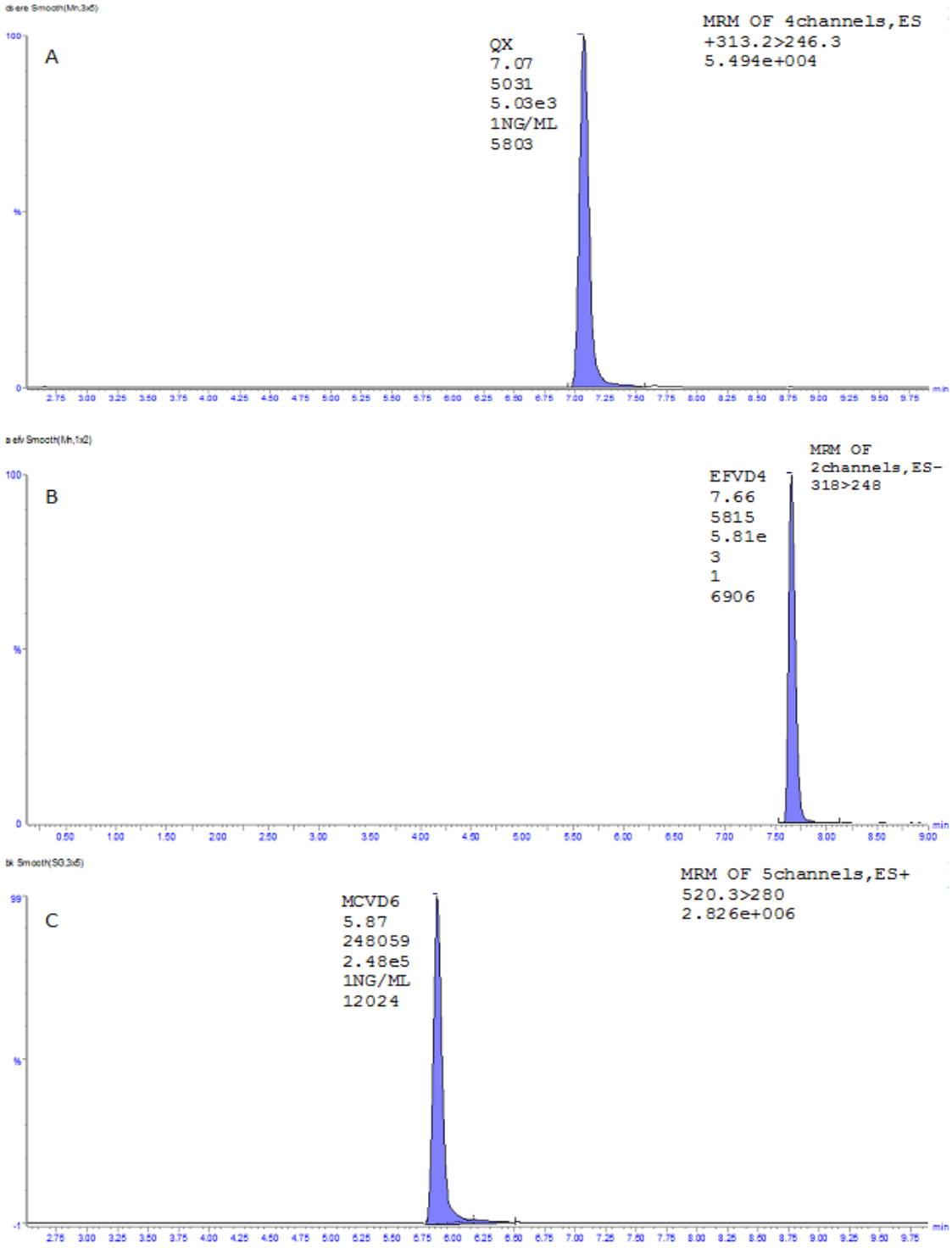

Figure 7. Representative MRM chromatograms of internal standards: QX (A), EFV-D4 (B), and MVC-D6(C)

a. Linearity, Precision, and Accuracy

The assay showed excellent linearity over the studied concentration ranges. The mean coefficient of determination (r^2) of all calibration curves was more than 0.998 across the 6 validation standard curves. Mean calculated concentrations over 6 runs of all ARV drugs did not deviate by more than ± 15% from nominal concentrations for the non-zero calibration standards. The LOQ was established at 5 ng/mL for MVC, 10 ng/mL for IDV, RGV, SQV, 15 ng/mL for RTV, 20 ng/mL for ATV, 50 ng/mL for APV,EFV,NFV,NVP, with a CV% and accuracy ranged from 2.7 % to 14.8 % and 92 % to 116% respectively (Table 8). The % CV and accuracy of the LOQ, for each ARV drug, were within the limits of ± 20% and 80% to 120% respectively as recommended by the U.S. Food and Drug Administration guidelines (Table 8) . LOQ intra-day % CV and accuracy ranged from 2.7 % to 14.0 % and from 92.1% to 108.2 % respectively whereas inter-day % CV and accuracy ranged from 7.5 % to 14.6 % and from 93.8% to 107.6 % respectively. The results of assay precision and accuracy, assessed at 3 QC concentrations for all ARV agents, are summarized in **Table 8**.

Low QC's intra-day % CV and accuracy ranged from 8.1 % to 13.1 % and from 87.6 % to 111.2 % respectively whereas inter-day % CV and accuracy ranged from 5.6 % to 13.57 % and from 86.4 % to 103.0 % respectively. Mid QC's intra-day % CV and accuracy ranged from 2.53 % to 14.8 % and from 88.3 % to 102.8 % respectively whereas inter-day % CV and accuracy ranged from 2.27 % to 14.5 % and from 90.3 % to 107.9 % respectively. High QC's intra-day % CV and accuracy ranged from 4.82 % to 11.4 % and from 87.4 % to 103.0 % respectively whereas inter-day % CV and accuracy ranged from 1.3 % to 10.78 % and from 89.4 % to 113.1 % respectively. All observed data for the intra-assay and inter-assay were less than ± 15.0%.

Table 8. Intraday and Interday Precision study of LC-MS/MS method

Within day	**ATV**	**APV**	**EFV**	**IDV**	**MVC**	**NFV**	**NVP**	**RGV**	**RTV**	**SQV**
LOQ										
Theoretical concentration (ng/mL)	20	50	50	10	5	50	50	10	15	10
Mean ±SD (ng/mL)	21±3	51±7	53 ±7	11±1	5±1	53±8	53±5	11±0	14 ±2	11±1
CV,%	13.6	14.0	13.8	9.4	13.2	14.8	10.3	2.7	12.9	9.8
Accuracy,%	106.0	101.6	106.4	107.2	97.2	106.8	106.0	108.2	92.1	106.2
Low QC										
Theoretical concentration (ng/mL)	60	80	80	200	10	100	80	30	100	200
Mean ±SD (ng/mL)	58.4±7.2	80.0±8	88.9±4.6	187.0±21	9.0 ±1	97.0±9	70.0±9	32.4±2.4	105.0±8.1	193.0±23
CV,%	12.38	10.4	5.17	11.1	8.1	9.7	13.1	7.31	7.74	11.8
Accuracy,%	97.3	100.2	111.2	93.6	88.8	97.4	87.6	107.9	105.0	96.5
Medium QC										
Theoretical concentration (ng/mL)	600	800	800	1000	90	1000	800	200	500	1000
Mean ±SD (ng/mL)	608.6±46.9	801±86	742.8±34.2	893±58	92±4	922±13.6	765±65	186.7±4.7	513.0±66.0	883±69
CV,%	7.7	10.7	4.6	6.5	4.2	14.8	8.5	2.53	12.85	7.8
Accuracy,%	101.4	100.2	92.9	89.3	101.7	92.2	95.6	93.34	102.8	88.3
High QC										
Theoretical concentration (ng/mL)	4000	8000	8000	8000	700	5000	7000	2000	2000	4000
Mean ±SD(ng/mL)	3869.4±366.5	8124±811	7583.7±233.5	8039±496	727±57	4370±497	6022±436	1969±94.9	2059±191.9	3966±299
CV,%	9.47	10.0	3.08	6.2	7.9	11.4	7.2	4.82	9.31	7.5
Accuracy,%	96.7	101.6	94.8	100.5	103.8	87.4	96.0	98.5	103.0	99.1
Between day	**ATV**	**APV**	**EFV**	**IDV**	**MVC**	**NFV**	**NVP**	**RGV**	**RTV**	**SQV**
LOQ										
Theoretical concentration (ng/mL)	20	50	50	10	5	50	50	10	15	10
Mean ±SD (ng/mL)	21±2	53±6	54±8	10±1	5±1	52±6	47±6	10±1	14±1	9±1
CV,%	7.5	11.1	14.6	9.2	10.2	12.5	13.6	8.8	8.3	7.5
Accuracy,%	102.7	105.2	107.6	98.8	102.8	103.2	94.4	102.2	94.9	93.8
Low QC										
Theoretical concentration (ng/mL)	60	80	80	200	10	100	80	30	100	200
Mean ±SD (ng/mL)	54.7±5.4	76±4	78.3±10.6	193±24.4	8.6±0.6	97±12.0	81±11	30.5±2.8	103.0±14.0	193.6±22.8
CV,%	9.98	5.6	13.5	12.6	6.6	12.37	13.9	9.28	13.57	11.78
Accuracy,%	94.2	94.4	97.9	96.6	86.4	97	101.7	101.7	103.0	96.8
Medium QC										
Theoretical concentration(ng/mL)	600	800	800	1000	90	1000	800	200	500	1000
Mean ±SD (ng/mL)	553.3±50.2	809±52	791.2±48.1	903.4±24.6	89.0±13	1040±74.3	864±69	197.6±16.8	532.4±67.9	938.2±94.4
CV,%	9.08	6.4	6.07	2.27	14.5	7.14	8.0	8.52	12.76	10.0
Accuracy,%	92.2	101.1	98.9	90.3	99.4	104.0	107.9	98.5	106.5	93.8

High QC Theoretical concentration(ng/mL) Mean ±SD (ng/mL) CV,% Accuracy,%	4000 4006.8±325.7 8.13 100.2	8000 9049±332 3.7 113.1	8000 7815.4±359.0 4.59 97.7	8000 8017.2±536.1 6.69 100.2	700 719±46 6.4 102.7	5000 4468±259 5.8 89.4	7000 6338±85 1.3 91.5	2000 2022±59.6 2.92 101.1	2000 2230.2±240.3 10.78 111.5	4000 3927±423.0 10.77 98.2

b. Selectivity and Specificity

After injection of blank plasma or plasma extract containing different ARV drugs , no significant interference with ARV agents concomitantly administered to the patients was observed.

c. Matrix Effect

Matrix effect of all analytes was evaluated as per the European medicine agency guidelines on bioanalytical method validation 2012. A ratio was calculated and typical matrix effect observed showed a negligible ion enhancement or no matrix effect for all ARV's.

