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REVIEW ARTICLE

Bio-analytical Assay Methods used in Therapeutic Drug Monitoring of Antiretroviral Drugs-A Review

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Abstract: ***Background*:** Several clinical trials, as well as observational statistics, have exhibited that the advantages of antiretroviral [ARV] treatment for humans with Human Immunodeficiency Virus / Acquired Immune Deficiency Syndrome HIV/AIDS exceed their risks. Therapeutic drug monitoring [TDM] plays a key role in optimization of ARV therapy. Determination of ARV's in plasma, blood cells, and other biological matrices frequently requires separation techniques capable of high effectiveness, specific selectivity and high sensitivity. High-performance liquid chromatography [HPLC] coupled with ultraviolet [UV], Photodiode array detectors [PDA], Mass spectrophotometer [MS] detectors *etc*. are the important quantitative techniques used for the estimation of pharmaceuticals in biological samples.

***Objective*:** This review article is aimed to give an extensive outline of different bio-analytical techniques which have been reported for direct quantitation of ARV's. This article aimed to establish an efficient role played by the TDM in the optimum therapeutic outcome of the ARV treatment. It also focused on establishing the prominent role played by the separation techniques like HPLC and UPLC along with the detectors like UV and Mass in TDM.

***Method*:** TDM is based on the principle that for certain drugs, a close relationship exists between the plasma level of the drug and its clinical effect. TDM is of no value if the relationship does not exist. The analytical methodology employed in TDM should: 1) distinguish similar compounds; 2) be sensitive and precise and 3) is easy to use

***Results*:** This review highlights the advancement of the chromatographic techniques beginning from the HPLC-UV to the more advanced technique like UPLC-MS/MS. TDM is essential to ensure adherence, observe viral resistance and to personalize ARV dose regimens. It is observed that the analytical methods like immunoassays and liquid chromatography with detectors like UV, PDA, Florescent, MS, MS/MS and Ultra performance liquid chromatography (UPLC)-MS/MS have immensely contributed to the clinical outcome of the ARV therapy. Assay methods are not only helping physicians in limiting the side effects and drug interactions but also assisting in monitoring patient's compliance.

***Conclusion*:** The present review revealed that HPLC has been the most widely used system irrespective of the availability of more sensitive chromatographic technique like UPLC.

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INTRODUCTION

The idea of overseeing pharmacotherapy based on plasma drug concentration has been utilized for quite a long time in an assortment of clinical settings. The curiosity in studying therapeutic drug monitoring [TDM] of ARV drugs has developed considerably since highly active ARV therapy [HAART] turned into a prime therapeutic intervention of care in clinical practice [1, 2]. The drugs used in the treatment of HIV infection are known as ARV drugs and there are around 24 of them approved by the FDA. Although, the ARV drugs do not cure the patients, when taken in combination, they can prevent the growth of the virus and further damage to the immune system. HIV attaches itself to the CDR5 receptor of the CD4 immune cells and empties its contents inside the cell. Once inside the HIV, the enzyme called reverse transcriptase builds the HIV DNA on HIV RNA through the process called as reverse transcription. HIV DNA then integrates with host chromosome using the HIV enzyme called as integrase, thereby establishing the HIV infection inside the cell. HIV DNA segment in the host chromosome is activated to produce the HIV proteins when infected cells reproduce. Later, all the HIV protein comes together to bud out of the cells as an immature virus. Protease enzyme then cuts the long polypeptide into small functional units to form the mature virus. Till date, ARV drugs have immensely contributed to increasing the life expectancy of the patient. Adherence to the dose regime is the key to success of the ARV therapy [3]. However, irrespective of adherence to routine regime and diet, bioavailability of the ARV drugs in HIV infected patients is significantly affected by the genetically determined difference in drug distribution and elimination. Various studies have already been established based on inter-individual variability in plasma concentrations of ARVs, lesser virological failure rates as well the adverse reactions that occur when optimal concentrations of ARV drugs are accomplished. Thus, TDM can help streamline ARV treatment [4].

TDM of ARV drugs is not only the technique to study the drug-drug interactions but also is an important tool to optimize the therapy in routine clinical practice. Moreover, pieces of evidence are also available showing that inadequate plasma drug concentrations can favour the development of drug resistance mutations and imperil present and future treatment choices. Various clinical trials have shown that drug serum concentrations are an essential component for the advancement in response to therapy for HIV [5]. The main goal of TDM of ARV drugs is to optimise treatment and its acceptability and to minimise drug-related toxicity and resistance.

Investigational studies in adults suggest that altered doses and regimen choices are primarily based on TDM accomplished *via* ARV drug concentrations which are related to optimal clinical response and/or acceptability [6-12]. The application of TDM requires, however, the availability of feasible and reliable techniques. This review is focused on different bio-analytical methods developed on HPLC in conjugation with UV-PDA and Mass detectors. For this review, we have extensively used all the important literature which dealt with the quantitative estimation and which contributed immensely to the clinical outcome of the patients infected with HIV.

ANALYTICAL TECHNIQUES IN TDM

As new drugs are constantly approved and introduced in HAART regimen, the need for TDM is also growing. Chromatography coupled with various spectrophotometry detectors such as UV, PDA, and MS is the instrument of choice of the most clinical pharmacology laboratories [13]. Alternatively, immunoassays which are commercially available for various drugs are also becoming available for the TDM of ARVs [14].

Immunoassays in TDM

TDM of clinical agents and drugs of abuse testing are accomplished by automated immunoassay techniques. Several immunoassays are also available for the quantitative estimation of the ARVs [15]. Most immunoassay techniques use samples without any prior treatment and analysed on fully computerized, random access systems. The immunoassays require a very small quantity of sample reagents [mostly <100µL], readily available in the instrument and concentration of unknown samples is extrapolated using calibration curves stored in the system. In immunoassays, the analyte is identified by its complexation with a particular binding molecule, which in maximum instances is an analyte-specific antibody [16]. These immunological reactions are further utilized to format and prepare various labels giving rise to the diverse arrays of immunoassays. On the basis of assay design, im-

munoassays are classified as competition and immunometric [17]. Competition immunoassays are mostly used for analytes with small molecular weight and require single analyte-specific antibody whereas, immunometric immunoassays work better for analytes with larger molecular weight [*e.g.* proteins or peptides] and use two distinct antibodies [18].

High-Performance Liquid Chromatography [HPLC]

Although gas chromatography (GC) is used as a separation technique, its use is limited to volatile molecules. Liquid chromatography, on the other hand, can be used for separation of both polar and nonpolar molecules Unlike gas chromatography, derivatization of the analyte is not necessary for HPLC analysis [19]. On the basis of mode of separation, HPLC is classified into two types' viz., normal phase and reverse phase chromatography. In normal phase chromatography, the stationary phase is polar [hydrophilic] and the mobile phase is nonpolar [hydrophobic] whereas reverse phase chromatography is the inverse of this. Several detectors can be used for checking peak elution from HPLC column including UV-Vis, PDA, refractive index detectors, mass spectrometer, conductivity detectors, fluorescence detectors, *etc*. In clinical laboratories, UV detection is most commonly preferred even though other detection techniques including fluorescence and electrochemical methods are also available.

Liquid Chromatography-Mass Spectrometry [LCMS]

LCMS is a modern analytical technique similar to gas chromatography (GC)-MS which finds its application in clinical toxicology laboratories for screening, detection and quantification of drugs of abuse in biological fluids. LCMS is widely used due to its high sensitivity and specificity. LCMS is preferred over GCMS as it enables analysis of polar, charged and thermally labile compounds that require derivatization with GC. LCMS also has an advantage regarding its use in clinical toxicology laboratories for analysing very low concentration of drugs [ng/ml] in biological matrices and high molecular weight compounds [20]. In LCMS system, the individual components in a mixture are first separated on the column, transferred to the ionization chamber and then moved to MS for fragmentation through an interface. Amongst the popular interfaces like electrospray ionization, atomic pressure chemical ionization, atomic pressure photo-ionization, the former *i.e*. electrospray ionization is most commonly utilized in clinical laboratories. On the basis of mass/charge ratio, the ions are separated and analyzed by MS to give a mass spectrum. The major types of mass spectrometric analyzers are quadrupole analyser, ion trap analysers, time-of-flight analyser and hybrid analysers [21-23].

Ultra-Performance Liquid Chromatography [UPLC]

UPLC is an advanced version of HPLC with the same working principle but with enhanced resolution, speed and sensitivity for the detection of drugs. The key focus of this technology is to deliver the mobile phase at high pressure [about 15,000 psi] and column packing with the smaller particle size [1.7-1.8 µm]. The key advantages of UPLC include lesser runtime, reduced operation cost and less solvent consumption.

The principle of UPLC is based on Van Deemter equation that describes the connection between linear velocity [flow rate] and plate height [HETP *i.e*. Height Equivalent to the Theoretical Plate or column efficiency] [24]. As one of the variables is microparticle size, the efficiency of UPLC does not reduce at increased flow rates. UPLC is widely accepted in clinical laboratories worldwide as this technology provides highly robust, dependable and reproducible results. Various detectors like fluorescence, UV, PDA, electrochemical and MS are used with UPLC of which UPLC/MS or UPLC/MS/MS has been adopted successfully in clinical laboratories [25, 26].

SAMPLING TECHNIQUES IN TDM

Routinely, ATV concentrations are measured in blood plasma obtained by venepuncture techniques. However, venous sampling is difficult for repeated sampling and in some populations, such as neonates, there are always logistical challenges when nonhospital-based sampling is required. A proper bio-analytical method is crucial for the measurements of drug level in matrices suitable for the patient. Special attention must be paid to bio-analytical methods in these patient-friendly matrices. Patient-friendly drug monitoring of antiretroviral drugs includes dried blood spots (DBS), hair, and saliva. The discussion of the sampling technique is out of the scope of this re-

view and hence we recommend the readers to refer to the article "Bio-analytical issues in patient-friendly sampling methods for therapeutic drug monitoring: focus on antiretroviral drugs" [24].

ANALYTICAL METHODS FOR TDM OF ARV

At present, there are five major classes of ARV drugs viz. nucleoside reverse transcriptase inhibitor [NRTI], nonnucleoside reverse transcriptase inhibitor [NNRTI], Protease inhibitors [PI], fusion inhibitor and integrase inhibitor [IIs].

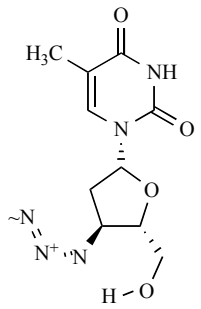
Nucleosidereverse Transcriptase Inhibitors (NRTIs)

The first generation of ARV drugs is NRTIs permitted to treat HIV [25]. Reverse transcriptase is an HIV enzyme that converts viral RNA into DNA in host CD4 cells and the process is known as reverse transcription. NRTIs inhibit the enzyme reverse transcriptase and prevent the synthesis of DNA. Without reverse transcriptase, HIV cannot replicate and infection cannot spread. Nucleoside analogues possess structural similarity with the natural building blocks of DNA and have to undergo phosphorylation to become active in the body. NRTIs are falsely chosen by reverse transcriptase to build the faulty DNA that denies further addition of natural nucleotides. Thus, the new DNA built incorrectly led to halt HIV replication [27]. Following are some NRTIs used for HIV therapy: Zidovudine [ZDV/AZT], Didanosine [DDI], Stavudine [d4T], Lamivudine [3TC], Abacavir[ABC], Emtricitabine [FTC], Tenofovir disoproxil fumarate [TDF], Adefovir [ADV].

Zidovudine [AZT]

AZT is a thymidine analogue NRTI approved in March 1987. Chemically, it is an 1-[[2R,4S,5S]-4-azido-5-[hydroxymethyl]oxolan-2-yl]-5-methyl-pyrimidine-2,4-dione, the solubility of which in water is 20100 mg/L [at 25 °C] with a logP value of 0.05. It was the first FDA approved ARV for the treatment of HIV.

Various methods have been developed for the quantitative determination of AZT including chromatography, fluorescence polarisation immunoassay, using diamond paste based immune sensor, fluorescence spectroscopy and voltammetry [26-38]. Castro and co-workers described a new selective and sensitive adsorptive stripping voltammetry method for AZT determination at the submicromolar concentration levels [39]. This technique was based on the controlled adsorptive accumulation of AZT at the mercury film electrode, followed by a linear-sweep adsorptive stripping voltammetry analyses. However, the first quantitative determination of AZT on HPLC is credited to Klecker *et al.* who, during the clinical pharmacokinetic studies quantified AZT levels in plasma, cerebrospinal fluid and urine by isocratic evaluation on C_{18} reverse-phase column [40]. Later, AZT became a pillar in the drugs combination called HAART, which contains a minimum of two NRTIs and one NNRTI in a fixed dose combination. Combination of the different classes of ARV drugs is used together to prolong the survival of HIV patients. Due to the increased prevalence of drug-drug interaction in and with HAART regime, several assay methods for multi and single - component quantitative analysis are developed. Jung *et al.* in 2007 developed LCMS assay for simultaneous estimation of 17 ARV drugs in human plasma for quantitative analysis while Nandi *et al.* in 2013 reported an assay for simultaneous quantification of AZT, 3TC, and NVP in human plasma with HPLC-UV method [41,42]. Similarly, simultaneous quantitation of 3TC, AZT and NVP in human plasma by LC-MS/MS assay was reported by Krishna *et al.* [43].



Zidovudine

Inside the cell, AZT undergoes phosphorylation reaction to give active AZT triphosphate metabolite. This active metabolite blocks the DNA synthesis by inhibiting the reverse transcriptase enzyme and chain termination process [44]. Hence, several methods have been developed to determine AZT concentration inside the cells which are described in Table **1** of this review.

Lamivudine [3TC]

Lamivudine [2'-deoxy-3'-thiacytidine, 3TC], approved in 1997, is a cytidine analogue NRTI

Table 1. Multicomponent methods to assay the concentration of ARV drugs.

Year/ Ref	Analytes [Extraction Method]	Separation-Detector	Column	Matrix	Run time [min]	Elution	Advantage
1998 / [264]	APV, IDV, NFV, RTV and SQV. [SPE]	HPLC-UV [210 and 239 nm]	ZorbaxÒ SB-C column [75 x 4.6 mm I.D. /particle size 3.5 µm; protected by a Chromguard column [1033 mm I.D.]	P	20	Isocratic , flow rate: 1.5 ml/min, Mobile Phase: CH_3CN plus distilled water containing 25 mM sodium acetate and 25 mM hexane-1-sulfonic acid and adjusted to pH 6.0 with hydrochloric acid 37% [40.5: 59.5, v/v].	This assay meets all current requirements of the validation of a bio-analytical methodology and covers the concentration ranges of interest for use in pharmacokinetic Studies.
2000/ [150]	APV, EFV, IDV, NFV, RTV, SQV, ABC, Didanosin, 3TC, NVP, D4T and AZT. [SPE]	HPLC-UV [260 nm]	Symmetry Shield 5 µm C18 column [250 x 4.6 mm I.D.] [Waters] protected by a 2 µm Upchurch filter	P	30	Gradient, flow-rate : 1.3 ml/min, Mobile phase is composed of 0.04 M NaHPO buffer with 4% [v/v] OSA and CH_3CN [50: 50, v/v].	This is a sensitive, specific and validated assay for the simultaneous quantitative determination of 12 ARV drugs from different therapeutic classes.
2000/ [151]	IDV, APV, SQV, RTV, NFV EFV. [SPE]	HPLC-UV [201 nm]	ChromCart cartridge column [125 x 4 mm I.D.] filled with Nucleosil 100, 5 µm C18 equipped with a guard column [8 x 4 mm I.D.] filled with the same packing material.	P	47	Gradient, flow rate: 1 ml /min, Mobile phase A: pure CH_3CN. Mobile phase B: 11.8 ml 8.5% H_3PO and 0.2 g sodium heptanesulfonate to 988. Mobile phase C: 0.3% AcOH in CH_3CN.	This optimised HPLC method provides a fairly simple procedure for simultaneously determining five currently used PIs and EFV in plasma of HIV-infected patients.
2001/ [221]	APV, IDV, NFV, RTV, SQV and NVP. [liquid–liquid extraction]	HPLC-UV [239 to 320]	Symmetry 5 µm C18 column [250 x 4.6 mm I.D.] protected with a Symmetry 5 µMC $_{18}$precolumn [20 x 3.9 mm I.D.].	P	35	Gradient, flow rate: 0.9 ml/min Mobile phase A: CH_3CN and 0.025 M tetramethylammonium perchlorate in 0.2% aqueous trifluoroacetic acid [55: 45 [v/v]], Mobile phase B: $CH_3OH$and 0.025 M tetramethylammonium perchlorate in 0.2% aqueous trifluoroacetic acid [55: 45 [v/v]].	This method allows simultaneous estimation of PIs and NVP for TDM in blood plasma.
2001/ [122]	I.S, IDV, AMP, RTV, SQV, NFV, EFV, NVP and DLV. [liquid–liquid extraction]	HPLC-MS [SIM]	Supelcosil LC-18-DB, 7.5 cm × 4.6 mm, 3µm analytical	P	10	Gradient, flow rate: 0.8 mL/min, Mobile phase A: H_2O, Mobile phase B: CH_3CN.	The very short analysis time [10 minutes], liquid–liquid extraction, simple mobile phase without buffer and small sample size are the key advantages of this method.
2001/ [61]	Zalcitabine, 3TC, d4T, didanosine, AZT and ziagen , IDV, NFV, SQV, RTV NVP, Delavirdin and EFV. [SPE]	HPLC-UV [250]	Luna C18 columns 150 x 4.6 mm.	P	55	Gradient, flow rate: 0.85 ml/min Method A: Mobile Phase A: HPLC grade CH_3CN, Mobile Phase B: HPLC grade H_2O Method B: The gradient was formed between 0.004 M sulfuric acid and CH_3CN [8 to 63% CH_3CN in 45 min with a 5-min hold at 63%].	Two rapid and simple SPE–HPLC methods presented in this paper covering analysis of 13 drugs.

(Table 1) contd….

Year/ Ref	Analytes [Extraction Method]	Separation-Detector	Column	Matrix	Run time [min]	Elution	Advantage
2003/ [225]	IDV, APV, RTV, LPV, SQV, NFV and NFV metabolite M8. [liquid–liquid extraction]	HPLC-UV[210 and 239nm]	Nova-Pak C18 column, 3.93150 mm.	P	30	Gradient, Flow rate : 1 ml /min, Mobile Phase A: 140 ml of CH_3CN, 75 ml triethylamine, Mobile phase B: 160 ml of 5 mM sodium dihydrogen phosphate, pH 6.	This assay was quality controlled by an international interlaboratory quality control program for the TDM in HIV infection. More than 13 laboratories from Europe, Canada, USA and Australia participated in this programme.
2003/ [62]	Zalcitabine, 3TC, Didanosine,D4T, AZT, ABC and NVP. [SPE]	UV, 269 nm [0–11 min], 250 nm [11–14 min], 271 nm [14–24 min] [24–33 min].	Polarity dC [150 x 33.9 mm, 5.0-µm particle size, Waters] with a guard column of Polarity dC [20 x 33.9 mm, 5.0-µm particle size, Waters].	P	33	Gradient, flow-rate : 1.1 ml/min, Mobile Phase A: 10 mM ammonium acetate buffer [pH 6.5], Mobile Phase B: 200 ml of mobile phase A [pH 6.5] mixed with 500 ml of CH_3CN and 300 ml of CH_3OH.	This method is useful for simultaneous determination of six NRTIs and NVP.
2003/ [166]	IDV, APV, EFV, SQV, NFV, NVP, RTV and LPV. [liquid–liquid extraction]	LC-MS	Nucleosil C18 HD, 5-mm particle size column [12.5 cm x 2mm] , protected with a guard column [8 x 2 mm].	P	21	Gradient, flow Rate: 200 ml /min. Mobile Phase A: CH_3CN containing 30% methanol and ammonium carbonate buffer pH 9.3 [5: 95, v/v], Mobile Phase B: CH_3CN containing 30% CH_3OH and ammonium carbonate buffer, pH 9.3 [95: 5, v/v].	The method is simple, fast, accurate and suitable for PIs [APV, IDV, LPV, NFV, RTV, SQV] and NNRTIs [EFV, NFV].
2004/ [158]	NVP, IDV, M8 APV, NFV, SQV, RTV, EFV ATV and LPV. [liquid–liquid extraction]	HPLC-UV [320 nm before 8 min and 259 nm after 8 min]	WATERS Symmetry® 5µm C18 column [250×4.6mm i.d.] protected with a WATERS Symmetry® 5µM C18 pre-column [20×3.9mm i.d.]	P	35	Gradient, flow rate: 0.9 ml/min, Mobile Phase A: CH_3CN and 0.025M tetramethylammonium perchlorate in 0.2% aqueous trifluoroacetic acid [55: 45 [v/v]], Mobile phase B: CH_3OH and 0.025M tetramethylammonium perchlorate in 0.2% aqueous trifluoroacetic acid [55: 45 [v/v]].	This method is based on a HPLC assay coupled with UV detection that allows the monitoring of PI and NNTRI with limits of quantitation which are consistent with therapeutic through plasma concentrations of PI and NNRTI.
2005/ [110]	3TC, didanosine, d4T, AZT and ABC [SPE]	UV [260 nm]	Symmetry Shield RP C18 column [150mm × 4.6mm i.d./particle size 3.5 µm] protected by a Symmetry Shield RP18 guard column [20mm×3.9mm i.d./ particle size 3.5µm].	P	24	Gradient, flow rate 1.0 ml/min, Mobile phase A: acetate buffer: CH_3CN [95: 5, v/v]. Mobile phase B: acetate buffer: CH_3CN [76: 24, v/v].	This approach requires less plasma sample with assay accuracies of about 92–102%.
2005/ [367]	NVP, IDV, M8, SQV, NFV, APV, ATV, RTV, LPV and EFV. [liquid–liquid extraction]	HPLC-UV [210 nm]	Symmetry Shield 3.5 µm RP18 column [15 cm 34.6 mm ID] equipped with a 2-cm pre-column packed with the same material [Waters].	P	60	Gradient, flow rate: 1 mL/min, Mobile phase A: 15 mM phosphate buffer, pH 4.2, Mobile phase B: CH_3CN.	The required plasma sample is 500 µL. This procedure allows the simultaneous determination of the different PIs plus M8, and the NNRTIs with good precision and accuracy in the range 50 to 10,000 ng/mL. Because all compounds are monitored at a single wavelength [210 nm], this HPLC method does not require the use of an expensive photodiode array detector.

(Table 1) contd….

Year/ Ref	Analytes [Extraction Method]	Separation-Detector	Column	Matrix	Run time [min]	Elution	Advantage
2005/ [142]	APV, NFV, IDV, LPV, SQV, RTV, ATV, NVP and EFV. [Protein precipitation]	LC-MS/MS [MRM]	Phenomenex 1 Synergy Max RP LC column [150 x 2 mm, 4m].	BS	8	Gradient, Flow rate : 0.5 mL/min, Mobile phase A: [97: 3 CH_3OH/H2O [v/v], 0.1% acetic acid, 10mM NH4Oac], Mobile Phase B: [10:90 MeOH/H2O [v/v]].	This method described a robust LC-MS/MS method for multi component analysis of PI and NNRTI drugs in patient plasma and for the first time in DBS.
2006/ [63]	3TC, Zalcitabine, FTC, Didanosine,D4T, ABC, AZT, NVP, IDV, SQV, APV, NFV, RTV, LPV, EFV and ATV. [SPE]	UV [240 and 260 nm]	C18 Symmetry column [250mm×4.6mmI. D.] Particle size of 5.0µm [Waters] with a Waters guard column [20×3.9mm I.D.].	P	35	Gradient, flow rate: 1.0 mL/min, Mobile phase A: 0.01M KH_2PO_4], Mobile Phase B: [CH_3CN].	This method id useful in simultaneous determination of the large number of anti-HIV drugs.
2007/ [41]	EFV, NVP, ZDV, d4T, ABC, 3TC, DDC, DDI, IDV, NFV, RTV, ATZ, TNF, SQV,LPV, APV and Delavirdine . [liquid–liquid extraction]	HPLC-MS/MS	Aquasil® C18, 50 x 2.1 mm column, with a particle size of 5 µm.	P	18	Gradient, flow rate: 0.5 mL/ Min, Mobile phase A: 0.05% formic acid in H_2O [v/v], Mobile phase B: CH_3OH[v/v] with 0.05% formic acid.	This is the first LC-MS/MS method that simultaneously quantifies 17 ARV agents in small volumes [50 µL] of plasma.
2007/ [179]	IDV, NVP, M-8, IS, NFV, SQV, ATV, APV, DRV, RTV, LPV, EFV and TPV. [liquid–liquid extraction]	MS	Atlantis dC-18 3µ column [150mm×2.1mm i.d.] Guarded with C18 [4.0mm×3.0mm i.d.] pre-column.	P	20	Gradient, flow rate: 100 l/h. Mobile phase A: HPLC grade water with 0.05% formic acid, Mobile Phase B: HPLC grade CH_3CN with 0.05% formic acid].	Small volumes [50µl] of plasma are required for assessment. High extraction efficiency and low limit of quantification are the additional characteristics.
2007/ [200]	APV, IDV, ATV, RTV, LPV, SQV, NFV and M8-NFV metabolite. [SPE]	HPLC-UV [215 nm] fluorescence detector [280 and 340 nm]	Allsphere hexyl 5µ column [150mm×4.6mm i.d.].	P	60	Isocratic, flow rate : 1 ml/min, The mobile phase was a mixture of CH_3CN, methanol and 15mM sodium dihydrogen phosphate buffer pH 4.5 [35/20/45, v/v/v].	Few of the advantages of this method includes 1) isocratic separation of all presently available PIs; 2) specific and fast determination of all compounds with UV and/or fluorescence detection; 3) no interferences with co-medications or metabolites.
2007/ [163]	NVP, IDV, APV, SQV, ATV, RTV, LPV, EFV and NFV. [liquid–liquid extraction]	HPLC-UV [212nm]	S-3, 3.0mm×150mmY MC-Pack Octyl C8 column [Waters] protected by the addition of a 0.5 µm filter apparatus.	P	18	Isocratic, flow rate: 0.4 ml/min The mobile phase consisted of 52% 25 mM monobasic potassium phosphate [pH 4.90], 48% CH_3CN, vol: vol.	This assay is entirely isocratic involving only two stable solvent components. This method is short and requires only 200µl of plasma for the analysis.

(Table 1) contd….

Year/ Ref	Analytes [Extraction Method]	Separation-Detector	Column	Matrix	Run time [min]	Elution	Advantage
2007/ [66]	Triphosphate metabolites of AZT, 3TC and ABC [SPE]	MS/MS [SRM]	Aquasil C18 column [2.1mm×50mm, 3µm particle size] with a Uniguard Aqusil C18 guard column [2.1mm×10mm,3µm particle size].	PBMC	3	Gradient, flow rate: 200µl/min, Mobile phase A: H_20 with 0.1% acetic acid, Mobile phase B: CH_3OH with 0.1% acetic acid.	This assay method has the advantage of allowing the measurement of ZDV-TP, 3TC-TP and CBV-TP either together or individually in samples extracted from PBMC.
2008/ [112]	D4T, 3TC, DDI, FTC, AZT, ABC and TDF. [SPE]	MS/MS [SRM]	Atlantis® T3 column [100mm×2.1mm, 3-µm particle diameter, Waters].	P	14	Gradient, flow rate: 250µL/min, Mobile Phase A: Water with 0.05% formic acid, Mobile Phase B: CH_3OH with 0.05% formic acid.	This method is simple and short, applicable for determining 7 N[t]RTIs, requires small plasma sample volume which suits well for the quantification of zidovudine in premature infants and neonates.
2008/ [85]	TDF and FTC. [SPE]	LC-MS/MS [multiple reaction-monitoring]	Chromolith Speed Rod RP18 column [50mm×4.6mm].	P	2	Isocratic, Flow rate: 0.7 ml/min. Mobile phase consist of CH_3CN and ammonium acetate [pH 3.0, 40mM] [20: 80, v/v].	This method provides excellent specificity and linearity with a limit of quantification range between 10 ng/ml to 25 ng/ml.
2008/ [203]	NVP, IDV, M8, RGV, SQV, NFV, APV, DRV, ATV, RTV, EFV, LPV, ETV and TPV. [SPE]	HPLC-PDA [210–320 nm]	Luna 5m C18 column [150 x 4.6 mm], protected with a C18 guard column [4.0 x 3.0 mm].	P	28	Gradient, flow rate: 1 mL/min; Mobile phase A: KH_2PO_4 50 mM with orthophosphoric acid, pH: 3.23 . Mobile phase B: CH_3CN.	This assay relies on SPE coupled with a new high-sensitive Waters 2998 PDA detector, which is simple, reliable, sensitive, and not expensive. Multiple channel acquisition in the range of 210–320 nm with a PDA detector which allows peaks purity, ensuring the absence of interferences.
2009/ [368]	AZT and NVP. [liquid–liquid extraction]	UV-PDA [246 nm]	Phenomenex LUNA C18, column [250×4.6 mm i.d., 5µm].	P	10.2	Isocratic, flow rate of 0.9 mL/min Mobile phase A: H_2O, pH 3.0, adjusted with orthophosphoric acid], Mobile Phase B: CH_3CN [73: 27, v/v].	This method requires small plasma sample. It is based on the rapid protein precipitation extraction.
2009/ [178]	DRV, RTV and ETR. [Liquid – Liquid extraction]	LC–MS [Single ion monitering]	Agilent Zorbax® XDB C8 [50mm×3.0mm, 1.8µm] column with an Agilent RRLC in-line filter.	P	8	Gradient, flow rate: 0.65 to 0.75 mL/min. Mobile phase A: Water with 0.01% formic, Mobile phase B: CH_3CN with 0.01% formic acid.	This method was optimized using a sub-2µm column, and is quick, accurate, sensitive, specific, and highly reproducible. It demonstrates good linearity, precision and accuracy within a wide concentration range [0.002–2.0µg/mL].
2009/ [169]	RAL, ETR, APV, ATV, DRV, EFV, IDV, LPV, RTV, SQV and TPV. [liquid–liquid extraction]	LC-MS/MS [MRM]	Sunfire C18 100mm x 32.1 mm, 3.5 mm HPLC column.	P	8	Gradient, flow rate: 0.3 mL/min, Mobile phase A: H_2O + ammonium acetate 2 mM with formic acid 0.1%, Mobile phase B: CH_3OH with formic acid 0.1%.	This method requires a small volume of plasma for analysis [100 mL].