d. Stability

The stability of PI's and NNRTI's at different conditions has been demonstrated previously in several articles [158–161]. Stability under various conditions for all analytes including IS's (freeze-thaw, heat deactivation, room temperature, solution, and long-term storage stabilities) has also been done [41]. During our validation, all analytes including IS's was stable in plasma after 3 freeze thaw cycles. Stock solutions and QC samples of ARV's were stable at -20^0C for at least 12 months. Taking into account the analytical variability, the processed samples were found to be stable throughout the analysis, which was always completed within 24 hours.

e. Clinical Sample Analysis

This LC-MS/MS method has been successfully applied for routine TDM in our laboratory. We determined trough plasma concentrations with the method reported here using samples from patients with HIV-1 treated with drugs mentioned in this method. Box-plot distribution of APV (n=154, panel A), ATV (n=991, panel B), EFV (n=183, panel C) , MVC (n=38, panel D), NVP (n=251, panel E), RGV (n=408, panel F), RTV (n=102, panel G), SQV (n=13, panel H) is shown in **Figure 8**. The developed method was applied for the determination of NVP plasma concentration in 279 samples of which 10.0 % were found to have levels less the LOQ. Measured trough concentrations ranged from 164 to 16896 ng/mL in 251 samples, with an inter-individual coefficient of variation (CV%) of 45.3 % . ATV plasma concentration in 1021 samples were analysed of which 2.93 % were found to have concentration less the LOQ. Measured trough concentrations ranged from 20 to 6144 ng/mL in 991 ATV samples, with an inter-individual CV% of 107.0 % . APV concentration found less then LOQ was 12.9 % in 177 patient samples. Measured APV trough concentrations ranged from 52 to 11225 ng/mL in 154 APV samples, with an inter-individual CV% of 122.6. RGV concentration found less then LOQ was 15.4 % in 408 patient samples. Measured trough concentrations ranged from 17 to 9814 ng/mL in 345 RGV samples, with an inter-individual CV% of

160.8. RTV concentration found less then LOQ was 12.0 % in 116 patient samples. Measured trough concentrations ranged from 15 to 1968 ng/mL in 102 RTV samples, with an inter-individual CV% of 120.9. MVC concentration found less then LOQ was 17.4 % in 46 patient samples. Measured trough concentrations ranged from 11 to 2816 ng/mL in 38 MVC samples, with an inter-individual CV% of 191.0 %. EFV concentration found less then LOQ was 11.6 % in 207 patient samples. Measured EFV trough concentrations ranged from 50 to 10078 ng/mL in 183 EFV samples, with an inter-individual CV% of 66.6%. SQV concentration found less then LOQ was 43.5 % in 23 patient samples. Measured EFV trough concentrations ranged from 52 to 3433 ng/mL in 13 SQV samples, with an inter-individual CV% of 87.12 %.

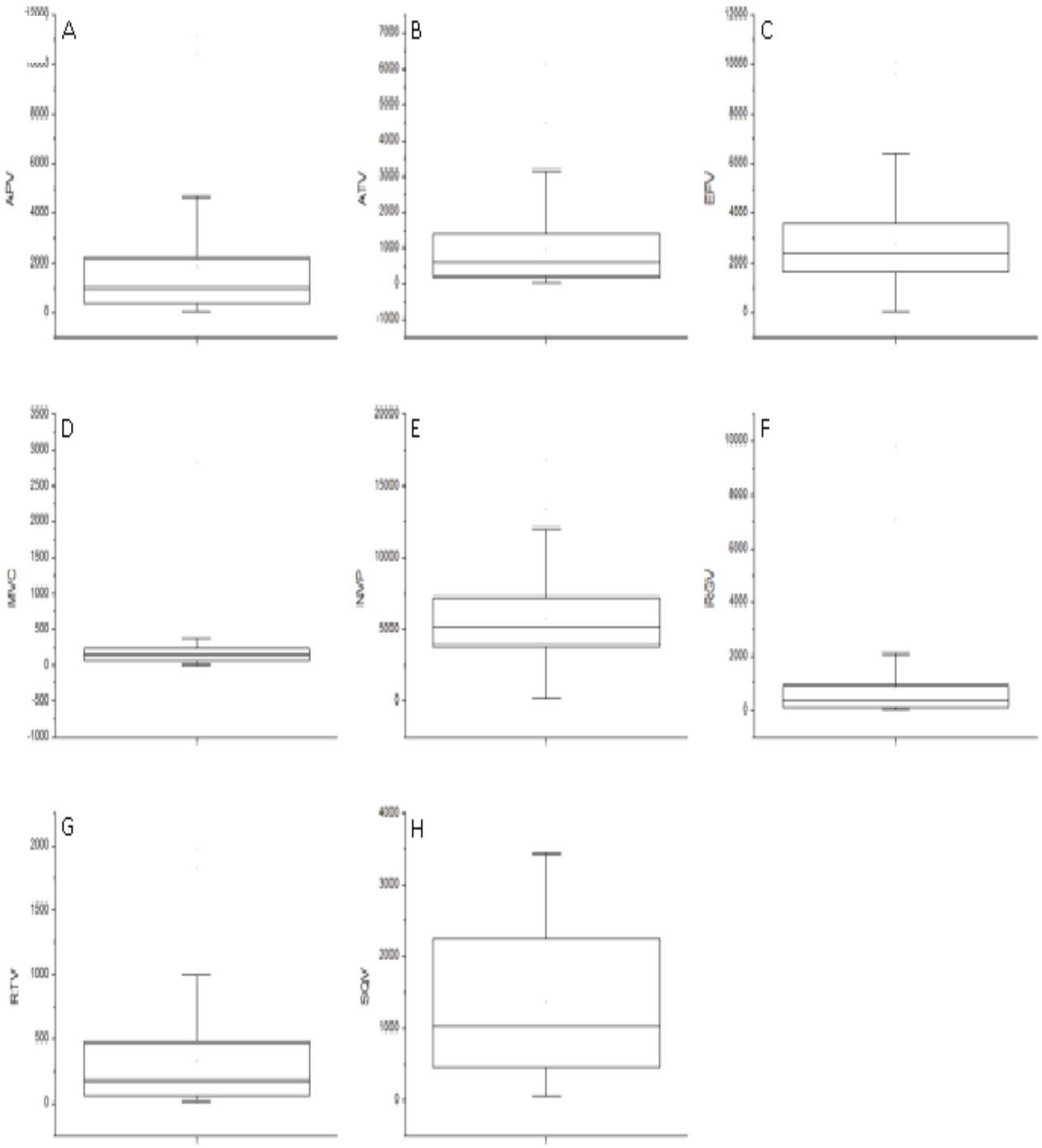

Figure 8. Box-plot of APV(n=154, panel A), ATV (n=991, panel B) , EFV (n=183, panel C) , MVC (n=38, panel D), NVP (n=251, panel E), RGV (n=408, panel F) , RTV (n=102, panel G), SQC (n=13, panel H)

2.3. A pharmacokinetic viewpoint on conventional antiretrovirals doses and possibility of dose reduction

In this part of the study, experience with TDM of ARV agents carried out as day-by-day clinical practice for the optimization of drug dosing in HIV-infected patients has been described. As mention in the introduction ARV therapy is not only associated with drug related side effects but also include drug costs economics which accounts for as much as 60% of ARV treatment program costs in several countries [162]. Hence several authors published clinical studies and critical reviews advocating for dose reduction of different ARV's to be urgently explored as one of the possible options to help and sustain ART roll-out in developing countries [163–167].

ARV drug dosing has not been unchangeable in the past, ZDV (reduced from 1500 to 500 mg daily) being the clearest example. Indeed ARV doses have often been initially chosen on a fairly arbitrary basis: for several ARV drugs, phase 2 data had shown not only no difference in efficacy between different doses but also better tolerability of lower doses [168,169]. Nonetheless, higher doses were chosen for further trials.

Stavudine is a particularly interesting example of dose reduction. A meta-analysis of dose-ranging studies showed that a 30 mg twice daily dose had the same efficacy of the 40 mg twice daily dose and a reduced risk of peripheral neuropathy, the drug's main side effect [170]. The dose of this nucleoside analogue was then reduced to 30 mg twice daily. One small study showed that stavudine given at 20 mg twice daily can maintain viral suppression while reducing lipoatrophy and other mitochondrial adverse effects [171], and this further reduced dose could be worthy of a trial.

EFV, a common component of first-line treatment regimens the world over, is used at 600 mg once daily. However an initial phase 2 trial showed no difference in efficacy between 200, 400 or 600 mg once daily dose, combined with ZDV/lamivudine [172] and plasma drug levels can be much higher in patients with certain CYP2B6 allelic variants (present mostly in Africans) [104], or in individuals with low body weight. The ENCORE 1 trial comparing EFV (plus TDF and emtricitabine) at the standard 600 mg once daily dose vs. 400 mg once daily for 96 weeks in 630 naive HIV-infected patients started in September 2011 and results were available in 2014 [173]. A small study in 33 HIV-infected patients treated with two NRTI's plus EFV at reduced dose has shown no virological failure with a minimum follow-up of 15 months [174].

ATV is a PI currently administered at the dose of either 300 mg with boosted RTV or, less frequently, 400 mg once daily. However a pilot study of ATV/RTV at a dose of 200/100 mg daily in Thai patients showed the same efficacy and plasma ATV drug levels as in Cauca-

sians on 300/100 mg daily [175], and the same lower dose guided by TDM was effective in 31 Caucasians[176].

DRV was initially used at a 600/100 (RTV) mg twice daily dose in ARV-experienced patients. Subsequently, the ARTEMIS [177] ,MONET [178] and ODIN[179] trials have all shown the efficacy of a DRV/ RTV 800/100 mg daily dose, which has also a more favorable safety profile, in both naive patients and in patients with limited ARV experience. In a small number of ARV-highly experienced patients a further reduced dose of DRV/ RTV (600/100 mg once daily)[180] was tested positively. LPV/RTV is commonly used at the dose of 400/100 mg twice daily but during its development lower doses have shown similar efficacy to the presently used one. In particular the Abbott 720 trial conducted in naive patients evaluated three doses: 200/100 mg twice daily, 400/100 mg twice daily and 400/200 mg twice daily. The 200/100 dose showed a very good efficacy [168] but the 400/100 mg was chosen for phase 3 development. A meta-analysis estimating the effects of LPV dose vs. RTV dose on LPV pharmacokinetics in five pharmacokinetic trials of LPV/RTV indicated that a 200/150 mg twice daily dose of LPV/RTV could maintain very similar LPV plasma levels to the standard 400/100 mg dose [78].

In a recent small study in Thai HIV-infected patients, inadequate plasma concentrations were observed with LPV/RTV used at an even lower dose (200/50 mg twice daily)[181]. However, at week 12, all patients had undetectable plasma HIV-1 RNA [181]. Researchers and clinicians have also used 200/50 mg LPV/RTV twice daily in six HIV-infected patients and observed C_{trough} of LPV below the suggested minimum concentration of 1000 ng/ml; however again, all patients continue to have undetectable HIV-RNA levels after 12–20 months on this low dose [182].