(Table 1) contd….

Year/ Ref	Analytes [Extraction Method]	Separation-Detector	Column	Matrix	Run time [min]	Elution	Advantage
2009/ [176]	RAL, MVC, DRV, and ETR. [SPE]	LC-MS/MS [SRM]	Waters Atlantis, 2.1mm×50mm, dC18 3µm.	P	10	Gradient , flow-rate : 0.3 ml/min, Mobile Phase A: 2mM ammonium acetate in ultrapure water containing 0.1% formic acid of pH 2.8, Mobile Phase B: MeCN 0.1% formic acid.	This method enables the simultaneous assessment of RAL–glucuronide. This method is suitable for the TDM service of new regimen combinations administered as salvage therapy to patients having experienced treatment failure, and for whom exposure, tolerance and adherence assessments are critical.
2010/ [141]	NVP, SQV, ATV, APV, DRV, RTV, LPV, EFV and ETV. [liquid–liquid extraction]	LC-MS selected ion recording [SIR]	Atlantis T3 C18 3µm column [150mm×2.1mm i.d], protected by a Security Guard with C18 [4.0mm×3.0mmi.d.] pre-column.	PS	19	Gradient, flow rate: 0.3 ml/min, Mobile phase A: HPLC grade water with 0.05% formic acid, Mobile phase B: HPLC grade CH_3CN with 0.05% formic acid.	This method utilized glass filter papers, which can be safely sent by-mail, promoting the practice of TDM.
2010/ [177]	MVC, SQV, RPV, NVP, APV, DRV, ATV, RTV, LPV and ETR. [liquid–liquid extraction]	HPLC–MS/MS [SRM]	Ascentis C18 column [3µm: 100mm×2.1mm] with a 2µm guard column, C18 Quest column saver.	P	5	Gradient, flow rate: 400µl/min, Mobile phase A: CH_3CN, Mobile phase B: H_2O with 0.05% formic acid.	This assay offers high sensitivity for all compounds [LLQ = 5–16 ng/ml] over a dynamic concentration range, and therefore has potential application for numerous pharmacokinetic analyses. It requires a relatively small volume of plasma for analysis [100µl].
2011/ [79]	IDV, NVP M-8, NFV RGV, SQV APV, DRV ATV, RTV EFV, LPV ETV and TPV. [liquid–liquid extraction]	LC-MS [SIR]	Atlantis T3 C18 3 µ column [150mm×2.1mm I.D.] Guarded with C18 [4.0mm×3.0mm I.D.] pre-column.	PBMC	28	Gradient, flow rate: 0.3 mL/min. Mobile phase A: mixture of water [0.05% formic acid], Mobile phase B: CH_3CN [0.05% formic acid].	Useful for routine determination of PIs, NNRTIs and RGV in PBMC. Low blood volume, good extraction efficiency and a low limit of quantification make this method suitable for use in clinical trials.
2012/ [43]	3TC, AZT and NVP. [SPE]	MS/MS [MRM]	HypurityadvanceC18 column[50mm x 4.6 mm,5 mm]	P	3.5	Isocratic, flow rate: 0.8 mL/min. Mobile phase consisting of CH_3CN with 0.1% formic acid [74: 26, v/v].	Simple sample preparation with adequate sensitivity and short run time are the advantage of this assay method.
2012/ [211]	3TC, LPV, RTV and AZT. [SPE]	MS/MS [MRM]	Vydac column [250 3 1 mm i.d.] packed with 3.0-µlm C18 particles.	P	16	Gradient , flow rate: 70 µL/min, Mobile phase A: CH_3CN, Mobile phase B: ultrapure water , both solvents contained 0.2% [v/v] formic acid.	Use of quadrupole-quadrupole-time-of-flight mass analyser offers advantages of obtaining very high specificity towards the selected analytes.
2013/ [42]	AZT, 3TC and NVP. [liquid–liquid extraction]	UV [270nm]	Hypersil BDS, C18 column.	P	10	Isocratic , flow rate: 0.85 ml/min, Mobile phase : mixture of buffer [0.1M ammonium acetate in 0.5% acetic acid, v/v] and methanol [40: 60, v/v].	Simple mobile phase composition, short run time, and liquid-liquid extraction are the key advantage of this assay method.

(Table 1) contd….

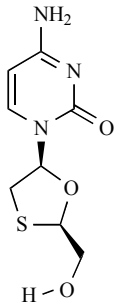
Year/ Ref	Analytes [Extraction Method]	Separation-Detector	Column	Matrix	Run time [min]	Elution	Advantage
2013/ [89]	AZT, ABC, FTC, 3TC, TDF and ribavirin. [liquid–liquid extraction]	LC-MS/MS [multiple reaction-monitoring	Synergy Polar reversed phase C18 column [150 mm × 2.0 mm ID, 4 µm particle size, protected with an inline filter.	P	11	Gradient, Flow rate: 300 µL/min , Mobile phase A: 0.1% [v/v] formic acid in water, Mobile phase B: 0.1% [v/v] formic acid in methanol.	This was the first method describing the simultaneous quantification of the anti-HCV drug ribavirin together with the anti-HIV drugs AZT, ABC, FTC, 3TC and TDF. The method is simple, sensitive, specific and reproducible and can be used in clinical practice to monitor N[t]RTI plasma concentrations in selected cases to optimize therapy.
2013/ [215]	APV, ATV, Boceprevir, DRV, EFV, EVG, ETR, IDV, LPV, MVC, NVP, RAL, RTV, SQV, TDF and TPV. [liquid–liquid extraction]	UPLC-MS/MS [MRM]	Waters AcquityHSST3 1.8µM [2.1×50mm] UPLC column[Waters Corp., Milford, MA, USA].	P	4.2	Gradient, flow rate of 0.6ml/min, Mobile phase A: H_2O with formic acid 0.1% [V/V], Mobile phase B CH_3CN with formic acid 0.1% [V/V].	Useful for simultaneous quantification of EVG, RAL, MVC, ETR, TDF as well as 10 other ARV agents. This assay required small volumes of plasma.
2014/ [147]	ATV, ATV-IS, DRV, EFV, EFV-IS, LPV, RTV and RTV-IS. [SPE]	UPLC-MS/MS [MRM]	ACE C18 column [3 x 100 mm].	PBMC	5	Gradient, flow rate: 500 µl/min, Mobile phase consisted of CH_3CN: water: formic acid [60: 40: 0.1].	The novelty of this assay lies in the sensitivity attained with the small sample volume. This is an important assay to study intracellular pharmacology of ARV drugs.
2014/ [369]	APV, ATV, DRV, IDV, LPV, NFV, RTV, SQV, TPV, FTC, 3TC, TFV, AZT, EFV and NVP. [liquid–liquid extraction	LC-MS [SRM]	Thermo Scientific Hypersil Gold PFP, 100× 3mm column.	P	16	Gradient, flow rate: 500 µl/min, Mobile phase A: H_2O with 0.1% acetic acid, Mobile phase B: CH_3CN with 0.1% acetic acid.	This method is validated, for the qualitative monitoring of 15 ARV agents in human plasma. The method requires minimal sample pre-treatment, and based on the high mass accuracy of the Exactive-MS.
2014/ [216]	IDV,SQV, NFV, APV, DRV, ATV, RTV and LPV. [liquid–solid extraction]	Parallel UPLC-MS/MS [MRM]	Kinetex XB-C18 column [100 × 2.1 mm I.D., 1.7 µ m, with Krud Katcher Ultra filter, Phenomenex, Torrance, CA, USA].	PS	4	Gradient, flow rate: 0.6 ml/min to 0.4 ml/min, Mobile phase A: 0.1% acetic acid in water, Mobile phase B: 0.1% acetic acid in CH_3CN.	Inherent advantages of this DBS based assay is the simplify sample collection. This method uses a parallel UHPLC–MS/MS platform which was developed and validated for the analysis of eight PIs in dried spots of plasma on filter paper. One sample can be injected every 4 min by alternating columns, however the assay can also run on a single column without the need of revalidation but at the expense of 50% increased analysis time.

(Table 1) contd….

Year/ Ref	Analytes [Extraction Method]	Separation-Detector	Column	Matrix	Run time [min]	Elution	Advantage
2014/ [189]	DTG and RPV. [SPE]	LC-MS/MS [MRM]	Phenomenex Luna 5 µm Phenyl-Hexyl, 2 mm × 50 mm.	P	2.5	Gradient, Mobile Phase A : 100% H_2O, 10 mM ammonium acetate, 0.1% acetic acid, Mobile Phase B: 100% CH_3CN.	This method allows accurate and precise determination of plasma concentrations of two recently licensed ARV agents including the novel INSTI DTG. The limits of quantification are consistent with trough plasma concentrations of ARV agents whereas use of deuterated-internal standard prevents matrix effect.
2016/[191]	ATV, DTG, EFV, ETV, LPV, RGV DRV RPV and TPV. [SPE]	HPLC-UV	XBridge C18 [4.6 mm×150 mm, 3.5 µm; Waters] column.	P	25	Gradient: Flow rate: 1 mL/min, Mobile phase A: Acetonitrile 50 mM, Mobile phase B; Acetate buffer at pH 4.5.	Good extraction efficiency and low limit of quantification makes this method suitable for use in clinical trials and for TDM.

Note: P: Plasma, DBS: Dried Blood spot, DPS: Dried Plasma Spot, PBMC: Peripheral blood Mononuclear Cell, SPE: Solid Phase Extraction.

effective against both HIV [type I and II] and hepatitis B virus [43, 44]. Chemically, it is an 4-amino-1-[[2R,5S]-2-[hydroxymethyl]-1,3-oxathiolan-5-yl]pyrimidin-2-one, the solubility of which in water is 70 mg/ml with a logP value of -1.4.



Lamivudine

It is a prodrug and metabolized intra-cellularly to its active 3TC triphosphate metabolite by cellular kinases enzymes [45]. Because of the polypharmacy dosage regimen, several drug-drug interactions of 3TC are noted in the literature [46]. Monitoring NRTI base therapy could be very vital for drug-drug interaction studies. Hence, several quantitative assay methods are reported in the literature for 3TC. Harker *et al.* reported the first validated assay for the quantification of 3TC in human serum and urine [47]. This method was developed for monitoring clinical investigations and getting pharmacokinetic information. Hsyu *et al.* proposed another assay for quantitative determination of 3TC on HPLC [48]. A radioimmunoassay for the quantitation of 3TC in ng/mL concentrations was developed and validated to determine intracellular phosphorylated 3TC [49]. However, the specified radio-ligand and antiserum are not economically accessible for this radioimmunoassay.

Plumb *et al.* developed an HPLC assay for the sulphoxide metabolite of 3TC in human urine and observed that it is the significant metabolite in rat and dog urine on oral administration [50]. It was then hypothesized that this sulphoxide metabolite might also be available in the urine of volunteers receiving 3TC orally/IV route. Zhou *et al.* developed a rapid assay for the quantitation of 3TC in human serum by HPLC with UV detection, which was found to be more desirable since it uses only 100 µl of serum for analysis as compared to Plumbs *et al.* method which needs 1 ml of the sample [51]. Hoetelmans *et al.* reported the assay for the determination of 3TC in human plasma, saliva, and cerebrospinal fluid in ng/mL concentration using HPLC with UV detection [52].

Until 1998, all the methods reported for 3TC were based on HPLC with UV detection. No method on HPLC with tandem mass spectrometry for the simultaneous determination of 3TC with other anti-viral drugs in human serum was reported. Kenney *et al.* in 2000, Estrela *et al.* in 2004 and Rower *et al.* in 2012 developed assays for simultaneous determination of AZT and 3TC in human serum and followed by 3TC and AZT in the seminal fluid by Pereira *et al.* on HPLC with tandem mass spectrometry [53-56]. Considering the cost of mass detector, Verma *et al.* and

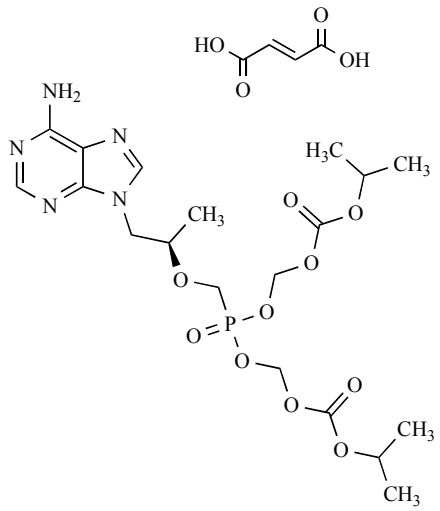
Wattananat *et al.* separately developed simultaneous assay methods for 3TC and AZT on HPLC with UV detector [57, 58]. Fan *et al.* reported validated HPLC assay for simultaneous determination of 3TC, d4T and EFV in human serum using gradient elution on HPLC with triple quadrupole mass spectrometry detection with ESI [59]. Ionisation polarity switch was used in this method as the antiviral combination including drugs from NRTI and NNRTI class that possess varying physiochemical characteristics like polarity and solubility. Aymard *et al.*, Simon *et al.*, Thiam *et al.*, Rezk *et al.*, Notari *et al.*, Tarinas *et al.* and Pynnonen *et al.* separately developed simple bio-analytical assay methods to quantitatively determine plasma concentrations of several ARV drugs including 3TC in a single blood sample [60-65].

Efficacy of NRTI depends on many variables including pharmacokinetics, metabolism, inter-individual genetic variability in enzymatic and transportation processes and intracellular kinase base activation of drugs [66]. These complexities in the interactions and variability show how difficult it is to predict the correlation between plasma drug and intracellular triphosphate concentrations. Since all the NRTIs undergo phosphorylation to form active NRTI triphosphate, it is of concern to determine the triphosphate levels in the target cells mainly inside the peripheral blood mononuclear cell [PBMC]. Few assay methods describing the quantification of phosphorylated 3TC in human PBMC's were reported separately by Solas *et al.*, Becher *et al.* and Robbins*et al.* [66-68].

Nirogi *et al.* for the first time reported a simple, sensitive, selective, precise and reproducible triple quad MS method for the simultaneous quantification of 3TC, d4T and NVP in DBS cards for rodent pharmacokinetic studies [69]. The benefits of this technique over the other revealed strategies include: [1] Use of less matrix [20 mL] for spotting clearly indicates the volume of the sample required at a time reduced appreciably; [2] sensitive technique with lower limit of quantification [3TC: 1 ng/mL, d4T: 1 ng/mL, NVP: 10 ng/mL]; [3] minimized biohazard threat; [4] less cost of sample storage and transport; [5] simple and precise evaluation technique in all matrices including whole blood, plasma and DBS.

Tenofovir Disoproxil Fumarate [TDF]

TDF is the acyclic nucleotide analogue of adenosine monophosphate approved for HIV treatment in 2004 [70]. Chemically, it is an [[2R]-1-[6-aminopurin-9-yl]propan-2-yl]oxymethylphosphonic acid, the solubility of which in water is 13.4 mg/mL at 25 ^{0}C with a logP value of -1.6. TDF is phosphorylated twice to the active diphosphate form.