The goal of this study was to quantify how many HIV-infected patients treated with conventional doses of ARV's had plasma drug trough concentrations – taken as a real-life surrogate marker of daily drug exposure – exceeding the upper therapeutic thresholds defined by the literature.

2.3.1. Experimental

This study is based on a retrospective analysis of routine TDM of ARV concentrations carried out as day-by-day diagnostic service by the Unit of Clinical Pharmacology at the Luigi Sacco University Hospital, Milan, Italy, between January 2010 and April 2015. We focused on the most frequently prescribed drugs, namely ATV, DRV, ETV, LPV, NVP, RAL, EFV, MVC and TDF as well as less used agents, such as FOS-APV, SQV, TPV etc . Each patient contributed to only one single drug measurement; if more than one assessment is available, we considered the first assessment (provided it was collected at a steady state). Blood trough samples drawn into EDTA-containing vacutainers were collected from all patients immediately before the next drug intake (trough samples), that is 24 or 12 h after the

last ARV administration in patients given the drug once (q.d.) or twice daily (b.i.d.), respectively. All samples were centrifuged at 3000 g (þ48C), then plasma was separated and stored at -20^{0}C. Plasma drug concentrations were determined by validated HPLC methods coupled either with tandem mass-spectrometric detection or with UV detection. Written informed consent with respect to patients' management was collected at the first outpatient visit. In compliance with privacy laws, the patients' identification code was encrypted before performing the statistical analyses.

2.3.2. Results

Measured ARV plasma trough concentrations were stratified according to the therapeutic windows of drug concentrations suggested by the DHHS guidelines [183]or by available literature, as detailed below. Therapeutic windows considered were 50–180 ng/ml for TDF [184–186], 150–800 ng/ml for ATV [183] , 1000–7000 ng/ml for LPV [183,187,188], 1000–4000 ng/ml for EFV [183,189,190] and 3000– 6000 ng/ml for NVP [183,191]. For other drugs, only the lower therapeutic thresholds of drugs concentrations were retrievable from the literature, namely more than 2000 more than 50 and more than 300 ng/ml for DRV [192,193], MVC [183] and ETV [194], respectively.

As shown in **Figure 9 and Table 9,** a wide distribution in the plasma trough concentrations was observed for most drugs, resulting in different patterns of mean interpatient variability (expressed as a percentage of coefficient of variation). The developed method was applied for the determination of DRV plasma concentration in 371 samples of which 7.8 % were found to have levels less the LOQ. Measured trough concentrations ranged from 205 to 31488 ng/mL in 342 DRV , with an inter-individual coefficient of variation (CV%) of 70.6 % (**Figure 9**: panel A, **Table 9**). ATV plasma concentration in 1022 samples were analysed of which 13.5 % were found to have concentration less the LOQ. Measured trough concentrations ranged from 60 to 6144 ng/mL in 885 ATV samples, with an inter-individual CV% of 96.8% (**Figure 9**: panel B, **Figure 10**, **Table 9**). ETV concentration found less then LOQ was 8.1 % in 74 patient samples. Measured ETV trough concentrations ranged from 53 to 3158 ng/mL in 68 ETV samples, with an inter-individual CV% of 85.6% (**Figure 9** : panel C, **Table 9**). LPV concentration found less then LOQ was 16.7 % in 209 patient samples. Measured trough concentrations ranged from 176 to 26125 ng/mL in 174 LPV samples, with an inter-individual CV% of 66.6% (**Figure 9** : panel D, **Figure 10**, **Table 9**). EFV concentration found less then LOQ was 12.6 % in 207 patient samples. Measured trough concentrations ranged from 454 to 10078 ng/mL in 183 EFV samples, with an inter-individual CV% of 65.5% (**Figure 9**: panel E, **Table 9**). RGV concentration found less then LOQ was 17.1 % in 408 patient samples. Measured trough concentrations ranged from 42 to 9814 ng/mL in 338 RGV samples, with an inter-individual CV% of 158.63 (**Figure 9**: panel F, **Table 9**). TPV concentration found less then LOQ was 58.3 % in 12 patient samples. Measured EFV trough concentrations ranged from 14450 to 28065 ng/mL in 5 TPV samples, with an inter-

individual CV% of 29.3% (**Figure 9**: panel G, **Table** 9). Measured RPV trough concentrations ranged from 22 to 173 ng/mL, with an inter- CV% of 48.0% (**Figure 9**: panel H, **Table** 9) in 48 samples and no samples found below LOQ level. The developed method was also applied for the determination of NVP plasma concentration in 279 samples of which 10.0 % were found to have levels less the LOQ. Measured trough concentrations ranged from 164 to 16896 ng/mL in 250 samples, with an inter-individual coefficient of variation (CV%) of 45.3 % (**Figure 9** : panel I, **Figure 10**, **Table** 9). APV concentration found less then LOQ was 12.9 % in 177 patient samples. Measured APV trough concentrations ranged from 52 to 11225 ng/mL in 154 APV samples, with an inter-individual CV% of 122.6 (**Figure 9**: panel J, **Table 9**). RTV concentration found less then LOQ was 12.0 % in 116 patient samples. Measured trough concentrations ranged from 15 to 1968 ng/mL in 102 RTV samples, with an inter-individual CV% of 120.9 (**Figure 9** : panel K, **Table** 9). MVC concentration found less then LOQ was 17.4 % in 46 patient samples. Measured trough concentrations ranged from 11 to 2816 ng/mL in 38 MVC samples, with an inter-individual CV% of 191.0 % (**Figure 9**: panel L, **Table 9**). SQV concentration found less then LOQ was 43.5 % in 23 patient samples. Measured SQV trough concentrations ranged from 52 to 3433 ng/mL in 13 SQV samples, with an inter-individual CV% of 87.12 % (**Figure 9** : panel M, **Table 9**) . Measured DTG trough concentrations ranged from 649 to 2878 ng/mL with an inter-individual variability of 60.8% in 3 samples. The same distribution was observed also for the other sampling times: DTG concentrations at 1, 2, 3 and 4 hours after the morning drug intake ranged from 1835 to 3420 ng/mL (CV% 30.4%;), from 2112 to 5666 ng/mL (CV% 45.1%); from 2836 to 6462 ng/mL (CV% 40.2%) and from 2167 to 5195 ng/mL (CV% 40.1%), respectively (**Figure 9** : panel N, **Table** 9).

Table 9. ARV'S plasma distribution

Sr no	**Analyte**	**Total**	**Below LOQ**	**Above LOQ**	**CV%**	**Concentration range**	**Assay Method**
1	DRV	371	29(7.8%)	342	70.6	205-31488	HPLC-UV
2	ATV	1022	(13.5%)	885	96.8	60-6144	HPLC-UV
3	ETV	74	(8.1%)	68	85.6	53-3158	HPLC-UV
4	LPV	209	(16.7%)	174	66.6	176-26125	HPLC-UV
5	EFV	207	(12.6%)	183	65.5	454-10078	HPLC-UV
6	RGV	408	(17.1%)	338	158.6	42-9814	HPLC-UV
7	TPV	12	(58.3%)	5	29.3	14450-28065	HPLC-UV
8	RPV	48	(0.0 %)	48	48.0	22-173	HPLC-UV
9	NVP	279	(10.0%)	250	45.3	164-16896	HPLC-UV
10	APV	177	(12.9%)	154	122.6	52-11225	LC-MS/MS
11	RTV	116	(12.0%)	102	120.9	15-1968	LC-MS/MS
12	MVC	46	(17.4%)	38	191.0	11-2816	LC-MS/MS
13	SQV	23	(43.5%)	13	87.12	52-3433	LC-MS/MS
14	DTG $C_{through}$	3	(0.0 %)	3	60.8	649-2878	HPLC-UV
	DTG 1 hr	3	(0.0 %)	3	30.4	1835-3428	HPLC-UV
	DTG 2 hr	3	(0.0 %)	3	45.1	2112-5666	HPLC-UV

	DTG 3 hr	3	(0.0 %)	3	40.2	2836-6462	HPLC-UV
	DTG 4 hr	3	(0.0 %)	3	40.1	2167-5198	HPLC-UV

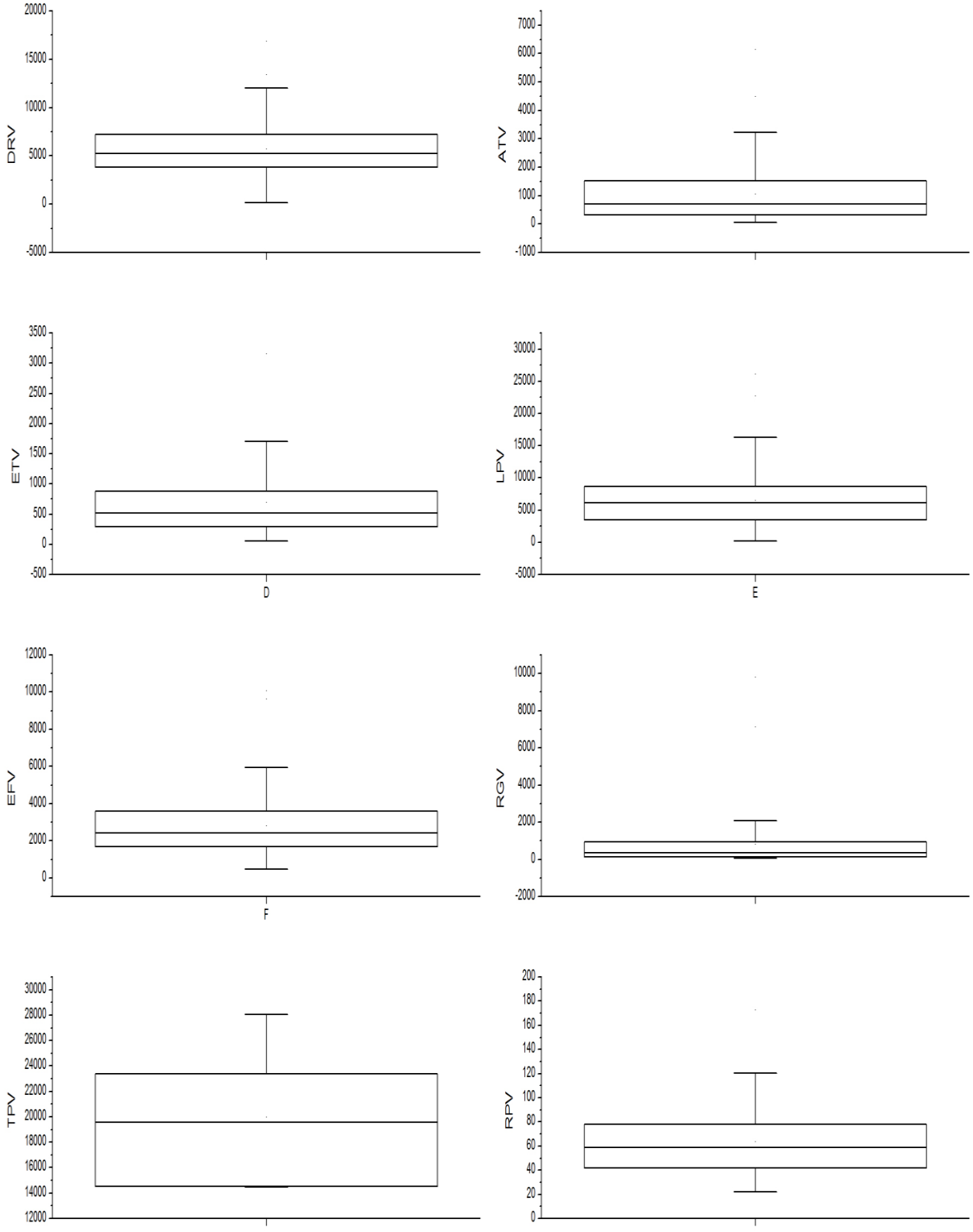

Figure 9. Box-plot of DRV (n=342), ATV (n=885), ETV (n=68), LPV (176), EFV (n=183), RGV (n=338), TPV (n=5), RPV (n=48), NVP (n=251), APV(n=153), EFV (n=183, panel C) , RTV (n=102), MVC (n=38), SQC (n=13), DTG (n=3)