Tenofovir disoproxil fumarate

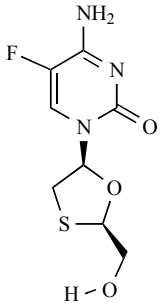
High fat meal increases the bioavailability of TDF and remains unaffected by normal meal [71]. As TDF is eliminated through the kidney and is not a substrate for CYP45, its dosage regimen needs to be modified in renal complications [72-77]. Gervasoni *et al.* showed that HIV-infected females with diminished body weight are in danger to be exposed to elevated TDF plasma trough concentrations, eventually bringing about a huge threat to produce long-term TDF complications [72]. All these factors made TDF an ideal candidate for TDM.

Several simultaneous multi-component assay methods for TDF on HPLC coupled with fluorescence, UV and mass detection have been described in the literature and discussed in Table **1**. Delahunty *et al.* developed and validated a sensitive assay for quantifying plasma TDF concentrations by using LC/MS/MS [73]. However, before Delahunty *et al.*., another research group of Sentenac *et al.* developed an assay for the determination of TDF in human plasma samples using reverse phase HPLC [RP-HPLC] technique and Rolf *et al.* developed LC assay for TDF in plasma using derivatisation with chloroacetaldehyde [74, 75]. During the same time, Jullien *et al.* in 2003 published a short communication regarding the determination of TDF in human plasma by HPLC with spectrofluorimetric detection [76]. In 2006, King

et al. developed LC-MS/MS assay for the determination of TDF-diphosphate in human PBMC's [77]. Barkil *et al.* in 2007 developed an assay for the determination of TDF in human plasma using combined UV and single MS detector for TDM [78]. Considering the importance of intracellular NRTIs concentrations, King *et al.* in 2006, D'Avolio *et al.* and Coulier *et al.* separately developed methods for the determination of TNF in human PBMC's which are detailed in Table **1** [79, 80].

Emtricitabine [FTC]

FTC is a synthetic fluoro derivative of thiacytidine with potent antiviral activity approved in 2003. Chemically it is an 4-amino-5-fluoro-1-[[2R,5S]-2-[hydroxymethyl]-1,3-oxathiolan-5-yl]pyrimidin-2-one, the solubility of which in water is 112 mg/mL with logP value of -1.4. FTC is phosphorylated to form FTC 5'-triphosphate within the cell.



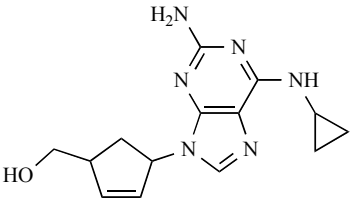
Emtricitabine

FTC, when combined with TDF, has shown together greater HIV RNA suppression compared to the combination of AZT and 3TC [81, 82]. Co-administration of FTC/TDF with antiviral drugs that eliminate through kidney by means of active tubular discharge may enhance plasma TDF or FTC concentrations and/or those of simultaneously given drugs [83]. Hence, an analytical assay method competent to determine FTC and TDF simultaneously in blood plasma was required. For this combination, in 2005, Rezk *et al.* developed an assay on HPLC after solid phase extraction process whereas Gomes *et al.* reported LC–MS/MS method for simultaneous determination of TDF and FTC in human plasma [82-85].

FTC undergoes phosphorylation to form active FTC triphosphates metabolite using cellular kinase enzymes. FTC and phosphorylated metabolite give varying pharmacokinetic results [86]. Thus, intracellular FTC nucleotide levels are monitored using assays developed and validated on HPLC–UV and LC–MS detection [87]. In 2010, Jansen *et al.* developed an analytical method for the quantification of FTC monophosphate, FTC diphosphate and FTC triphosphate in combination with TDF, TDF monophosphate and TDF diphosphate in human PBMC's [88]. For simultaneous analysis of FTC with other ARV drugs, Kromdijk *et al.* developed LC- MS/MS method for simultaneous determination of FTC with other antiviral agents like AZT, ABC, 3TC, TDF and ribavirin in human plasma [89].

V. Abacavir [ABC]

Abacavir [carbocyclic 2′-deoxyguanosine, ABC] approved in 1998, is a guanosine analogue NRTI which is used in the therapy of HIV infections [90]. Chemically, it is an [[1S,4R]-4-[2-amino-6-[cyclopropylamino]purin-9-yl]cyclopent-2-en-1-yl]methanol, the solubility of which in water is 77 mg/mL with logP value of 1.2. Only 2% of ABC is excreted unchanged in urine while the remaining 98% is metabolized by the liver [91, 92]. The half-life of ABC is about 1.5 h [93,94]. Carbovir triphosphate [CBV-TP] is an active metabolite of ABC that gives antiviral effect [95]. This CBV-TP showed extended half-life elimination [> 20 h] on once daily dosing.



Abacavir

ABC is not appreciably metabolized by cytochrome P450 [CYP] enzymes, and hence it does not show any major drug interactions with 3TC, ZDV, ethanol or methadone [96-99]. To satisfy the needs of clinical pharmacokinetic and drug-drug interaction studies, various rapid, selective, and robust analytical methods were developed for ABC concentration determination. Seshachalam *et al.* developed a validated RP-HPLC method for the analysis of ABC and related impurities over the range of 0.005–0.2 mg/mL [100]. Uslu *et al.* reported anodic voltammetry assay for the determination of ABC in pharmaceuticals and serum samples [101]. Two different assays have predicted ABC in human plasma using HPLC–UV with a sensitivity of 50 ng/mL and 20 ng/mL, respectively [102,103]. Both the techniques em-

ployed substantial plasma volume [≥ 300 µL] for test sample preparation and extended chromatographic run time [≥ 10min]. Clark *et al.* have reported the determination of ABC in maternal plasma, amniotic fluid and foetal and placental tissues by a polarity-switching LC/MS/MS technique [104]. The recovery of ABC was found in the range of 53-87%, with the limit of detection [LOD] being 1 ng/mL and retention time 5.1 min. Ravitch *et al.* reported an HPLC–UV assay for the determination of ABC and its metabolites in human urine and cerebrospinal fluid with a limit of quantitation [LOQ] ≥ 61 ng/mL and extended retention time of 35 min [105]. Similarly, ABC and its active metabolites from human PBMC's were analysed by Fung *et al.* using LC/MS/MS techniques [106]. Liquid chromatographic analysis method for the simultaneous determination of ABC and mycophenolic acid in human plasma and ABC and ZDV in rat tissues has also been reported [107,108]. Both these methods are least responsive and showed extended chromatographic run time of 20 min. Two different techniques describe the simultaneous determination of triphosphate metabolites of ABC,ZDV, 3TC, and TDF diphosphate in human PBMC by LC/MS/MS [109]. Other techniques such as LC/MS/MS, HPLC and MALDI-TOF/TOF describe simultaneous determination of ABC with commonly prescribed ARVs in human plasma [110-113].

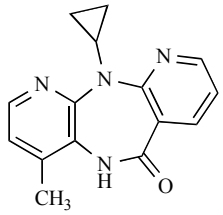
A potentially useful assay method has been illustrated by Jung *et al.* to determine ABC and 16 other ARV drugs in human plasma [50 µL] by polarity-switching LC/MS/MS [41]. In this method, they utilized a combination of liquid–liquid extraction and protein precipitation to extract all the drugs with at least 75% recovery. However, the linear dynamic range established in this assay method for ABC was very limited [1–500 ng/mL]. Hence, Saux *et al.* have estimated seven NRTIs including ABC in human plasma by LC/MS method [112]. In this assay, plasma samples were precipitated with acetonitrile to get a mean recovery of 87.9% and LOQ as 20 ng/mL for ABC with a runtime of 14 min for the separation of all seven drugs. The limit of quantitation achieved in this assay was 20 ng/mL for ABC, and the separation of all seven drugs was possible in a runtime of 14 min. Notari *et al.* used standard addition analysis to estimate ABC and five more ARVs in the plasma by MALDI-TOF/TOF technology reported LOQ as 0.01 pmol/µL for all the drugs [113].

NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR [NNRTI]

NNRTIs restrain the process of viral DNA synthesis by directly binding to the hydrophobic pocket of reverse transcriptase enzyme [25]. NNRTIs are classified as 1st generation and 2nd generation NNRTIs. 1st generation NNRTIs include Nevirapine [NVP] and Efavirenz [EFV] and 2nd generation NNRTIs are Etravirine [ETV] and Rilpivirine [RPV]. HIV-2 is naturally resistant to NNRTIs.

Nevirapine [NVP]

NVP is the first member of NNRTI class which was approved in the USA in 1996. It is a benzodiazepine analogue. Chemically, it is an 11-cyclopropyl-4-methyl-5H-dipyrido[2,3-e: 2',3'-f][1,4] diazepin-6-one, the solubility of which in water is 100 mg/l at neutral pH with logP value of 2.5.



Nevirapine

NVP is an inducer of the CYP450 enzyme system and also an autoinducer of its own metabolism. Administration of this drug for a period of 15 days results in enhancement of its oral clearance by two-folds and decrease in the half-life from 45 to 30 h [114, 115]. This helps NVP in reducing the level of other co-administered ARVs which are metabolized *via* CYP. These possible drug-drug interactions make NVP an ideal candidate for TDM.

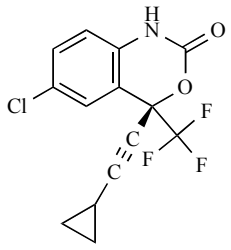
Heeswijk *et al.* first reported the assay method to determine NVP in human plasma by ion-pair RP-HPLC with UV detection [116]. Pav *et al.* of Boehringer Ingelheim Pharmaceuticals, USA presented a validated assay method which described HPLC-UV technique for the estimation of NVP in human plasma, serum, milk and cerebrospinal fluid following solid phase extraction [117]. This technique was useful in advanced clinical studies of NVP for better therapeutic potential. Few other methods have been described for NVP determination by HPLC [118-121]. Single mass detector was first used to detect the NVP plasma concentration

by Villani *et al.* who coupled liquid chromatography to a single MS [LC/MS] using ion-trap technology [122]. Laurito *et al.* developed a precise, sensitive and rapid LC-MS/MS technique for estimating NVP in human plasma using dibenzepine as the internal standard [IS] [123]. Similarly, Chi *et al.* reported another LC-MS/MS assay for the estimation of NVP in human plasma which required only 50 µl of the sample and hence advantageous for use with paediatric samples [124].

Till 2007, no analytical methods were available for the simultaneous estimation of NVP and its metabolites. Liu *et al.* in 2007 first developed and validated highly specific and sensitive LC-MS/MS assay for the quantification of NVP and its two oxidized metabolites, 2 hydroxy nevirapine and nevirapine 4-carboxylic acid [125]. Vogel *et al.* in 2010 came up with the first assay method of NVP on GCMS which has an advantage in terms of peak overlapping [126]. Kromdijk *et al.* in 2012 used DBSs for the estimation of plasma concentrations of NVP and EFV that offer patient-friendly and easy sampling alternative [127]. Other than the above-mentioned assay methods, several other simultaneous assay methods with other ARVs were developed and have been described in Table **1**.

Efavirenz [EFV]

EFV is a benzoxazin analogue approved by FDA in 1998 for the treatment of patients infected with HIV [128]. Chemically, it is an [4S]-6-chloro-4-[2-cyclopropylethynyl]-4-[trifluoromethyl]-1H-3,1-benzoxazin-2-one, the solubility of which in water is 0.093 mg/L at 25^{0}C with logP value of 4.6.



Efavirenz

The dosing of EFV is once-daily due to its long half-life. EFV is usually preferred to treat HIV patients co-infected with tuberculosis [TB]. Both the diseases are life-threatening and treatment becomes very difficult due to drug-drug interactions between EFV and rifampicin [129, 130]. Side effects of EFV are found to be associated with the EFV plasma concentration and hence, several analytical methods have been developed to estimate its plasma concentration. Various side effects are associated with high and low plasma levels of EFV particularly in HIV-TB co-infected patients for which TDM studies become necessary. EFV levels are directly correlated with optimum therapeutic output and central nervous system side effects. Therefore, TDM of EFV in clinical practice is essential for optimum therapeutic output, especially in HIV-TB co-infected patients who are under treatment with the combination of EFV and rifampicin.

On the other hand, EFV possesses high protein binding property [>99%] and thus gets penetrated into male genital tract through blood. High penetration in male genital tract makes it an important candidate to study its concentration for prophylaxis use. Hence, Avery *et al.* developed LC-MS/MS spectrometric assay for the estimation of protein free and bound EFV in human seminal and blood plasma [131]. The first HPLC assay method for EFV and its hydroxyl metabolites was reported in 1996 by Lee *et al.* of Merck research laboratory, USA for pre-clinical studies on mouse, monkey, rats and dogs [132]. Thereafter, several other HPLC methods have been published for HPLC with UV and mass detectors [133-140].

Techniques like DBS propose a patient-friendly alternative wherein blood cards are prepared by sampling blood from the patient with an easy simple finger prick. Thereafter, various simultaneous assay methods were reported in the literature for ARV determination form DBS using Mass and UV detectors [141-143].

HIV replication took place inside the cell, so ARV drugs have to enter the cells at an adequate concentration to restrain viral replication. Subsequently, studying intracellular drug concentration is a valuable tool to ascertain effective levels of ARVs in target cells mainly in virological failure regardless of efficient plasma level concentrations. Several analytical methods for intracellular quantification of EFV on HPLC with UV and mass detectors are available in recent literature [144-147]. Also as an alternative matrix other than plasma, saliva and CSF few methods are also available in the literature describing the EFV assays from DBS [148].

Proust*et al.* separated and analysed EFV and other ARV drugs by RP-HPLC on a C_{18} column with spectrophotometric detection at 260 nm

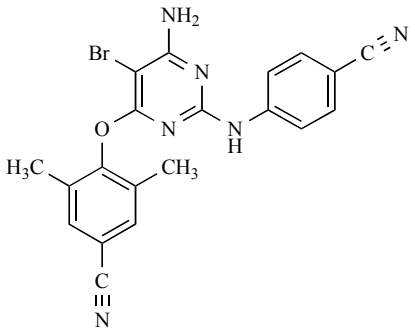
[149]. Aymard *et al.* determined twelve ARV agents in human by validated HPLC-UV assay technique where separation was carried out on C_{18} symmetry shield column and the analyte was detected using UV detector which was set at a single wavelength [260 nm] [150]. Marzolini *et al.* simultaneously determined protease inhibitors IDV, APV, SQV, RTV, NFV and the NNRTI EFV by HPLC after solid-phase extraction [151]. Similarly, various other simultaneous assays were reported on UV detectors in which EFV was determined with rifampicin and ATVs [152-164].

As the assay method with mass detectors is more specific, sensitive and fast, several HPLC-MS/MS methods are also reported for EFV with other ARVs. Volosov *et al.* developed a simple rapid assay for estimation of ARVs including EFV by LC-MS/MS with a runtime of 4.5 min [165]. Rentsch *et al.* separated and detected ARVs including EFV using LCMS and atmospheric pressure chemical ionisation. Following solid-phase extraction, the ARVs were separated within 21 min using gradient elution [166].