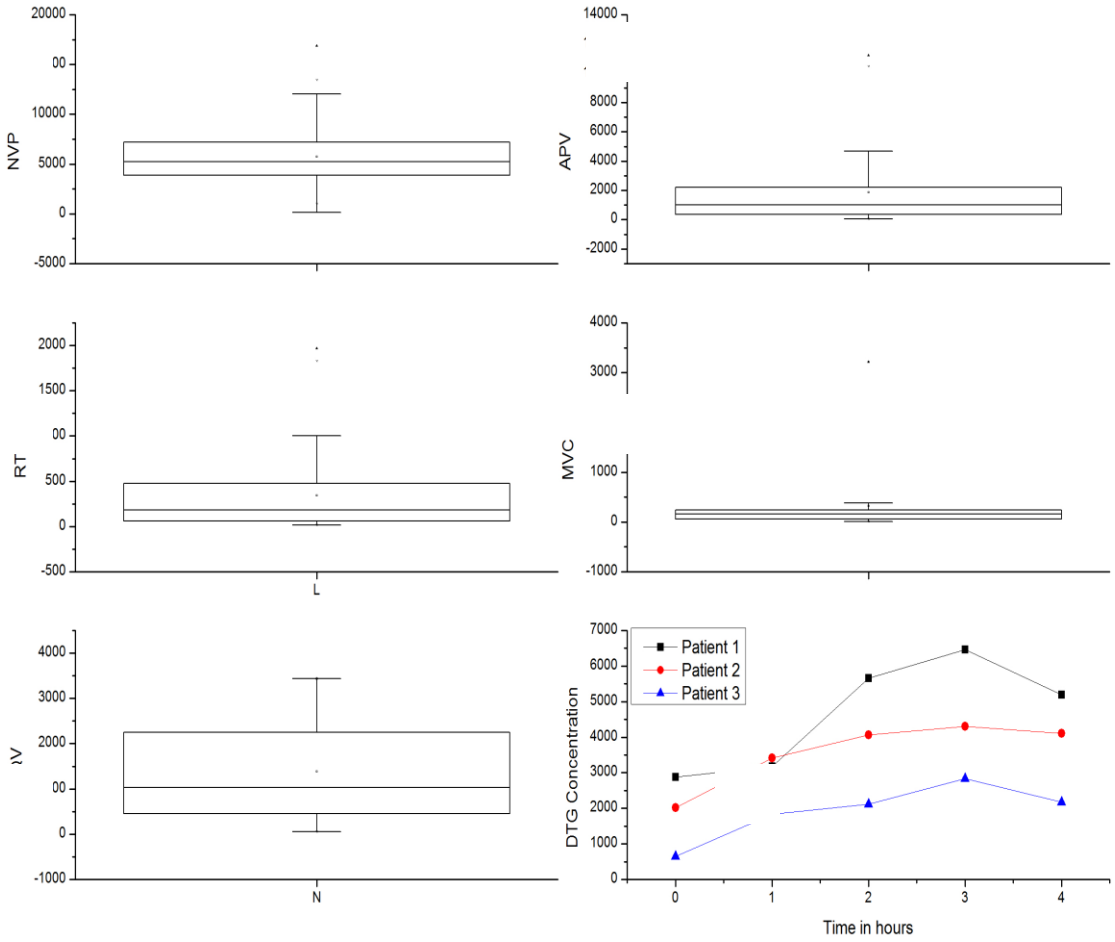

Figure 9. (Continued).

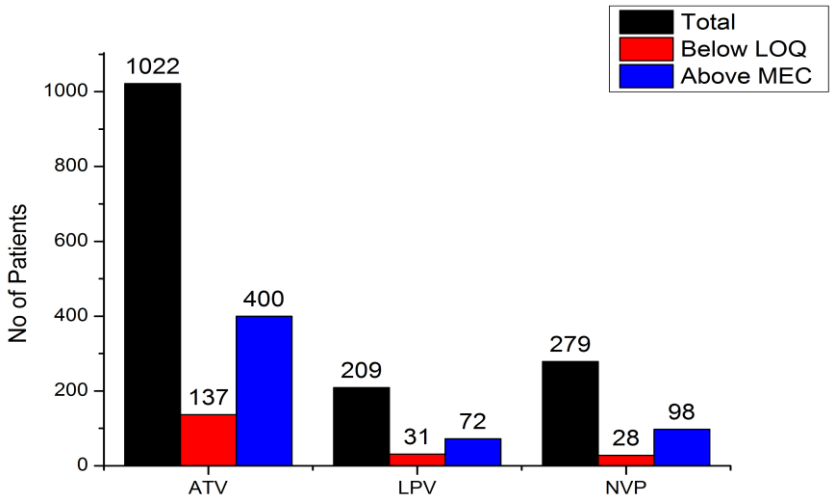

Figure 10. Plasma distribution of ATV, LPV and NVP

2.4. Metabolic and kidney disorders correlate with high atazanavir concentrations in HIV infected patients

RTV-boosted ATV (ATV/r) is one of the PI's selected as the preferred choices in the American Department of Health and Human Services (DHHS) and European guidelines for the initial treatment of patients infected with human immunodeficiency virus-1 (HIV-1) [178,183]. This drug is relatively well tolerated in most patients; however, side effects including hyperbilirubinemia, which may result in visible jaundice or scleral icterus, dyslipidemias, nephrolithiasis and cholelithiasis have been reported in the medium and long term [195–198]. RTV enhanced ATV concentrations and improved virologic activity more than unboosted ATV [199] . Nevertheless, unboosted ATV may be selected for some patients because it has fewer gastrointestinal adverse effects, less hyperbilirubinemia and less impact on lipid profiles than ATV/r. TDM of ATV plasma trough concentrations is adopted for the routine management of patients only in a minority of centre's worldwide. However, considerable inter-individual variability has been observed in plasma concentrations of ATV (**Table 9**) after standard dosing, mainly related to drug-to-drug interactions and inherited differences in the hepatic metabolism [200–204]. Significant correlations have been reported between plasma ATV trough concentrations and clinical outcome. In treatment-experienced as well as naive HIV patients the highest probability of achieving undetectable viral load has been associated with ATV plasma concentrations >150 ng/mL [203–205]. Accordingly, this threshold concentration is currently recommended by international guidelines for the optimal management of patients on ATV-based ARV regimens [183]. More scanty data are available concerning the relationships between ATV exposure and toxicity. A threshold ATV concentration of 800 ng/mL has been proposed as a risk factor for hyperbilirubinemia [206,207], whereas no specific associations have been reported for other ATV-related complications.

In the present study we sought to: I) assess the distribution of ATV plasma trough concentrations in HIV-infected patients according to drug dosage and II) verify a direct association between ATV plasma concentration and the degree of hyperbilirubinemia. As an exploratory analysis we also investigated the potential relationship between ATV concentrations and other drug-related adverse events (nephrolithiasis and dyslipidemia).

2.4.1. Experimental

a. Study population

Male and female HIV-infected patients on ATV-based ARV therapy who underwent TDM of ATV concentrations referring to the Department of Infectious Diseases at Luigi Sacco University Hospital, Milan, Italy were enrolled in the present study. Paediatric subjects, patients with severe hepatic impairment (defined as Child-Pugh Class B or C) or with history

of kidney diseases (including previous episodes of nephrolitiasis before initiation of ATV) were excluded from the present study. Written informed consent to patients' management (that is consent for diagnostic evaluations, drug administration and all other medical procedures/interventions performed exclusively for routine treatment purposes) was collected to the first outpatient visit. Patients provided also written informed consent for their records (anonymized) to be used for future research purpose. In compliance with privacy laws, the patients' identification code was encrypted before performing the statistical analyses. Given the retrospective observational nature of the present investigation, no formal approval from the local ethics committee was required according to the legislation of the national drug agency.

Adherence of patients to therapy was verified through direct questioning during every outpatient visits. Data on self-reporting adherence were matched with data from our Pharmacy Department in order to verify that patients have accepted the package with the ARV's dose required to fully cover the time between two visits. Only patients with high adherence to ARV medications (above 95% of the doses) were considered.

b. Study design

This study is based on a retrospective analysis of routine TDM of ATV carried out as day-by-day clinical practice for the optimisation of drug dosing in HIV-infected patients between January 2010 and May 2013. HIV-infected patients treated with ATV for at least three months and with one assessment of ATV plasma trough concentrations were included. ATV plasma trough concentrations were stratified as below, within or above the therapeutic window, which was set at 150–800 ng/mL according to the available literature[206,207]

Clinical information on ATV-related complications were recorded in each patient as follows. Hyperbilirubinaemia was scored from grade 0 to grade 4 in accordance with the AIDS Clinical Trials Group Guidelines for total bilirubin levels [208]: grade 0 (<1.3 mg/dL); grade 1 (1.3–1.9 mg/dL); grade 2 (1.9–3.1 mg/dL); grade 3 (3.1–6.1 mg/dL); and grade 4 (>6.1 mg/dL). Patients with the homozygous UGT1A1*28 genotype were excluded from the analysis (higher risk to experience hyperbilirubinemia irrespective of ATV plasma concentrations). Dyslipidemia was defined by the concomitant presence of all these conditions: 1) total cholesterol >200 mg/dL or serum triglycerides >180 mg/dL; 2) at least a 20% increase in serum cholesterol or serum triglyceride levels compared to baseline; 3) normal serum lipid levels at the last visit before starting ATV. Nephrolitiasis was defined as episode of acute flank pain plus one of the following: 1) new-onset hematuria confirmed by urine analysis, 2) documented presence of stones or radiological findings suggestive of renal stones 3) stone passage confirmed by either the patient or attending physician; in the absence of urinary oxalate or urate crystals and with normal value of serum uric acid. Patients with history of nephrolitiasis before starting treatment with ATV were not included in the present study.