Heine *et al.* developed and validated an RP-HPLC assay method for the estimation of EFV. In this method, separation was carried out on a C_{18} column with gradient elution at a flow rate of 0.25 mL/min. The mobile phase comprised of acetate buffer [pH 5] and methanol and the runtime was of 10 mins. Triple quadrupole mass spectrometer operated in positive ion mode was used for fragmentation and quantification [167]. Another validated HPLC/positive ion electrospray tandem mass spectrometry assay for the simultaneous estimation of EFV, FTC, and TDF in human plasma was reported by Nirogi*et al.* [168]. Quaranta *et al.* reported a fast, precise, and sensitive LC-MS/MS assay technique for estimation of Raltegravir [RAL], etravirine [ETR], EFV and 8 other ARVs inhuman plasma effectively implemented for routine TDM [169]. Heine *et al.* also developed and validated an assay method for rapid estimation of 13 ARVs in plasma using LC-MS [170].

Etravirine [ETR]

ETR is diarylpyrimidine analogue approved by FDA in 2008 [171]. Chemically, it is an 4-[6-amino-5-bromo-2-[4-cyanoanilino]pyrimidin-4-yl]oxy-3,5-dimethylbenzonitrile, the solubility of which in water is 0.07 mg / ml at 25 °C with logP value of 4.5.



Etravirine

It is a second generation NNRTI possessing wide range of activities that are attributed to its potential by binding to reverse transcriptase in multiple ways [171]. It possesses an extended half-life [30-40 h] and its metabolism is catalysed by CYP3A4, CYP2C9, and CYP2C19 isoenzymes [172].

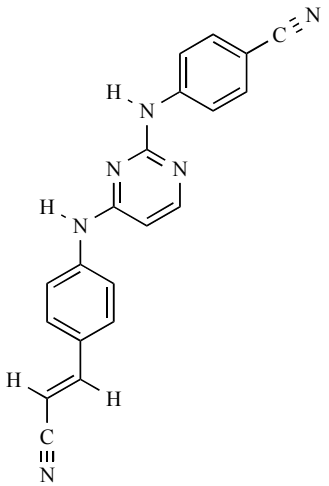
With ETR being the substrate for CYP3A4, the possibility of drug interactions with PI is very high and hence it is very important to monitor the plasma levels of ETR. Various LC–MS/MS techniques have been reported for the assay of ETR in human plasma. Heine *et al.* in 2009 developed an LC–MS/MS assay to estimate ETR in human plasma, dry blood spots, and PBMC by including ETR in their existing method and latter validating it [173, 174]. Abobo *et al.* reported LC–MS/MS estimation assay of ETR in rat plasma [175]. Several LC–MS/MS assay techniques are also available for the simultaneous estimation of ETR with other NNRTIs and PIs in human plasma [176-178].

A method developed by D'Avolio *et al.* is of wide interest because of the use of DBS on the glass filter [141]. Here, authors have developed and validated a new analytical technique, altering their previous HPLC–MS chromatographic run, for simultaneous estimation of nine different ARV drugs like PIs [SQV, ATV, APV, DRV, LPV, RTV] and NNRTIs [ETR, EFV,NVP] in dried plasma spot [179]. Also, short and long-term stability of drugs at varying storage conditions [room temperature, 4°C and −20°C] was reported. In this assay, 100 µL of plasma was used for investigation which was spread over a glass paper filter and drugs were extracted using tertbutylmethylether. After drying, the extract was reconstituted and chromatic separation was achieved on RP- C_{18} columns and analyte were identified and quantified using quadruple mass detector. As the assay was validated at the concentration levels more fre-

quently encountered in clinical practice, it is applied for routine TDM. The most significant finding of this assay is the long stability of dried plasma spot that allows easy transfer of samples at room temperature for TDM and pharmacokinetic studies.

Rilpivirine [RPV]

RPV, a diarylpyrimidine analogue, is a second-generation NNRTI approved in 2011. Chemically it is an 4-[[4-[4-[[E]-2-cyanoethenyl]-2,6-dimethylanilino]pyrimidin-2-yl]amino]benzonitrile, the solubility of which in water is <0.1mg/ml with logP value of 4.86.



Rilpivirine

It prevents the replication of HIV by directly binding to a hydrophobic pocket of reverse transcriptase and thereby inhibits enzyme activity [180]. RPV possesses high protein binding property [>99%] and hence has a long half-life and given in once-daily dosing [181].

Various studies have evaluated the pharmacokinetic correlation between RPV and other ARVs. An important finding suggests that the increased exposure of RPV when co-administered, RPV [150 mg]with DRV/RTV [800/100 mg] in once-daily dosing in 16 HIV-negative volunteers was due to CYP 3A4 inhibition [182]. Co-administration of RPV [150 mg] and rifampicin [600 mg] in once-daily dosing in 16 HIV-negative volunteers for 7 days was found to diminish AUC_{24h}, C_{max}, and C_{min} by 80%, 69%, and 89%, respectively for RPV [183]. Drug interactions with several co-administered drugs have been reported in the literature and hence, several analytical assay methods have been developed [184-192]. Studies revealed that RPV [150 mg] when administered with ketoconazole [an antifungal agent known to inhibit CYP 3A4 enzyme; 400 mg] in once-daily dosing in 16 HIV-negative volunteers resulted in enhancing AUC_{24h}, C_{max}, and C_{min} by 49%, 30%, and 76%, respectively, for RPV [193]. Looking at all these drug-drug interactions, TDM is recommended for RPV.

Until 2011, a single analytical assay method [multicomponent analysis] was reported for the estimation of RPV in human plasma by LC/MS [177]. However, the method was not precise for the determination of RPV and was multifaceted, with gradient elution, mobile phase and extended chromatographic run time. Also, the technique was not adequately sensitive for pharmacokinetic or bioequivalence studies. Hence, Heeswijk *et al.* in 2006, Burugula *et al.* and Shibata *et al.* in 2013 developed a single component assay method on LC-MS [184-186]. In the year 2013, Aouri *et al.* reported a validated assay on LC-MS/MS for the multicomponent analysis of RPV and EVG in plasma, over clinical concentrations ranges, by using corresponding isotopically stable-labelled internal standard [187]. Gupta *et al.* and Grégoire *et al.* in 2014 presented separately LC-MS/MS assay methods for routine measurement of RPV in plasma samples [188, 189]. Gupta *et al.* assay technique utilizes small plasma volume [50 µL] for processing and also employed isotopically stable-labelled internal standard whereas assay developed by Grégoire *et al.* also employed deuterated-internal standard but also allows simultaneous determination of DTG and RPV in plasma in a single analysis. Cozzi *et al.* and Charbe *et al.* in 2016 also developed and validated the HPLC-UV method for the simultaneous estimation of most commonly prescribed ARV drugs [190, 191].

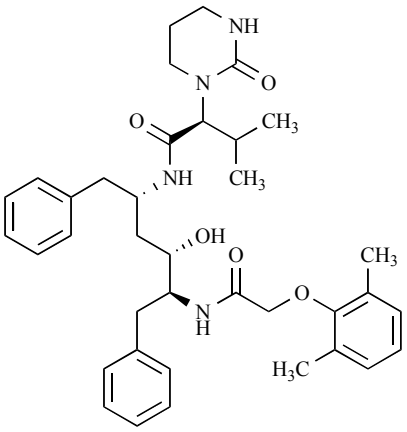
Protease Inhibitors (PIs)

PIs are the class of the antiretroviral drugs which inhibit the protease, an enzyme essential for the formation of mature virions after budding out from the host cell membrane. Protease inhibitors prevent the essential steps of virion maturation *i.e* cleavage of gag and gag/pol precursor proteins [192]. Virions produced in which the gag/pol precursor proteins are not cleaved hence they are faulty and mostly non-infectious. Presently, the most commonly recommended PIs are LPV, IDV, NFV, APV and RTV, DRV and ATV. As resis-

tance to some of the widely prescribed PIs is high, 2nd generation PIs have been developed that are effective against otherwise resistant HIV variants.

Lopinavir [LPV]

LPV approved in 2000 is a peptidomimetic HIV PI used in combination with RTV. Chemically, it is an [2S]-N-[[2S,4S,5S]-5-[[2-[2,6-dimethylphenoxy]acetyl]amino]-4-hydroxy-1,6-diphenylhexan-2-yl]-3-methyl-2-[2-oxo-1,3-diazinan-1-yl]butanamide which is practically insoluble in water and freely soluble in methanol and ethanol with logP value of 5.94.



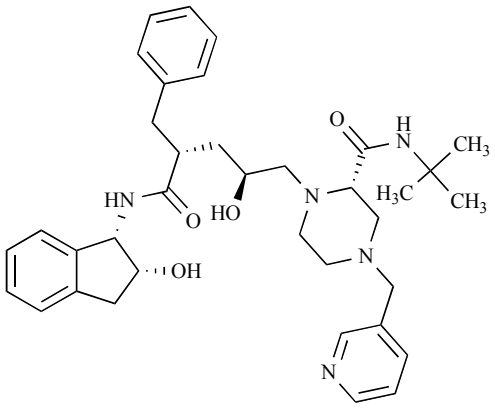
Lopinavir

LPV is exclusively metabolised by CYP3A [193]. LVP monotherapy does not yield effective concentration, however when boosted with RTV, plasma levels found to exceed the serum protein binding-adjusted 50% inhibitory concentration [IC50] for wild-type HIV type 1 by at least 50-fold [194]. The LPV-RTV combinations are available in capsules dosage formulation with a dose of 133 mg of LPV and 33 mg of RTV per capsule. LPV is the CYP3A4 substrate whereas RTV inhibits CYP3A resulting in sustained and elevated plasma LPV levels. LPV is metabolized by CYP3A, hence interaction with anti-tuberculosis drugs like rifampin is more likely and hence LPV is considered as a perfect compound for TDM [195].

The first chromatographic simultaneous assay method for LPV was reported by Leibenguth *et al.* [196]. This RP-HPLC assay allows simultaneous quantification of six PIs [IDV, RTV, SQV, NFV, APV, and LPV] using carbamazepine as an internal standard. Thereafter, several simultaneous assays on HPLC with UV PDA, Diode array and MS detectors have been made available in the literature [197-216]. Although not much in use, Azoulay *et al.* in 2004 reported an immunoassay for LPV [217]. PIs exert their action inside cells where they are present in very minute quantity. Determination of the intracellular levels of drugs is an analytical challenge; overcoming this limitation, few analytical methods are available in the literature to determine the intracellular concentrations of these drugs. For routine TDM, plasma and saliva are the common biological matrixes, however, for various PIs including LPV and NNRTIs, multicomponent analytical methods based on DBS have been reported in the literature. TDM based on DBS sampling technique is not only advantageous in reducing the infection risk of HIV/AIDS samples but also allows regular mailing of patient samples.

Indinavir [IDV]

IDV approved in 1996 is a synthetic hydroxyaminopentane amide agent that selectively inhibits the protease of both HIV 1 and 2. Chemically it is an [2S]-1-[[2S,4R]-4-benzyl-2-hydroxy-5-[[[1S,2R]-2-hydroxy-2,3-dihydro-1H-inden-1-yl] amino]-5-oxopentyl]-N-tert-butyl-4-[pyridin-3-ylmethyl]piperazine-2-carboxamide which is highly soluble in water [0.015 mg/ml] and ethanol with logP value of 2.9.



Indinavir

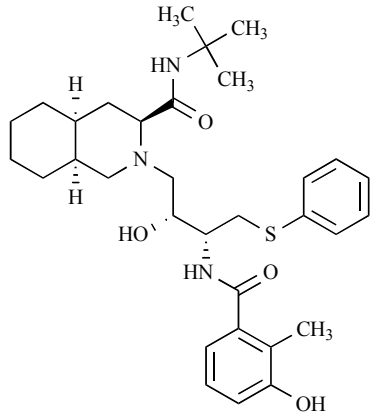
HIV PIs, being the substrates and inhibitors of CYP3A, demonstrate high probability of pharmacokinetic interactions with co-administered drugs [218, 219]. Concurrent use of PIs with CYP3A inducers like phenytoin ritonavir rifampin, or inhibitors like telithromycin, nefazodone, itraconazole or ketoconazole may increase the probability of diminished efficacy or serious drug-related side effects and toxicity [219]. All these possible drug-drug interactions make IDV a good target for TDM.

Several HPLC assays, using UV detection, have been reported for the determination of IDV in se-

rum/plasma either alone or together with its metabolites [220-227]. With the aim to develop an assay method to analyse IDV in serum, urine, and cerebrospinal fluid, *Svensson et al* reported rapid and simple determination using HPLC [220]. Due to the higher therapeutic range, HPLC with UV or PDA detector is considered sensitive enough to quantify IDV in plasma. However, *Woolf et al* in 1997 quantified IDV and deuterated IDV in human plasma using triple quadrapole Mass detector [228]. Method published in 2001 by *Armbruster et al* explains the estimation of IDV and SQV within the cells using an *in vitro* system of human umbilical venous endothelial cells [229]. Various other simultaneous assay methods as detailed in Table **1** have been developed for the estimation of IDV either alone or in combination in human blood plasma samples.

Nelfinavir [NFV]

Approved in 1997, NFV is a synthetic antiviral agent that selectively inhibits HIV protease enzyme [230]. Chemically it is an [3S,4aS,8aS]-N-tert-butyl-2-[[2R,3R]-2-hydroxy-3-[[3-hydroxy-2-methylbenzoyl]amino]-4-phenylsulfanylbutyl]-3,4,4a,5,6,7,8,8a-octahydro-1H-isoquinoline-3-carboxamide which is slightly soluble in water and highly soluble in methanol, ethanol, and acetonitrile with logP value of 6.

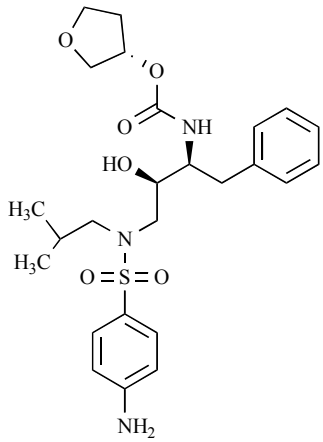


Nelfinavir

NFV is primarily metabolised by CYP3A4 [more than 50%], 2D6, 2C19, and 2C9 [231]. The first assay method on HPLC with UV detector reported by Wu *et al.* did not include M8 metabolite [232]. Therefore, Lamotte *et al.* in 1999, Janoly *et al.* in 2002, Hirabayashi *et al.* in 2006 and Justesen *et al.* in 2013 determined NFV along with its active metabolite M8 in human plasma using HPLC [233, 234]. In 2005, not much in use, automated microparticle agglutination immunoassay for the TDM of NFV was reported by Lawrence *et al.* [235]. Uglietti *et al.* 2007 developed an ELISA method which is an immunoenzymatic test based on the use of polyclonal rabbit antibodies [236]. In this study, the authors also evaluated the correlation between HPLC and TDM-ELISA® results of NFV and M8 plasma concentrations in blood samples of HIV-positive patients treated with NFV-containing combination therapy. Several other simultaneous assays of NFV with PI, NNRTI, II on HPLC with MASS, UV and PDA in plasma and DBS has been developed and discussed in Table **1**.