c. Pharmacokinetic evaluations

Blood trough samples drawn into EDTA-containing vacutainers were collected from all patients immediately before the next drug intake, that is 24h or 12h after the last ATV administration in patients given the drug qd or bid, respectively (a time window of ± 30 min was directly verified by the nurse staff and considered as acceptable). All samples were centrifuged at 3000 g (+4°C), then plasma was separated and stored at -20°C. Plasma ATV concentrations were determined by a validated HPLC method coupled with tandem mass-spectrometric detection (LC-MS/MS) (**Section 3.3**). Briefly, 100 microL of plasma sample was extracted by protein precipitation with acetonitrile. After centrifugation an aliquot of the supernatant was analysed by the LC-MS/MS system consisting in a Waters Alliance 2695 coupled with a Quattro Premier XE triple quadrupole (Waters, Italy). Chromatographic separation was performed under gradient conditions on a reversed phase C18 column (Xbridge, 2.1X100 mm 3.5 microm particle sizes, Waters, Milan, Italy) maintained at 50°C. Mass-spectrometric analysis was performed in positive electron spray ionisation mode. For quantification, multiple-reaction monitoring (MRM) mode was applied to monitor the transitions from the precursor ion (M+H)+ to the product ion (m/z 705.35 to m/z 335.3 for ATV). The method was linear over the ATV concentration ranges of 20 to 9000 ng/mL. The lower limit of quantification of the method set at 20 ng/mL. Between and within-day imprecision and inaccuracy were less than 15%, as requested by Consensus Guidelines on Bioanalytical Method Validations [157].

d. Statistical analyses

Results were given as mean (± standard deviation) or median (plus interquartile range) according to distribution of the data based on results of the Kolmogorov-Smirnov normality test. ATV plasma trough concentrations were stratified according to drug dosage (ATV dose, frequency of daily drug administration and concomitant RTV use) and to therapeutic window (set at 150–800 ng/mL). All comparisons between two groups were carried out using the unpaired t test or Mann-Whitney test, whereas the ANOVA or Kruskall-Wallis tests were considered when comparing more than 2 groups, according to the distribution of the data.

The independent association between plasma ATV concentrations and dyslipidemia was assessed by means of uni- and multi-variate regression analyses using the event (toxicity yes or no) as dichotomic dependent variable and as independent covariates clinical, demographic and pharmacologic data that resulted significantly associated with the event in the univariate models (MEDCALC, Software). A p value of less than 0.05 was considered as statistically significant.

2.4.2. Results

a. Patients' characteristics

Two-hundred forty HIV-infected adult patients were included in the present study. Demographic and clinical data at the time of TDM are listed in **Table 10**. Patients were at 901 ± 870 days of therapy with ATV, mainly treated at the conventional ATV/r dosage 300/100 mg qd (68%), given concomitantly with TDF-based ARV regimens (56%). Ninety-two percent of them were Caucasians. Thirty-nine (16%) out of the 240 patients evaluated (28 on ATV 300/100 mg and 11 on ATV 400 mg) had detectable viral load at the time of TDM (median, interquartile range 2.36, 1.97–3.25 log cp/mL).

Table 10. Main demographic, hematologic and biochemical characteristics of HIV-positive patients receiving atazanavir as part of their ARV regimen

Parameters	**All Patients (n=240)**	**ATV/r 300/100 (n=163)**	**ATV 400 (n=77)**
Male gender	% 68%	74%	56%
Age, years	46±11	45±10	49±13
Naïve, %	23%	29%	11%
ATV therapy, days	901±870	846±886	1021±851
HAART, %	56% TDF-based	60% TDF-based	47% TDF-based
	24% ABC-based	24% ABC-based	22% ABC-based
	10% RAL-based	7% RAL-based	18% RAL-based
	10% others	9% others	13% others
Weight, Kg	69±14	70±14	68±14
Body mass index, Kg/m^2	24±4	24±4	24±4
Creatinine, mg/dL	0.9±0.4	0.9±0.3	0.9±0.7
GGT, IU/L	72±114	85±134	47±48
ALT, IU/L	47±49	49±53	42±40
Total bilirubin, mg/dL	2.1±1.6	2.3±1.7	1.7±1.2
Total cholesterol, mg/dL	187±46	191±48	178±39
HDL cholesterol, mg/dL	50±29	50±33	49±18
Triglycerides, mg/dL	167±124	179±132	143±104
CD4, cells/mL	593±247	572±249	639±239
Pts with VL>100 cp/mL, %	12%	13%	11%
HCV/HBV coinfection, %	34%	36%	26%

RAL: raltegravir; GGT: gamma-glutamyltransferase; ALT: alanine aminotransferase; HCV: hepatitis C virus; HBV: hepatitis B virus; VL: viral load

b. Distribution of ATV plasma trough concentrations

A wide distribution was observed in the ATV plasma trough concentrations, with median values of 546 [204–1030] ng/mL, resulting in a mean inter-patient variability of 110.3%.

Four percent of the determinations resulted <20 ng/mL. As expected, mean ATV plasma concentrations were higher in patients using RTV-boosted ATV than in patients using unboosted ATV (given at 400 qd or 200 bid) (650 [357–1204] vs. 238 [99–551] ng/mL, p<0.0001).

As shown in **Figure 11**, a significant proportion of patients with ATV plasma trough concentrations out of the therapeutic drug window were identified. 43.9% of patients treated with ATV 300/100 qd had ATV concentrations exceeding the upper therapeutic threshold, whereas unboosted ATV 400 mg was associated with the highest proportion of values <150 ng/mL (35.5%,**Table 11**).

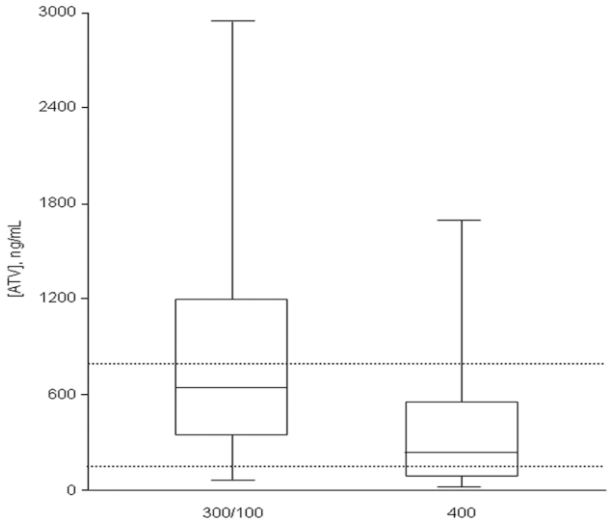

Figure 11. Box-plot of atazanavir (ATV) plasma trough concentrations clustered according to drug dosage

Dashed lines represent the upper and lower limits of the therapeutic window of ATV concentrations (150–800 ng/mL)

Table 11. Distribution of atazanavir (ATV) plasma trough concentrations according to daily drug dosage

	ATV ng/mLmedian [IQR]	**Samples < 150 ng/mL**	**Samples150–800 ng/mL**	**Samples >800 ng/mL**
All evaluations (n = 240)	546 [204–1030]	21.2%	44.3%	34.4%
ATV/r 300/100 mg qd (n = 164)	650 [357–1204]	11.6%	44.5%	43.9%
ATV 400 mg* (n = 76)	238 [99–551]	35.5%	44.7%	19.8%

**given either as 400 mg qd or 200 mg bid; IQR: interquartile range*

c. ATV plasma concentrations and hyperbilirubinemia

One-hundred forty-seven (61%) out of the 240 HIV-positive patients enrolled in the present study experienced hyperbilirubinemia of grade ≥1. As shown in **Figure 12**, a significant and direct association has been observed between the severity of hyperbilirubinemia and ATV plasma trough concentrations (ATV concentrations: 271 [77–555], 548 [206–902], 793 [440–1164], 768 [494–1527] and 1491 [1122–1798] ng/mL in patients with grade 0, 1, 2, 3 and 4 hyperbilirubinemia, respectively, with $p<0.01$ of grade 2, 3, and 4 vs. grade 0. The same trend was confirmed also when repeating the above mentioned comparisons stratifying data according to boosted or unboosted ATV intakers (**Table 12**).

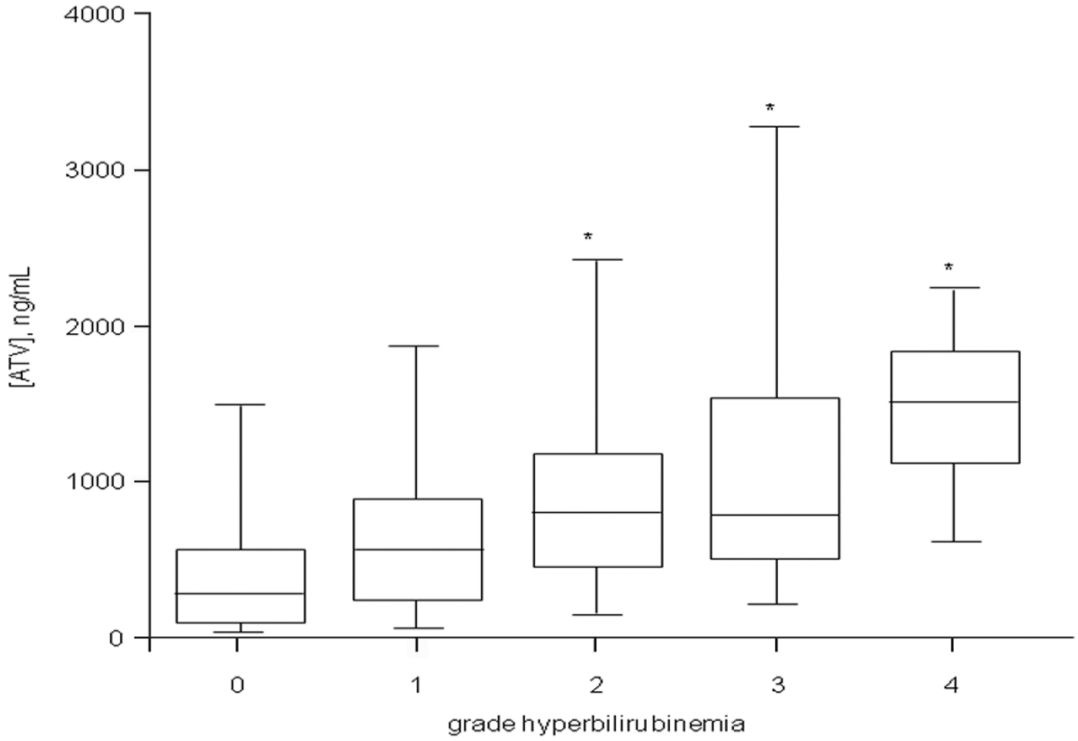

Figure 12. Box plot of ATV plasma trough concentrations clustered according to the grade of hyperbilirubinemia (scored as grade 0, 1, 2, 3 or 4 based on total bilirubin concentrations below 1.3 mg/dL, between 1.3–1.9, 1.9–3.1, 3.1–6.1, or above 6.1 mg/dL, respectively).