Amprenavir [APV]

APV approved in 1999 is a synthetic derivative of hydroxyethylaminesulfonamide that selectively inhibits HIV protease [237]. Chemically, it is an [[3S]-oxolan-3-yl] N-[[2S,3R]-4-[[4-aminophenyl] sulfonyl-[2-methylpropyl]amino]-3-hydroxy-1-phenylbutan-2-yl]carbamate, the solubility of which in water is 40 mg/l at 25 ^{0}C with logP value of 2.2.



Amprenavir

APV is metabolised primarily by the isoenzyme CYP3A4 which is also the major substrate for various inducers and inhibitors. When prescribed as a mono-therapy, APV is not able to achieve efficient plasma levels without side effects, however as proved in a preclinical study in rats, APV when boosted with RTV, several fold growth in the area under the curve from 0 to 8 h [AUC0–8] of APV was observed. Similarly, when APV was co-administered in humans with CYP3A4 inhibitors [*e.g.* ketoconazole], the surge in the AUC of APV was observed [238].

At the suggested dose, APV is effective in reducing the seminal and blood plasma load of HIV but studies revealed that it exhibits limited access

to CNS and into the male genital tract [239]. A better study on the ARV drug distribution into male genital tract could help formulate the effective drug and dose regime not only to reduce the viral load but also to reduce the sexual transmission [240, 241]. Therefore, Pereira *et al.* developed a method for the determination of APV in human seminal plasma using HPLC–tandem MS whereas Sparidans *et al.* developed a sensitive LC assay for APV in human plasma, cerebrospinal fluid and semen [242, 243].

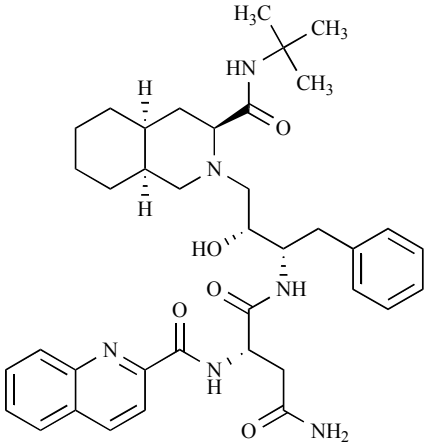
In the year 2007, to quantify the drugs like LPV and RTV in plasma and PBMC's, an immune assay based ELISA method was developed [217]. In 2007, Bastiani *et al.* developed and evaluated an immunoassay for the monitoring of APV [244]. In this assay, a diazo-conjugate of APV and bovine serum albumin was utilized to evoke polyclonal anti-APV rabbit sera, which were then used in a competitive ELISA assay carried out in microplates coated with the anti-APV polyclonal antibodies *via* a capture anti-rabbit antibody. The competition between free APV and a conjugate of the drug with horseradish peroxidase allowed the development of a fast and easy to use ELISA [244].

Two individual methods for assaying APV in human plasma, cerebrospinal fluid semen and in human seminal plasma using LC-MS/MS are available [242, 243]. Several other simultaneous HPLC assays with UV detection, photodiode, PDA, and MS have been reported in the literature [244-250]. Marzolini *et al.* developed and validated a HPLC method for the simultaneous quantification of IDV, APV, SQV,RTV, NFV, and EFV from the plasma of HIV infected patient with UV detector set at 201 nm [151]. Various simultaneous methods have been reported for APV with other ARVs, which mostly use UV or MS detection system. Proust *et al.* separated six ARV agent by reversed-phase LC on a C_{18} column with spectrophotometric detection at 260 nm [156].

Monitoring drug concentrations inside the cell at the target proteins is used to guarantee the therapeutically effective levels is important particularly in case of virological failure regardless of effective drug concentrations. Hence, few simultaneous assay methods for ARV quantification from PBMC have been reported in the literature for example, Elens *et al.* proposed a UPLC–diode array detection assay for the simultaneous quantification of the PIs like APV, ATV, IDV, LPV, NFV, RTV, SQV, and TPV [TPV], and NNRTIs like EFV and NVP [79, 144, 145, 204]. Similarly, Djerada *et al.* useed UPLC-MS/MS, to concurrently quantify EVG, RAL, MVC, etravirine, TDF and 10 other ARV agents [APV, ATV, darunavir, EFV, IDV, LPV, NVP, RTV, SQV, TPV] and boceprevir [215].

Saquinavir [SQV]

SQV approved in 1995 is a peptidomimetic inhibitor of HIV protease enzyme. Chemically it is an [2S]-N-[[2S,3R]-4-[[3S,4aS,8aS]-3-[tert-butylcarbamoyl]-3,4,4a,5,6,7,8,8a-octahydro-1H-isoquinolin-2-yl]-3-hydroxy-1-phenylbutan-2-yl]-2-[quinoline-2-carbonylamino]butanediamide which is insoluble in water but soluble in DMSO with logP value of 3.8.



Saquinavir

SQV is a decent substrate for CYP3A isozymes and is utilized as part of a combination with RTV for the treatment of HIV. SQV is also the good inhibitors and substrates for p-glycoprotein [250-252]. As bioavailability of SQV is not sufficient for a therapeutic response without side effects, it is generally boosted by RTV at a lower dose. On the drug interaction front, any drug, inducing CYP3A4 activity can stimulate SQV metabolism and could reduce its plasma levels [253, 254]. For example, Rifampin is the potent inducer of CYP3A4 and hence could reduce AUC of SQV by around 80 %. Rifabutin, which is also a known cyp3A4 inducer, could exert a similar effect and could reduce AUC of SQV by 40 %. Other CYP3A4 inducers like phenobarbital, phenytoin, dexamethasone, and carbamazepine were also found to reduce the SQV concentration in patient

plasma [255]. Contrary to this, drugs like ketoconazole and Grapefruit juice, which are known inhibitors of the CYP450 may increase the AUC of SQV [256]. Infact, Grapefruit juice was found to increase the AUC of SQV by around 220% [257]. All these drug-drug interactions make SQV an ideal candidate for TDM.

Various analytical methods were developed for clinical monitoring for SQV. Knebel *et al.* developed and validated HPLC-MS assay method for the determination of SQV in human plasma. Use of MS detector enables Knebel *et al.* to quantify SQV in the linear range from 0.4 to 200 ng/ml [258]. SQV was then later determined by Hoetelmans *et al.* in human plasma, saliva and cerebrospinal fluid by ion-pair HPLC-UV and by Ha *et al.* in plasma by HPLC-UV [52, 259]. Frappier *et al.* developed a simultaneous quantification method for the determination of RTV and SQV in human serum by HPLC [260]. Armbruster *et al.* performed a series of experiments on HPLC-UV to determine intracellular levels of IDV and SQV, using an *in vitro* model of human umbilical venous endothelial cells. In this experiment, Armbruster *et al.* also evaluated the influence of these agents on the expression of adhesion molecules on these cells [229].

Strategies depicted here for the estimation of SQV involved tiresome and lengthy sample preparation like liquid–liquid or solid–liquid extraction. Such methods not only require a higher time of extraction but are also expensive as compared to the other available methods. Considering this, Campanero *et al.* developed a bio-analytical assay using HPLC to quantify SQV plasma concentrations in a short period of time [261].

The oral bioavailability of SQV is around 4% after a meal and even lower when taken in the fasted state. The low oral bioavailability of SQV is attributed to fast metabolism, rapid first-pass clearance and poor transport across the intestinal mucosa [262].

To study the effects of all the parameters which affect the oral bioavailability of SQV, Ucpinar *et al.* developed and validated an HPLC-UV assay method for the in-vitro quantification of SQV using Caco-2 cell monolayer as a model [263]. Plasma is considered for routine TDM of PI and NNRTIs whereas saliva is considered as the alternative body fluid for determining the plasma concentration.

For the first time, Koal *et al.* developed an LC-MS/MS method for simultaneous multicomponent analysis of PI's and NNRTI's including SQV in dried blood [142].

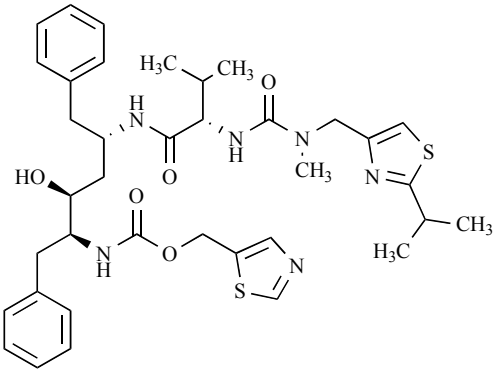
As the bioavailability of SQV is very low, two HPLC–UV assays were developed with a detection limit close to 1 ng/mL plasma. Few studies have shown simultaneous quantification of SQV with other PIs with higher limits of quantification [264, 265]. In addition, SQV was also simultaneously assayed with the NNRTI's like delavirdine and EFV [149, 151]. Other important methods like immunoassay and LC–MS/MS for the simultaneous quantification of SQV have been developed [266]. The HPLC–UV assay [limit of quantification 1 ng/mL] was compared with a radioimmunoassay [LOQ 0.5–1.0 ng/mL] and validated against an LC–MS–MS assay [LOQ 0.4 ng/mL] [266, 267]. The LC–MS/MS assay used solid-phase extraction [SPE] for sample cleaning and separation was carried out on a short LC column in the selected reaction monitoring mode [SRM]. The assay was validated and is useful to determine the SQV in range of 0.4–200 ng/mL. An isotopically labelled form of SQV was used as the internal standard to normalize the possible sample loss during extraction. Another highly useful assay for low level determination of SQV in plasma, urine, and saliva samples using LC-MS/MS was reported in 1997 and 2003 [259, 268].

Ritonavir [RTV]

RTV, a peptidomimetic agent useful in inhibiting both HIV-1 and HIV-2 proteases, was approved as monotherapy, or for co-administration in patients with advanced HIV infection [269]. Chemically, it is an 1,3-thiazol-5-ylmethyl N-[[2S,3S,5S]-3-hydroxy-5-[[[2S]-3-methyl-2-[[methyl-[[2-propan-2-yl-1,3-thiazol-4-yl]methyl]carbamoyl] amino]butanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate which is insoluble in water but freely soluble in methanol and ethanol and isopropanol with logP value of 3.9.

Although, RTV was developed as a PI, it was later found to be a very potent inhibitor of CYP3A4 and CYP2D6 hence nowadays, it is used as a pharmacokinetic booster to boost the plasma levels of other PIs mostly metabolised by these isoenzymes [270].

A bioassay method for the quantification of RTV in human samples was first briefly described



Ritonavir

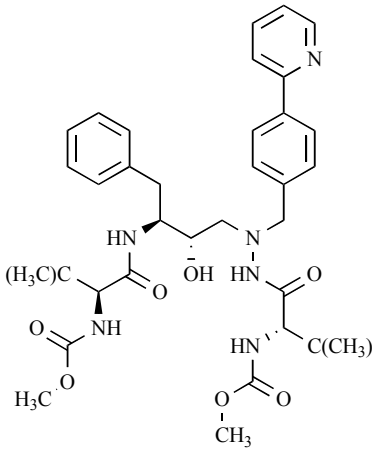
by Hsu *et al.* [271]. Later, an assay method was reported by Marsh *et al.* of Abbott Laboratories to determine RTV concentration in biological samples using HPLC, whereas Hoetelmans *et al.* published an HPLC method for the determination of RTV in human plasma, cerebrospinal fluid, and saliva [272, 273]. Granda *et al.* modified a previously described method and applied it for the determination of RTV concentrations in human plasma and in mouse serum, liver, and brain [272, 274]. Penzak *et al.* in 2001, developed a rapid HPLC assay for the quantitation of RTV in human plasma [275].

Intracellular concentrations of ARV drugs are not only influenced by their physical and chemical properties but patient genetic also plays important role in the proper therapeutic outcome. The assay's quantifying PIs and NNRTIs levels at the site of their pharmacological action are an essential tool for the on-going therapeutic drug monitoring aimed at averting ARV therapy failure and toxicity. Few such simultaneous assay methods have been reported in the literature which determines the intracellular concentration of ARVs along with RTV [147, 276].

Akeb *et al.* in 2002 described the first one-step competitive ELISA for RTV quantification using RTV–acetylcholinesterase as a tracer [277]. This method demonstrated 15 times lower threshold sensitivity than HPLC–UV and permitted sensitive routine determination of RTV in methanol-extracted plasma and from HIV patients receiving multidrug therapy. Frappier *et al.* reported simultaneous quantification method for RTV and SQV. This method involved extraction of RTV and SQV from serum on solid-phase extraction using C_{18} cartridges followed by HPLC separation on C8 column with a UV detector set at 240 nm [260]. Several other sensitive simultaneous assay methods were reported with UV and MS detectors for RTV [202, 205, 209, 211, 264, 265, 278].

Atazanavir [ATV]

Approved in 2003, ATV is an aza-dipeptide analogue with a bis-aryl substituent on the [hydroxethyl]hydrazine moiety useful against wild and mutant forms of HIV protease [279]. Chemically it is an methyl N-[[2S]-1-[2-[[2S,3S]-2-hydroxy-3-[[[2S]-2-[methoxycarbonylamino]-3,3-dimethylbutanoyl]amino]-4-phenylbutyl]-2-[[4-pyridin-2-ylphenyl]methyl]hydrazinyl]-3,3-dimethyl-1-oxobutan-2-yl]carbamate which is slightly soluble in water but freely soluble in methanol and ethanol with logP value of 4.5.



Atazanavir

ATV is not only a substrate and inhibitor of CYP P450 3A but is also an inhibitor and inducer of transporter protein like P-glycoprotein [280]. Hence to avoid the drug interactions, ATV has to be used with care in patients taking strong and moderate CYP3A4 substrates, inhibitors and inducers. Because of the possible drug-drug interaction and common disposition enzymes involved, ATV is considered as the major TDM target in ART. The first ATV plasma assay was reported by Schuster *et al.* whereas Jemal *et al.* of Bristol-Myers Squibb Pharmaceutical Research Institute, USA developed an assay for ATV quantification in PBMC [281, 282]. Colombo *et al.*, reported a simple ATV assay for quantification in human plasma using offline solid-phase extraction followed by separation using HPLC, which was coupled with UV-diode array detector [283]. Sparidans *et al.* developed an isocratic LC-UV method dedicated to ATV with a sample pre-treatment [284]. Cateau *et al.* determined ATV levels in human plasma using solid-phase extraction and

HPLC [285]. Loregian *et al.* in 2006, Cattaneo *et al.* in 2008 and Muller *et al.* in 2010 developed a simple HPLC-UV method for ATV determination in human plasma [286-288].

As mentioned previously, serum and especially plasma are the preferred matrices whereas saliva is considered as an alternative body fluid for TDM [289]. TDM using DBS samples is advantageous as compared to plasma because it reduces the risk of infection to a minimum and also enables regular mailing of blood samples and easy handling in pathology. Considering this, Koal *et al.* in 2005, D'Avolio *et al.* in 2010 and recently Watanabe *et al.* in 2014 quantify ARV drugs including ATV in DBS samples using LC-MS/MS [141, 142, 216].