**p<0.01 vs grade 0.*

Table 12. Atazanavir (ATV) plasma trough concentrations measured in patients that did or did not experienced drug-related adverse events

Adverse event	**N**	**Overall patients**	**N**	**ATV/r 300/100**	**n**	**ATV 400**
Hyperbilirubinemia						
- Grade 0	86	271 (77–555)	52	347 (121–785)	34	141 (56–293)
- Grade 1	45	548 (206–902)	30	634 (355–962)	15	194 (106–358)
- Grade 2	54	793 (440–1164)*	40	909 (586–1264)**	14	466(260–804)**
- Grade 3	41	768 (494–1527)*	31	825 (531–1368)**	10	540(394–1979)*
- Grade 4	7	1491 (1122–1798)*	6	1363 (1066–1693)**	1	1836
Dyslipidemia	55	582 (266–1148)*	44	746 (305–1313)**	11	250 (106–486)
Nephrolitiasis	11	1098 (631–1238)*	9	9 1098 (652–1285)**	2	742 (293–1191)
Controls^	66	218 (77–541)	39	343 (125–810)	27	141 (50–259)

Data were stratified also according to ritonavir use.
^patients that did not develop ATV-related adverse events;
**p < 0.01 and*
***p < 0.05 vs. Controls*

d. ATV plasma concentrations and other drug-related adverse events

Overall, 55 out of the 240 HIV-positive patients developed dyslipidemia, namely hypercholesterolemia (n = 26, mean increase 46±22%) or hypertriglyceridemia (n = 45, mean increase 114±76%). Patients with dyslipidemia had ATV concentrations significantly higher as compared with patients with no ATV-related complications (582 [266–1148] vs. 218 [77–541] ng/mL, $p<0.01$). This was confirmed also when repeating the above mentioned comparisons according to RTV or not administration (**Table 12**). Moreover, in order to demonstrate a direct relationship between ATV concentrations and lipid increase, we attempted to correlate ATV concentrations with the magnitude of lipid levels increase as compared to baseline. Using this approach we found a direct association between ATV concentrations and the percentage increase in serum triglycerides levels (**Figure 13**). A similar, but not significant trend was also observed for cholesterol.

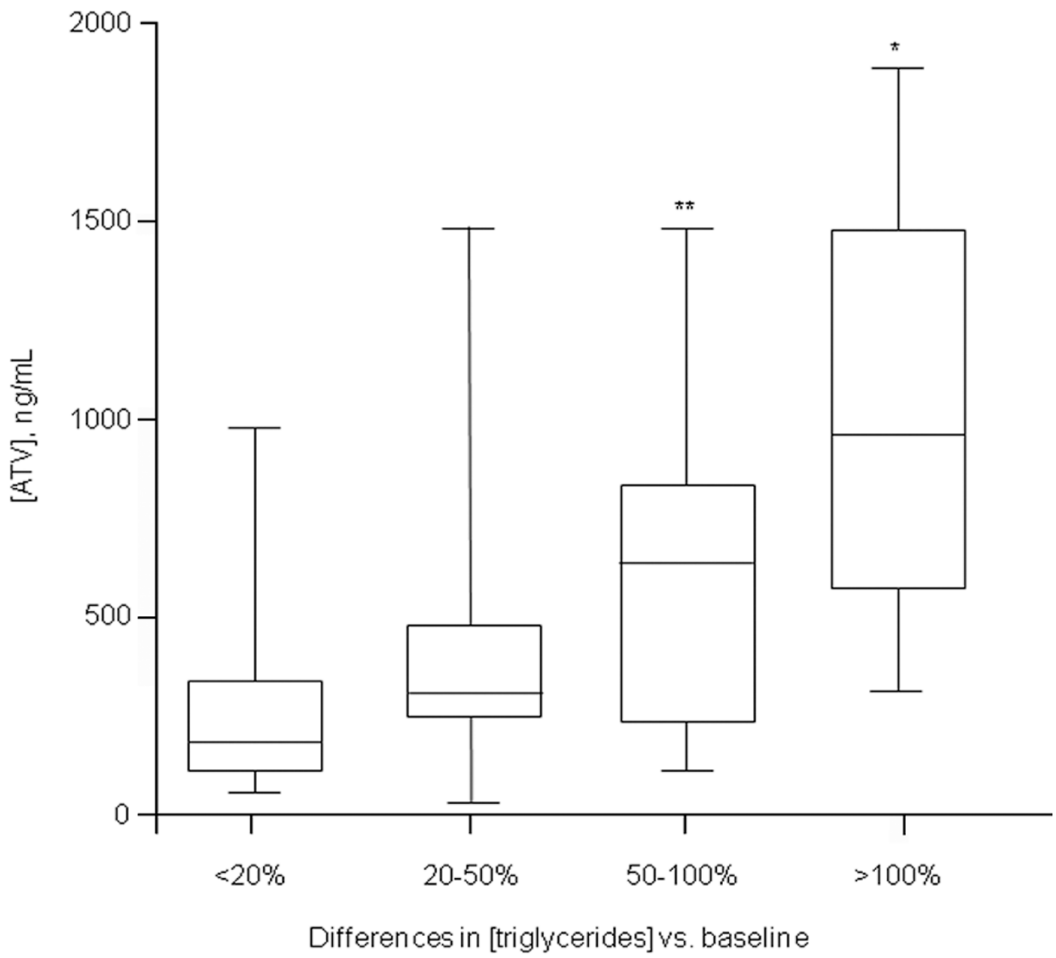

Figure 13. Box plot of ATV plasma trough concentrations clustered according to the magnitude of triglyceride concentrations increase as compared to values measured at the last visit before starting ATV

**p<0.01 and **p<0.05 vs. patients experiencing ≤20% increase (first column from the left)*

At univariate analysis ATV concentrations ($p<0.001$), concomitant HAART ($p = 0.079$), RTV use ($p = 0.009$) and patients' gender ($p = 0.046$) were associated with the development of dyslipidemia, while no association was found with other clinical covariates (**Table 13**). In the multivariable regression analysis only ATV concentrations ($p<0.01$) and RTV use ($p = 0.02$) remained independently associated with dyslipidemia.

Table 13. Univariate and multivariate regression analysis of clinical, demographic and pharmacological covariates associated with the development of atazanavir (ATV)-related dyslipidemia

	Univariate		**Multivariate**	
Variable	**r-value**	**p-value**	**r- value**	**p-value**
Gender	0.14	0.046		
Age	0.04	0.502		
Body weight	0.04	0.469		
Serum creatinine	0.01	0.616		
ALT	<0.01	0.276		
GGT	0.01	0.172		
HCV/HBV Coinfection	0.01	0.873		
CD4 count	0.02	0.336		
Concomitant ARV drugs	0.12	0.079		
Days of ATV therapy	<0.01	0.980		
ATV concentrations	0.35	<0.001	0.33	<0.01
Ritonavir use	0.18	0.009	0.18	0.02

r-value: correlation coefficient; GGT: gamma-glutamyltransferase; ALT: alanine aminotransferase; HCV: hepatitis C virus; HBV: hepatitis B virus; ATV: atazanavir

Eleven out of the 240 HIV-positive patients enrolled in the present study experienced episodes of nephrolitiasis indirectly attributed to ATV after having excluded other clinical conditions predisposing to kidney stones. As shown in **Table 12** these patients were mainly treated with RTV (82%) and had ATV concentrations significantly higher as compared with patients with no ATV-related complications (1098 [631–1238 vs. 218 [77–541] ng/mL, p<0.01). Only one out of 240 patients experienced an episode of cholelithiasis 226 days after starting treatment with ATV (ATV plasma trough concentrations: 438 ng/mL).

2.5. A case study on suspected pharmacokinetic interaction between raltegravir and the 3D regimen of ombitasvir, dasabuvir and paritaprevir/ritonavir in an HIV-HCV liver transplant recipient.

After the introduction of the HAART, HIV infection is no longer considered an absolute contraindication for solid organ transplantation in patients with end-stage liver disease [209,210]. However, recurrence of hepatitis C virus (HCV) infection after liver transplantation is frequent and may lead to allograft loss [211,212]. Thus, safe and effective treatment of HCV in the post-transplant setting is a mandatory task that needs to be addressed properly. The combination of ombitasvir, dasabuvir and paritaprevir/RTV (referred as 3D regimen) has been proven to be associated with high sustained virologic response in HIV-HCV co-infected patients [213,214] and optimal tolerability when co-administered with immunosuppressive

drugs in healthy volunteers [215]. Theoretically, 3D regimen has also a limited propensity to interact with RAL that is usually given to HIV-infected patients undergoing solid organ transplantation to limit drug to- drug interactions between HAART and immunosuppressive agents [216,217].

A 57-year-old HIV-infected patient received orthotopic liver transplantation in 2008 for HCV and hepatocellular carcinoma. Post-transplant, he had recurrence of chronic infection with the HCV genotype 1a. He was on maintenance HAART with ABC, lamivudine and RAL since 2008 with an optimal virologic response (HIV RNA <37 cp/mL) and CD4 count of 272 cells/μL. Long-term immunosuppressive regimen was of 50 mg cyclosporine monotherapy twice daily with trough concentrations ranging from 80 to 140 ng/mL. On July 2014, 3D treatment was started. His Child-Pugh class was B with a score of 8 with minimal alterations in serum transaminases (aspartate aminotransferase 93 IU/mL, alanine aminotransferase 38 IU/mL) and with surveillance abdominal imaging negative for the recurrence of hepatocellular carcinoma. In agreement with the available literature [215], the dose of cyclosporine was initially reduced to 20 mg qd and subsequently increased up to 30 mg bid on the basis of trough drug concentrations. TDM of RAL plasma concentrations, performed by the collection of blood samples immediately before and at 1, 2, 3 and 4 h after the morning drug dose, was carried out before, during and at the end of 3D treatment (two assessments for each time period). RAL plasma concentration was determined by using validated HPLC-UV assay method (**Section 3.1**). RAL area under curve over 12 h (AUC0–12) was estimated using a previously validated algorithm [218]. As shown in **Figure 14**, a dramatic increase in the RAL AUC0–12 was observed during co-administration of the 3D regimen (from 8.68±4.48 to 31.72±0.45 μgxh/mL; +265 %). No other drugs known to potentially interfere with RAL pharmacokinetics were given during the 3D treatment period. The patient did not experience alterations in serum transaminases or creatine phosphokinase levels and was able to complete the 6-month scheduled 3D therapy. At the conclusion of the antiviral treatment, the patient exhibited sustained viral clearance with tests negative for HCV RNA at week 12 post-therapy.