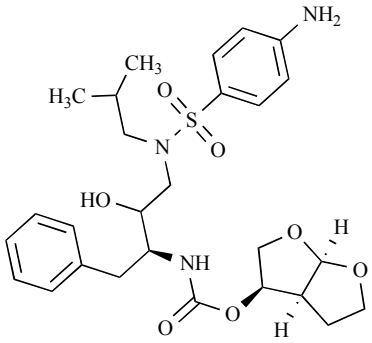
Fayet *et al.* developed a method to determine unbound ARV drug concentrations. This method is useful for the assessment of inter-individual variability in ARV drug-free fractions and their correlation with albumin and glycoprotein levels in patients with HIV undergoing TDM [290].

Intracellular levels of ARV drugs are not only influenced by their physical and chemical properties but patient genetic factors are actively involved in maintaining the drug levels inside the cells [291, 292]. Therefore, assays measuring ARV levels at the site of their pharmacological action appear to be an essential tool for accurate TDM. Such assay methods describing the mononuclear blood cell concentration were first developed by Colombo *et al.* in 2005 and later by D'Avolio *et al.* in 2011 and Podany *et al.* in 2014 [79, 145, 147].

Other than the above-mentioned methods for ATV, several other simultaneous assay methods on HPLC with UV Diode array and PDA detectors are available in the literature [200-203, 293]. MS detectors are considered to be highly sensitive and LC-MS assays are fast and suitable for high-throughput TDM. Hence, several simultaneous chromatography assay methods have been developed for ARTs including ATV with MS detection systems [169, 170, 177, 179, 246, 249, 294].

Darunavir [DRV]

DRV was designed to overcome high genetic barrier to the development of resistance [295]. Chemically, it is an [[3aS,4R,6aR]-2,3,3a,4,5,6a-hexahydrofuro[2,3-b]furan-4-yl] N-[[2S,3R]-4-[[4-aminophenyl]sulfonyl-[2-methylpropyl]amino]-3-hydroxy-1-phenylbutan-2-yl]carbamate, the solubility of which in water is 0.15 mg/mL at 20 °C but is freely soluble in methanol with logP value of 1.8.



Darunavir

Several studies have shown that DRV is useful against wild-type HIV-1 and PI-resistant viruses [295, 296]. Co-administration of DRV is with low-dose RTV [DRV/r]; the 600/100 mg twice daily dose has now been approved in many countries for treatment-experienced adult patients, such as those with HIV-1 strains resistant to more than one PI whereas 800/100 mg qd dose is now recommended for naive patients [297]. DRV has an intermediate-to-high absorptive permeability in Caco-2 monolayers, indicating that DRV exhibits sufficient membrane permeability to obtain adequate intestinal absorption. DRV was found to bind 95% to α1-acid glycoprotein and was also shown to bind to albumin, but to a lesser extent than to α1- acid glycoprotein [298].

Results from an in-vitro study of human liver microsomes indicate that DRV primarily undergoes oxidative metabolism and is extensively metabolised by CYP450 enzymes, mainly CYP3A [297, 298]. DRV is metabolised by and also inhibits CYP3A. However, in combination with RTV, a more potent inhibitor of CYP3A, there is a marked increase in DRV plasma concentrations. In a study of HIV-negative healthy volunteers, the absolute bioavailability of DRV [600 mg once daily] was increased to 82% in the presence of RTV [100 mg twice daily] compared with 37% when DRV was administered alone [299].

The $t_{1/2}$ [terminal elimination half-life] of DRV, when combined with RTV [1,200/100 mg once daily] in a dose-ranging study of healthy volunteers was found to be 15 h [4]. Following intravenous administration of DRV [150 mg; single 1 h infusion] alone or in the presence of low-dose RTV [100 mg twice daily] in HIV-negative

healthy volunteers, the mean systemic clearance was 32.8 l/h and 5.9 l/h, respectively [299].

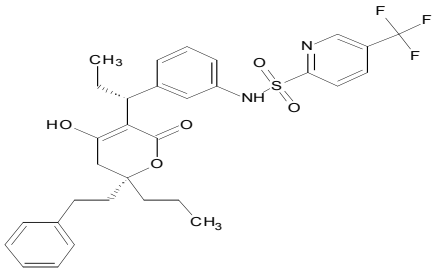
Various simultaneous and mono-component assays have been published to quantify DRV in different biological matrices. Other ARV drugs assayed with DRV include other PIs, NRTI and NNRTI, integrase inhibitor, RAL and an entry inhibitor, MVC [299-302]. DRV was assayed in cerebrospinal fluid and human plasma with a limit of quantitation ≥5.0 ng/mL. DRV and RTV were assayed in human plasma using tandem mass spectrophotometer with a sensitivity of 10 ng/mL [300-302].

Another important simultaneous assay was described to quantify RTV, ETR,ABC and DRV [178]. Simple liquid-liquid extraction in tert-butyl methyl ether was employed to extract DRV from 100µL of plasma. The separation was carried out in 7 mins on a column having 2µm particle under gradient elution. This method was further validated for the plasma concentration range of 2–2000 ng/mL [178]. To study drug-drug interactions, Fayet *et al.* developed a simultaneous assay for DRV and three new ARV agents namely RAL, MVC, and ETR [176]. Few other simultaneous methods for the determination of DRV along with six or more ARVs in DBS, human plasma and PBMCs [intracellular concentration] by LC–MS/MS were also published [79, 141, 174, 177, 249].

Considering the convenience of DBS, Heine *et al.* developed a simultaneous assay to quantify four PIs and two NNRTIs from DBS [174]. Few other more sensitive simultaneous assay methods were developed to quantify DRV and most commonly prescribed ARV's from blood plasma samples with ≥15 ng/mL LOQ [141, 177, 249]. To quantify DRV and other ARV's at the site of action, Heine *et al.* simultaneously assayed nine PIs, NFV metabolite and two NNRTs in lysate of PBMC's. [173] Another highly sensitive simultaneous HPLC–MS assay for various ARV's including DRV in PBMC of HIV infected patients was developed [79,147]. The LOQ of all the ARV ranged from 0.1 to 32 ng/mL. The separation was carried out for 15 mins on Atlantis T3 C_{18} column. Due to the large number of samples, robust assays with short run time are in demand, hence, Gupta *et al.* published short and robust UPLC–MS/MS assay for DRV determination in human plasma samples [303]. On the same line, Djerada *et al.* developed and validated a simple robust method for TDM of 15 ARVs on UPLC-MS/MS [215]. Very recently, Watanabe *et al.* also developed another sensitive UPLC–Mass assay for the quantification of HIV PIs from DBS sample [216].

Tipranavir [TPV]

TPV approved in 2005 is a non-peptidomimetic agent that inhibits wild and drug resistant forms of HIV protease [304]. Chemically, it is an N-[3-[[1R]-1-[[2R]-4-hydroxy-6-oxo-2-[2-phenylethyl]-2-propyl-3H-pyran-5-yl]propyl]phenyl]-5-[trifluoromethyl]pyridine-2-sulfonamide, the solubility of which is insoluble in water but soluble in dehydrated alcohol with logP value of 6.9.



Tipranavir

TPV is typically a substrate of CYP3A4 and hence for effective plasma concentration in plasma, TPV 500 mg is commonly boosted by 200 mg of RTV [305]. The combination of TPV with RTV has inducing and inhibiting effects on several CYP isoenzymes [306]. As a result, drug interactions are more common with these combinations which essentially make TPV an ideal candidate for TDM.

To study the TPV drug interactions and TDM, Crommentuyn *et al.* were the first group who published a simultaneous assay to quantify TPV with ATV using tandem MS detector coupled with LC [294]. Typically, TPV clinically significant plasma concentration ranges between 1 and 50 mg per millilitre and hence it does not need very highly sensitive assay. Therefore, Keil *et al.* , Giraud *et al.*, Dailly *et al.* and Colombo *et al.* separately in 2006 and D'Avolio *et al.* in 2007 reported HPLC-UV assay for TPV [307-311]. In 2008, Langmann *et al.* developed another HPLC-UV method for the determination of TPV in plasma [312]. Several other simultaneous quantification assay methods in various biological matrixes on HPLC coupled with mass, UV, fluorescence detectors have been mentioned in various literature and details of the same are given in Table **1**.

ENTRY INHIBITORS AND FUSION INHIBITORS

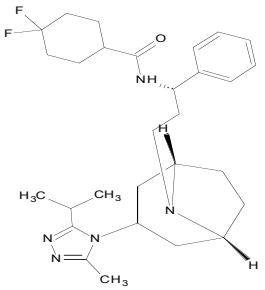
Entry inhibitors [EI's] work by preventing the HIV entry in healthy CD4 cells [313]. Entry inhibitors work by blocking CCR5 or CXCR4 receptor proteins of CD4 cells or GP120 or GP41 proteins on the HIV surface [314]. In order to get entry into the CD4 cells, GP120 or GP41 proteins present on the HIV surface must dock with the CCR5 receptor proteins present on the CD4 cells. EI's essentially work by preventing this to occur. Some EI's block gp120 or gp41 proteins whereas, some work by blocking CCR5 or CXCR4 receptors on a CD4 cell's surface [315]. Successful blocking of these entry assisted proteins makes binding of HIV with the surface of CD4 cells difficult and ultimately prevent the HIV entry into the immune cells [316]. Two entry inhibitor's, Roche's *Fuzeon* [enfuvirtide, ENF: targets the gp41 protein on HIV's surface] and Pfizer's *Selzentry* [MVC: targets CCR5 protein on CD4 cells] have been approved by the U.S. Food and Drug Administration [FDA] in March 2003 and August 2007, respectively.

Enfuvirtide [ENF]

ENF is a high molecular weight polypeptide made up of 36-amino acid [317]. Although HPLC and UPLC coupled with UV, Mass *etc* detectors are the techniques of choice to quantify a large number of drugs from various biological matrices. Quantification of peptides like ENF on HPLC columns is a difficult task. Challenges like column blocking, sample extraction, sticking to tubing and container walls and limited separation limit HPLC use in TDM of ENF. Assays for such peptides usually depend on the automated immune assays. With all the challenges, Lawless *et al.* and Chang *et al.* of Hoffmann-La Roche, USA successfully developed an assay for ENF and its metabolite in human plasma using LC–MS/MS and HPLC with fluorescence detector respectively [318, 319].

Maraviroc [MVC]

MVC which was approved in 2007 is a chemokine co-receptor 5 [CCR5]antagonist. Chemically it is an 4,4-difluoro-N-[[1S]-3-[[1S, 5R]-3-[3-methyl-5-propan-2-yl-1,2,4-triazol-4-yl]-8-azabicyclo[3.2.1]octan-8-yl]-1-phenylpropyl] cyclohexane-1-carboxamide which is fairly soluble in water and methanol with logP value of 5.1. It is used in combination with other anti-HIV drugs for the treatment of HIV-1 infection.



Maraviroc

It works by inhibiting the entry of HIV into CD4 cells by blocking CCR5 receptor on CD4 cells [320]. MVC undergoes N-dealkylation and oxygenation before excretion [321]. N-dealkylation is chiefly facilitated by CYP3A4 which is also involved in the metabolism of several drugs of different category thereby making MVC an important candidate for TDM [322].

To facilitate TDM and drug interaction studies of MVC, Fayet *et al.* and Martin *et al.* in 2009 separately reported the assays to quantify MVC using LC-MS/MS with other commonly prescribed ARV drugs [RAL, MVC, DRV, and ETR] [176, 249]. Notari *et al.* reported a simple simultaneous assay to quantify MVC and RAL in human plasma samples using HPLC-UV [323]. Avolio *et al.* developed and validated an efficient simple HPLC-UV assay to quantify MVC in the plasma sample of HIV infected patients [324]. On a similar line, Takahashi *et al.* validated a robust LCMS assay to determine plasma MVC levels in human blood plasma samples [325]. The principal benefit of Takahashi's assay is the rapid liquid-liquid sample extraction from plasma and the use of an internal standard to normalize the sample loss.

In 2010, Else *et al.* developed and validated a fast and sensitive LC–MS/MS assay for simultaneous quantification of PI's like APV, ATV, DRV, LPV, RTV, SQV, NNRTI's like NVP, CCR5 antagonists like MVC and the "second generation" NNRTI's like ETR and RPV [177]. For sample extraction, IS [quinoxalone; QX] was added to 100µl plasma aliquots prior to protein precipitation. 500 µl of acetonitrile [500µl] and 0.05% formic acid were used for the protein precipitation. Analyte separation was achieved on a reverse-phase C_{18} column with gradient elution [acetonitrile and 0.05% formic acid]. All the analytes including IS were identified and quantified

by operating triple-quadrupole in selective reaction monitoring and positive ionisation mode.

To extend the study of distribution of MVC to various body compartments, Brewer *et al.* of Pfizer, UK developed and validated an assay to quantify MVC in human plasma, urine, and cerebrospinal fluid [326]. This assay comprises of the simple protein precipitation extraction followed by LCMS using TurboIon- Spray® and multiple-reaction monitoring. This assay was also validated to quantify one of the major metabolites of MVC's [UK-408,027] in plasma. MVC-d5 was used as an internal standard. The assay enabling the measurement of IIs, PIs and NNRTIs concentration at the site of the pharmacological action is an essential tool for preventing ARV therapy failure and drug-related side effects and toxicity. Thus, to determine the exact intracellular levels of ARV, D'Avolio *et al.* in 2011 came up with the article, titled as "A HPLC–MS method for the simultaneous quantification of fourteen ARV agents in peripheral blood mononuclear cell of HIV infected patients optimized using medium corpuscular volume evaluation". This assay was highly sensitive and selective to determine the simultaneous quantification of14 ARV drugs in PBMC's for HIV-infected [79]. PBMCs were isolated by Ficoll density gradient centrifugation. Drugs from PBMC's pellets were extracted with methanol: water [70: 30, v/v], and quinoxaline was used as an IS. The supernatant obtained was dried and resuspended in water/acetonitrile [60/40, v/v], before separation on 2.1 mm × 150 mm Atlantis® T3 3µ chromatographic column. Chromatographic separations were performed using gradient elution. Analyte quantification was performed using electro-spray ionisation–single quadrupole MS operating in selected ion recording [SIR] detection mode.

PIs [IDV, SQV, NFV, NFV M8 metabolite, APV, DRV, ATV, RTV, LPV, TPV] integrase inhibitor [II] [RAL] and the NNRTIs [NVP and ETR] were identified in positive ionisation mode, while EFV was quantified in negative ionisation mode. The calibration curves were obtained using blank PBMCs spiked with ARV drugs at concentrations ranging from 0.1 to 32 ng/mL [1–320 ng/mL for TPV] and fitted to a quadratic regression model weighted by 1/*X*.