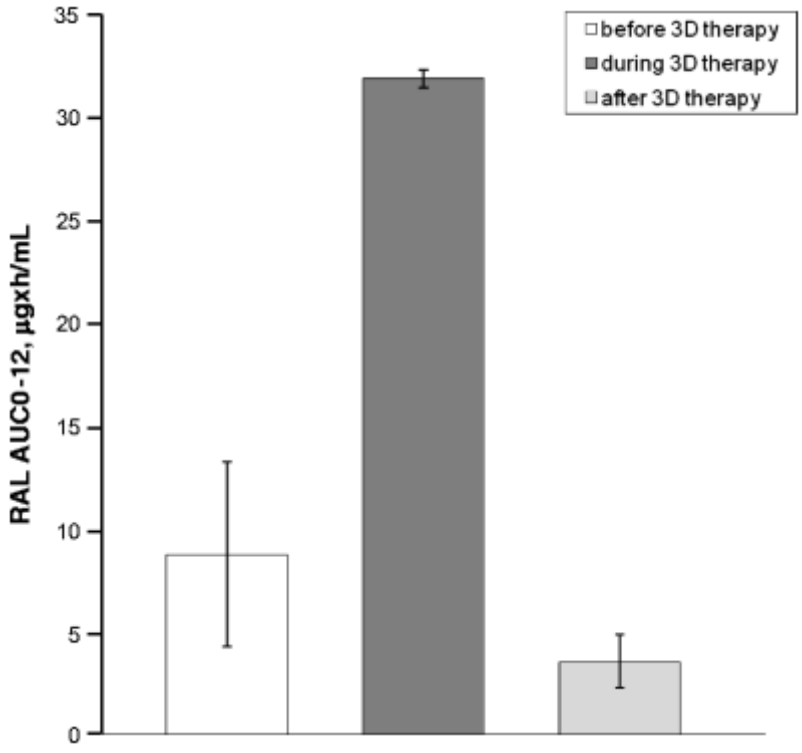

Figure 14. Mean RAL AUC0–12 values measured before, during and at the end of the 3D therapy (n = 2 for each observational period). Vertical bars represent the standard deviation

Two RAL pharmacokinetic assessments performed 6 to 9 months later revealed a reduction in the RAL $AUC_{0–12}$ (3.50±1.29 µgxh/mL). Worthy of mention, at the last available follow-up, HIV RNA was undetectable, serum transaminases were in the normal range and cyclosporine trough concentration was 115 ng/mL.

3. Discussion and Conclusions

3.1. An HPLC-UV Method for the Simultaneous Quantification of Nine Antiretroviral Agents in the Plasma of HIV-Infected Patients

a. Discussion

In this part of the study we report the development and validation of an HPLC-UV assay to quantify simultaneously nine ARV compounds with accuracy and precision. This bioanalytical method is now being successfully applied to measure ARV plasma concentrations for therapeutic purposes.

Under our laboratory conditions all drugs including IS appeared to be stable when subjected to heat inactivation and freeze/thaw cycles, in comparison to freshly prepared controls. Moreover, long-term stability data suggested that all analytes remained sufficiently stable under our current storage conditions (−20 ^{0}C). The stability of extracted samples at room temperature (24 ^{0}C) or within the auto-sampler (which is maintained at 10 ^{0}C) was found to be stable over the period of analysis. This HPLC-UV SPE assay has a short run time of 25 min per cycle with simple gradient making it suitable for a high-throughput TDM whereby large numbers of samples are processed quickly and efficiently. This is of high value in any clinical setting where laboratory analyses have to be optimized combining time-efficiency and reliability.

Emergence of drug-resistant HIV-1 strains and problems of long-term tolerability of some anti-HIV compounds can jeopardise efficacy and acceptability of existing anti-HIV medications; Hence the need to develop new drugs. DTG and RPV are among the most recent and promising anti-HIV molecules. Both these drugs have been shown to be highly effective in multi treated patients and found to be well tolerated. DTG and, especially, RPV which is the substrate and inhibitor of P450 (CYP)3A4 have a high potential for interactions with other ARV agents , notably with PI and MVC . RGV and ETV also have a high potential for drug interactions, being substrates and inducers of CYP3A4[93]. Patients administered DTG and/or RPV are also given other ARV compounds; therefore, measurement of plasma concentrations of the different drugs associated in the same ARV regimen is necessary. Our method is of particular clinical value as it allows a quick and reliable assessment of the plasma level of these drugs, although the clinical usefulness of TDM of DTG and RPV and their interactions with other co-administered drugs still needs to be investigated further.

Our assay method included widely prescribed PI's, NNRTI's and integrase inhibitor's. The choice for the limits of their ranges was based on the highest values reported in the clinical reports and PK studies. Reliability, costs, ruggedness, sensitivity and reproducibility are key points of measurement of drug plasma concentrations. Our assay, relying on SPE coupled with a high-sensitive Waters UV detector, was simple, reliable, sensitive, and less

expensive in terms of consumables and instrumentation when compared with other methods developed for LCMS or LC-MS/MS.

The relative accuracy of all QC's at three different concentration levels and intraday and interday precision supports both the accuracy and precision of our procedure. The choice of a specific wavelength for each drug (**Table 2**) was based on the need for adequate sensitivity and high specificity. An example is ETV and RPV for which quantification at 305 nm was chosen to ensure the absence of interferences and better sensitivity. The absence of interfering peaks, excluding the overlapping of DTG with RGV and EFV with LPV, allowed accurate measurement of drugs plasma concentrations. The Co-elution of DTG and RGV, occurring with our method because of similar retention time, is not an issue as both drugs belong to the same class and hence are highly unlikely to be prescribed together. Co-elution of LPV with EFV can be tackle by running two different assays or by analysing either one of them in single assay.

b. Conclusion

The SPE and HPLC-UV methods described here allow accurate and reproducible simultaneous quantification of nine ARV agents in plasma by a single assay. Good extraction efficiency and low limit of quantification make this a suitable method for use in clinical trials and for TDM. This method has been successfully applied for our routine TDM studies in HIV-infected patients.

3.2. Development and validation of electrospray ionization LC-tandem mass assay for the simultaneous measurement of ten antiretroviral agents in human plasma samples

a. Discussion

In this part of the study we reported the development and validation of an electrospray ionization LC-tandem mass assay for the simultaneous measurement of ten ARV agents in human plasma samples. This bio-analytical method is now being successfully applied to measure ARV plasma concentrations for therapeutic purposes.

Under our laboratory conditions all drugs including IS appeared to be stable when subjected to heat inactivation and freeze/thaw cycles, in comparison to freshly prepared controls. Moreover, long-term stability data suggested that all analytes remained sufficiently stable under our current storage conditions (−20 ^{0}C). The stability of extracted samples at room temperature (24 ^{0}C) or within the auto-sampler (which is maintained at 10 ^{0}C) was found to be stable over the period of analysis. This LC-MS/MS assay has a short run time of 8 min

per cycle with simple gradient making it suitable for a high-throughput TDM whereby large numbers of samples are processed quickly and efficiently. The assay showed excellent linearity over the studied concentration ranges. The mean coefficient of determination (r^2) of all calibration curves was more than 0.998. The % CV and accuracy of the LOQ, for each ARV drug, were within the limits of ± 20% and 80% to 120% respectively as recommended by the U.S. Food and Drug Administration guidelines. The results of assay precision and accuracy, assessed at 3 QC concentrations for all ARV agents are within the acceptable limits. In terms of selectivity and specificity no significant interference with ARV's concomitantly administered to the patients was observed .This is of high value in any clinical setting where laboratory analyses have to be optimised combining time-efficiency and reliability.

This LC-MS/MS method has been successfully applied for routine TDM in our laboratory. We determined trough plasma concentrations with the method reported here using samples from patients with HIV-1 treated with drugs mentioned in this method.

b. Conclusion

The development and validation of this assay were performed to quantify ATV, APV, EFV, IDV, MVC, NFV, NPV, RTG, RTV, SQV, for routine TDM in patients with HIV-1 treated with multiple ARV regimens. The assay requires a small volume of plasma for analysis (100 µL) and combines a short time of analysis of 8 minutes per sample, following quick and simple protein precipitation. 20 µL of each sample were injected onto the analytical column except 40 µL for EFV. In conclusion, a rapid, specific, and sensitive LC-MS/ MS method for the quantification of 10 currently approved ARV's in human plasma was developed and has been successfully applied for routine TDM and pharmacokinetic studies in patients with HIV.

3.3. A pharmacokinetic viewpoint on antiretrovirals dosing and possibility of dose reduction

a. Discussion

The large majority of patients treated with DRV, ETV and MVC had concentrations largely exceeding the minimum effective concentrations reported in the literature **Figure 9**. As no evidence is available to date on potential associations between plasma concentrations of DRV, ETV and MVC and drug-related toxicities, we were not able to estimate the percentage of patients potentially overexposed to these drugs. A very wide distribution in the measured RAL plasma trough concentrations (n 408) was observed [median, (interquartile range) concentrations: 338 (42 to 9814) ng/ml corresponding to an inter-patient coefficient of variation of 158.63 , with 17.1 % of samples below the tentative minimum effective concen-

tration of 40 ng/ml [20]. No upper therapeutic threshold of RAL concentrations associated with drug tolerability is defined currently.

A large percentage of patients treated with in label doses of LPV (34.4 % patients above 7000 ng/ml), ATV (45.1 % patients above 800 ng/ml), and NVP (35.1 % patients above 6000 ng/ml), were found to have trough concentrations largely exceeding the upper therapeutic threshold (Error! Reference source not found.), therefore patients potentially overexposed to ARV therapies and hence increased risk to drug-related toxicity. Indeed, evidence is available showing that high concentrations of TDF, LPV, ATV, EFV and NVP correlated with treatment tolerability. It is more difficult to confirm this trend for novel drugs – namely DRV, ETV and MVC – simply because no clinical evidence is available to date correlating their plasma concentrations with toxicity. Similarly, no conclusion can be drawn for RAL. As confirmed by this and our previous findings, trough concentrations of RAL were associated with a very wide interpatient variability [129]. In our clinical practice, we discourage monitoring RAL by measuring trough level because this concentration does not correlate with RAL area under the curve (AU0–12 h) [129]. Rather, we suggest considering limited sampling strategies on the basis of the collection of four to five plasma samples in the first 3–4 h after drug administration to predict q.d. RAL exposure [218]. In the present study, we were not able to assess intrapatient variability because we considered only one sample per patient. However, emerging evidence is now available showing that intrapatient variability of RAL pharmacokinetics is much higher than PI's and nonnucleoside reverse trascriptase inhibitors [129,219–221].

b. Conclusion

The main limitation of our investigation is represented by the lack of clinical information on patients' characteristics, their immune-virologic status, concomitant medications and treatment tolerability. Here, we would like just to give a picture of exposure of HIV-infected patients to ARV treatments in a real-life scenario, providing a pharmacokinetic-based rationale for TDM-guided dose reduction of current therapies. Further studies are needed to confirm our hypothesis with strong clinical endpoints.

In conclusion, in this study, we document that a significant proportion of patients treated with some of the ARV's at marketed doses had plasma concentrations exceeding the upper therapeutic threshold. Such selected patients, who might have the highest risk of experiencing drug-related complications, may benefit from TDM-driven adjustments in ARV doses with potential advantages in terms of costs and toxicity This approach, however, may be difficult to be applied in an era of fixed-dose regimens and fixed-dose combinations. Further studies are, therefore, needed to identify good candidates/drugs for TDM.