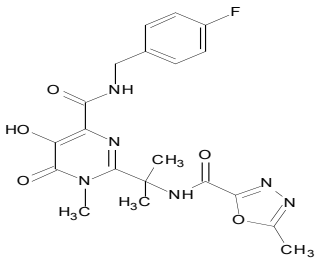
To reduce the time of analysis and solvent consumption in 2013, Djeradaet *al.* developed UPLC-MS/MS assay for simultaneous analysis of EVG, RAL, MVC, ETV, TFV, boceprevir and 10 other ARV agents in human plasma samples [215]. Very recently in 2014, Simiele *et al.* published new HPLC-MS assay to determine MVC plasma levels [327]. In this assay 6,7-Dimethyl-2,3-di[2-pyridyl] quinoxaline was used as an internal standard and added to 100 µL of plasma. Protein precipitation method to extract the analyte from the sample was employed. Protein precipitation of plasma sample was carried out using 500 µL of acetonitrile. All the analyte was separated on a T3 Atlantis column [150 mm × 4.6 mm] with a particle size of 5 µm. Fragment Ions were detected at m/z 257.5 and 313.3 for MVC and quinoxaline respectively.

INTEGRASE INHIBITORS [II'S]

II's also known as integrase strand transfer inhibitors (INSTIs), are a class of ARV drugs developed to block the action of an important enzyme called integrase. Integrase is a viral enzyme that facilitates the insertion of the viral genetic material into the DNA of the CD4 cell [328]. Since integration is an important step in replication, blocking it can stop further formation and spread of the virus. II's were originally developed for the treatment of HIV infection, but later found their use in the other retrovirus infections. Since II's target a distinct and important phase in the retroviral life cycle, they may be very good candidates in combination therapy to minimize drug development resistance.

Raltegravir [RAL]

RAL which was approved in 2007 is a pyrrolidinone derivative used in combination with other anti-HIV agents [329,330]. Chemically, it is an N-[2-[4-[[4-fluorophenyl]methylcarbamoyl]-5-hydroxy-1-methyl-6-oxopyrimidin-2-yl]propan-2-yl]-5-methyl-1,3,4-oxadiazole-2-carboxamide which is soluble in methanol with logP value of 0.4.



Raltegravir

Other than the P-gp substrate, the major mechanism of clearance of RAL in humans is UGT1A1-mediated glucuronidation [331, 332]. Being the first member of its class and substrate

for SLC22A6 and P-gp, RGV is considered as an important candidate for TDM.

The first RAL assay was on HPLC-MS/MS which was reported by Merschman *et al.* [333]. As the assay developed by Merschman *et al.* was intended for clinical studies at Merck, this method was not easily transferable to routine clinical practice because of the use of stable radioisotope of RAL and mass detector. To overcome this issue, Poirier *et al.* and Goldwirt *et al.* separately came up with the simple method for the quantification RAL in human plasma samples using HPLC with fluorescence detection, whereas Long *et al.* published a sensitive HPLC-MS assay for RAL analysis [334-336].

Poirier *et al.* and Merschman *et al.* used a similar liquid–liquid extraction method which was not easily available in all the bio-analytical laboratories. There was still a need for a standard universal assay that can be applied to bio-analytical laboratories globally. Therefore in 2008, Rezk *et al.* developed and validated an accurate and precise HPLC-UV method for rapid determination of RAL in human blood plasma after solid phase extraction [337].

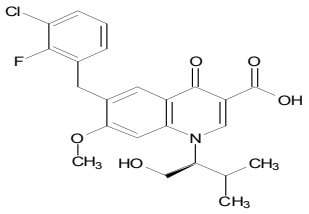
As described previously, with an added advantage of DBS sampling, Heine *et al.* developed and validated the simple assay for the quantification of RAL in plasma, DBS and PBMC lysate by means of LC-MS/MS [338]. This assay is very handy for the pharmacokinetic studies in children and neonates and also enables self-sampling for purposes of TDM.

Prophylactic use of RAL depends on its capacity to hoard in the secretion of reproductive organs. Therefore, to be an effective pre- and post-exposure prophylaxis agent against HIV infection, the pharmacokinetics of RAL in female genital tract secretions is needed to be understood. To support the PK studies of RAL in female genital tract secretion, Talameh *et al.* developed an HPLC assay to quantify RAL in CVF using a previously validated solid-phase extraction assay [337, 339]. In 2012, Robbins *et al.* developed and validated UPLC-MS assay to quantify RAL in human plasma and similarly, Jean-Francois *et al.* developed a simultaneous assay to quantify azole antifungals, antibiotics, imatinib, and RAL in human plasma by two-dimensional HPLC-MS [340, 341]. MS is an expensive and chromatographic method generally requiring time-consuming extraction and clean-up steps prior to chromatographic separation. To overcome this issue, Peris-vicente *et al.* developed a micellar liquid chromatography assay for the determination of ABC, 3TC and RAL in plasma which does not need tedious and time-consuming extraction clean-up steps to develop a well-resolved chromatogram [342].

Another very useful UPLC-MS/MS method was developed by Djerada *et al.* for the determination of the commonly prescribed PI's like APV, ATV, DRV, IDV, LPV, RTV, SQV and TPV, TDF a NRTI, non-NRTI such as EFV, NVP, ETR, MVC RAL, EVG and direct acting anti-HCV boceprevir [215]. Several other simultaneous assay methods for RAL are mentioned in Table **1**.

Elvitegravir [EVG]

EVG, approved in 2012 is a modified quinolone antibiotic with activity against HIV1. Chemically it is an 6-[[3-chloro-2-fluorophenyl]methyl]-1-[[2S]-1-hydroxy-3-methylbutan-2-yl]-7-methoxy-4-oxoquinoline-3-carboxylic acid which is slightly soluble in water but highly soluble in methanol with logP value of 5.3.



Elvitegravir

It is prescribed as a combination in a once-daily single-tablet regimen, compromising 150 mg of EVG, 150 mg cobicistat, 200 mg FTC, and 300 mg TDF disoproxilfumarate [TDF] [343]. Cobistat being the CYP3A inhibitor, acts as a pharmaco-enhancer to boost the effective plasma levels of EVG [344]. EVG is primarily metabolised by CYP3A4 and a small part of EVG is also metabolised *via* glucuronidation mediated by UGT1A1 [345]. In a 10-day monotherapy study which was performed in patients with HIV infection EVG 800 mg/day, 200, 400 and 800 mg twice daily and 50 mg boosted with RTV 100 mg/day provided a maximum mean change of viral load from baseline [346, 347].

The drug interactions between AZT, didanosine, d4T, ABC, ETR and RTV-boosted EVG were studied. The results showed that these drugs can be co-administered regardless of dose adjust-

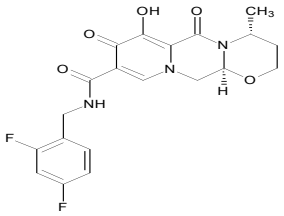
ment [348, 349]. A comparative study of EVG/ cobicistat/ FTC/TDF disoproxilfumaratevs EFV/ FTC/TDF disoproxilfumarate in the treatment of naïve patients showed that 90 and 83% of patients attained a virus load lower than 50 copies/mL at 24 and 48 weeks, respectively. Psychiatric side effects were observed in the EVG/ cobicistat/FTC/ TDF disoproxilfumarate group when compared with that of the EFV/FTC/TDF disoproxilfumarate arm [350]. Effect of the protein-rich drink or a standard meal on the pharmacokinetics of the EVG, Cobicistat, FTC and TDF in healthy Japanese male subjects was studied by Ishikawa *et al.* [351]. Authors observed that the EVG and TDF component of the combination was found to decrease by 50% and 28%, respectively when administered in a fasted state whereas the EVG and TDF levels were comparable following administration with a nutritional protein-rich drink and with a standard breakfast. Food or a nutritional drink, on the other hand, did not lower the bioavailability of Cobicistat or FTC.

As co-morbidities like dyslipidemia are an important factor to be considered in ART, a study was conducted on an interaction between cobicistat induced EVG and antilipidemic agent Rosuvastatin [352]. The study revealed that although the rosuvastatin concentrations increased when co-administered with EVG/co, no clinically relevant interactions were observed between EVG/co and rosuvastatin.

Risk of lower exposure to EVG even when boosted with RTV has to be considered in association with drugs or environmental factors that strongly induce CYP3A activity. Hence monitoring EVG plays the crucial role in the success of ART. An assay for EVG using an SPE has been reported as a part of clinical studies, however, with a limited calibration range [only up to 1000 ng/ml] and without much details of the validation [353]. To date, two simultaneous assays are available in the literature for the quantifications of EVG which can apply for routine TDM service for patients follow-up and for clinical research projects [187, 215].

Dolutegravir [DTG]

DTG is an HIV integrase strand transfer inhibitor approved by FDA in 2013. Chemically it is an [4R,12aS]-N-[[2,4-difluorophenyl]methyl]-7-hydroxy-4-methyl-6,8-dioxo-3,4,12,12a-tetrahydro-2H-pyrido[5,6]pyrazino[2,6-b][1,3]oxazine-9-carboxamide which is slightly soluble in water but highly soluble in methanol with logP value of 2.2.



Dolutegravir

It is the first second-generation approved drug that blocks the enzyme integrase necessary for HIV viral replication. DTG can be used for treating HIV patients who have either taken [treatment-experienced] or never taken [treatment-naive] HIV treatment previously [354]. The efficiency and toxicity levels of DTG are studied during various clinical trials and proved to be safer in both treatment naïve and experienced patients [355].

Phase-I clinical trial studies were carried out on 34 healthy volunteers and various kidney parameters like glomerular filtration rate, effective renal plasma flow, and creatinine clearance were evaluated [356]. The study of DTG over a period of 14 days revealed that the dose of DTG [50 mg] once or twice daily had no prominent effect on GFR as compared with placebo. This study demonstrated a reversible increase in the creatinine to the non-pathological levels. The increased creatinine levels are attributed to the inhibition of the organic cation transporter protein 2, specifically in the proximal renal tubule of a nephron.

Letendre *et al.* in 2013 studied TDM of DTG in the CSF of 12 ARV-naive HIV-infected patients getting DTG 50 mg daily co-administered with ABC/3TC regimen [357]. The overall spread of DTG in the CSF was just like the unbound DTG in blood plasma at week 2 [mean CSF DTG = 16.2 ng/ml, mean plasma unbound DTG = 16.8 ng/ml] and at week 16 [mean CSF DTG = 12.6 ng/ml, mean plasma unbound DTG = 23 ng/ml]. Moreover, the spread of DTG in the CSF for all patients at week 16 ranged from 18 to 90-fold higher than the *in vitro* IC_{50} for wild-type virus [0.2 ng/ml] [357, 358]. These studies illustrated that the DTG can pass freely across the blood–brain barrier.

In a phase-III non-inferiority single trial carried out on 833 patients, the adequacy of DTG 50 mg once daily co-administered with ABC/3TC compared with EFV/TDF and TDF/FTC once daily was carried out [359]. The study revealed that

DTG regimen was statistically better to the EFV/TDF and TDF/FTC combinations [359].

At clinically relevant concentration, DTG neither induced nor inhibited CYP or UGT isoenzymes [360]. The interaction potential of NRTIs with DTG is low as NRTIs do not undergo hepatic transformation following CYP metabolic pathway [361]. EFV, when co-administered with DTG may reduce DTG plasma concentration due to its ability to induce /inhibit CYP2B6 or UGT1A1 isoenzymes respectively. CYP2B6 and UGT1A1 are the major enzymes which metabolize the DTG and hence TDM of DTG is essential when co-administered with EFV [362].

PIs are known UGT1A1 inhibitors. ATV being one of them, possesses the ability to enhance DTG plasma concentrations. Conversely, RTV is a well-known inducer of the UGT1A1 pathway and hence has the ability to reduce DTG plasma concentrations [363]. DRV and LPV are well-known inhibitors of the CYP3A4 pathway and hence may enhance DTG plasma concentrations [364]. This ATV interaction also suggests that DTG is a good candidate for the TDM when co-administered and boosted with PI's.

To help further understand the DTG plasma pharmacokinetics, Bennetto-hood *et al.* have developed assays for DTG using an LC–MS/MS whereas Charbe *et al.* and Cozzi *et al.* developed simultaneous HPLC-UV assay method for the quantification of DTG [191, 365, 366]. Currently, one other assay developed by Grégoire *et al.* showed simultaneous determination of the plasma concentration of DTG and RPV using deuterated-internal standards [189].

CONCLUSION

This review is aimed at focusing on the role of liquid chromatographic techniques in TDM of ARVs and to give a comprehensive literature survey of the various ARV assays methods. This review also highlights the advancement of the chromatographic techniques beginning from the HPLC-UV to the more advanced technique like UPLC-MS/MS. TDM is essential to ensure adherence, observe viral resistance and to personalise ARV dose regimens. Analytical methods like immunoassays and liquid chromatography with detectors like UV, PDA, Florescent, MS, MS/MS and UPLC-MS/MS have immensely contributed to the clinical outcome of the ARV therapy. Assay methods are not only helping physicians in limiting the side effects and drug interaction but also assisting in monitoring the patient's compliance.

Literature survey revealed that HPLC has been the most widely used system irrespective of the availability of more sensitive chromatographic technique like UPLC. Because of smaller particle-sized of stationary phase, shorter column length with higher flow rates under high pressure, UPLC offers more advantage in terms of efficiency. With UPLC, it is now possible to develop more resolved methods. Resolved peaks per unit time are higher with UPLC which ultimately enhance the data quality. The main focus of any research institute or of the pharmaceutical industry is to reduce the R & D cost and time. Quality data in a shorter time can help the industry to save on the method development cost. As the UPLC column size is shorter, the re-equilibrium time is also short which ultimately save on solvents. The only disadvantage of UPLC is the higher price of the instrument and reduced column life because of the increased back pressure. But the faster separation, reduced solvent consumption, and better resolution can counterbalance the disadvantages. Seeing the advantages of UPLC, in near future, it is quite possible that methods developed on HPLC will be transferred on to the UPLC. It has been also revealed that UV is commonly used as a detector with HPLC. If the sample is adequate, UV detector assures the detection of all the UV-absorbing components. Over a certain period of time, most researchers preferred UV detectors with RP-HPLC techniques due to its consistency and sensitivity. However, due to the limitations associated with HPLC-UV (*e.g* increased solvents utilization and lack of reproducibility), more sensitive detectors like MS or MS/MS are in demand. These detectors have gained more popularity owing to their enhanced sensitivity, specificity and shorter analysis time. LC–MS/MS plays a significant role in TDM of HIV drugs in different matrices such as plasma, urine, PBMCs, CSF, and hair. It is the most preferred alternative to immunoassays and HPLC-UV technique due to its advantage of simultaneous estimation of the number of drugs in picomol concentrations.

Although matrix effect is the challenge in validation of HPLC-MS assay methods, proper sample preparation and use of appropriate chromatographic technique and internal standard give near errorless result. In the literature review, it is

also observed that most of the ARV drugs are either water-soluble or are soluble in polar solvents like methanol, ethanol or DMSO which perfectly fits in the basic requirement of the method developments on RP-HPLC.

Advancement in the column and pump technology leads to the development of the automatic rapid UPLC-MS/MS system. With the advantage of higher speed, sensitivity, reproducibility, and specificity over HPLC, UPLC can be used to simultaneously separate and determine multicomponent ARVs in different matrices. UPLC with mass detectors clearly has huge prospective for metabolite detections and is becoming an important tool in clinical pharmacology laboratories. Analytical and clinical laboratories will be continuously tested with blood samples containing a wide range of ARVs in extremely low concentrations. Hence, the highly sensitive and specific identification methods like LC–MS/MS and UPLC-MS/MS appear to be the technique of the preferred technique of future.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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