3.4. Metabolic and kidney disorders correlate with high atazanavir concentrations in HIV infected patients

a. Discussion

In this study, we document that a significant proportion of HIV-infected patients treated with ATV at the standard 300/100 mg qd dosage had plasma drug concentrations largely exceeding the upper therapeutic threshold, whereas nearly one third of patients treated with unboosted ATV had drug concentrations below 150 ng/mL, considered as the minimum concentration to be reached for the optimal management of patients on ATV-based ARV regimens [183] .

More than 60% of HIV-positive patients enrolled in the present study experienced hyperbilirubinemia of grade 1 or more, allowing us to deeply investigate the potential contribution of ATV concentrations on this drug-related adverse event. In our study we firstly confirmed the previously reported relationship between ATV exposure and the increase in total bilirubin concentrations[183,206,207]. Moreover, we extended previous findings by documenting a direct and linear correlation between ATV trough concentrations and the severity of hyperbilirubinemia. Interestingly, such relationship was established also when excluding patients homozygous for the UGT1A1*28, the genotype associated with a defect in the metabolism of bilirubin[222,223]. Using this approach, we demonstrated that association of ATV exposure with hyperbilirubinemia still persisted also in patients with the favourable UGT genotype. Accordingly, it could be speculated that in these patients, lacking of an inherited risk to experience the event, ATV-related hyperbilirubinemia might be eventually reversed by reducing ATV doses.

The potential effect of ATV on lipid profile is still a matter of debate. Meta-analyses have reported that plasma lipid concentrations were lower with ATV/r than with other RTV-boosted PI regimens [224] . Nevertheless, evidence is also available showing that ATV has a worst atherogenic lipid profile compared with NNRTI's[198]. Our exploratory analyses study extended previous findings by documenting that dyslipidemia might be associated with ATV plasma trough concentrations. In order to demonstrate a direct relationship between ATV exposure and lipid increase, we correlated ATV concentrations with the magnitude of lipid levels increase as compared to baseline. Using this approach we found a linear and significant association between ATV concentrations and the percentage increase in serum triglycerides levels, but not for cholesterol, probably because of the less number of events and the narrow distribution in the percentage increased compared with triglyceride concentrations. Among the different ATV-based regimens, lipid alterations were more frequently found in patients on ATV/r that in those given ATV alone [199] , suggesting a key role of RTV [225] . This has been fairly confirmed also by our study by documenting that: I) patients experiencing ATV-related complications were more frequently receiving RTV; II) the signif-

icant association between RTV and ATV-related dyslipidemia was confirmed by multivariate analyses. In order to avoid the potential bias related to RTV use associated to the fact that a higher proportion of patients in the "adverse effect group" were treated with boosted ATV compared with control patients (those who did not experienced ATV-related complications), we repeated our comparisons stratifying data according to RTV boosted/unboosted intakers. Also using this approach, we documented that among patients given ATV/r 300/100, those experiencing dyslipidemia have significantly higher ATV concentrations compared with those not experiencing drug-related complications. A similar, but not significant, trend was found also in patients treated with ATV 400 mg. The small number of ATV unboosted intakers did not allow us to reach definitive conclusion on this topic. We are, however, confident that our preliminary findings could provide the rationale for ad-hoc prospective studies aimed at investigating the impact of ATV exposure on dyslipidemia in unboosted regimens. We also acknowledged that, as an important additional limitation, no information were available on RTV exposure, because RTV concentrations are not assessed in the routine management of HIV patients. Accordingly, the potential contribution of RTV co-administration on episodes of dyslipidemia in patients treated with ATV cannot be completely ruled out.

Cases of complicated and uncomplicated ATV-associated nephrolithiasis have been described, sometimes leading to obstructive uropathy and acute renal failure [226–229]. The association between exposure to ATV and increased incidence kidney stones has been subsequently confirmed also by epidemiological studies [230] . To the best of our knowledge, our is the first study showing that overexposure to ATV is associated also with increased risk of nephrolitiasis. It should be, however, acknowledged that as potential limitation of the present observation, the presence of ATV in the stones was not directly verified, and that episodes of drug-related nephrolitiasis were diagnosed indirectly after excluding the presence of oxalate or urate crystals in urinalysis and high value of serum uric acid.

The potential association between exposure to ATV and increased risk to cholelithiasis is still a matter of debate , however Rakotondravelo and co worker have reported 14 case of ATV releated cholelithiasis [231]. In our cohort, we found only one episode of cholelithiasis in a patient treated with ATV for less than one year.

b. Conclusion

In conclusion, in this study we documented that significant proportion of patients treated with conventional ATV dosages had plasma concentrations exceeding the upper therapeutic threshold. A likely possibility is an inherited deficit in ATV clearance[232] and/or ATV metabolism; in particular, ATV is a dedicated CYP3A substrate, which includes 3A4 and 3A5, two polymorphic genes [200–202]. The administration of unboosted ATV to healthy subjects carrying the defective CYP3A5*3 resulted in significantly higher ATV concentrations compared with values measured in patients expressing CYP3A5 [222]. Assuming that over 90% of patients in our study were Caucasians with a high prevalence of carriers of

CYP3A5 *3, it is likely that the observed overexposure to ATV concentrations is the result of excessively high dose of RTV-boosted ATV or of needless boosting with RTV. This is a first important conclusion of our study that raises concerns on the need of full dose of RTV-boosted ATV in Caucasian patients and opens new questions about the ATV dosages that should considered correct (or in label and off label in Europe). This may be particularly relevant in light of the recent findings showing that RTV may accumulate at intracellular level at higher degree than other PI's providing additional antiviral activity and eventually allowing the use of reduced PI doses[233]. We also found that such patients have the highest risk of experiencing ATV-related complications and may benefit from TDM-driven adjustments in ATV dosage with potential advantages in terms of costs and toxicity.

3.5. Suspected pharmacokinetic interaction between raltegravir and the 3D regimen of ombitasvir, dasabuvir and paritaprevir/ritonavir in an HIV-HCV liver transplant recipient

a. Discussion

This study show that the 3D regimen of ombitasvir, dasabuvir and paritaprevir/RTV is safe and efficacious in HIV-HCV co-infected patients [213,214]. Studies in healthy volunteers have also provided indirect evidence that 3D regimen can be safely given to liver transplant recipients provided that the doses of immunosuppressive agents are reduced and their concentrations strictly monitored[215]. However, no data are available to date on the use of the 3D regimen in HIV-HCV co-infected liver transplant recipients given RAL based HAART. Here, we confirm the optimal safety of this combination in our patient. At the same time, we observed a nearly 300 % increase in the RAL $AUC_{0–12}$ during concomitant 3D treatment that returned to baseline values at the end antiviral therapy. Hence, it cannot be excluded that this increment might become clinically relevant in patients that are more prone to experience RAL-related hepatic or muscle toxicities[234] . In these circumstances, TDM-guided early RAL dose adjustments during 3D therapy may improve the tolerability of patients to both HCV and HIV therapies.

The mechanisms explaining this unanticipated drug-to drug interaction are unknown. A potential contribution of improved liver function and ability to eliminate RAL after clearance of HCV is unlikely according to the recent results by Barau et al. [235]. We can speculate that the observed increase in RAL exposure might be due to UGT1A1 inhibition by paritaprevir/RTV, ombitasvir and/or dasabuvir, by an effect of 3D on transport proteins involved in the disposition of RAL [236,237].

b. Conclusion

In conclusion, a threefold increase in RAL exposure in HIV-HCV co-infected liver transplant recipients treated with the 3D regimen for recurrent HCV infection was described. Such suspected interaction, which might be clinically relevant in selected patients, is easily manageable through TDM of RAL concentrations, eventually improving the safety of this drug.

4. Abbreviations

% CV	Coefficient of variation
3TC	lamivudine
ABC	Abacavir
APV	Amprenavir
ARV	Antiretroviral
ARV's	Antiretrovirals
ATV	Atazanavir
b.i.d.	Twice (two times) a day
Cmax	Maximum (or peak) serum concentration
d4T	Stavudine
ddI	Didanosine
DLV	Delavirdine
DRV	Darunavir
DTV	Dolutegravir
EFV	Efavirenz
EI	Electron ionisation
ETV	Etravirine
ETV	Etravirine
EVG	Elvitegravir
FOS-APV	Fosamprenavir
FTC	Emtricitabine
GC	Gas chromatography
GC/MS	Gas chromatography/ mass spectrophotometer
HAART	Highly active antiretroviral therapy
HPLC	High performance thin layer chromatography
IDV	Indinavir
LC-MS/MS	Liquid chromatography-tandem mass spectrophotometer
LOQ	Limits of quantification
LPV	Lopinavir
MS	Mass spectrophotometer
MS/MS	Tandem mass spectrophotometer
MVC	Maraviroc
NFV	Nelfinavir
NNRTI's	Non nucleoside reverse transcriptase inhibitors
NRTI's	Nucleoside reverse transcriptase inhibitors
NVP	Nevirapine
PBMC	Peripheral blood mononuclear cells
PDA	Photo diode array
PI's	Protease Inhibitors
q.d.	Once (one time) a day
QC's	Quality controls
RPV	Rilpivirine
RTV	Ritonavir
SQV	Saquinavir
T-20	Enfuvirtide
TDF	Tenofovir
TDM	Therapeutic drug monitoring

TPV	Tipranavir
UPLC	Ultra performance liquid chromatography
UV	Ultra violet
ZDV	Zidovudine

5. Publication and conferences

Publications

1. Dario Cattaneo, Sara Baldellia, Nitin Charbe, Simone Castoldi, Valeria Cozzi SF and, Clementib E. Is it time to revise antiretrovirals dosing? A pharmacokinetic viewpoint. AIDS. 2014;130(3):392–3.

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6. Valeria Cozzi , Nitin Charbe , Sara Baldelli , Simone Castoldi , Chiara Atzori , Dario Cattaneo , Emilio Clementi . Development and validation of a chromatographic UV method for the simultaneous quantification of dolutegravir and rilpivirine in human plasma" , Submitted to *Therapeutic Drug Monitoring*.

7. Nitin Charbe ,Valeria Cozzi , Dario Cattaneo , Sara Baldelli , Simone Castoldi , Emilio Clementi . Comprehensive review of the chromatographic quantitative assay methods and their use in therapeutic drug monitoring of HIV patients, submitted to *HIV and AIDS review.*

8. Nitin Charbe ,Valeria Cozzi , Dario Cattaneo , Sara Baldelli , Simone Castoldi , Emilio Clementi . An HPLC-UV Method for the Simultaneous Quantification of Nine AntiretroviralAgents in the Plasma of HIV-Infected Patients, submitted to Journal of pharmaceutical sciences and biomedical analysis.

9. Nitin Charbe ,Valeria Cozzi , Dario Cattaneo , Sara Baldelli , Simone Castoldi , Emilio Clementi Development and validation of electrospray ionization LC-tandem mass assay for the simultaneous measurement of ten antiretroviral agents in human plasma samples. Manuscript under review for submission.

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